INTERACTIONS OF DIETARY AND WATERBORNE COPPER IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

By

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COPPER METABOLISM IN FRESHWATER FISH

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ABSTRACT

In mammals, copper is an essential yet potentially toxic trace element if accumulated in excess of cellular requirements. These conflicting properties demand a tight regulation of Cu, both at the cellular and organismal levels. Unlike mammals, fish can assimilate significant amounts of Cu from the water via the gills, in addition to the traditional dietary uptake via the gastrointestinal tract. The relative contributions of branchial and gastrointestinal routes of uptake in Cu homeostasis and toxicity in fish remain largely unknown. Therefore this thesis examined the interactions between dietary and waterborne routes of Cu uptake as they relate to homeostasis and toxicology of Cu using the freshwater rainbow trout, *Oncorhynchus mykiss,* as a model system.

Copper is clearly essential to fish based on both growth response and body Cu status in rainbow trout. Direct measurements of unidirectional Cu uptake following exposure of rainbow trout to conditions depleted and elevated in Cu showed up-regulation and down regulation of branchial Cu uptake, respectively. Thus Cu uptake from the water responds to body Cu status and there is an interaction between dietary and waterborne Cu uptake in the maintenance of body Cu balance. Under conditions of background water and dietary Cu levels, diet is the more important route of Cu uptake, accounting for about 90% of the Cu intake. However, during deficiency, waterborne Cu uptake may contribute up to 60% of the Cu requirement. Interestingly, while waterborne Cu uptake responds to acclimation to elevated waterborne Cu, as well as elevated dietary Cu, gastrointestinal Cu uptake does not. It thus appears that the gut serves for bulk acquisition of Cu while the gill performs homeostatic fine-tuning via

adjustment of branchial Cu transport mechanisms. Moreover, the response of branchial uptake mechanisms to Cu acclimation suggests the presence of at least two types of Cu transporters at the gill epithelium.

A linkage between Cu and sodium (Na^+) uptake and homeostatic mechanisms in fish gills was unveiled. Elevating dietary Na^+ reduced unidirectional uptake rates of Cu, and up to 95% of the reduction in Cu uptake was explained by parallel reduction in waterborne Na^+ uptake. This co-variation between Na^+ and Cu uptake strongly suggests that Cu and Na^+ share uptake pathways, possibly the apical Na^+ channel. Furthermore, long-term exposure to elevated dietary Na^+ reduced Cu accumulation in the liver as well as the short-term binding of Cu to the gills. These findings have important implications including the use of dietary Na^+ to protect against Cu toxicity both in fish and humans.

From a toxicological perspective, this thesis research uncovered new areas for consideration in the development of water quality criteria for Cu. One method currently under development for determining realistic site-specific water quality guidelines is the Biotic Ligand Model (BLM). The US Environmental Protection Agency has provisionally adopted the BLM as a site-specific modification to the current acute Ambient Water Quality Criteria for Cu. The tenet of BLM is prediction of the metal bound to the gill that results in toxicity in water of known chemistry. Several water chemistry parameters including Ca^{2+}, Na^+, dissolved organic matter, and pH greatly modify metal binding to the gill and hence toxicity. This thesis shows that dietary quality factors such as Cu and Na^+ content, and acclimation to waterborne Cu influence

the binding of Cu to the gill biotic ligand. Future development of the BLM should therefore consider dietary factors and acclimation effects.

Lastly, this thesis provides direct evidence that dietary metal uptake and diet quantity are important in toxicology and environmental risk assessment for metals. Food quantity influences fish growth and metal uptake thereby affecting whole body and tissue Cu concentrations such that lower Cu concentrations do not necessarily reflect lower Cu accumulation but can, in fact, represent growth dilution. Therefore, using body metal burdens for biomonitoring and risk assessment is meaningful only if dietary metal intake and feeding regimes are taken into account.

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Finally, I thank most sincerely my beloved wife Teresa, and sons, Ken and Dennis, for their love, understanding and undying support. At last I am done and I dedicate this thesis to you.

THESIS ORGANIZATION AND FORMAT

This thesis is organized in an "open-faced" format approved by McMaster University and with the recommendation of my Supervisory Committee. The thesis consists of eight chapters. Chapter 1 comprises an introduction and a summary of the major findings and significance of the research carried out. Chapters 2-7 are made up of discrete manuscripts published or accepted for publication in peer-reviewed scientific journals. Chapter 8 summarizes Cu metabolism in fish and highlights directions for future research.

Chapter 1: General introduction and scope of the study.

Chapter 2: Copper metabolism and gut morphology in rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal dietary copper exposure.

Authors: C. N. Kamunde, M. Grosell, J. N. A. Lott, and C. M. Wood.

Accepted: November 2000.

Journal: Canadian Journal of Fisheries and Aquatic Sciences **58**, 293-305.

Comments: This study was performed by CNK with input by MG, under the supervision of CMW. Gut morphology was done as part of partial requirement of a graduate course supervised by JNAL.

Chapter 3: **Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake.**

Authors: C. N. Kamunde, M. Grosell, D. Higgs, and C.M. Wood.

Journal: Journal of Experimental Biology **205**, 279-290.

Accepted: October 2001.

Comments: This study was carried out and written by CNK with significant contribution by MG, under the supervision of CMW. DH made the diets.

Chapter 4: **Waterborne vs. dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure.**

Authors: C. N. Kamunde, C. Clayton, and C. M. Wood.

Journal: American Journal of Physiology **283**, R69-R78.

Accepted March 2002.

Comments: This study was done by CNK under the supervision of CMW. CC then a summer student in the lab made significant contributions in the study and sample analysis.

Chapter 5: **The influence of ration size on copper homeostasis during sublethal dietary copper exposure in rainbow trout, *Oncorhynchus mykiss.***

Journal: Aquatic Toxicology.

Authors: C. N. Kamunde and C. M. Wood.

Accepted: June 2002.

Comments: This work was done and written by CNK under the supervision of CMW.

Chapter 6: Dietary sodium inhibits aqueous Cu uptake in rainbow trout (*Oncorhynchus mykiss*).

Authors: G. Pyle, C. N. Kamunde, D.G McDonald, and C.M. Wood.

Journal: Journal of Experimental Biology.

Accepted: July 2002.

Comments: This work was done jointly by CNK and GP under supervision of CMW and DGM. The manuscript was written by GP with input from CNK.

Chapter 7: Influence of dietary sodium on waterborne copper toxicity in rainbow trout, *Oncorhynchus mykiss*.

Authors: C. N. Kamunde, G. Pyle, D. G. McDonald, and C. M. Wood.

Journal: Environmental Toxicology and Chemistry.

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Chapter 8: Summary.

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE STUDY

BACKGROUND

Copper is an essential transition metal required for the survival of all organisms ranging from bacteria to humans (Linder, 1991). Because of its ability to cycle between a stable oxidized Cu^{2+} and a reduced unstable Cu^{+}, copper serves as a catalytic cofactor for several proteins involved in key biological redox pathways essential for life (see Table 1 for a list of some cupro-proteins and their functions). However, the redox cycling of Cu is also partly the basis for its toxicity because it can lead to the formation of highly reactive oxygen species (Halliwell and Gutteridge, 1984) which can cause devastating cellular damage including oxidation of proteins, membrane lipid peroxidation, and cleavage of DNA and RNA molecules. In addition, Cu interferes with the homeostasis of other metals and inactivates proteins by strongly binding to cysteine, histidine, and methionine residues. The need to acquire and deliver Cu to essential enzyme systems without resultant toxicity necessitates the presence of tightly regulated homeostatic mechanisms at the organismal, tissue, and cellular levels (Harris, 1991; Vulpe and Packman 1995; Linder and Hazegh-Azam, 1996). Despite a concerted research effort, Cu homeostatic mechanisms in animals at the cellular through organismal levels remain relatively poorly defined.

The maintenance of physiological levels of Cu is achieved through a Cu homeostasis system that entails regulated uptake, transport, storage/sequestration, and efflux/excretion (Turnlund *et al.*, 1998; Camakaris *et al.*, 1999). In particular, mammalian body Cu balance is maintained almost entirely by gastrointestinal absorption and biliary excretion (Schaefer and Gatlin, 1999). The importance of the maintenance of Cu homeostasis is underscored by the existence of two genetic conditions in humans, the Menke's and Wilson's diseases (DiDonato and Sakar, 1997; Schaefer and Gatlin, 1999). These diseases arise from dysfunctional mutations of genes encoding for specific Cu transporters (in the family of P-type ATPases) within the membrane of cells or cell organelles. Menke's disease is an X-linked recessive disorder of Cu metabolism that manifests as Cu deficiency due to the failure of Cu export mechanisms in gut mucosal cells (i.e., the export step from the cytoplasm to the extracellular fluid), leading to inactivity or reduced activity of cupro-enzymes, and accumulation of Cu in the gut. Wilson's disease on the other hand is an autosomal recessive disorder of Cu metabolism and manifests as Cu overload and toxicity due to failure of Cu excretion from the liver, both as incorporation of Cu into ceruloplasmin, and export for biliary excretion. Since biliary excretion is the main mechanism of Cu excretion in humans, massive accumulation of Cu occurs in the liver leading to necrosis of hepatocytes and liver cirrhosis. Necrotic hepatocytes release Cu into plasma, which is then deposited in extrahepatocytic tissues including the brain, resulting in cerebral pathology.

In contrast with the mammalian model in which Cu metabolism involves the regulation of uptake primarily via one (gastrointestinal) route of uptake and one route of

excretion (hepatobiliary) (Linder and Hazegh-Azam, 1996; Schaefer and Gatlin, 1999), fish possess two principal routes of Cu uptake, i.e., gut and the gills. Multiple pathways of excretion possibly exist in fish including branchial, hepatobiliary, and renal routes. A large body of scientific literature exists on exposure of fish to high Cu levels via either route (McDonald and Wood, 1993; Handy, 1996; Wood, 2001). Most importantly, waterborne Cu has been shown to be a branchial toxicant inhibiting Na^+ balance by effects on Na^+ uptake and gill diffusive permeability. In contrast, dietary Cu is far much less toxic although acute toxicity may well be due to interference with Na^+ balance; indeed an interaction of Cu and Na^+ uptake in mammalian gut has been demonstrated (Wapnir, 1991). Nonetheless, only a few studies have focused on the interactions between branchial and gastrointestinal uptake mechanisms in the homeostatic maintenance of metal balance. For example, Miller *et al.* (1993) reported that tissue Cu levels in rainbow trout increased with increasing dietary Cu, but waterborne Cu became increasingly more important as its concentration rose. Therefore, the importance of the gastrointestinal tract in metal uptake appears to increase in the presence of conditions that reduce branchial uptake such as low waterborne metal levels, and conditions that decrease the bioavailability of waterborne metal. However, this remains to be directly demonstrated by simultaneous measurements of Cu uptake from the diet and the water. Furthermore, the response of waterborne Cu uptake to dietary loading and deficiency remains undefined.

OVERVIEW OF THE THESIS

This thesis examined interactions between dietary and waterborne routes of Cu uptake in the homeostasis and toxicity of both dietary and waterborne Cu in a freshwater fish, rainbow trout. It is made up of eight chapters. The first chapter provides a brief introduction to the thesis including the objectives and major findings of the research carried out. An overview of the present state of the knowledge relating to Cu homeostasis and toxicity in animals, and especially fish, is presented. Chapters 2-7 comprise papers from six separate studies each testing specific hypotheses. The overall goal was to improve knowledge on the interactions between dietary and waterborne Cu uptake in Cu metabolism and toxicity in fish. Chapter 8 summarizes Cu homeostasis and identifies gaps in knowledge for future research. The thesis covers three broad topic areas namely: i) essentiality and toxicity of Cu; ii) waterborne *versus* dietary Cu uptake; and iii) effects of dietary quantity and quality on Cu metabolism. These are outlined in the following summary.

Essentiality and toxicity of copper

The fundamental issues in Cu metabolism namely, essentiality and toxicity, are examined in the first two papers (Chapters 2 and 3). In Chapter 2 juvenile rainbow trout were exposed to elevated dietary Cu levels (300 and 1000 $\mu g\ g^{-1}$) for 28 days, and endpoints indicative of toxicity evaluated. The primary goal here was to determine the toxic level of dietary Cu and to further characterize the effect of dietary Cu on subsequent uptake of waterborne Cu. At this point in time, data on dietary Cu exposures

to fish were few. Although a previous study on rainbow trout had reported that the toxic level of dietary Cu is about 664-730 $\mu g\ g^{-1}$ (Lanno *et al.*, 1985), 1000 $\mu g\ g^{-1}$ did not cause any effect on growth, suggesting that the toxic levels of Cu in the diet for trout may be much higher.

The other objective of this Chapter was to evaluate the morphopathological impact of dietary Cu by performing a detailed ultrastructural analysis of the intestine during dietary Cu exposure. Exposure to elevated dietary or waterborne metals is often accompanied by morphological changes at the primary target site of exposure as well as in internal tissues. While the structural impact of metals on the fish gill is fairly well characterized (Baker, 1969; Mallatt, 1985; Taylor *et al.*, 1995) few studies have evaluated the effect of dietary Cu exposure on gut structure. Woodward *et al.* (1995) reported gut impaction, swelling, and degenerative changes consisting of vacuolation and sloughing of mucosal cells while Berntssen *et al.* (1999) observed increased apoptosis and cell proliferation in Atlantic salmon ileum. In this study, despite the high dietary level of Cu exposure and heavy Cu accumulation in gut tissues, the morphological impact of Cu was modest, comprising mainly increased apoptosis and cell division, proliferation of eosinophilic granule cells, and appearance of lamellated bodies. Since fish exhibiting these ultrastructural changes had similar growth rate to the controls, it was concluded that the cost, if any, associated with tissue repair was small.

The specific objectives of Chapter 3 were first to determine the conditions under which Cu deficiency could be induced in fish and to assess whether fish could obtain their Cu requirements from water. Second, the effect of restricted Cu intake via both the

diet and water, and excess Cu intake via the diet on body and tissue Cu levels was assessed to delineate deficient, normal, and toxic levels of Cu in trout. Third, direct measurements of Cu uptake from the water using ^{64}Cu were done not only to evaluate the effect of deficiency and excess Cu intake on waterborne Cu uptake kinetics, but also to enable quantification of the relative contributions of dietary and waterborne Cu uptake. The results clearly demonstrated a requirement of Cu for normal growth and development of rainbow trout based on depressed growth and low Cu status in fish exposed to Cu-deficient conditions. Direct measurements of unidirectional Cu uptake showed for the first time that: (i) waterborne Cu uptake rates are regulated by whole-body Cu status; ii) branchial Cu uptake is down-regulated during exposure to elevated dietary Cu and up-regulated during Cu deficiency; and iii) waterborne Cu uptake can contribute up to 60% of total body Cu content during deficiency. Thus there is an interaction between dietary and waterborne Cu uptake geared toward maintaining Cu balance, with the gill playing a key role in the process. No toxic signs were observed in response to dietary Cu loading, despite a nearly 20-fold increase in whole-body Cu concentration, suggesting that the excess Cu in the fish was held in non-toxic form and that whole-body and tissue Cu concentrations are not directly related to toxicity.

Dietary *versus* waterborne copper uptake

This section, the paper presented as Chapter 4, reports a comparative analysis of uptake rates of dietary and waterborne Cu and the effect of waterborne Cu acclimation on these rates. As a consequence of the studies reported in Chapters 2 and 3, a fairly

clear picture of the effect of dietary Cu exposure on whole-body Cu metabolism had emerged. However, an important aspect of dietary *versus* waterborne Cu interaction that had not been previously addressed was the effect of waterborne Cu acclimation on subsequent uptake and distribution of both dietary and waterborne Cu. Furthermore, there had yet been no quantitative comparative analysis of the relative importance of waterborne *versus* dietary Cu uptake in fish using direct measurements of uptake via both routes. This formed the basis of the study reported in Chapter 4. The hypotheses tested here were built on the premise that Cu homeostasis is centrally regulated and that exposure to Cu via one route would affect uptake via the other route. Therefore the uptake of both dietary and waterborne Cu was assessed using ^{64}Cu radio-labeled diets or water in 48-h long fluxes during the course of waterborne Cu acclimation. The specific aims of the study were: (i) to determine the relative quantitative contributions of waterborne *versus* dietary uptake rates; (ii) to characterize the effects of waterborne Cu acclimation on subsequent uptake of both dietary and waterborne Cu; (iii) to quantify waterborne and dietary Cu turnover and exchangeable pools; and (iv) to assess the effect of acclimation on gill Cu-binding kinetics.

Acclimation as applied in toxicology denotes an increased tolerance to an elevated concentration of an otherwise lethal toxicant arising from long-term exposure to sublethal concentration of the toxicant (McDonald and Wood, 1993). The phenomenon is characterized by reduction in the extent of physiological disturbances following challenge with a higher concentration of the toxicant (McDonald and Wood, 1993), and/or increases in LC50 (concentration required to kill 50% of a population in a

given time) (Bradley *et al.*, 1985) or LT50 (time to 50% mortality at single lethal concentration) (McDonald *et al.,* 1991). These effects may be associated with increased resistance of the metal-sensitive processes. For example, it has been demonstrated that chronic sublethal exposures to Cu result in increased synthesis of Na^+,K^+-ATPase (Stagg and Shuttleworth, 1982; Lauren and McDonald, 1987ab). The most controversial effect of acclimation relates to gill Cu uptake. Apparently, prolonged exposure to sublethal concentrations of Cu may reduce (Grosell *et al.,* 1996, 1997), increase (Grosell *et al.*, 1998; Taylor *et al.*, 2000) or not change (McCarter and Roch, 1984; Grosell *et al.*, 1996) branchial Cu uptake. The data presented in Chapter 4 show opposite effects of acclimation on the subsequent uptake of waterborne Cu dependent on the exposure water Cu concentration, which probably explains the variable data reported by previous authors. Furthermore, the dichotomous response of gill Cu-binding to acclimation points to the existence of at least two Cu transporters at the gill that respond differently to acclimation to waterborne Cu. The nature of the gill Cu transporters is considered further in Chapter 6 and 7.

The second focus of Chapter 4 was on dietary Cu uptake, and the effect of acclimation to waterborne Cu on the uptake of dietary Cu. In mammals, dietary Cu is absorbed in the stomach and the intestines (Linder, 1991; Pena *et al.*, 1999). Low pH in the upper parts of the intestines (duodenum) makes this region the main site for Cu absorption. Generally, the efficiency of Cu absorption decreases with increasing Cu intake (Linder, 1991; Turnlund *et al.*, 1989). At the cellular level, the mechanisms of Cu absorption in the gut are not well understood. However, there is evidence that

absorption of Cu across the brushborder into the enterocyte, and the subsequent transfer across the basolateral membrane into the blood occur by different mechanisms. The transfer of Cu across the apical side of the mucosal cells of the small intestine was originally thought to occur solely by non-energy-dependent diffusion as Cu^{2+} ions (Linder, 1991; Linder and Hazegh-Azam, 1996) or by pinocytosis as Cu-protein complexes. However, Pena *et al.* (1999) argued that diffusion alone would not completely account for this uptake given the refined nature of Cu uptake by unicellular organisms such as yeast. A role for a high affinity Cu transport protein, denoted hCtr1, in the apical Cu uptake in mammalian intestine was proposed and later demonstrated (Zhou and Gitschier, 1997; Lee *et al.*, 2000, 2001, 2002; Harris, 2001). In addition, the natural resistance-associated macrophage protein (Nramp2) system is thought to mediate uptake of Cu through an energy independent mechanism (Rolfs and Hediger, 1999). Nramp2 is located in the apical region of the intestinal epithelial cells and functions to transport iron across the plasma membrane (Canonne-Hergaux *et al.*, 1999). However, this protein also mediates the translocation of several divalent cations including Zn^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, and Pb^{2+} and has therefore been referred to as DCT1 (divalent cation transporter) (Gunshin *et al.,* 1997) or DMT1 (divalent metal transporter) (Andrews, 1999). Once in the mammalian intestinal cell, Cu may be incorporated into Cu-requiring proteins, whereas basolateral efflux is effected by the Menke's (MNK) protein, a Cu-translocating P-type ATPase (Camakaris *et al.,* 1999; Pena *et al.*, 1999). Consistent with demonstrable expression of MNK in normal intestine, Menke's disease patients do not express this gene, and therefore accumulate Cu in intestinal mucosal

cells. The MNK protein is involved in both providing Cu to secreted Cu-metalloproteins and in the efflux of Cu from intestinal epithelial cells. The localization of MNK protein is regulated by Cu concentrations (Petris *et al.*, 1996). At low cellular Cu concentrations the MNK protein is localized in the trans-golgi network (TGN) where it delivers Cu to the secretory pathway. At elevated concentrations, Cu induces the trafficking of MNK protein from TGN to the plasma membrane where it is involved in Cu efflux to protect cells from potentially toxic Cu levels. Cu-regulated trafficking of the MNK protein to the basolateral membrane of gut epithelial cells is thought to be responsible for Cu absorption into the blood. Transfer of Cu across the basolateral membrane (possibly via the MNK protein) is the rate-limiting step in intestinal Cu absorption (Linder and Hazegh-Azam, 1996). Once absorbed into blood, Cu is bound to plasma albumin and histidine (Linder, 1991) for transportation to the liver where it is incorporated to ceruloplasmin and transported via the blood to other organs (Goode *et al.*, 1989). Excess Cu is excreted into the bile. The Wilson's protein (another P-type Cu-ATPase) mediates both these processes, and in its absence (Wilson's disease), toxic levels of Cu accumulate in the liver. A small proportion of circulating Cu is associated with amino acids (Cousins, 1985) and transcuprien (Linder, 1991).

Specific studies on uptake mechanisms of dietary Cu in fish have been rarely undertaken, but mechanisms of absorption similar to those in mammals are likely. The fish stomach environment is acidic (Fange and Groves, 1979), and therefore may free Cu from the food with subsequent partial gastric absorption as in mammals. The short-term ^{64}Cu absorption data presented in this Chapter suggest a role of fish stomach in Cu

absorption. However, the posterior intestine accumulated the highest levels of new Cu consistent with recent findings by Clearwater *et al.* (2000), suggesting that this region of the fish intestine is the most important for Cu absorption. Recently, Bury *et al.* (2001) demonstrated that Fe^{2+} was also preferentially transported in the posterior intestine. Intestinal uptake of Cu in fish is thought to occur via both simple diffusion for apical entry and biologically mediated transport for basolateral exit (Clearwater *et al.*, 2000; Handy *et al.*, 2000). Nonetheless, the involvement of some high affinity transporter homologous to the hCtr1 cannot be ruled out; an analogue of hCTr has recently been cloned from zebrafish embryos (M. Allende, personal communication). Handy *et al.* (2000) postulated that the basolateral limiting step in Cu uptake in fish is via a Cu/anion symport and/or possibly a Cu-ATPase. Thus there appear to be similarities between mammalian and piscine gastrointestinal Cu uptake and transport.

On a comparative basis, this study on waterborne *versus* dietary Cu uptake showed for the first time that at background Cu levels uptake and turnover rates of dietary Cu are more than tenfold higher than those of waterborne Cu, highlighting the importance of gastrointestinal route relative to branchial route of Cu uptake. However, both the gill and gut tissues saturated with Cu during 48-h waterborne or dietary Cu flux periods, suggesting the presence of homeostatic mechanisms that bring the rates of uptake and export into equilibrium at both sites. Interestingly, acclimation to elevated waterborne Cu reduced the uptake rates and exchangeable Cu pools of waterborne but not dietary Cu and caused important changes in gill Cu-binding characteristics.

Effects of dietary quantity and quality on copper metabolism

Diet-related variables such as dietary quantity and quality have marked effects on fish physiology and metabolism (Cowey and Sargent, 1979). Several studies have suggested that environmental pollutants affect appetite of the fish resulting in alteration in the dynamics of metal handling (Jimnez *et al.,* 1987; Lanno *et al.*, 1989; Wilson *et al.*, 1994; D'Cruz *et al.*, 1998). Generally, bioenergetic effects of Cu toxicity include depressed appetite, reduced growth, and altered food conversion efficiency (Buckley *et al.,* 1982; Lanno *et al.*, 1985; De Boeck *et al.*, 1995). However, the possible effects of food-related variables on homeostatic regulation and toxicity of Cu are not well studied. This is in sharp contrast to the much better characterized effects of water quality variables such as pH (Cusimano *et al.*, 1985), Ca^{2+}, and Na^{+} (Pagenkopf, 1983; Laurén and McDonald 1986; Playle *et al.*, 1992; Erickson *et al.*, 1996) on the uptake of waterborne Cu.

Chapters 5 through 7 therefore investigated the modifying effects of ration size and dietary Na^{+} on the homeostasis and sublethal toxicity of Cu. In Chapter 5 juvenile rainbow trout were exposed to a consistent load of 0.24 μmol Cu g $fish^{-1}$ day^{-1}, delivered in 1.5, 3.0, and 4.5% ration for 35 days. One of the objectives was to assess whether fish maintained on a high ration have superior ability to regulate and mitigate the deleterious effects of dietary Cu exposure. Secondly, we sought to establish possible connections between tissue Cu accumulation, metal dose, and toxicity for purposes of risk assessment in aquatic toxicology. Finally, we evaluated the effect of dietary Cu exposure on tolerance to waterborne Cu. The main finding was that the Cu-exposed fish

accumulated the same total metal load irrespective of ration, and the differences in metal concentration in tissues were a reflection of the fish size, and thus represented growth dilution rather than greater metal retention. This finding was consistent with the findings in Chapter 2 in which most of the change in tissue Cu concentration during deficiency was explained by growth dilution rather than loss of Cu. Likewise, Saari *et al.* (1993) found that food restriction in rats improved liver Cu status during Cu deficiency since slower growth of the liver in deficient rats left the Cu more concentrated. These finding have great implications when using body metal burden as an indicator of exposure for risk assessment. Yet another interesting finding from this study was that Cu uptake rates were higher in faster growing fish (fish on high ration), possibly indicating a greater need for Cu in rapidly growing fish. Interestingly and contrary to one previous report (Miller *et al.*, 1993), dietary Cu loading did not change the 96-h waterborne Cu LC50.

The mechanisms of Cu uptake at the fish gill had not been well characterized previously. Most studies examined the toxic effects during waterborne Cu exposure with little emphasis on the mechanisms of uptake and transport (McDonald and Wood, 1993; Wood, 2001). Where mechanistic approaches have been employed, the focus has been to understand toxicity rather than Cu uptake and transport *per se* (Laurén and McDonald, 1985, 1986, 1987ab; McDonald and Wood, 1993; Wood, 2001). A fundamental discovery accruing from these toxicological studies was that waterborne Cu interferes with Na^+ metabolism in the fish gills. The recent demonstration of vanadate sensitivity of branchial Cu uptake (Campbell *et al.*, 1999) suggested the presence of a P-

type Cu transporting ATPase at the gills. Indeed, a putative Cu-ATPase was recently partially cloned in gill tissue (Grosell *et al.*, 2001a). Furthermore, identification of a phenamil-sensitive, bafilomycin-sensitive high affinity Cu uptake pathway in trout gills (Grosell and Wood, 2002) similar to that for Ag (Bury and Wood, 1999) implicates the apical Na^+ channel/H^+-ATPase in Cu uptake.

In Chapters 6 and 7, therefore, the effect of elevated dietary NaCl (a dietary quality parameter) on Cu homeostasis and waterborne Cu toxicity were examined. Sodium as a dietary quality variable was chosen because Cu toxicity arises primarily from the disturbance of Na^+ balance (Stagg and Shuttleworth, 1982; Laurén and McDonald, 1986, 1987a and b). Chapter 6 examined the interaction between dietary Na^+, waterborne Na^+, and waterborne Cu using short-term exposures to Na^+ and Cu. Juvenile rainbow trout were exposed to a range of dietary Na^+ from control to 3.0% for seven days, and waterborne Na^+ and Cu uptake assessed in 6-h exposures to ^{22}Na and ^{64}Cu. This experiment demonstrated for the first time that dietary Na^+ reduces waterborne Cu uptake. Consequently the conclusion drawn was that Na^+ and Cu share uptake pathways at the gill. Additional evidence of such a shared pathway was experimentally demonstrated in a recent pharmacological and cation competition study (Grosell and Wood, 2002).

Chapter 7 extended studies on the Na^+/Cu interactions by investigating the effects of simultaneous long-term exposure to elevated dietary Na^+ and waterborne Cu. Here juvenile rainbow trout were exposed either to background (19), 55, or 118 nmol l^{-1} Cu in combination with either control (0.6%) or 3.0% dietary Na^+ for 21 days.

Indicators of Cu and Na^+ homeostasis including body burdens and uptake and distribution of new Na^+ and Cu were assessed on a weekly basis. Monitoring mortalities and growth throughout the experiment assessed toxicity. Possible modifications of gill transporters/binding sites were evaluated by performing a 3-h gill Cu-binding test at the end of the experiment. Novel data emanating from this study were that dietary Na^+ markedly reduced the accumulation of Cu in the liver but only modestly affected gill and whole body Cu levels. Whole-body unidirectional Cu and Na^+ uptake rates were strongly correlated with up to 95% of the Cu uptake being explained by Na^+ uptake, underscoring the earlier observations of Chapter 6 and Grosell and Wood (2002) that Na^+ and Cu share uptake pathways. The interesting observation made during gill Cu-binding assay was that waterborne Cu pre-exposure decreased gill Cu-binding at the low ambient Cu concentrations while the combined dietary Na^+ and waterborne Cu exposure reduced gill Cu binding throughout the entire range of Cu concentration tested. Thus Cu transporters at the gill are sensitive to both waterborne Cu and dietary Na^+ pre-exposure.

With respect to Cu toxicity and environmental issues, this thesis provides data that dietary route of exposure is an important source of Cu contamination. Water quality criteria/guidelines to protect aquatic life from metal impact have for long been based on waterborne metal exposure only (USEPA, 1986; CCME, 1995), although in polluted waters metals are likely available to fish through both water and contaminated food. Realistic water quality criteria should therefore take into account metal exposure via the dietary route and the interactions of waterborne and dietary metals. This thesis provides data that dietary Cu exposure is important due to both the direct effect of metal uptake

from the gut, and secondary modification of gill Cu-binding characteristics. Furthermore, both dietary quality (Na^+) and quantity modify gill Cu-binding. The US EPA is currently developing new ambient water quality criteria for Cu and other metals based on Biotic Ligand Modeling of fish gill-metal interactions for site-specific testing (Di Toro *et al.*, 2001; Santore *et al.*, 2001). The tenet of the BLM is to predict the degree of metal accumulation at the gill that causes mortality MacRae *et al.* (1999). A number of dietary quality and quantity factors, as well as waterborne Cu pre-exposure, modify Cu-binding to the gills and thus could alter the toxic effects of Cu on fish. These factors therefore warrant consideration in the future development of a chronic BLM for Cu.

In conclusion, this thesis provides important data on dietary and waterborne Cu exposure that are important to the understanding of Cu metabolism and toxicity in fish. Clearly, diet is the major route for Cu uptake while the gill fine-tunes the Cu homeostatic system based on requirements. Transporters for Cu, both specific and non-specific, apparently exist at both the gills and the gut and work in concert to maintain Cu homeostasis.

Table 1-1. Some copper requiring proteins and their biological functions.

Cupro-protein	Function
Cytochrome c oxidase	Mitochondrial electron transfer
Ceruloplasmin	Ferroxidase, Cu transport
Cu/Zn superoxide dismutase	Free radical detoxification
Dopamine β-hydroxylase	Catecholamine production
Metallothionein	Metal sequestration
Lysyl oxidase	Cross-linking of collagen and elastin
Hephaestin	Iron efflux in intestine
Tyrosinase	Melanin production
Peptidylglycine monooxygenase	Activation of peptide hormones

CHAPTER 2

COPPER METABOLISM AND GUT MORPHOLOGY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) DURING CHRONIC SUBLETHAL DIETARY COPPER EXPOSURE

ABSTRACT

Juvenile rainbow trout *(Oncorhynchus mykiss)* were exposed to 11 (control), 300 (medium), and 1000 μg g^{-1} (high) Cu (as $CuSO_4 \cdot 5H_2O$) in the diet for 28 days at a daily ration of 4% wet body weight, with a background waterborne Cu concentration of 3 μg l^{-1}. There was no effect of dietary Cu on growth, condition factor, or food conversion efficiency. Whole-body Cu content increased continuously over the exposure period in all groups and was 2-fold and 4-fold higher than control at day 28 for the medium and high Cu diets respectively. Cu accumulated mainly in liver and gut tissue with the latter stabilizing by day 14. Accumulation also occurred in gill, kidney, and carcass. Plasma Cu concentration was not different from the controls whereas Cu in bile was greatly elevated, an indication of increased hepatobiliary excretion. Dietary Cu pre-exposure decreased the uptake of waterborne Cu across the gills providing the first evidence of homeostatic interaction between the two routes of uptake. Electron microscopic observations of the mid-intestine revealed numerous mitochondria, lysosomes, lamellated bodies, and extensive lamellar processes in the enterocytes. Apoptosis, mitosis, and eosinophilic granule cells were more apparent in Cu-exposed fish.

INTRODUCTION

Fish assimilate Cu from both water and diet (Dallinger *et al.,* 1987). While the uptake and effects of waterborne Cu are fairly well understood (McDonald and Wood, 1993), effects of dietary Cu in fish have been less thoroughly investigated (Handy, 1996). A few studies have highlighted a micronutrient requirement for Cu (Murai *et al.*, 1981; Ogino and Yang, 1980) while others have recognized dietary exposure as a source of Cu intoxication in fish (Woodward *et al.*, 1994, 1995; Handy, 1996). Available dietary Cu exposure data indicate that bioavailability is very low (3% in rainbow trout, *Oncorhynchus mykiss*) and toxic effects occur at much higher exposure levels relative to waterborne exposures (Lanno *et al.*, 1985; Julshamn *et al.*, 1988; Handy, 1992). It has been argued that the gastrointestinal tract offers a strong barrier to dietary toxicants and effectively regulates metal uptake (Berntssen *et al.*, 1999). Although the majority of the studies on dietary Cu exposure in fish have assessed growth and tissue-specific accumulation, results have been rather variable (Handy, 1992; Mount *et al.,* 1994; Berntssen *et al.,* 1999) and probably reflect the differences in experimental designs, diet variables such as Cu concentrations and bioavailability, and the length of the exposure.

Interactions between dietary and waterborne Cu uptake are likely important in Cu homeostasis in fish. Unfortunately nothing is known about the effects of dietary Cu pre-exposure on subsequent waterborne Cu uptake or vice versa. Previous studies that attempted to delineate effects of waterborne and dietary Cu exposure (Miller *et al.*, 1993; Farag *et al.,* 1994) focused mainly on tissue metal accumulation attending Cu exposure through the two routes. The problem with such studies is distinguishing Cu of

dietary uptake from that of waterborne uptake. Application of direct measurements of Cu uptake using radioisotope methodologies (Wood, 1992) not only permits this but also distinguishes Cu of recent uptake from Cu accumulated over the long-term.

Exposure to elevated dietary metal levels is often accompanied by morphological changes in the gut, but there are only few studies on Cu. Effects of a dietary metal mixture (including Cu) on rainbow trout gut morphology as described by Woodward *et al.* (1995), include gut impaction, swelling, ulceration and epithelial lifting. Recently Berntssen *et al.* (1999) reported increased apoptosis and cell proliferation in the ileum of Atlantic salmon (*Salmo salar* L) parr exposed to elevated dietary Cu. Additional morphological studies are desirable to more fully describe the impact of dietary Cu on the fish gut.

In the present study we evaluated tissue-specific partitioning and bioaccumulation of Cu following dietary exposure at environmentally realistic sublethal levels. Secondly, we assessed the impact of dietary Cu exposure on subsequent uptake and distribution of waterborne Cu using a sensitive radioisotope methodology which distinguishes recently accumulated Cu from that already present in tissues prior to exposure. Finally, we described the morphological changes occurring in the mid-intestine during chronic elevated dietary Cu exposure.

MATERIALS AND METHODS

Experimental animals

One hundred and twenty juvenile rainbow trout (weight and length, (means ± SEM) = 37.24 ± 1.20 g and 14.86 ± 0.20 cm, respectively) were transferred from laboratory stock (originally from Humber Springs Trout Farm, Ontario) and equally divided into three 80-l experimental tanks supplied with a through-flow of aerated dechlorinated Hamilton City tap-water (Na^+, 13.8 mg l^{-1}; Cl^-, 24.8 mg l^{-1}; Ca^{2+}, 40 mg l^{-1}; HCO_3^-, 115.9 mg l^{-1}; pH, 7.9-8.2; background Cu, 3 μgl^{-1}) at flow rates of 1.2 l min^{-1}. Seven days before starting the experiment, all the fish were anesthetized with 0.1% tricaine methanesulphonate (MS-222) and individually marked by introducing alcian blue dye (0.06 g ml^{-1} in nanopure water) spots on their undersides with Panjet (Wright Dental Manufacturing Co, Dundee). This facilitated fish identification and determination of specific growth rates.

Diet preparation

Cu-enriched diets were 'made' in-house by grinding commercial trout chow [5 point Regular Trout Grower Pellets, composition: 40% crude carbohydrate, 42% crude protein, 1% calcium, 0.75% phosphorus and 0.35% sodium (Martin's Feed Mill Ltd., Elmira, Ontario)] and mixing it with Cu (as $CuSO_4 \cdot 5H_2O$, Fisher Scientific, Toronto, ON) to give nominal concentrations of 300 (medium) and 1000 (high) $\mu g\ g^{-1}$ Cu-diet. The food was then extruded through a pasta maker, air-dried, and broken into small pellets (approximately 8 mm^3) by hand. The actual Cu concentrations of the diets

(means ± SEM) as determined by atomic absorption spectroscopy were 277.8 ± 5.9 (n = 5), and 1041.9 ± 17.5 (n = 5) μg g^{-1} diet for medium and high Cu diets respectively. These dietary levels of exposure were chosen to fall within environmentally realistic levels (Dallinger and Kautzky, 1985) and above maximum tolerable levels (Lanno *et al.*, 1985) respectively. Control diet consisted of the same commercial fish chow treated in the same way but not supplemented with Cu. Cu concentration of the control diet was 11.4 ± 0.2 μg g^{-1} (n = 5).

Feeding, pre-sampling procedure, and sampling regime

Fish were fed twice daily in the morning (08:00-09:00 h) and evening (18:00-19:00 h) at 2% wet body weight, totaling 4% day^{-1}, and water temperature was maintained at 14 ± 1 °C throughout the experimental period. Visual examination during feeding revealed that all fish readily ingested the diets; there was no avoidance of the high Cu diet. Fecal material was siphoned within 1 h of feeding and the flow-through system continuously flushed away any fecal matter deposited between the feeding times. Copper concentrations in the exposure tanks before and after feeding ranged from 2.89 μg l^{-1} to 3.21 μg l^{-1} in all groups; there were no differences before and after feeding. Sampling was done at the start of the exposure (day 0) and at days 14 and 28. Prior to sampling all the fish were anesthetized with 0.1% MS-222, identified, and their weights and lengths taken individually. After recovery from the anesthesia the fish were returned into their respective experimental tanks and starved for 24 h to clear the gut of ingesta. During the starvation period, fecal material was siphoned from the tanks twice

every 12 h to minimize any chance of coprophagy.

Waterborne ^{64}Cu exposure

Measurement of waterborne Cu uptake, by means of ^{64}Cu flux determination, preceded terminal sampling at all sampling times. The radioisotope ^{64}Cu, (as $CuNO_3$; $t_{1/2}$, 12. 65 h) was prepared at the McMaster Nuclear Reactor, Hamilton, Ontario. Fish (n = 10) from the control group and the two dietary Cu exposed groups were moved into separate tanks containing 20 l of static aerated dechlorinated Hamilton city tap-water and allowed to settle for 1 h. Subsequently 0.7 μCi l^{-1} of ^{64}Cu (specific activity 0.35 μCi g^{-1}) (1 μCi = 37 kBq) was introduced into each tank and the exposure carried out for 12 h. A time course study demonstrated that 12 h of exposure was adequate to allow measurable uptake of the Cu into the target organs and whole body. Furthermore, whole-body uptake was linear over the 12 h period, demonstrating that a 1-h settling period prior to exposure was adequate, and that there was no deterioration of conditions that affected Cu uptake over the 12-h. The radioisotope dosage administered added a total concentration of 0.2 μg l^{-1} Cu in the water, and was chosen to ensure that the total water Cu was not substantially elevated above the ambient level of 3 μg l^{-1}.

During the 12-h static ^{64}Cu flux, excretory Cu significantly elevated the water Cu levels above the ambient concentration. To overcome this problem, a separate ^{64}Cu flux experiment was performed on fish that had been exposed to either control or high Cu diet for 14 days. In this experiment, 50 individually marked fish (25 control and 25 high dietary Cu-exposed) were exposed to ^{64}Cu for 12 h in the same flux chambers so that

total Cu concentrations in the water would be identical for the two groups. Five different water Cu concentrations (1.3, 2.5, 5.0, 6.2, and 12.0 $\mu g\ l^{-1}$) were tested with five fish from each treatment in each flux chamber.

When it became evident that waterborne Cu uptake was strongly influenced by body size, an additional experiment was performed with 144 juvenile rainbow trout ranging in body mass from 0.5 to 77 g. ^{64}Cu flux was measured under control conditions (control diet, water Cu approximately 3 $\mu g\ l^{-1}$) using methods identical to those outlined above.

Sampling

Fifteen minutes after introduction of ^{64}Cu to the flux tanks, a 10-ml water sample was taken from each tank. After 12 h of exposure to ^{64}Cu, a second 10-ml water sample from each tank was taken. Subsequently the fish were anesthetized with 0.1% MS-222 and a blood sample obtained by caudal puncture into heparinised 1-ml syringes fitted with 23 gauge needles. An aliquot of 50 µl of each blood sample was immediately centrifuged for 4 minutes at 13 000 x *g* to separate plasma. The fish were then killed by a blow to the head and the entire gill baskets, liver, gut, kidney, muscle and the rest of the carcass dissected out and collected into separate pre-weighed scintillation vials or Eppendorf tubes. Before dissecting out the liver, bile was collected by aspiration from the gall bladder into a 1-ml syringe fitted with a 23-gauge needle. In addition, the carcass and the gills were rinsed in nanopure water to remove surface bound radioactivity.

Analysis

^{64}Cu activity in the tissue and water samples was measured on a Canberra-Packard Minaxi Gamma counter with an on-board program for correcting for decay. Subsequently the tissues were digested overnight at 70 °C with 6 volumes of 1 N nitric acid (Fisher Scientific, trace metal grade), and then centrifuged for 4 minutes at 13 000 x *g*. A sub-sample of the supernatant was diluted suitably with 0.5% nitric acid. Total tissue Cu concentration was determined by atomic absorption spectrophotometry (AAS; Varian AA-1275 with GTA furnace atomizer, using 10-μl injection volume and the operating conditions specified for Cu by the manufacturer. Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range. Under the conditions of the analyses (taking dilution factors into account) the detection limits for water and tissue were 0.3 $\mu g\ l^{-1}$ and 0.6 $\mu g\ g^{-1}$ respectively, far below experimental values measured in this study.

Calculations

Whole-body total Cu concentration was calculated by dividing the sum of Cu contents (concentration multiplied by weight or volume) of all the tissues plus the carcass by the sum of weights of all the tissues plus carcass. Fish Cu content was calculated by multiplying whole body (fish) Cu concentration by the fish weight.

The ^{64}Cu uptake of the tissues was calculated on individual fish basis using the equation:

(1) $$a(bc^{-1})^{-1}$$

where *a* is the ^{64}Cu cpm g^{-1} of tissue, *b* is the ^{64}Cu cpm l^{-1} of water and *c* is the total Cu concentration in water in $\mu g\ l^{-1}$. Proportional distribution was calculated from the product of eq. 1 for each tissue and that tissue weight divided by the sum of these values for all tissues plus carcass.

"Previous compartment analysis", as outlined by Grosell *et al.* (1997) was used to trace the movement of Cu between different body compartments. Newly accumulated copper in each tissue or organ was calculated on an individual fish basis using the equation:

(2) $$a(de^{-1})^{-1}$$

where *a* is ^{64}Cu cpm g^{-1} of tissue, *d* is ^{64}Cu counts in the previous compartment (cpm l^{-1}) and *e* is the total Cu concentration in the previous compartment ($\mu g\ g^{-1}$ or $\mu g\ l^{-1}$, respectively). Previous compartments, based on the criteria of Grosell *et al.* (1997) were: water for gill; gill for plasma; plasma for liver, gut tissue, kidney, muscle and carcass; and liver for bile.

Specific growth rate (SGR) was calculated for each 2-week period (0-14 days and 15-28 days) using the formula:

(3) $$SGR = 100\ (\ln(wt_2) - \ln(wt_1)) \cdot t^{-1}$$

where wt_1 and wt_2 are individual fish weights at the start and end of the each growth period, respectively, and t is the time interval in days. Fish were identified by their distinctive marks and weighed individually.

Condition factor (k) for individual fish was calculated using the formula:

(4) $$k = 100(\text{weight x length}^{-3})$$

Food conversion efficiency (FCE) on a per tank basis was calculated as:

(5) $$\text{FCE (\%)} = 100(\text{weight gain x food eaten}^{-1})$$

Transmission electron microscopy (TEM)

At each sampling time (0, 14, and 28 days), three additional fish per treatment (27 in total) were killed with an overdose of MS-222 and the gastrointestinal tract exposed. Control and experimental fish were processed simultaneously. The mid-intestine was identified as the section of the intestine between the last pyloric cecum and the start of the distal intestine (darker region with larger diameter and annular rings), dissected out, immediately flushed with Cortland saline (Wolf, 1963) and fixed by immersion for 2 h in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, osmolarity, 230 mosmol. Subsequently it was cut into 4 equal regions, which were further subdivided into 4 medium sized blocks. Each block was then diced into small

pieces, approximately 1 mm^3 in size. These pieces were then fixed further overnight in greater than 10 times volume of the same solution. Ten such pieces per fish were selected randomly and post-fixed in 1% buffered osmium tetraoxide for 4 hours at 4 °C. The tissues were subsequently dehydrated in a series of ethanol from 50% through 100% and embedded in Spurr's resin. Five-micron thick semi-thin monitor sections were obtained and stained with toluidine blue for light microscopic observation. Thin sections (80 nm thick) were then obtained and mounted on copper grids, stained with uranyl acetate and lead citrate and viewed under a JEM-1200EXII transmission electron microscope at 80 kV.

Statistical analysis

All data except the qualitative microscopic observations and FCE are presented as means ± SEM. Effects of different oral exposure of Cu on growth, tissue Cu concentration and subsequent waterborne copper uptake at each sampling point were assessed using a two-way analysis of variance (ANOVA) with time and diet Cu concentration as variables. Percentage data were subjected to arcsine transformation prior to statistical testing. Significance was set at $p < 0.05$. Student-Newman-Keuls pair-wise multiple comparison procedure was used to make comparisons between measurements.

RESULTS

Growth and mortality

All the fish maintained high rates of growth during the exposure. Mean weights rose from about 37 g at the start of the exposure to about 70 g whilst length increased from 15 cm to 18 cm (Table 1). Under our conditions of exposure, dietary copper concentration as high as 1042 $\mu g\ g^{-1}$ had no significant effect on specific growth rate, condition factor, and food conversion efficiency. However, non-significant growth stimulation and inhibition were observed with medium- and high-Cu diets respectively. A similar trend was evident for condition factor and food conversion efficiency (Table 1). No mortality occurred during the exposure.

Copper bioaccumulation and partitioning

When expressed on an individual fish basis, Cu content increased continuously with normal growth throughout the experiment in the control fish as well as in the Cu-exposed fish (Fig. 1). However, by day 28 total Cu content per fish was about twofold higher in the medium diet group and fourfold higher in the high diet group relative to the simultaneous controls. Since growth rates were unaffected by these treatments, whole-body Cu concentrations exhibited similar trends (Table 2). However, Cu concentrations in the gut tissue rose to a much greater extent, from about 1 $\mu g\ g^{-1}$ in controls to approximately 5 $\mu g\ g^{-1}$ for the medium-Cu diet and 25 $\mu g\ g^{-1}$ for the high-Cu diet on days 14 and 28. Uptake and transport of Cu to internal organs occurred with

significant accumulation in the liver, bile, kidney, gills, muscle, and carcass. Only in the gut tissue and bile did Cu accumulation appear to reach steady state. Despite this pattern of widespread accumulation, plasma Cu remained constant between 0.5 and 0.7 $\mu g\ ml^{-1}$ throughout the exposure.

The proportional distribution of total Cu in the organs and tissues sampled is shown in Table 3 based on measurements of the absolute amounts of Cu in the different organs and in the whole animal. The key organs and tissues for Cu accumulation were the liver, gut and carcass (which includes muscle). In control animals the liver and carcass together contained 75-85% of the Cu while all the other organs together contained 15-25% of the Cu. Following dietary Cu exposure, the contribution of the carcass decreased and Cu was distributed mainly in the liver and gut tissue. The contribution of these two tissues to total body Cu was 65-75% in fish exposed to the medium diet and 85% in fish exposed to the high copper diet. Gills contributed less than 3.5% of the total Cu in control fish, and fish exposed to dietary Cu had a significantly lower proportion of the Cu in gills. The kidney accounted for less than 2% of the total Cu in all the groups.

Waterborne ^{64}Cu uptake

Exposure to waterborne ^{64}Cu for 12 h resulted in detectable levels of radioactivity in all the body tissues sampled (Fig. 2A). In the early stages of the exposure, gills showed high levels of radioactivity but with time greater radioactivity was registered in internal organs especially the liver. By the end of the 12 h the ^{64}Cu

concentrations were highest in the liver followed in decreasing order by the gill, gut and carcass. Uptake into whole-body (sum of activities in all the tissues over total weight of all the tissues) increased linearly with time over the 12-h period (Fig. 2B).

Two complications arose in the measurement of ^{64}Cu uptake from the water. Firstly, weight specific uptake clearly declined with body mass. Secondly, levels of Cu in the water varied among different trials due to differential Cu excretion by the fish as outlined below. Therefore two additional experiments were performed. In the first, analysis of Cu uptake measurements taken from 144 unexposed fish of different sizes ranging from 0.5 g to 77 g showed a clear non-linear dependence of mass-specific uptake (y, ng g^{-1}) on body mass (x, g) that was best described by a negative exponential relationship (Fig. 3A): $y = 0.179\exp(-0.0743x)$, $r^2 = 0.45$, $p < 0.0001$. This relationship was used to correct the data for all fish in the second experiment to a common weight of 10 g.

In this experiment control and high dietary Cu exposed fish were exposed to ^{64}Cu in the same flux chambers at a range of identical water Cu concentrations. The results (Fig. 3B) demonstrate that dietary Cu pre-exposure significantly reduced subsequent branchial ^{64}Cu uptake. In addition, Cu uptake increased linearly with water Cu concentration over the range of water Cu tested in both control and experimental groups.

During the initial 12-h flux experiments where the control and Cu-treated fish were kept in separate chambers, net excretion of Cu from the medium and high dietary Cu-exposed groups significantly elevated the water Cu concentration above the ambient

level of 3 μg l^{-1}. Furthermore, in different trials the extents of these elevations were different. Thus, absolute rates of Cu uptake from the water could not be validly compared between treatments, and the ^{64}Cu data could only be used to compare relative internal distribution of ^{64}Cu with that of total Cu. Treatment-related differences for ^{64}Cu distribution at day 28 were relatively minor, but there were major differences in comparison to total Cu distribution (Fig. 4). In particular, the gills contained approximately 20% of ^{64}Cu but only 2% of the total Cu. Furthermore the carcass contained about 60% of the ^{64}Cu but only 20% of the total Cu. The other major differences were in gut and liver which each contained about 5-10% of the ^{64}Cu whereas about 50% of total Cu was in the liver and 25% in the gut. Similar patterns were seen at day 14 (data not shown).

Newly accumulated Cu

Newly accumulated Cu in each of the tissues sampled calculated using the previous compartment analysis of Grosell *et al.* (1997) is shown in Table 4. In both the medium and high dietary Cu exposed groups, all the tissues analyzed exhibited newly accumulated Cu concentrations which were similar to the respective controls, except for the liver of the high dietary Cu group at day 28, and the gut of the medium diet at day 14. In all cases, the level of newly accumulated Cu was 1-2 orders of magnitude lower than the corresponding total Cu concentration (note different units in Tables 2 and 4) except for bile and plasma. For bile and the plasma, the calculated values of newly accumulated Cu were in excess of the corresponding total Cu concentrations, indicating

the dynamic turnover in these two compartments.

Mid-intestinal morphology

Representative electron micrographs of mid-intestine from control (n = 15) and Cu exposed (n = 12) rainbow trout are shown in Fig. 3. Normal mid-intestinal epithelium comprised absorptive cells, the enterocytes, and mucus cells (Fig. 4A and B). Enterocytes possessed numerous closely packed microvilli (brush border) and were joined by typical epithelial cell junctional complexes whilst the mucus cells were densely packed with secretory granules of variable size and electron density.

No differences were noted in mid-intestinal structure between the medium and high dietary Cu exposed groups or between day 14 and day 28. The most prominent effect of high dietary Cu was the appearance of numerous mitochondria in the apical region of the enterocytes (Fig. 5C). A number of cells showed characteristics of apoptosis (Fig. 5D). Apoptotic cells were shrunken and possessed many electron dense bodies and very few microvilli and mitochondria. The plasma membrane appeared intact and showed no signs of rupture as would occur in necrotic cells. In addition, the mid-intestine of fish exposed to elevated dietary Cu showed dramatic organellar changes marked by formation of numerous lamellated bodies (Fig. 6A and B). The lamellated bodies were composed of a whorl of circular membranous lamellae surrounding electron dense granules and occurred in the cytoplasm, or within mitochondria and lysosomes. As well, cell renewal was evidenced by observation of mitosis and immature cells (Fig. 6C). Immature mucus cells contained extensive rough endoplasmic reticulum, well-

developed Golgi apparatus, and secretory granules at various stages of formation (Fig. 6C). In the lamina propria and the stratum compactum there was an apparent increase in the number of eosinophilic granule cells (EGCs) (Fig. 6D). The eosinophilic granule cells contained electron dense granules of different sizes and occasional myelin bodies.

DISCUSSION

Growth

Within the range of concentrations tested here, inhibitory growth effects of dietary Cu have been reported in trout, *Salmo gairdneri* (Lanno *et al.,* 1985) and channel catfish (Murai *et al.,* 1981) while lack of effect has been reported in channel catfish (Gatlin and Wilson, 1986), rainbow trout (Mount *et al.,* 1994; Handy *et al.,* 1999), and Atlantic salmon (Berntssen *et al.,* 1999). Interestingly Mount *et al.* (1994) reported mortalities in the absence of growth effect. In the present study, no significant growth effects or mortalities were manifest even in fish on a dietary Cu exposure as high as 1042 $\mu g\ g^{-1}$. According to Lanno *et al.* (1985), dietary Cu concentration of 664-730 $\mu g\ g^{-1}$ and above would cause poor food conversion and retard growth in rainbow trout. Although the discrepancy amongst these data sets could be due to the different exposure periods, it is also possible that dietary Cu levels necessary to cause toxicity are much higher than previously thought. Furthermore, the amount of Cu absorbed, and hence effects, may depend on the bioavailabilty of the Cu in different food formulations.

Bioaccumulation and partitioning of Cu

Gut tissue Cu accumulation exhibited saturation with time, and dose-dependence. Seemingly, elevated dietary Cu level within the non-toxic range was well regulated. On a local basis, small but significant Cu accumulation occurred in gut tissue during exposure to the medium-Cu diet, and internally, increases in most tissue compartments were modest. Therefore, 300 $\mu g\ g^{-1}$ Cu in the diet appears to be well tolerated and may well be in the nutritional range since it stimulated growth slightly and did not cause severe accumulation of Cu. In contrast, the high-Cu diet, representing a threefold increase in dietary Cu concentration relative to the medium diet, resulted in a dramatic twenty-fold increase (relative to day 0) in gut tissue Cu concentration, a marked increase in Cu concentration in most internal compartments (especially liver) and a non-significant tendency for reduced growth, suggesting that it was beyond the nutritional range.

The general trend at both levels of exposure was that during the early stage of exposure (14 days), a greater proportion of the Cu was retained in the gut tissue than at the later stage (day 28). This is probably due to the mobilization of the Cu from the primary site of uptake to other tissues, especially the liver. Furthermore, dietary Cu exposure resulted in continuous Cu accumulation in whole body in contrast with waterborne Cu exposure where stable whole body Cu was reported in rainbow trout (Marr *et al.,* 1996; Taylor *et al.,* 2000) following 1-2 months of waterborne Cu exposure. This probably indicates that dietary Cu uptake is less tightly controlled than

waterborne Cu uptake.

Liver Cu accumulation was linear over time and dose-dependent and was higher than all the other tissues in agreement with previous studies (Julshamn *et al.,* 1988; Handy, 1993). The response of the liver to the 300 $\mu g\ g^{-1}$ diet is important with regard to the threshold of dietary Cu to cause significant accumulation in the liver. Handy (1992) reported that 200 $\mu g\ g^{-1}$ dietary Cu did not cause significant accumulation in trout liver. Results from this study show that 300 $\mu g\ g^{-1}$ Cu caused significant accumulation in the liver early in the exposure but not later in the exposure, indicating that regulatory mechanisms had checked further accumulation. It can therefore be deduced that the maximum dietary level of Cu to cause sustained significant accumulation in juvenile rainbow trout liver at 4% body weight ration is greater 300 $\mu g\ g^{-1}$.

Copper concentration in the gills was significantly elevated at both dietary levels in agreement with previous reports on exposure to sublethal dietary Cu for 32 days (Handy, 1992) and 42 days (Miller *et al.,* 1993). Accumulation of dietary Cu in the gill underscores the unique anatomical location of fish gills and their potential importance in Cu metabolism and toxicity. The gill Cu levels obtained in this study are comparable to those associated with death from waterborne Cu exposure (MacRae *et al.,* 1999). Although these authors reported that 1.4 μg Cu g^{-1} gill tissue accumulated in 12 h caused 50% mortality in rainbow trout within 5 days, 1.37 μg Cu g^{-1} gill tissue accumulated in 28 days of exposure to the high-Cu diet resulted in no mortality. It is possible that the majority of the Cu binding to gills during waterborne Cu exposure is the toxic Cu^{2+}

species while that accumulated in gills after dietary exposure is protein-bound (after passage through the liver and basolateral membranes of the gill cells) and probably non-toxic.

Muscle Cu concentration was not affected by dietary exposure except on day 28 when fish exposed to the high dietary Cu showed a significant accumulation. Thus muscle Cu concentration appears to be homeostatically regulated. Total plasma Cu levels also remained remarkably constant throughout the exposure in agreement with previous studies (Miller *et al.,* 1993; Berntssen *et al.,* 1999). In one study where elevated plasma Cu was reported (Handy, 1992), sampling was not preceded by a substantial period of starvation.

One of the regulatory responses associated with acclimation to waterborne Cu exposure in rainbow trout is increased hepatobiliary Cu excretion (Grosell *et al.*, 1997, 1998). In the present study, increased biliary Cu concentration was observed at day 14, which remained elevated to the same level at day 28, indicating that a balance between Cu uptake into the liver and excretion into bile had been established. It would appear that biliary excretion of Cu, perhaps acting in concert with other regulatory mechanisms not assessed in this study, prevents serious changes in Cu distribution at dietary Cu concentration of 300 $\mu g\ g^{-1}$. However, the regulatory capacity of this system is overcome at the high exposure dose (1000 $\mu g\ g^{-1}$), resulting in Cu accumulation in the liver and other tissues.

Waterborne Cu uptake

Branchial uptake of waterborne Cu increased with fish mass but when plotted on a per unit body mass basis, a negative exponential relationship was evident. When the allometric equation of Hughes (1984) was used to calculate gill surface area of the fish, it was clear that a difference in gill surface area explained only a small portion of the relationship. While Cu uptake per unit body weight changed approximately eightfold over the weight range, gill surface area changed by less than 1.5-fold. Thus other factors are probably also involved. For example, variations in Cu transporter activities and metabolic rates among fish of different size could affect Cu uptake independent of gill surface area.

Absolute rates of Cu uptake are probably affected by the speciation and therefore the bioavailability of Cu in the water. In the moderately hard, moderately alkaline water of our experiments, Cu largely exists as $Cu(OH)_x$ and $CuCO_3$ which are less bioavailable than Cu^{2+}. For example, Taylor *et al.* (2000) reported that Cu was more bioavailable to rainbow trout in moderately acidic soft water than in the present water quality.

In a previous study on the interaction between dietary and waterborne Cu, Miller *et al.* (1993) reported that the uptake of dietary and waterborne Cu were independent. Our observations using a sensitive radioisotope methodology demonstrate that pre-exposure of Cu through the diet decreased uptake of waterborne Cu. Thus, we provide the first evidence of homeostatic interaction between the two routes of uptake. Elevated body (including gill) Cu burden negatively influences Cu uptake, perhaps by

occupying potential new Cu binding sites or causing down-regulation of Cu transport proteins, to check further increase in tissue Cu concentrations. The presence of a Cu-ATPase in trout gill has recently been suggested (Campbell *et al.,* 1999) based on inhibition of Cu uptake by serosal vanadate in perfused rainbow trout head preparations. Although the mechanisms of Cu uptake across the gills are yet to be clearly explained, previous Cu exposure appears to play some role in the regulatory mechanisms.

The newly accumulated Cu data indicate that the short-term exchangeable pool of Cu within the fish is very small relative to the total body Cu. In all the tissues sampled, except for the bile and plasma, the newly accumulated Cu was one to two orders of magnitude lower than the total Cu. However, in bile and plasma, the calculated values were higher than the total Cu. The apparent overestimation of newly accumulated Cu in the plasma and bile can be explained by high total Cu levels coupled with low specific activities in the previous compartments (gill and liver for plasma and bile, respectively), and lack of equilibrium between ^{64}Cu and total Cu pools. Such a scenario means that the ^{64}Cu is more available than total Cu for exchange in the plasma and in the bile. In this study, although ^{64}Cu activity was reduced in fish pre-exposed to dietary Cu, newly accumulated Cu in the tissues sampled (taking into account the dilution of ^{64}Cu within the branchial, plasma and hepatic compartments) was not different among the treatment groups.

However, the distribution pattern of ^{64}Cu among various tissues differed greatly from that of total Cu (see Fig. 4). While the higher gill and lower gut incorporations were not surprising considering that ^{64}Cu uptake was measured from waterborne

exposure, the very high carcass incorporation was remarkable. The carcass is mainly composed of white muscle and Grosell *et al.* (2001) have recently shown that white muscle serves as an important short-term buffer compartment when trout are infused with radio-labeled Cu.

Mid-intestinal morphology

The morphology of the mid-intestine in control fish was similar to that described previously for normal trout (Ezeasor and Stokoe, 1980; Nonnotte *et al.,* 1986). The impact of dietary Cu on mid-intestinal structure could be categorized broadly into cellular and subcellular responses. The cellular response was characterized by an increase in apoptosis and cell division in the intestinal epithelium, similar to that reported in previous studies on lead and cadmium (Crespo *et al.*, 1986) as well as Cu (Berntssen *et al.,* 1999). These changes are therefore not specific to Cu. Assuming that the dying epithelial cells have the same or higher Cu content than the normal ones, evacuation of these dead cells in feces or intestinal mucus would provide a significant route for excretion of dietary Cu. In addition, many of the immature cells were mucus cells suggesting an increased need for mucus and its attendant protective role. Although metal binding properties of gastrointestinal mucus have not been assessed in fish, mammalian studies have shown that intestinal mucus strongly binds divalent ions (Colman and Young, 1979).

The appearance of numerous lamellated bodies and multivesicular bodies in intestinal epithelium has not been previously reported for dietary Cu exposure in fish. In

mammalian studies, lamellated bodies are associated with regressive changes affecting cell organelles, e.g., in lysosomes during treatment with drugs and in mitochondria during exposure to toxic agents such as chloroform (Lüllmann-Rauch, 1979; Guastadisegni *et al.*, 1999). It is conceivable that abnormally high intracellular Cu causes damage and re-arrangement of organelle membranes. The lysosomes observed in the enterocytes would possibly play a role in intestinal Cu sequestration and detoxification similar to their role in the liver during exposure to high Cu levels (Lanno *et al.*, 1987; Weiss *et al.*, 1986). The apparent increase in the number of mitochondria in mid-intestinal enterocytes suggests increased energy demand. It is possible that the uptake and (or) local regulation of Cu occur by energy requiring processes and mitochondria supply the necessary energy to drive these processes. Conversely, Cu may impair energy-requiring processes in the enterocytes and the animal responds by increasing the number of mitochondria as a compensatory measure. Finally, dietary Cu stimulated an increase in eosinophilic granule cell (EGC) infiltration in the lamina propria and stratum compactum. EGCs have been hypothesized to constitute a composite defense mechanism, both humoral and mechanical, which develops in response to environmental demand (Ezeasor and Stokoe, 1980).

The structural changes observed in the mid-intestine might be expected to have a metabolic cost, though only a marginal decrease in growth was evident at the high exposure dosage. More sensitive parameters, e.g., oxygen consumption or protein synthesis rates, might be more appropriate to assess the cost of Cu exposure. Nonetheless, it is noteworthy that dietary Cu only modestly affected gut morphology.

The bioaccumulation data indicate that, unlike the other tissues, gut tissue Cu was regulated and concentration stabilized by day 14. The absence of growth effect suggests that nutrient absorption in the gut was not significantly compromised, a persuasive indication that dietary Cu did not severely impact gut structure and function.

In conclusion, rainbow trout regulated dietary Cu at the level of the gut by increasing clearance to other tissues, at the liver by increasing biliary Cu excretion, and at the gill by reducing waterborne uptake in response to dietary exposure. The modest morphological changes in the intestinal tract suggested high cell and organelle turnover and local regulation of Cu. In spite of possible increased energy demand for regulation and tissue repair there was no significant growth inhibitory effect.

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Table 2-1. Means ± SEM (n) starting and final wet weights (g) and lengths (cm), specific growth rate (SGR), condition factor (*k*), and food conversion efficiency (FCE) for control and Cu-exposed rainbow trout. * Significant difference ($p < 0.05$) from respective starting values.

Biological Index		Diet Cu content	
	Control (11 μg g^{-1})	Medium (300 μg g^{-1})	High 1000 μg g^{-1})
Starting weight (g)	36.99 ± 1.93(40)	37.68 ± 2.16(41)	37.04 ± 2.219(40)
Final weight (g)	63.32 ± 5.73(17)*	75.99 ± 4.98(18)*	65.69 ± 5.15(17)*
Starting length (cm)	14.67 ± 0.40(41)	14.94 ± 0.33(41)	14.99 ± 0.33(40)
Final length (cm)	17.57 ± 0.49(17)*	18.72 ± 0.34(18)*	18.53 ± 0.84(17)*
SGR (% day^{-1}): Day 0-14	2.44 ± 0.12(25)	2.69 ± 0.14(27)	2.16 ± 0.14(27)
Day 15-28	2.41 ± 0.12(14)	2.50 ± 0.23(13)	2.40 ±0.16(13)
k Day 0	1.14 ± 0.06(40)	1.11 ± 0.04(41)	1.07 ± 0.03(40)
Day 14	1.10 ± 0.02(30)	1.15 ± 0.02(31)	1.09 ± 0.01(30)
Day 28	1.11 ± 0.01(17)	1.13 ± 0.02(18)	1.06 ± 0.06(17)
FCE (%): Day 0-14	72.8(25)	81.1(27)	64.2(27)
Day 15-28	69.0(14)	69.9(13)	66.7(13)

Table 2-2. Total Cu concentrations (means ± SEM; μg g^{-1} wet weight or μg ml^{-1}, n = 10 per treatment) in tissues and whole-body of control and dietary Cu exposed rainbow trout after 0, 14, and 28 days of exposure. * Significant difference ($p < 0.05$) relative to the respective control.

	Gill	Liver	Gut	Kidney	Muscle	Carcass	Bile	Plasma	Whole body
Day 0									
Control	0.69 ± 0.04	16.85 ± 1.89	1.26 ± 0.13	1.57 ± 0.09	0.36 ± 0.02	0.48 ± 0.05	13.94 ± 1.11	0.70 ± 0.07	0.63 ± 0.06
Day 14									
Control	0.54 ± 0.02	18.57 ± 2.12	2.29 ± 0.23	2.77 ± 0.25	0.31 ± 0.02	0.34 ± 0.04	7.04 ± 0.76	0.56 ± 0.03	0.93 ± 0.06
Medium	0.65 ± 0.06*	29.91 ± 3.71*	3.20 ± 0.62	4.48 ± 1.09	0.27 ± 0.01	0.51 ± 0.07*	16.36 ± 1.7*	0.52 ± 0.03	1.84 ± 0.32*
High	0.94 ± 0.16*	62.67 ± 12.28*	25.96 ± 2.42*	5.31 ± 1.12*	0.37 ± 0.04	0.48 ± 0.03*	28.54 ± 3.01*	0.52 ± 0.02	3.74 ± 0.16*
Day 28									
Control	0.91 ± 0.07	38.40 ± 6.43	2.19 ± 0.73	1.70 ± 0.32	0.26 ± 0.02	0.36 ± 0.04	10.75 ± 0.99	0.50 ± 0.04	1.46 ± 0.24
Medium	1.22 ± 0.04*	45.18 ± 6.87	5.41 ± 1.03*	1.73 ± 0.16	0.32 ± 0.04	0.45 ± 0.03	15.39 ± 1.7*	0.52 ± 0.01	1.72 ± 0.15
High	1.37 ± 0.09*	100.27 ± 22.51*	23.58 ± 2.44*	9.98 ± 3.3*	0.32 ± 0.01*	0.70 ± 0.12*	27.73 ± 2.89*	0.50 ± 0.01	5.24 ± 0.53*

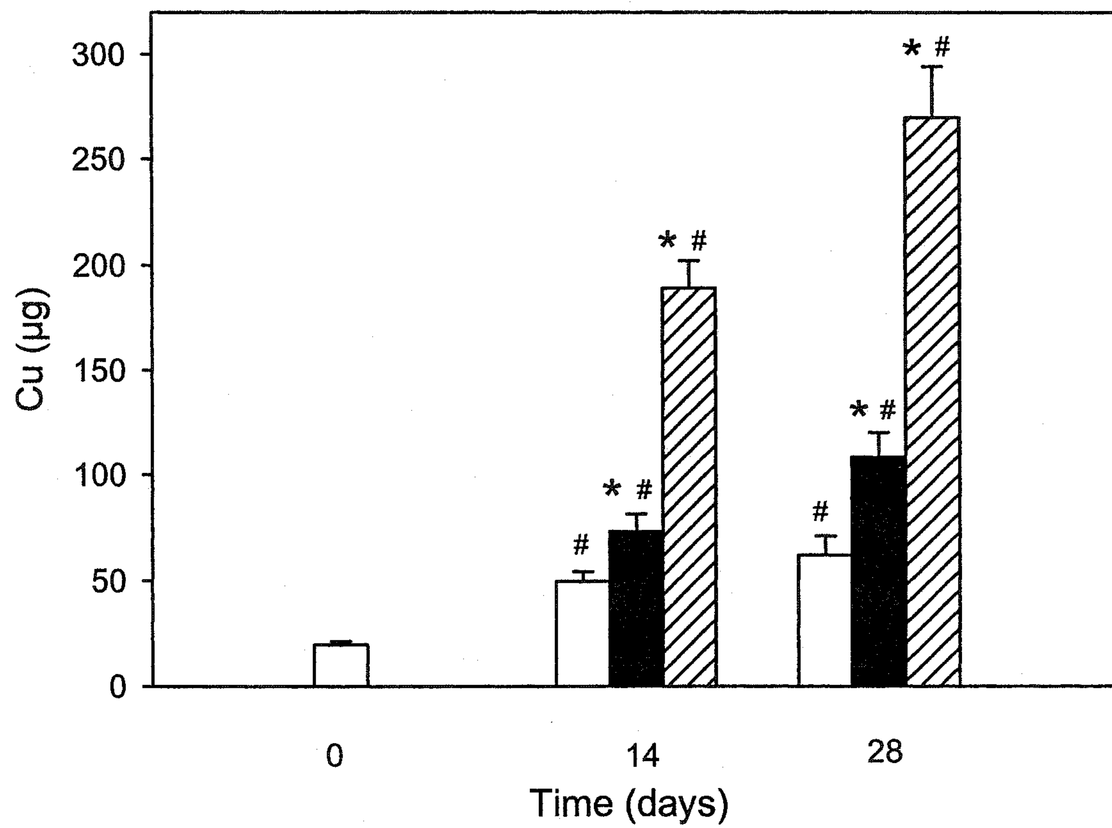
Table 2-3. Proportional distribution (%, means ± SEM) of total body Cu in control, medium and high dietary Cu-exposed rainbow trout. **Note**: Statistical analysis was performed on arcsine transformed proportional data. * Significant difference ($p < 0.05$) from respective control; n = 30 for day 0 control and n = 10 per tissue in all other treatments.

	Gill	Liver	Gut	Kidney	Carcass
Day 0					
Control	3.16 ± 0.24	27.16 ± 2.24	12.28 ± 1.47	1.30 ±0.08	56.10 ± 2.18
Day 14					
Control	1.90 ± 0.13	46.86 ± 2.03	19.14 ± 2.12	1.77 ± 0.19	28.50 ± 1.64
Medium	1.37 ± 0.15*	50.70 ± 5.47	14.10 ± 2.44	1.87 ± 0.54	28.82 ± 4.59
High	0.77 ± 0.13*	37.99 ± 4.48	47.78 ± 4.19*	0.74 ± 0.16*	11.07 ± 0.72*
Day 28					
Control	2.26 ± 0.45	59.35 ± 5.32	13.73 ± 3.36	0.79 ± 0.15	23.87 ± 3.33
Medium	2.03 ± 0.19	49.66 ± 3.56	23.11 ± 3.39	0.64 ± 0.07	24.56 ± 2.16
High	0.83 ± 0.09*	51.00 ± 3.91	35.05 ± 3.89*	1.10 ± 0.28	12.01 ± 1.95*

Table 2-4. Newly accumulated Cu (means ± SEM, ng g^{-1} wet weight or µg ml^{-1} n = 30 for day 0 samples and n = 10 for day 14 and 28 samples. * Significant difference ($p < 0.05$) from respective control.

	Gill (ng g^{-1})	Liver (ng g^{-1})	Gut (ng g^{-1})	Kidney (ng g^{-1})	Muscle (ng g^{-1})	Carcass (ng g^{-1})	Bile (µg ml^{-1})	Plasma (µg ml^{-1})
Day 0								
Control	8.14 ± 0.43	284.33 ± 78.60	89.14 ± 49.79	35.25 ± 13.96	82.11 ± 40.83	15.37 ± 5.06	33.34 ± 9.74	1.48 ± 0.59
Day 14								
Control	2.73 ± 0.27	198.71 ± 46.67	52.18 ± 20.90	177.83 ± 61.68	142.99 ± 59.52	31.00 ± 7.16	56.67 ± 14.15	0.48 ± 0.09
Medium	2.82 ± 0.42	485.20 ± 152.17	403.12 ± 260.54	376.20 ± 103.21	261.57 ± 102.78	57.73 ± 20.99	215.54 ± 141.62	0.61 ± 0.12
High	2.22 ± 0.18	419.72 ± 129.55	190.93 ± 54.51*	233.05 ± 58.52	183.76 ± 62.25	37.81 ± 11.05	139.23 ± 39.47	1.10 ± 0.35
Day 28								
Control	1.86 ± 0.10	61.76 ± 9.05	167.64 ± 24.90	36.85 ± 6.38	53.18 ± 8.67	30.01 ± 5.50	262.82 ± 104.85	1.18 ± 0.29
Medium	1.96 ± 0.30	272.41 ± 134.11	135.28 ± 19.12	38.40 ± 7.71	34.59 ± 7.44	33.66 ± 5.08	52.65 ± 14.20	1.75 ± 0.37
High	2.41 ± 0.27	325.11 ± 71.73*	177.07 ± 31.50	170.74 ± 88.98	32.44 ± 5.55	45.77 ± 6.71	132.06 ± 45.54	1.59 ± 0.26

Figure 2-1. Total Cu content per fish in control and dietary Cu exposed rainbow trout at days 0, 14, and 28. Open bars, control; solid bars, 300 μg Cu g^{-1} diet; hatched bars, 1000 μg Cu g^{-1} diet. Values are means ± SEM, n = 10 per treatment per time. An asterisk indicates significant difference from the corresponding control; a pound sign indicates significant difference from day 0 control.



Cu (µg)
300
250
200
150
100
50
0
*#
#
0
14
28
Time (days)

Figure 2-2. Time course of waterborne ^{64}Cu uptake (means ± SEM) in previously unexposed rainbow trout on the control diet into the (A) gill, liver, gut, and carcass and (B) whole-body (n = 5 per data point). Water Cu concentrations were 2.64 ± 0.21 μg l^{-1}, n = 4 and 2.81 ± 0.14 μg l^{-1}, (n = 4) before and after the flux, respectively.

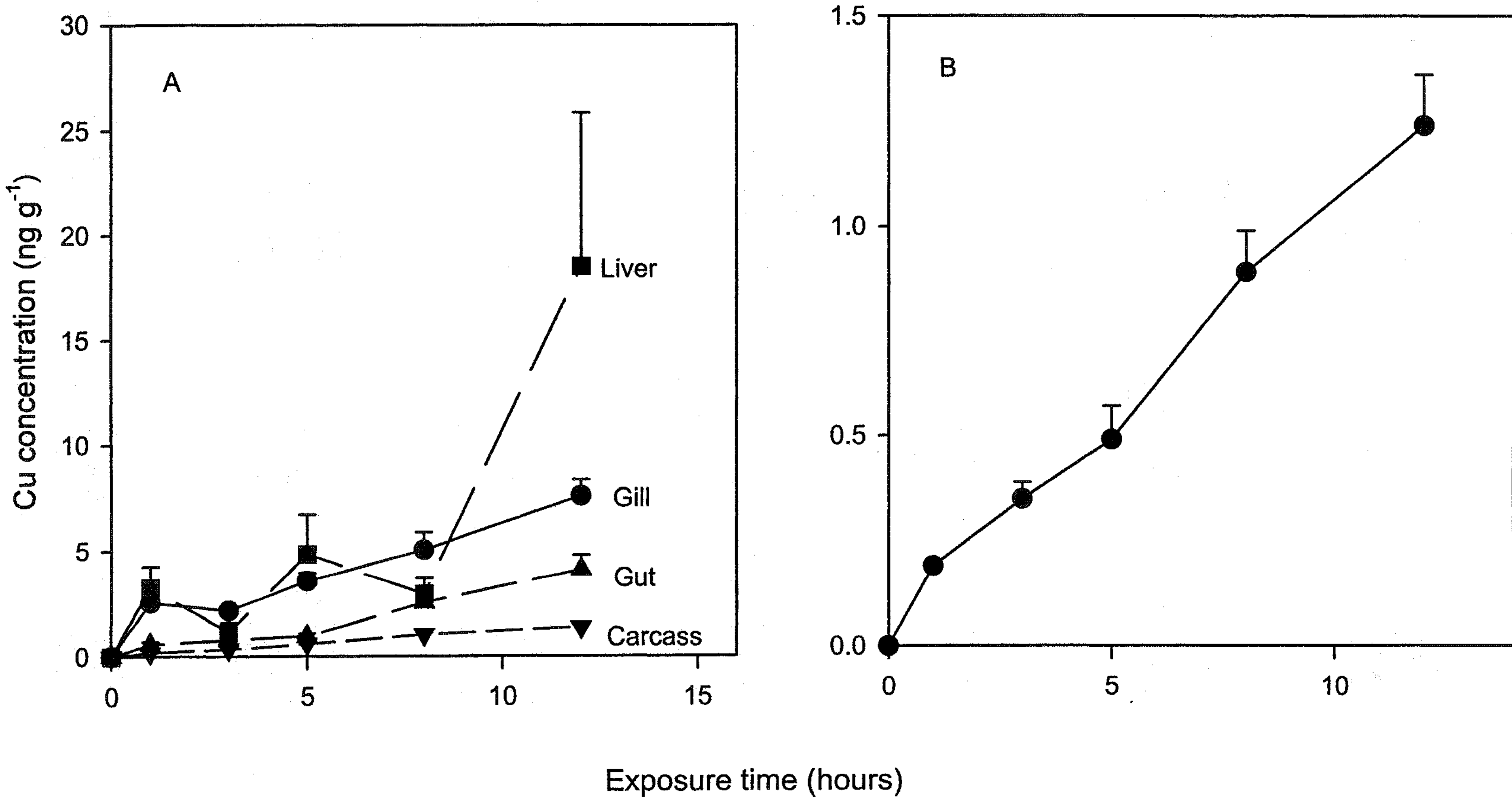

A
B
Liver
Gill
Gut
Carcass
Cu concentration (ng g-1)
Exposure time (hours)
30
25
20
15
10
5
0
1.5
1.0
0.5
0.0

Figure 2-3. (A) Relationship between body mass (x) and waterborne ^{64}Cu uptake (y) across the gills in previously unexposed rainbow trout on control diet (n = 144). The relationship is best described by the negative exponential equation $y = 0.179\exp(-0.0743x)$, $r^2 = 0.45$, $p < 0.0001$. (B): Cu uptake rate measured with ^{64}Cu, at different water Cu concentrations in unexposed fish (open circles) and fish exposed (solid circles) to high Cu diet for 14 days. Values are means ± SEM, n = 5 per group per sampling time. The equation in Fig 3A was used to correct for size differences; rates are expressed for 10-g fish. An asterisk indicates a significant difference.

A Cu uptake and body mass
$y = 0.179\exp(-0.0743x)$
Cu uptake (ng g^{-1} h^{-1})
0.0
0.1
0.2
0.3
0
20
40
60
80
100
Body mass (g)
B New Cu accumulation
Exposed
Unexposed
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14
Water Cu (µg l^{-1})

Figure 2-4. Proportional distribution of (A) total Cu and (B) waterborne ^{64}Cu in carcass (horizontal hatch), kidney (gray), gut (diagonal hatch), liver (solid), and gill (open) of control and dietary Cu-exposed rainbow at day 28 (n = 10 per treatment).

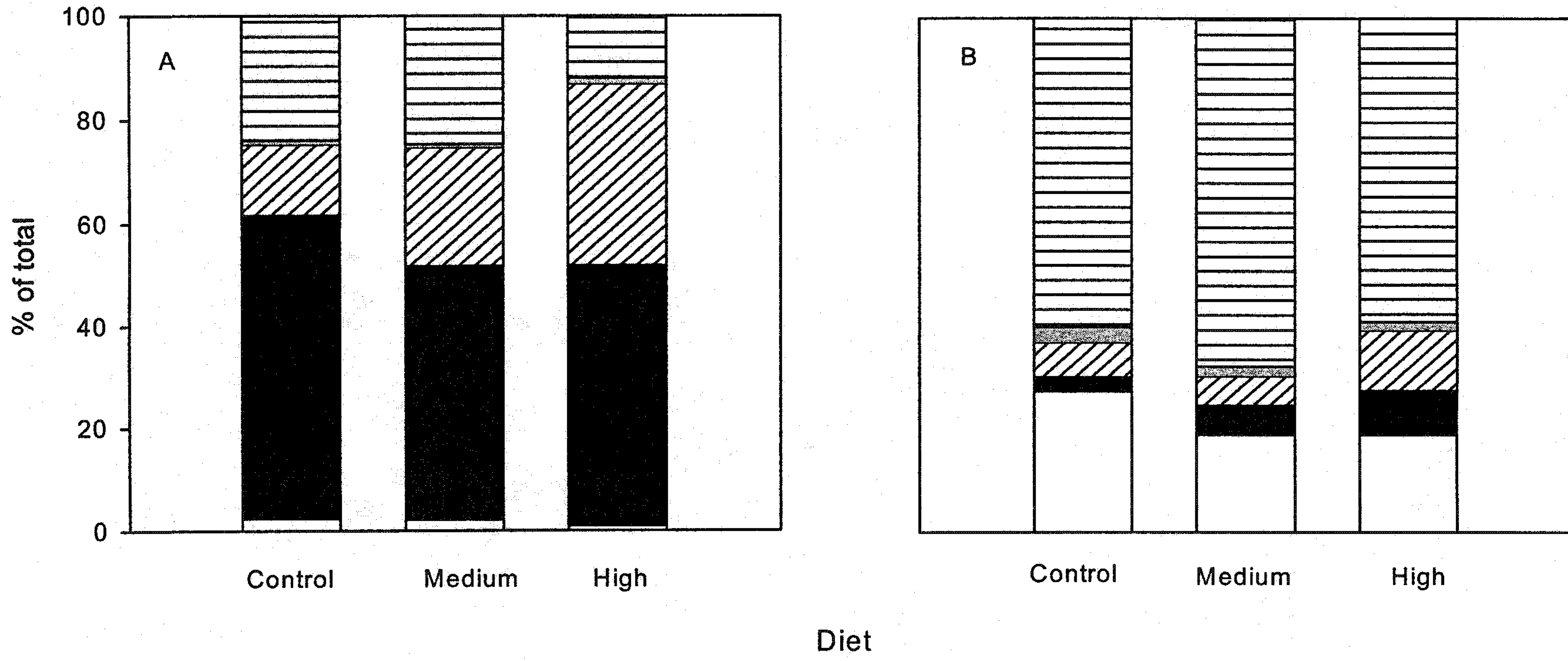
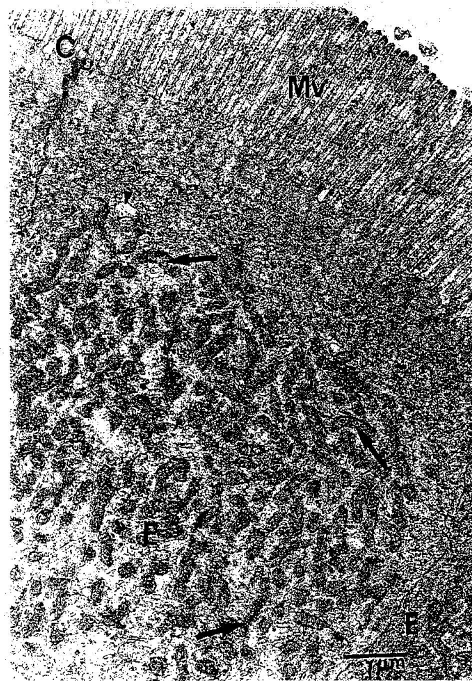

% of total
100
80
60
40
20
0
A
Control
Medium
High
B
Control
Medium
High
Diet

Figure 2-5. Representative electron micrographs of the apical region of mid-intestine in control and dietary Cu-exposed rainbow trout. (A) Control. E, enterocyte; L, lumen; Mv, microvilli; Ld, lipid droplet; m, mitochondrion; small arrow head, tight junction; large arrowhead, desmosome. (B) Control. mc, mucus cell, sg, secretory granule. (C) Cu-exposed. Arrows, mitochondria; arrowhead, multivesicular body. (D) Cu-exposed. AE, apoptotic enterocyte; E, normal enterocytes; arrows, electron dense granules in apoptotic cell; small arrowheads, multivesicular body; large arrowhead, denuded mirovillus.

A
L
Mv
Ld
E

B
E
Ld
mc
sg
1 µm


C
Mv
E

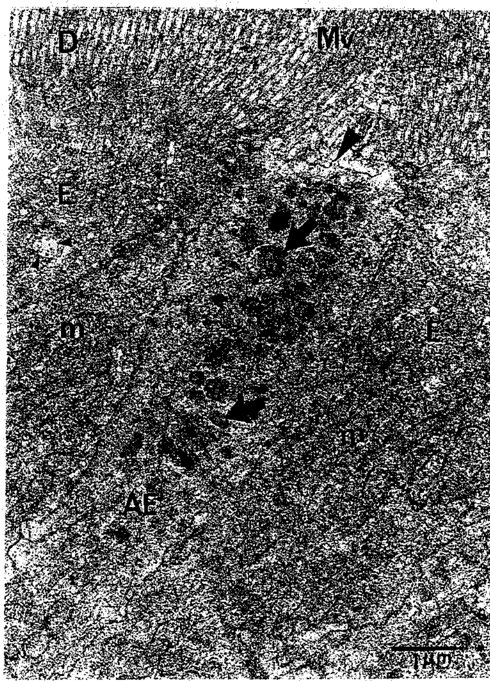
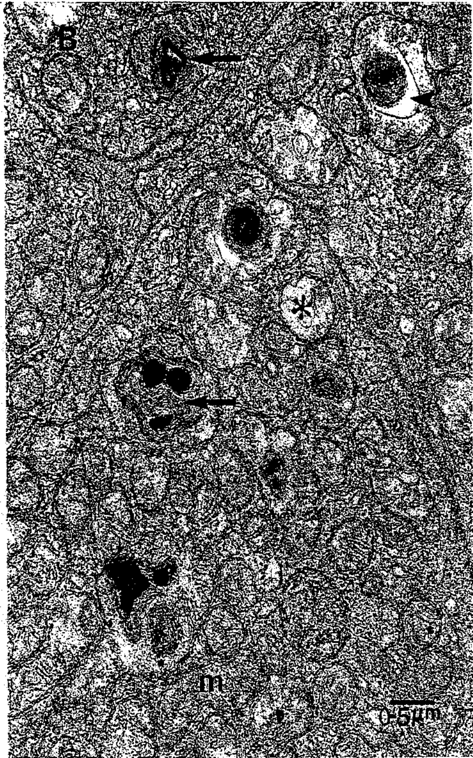
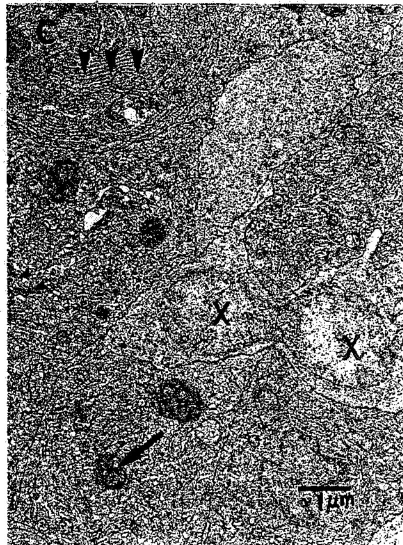
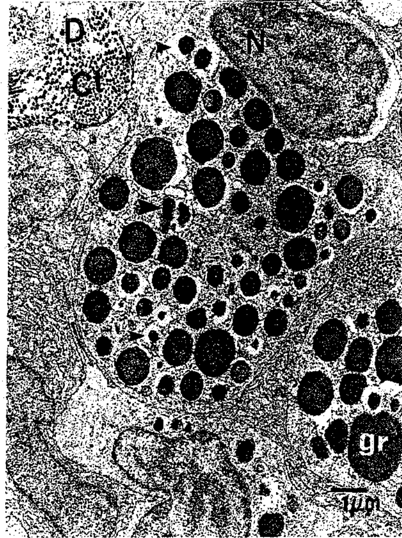

D
Mv
E
m
AE

Figure 2-6. Representative electron micrographs of enterocytes in a part of mid-intestine of rainbow trout exposed to elevated dietary Cu. (A) Changes within enterocytes. Mv, microvilli on the apical side of an enterocyte; mc, mucus cell; large arrowheads, lamellated bodies, small arrowheads, secondary lysosomes. Note numerous mitochondria (double arrowheads). (B) Close-up of inset in Fig. 6A. Arrows, lamellated bodies with electron dense granules; m, mitochondrion; asterisk, vacuole; arrowhead, halo. (C) Mitotic activity. Small arrowheads, Golgi apparatus; asterisk, newly formed secretory granule; large arrowheads, rough endoplasmic reticulum; X, dividing cell nucleus; arrow, secondary lysosome. (D) Eosinophilic granule cell. N, nucleus; gr, granule; large arrowhead, myelin body; small arrowheads, halo; Ct, connective tissue.

A
Mv
mc
2 µm


B
m
0.5µm


C
X
X
1µm


D
N
Cl
gr
1µm

CHAPTER 3

COPPER METABOLISM IN ACTIVELY GROWING RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): INTERACTIONS BETWEEN DIETARY AND WATERBORNE COPPER UPTAKE

ABSTRACT

Juvenile rainbow trout were exposed to diets with low (12.6), normal (50.4), or elevated (4437.5 nmol g^{-1}) copper (Cu) concentrations in combination with either low (5.8) or normal (48.5 nmol l^{-1}) waterborne Cu levels over a 50-day period during which body mass increased up to fivefold. A nutritional requirement for Cu was demonstrated based on growth response and whole-body and tissue Cu status. Simultaneous low Cu in both the water and the diet depressed growth by 31% over 7 weeks. There were reductions in both specific growth rate (SGR, 1.95 *versus* 2.55% day^{-1}) and food conversion efficiency (FCE, 53-59% *versus* 75-80%) over weeks 0-4, but these effects disappeared in weeks 4-7. Elevated concentration of dietary Cu did not affect SGR or FCE. Low levels of dietary and waterborne Cu decreased, and high levels of dietary Cu increased Cu concentrations in whole-body, liver, carcass, gut and gills. Copper levels in the liver strongly reflected the exposure conditions with a corresponding fivefold decrease and a 22-fold increase in Cu concentration. Restricting available Cu caused an

exponential decline in whole-body Cu concentration from .0175 to .0069 μmol g^{-1} and increased the uptake of waterborne Cu (measured with ^{64}Cu) by the gills. Conversely, high levels of dietary Cu caused a linear increase in whole-body Cu concentration to about 0.170 μmol g^{-1} and depressed the uptake of waterborne Cu. Waterborne Cu uptake contributed the majority (60%) of the body's Cu accumulation under Cu-deficient conditions while dietary Cu contributed the majority (99%) at high dietary levels of Cu. True bioavailability of dietary Cu decreased with increasing dietary Cu concentration although the absolute amount retained increased. These findings demonstrate an important interaction between dietary and waterborne Cu uptake in fish and provide compelling evidence of a key role of the gill in Cu homeostasis.

Keywords: Cu homeostasis, Cu deficiency, waterborne Cu uptake, dietary Cu uptake, gills, rainbow trout

INTRODUCTION

Copper is essential for the survival of all organisms, including fish (Ogino and Yang, 1980; Satoh *et al.,* 1983). It is a cofactor for several proteins that carry out fundamental functions in growth and development (Linder, 1991; Fairweather-Tait, 1997; Uauy *et al.,* 1998). However, Cu is also a very potent toxicant when allowed to accumulate in excess of cellular needs (Harris, 1991; Pena *et al.*, 1999). Consequently body Cu levels should be subject to tight homeostatic control to guard against deficiency and toxicity. The maintenance of Cu balance involves the strict regulation of uptake, distribution, detoxification, and excretion. Two genetic diseases of Cu metabolism in man, the Menke's and Wilson's diseases (Linder and Hazegh-Azam, 1996), manifest as failure of these processes. Susceptibility to Cu (as well as other trace elements) deficiency or toxicity depends on species, age, and diet, a reflection of variation in efficiency of absorption and excretion (Baker, 1986; Bremner, 1998; Uauy *et al.,* 1998). Young animals are apparently more prone to deficiency or toxicity due to increased demands for growth and the coupling of a high efficiency of absorption with the immaturity of the excretion system respectively.

Despite extensive studies (Harris, 1991; Linder and Hazegh-Azam, 1996; Pena *et al.,* 1999), the exact mechanisms of Cu homeostasis in mammals are still not well understood. Much less is known about Cu metabolism and regulation in fish although in contaminated environments fish may take up Cu through both the gut and the gills (Dallinger *et al.,* 1987). Substantial literature pertaining to Cu uptake via either gills or gut exists (McDonald and Wood, 1993; Handy, 1996) but the interactions between the

two routes of uptake are yet to be clearly determined. One study (Miller *et al.,* 1993) did examine this potential interaction in rainbow trout but started with the assumption that uptake from the water was zero at control (low) waterborne Cu levels of 79–205 nmol l^{-1}, an assumption which is not substantiated by the present study. The assessment of Cu requirement is much more complex in fish relative to mammals due to this potential for extra-intestinal Cu uptake via the gills, and the fact that Cu is ubiquitously present in the aquatic environment as a result of both natural and anthropogenic processes. While acknowledging a possible complication due to branchial Cu uptake, previous studies that have determined Cu requirements (Ogino and Yang, 1980; Satoh *et al.,* 1983; Lorentzen *et al.,* 1998) failed to assess the potential contribution of waterborne Cu.

Although previous studies have independently assessed toxic effects (see McDonald and Wood, 1993; Handy, 1996 for reviews) or nutritional requirements (Ogino and Yang, 1980; Murai *et al.,* 1981; Satoh *et. al.*, 1983; Lorentzen *et al.,* 1998), no study has simultaneously investigated Cu metabolism in states of experimental deficiency and sublethal loading in fish. Particularly, the interaction of dietary and waterborne Cu uptake has yet to be unequivocally demonstrated, a finding which would allow the determination of the relative contributions of waterborne and dietary Cu in nutrition and toxicity.

This study was therefore conducted to investigate Cu metabolism during Cu restriction and elevated dietary Cu exposure in juvenile rainbow trout. Firstly, we set out to establish conditions under which Cu deficiency could be induced in fish and to

determine whether fish could obtain their Cu requirement from water. Secondly, we assessed the effects of Cu restriction and excess levels of dietary Cu exposure on growth and whole-body and tissue Cu reserves. Thirdly, we used direct measurements of ^{64}Cu fluxes to quantify waterborne Cu uptake in fish in which whole-body Cu had been depleted or elevated, and thereby were able to quantitatively partition Cu uptake from diet and from water in order to determine their relative contributions. Finally, we assessed possible interactions between dietary and waterborne Cu uptake.

MATERIALS AND METHODS

Experimental animals and diet

Fingerling rainbow trout were obtained from Humber Spring Trout Hatchery, Mono Mills, Ontario. Prior to beginning the experiment, the fish were acclimated to laboratory conditions by holding them in one large tank supplied with aerated flow-through Hamilton tap water (moderately hard water from Lake Ontario, Na^+ 0.6 mmol l^{-1}, Cl^- 0.7 mmol l^{-1}, Ca^{2+} 1.0 mmol l^{-1}, HCO_3^- 1.9 mmol l^{-1}, pH 7.9-8.2; dissolved organic carbon (DOC) 3 mg l^{-1}, background Cu 4.72 nmol l^{-1} (3 μg l^{-1}) at 14 °C. The fish were maintained on a commercial fish starter-diet at a daily ration of 4% wet body weight ration and attained the targeted starting weight of 0.5 g wet weight within one month. By the end of this period the fish had become used to the laboratory diet and were consuming all of it within 1 hour. The pre-experimental diet was a regular commercial

trout starter diet (Martin Feed Mills) that contained 330 ± 10 nmol g^{-1} Cu (20.95 ± 0.64 μg g^{-1}).

Cu-supplemented and Cu-deficient diets were prepared at the West Vancouver Laboratory, Department of Fisheries and Oceans, West Vancouver, British Columbia. The diet composition (Table 1) was based on known requirements for rainbow trout (NRC, 1993) and the only variable was the Cu content. This diet fulfilled the criteria spelled out for diets intended for nutrient requirement studies (Baker, 1986).

Experimental protocol

The experimental design consisted of 3 dietary Cu levels (low, normal and high) and 2 waterborne Cu levels (low and normal). The exposure system comprised a battery of fifteen 3-l tanks allowing for triplicates of five treatments of combinations of waterborne and dietary Cu concentrations: low waterborne Cu + low dietary Cu, low waterborne Cu + normal dietary Cu, normal waterborne Cu + low dietary Cu, normal waterborne Cu + normal dietary Cu, and normal waterborne Cu + high dietary Cu; measured concentrations are shown in Table 2. The experimental water used for both the low and normal waterborne Cu levels was generated by reconstituting de-ionized water produced by reverse osmosis with $NaHCO_3$ and $CaCl_2$ to bring the levels of these ions to those of Hamilton tap water used during the 1 month acclimation period. The de-ionized water contained 0.4 mg l^{-1} DOC. For low water Cu, the Cu concentration was 5.82 ± 0.47 nmol l^{-1} (0.37 μg l^{-1}), the level remaining after reverse osmosis

treatment. For normal waterborne Cu levels, Cu was added as $CuSO_4 \bullet 5H_20$ to raise the level to 48.47 ± 0.94 nmol l^{-1} (3.1 µg l^{-1}), the ambient Cu level in Hamilton tap water. Replacement Cu and salts were delivered from separate Mariotte bottles into two header tanks that supplied the experimental tanks. The tanks were supplied with flow-through aerated water thermostatically maintained at 14 ± 1 °C throughout the 50-day experimental period. Flow rates to all the experimental tanks were set at 60 ml min^{-1}, which provided a 50% turnover time of 34.7 min in the 3-l tanks. At the beginning of the experiment, fish were randomly separated into groups of 40 in each of the 15 tanks. All the groups were fed the designated diet (low Cu, normal Cu or high Cu) at 4% wet body weight ration delivered in two equal portions twice a day. All food was consumed within 1 h. Faecal material was siphoned after 1 hour of feeding.

Sampling

Sampling was done at the start of the exposure (week 0) and subsequently at weeks 2, 4 and 7 to assess tissue and whole-body Cu status. A sampling time interval of 2-3 weeks was used to provide adequate period for physiological adjustments (e.g., acclimation) to occur within each exposure group before the subsequent sampling. Prior to sampling, all the fish were bulk-weighed on a per tank basis and starved for two days. During the starvation period, faecal material was siphoned from the tanks twice every 12 h to minimize any faecal ingestion. For weeks 0, 2 and 4, five fish per replicate (15 fish per treatment) were randomly netted from the experimental tanks and killed with an

overdose of MS-222. Gills, liver, gut (washed free of its contents) and the rest of carcass were weighed and collected into separate pre-weighed scintillation vials or Eppendorf tubes. Additionally, 2 fish per tank were collected at each sampling time for moisture content analysis, by drying to a constant weight at 70 °C. For week 7, a 12-h measurement of Cu uptake using ^{64}Cu preceded sampling as described below.

Waterborne Cu uptake kinetics

The effect of the exposure conditions on waterborne Cu uptake kinetics by gills was assessed at week 7 (day 50) over a range of waterborne Cu concentrations. Each treatment was divided into five groups (n = 9); each group was then exposed to waterborne ^{64}Cu at a nominal total Cu concentration of either 31, 47, 79, 94 or 126 nmol l^{-1}. The radioisotope ^{64}Cu (as $CuNO_3$) was prepared at the McMaster University Nuclear Reactor. On the day of the experiment, 0.7 μCi l^{-1} of ^{64}Cu (specific activity 0.35 μCi μg^{-1}) was introduced into each experimental tank; the tanks had been pre-dosed with $CuSO_4 \bullet 5H_2O$ to bring the concentration to the nominal level. The radioisotope dosage administered added a total concentration of 3 nmol l^{-1} (0.2 μg l^{-1}) Cu to the water, and therefore did not substantially elevate the water Cu concentration. The fish were then exposed to the ^{64}Cu for 12 h under static water conditions. A 10-ml water sample was taken from each tank 15 minutes after introduction of ^{64}Cu and again after 12 h. Over this period the water ^{64}Cu activity and total Cu concentration changed by no more than 6.5%.

Analysis

Cu concentrations in water, tissue, and food samples were determined by atomic absorption spectroscopy (AAS; Varian AA-1275 with GTA furnace atomizer) using a 10-µl injection volume and the operating conditions specified for Cu by the manufacturer. Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range. Water samples were acidified (0.5% nitric acid), while solid samples were weighed and digested overnight at 70 °C with 6 volumes of 1N nitric acid (Fisher Scientific, trace metal grade), and then centrifuged for 4 min at 13 000 x *g*. A sub-sample of the supernatant was diluted appropriately with 0.5% nitric acid. For day 50, the tissues and water samples were first measured for ^{64}Cu activity on a Canberra-Packard Minaxi Gamma counter with an on-board program for decay correction, and then analyzed as described above for determination of total Cu concentrations.

Calculations

Whole-body total Cu concentration was calculated by dividing the sum of Cu contents (concentration multiplied by weight) of all the tissues plus the carcass by the sum of weights of all the tissues plus carcass.

Whole-body uptake of waterborne ^{64}Cu was calculated by adding up ^{64}Cu activities (cpm) in all tissues plus carcass. Fish weights were determined by summing

up the weights of liver, gills, gut tissue (washed), and carcass for each fish. Whole-body Cu uptake was then calculated using the equation:

$$a(bc^{-1})^{-1} \quad (1)$$

where *a* is the ^{64}Cu cpm g^{-1}fish, *b* is the ^{64}Cu cpm l^{-1} of water and *c* is the total Cu concentration in the water in nmol l^{-1}. The uptake was then divided by the time of exposure (12 h) to convert it into a rate. The resulting values were rather small hence they are reported as pmol g^{-1} h^{-1}.

Specific growth rate (SGR) was calculated on a per tank basis for 3 growth periods of 2 or 3 weeks using the formula:

$$SGR = 100[(\ln(m_2) - \ln(m_1))/(t)] \quad (2)$$

where m_1 = mass at beginning of growth period (g), m_2 = mass at end of growth period (g), t = duration of growth period in weeks.

Food conversion efficiency (FCE) was calculated on a per tank basis for growth periods 0-2, 2-4, and 4-7 weeks:

$$FCE\ (\%) = 100(\text{weight gain per tank/food eaten per tank}) \quad (3)$$

To calculate true bioavailability of dietary Cu, we first estimated Cu uptake from water over 7 weeks by adjusting waterborne Cu uptake rates measured at the end of week 7 for size using mean fish weights determined for weeks 0-2, 2-4, and 4-7 using the Cu uptake rate *versus* body mass relationship determined by Kamunde *et al.* (2001). It was assumed that all the Cu accruing from waterborne uptake was accumulated.

True bioavailability of dietary Cu (%), defined as the % retention of Cu ingested via diet after subtracting the accumulation that occurred by waterborne uptake, was then calculated as:

$$100\ ((totCu_f - totCu_0) - Cu_{water})/Cu_{diet} \qquad (4)$$

where $totCu_f$ and $totCu_0$ are whole-body total Cu at the end and beginning of the experiment respectively, Cu_{water} and Cu_{diet} are the total Cu taken up from the water and the total Cu ingested with the diet over the experimental period. Visual observation during feeding showed that all the food was ingested. Thus, to calculate Cu_{diet}, total amount of food delivered (ration) and the Cu concentration of the food were used.

Relative contributions of dietary and waterborne Cu to the total body metal burden were calculated as:

$$\text{Relative contribution of water (\%)} = 100(Cu_{water}/(Cu_f - Cu_0)) \quad (5)$$

$$\text{Relative contribution of diet (\%)} = 100 - 100(Cu_{water}/(Cu_f - Cu_0)) \quad (6)$$

The assumptions for this calculation were as for the bioavailability calculation (Equation 4).

Somatic indices for liver, gill and gut were calculated as:

$$100(x/\text{wet body wt}) \quad (7)$$

where *x* is wet weight of the organ or tissue of interest.

For gill the entire gill basket was used whereas for the gut, the gut contents and extraneous tissues such as fat were removed.

Statistical analysis

Data are presented as means ± SEM (n). Effects of exposure conditions on growth, tissue Cu concentrations, and subsequent waterborne Cu uptake at each sampling point were assessed using a two-way analysis of variance (ANOVA) with time, diet, and waterborne Cu concentrations as variables. Percentage data were

subjected to arcsine transformation prior to statistical testing. In all the cases, significance was set at $p<0.05$. Student-Newman-Keuls pairwise multiple comparison procedure was used to make comparisons between measurements. One way ANOVA and Bonferroni's test were used to compare changes in FCE and SGR at $p<0.05$ and curve fitting for whole-body Cu concentration patterns over time was done with Statistica 5.1 using individual data points by the Quasi-Newton estimation method.

RESULTS

Growth

Over the 7-week period, fish wet body mass increased by up to fivefold. Mortality was less than 2% and was not related to conditions of exposure. Juvenile rainbow trout exposed to the combination of low levels of dietary and waterborne Cu were retarded in growth relative to all the other groups. Cumulative weight gain was lower at all times from 2 weeks onwards, and reduced by 31% (18g *versus* 26 g) over 7 weeks (Fig. 1A). Specific growth rate (data not shown) was significantly depressed at weeks 0 – 2 and 2 – 4 (approximately 1.95% day^{-1} *versus* 2.55% day^{-1} in both periods) though the effect had disappeared by weeks 4 – 7 (2.7% day^{-1} *versus* 2.8% day^{-1}). There were no significant differences in growth between any of the other treatment groups; fish receiving Cu via either one of the routes alone or in combination maintained normal growth. Growth retardation in the deficient group was associated with significantly

decreased food conversion efficiency (53-59% *versus* 75-80%) during the first 4 weeks (Fig. 1B).

There were no significant differences over time or between groups in whole-body moisture content, which remained between 74% and 76% throughout (data not shown). Hepatosomatic and gastrointestinosomatic indices increased from 1.3% to 2.0% and from about 9% to 11% respectively while the branchiosomatic index decreased from about 4.5% to 3.5% over the 7 weeks (data not shown). There were no treatment-related effects on these indices. Consequently allometric equations for the growth of liver, gill, gut, and carcass were derived from pooled data of these organs (Table 3). Body weight was well correlated with the weight of these organs and between 82% and 99% of the variance in growth of the organs could be explained by the change in body weight.

Whole-body Cu status

Whole-body Cu concentration (initially around 0.0175 μmol per g wet weight) declined slightly to around 0.010 μmol g^{-1} in fish exposed to normal Cu in water or diet either in combination or separately (Fig. 2). However, fish deprived of Cu or exposed to high dietary Cu levels exhibited much lower (0.0069 μmol g^{-1}) and higher (0.170 μmol g^{-1}) whole-body Cu concentrations by week 7.

Fig. 3A analyses the pattern of whole-body Cu concentration (y, μmol g^{-1}) over time (x, weeks) during exposure to the combination of low levels of Cu in water and in

diet, while Fig. 3B analyses the corresponding pattern during exposure to normal Cu levels in water and elevated Cu concentration in diet. In the former, the pattern was best explained by the negative exponential model, $y = 0.4268 + 0.6879\exp(-0.5868x)$, $r^2 = 0.80$, with $t_{1/2} = 1.18$ weeks. In contrast, during exposure to high dietary Cu, the data best fitted the linear model, $y = 0.7697 + 1.3392x$, $r^2 = 0.80$, indicating continuous Cu accumulation above normal body Cu concentration.

Tissue Cu status

In the intestinal tissue, Cu levels rose by week 7 from about 0.03 μmol g^{-1} to 0.3 μmol g^{-1} wet mass, a tenfold increase, in the fish exposed to high dietary Cu concentration and decreased fivefold to 0.007 μmol g^{-1} in the animals exposed to low Cu levels in both diet and water (Fig. 4A). In fish on normal Cu, either in the diet or water, gut tissue Cu was similar to control levels and remained between 0.015 and 0.025 μmol g^{-1}. As a proportion of the total (Fig. 4B), Cu in the gut tissue depended on level and period of exposure. For all the groups except the one on high Cu diet level, the proportion of Cu retained in the gut remained between 15 and 20%. In contrast the group on high Cu diet level held more than 40% of their total Cu in the gut tissue early in the exposure, but this declined to about 20% later in the exposure.

The liver showed dramatic changes in Cu levels (Fig. 5A). Liver Cu concentration rose 22-fold in the high dietary Cu exposed fish and fell by 80% in fish on low dietary and waterborne Cu relative to the values at the start of the experiment.

Accumulation of Cu in the liver was continuous throughout the exposure whereas Cu depletion was initially rapid and slowed down over time. The proportion of whole-body total Cu retained in the liver (Fig. 5B) gradually increased from about 20% to about 75% in the fish on high Cu concentration in the diet. In all the other groups the proportion of Cu retained in the liver ranged between 10 and 30%.

Gill Cu content (Fig. 6) was quite variable but was significantly elevated in the fish on high dietary Cu level and significantly lower in the Cu deficient group. The contribution of the gill to the total Cu was 3-5% in all the groups except the one on high dietary Cu concentration, where it fell to about 1%.

Carcass (whole-body less liver, gut, and gills) Cu concentration (Fig. 7A) rose during exposure to high dietary Cu levels from approximately 0.012 $\mu mol\ g^{-1}$ to about 0.017 $\mu mol\ g^{-1}$, and declined significantly in the fish exposed to conditions of Cu deficiency. In the groups receiving normal Cu *via* either or both routes, there were small but significant decreases in carcass Cu concentration at all the sampling times relative to day 0. The proportion of total Cu retained in the carcass (Fig. 7B) remained at approximately 60% in the fish receiving normal levels of Cu in the diet and water but rose to 75% in the Cu-deficient group. In contrast, the proportion of Cu in the carcass for the group on the high dietary Cu level declined to <10% of the total by the end of the experiment.

Waterborne Cu uptake kinetics

Waterborne Cu uptake rates *via* the gills measured using ^{64}Cu over a range of waterborne Cu concentrations at week 7 of exposure are shown in Fig. 8. Fish exposed to low Cu either in the water and/or the diet had elevated rates of uptake of waterborne Cu at all the waterborne Cu concentrations tested. Fish exposed to high dietary Cu concentration had decreased rates of waterborne Cu uptake. In all the groups the rate of waterborne Cu uptake *via* gills increased with the water Cu concentration, a trend that was more marked in the Cu-deficient group.

DISCUSSION

Growth and nutritional requirement for Cu

Growth of juvenile rainbow trout on normal waterborne and dietary Cu regimes was within the expected range for the feeding and temperature regime (Brett and Groves, 1979). Copper is clearly an essential trace element in rainbow trout, based on reduced growth associated with reduced food conversion efficiency in Cu-deficient animals. Copper deficiency in juvenile rainbow trout was induced by exposing fish to reduced Cu levels in both diet and water simultaneously. Ogino and Yang (1981) reported reduced growth in carp but not in rainbow trout exposed to low dietary Cu in normal water Cu levels, whereas Satoh *et al.* (1983) observed growth depression in rainbow trout fed 22 nmol g^{-1} Cu in the diet. Gatlin and Wilson (1986) and Murai *et al.* (1981) did not find growth retardation in channel catfish fed diets containing 24 nmol g^{-}

^{1} or 14 nmol g^{-1} Cu. A fundamental difference between the present study and the previous ones is that in addition to receiving low dietary Cu, fish were exposed to water which was also deficient in Cu. Secondly, our fish were much smaller (starting weight 0.5 g). Our results clearly indicate that to induce Cu deficiency, the experimental fish need to be young, hence with low basal Cu load. It is noteworthy that previous studies (Gatlin and Wilson, 1986; Murai *et al.,* 1981) that did not find depressed growth used much larger fish (starting weights 10 to 30-fold higher than in the present study). Based on growth response, 12.6 nmol g^{-1} of Cu in diet in normal water Cu level (48.5 nmol l^{-1}) supplied adequate amounts of Cu for normal growth but the same amount of dietary Cu is inadequate if the water Cu concentration is deficient. Therefore for determination of minimum dietary requirement of Cu in fish, the waterborne Cu concentration must be taken into account.

Whole-body Cu status

Copper concentration data were expressed on a wet weight basis since a previous study (Shearer, 1984) on rainbow trout of varying body size showed that whole-body wet weight concentrations are more useful for comparison of trace elements than dry weight concentrations. In fact, since there were no treatment-related or time-related effects on moisture content, the same trends would have been seen even if the data had been expressed on a dry weight basis.

Although an ideal biomarker of Cu status in mammals has yet to be identified (Milne, 1998), several indicators have been used by different authors to assess Cu nutritional status. These include growth, activities of cuproenzymes, and plasma Cu concentration (Baker, 1986; Gatlin and Wilson, 1986; Turnlund *et al.,* 1997, 1998). Based on previous studies (Grosell *et al.*, 1997, 1998, 2001b; Kamunde *et al.,* 2001), plasma Cu concentration cannot be used as a sensitive indicator of Cu status in fish since it is very tightly regulated during waterborne and dietary Cu exposure. In this study whole-body and liver Cu concentrations were sensitive indicators of Cu exposure. Baker (1986) pointed out that although growth data are in the long term the only defensible way to establish trace element requirement, the use of body stores also provides an important indicator in determining the nutrient requirement.

Whole-body Cu concentration declined exponentially over time during deficiency, but increased linearly during exposure to high dietary Cu levels. Laurén and McDonald (1987b) described the loss of whole-body Cu after 28 days of exposure to high waterborne Cu as linear. Although these authors used larger fish, there appears to be notable differences in the kinetics of elimination of abnormally high body Cu concentrations (depuration, as described by Laurén and McDonald, 1987b) and the decline of normal body Cu concentrations in the face of deficiency (as in the present study). For actively growing juvenile rainbow trout, simple growth dilution was evident and could account for most of the decline in whole-body Cu concentration. Fish weight increased by about 250% while whole body Cu concentration declined by 60% during the same period, almost exactly the percentage expected by growth dilution.

Furthermore, growth of all the organs and tissues sampled was well correlated with body weight, independent of treatment. Notably, body weight explained 90, 96, and 99% of the change in liver, gut, and carcass weight respectively (Table 3). Since these organs were the main Cu reservoirs, change in Cu concentration in whole-body due to growth dilution would reflect the change in these tissues. Overall, the decline in whole-body Cu concentration fitted a one compartment model (simple negative exponential), and the increase in whole-body concentration during dietary loading was linear, an indication that the latter is not a well-regulated phenomenon.

Interestingly, despite the decline in whole-body Cu concentration in the deficient fish, all the groups had significantly higher Cu levels per fish at the end of exposure compared to the levels at the beginning of the experiment (Fig. 9). Total Cu content in fish on the high dietary Cu level and normal water increased 65-fold, whilst for fish on inadequate Cu *via* both routes, only a twofold increase occurred (Fig. 9). For the groups receiving normal Cu levels in the diet or water in combination or separately, the Cu content increased fivefold. This increase occurred in absence of changes in whole-body moisture content. Thus the fish extracted Cu from the water and diet in all the treatment combinations although the amount obtained from the low diet and low water Cu levels was not adequate to meet the requirement for normal growth or the normal tissue concentration. Nonetheless, this observation illustrates that Cu uptake mechanisms both at the gills and gut are highly efficient.

Tissue Cu status

It has been demonstrated that the role of the liver is central in mammalian Cu metabolism (Cousins, 1985; Harris, 1991; Pena *et al.,* 1999), and it appears to have a similar role in fish. Accumulation of high amounts of Cu during dietary exposure has been reported (Julshamn *et al.,* 1988; Handy 1992, 1996; Kamunde *et al.,* 2001). In the present study, liver Cu content clearly reflected the level of exposure. The role of the liver in Cu metabolism in fish can be viewed as that of concentrating Cu when fish are exposed to large quantities and mobilizing it when inadequate quantities are present in diet and water. In male Sprague-Dawley rats fed a Cu-deficient diet, liver Cu concentration was reported to decrease at a slow rate of about 4% a week (Owen and Hazelrig, 1968). In this study, the decline in liver Cu concentration was relatively rapid with more than half the Cu content lost in about a week [$t_{1/2}$ = 1.18 weeks (8.25 days), Fig. 3A], a reflection of both inadequate uptake and growth dilution. The decline in liver Cu concentration continued throughout the experimental period, and only 20% of the initial Cu concentration remained at the end of the experiment.

The concentration of Cu in the liver strongly influenced whole-body Cu content although the liver represented only 1.3-2% of the body weight. At the beginning of the exposure about 20% of the body Cu burden was in the liver. This proportion remained between 10-30% in all the groups except the group on a high dietary Cu level, which held 75% of the body Cu in the liver by the end of the exposure. Chronic dietary Cu exposure is characterized by a continuous accumulation of Cu in the liver as seen in the present study and previous studies (Handy, 1993; Kamunde *et al.*, 2001). Although we

noted massive accumulation of Cu in the liver in this study, there was no indication of toxicity since the fish grew at the same rate as the controls.

Copper content of gut tissue was greatly elevated in fish exposed to high dietary Cu but appeared to level out over time, an indication that this tissue effectively regulates its internal Cu levels, as suggested in previous studies (Berntssen *et al.,* 1999; Kamunde *et al.*, 2001). Furthermore, Cu build-up in the gut tissue is diagnostic of dietary Cu exposure and does not occur during waterborne Cu exposure to any great extent (Kamunde *et al.,* 2001). A common trend with gut Cu uptake kinetics and accumulation is that early in the exposure, a high proportion of the metal burden is held within the gut tissue but subsequently this is mobilized into other tissues. Later in the exposure, the gut tissue attains steady state despite continued exposure to elevated dietary Cu, suggesting that prolonged exposure stimulates clearance of Cu from the gut to other tissue, increases loss through faeces and mucosal exfoliation, or decreases absorption. Our data suggest stimulated Cu mobilization into other tissues especially the liver, under these conditions.

During elevated levels of dietary Cu exposure in normal water, the gills accumulated significant amounts of Cu, in agreement with previous studies (Miller *et al.,* 1993; Kamunde *et al.,* 2001), thus pointing to a potential role of the gills in Cu excretion. Although the changes in carcass Cu concentration during deficiency and exposure to elevated dietary Cu levels were small, the change in Cu content was enormous given the large mass that the carcass comprises. This compartment held the

highest proportion of whole-body Cu burden in all the groups except in the group receiving a high dietary Cu level, in which the liver was the dominant Cu reservoir.

Whole-body waterborne Cu uptake

Copper uptake rates were measured after 7 weeks of continuous exposure to constant conditions of dietary and waterborne Cu, by which time any acclimation process would presumably be complete. Fish deprived of Cu in the water or diet together or separately had high uptake rates at the low waterborne Cu concentrations (<100 nmol l^{-1} Cu), which increased dramatically above this concentration (Fig. 8). Two types of Cu-binding sites, the high-affinity low-capacity binding sites, and the low-affinity high-capacity binding sites, have been recently described in trout gills (Taylor *et al.*, 2000). These authors demonstrated saturation of the high-affinity low-capacity sites at <315 nmol l^{-1} Cu, and recruitment of low-affinity high-capacity sites above this concentration. In the present study, which measured transport rather than binding, saturation of the high-affinity sites appeared to occur at much lower water Cu concentrations. The generally higher uptake rate at a waterborne concentration of 126 nmol l^{-1} may represent the point at which the low-affinity high-capacity sites start to be recruited. It appears that restriction of Cu in diet increases the capacity and affinity of both types of binding sites.

Uptake of waterborne Cu via gills has been studied mainly as it pertains to Cu toxicity (see review by McDonald and Wood, 1993), while a possible role for the gills in

normal Cu metabolism has been largely disregarded. Gills play vital roles in gaseous exchange, acid-base balance, and ionoregulation; the present study suggests an additional, novel role of the gills in trace metal nutrition and homeostasis. We report for the first time that exposure of fish to conditions deficient in Cu causes an up-regulation of branchial Cu uptake. Furthermore, there is reduced branchial uptake following pre-exposure to high dietary Cu (see also Kamunde *et al.*, 2001). Thus fish respond to different levels of dietary Cu by varying the rate of Cu absorption from water. This strategy may serve to minimize or prevent the development of Cu deficiency when intake is low and, conversely, Cu toxicity when intake is high, and indicate that Cu is under tight homeostatic control.

These observations possibly suggest the presence of a Cu transporter in the fish gills, that responds to body Cu status. Mammalian studies have shown several specific P-type ATPases which serve for Cu transport, e.g., the Menke's and Wilson's proteins, to be involved in Cu homeostasis (Bingham *et al.,* 1998; Roft and Hediger, 1999). For fish, Campbell *et al.* (1999) demonstrated vanadate-sensitive Cu transport (indicative of the involvement of a P-type ATPase) in perfused whole gills of rainbow trout, and Bury *et al.* (1999) reported an ATP-dependent silver uptake by trout gill basolateral membrane vesicles. Silver can substitute for Cu in bacterial Cu-ATPase (Solioz and Odermatt, 1995) and silver transport in rainbow trout gills could thus well be *via* a Cu-ATPase.

Interactions between dietary and waterborne Cu

Only a few studies have assessed the interaction between dietary and waterborne metal uptake in fish. Miller *et al.* (1993) argued that Cu assimilated from either route partitioned into functionally independent compartments in rainbow trout. Furthermore, using whole-body Zn burden, Spry *et al.* (1988) reported no interaction between dietary and waterborne Zn uptake in the same species. However, both these studies based their conclusions, at least in part, on the assumption of zero uptake from their control water Cu (79-205 nmol l^{-1}) and Zn (107 nmol l^{-1}) levels. The current data (Fig. 8) show that this is clearly not the case for Cu at least. Furthermore, these measurements revealed a marked interaction between dietary and waterborne Cu uptake geared toward maintaining Cu homeostasis during deficiency or excess Cu exposure.

We estimated the relative contribution of waterborne and dietary Cu uptake to whole total body Cu load using measured waterborne Cu uptake rates, feeding rates and dietary Cu concentrations (see Materials and Methods). At low dietary Cu, water was clearly the main source of Cu, contributing 60% of the total (Fig. 10A). With increasing dietary Cu, the contribution of dietary Cu increased whilst that of waterborne Cu decreased. In the group maintained on normal dietary and waterborne Cu, water contributed less than 10% of the body Cu. At the highest dietary Cu concentration, diet was clearly the main source of Cu (99%) and water contributed insignificant amounts to the total Cu burden. A previous study on relative contributions of waterborne and dietary Cu uptake to liver Cu concentration (Miller *et al.,* 1993) showed increasing contribution of waterborne Cu uptake as waterborne Cu concentration increased.

In turn, this analysis allowed estimation of the true bioavailability of dietary Cu (see Materials and Methods for definition), which decreased with increasing dietary Cu concentration (in general agreement with studies on humans - e.g., Turnlund *et al.,* 1997, 1998), both in water with normal Cu concentration and water with low Cu concentration (Fig. 10B and C). Furthermore, waterborne Cu had an apparent stimulatory effect on the bioavailability of dietary Cu and *vice versa*. Possible explanations for this could be that the up-regulation of gill uptake that occurs in the low Cu water entails a compensatory down-regulation of intestinal uptake processes, or that gastrointestinal Cu absorptive mechanisms require some threshold waterborne Cu for optimal performance and thus are less effective at low water Cu levels. It is noteworthy that Spry *et al.* (1988) observed a similar stimulatory effect of waterborne Zn on the retention of dietary Zn in rainbow trout.

Murai *et al.* (1981) noted that the responsiveness of catfish to graded levels of dietary Cu was less pronounced than in most terrestrial animals and argued that Cu metabolism in catfish may have been affected by waterborne Cu. However, these authors did not provide any waterborne Cu uptake data to support this insight. The present study not only provides this missing link (waterborne Cu uptake data) but also ascribes to the gills a key role in the normal Cu metabolism in fish. Branchial uptake contributed about 60% of the body Cu load during deficiency, but diet was the preferred source of Cu under normal dietary and waterborne conditions, contributing more than 90% of the body burden. These findings coupled with recent reports of branchial Cu excretion (Grosell *et al.,* 2001b; Kamunde *et al.,* 2001) persuasively underline a key role

of the gills in Cu homeostasis in fish and provide evidence of the gill as an organ of nutritional regulation.

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Table 3-1. Compositions of test diets containing different supplemental amounts of Cu. The diets received respectively either no supplemental Cu (low-Cu diet, i.e., ~3.15nmol g^{-1} from casein), the required dietary level of Cu of 47.21 nmol g^{-1} (normal-Cu diet) or a high dietary Cu level of 4720.69 nmol g^{-1} (high-Cu diet). Normal dietary Cu levels were based upon the known Cu needs of rainbow trout (NRC, 1993).

Ingredients (g kg^{-1} dry weight basis)	Diet*		
	Low-Cu	Normal-Cu	High-Cu
Copper premix; I-cellulose carrier[a]	0.0	37.3	37.3
$CuSO_4 \cdot 5H_2O$ (nmol g^{-1})	0.0	47.2	4720.7
I-cellulose	37.3	0.0	0.0

*All three diets received the following ingredients (g kg^{-1} dry wt): casein. (vitamin-free), 87.97; amino acid mix[b], 381.79; dextrin, 158.20; stabilized[c] sardine oil, 168.65; vitamin supplement[d], 39.87; choline chloride (60%), 4.98; ascorbic acid, 1.50; mineral supplement[e], 89.71; finnstim™, 14.95; Santoquin, 0.11; carboxymethyl cellulose, 14.95.

[a] Diet palatability enhancer supplied by Finnsugar Bioproducts, Helsinki, Finland.

[b] All three diets received the following levels of supplemental amino acids (g kg^{-1} dry diet): arginine-HCl, 26.32; histidine, 8.64; isoleucine, 16.24; leucine, 33.59; lysine-HCl, 31.98; methionine, 10.62; cysteine, 4.11; phenylalanine, 20.02; tyrosine, 13.76; threonine, 17.78; tryptophan, 3.47; valine, 22.47; glutamic acid, 37.30; glycine, 116.4; alanine, 2.45; proline, 16.64.

[c] Stabilized with 0.225 g BHA (butylated hydroxyanisole) kg^{-1} oil.

[d] The vitamin supplement provided the following levels of nutrients kg^{-1} dry diet: vitamin A acetate, 5000 IU; cholecalciferol (D_3), 2400 IU; DL-I-tocopheryl acetate (E), 300 IU; menadione, 18 mg; D-calcium pantothenate, 165 mg; pyridoxine HCl, 40 mg; riboflavin, 60 mg; niacin, 300 mg; folic acid, 15 mg; thiamine mononitrate, 50 mg; biotin, 1.5 mg; cyanocobalamin (B_{12}), 0.2 mg; inositol, 400 mg; p-amino-benzoic acid, 400 mg; butylated hydroxytoluene, 22 mg.

[e] The mineral supplement provided the following levels of minerals kg^{-1} dry diet: Ca (as $CaCO_3$ and $CaHPO_4$), 9989 mg; P (as KH_2PO_4 and $CaHPO_4$), 7361 mg; Mg (as $MgSO_4 \bullet 7H_2O$), 1500 mg; Fe (as $FeSO_4 \bullet 7H_2O$), 200mg; Zn (as $ZnSO_4 \bullet 7H_2O$), 96 mg; Mn (as $MnSO_4 \bullet H_2O$), 75 mg; Na (as NaCl), 2344 mg; K (as K_2SO_4, K_2CO_3, and KH_2PO_4), 8000 mg; I (as KIO_3), 10 mg; F (as NaF), 5 mg; Co (as $CoCl_2 \bullet 6H_2O$), 3 mg; Se (as Na_2SeO_3), 0.2 mg; Al (as $AlCl_3 \bullet 6H_2O$), 5 mg.

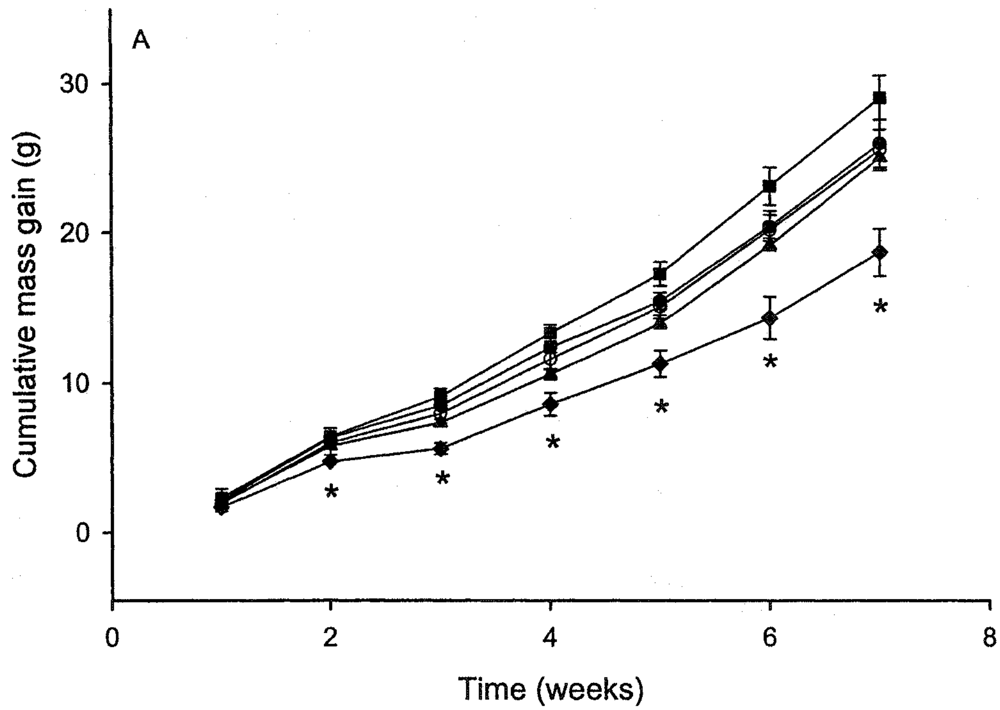
Table 3-2. Levels of Cu exposure and measured Cu concentrations (means ± SEM) in the water (nmol l^{-1} and μg l^{-1}) and diet (nmol g^{-1} and μg g^{-1}) for each level; n = 35 and 10 for each waterborne and dietary Cu level respectively.

	Water Cu			Diet Cu		
	Low	Normal		Low	Normal	High
nmol l^{-1}	5.82±0.47	48.47±0.94	nmol g^{-1}	12.60±0.55	50.40±0.74	4437.45±224.68
μg l^{-1}	0.37±0.03	3.08±0.06	μg g^{-1}	0.80±0.03	3.20±0.05	282.00±14.28

Table 3-3. Allometric equations and correlation coefficients for the relationships between wet body weight and weights of gill, liver, gut, and carcass; n = 144 per organ/tissue.

Organ/tissue	Allometric equation	r^2	P value
Gill	$0.0379W^{0.8662}$	0.82	<0.0001
Liver	$0.0182W^{1.2027}$	0.90	<0.0001
Gut	$0.1041W^{1.0682}$	0.96	<0.0001
Carcass	$0.8394W^{0.9928}$	0.99	<0.0001

Figure 3-1. A: Effects of exposure of juvenile rainbow trout to a combination of waterborne and dietary Cu ranging from deficient to excess on growth. Values represent cumulative weight gain per tank, means ± SEM, n = 3. ♦, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. B: Effects of the exposure conditions on food conversion efficiency in actively growing rainbow trout. Values are means ± SEM on a per tank basis, n = 3 per data point. LL, low waterborne Cu and low dietary Cu; LN, low waterborne Cu and normal dietary Cu; NL, normal waterborne Cu and low dietary Cu; NN, normal waterborne Cu and normal dietary Cu; NH, normal waterborne Cu and high dietary Cu level. * Indicates significant difference relative to group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$). No differences were observed with other comparisons of treatments.


A
Cumulative mass gain (g)
Time (weeks)
0
10
20
30
2
4
6
8
*

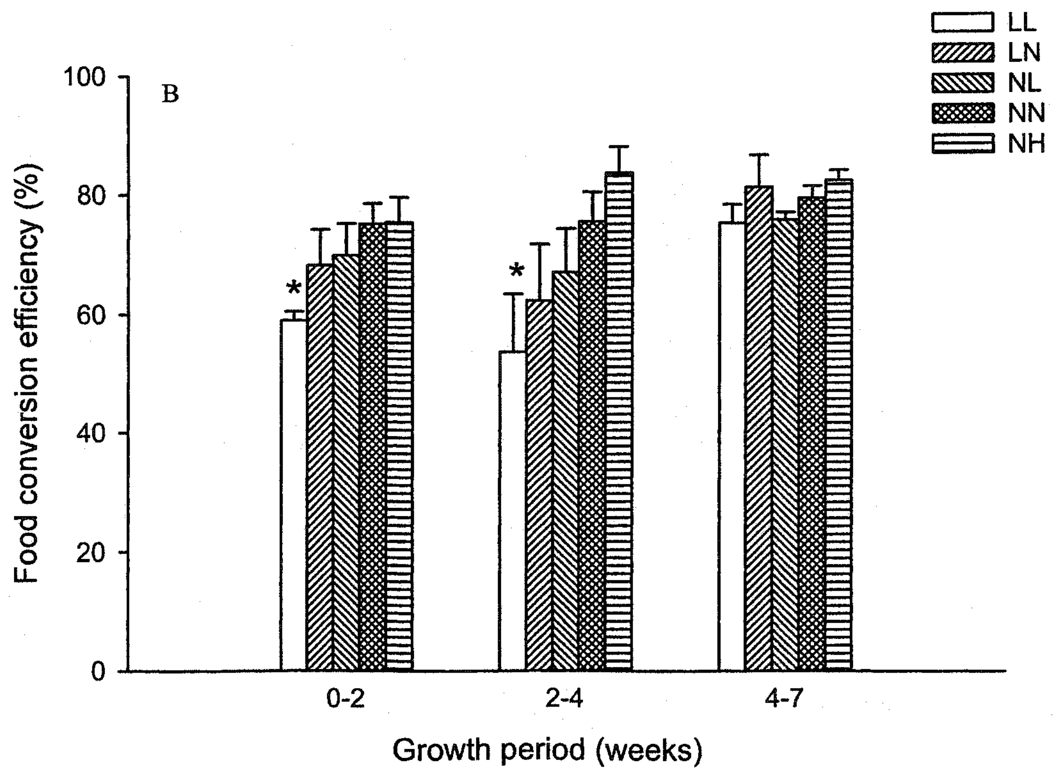

B
LL
LN
NL
NN
NH
Food conversion efficiency (%)
Growth period (weeks)
0-2
2-4
4-7
0
20
40
60
80
100
*

Figure 3-2. Effects of dietary and waterborne Cu exposure conditions on whole-body Cu concentration in actively growing rainbow trout. Values are means ± SEM, n = 15 for weeks 0, 2, and 4, and n = 9 for week 7 for each treatment. ◆, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. * Indicates significantly higher and # indicates significantly lower relative to group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$).

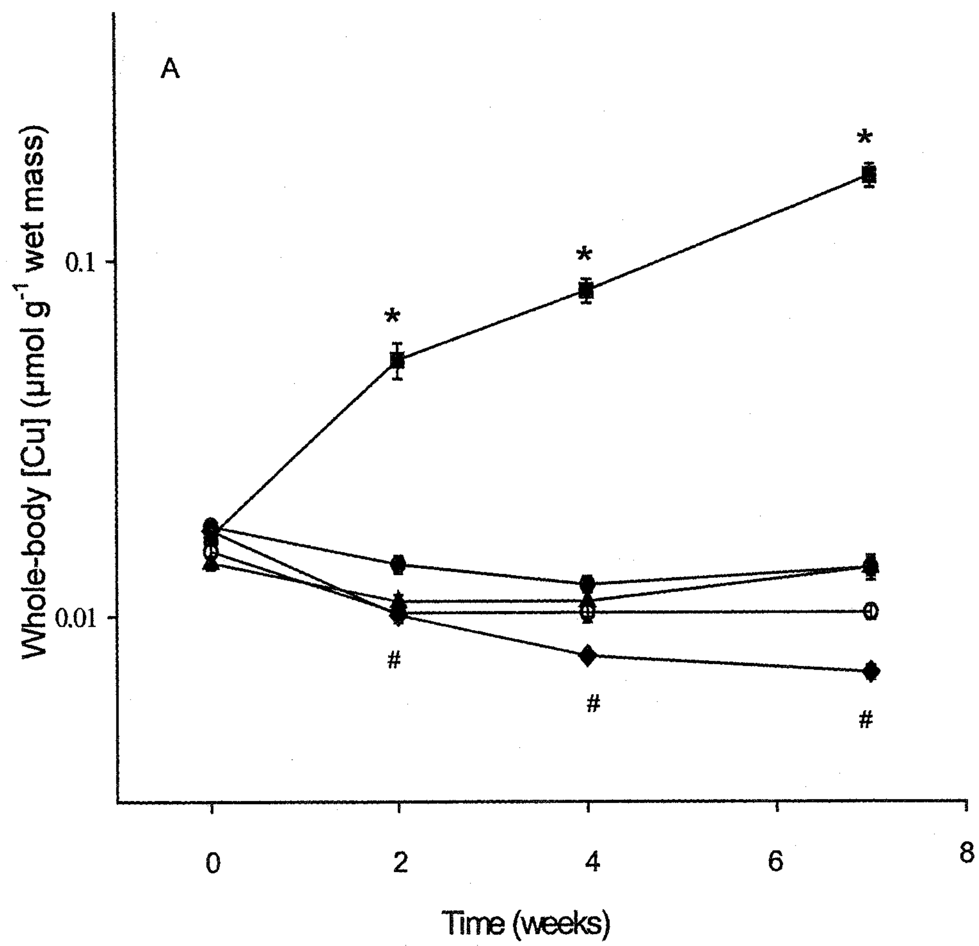
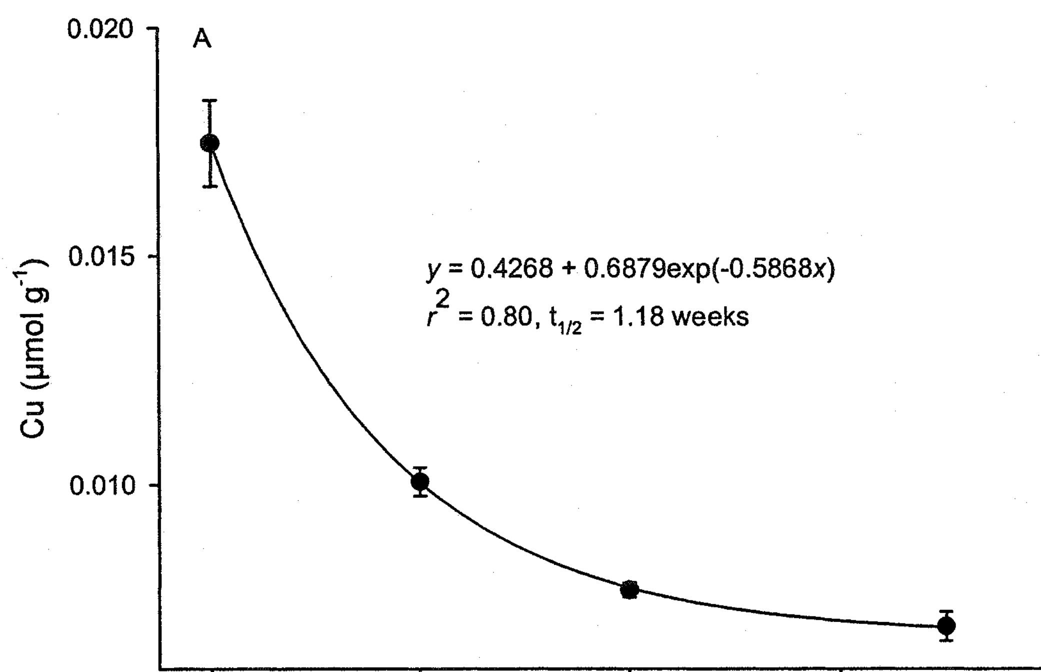

A
Whole-body [Cu] (μmol g-1 wet mass)
0.1
0.01
*
*
*
#
#
#
0
2
4
6
8
Time (weeks)

Figure 3-3. Temporal patterns in whole-body Cu concentration (y, μmol g^{-1}) with time (x, weeks) during exposure to low waterborne and low dietary Cu (A), and to normal waterborne and high dietary Cu level (B). Values are means ± SEM, n = 15 for week 0, 2, and 4, and n = 9 for week 7 for each treatment. In (A) the negative relationship is best described by the exponential equation $y = 0.4268 + 0.6879\exp(-0.5868x)$, $r^2 = 0.80$, $t_{1/2} = 1.18$ weeks, and in (B), the positive relationship is best described by the linear equation, $y = 0.5897 + 1.407x$, $r^2 = 0.80$. Equations were derived from individual data points.


A
0.020
0.015
0.010
Cu (µmol g-1)
y = 0.4268 + 0.6879exp(-0.5868x)
r2 = 0.80, t1/2 = 1.18 weeks

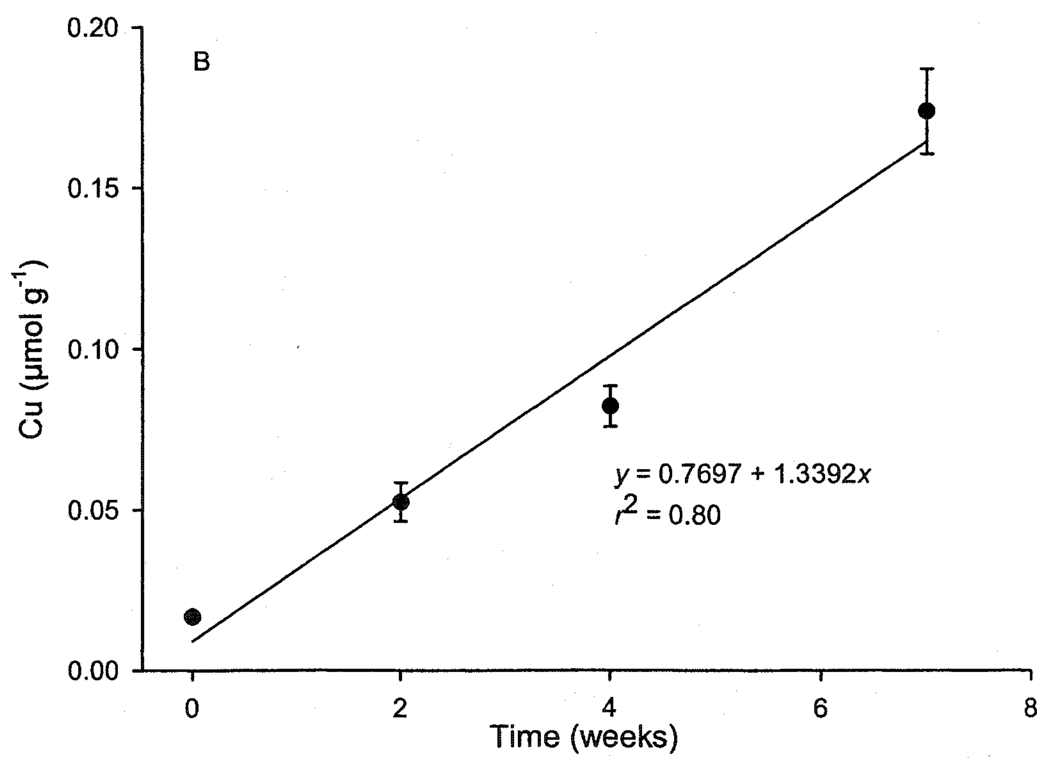
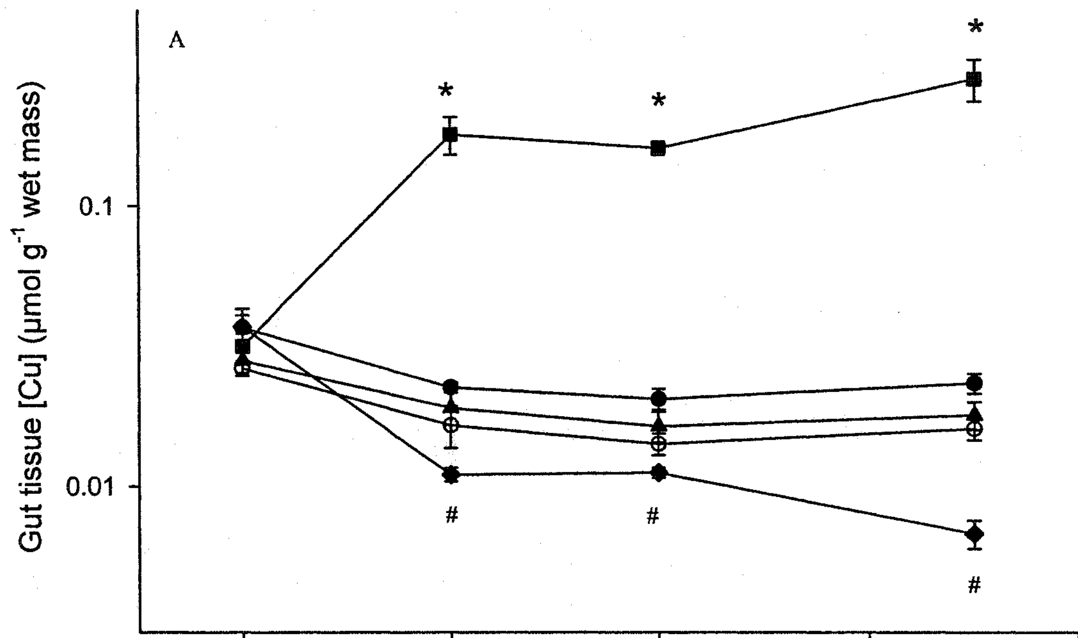

B
0.20
0.15
0.10
0.05
0.00
Cu (µmol g-1)
y = 0.7697 + 1.3392x
r2 = 0.80
0
2
4
6
8
Time (weeks)

Figure 3-4. Gut tissue Cu concentration (μmol g^{-1}) during the exposures and proportional contribution (%) to total body Cu burden. Percentage data were transformed to arc sin for statistical analysis. Values are means ± SEM; n = 15 for week 0, 2, and 4, and n = 9 for week 7 for each treatment. ◆, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. * Indicates significantly higher and # indicates significantly lower relative to group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$).


A
Gut tissue [Cu] (μmol g-1 wet mass)
0.1
0.01
*
*
*
#
#
#

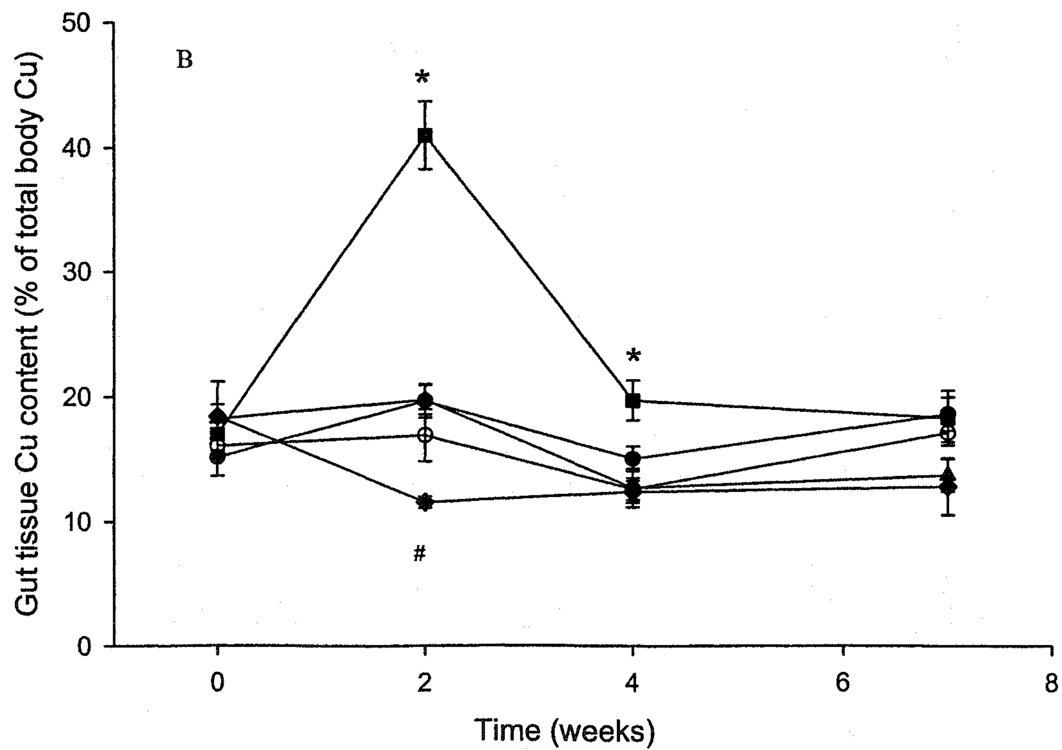
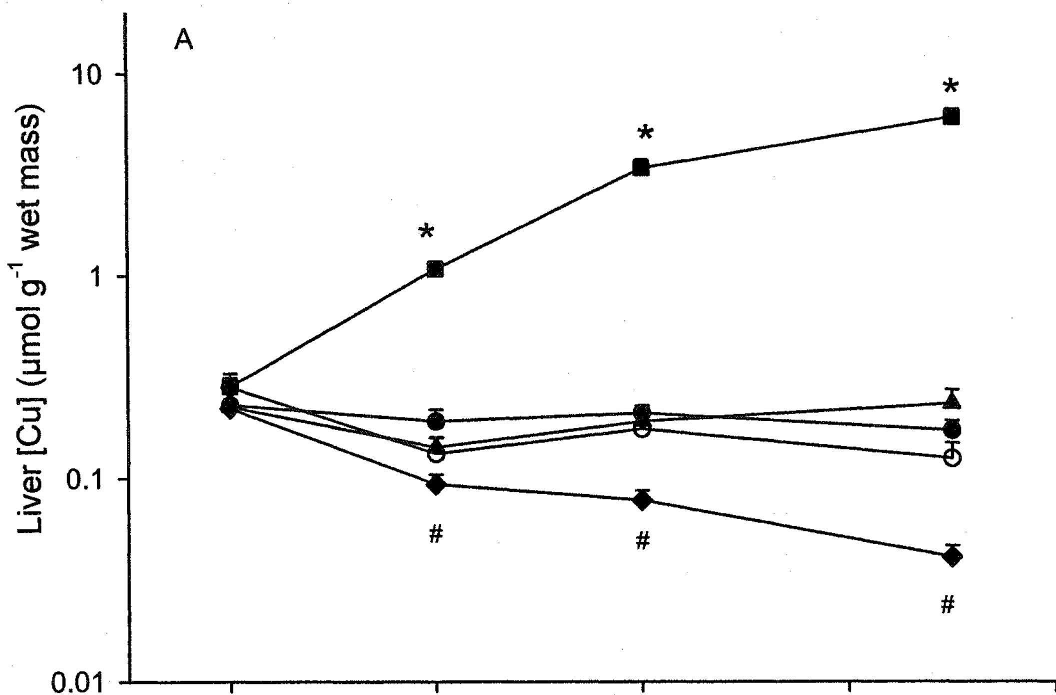

B
Gut tissue Cu content (% of total body Cu)
50
40
30
20
10
0
*
*
#
0
2
4
6
8
Time (weeks)

Figure 3-5. Liver Cu concentration (µmol g^{-1}) during the exposures (A) and proportional contribution of liver (B) to total body Cu. Percentage data were transformed to arc sin for statistical analysis. Values are means ± SEM; n = 15 for weeks 0, 2, and 4, and n = 9 for week 7 for each data point per treatment. ♦, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. * Indicates significantly higher and # indicates significantly lower relative to group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$).


A
Liver [Cu] (µmol g-1 wet mass)
10
1
0.1
0.01
*
*
*
#
#
#

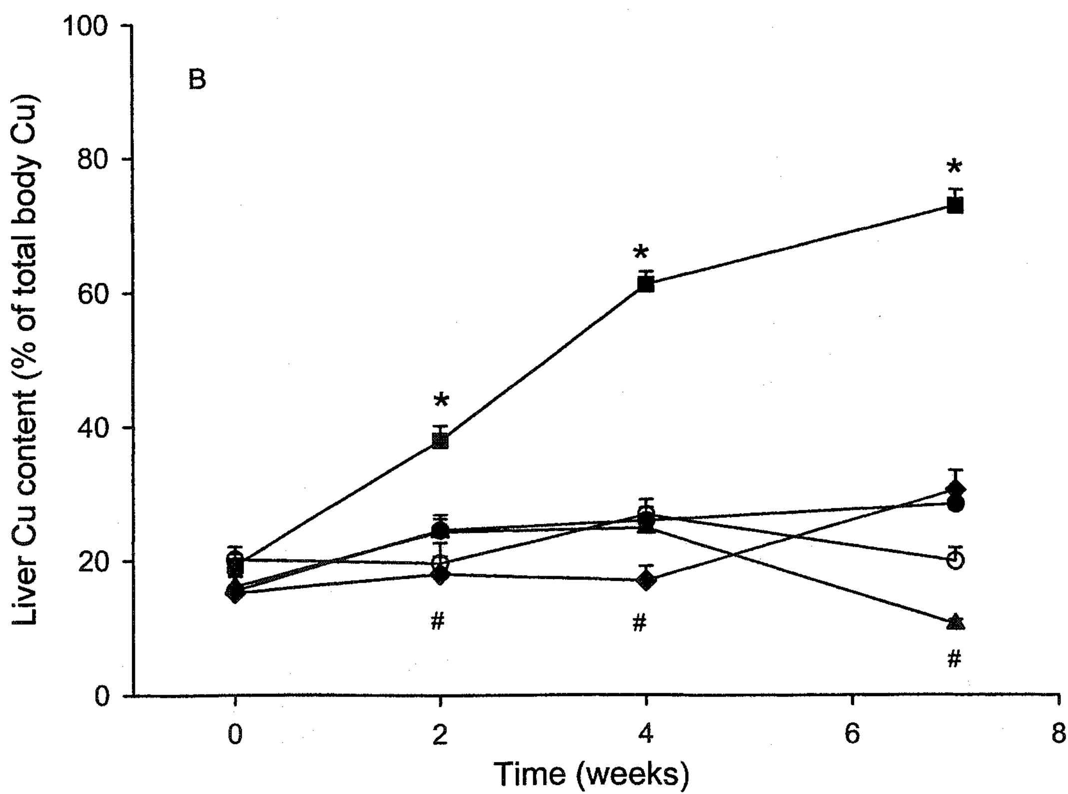
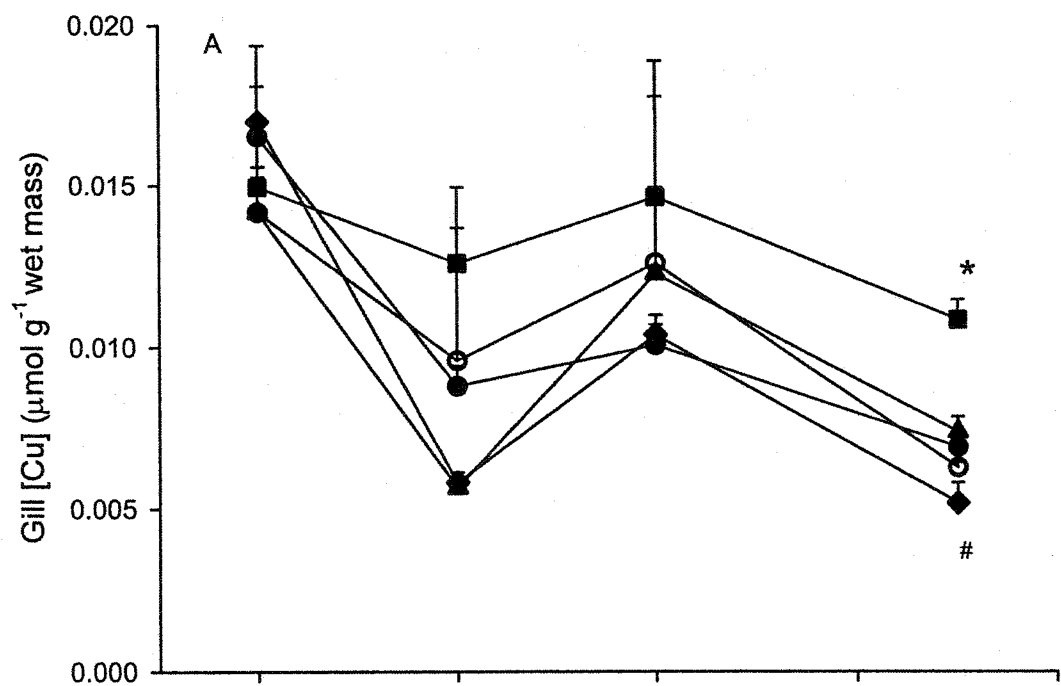

B
Liver Cu content (% of total body Cu)
100
80
60
40
20
0
0
2
4
6
8
Time (weeks)
*
*
*
#
#
#

Figure 3-6. Gill Cu concentration in (μmol g^{-1}) during the exposures (A) and percentage contribution of the gill (B) to total body Cu burden during the exposures. Percentage data were transformed to arc sin for statistical analysis. All the values are means ± SEM, n= 15 for week 0, 2, and 4, and 9 for week 7 for each of the treatment. ♦, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. * Indicates significantly higher and # significantly lower relative to group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$).


A
Gill [Cu] (μmol g⁻¹ wet mass)
0.020
0.015
0.010
0.005
0.000
*
#

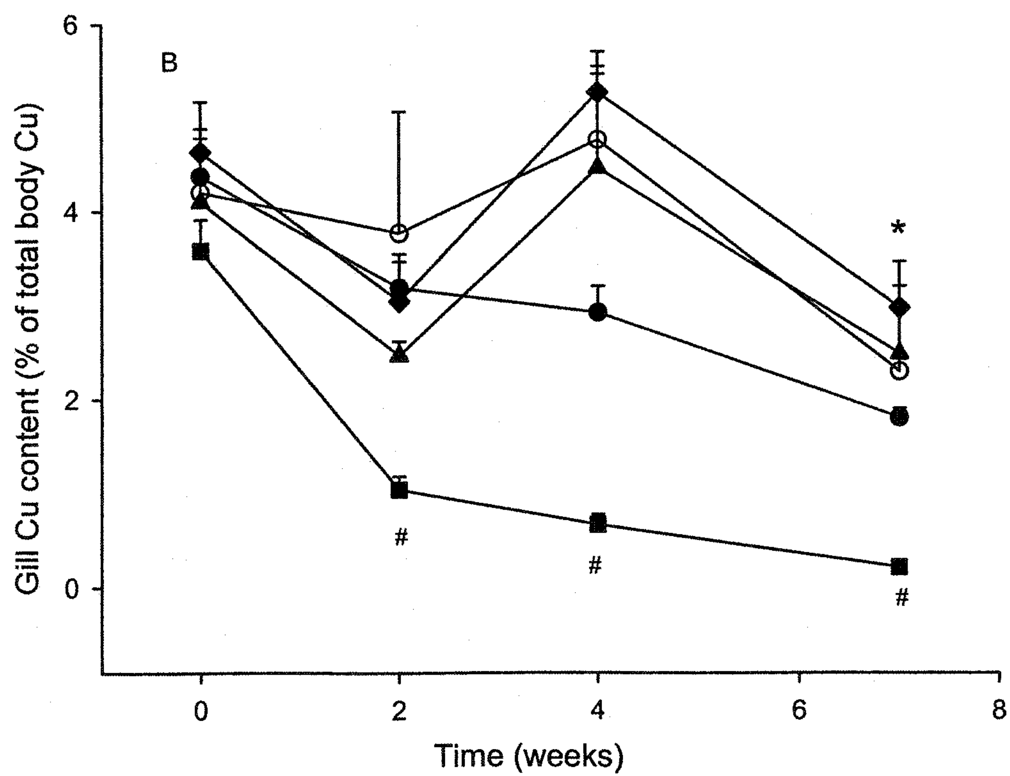
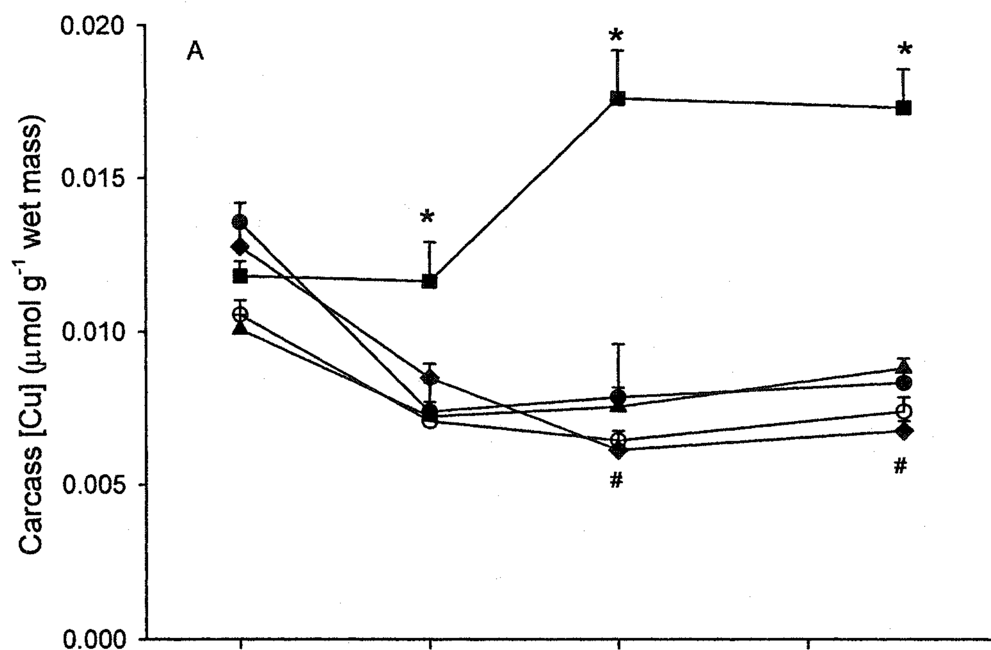
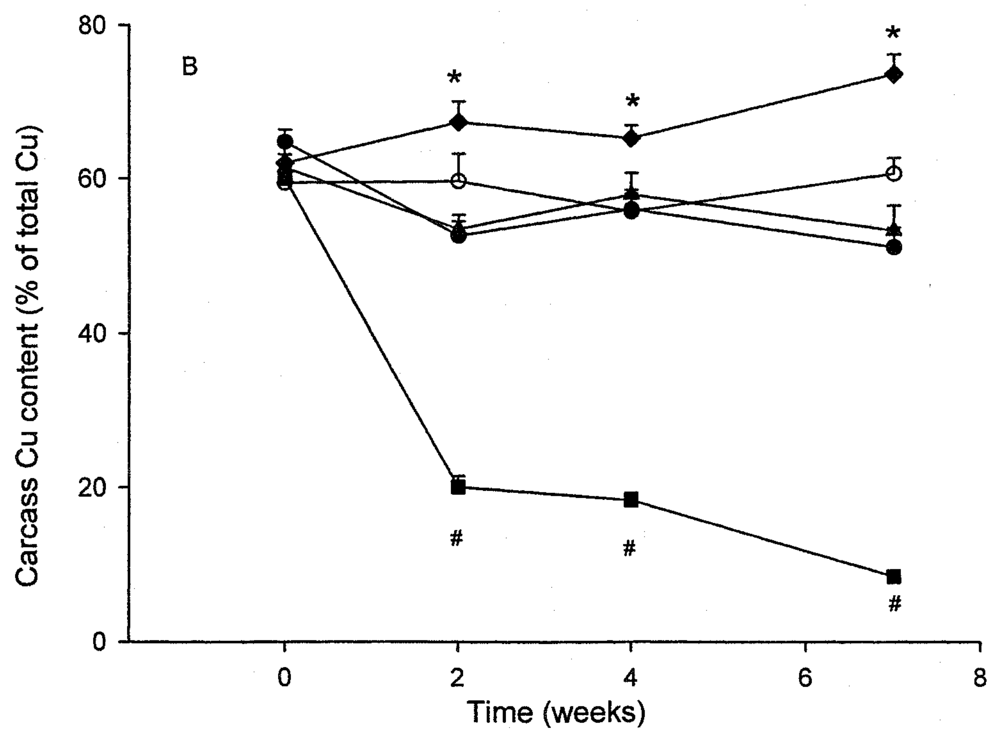

B
Gill Cu content (% of total body Cu)
6
4
2
0
*
#
#
#
0
2
4
6
8
Time (weeks)

Figure 3-7. Carcass Cu concentration (µmol g^{-1}) during the exposure (A) and proportional contribution of the carcass (B) to total Cu burden during the exposures. Carcass comprised whole body less gill, liver and gut. Percentage data were transformed to arc sin for statistical analysis. All the values are means ± SEM, n= 15 for week 0, 2, and 4, and n = 9 for week 7 for each of the treatment. ◆, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. * Indicates significantly higher and # indicates significantly lower relative to group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$).

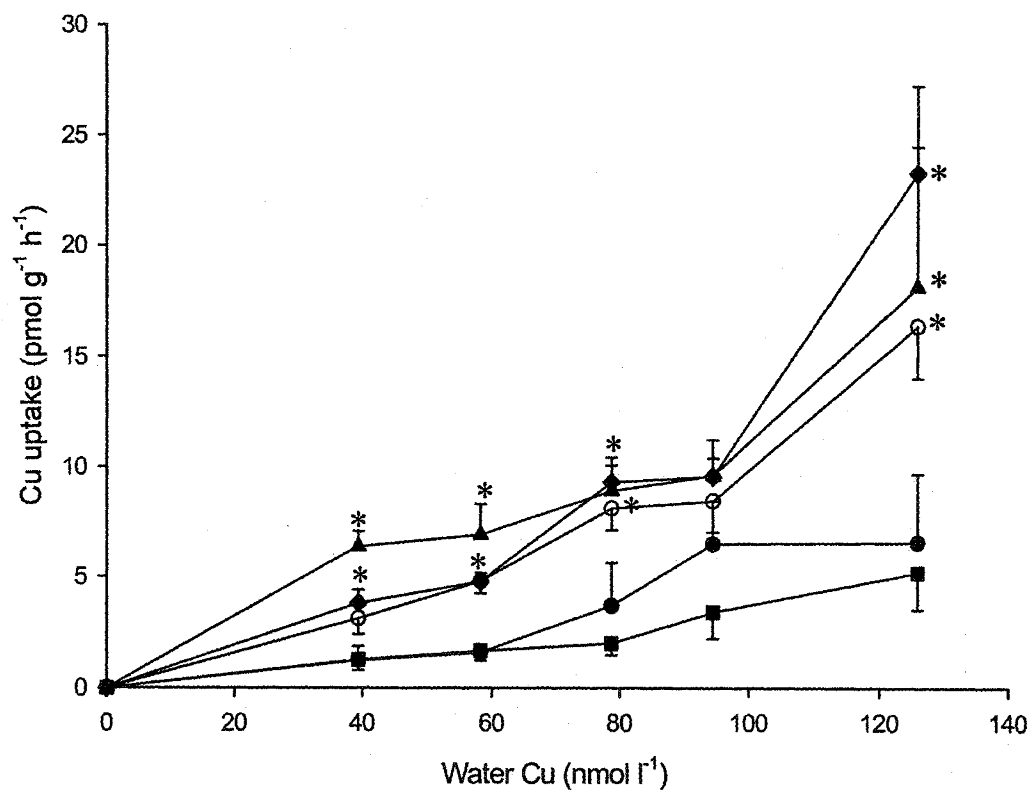


0.020
0.015
0.010
0.005
0.000
A
Carcass [Cu] (μmol g-1 wet mass)
*
#



80
60
40
20
0
B
Carcass Cu content (% of total Cu)
0
2
4
6
8
Time (weeks)
*
#

Figure 3-8. Waterborne Cu uptake rates (means ± SEM, pmol g^{-1} h^{-1}, n = 9 per data point), measured using ^{64}Cu at the end of the 7 week exposure for all the treatment groups. These measurements were carried out over a 12 hour period, at a range of waterborne copper concentrations. ♦, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. * Indicates significantly higher relative to the group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$).



Cu uptake (pmol g^{-1} h^{-1})
Water Cu (nmol l^{-1})
0
5
10
15
20
25
30
0
20
40
60
80
100
120
140

Figure 3-9. Effects of dietary and waterborne Cu exposure conditions on fish Cu content. Values are means ± SEM, n = 15 for weeks 0, 2, and 4, and n = 9 for week 7 for each treatment. ♦, low waterborne Cu and low dietary Cu; ▲, low waterborne Cu and normal dietary Cu; ○, normal waterborne Cu and low dietary Cu; ●, normal waterborne Cu and normal dietary Cu; ■, normal waterborne Cu and high dietary Cu level. * Indicates significantly higher and # indicates significantly lower relative to group on normal water Cu and normal dietary Cu (ANOVA, $p<0.05$).

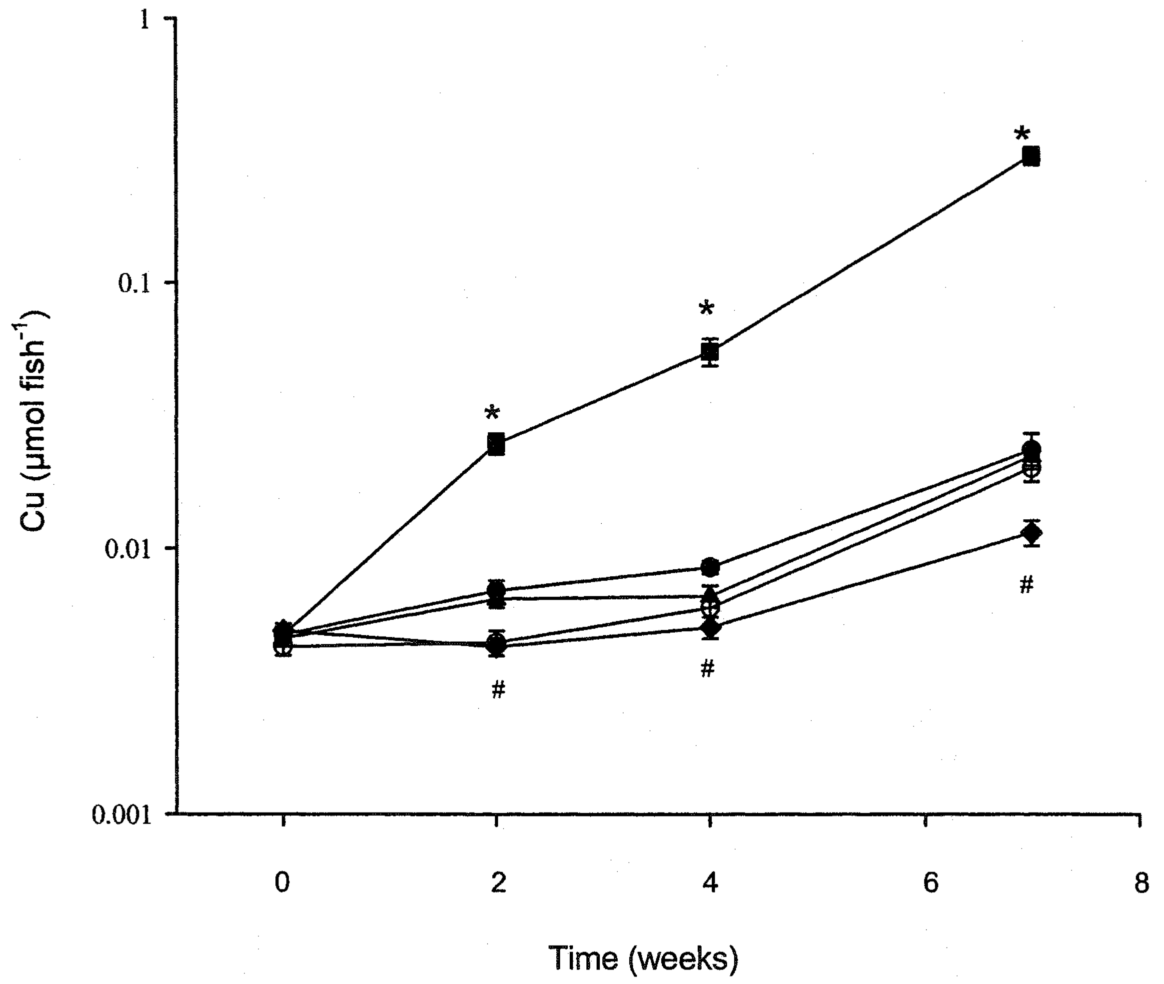
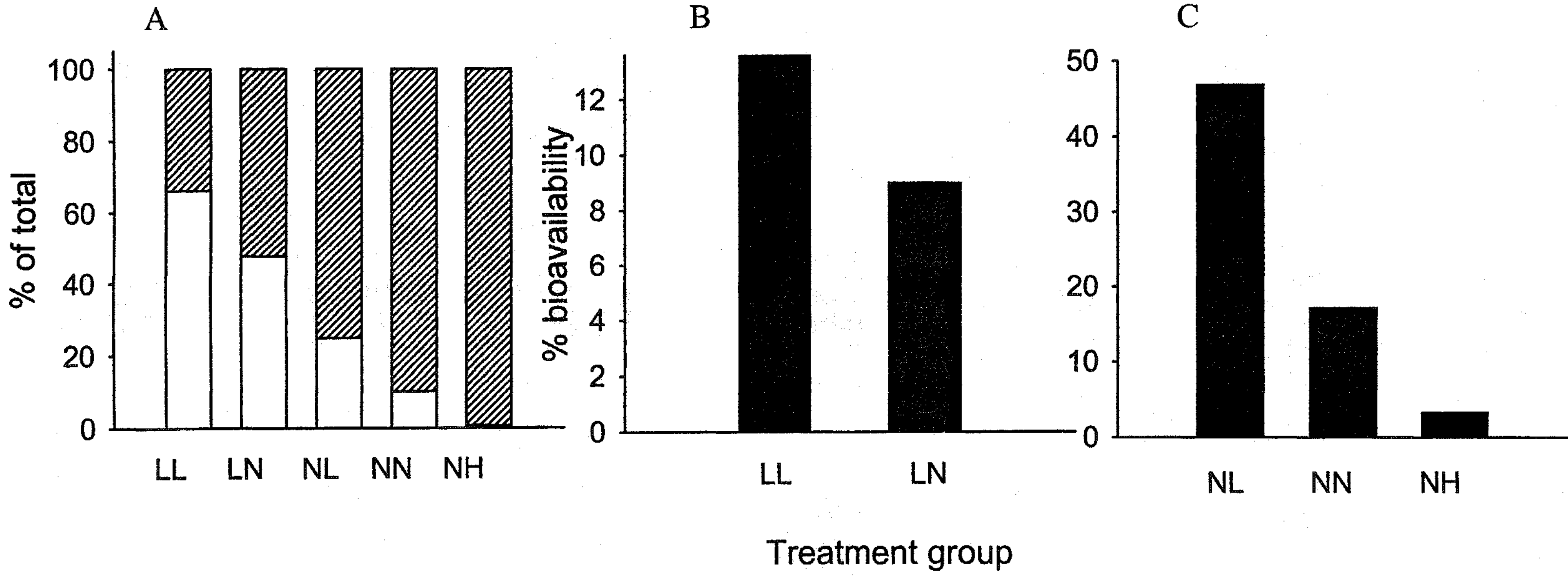

1
0.1
0.01
0.001
Cu (µmol fish⁻¹)
*
*
*
#
#
#
0
2
4
6
8
Time (weeks)

Figure 3-10. A: Relative contribution of dietary and waterborne Cu uptake to the total body Cu burden accumulated over 7 weeks of exposure to experimental regime. Open, bars, contribution of the waterborne Cu uptake; hatched bars, contribution of the dietary Cu uptake; LL, low waterborne Cu and low dietary Cu; LN, low waterborne Cu and normal dietary Cu; NL, normal waterborne Cu and low dietary Cu; NN, normal waterborne Cu and normal dietary Cu; NH, normal waterborne Cu and high dietary Cu level. B and C: True bioavailability of dietary Cu (% of Cu ingested in food and retained during the exposure period). B: bioavailability of dietary Cu in normal water. C: bioavailability of dietary Cu in low waterborne Cu. See text for details of the calculation.


A
B
C
% of total
% bioavailability
100
80
60
40
20
0
12
10
8
6
4
2
0
50
40
30
20
10
0
LL
LN
NL
NN
NH
LL
LN
NL
NN
NH
Treatment group

CHAPTER 4

WATERBORNE *VERSUS* DIETARY COPPER UPTAKE IN RAINBOW TROUT AND THE EFFECTS OF PREVIOUS WATERBORNE COPPER EXPOSURE

ABSTRACT

Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne Cu (22 μg l^{-1}) in moderately hard water for up to 28 days. Relative to control fish kept at background Cu levels (2 μg l^{-1}), Cu pre-exposed fish displayed decreased uptake rates of waterborne Cu via the gills but not of dietary Cu via the gut during 48-h exposures to ^{64}Cu radio-labeled water and diet, respectively. At normal dietary and waterborne Cu levels, the uptake rates of dietary Cu into the whole-body without the gut were between 0.40 and 0.90 ng g^{-1} h^{-1}, more than tenfold higher than those of waterborne Cu into the whole-body without the gills, which were between 0.02 and 0.07 ng g^{-1} h^{-1}. Previously Cu-exposed fish showed decreased new Cu accumulation in the gills, liver, and carcass during waterborne ^{64}Cu exposures and in the liver during dietary ^{64}Cu exposures. A 3-h gill Cu-binding assay showed down-regulation of the putative high affinity-low capacity Cu transporters and up-regulation of the low affinity-high capacity Cu transporters at the gills in Cu pre-exposed fish. Exchangeable Cu pools in all the tissues were higher during dietary ^{64}Cu exposures than waterborne ^{64}Cu exposures, and previous Cu exposure reduced waterborne exchangeable Cu pools in gill, liver, and carcass. Overall,

these results suggest a quantitatively greater role for the dietary than the waterborne route of Cu uptake, a key role for the gill in Cu homeostasis, and important roles for the liver and gut in the normal metabolism of Cu in fish.

Keywords: Cu metabolism; Cu pre-exposure; water; diet

INTRODUCTION

Copper (Cu) homeostasis in animals is tightly regulated because Cu is both essential and toxic to living systems. Mammalian studies have demonstrated that this homeostasis is primarily regulated at the hepato-gastrointestinal level (Schaefer and Gatlin, 1999; Turnlund *et al.,* 1998; Wapnir, 1998), but the situation in fish is confounded by the presence of two potential routes of uptake, the gill and the gastrointestinal tract. Although numerous studies have examined the effects of waterborne Cu exposure in fish, most have focused on Cu toxicity with little emphasis on the possible effects of such exposure on Cu homeostasis (McDonald and Wood, 1993; Wood, 2001). Nonetheless, significant progress has been made recently by Grosell and Wood (2001) and Grosell *et al.* (1996, 1997, 1998; 2001b) who investigated key aspects of Cu metabolism in fish during waterborne exposures, including branchial uptake, plasma clearance, and renal and hepatobiliary excretion. These studies demonstrated that Cu homeostasis in fish entails regulated uptake, transport, and excretion, as is the case for mammals (Turnlund *et al.,* 1998). However, to fully understand Cu homeostasis and toxicity in fish, a clear perception of the interactions between dietary and waterborne Cu uptake is necessary. To this end, we have recently demonstrated that dietary Cu pre-exposure down-regulates branchial uptake of waterborne Cu (Kamunde *et al.*, 2001, 2002c), whereas deprivation of Cu up-regulates branchial uptake (Kamunde *et al.,* 2002c). However, a key aspect of this interaction that has to date been ignored is the possible effect of waterborne Cu pre-exposure on subsequent uptake, distribution, and excretion of dietary Cu.

Acclimation to waterborne Cu is still a matter of controversy. McDonald and Wood (1993) defined acclimation as being characterized by increased tolerance to acute doses of metal following chronic exposure to sublethally toxic doses. This acclimation could be attributed to several factors, including changes in branchial cellular morphology and permeability to ions, changes in uptake and accumulation rate of the metal, and increased excretion, storage, and detoxification capacity. With respect to Cu there are contradictory reports on the effect of acclimation on the subsequent uptake of waterborne Cu. Constant uptake (Grosell *et al.,* 1996; McCarter and Roch, 1984), increased uptake (Taylor *et al.,* 2000), and decreased uptake (Grosell *et al.,* 1997) have all been reported. Detailed evaluation of Cu uptake following pre-exposure to waterborne Cu is therefore warranted in order to elucidate the effects of acclimation on uptake of waterborne Cu. We hypothesize that the reported differences in Cu uptake rates are due to different effects of acclimation on the two putative types of Cu-binding sites/transporters in the gills (Grosell and Wood, 2002; Taylor *et al.,* 2000).

The purpose of the present study was therefore to first determine the relative quantitative contributions of waterborne and dietary uptake rates for Cu. The second was to characterize the effects of waterborne Cu pre-exposure on Cu homeostasis and unidirectional uptake of waterborne Cu using direct ^{64}Cu flux measurements. Given the dual routes of Cu uptake in fish, the third objective was to evaluate the effects of waterborne Cu pre-exposure on the uptake and distribution of dietary Cu. Our hypothesis was that Cu homeostasis is regulated centrally and the exposure of fish to Cu via one route would impact Cu uptake via the other route. Finally, 3-h binding of

waterborne Cu at different concentrations to the gill determined using ^{64}Cu (cf. Playle *et al.,* 1992) was assessed in order to illuminate the effects of Cu pre-exposure on the two putative types of Cu-binding sites/transporters in the gills.

MATERIALS AND METHODS

Fish

About 900 juvenile rainbow trout, (*Oncorhynchus mykiss*, 9 - 11 g, Humber Springs Trout Farm, ON, Canada) were initially maintained for 3 weeks in one 500-l tank supplied with a flow-through of dechlorinated municipal (Hamilton, ON, Canada) tap water (Na^+ 0.6 mmol l^{-1}; Ca^{2+} 1.0 mmol l^{-1}; HCO_3^- 1.9 mmol l^{-1}; Cl^- 0.7 mmol l^{-1}; pH 7.7; dissolved organic carbon (DOC) 3mg l^{-1}; background Cu 2 μg l^{-1}; temperature 14 ± 1°C). Fish were fed a 2% daily ration (dry feed/wet body weight) of commercial trout chow (1 point, Martin's Feed Mill Ltd., Elmira, ON, Canada) containing (in %): crude protein 52 (maximum), crude fat 17 (minimum), crude fiber 2.5 (maximum), Na^+ 0.4 (actual), Ca^{2+} 1.4 (actual), phosphorus 1.0 (actual), and vitamins (in IU kg^{-1}): A 10000 (minimum), C 300 (minimum), D_3 3000 (minimum), and E 100 (minimum). Measured total concentration in the diet was 40 mg kg^{-1}.

Time course of acute waterborne and dietary ^{64}Cu uptake

An initial study examined ^{64}Cu uptake from the diet and water over time to determine the appropriate conditions for subsequent exposures. Sixty control fish (30 each for a waterborne and a dietary flux) were exposed to waterborne or dietary ^{64}Cu for

48 h and sampled after 3, 6, 12, 24, 36, and 48 h of exposure. The exposure conditions are outlined below. For both fluxes, gills, plasma, liver, gut, and the rest of the carcass were collected individually and analyzed for ^{64}Cu radioactivity. For the dietary flux, sampling was done from 6 h onward because fish retained substantial amounts of food in the esophagus within 3 h of feeding and often regurgitated it during terminal anesthesia, thereby contaminating other tissues, especially the gill.

Exposure to waterborne Cu and depuration

The rest of the fish were divided into four groups each of about 200 fish and placed in 150-l experimental tanks. Daily rations were increased to 4%. Fish in 2 of the tanks were exposed for 28 days to a nominal 20 $\mu g\ l^{-1}$ Cu achieved by delivering a concentrated stock solution of Cu (as $CuSO_4 \bullet 5H_2O$, analytical grade) from a Mariotte bottle to a head tank fed with dechlorinated Hamilton municipal tap water. Experimental tanks were supplied from the head tank at a flow rate of 1.1 $l\ min^{-1}$. The actual water [Cu] in the experimental tanks determined by atomic absorption spectrophotometry was $22.2 \pm 0.9\ \mu g\ l^{-1}$, (mean ± SEM, n = 29). At days 0, 7, 14, and 28, ten fish were removed for the determination of total Cu and measurement of Cu uptake from the water or diet using ^{64}Cu (see below).

Waterborne Cu exposure was terminated after 28 days and daily feeding reduced to 2% for the following 30 days. At days 10, 20, and 30 post-exposure, 10 control and 10 Cu-acclimated fish were randomly netted out from the tanks, terminally anesthetized

with 1 g l^{-1} tricaine methanesulfonate (MS-222), and the gill, liver, and the rest of the carcass were dissected out for total Cu analysis (see below).

Waterborne and dietary ^{64}Cu exposures

Measurements of unidirectional Cu uptake from the water via the gills and from the diet via the gut were made using 48-h ^{64}Cu exposures at days 0, 7, 14, and 28 of waterborne Cu exposure. Waterborne flux was measured in 30-l flux chambers under static conditions of dechlorinated aerated Hamilton municipal tap water. Fish were not fed during the flux to avoid fouling of the water. Water quality parameters including oxygen content, pH, and temperature monitored during the experiment remained close to levels measured in the flow-through tank water in which the fish were maintained. For day 0, the acute waterborne Cu exposure was performed using 10 fish at background water Cu concentration with 1 μg l^{-1} Cu added as ^{64}Cu [$CuNO_3$ (specific activity 3.3 μCi μg^{-1}, produced in the McMaster University Nuclear Reactor)]. Addition of ^{64}Cu brought the measured final water Cu concentration to 2.8 μg l^{-1}. For days 7, 14, and 28, flux measurements were performed at background water Cu concentration (2 – 3 μg l^{-1}) and 20 – 22 μg l^{-1} for both the controls (n = 10 at each time and concentration) and Cu-exposed fish (n = 10) to allow direct comparison of the Cu uptake rates. For each concentration the water was spiked with 1 μg l^{-1} ^{64}Cu (3.3 μCi μg^{-1} as $CuNO_3$). Water samples were taken 15 min after the start of the flux and thereafter every 12 h for 48 h for analysis of ^{64}Cu γ radioactivity and total Cu.

Similarly, dietary ^{64}Cu flux was measured at waterborne concentrations of 2 and 22 μg l^{-1} Cu for both the control (n = 10 at each time and concentration) and waterborne Cu pre-exposed fish (n = 10) except for day 0 when the flux measurement was performed only at background waterborne Cu concentration. For each dietary flux measurement day, a fresh ^{64}Cu-labeled diet was made by the method described in Kamunde *et al.* (2001), except that radioactive Cu was used. For the initial acute time-course study described above, 5 mg of ^{64}Cu labeled $CuNO_3$ (17 mCi) were introduced to 50 g of commercial trout chow, thus elevating the diet Cu concentration to 165 mg kg^{-1}. However, in subsequent experiments we elected to use a lower level of ^{64}Cu in the diet (5 mg ^{64}Cu = 17 mCi added to 250 g of commercial trout chow), thereby raising measured diet Cu concentration to only 65 mg kg^{-1}, closer to the acclimation diet Cu level (40 mg kg^{-1}). For all preparations, the addition of the ^{64}Cu was followed by 45 minutes of thorough mixing of the food in a commercial pasta maker. Thereafter the food was extruded and air-dried in an oven at 70 °C for about 1 h. Subsequently fish were fed a 4% daily ration of the radioactive food and allowed to feed for 1 h. Thereafter they were transferred into the 30-l flux tanks for 48 h. Food left over in the feeding tank was collected, dried, and weighed to estimate the exact amount consumed (typically 60 – 75% of the ration). Water in the flux tanks was continuously aerated and changed every 12 h to avoid build up of fecal waste.

Sampling

Fish were killed with an overdose ($1\ g\ l^{-1}$) of neutralized (MS-222) and rinsed with double distilled water after the 48-h waterborne and dietary ^{64}Cu exposures. Blood was immediately collected by caudal puncture and centrifuged at 13 000 x *g* to collect plasma. The fish were then dissected and bile was collected immediately by aspiration with 1-ml syringes. Subsequently, liver, gill, gut (separated into stomach, pyloric cecae + anterior intestine, mid-intestine, and posterior intestine), and the rest of the carcass were dissected out, rinsed with double distilled water, and weighed.

Analyses and calculations

The ^{64}Cu γ radioactivity was determined in water, diet, and all tissues collected using a Canberra-Packard MINAXI Auto-gamma counter with an on-board program for decay correction. Absolute whole-body uptake of waterborne and dietary ^{64}Cu was calculated by summing up ^{64}Cu activities (cpm) in all tissues plus carcass. Fish weights were determined by summing up the weights of liver, gills, plasma, gut tissue, bile, and carcass for each fish. Whole-body new Cu uptake from the water and diet was then calculated using the equation:

$$a(bc^{-1})^{-1}, \qquad (1)$$

where a is the ^{64}Cu cpm g^{-1} fish, b is ^{64}Cu cpm l^{-1} or g^{-1} in the water or diet, respectively, and c is the total Cu concentration in the water or diet ($\mu g\ l^{-1}$ or $\mu g\ g^{-1}$).

This equation has been widely used to calculate unidirectional waterborne (Grosell *et al.,* 1996, 1997, 1998, 2001b; Kamunde *et al.,* 2001, 2002c) and dietary (Clearwater *et al.,* 2000) Cu uptake in fish. For the purpose of comparison, the ^{64}Cu specific activities for the waterborne and diet were very similar. The time-course study showed that the uptake organs themselves (gills for waterborne ^{64}Cu exposure; gut for dietary ^{64}Cu exposure) tended to saturate over the 48-h exposure periods, whereas internal accumulation in the remainder of the body proceeded linearly with time. Therefore uptake rates were also calculated for the whole-body without the gills and for the whole-body without the gut for the waterborne and dietary exposures, respectively, to yield the rates of internal accumulation via the two routes.

Newly accumulated Cu into individual tissues or organs, by reference to the specific activity of Cu in the exposure system (water or diet), was calculated using the same equation with a being ^{64}Cu cpm g^{-1} tissue or organ.

For total Cu determination, all tissues were digested overnight at 70°C with 6 volumes of 1N nitric acid (Fisher Scientific, trace metal grade), and 1.5 ml aliquots were centrifuged for 4 min at 13 000 x *g*. A sub-sample of the supernatant was diluted appropriately with 0.5% nitric acid, and total Cu concentration was determined by atomic absorption spectroscopy (AAS; Varian AA-1275 with GTA furnace atomizer) using a 10-µl injection volume and operating conditions for Cu specified by the manufacturer. Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range.

For the purpose of comparison (see *Discussion*), the exchangeable pools of Cu after the 48-h waterborne and dietary ^{64}Cu exposures were calculated at day 28 from the total Cu and newly accumulated Cu for each tissue using 2 different methods. The first method was by reference to the specific activity of Cu in exposure system (diet or water) as in equation (1) above. This approach assumed that ^{64}Cu in the exposure medium was equally available for uptake into all the tissues. The second method was by reference to the specific activity of the previous compartment (Grosell *et al.,* 1997; Kamunde *et al.,* 2001), employing the following equation to calculate newly accumulated Cu.

$$a(de^{-1})^{-1}, \qquad (2)$$

where a is the cpm per gram tissue, d is ^{64}Cu cpm l^{-1} or $\mu g\ g^{-1}$ in the previous compartment, and e is the total Cu concentration in the previous compartment in $\mu g\ l^{-1}$ or $\mu g\ g^{-1}$. Here it was assumed that ^{64}Cu and total Cu in the previous compartment were in complete equilibrium and that the specific activity of the previous compartment was homogeneous throughout the compartment. For the waterborne exposures, the previous compartments were water for gill, gill for plasma, and plasma for all other tissues. For the dietary exposures, the previous compartments were diet for gut tissue, gut tissue for plasma, and plasma for all other tissues.

The percent exchangeable Cu was then calculated as:

$$100\ (Cu_{new} \bullet Cu_{tot}^{-1}), \qquad (3)$$

where Cu_{new} is newly accumulated Cu and Cu_{tot} is total Cu in the previous compartment, both in μg per g (or μg per ml for plasma).

Gill Cu-binding

Waterborne ^{64}Cu flux measurements during the Cu exposure experiment revealed decreased Cu uptake in Cu-exposed fish. Consequently, an additional 28-day exposure of juvenile rainbow trout to 22 μg l^{-1} Cu (n = 60) or background conditions (2 μg l^{-1}) (n = 60) was carried out. At the end of the 28-day period, a 3-h gill Cu-binding assay was performed for both the control and the Cu-exposed fish. To characterize the possible effects of Cu-exposure on the high affinity-low capacity, and low affinity-high capacity Cu-binding sites (Taylor *et al.,* 2000; Grosell and Wood, 2002), a wide range of waterborne Cu concentrations (2.6 to 84.9 μg l^{-1}) was used. For each concentration, the fish were exposed to the required concentration labeled with ^{64}Cu [total dose as ^{64}Cu ($CuNO_3$)] for 3 h. The fish were then terminally anesthetized in MS-222 (1 g l^{-1}) and gills were excised, rinsed in double distilled water, and counted for ^{64}Cu γ radioactivity as described above. Copper bound to the gills was calculated using an equation analogous to that for whole body Cu uptake (Equation 1).

Statistics

Values are means ± SEM. Linear and non-linear regression lines were fitted using SigmaPlot 2000 (SPSS Inc., Chicago, IL). Effects of exposure conditions on

tissue Cu concentration and subsequent waterborne and dietary Cu uptake at each sampling point were assessed using analysis of variance (ANOVA) with time, and waterborne Cu concentration, acclimation, or route of exposure as variables. Tukey's multiple comparison procedure for significant difference or paired Student's *t*-test (as appropriate) was used to make comparisons between measurements. In all cases, differences were considered significant at $p < 0.05$.

RESULTS

Time course of acute waterborne and dietary Cu uptake

Unidirectional Cu uptake over time into whole-body with and without the gill for the waterborne exposure is shown in Fig. 1A. New Cu accumulation into the whole-body tended to plateau, whereas "internal" uptake into the whole-body without the gills was linear over the 48 h. This observation is consistent with the linear Cu uptake into all the organs over time except the gills, in which there was an initial rapid uptake to about 40 ng g^{-1} newly accumulated Cu, followed by a decline and stabilization at about 25 ng g^{-1} (Fig. 2A). After 48 h, newly accumulated Cu concentration in the tissues were ranked in order as follows: liver > gill > gut > plasma > carcass.

Similarly for the dietary time course experiment, Cu uptake into the whole-body tended to saturate over time whereas "internal" uptake remained linear for the whole-body without gut over the 48 h (Fig. 1B). Again the saturable component was attributed to the uptake site, i.e., gut tissues (stomach, pyloric cecae + anterior intestine, mid-intestine and posterior intestine; Fig. 2C), while uptake into the other organs was linear

with time (Fig. 2B). At 48 h, the highest amount of Cu was found in the posterior intestine followed in decreasing order by the pyloric cecae + anterior intestine, mid-intestine, and stomach. In the other tissues new Cu accumulation was ranked in the following order: liver > plasma > gill > carcass. Generally new Cu accumulation into the whole-body and internal tissues was much higher during dietary ^{64}Cu exposure than during waterborne ^{64}Cu exposure.

Based on these results, 48 h exposures were employed in subsequent waterborne and dietary ^{64}Cu uptake determinations, and our measurements focussed on the linear internal accumulation rates (i.e., whole-body minus uptake organ). However, whole-body uptake rates were also calculated, yielding the same basic trends.

Growth, Cu accumulation, and Cu depuration

Exposure to 22 $\mu g\ l^{-1}$ Cu had no effect on growth. All the fish exhibited a twofold increase in weight from 9 - 11 to 23 - 24 g over 28 days and there was negligible mortality (2 deaths in 900 fish). Whole-body Cu concentration increased in the Cu-exposed fish from about 1.5 $\mu g\ g^{-1}$ to 2.0 $\mu g\ g^{-1}$. The increase in whole-body Cu concentration was due to a small Cu accumulation in the gill and the carcass and a large accumulation in the liver. There were no treatment-related changes in the Cu concentration in the plasma, kidney, gut, and bile. In particular, total plasma Cu remained within narrow margins between 0.65 and 0.86 $\mu g\ ml^{-1}$ for both the control and Cu-exposed fish.

During the depuration phase of the study (data not shown) loss of whole-body Cu after 28 days of waterborne Cu exposure was slow and could be attributed to the slow loss from the liver. Liver and whole-body Cu concentration returned to control levels only at day 30 of depuration. Gills exhibited more rapid clearance of the Cu burden and returned to the control levels within 20 days post-exposure.

Whole-body waterborne and dietary Cu uptake rates

At 2.8 μg l^{-1} waterborne Cu concentration, whole-body (without gills) unidirectional uptake rates of Cu from the water ranged from 0.02 to 0.07 ng g^{-1} h^{-1} and were significantly lower in the previously Cu-exposed fish at day 28 (Fig. 3A). The mass-specific uptake rates tended to decrease with time as fish increased in size. At 22 μg l^{-1} waterborne Cu concentration, whole-body Cu uptake rates were about tenfold higher compared to the values at background waterborne Cu level (2.8 μg l^{-1}) and ranged between 0.29 and 0.55 ng g^{-1} h^{-1} (Fig. 3B). Previously Cu-exposed fish had significantly lower rates of uptake relative to the controls on days 14 and 28. Absolute whole-body waterborne unidirectional Cu uptake rates shown in Table 1 followed similar trends but were quantitatively higher, a reflection of the branchial contribution.

Whole-body (without gut) unidirectional Cu uptake rates during the 48-h dietary ^{64}Cu exposure ranged from 0.40 to 0.90 ng g^{-1} h^{-1} for both control and Cu-acclimated fish (Fig. 4). Thus whole-body (without gut) dietary Cu uptake rates were more than tenfold higher than those of the whole body without the gills under background conditions (2-3 μg l^{-1} Cu), but comparable to waterborne uptake rates in fish exposed to

an elevated waterborne level (22 μg l^{-1}). Unlike waterborne Cu uptake, neither the ambient water Cu concentration nor the fish size affected the rate of Cu uptake from the diet. Absolute whole-body unidirectional Cu uptake rates (Table 1) followed the same trend but were up to twofold higher, reflecting a major contribution of gut tissue to whole-body ^{64}Cu accumulation during dietary exposures.

Newly accumulated Cu in tissues

Newly accumulated waterborne Cu in tissues of control and previously Cu-exposed fish are shown in Fig. 5. Newly accumulated Cu (acquired over 48 h exposure to waterborne ^{64}Cu) in the gills ranged from 6 to 16 ng g^{-1} in fish exposed to background Cu level (2.8 μg l^{-1}) and was significantly lower in previously Cu-exposed fish on days 14 and 28 (Fig. 5-1A). At 22 μg l^{-1} waterborne Cu (Fig. 5-1B), newly accumulated gill Cu ranged from 36 to 90 ng g^{-1} and was therefore about 6 times higher than in fish exposed to background (2.8 μg l^{-1}) waterborne Cu. Again, previously Cu-exposed fish had significantly lower values on day 28. In the liver, newly accumulated waterborne Cu was between 19 and 65 ng g^{-1} in the 2.8 μg l^{-1} exposure (Fig. 5-2A), whereas the figure increased to 240 – 860 ng g^{-1} at the high waterborne Cu (22 μg l^{-1}, Fig. 5-2B). Previously Cu-exposed fish had significantly lower new hepatic Cu accumulation compared to the controls on day 28 for the background Cu level, and on days 14 and 28 for the elevated Cu level. For plasma (Fig. 5-3A,B) and gut (Fig. 5-4A,B), Cu pre-exposure had no effect on the newly accumulated waterborne Cu. Plasma newly accumulated Cu was, however, strongly influenced by the water Cu concentration

during the exposure, rising from 6 - 28 ng g^{-1} during background exposures (2.8 μg l^{-1}) to 66 - 103 ng g^{-1} during high waterborne Cu exposures (22 μg l^{-1}). In the carcass, newly accumulated Cu ranged between 0.5 and 1.4 under background conditions (2.8 μg l^{-1} Cu, Fig. 5-5A), and was significantly elevated to 4 - 13 ng g^{-1} at high ambient water Cu concentration (22 μg l^{-1}, Fig. 5-5B). Newly accumulated Cu was lower in the carcass of Cu pre-exposed fish on day 28 under background Cu levels (2.8 μg l^{-1} Cu) and on days 14 and 28 under elevated Cu levels (22 μg l^{-1}).

For the 48-h dietary ^{64}Cu exposures, new Cu accumulation in the gills ranged from 15 to 27 ng g^{-1} and was higher on all days than that accumulated during waterborne ^{64}Cu exposures at background water Cu level (Fig. 5-1A). However, at 22 μg l^{-1} water Cu level (Fig. 5-1B), the newly accumulated dietary gill Cu was lower than during the comparable waterborne ^{64}Cu exposures on all days. Newly accumulated Cu in the liver during dietary exposures ranged from 680 to 1590 ng g^{-1}, and was therefore much higher than waterborne exposures (Fig. 5-2A,B). Previously Cu-exposed fish had significantly lower new Cu accumulation in the liver on day 28 in the exposures done at 2.8 μg l^{-1} waterborne Cu concentration. Plasma accumulated 45 to 100 ng ml^{-1} new Cu, and Cu pre-exposure and ambient water Cu concentration during the dietary exposures had no effect except on day 14 when acclimated fish had higher levels (Fig. 5-3A,B). Newly accumulated Cu in plasma was higher for all the dietary exposures compared to the values for the waterborne exposures carried out at 2.8 μg l^{-1} waterborne [Cu]. In the gut newly accumulated Cu ranged from 400 to 1200 ng g^{-1}, up to 200-fold higher than during waterborne exposures (Fig. 5-4A,B). Waterborne Cu pre-exposure had no effect

on new dietary Cu accumulation in the gut. New dietary Cu accumulation in the carcass was 8-22 ng g^{-1} and was not affected by pre-exposure to waterborne Cu (Fig. 5-5A,B). However, these values were generally higher than those of the waterborne ^{64}Cu exposures.

Gill Cu-binding characteristics

The short-term (3-h) gill Cu-binding assay revealed a biphasic effect of previous waterborne Cu exposure on gill Cu-binding kinetics (Fig. 6). At waterborne Cu below 10 μg l^{-1} waterborne Cu pre-exposed fish exhibited lower Cu binding compared to the controls (Fig. 6, inset). However, at higher waterborne [Cu], the reverse occurred: control fish exhibited much lower gill Cu-binding compared to the acclimated fish.

DISCUSSION

Dietary *versus* waterborne Cu uptake

Under the conditions of background levels of Cu in the water and diet, unidirectional Cu uptake rates into internal tissues, as well as into the whole-body, were more than tenfold higher during dietary than during waterborne ^{64}Cu exposure, suggesting that diet is a much more important source of Cu to fish than water. This finding is based on direct measurements, but considers only unidirectional uptake at the two sites, not net fluxes. Nevertheless, the finding is consistent with our recent conclusion, based on indirect calculations, that dietary uptake contributes more than 90% of body Cu content under normal waterborne and dietary Cu conditions (Kamunde

et al., 2002c). Interestingly, both uptake sites, i.e., gills for waterborne and gut for dietary exposures, saturated over time but the rest of the organs/tissues exhibited linear Cu accumulation via either route of exposure (Fig. 1). This suggests the presence of homeostatic mechanisms at both tissues that operate to bring rates of uptake and export (either to the internal tissues or to the medium) into equilibrium. The present study also demonstrates that Cu absorption from both water and diet, and transport across the gill and gut epithelial cells, are rapid processes, with Cu appearing in plasma within 3 h of exposure to ^{64}Cu via either route. For dietary uptake, this probably means that partial Cu absorption occurred in the fish stomach, as is the case in mammals (Wapnir, 1998). The appearance of ^{64}Cu radioactivity in all segments of the gut within 3 to 6 h of feeding (when ingested food was still all in the stomach) suggests that Cu movement and absorption along the gut are independent of diet movement and absorption.

The high accumulation of new Cu in the posterior intestine is consistent with recent findings by Clearwater *et al.* (2000) and suggests that this region of the fish intestine is the most important for Cu absorption. Intestinal uptake of Cu in fish is thought to occur via both simple diffusion for apical entry and biologically mediated transport for basolateral exit (Clearwater *et al.,* 2000; Handy *et al.,* 2000). Recently, Bury *et al.* (2001) demonstrated that Fe^{2+} was also preferentially transported in the posterior intestine. The apparent saturation of the gut tissues observed in the present study (Fig. 2C) is consistent with earlier reports of a strong Cu regulatory capacity of gut tissue (Berntssen *et al.*, 1999; Clearwater *et al.,* 2000; Kamunde *et al.,* 2001).

Effect of previous Cu exposure on waterborne and dietary Cu uptake

Studies that have assessed the effect of waterborne Cu exposure on subsequent uptake of waterborne Cu focussed primarily on uptake into the gills and reported variable results (Grosell *et al.,* 1996, 1997, 1998; McCarter and Roch, 1984; Taylor *et al.,* 2000). In the present study we also evaluated the unidirectional uptake of Cu into the whole-body and demonstrated a substantial reduction in the uptake rates of waterborne Cu after 2 weeks of continuous exposure to environmentally realistic waterborne Cu concentrations (e.g., acclimation). A comparison of waterborne and dietary Cu uptake rates into the whole-body excluding the gills or gut (Figs. 3 and 4) revealed a tenfold higher rate for dietary Cu uptake. Including the gill for the waterborne exposures increased whole-body waterborne Cu uptake rate by only about 10%, while including the gut for dietary exposures doubled the dietary Cu uptake rate (Table 2). Thus in absolute terms, whole-body dietary Cu uptake rates were up to 30 times higher than the waterborne Cu uptake rates.

Contrary to our original hypothesis, previous exposure to waterborne Cu had no effect on the uptake rates of dietary Cu even though pre-exposure to high dietary Cu decreases waterborne Cu uptake and pre-exposure to low dietary Cu increases waterborne Cu uptake in this species (Kamunde *et al.,* 2001; 2002c). In mammals, which have only one route of uptake, the rates and efficiency of dietary Cu absorption change in response to whole-body Cu status and level of dietary Cu exposure (Turnlund *et al.,* 1998). This effect has been explained on the basis that Cu regulates the proteins involved in Cu homeostasis, e.g., Cu-ATPases (Harrison and Dameron, 1998; Schaefer

and Gatlin, 1999). In fish, where there are two significant routes of Cu uptake, it is possible that the gut serves for bulk acquisition, while the gill performs homeostatic fine-tuning via adjustment of branchial Cu transport proteins.

Newly accumulated Cu in tissues

Previous waterborne Cu exposure decreased the accumulation of new Cu in the gills (Fig. 6), contrary to several previous reports (Grosell *et al.,* 1998; McCarter and Roch, 1984; Taylor *et al.,* 2000). Furthermore, the differential gill Cu binding response dependent on the ambient water Cu concentration (see *Gill Cu-binding*) points to the existence of at least two Cu uptake/transport mechanisms at the gills.

Waterborne Cu pre-exposure decreased new Cu accumulation from the water into the liver suggesting down-regulation of branchial export of Cu into the liver or occupation of potential Cu-binding/deposition sites in the liver by Cu accumulated during the acclimation. Interestingly, new Cu accumulation into the liver during 48-h dietary ^{64}Cu exposure was also decreased by previous exposure to waterborne Cu. However, even after accounting for differences in the rates of uptake by the 2 routes, newly accumulated liver Cu following dietary ^{64}Cu exposures was much higher than during waterborne exposures, indicating that dietary Cu was more readily available for hepatic uptake. Anatomical considerations may play an important role here since the liver is immediately downstream of the gastrointestinal tract and receives materials absorbed from the gut via the venous hepatic portal system. In contrast, Cu absorbed from the water via the gills is likely transported via arterial circulation and deposited in

other peripheral tissues prior to reaching the liver. The significant decrease in newly accumulated Cu in the carcass during 48-h waterborne ^{64}Cu exposures in Cu pre-exposed fish underlines the fact that Cu pre-exposure and the attendant elevation in tissue Cu concentration result in widespread decline in the demand for new Cu uptake in rainbow trout. Together, these data suggest that cellular Cu transport mechanisms respond to body Cu status during chronic waterborne Cu exposure in the same manner as during dietary exposures in fish (Kamunde *et al.,* 2001, 2002c) and in humans (Turnlund *et al.,* 1998).

Cu turnover and exchangeable Cu pools

The exchangeable Cu pools were estimated for both the control and Cu pre-exposed fish based on new Cu uptake into the tissues directly from the water or diet (Table 2A) or from the previous compartment (Table 2B) at 2.8 and 22 $\mu g\ l^{-1}$ water Cu. These calculations report the short-term exchangeable pool size – i.e., that percent of the total tissue Cu content that is exchangeable in a 48-h period – using two different assumptions as outlined in the methods. Using the system specific activity (i.e., a calculation that gives the fraction which is exchangeable with Cu in the exposure medium) revealed that ambient water Cu concentration was directly correlated with the exchangeable Cu pools during waterborne exposures: a tenfold increase in the ambient Cu concentration caused a similar increase in the exchangeable Cu pools. Waterborne Cu pre-exposure significantly reduced the exchangeable waterborne Cu pools in gills, liver, and carcass, consistent with increased total Cu concentration and reduced newly

accumulated Cu in these tissues. Previous studies have reported increased synthesis of Cu binding proteins, e.g., metallothionein, acid soluble thiols, and glutathione in fish tissues during chronic exposure to waterborne Cu (Dang *et al.*, 1999; Laurén and McDonald, 1987b; McCrater and Roch, 1984; Paris-Palacios *at al.,* 2000). It is likely that binding of Cu to such proteins in the Cu pre-exposed fish affected the movement of Cu among the tissues. Indeed, the slow loss of whole-body and hepatic Cu observed during depuration suggests that the Cu in fish tissues existed in slowly exchangeable form following chronic waterborne Cu exposure. For dietary exposures, the exchangeable Cu pools were higher than for the waterborne exposures in all the tissues except the gills. In particular, the gut exchangeable Cu pool from the diet was up to 200 times higher than the gut exchangeable Cu pool from the water, underlining the importance of this organ in dietary but not waterborne Cu uptake. Thus, Cu turnover in fish is much higher during dietary exposure.

Clearwater *et al.* (2000) and Laurén and McDonald (1987b) calculated exchangeable Cu pools in trout tissues using a similar method for dietary and waterborne Cu exposure, respectively. Our results are generally in agreement with Clearwater *et al.* (2000) for dietary exposure but are lower than those reported by Laurén and McDonald for waterborne exposure. The discrepancy with the latter study can be explained on the basis of the ambient Cu levels used in the present study (2.8 and 22 $\mu g\ l^{-1}$) *versus* 55 $\mu g\ l^{-1}$ in Laurén and McDonald.

Using previous compartment analysis (i.e., a calculation that gives that fraction that is exchangeable with the previous compartment, as defined in the methods) resulted

in much higher exchangeable Cu pools in all the organs for both exposure routes, except, of course, the gills for the waterborne exposures and gut tissue for the dietary exposures (Table 2). Except for the gills and the liver during waterborne exposures, ambient Cu concentration had no effect on the exchangeable pools calculated in this manner. Another noteworthy observation is the much lower plasma exchangeable Cu pool during the dietary exposures, again perhaps reflecting the fact that much of the newly absorbed Cu was directly shunted to the liver by the venous hepatic portal system, rather than mixing freely with the entire plasma volume. Overall, the present data demonstrate a greater accessibility of plasma (as well as gill and gut) Cu for turnover with tissue pools than dietary or waterborne Cu.

Gill Cu-binding

The gill Cu-binding assay was developed to evaluate adsorption of Cu on or in the gill surface at equilibrium (Playle *et al.,* 1992). Our data show that 3-h values actually represent a peak (Fig. 2A), and a modest decline may ensue thereafter, which is in agreement with Grosell *et al.* (1997, 1998) and Grosell and Wood (2002). Furthermore, significant Cu internalization also occurs within 3 h (Fig. 1A). Waterborne Cu pre-exposure had both a stimulatory and an inhibitory effect on the 3-h gill Cu-binding dependent on the ambient Cu (Fig. 6). At waterborne Cu below 10 $\mu g\ l^{-1}$, Cu-binding to the gill was decreased while at waterborne Cu above 10 $\mu g\ l^{-1}$, Cu-binding to the gill was increased in acclimated fish. Several different types of Cu binding sites have now been identified in trout gills. Taylor *et al.* (2000) described high affinity-low capacity

Cu-binding sites predominantly at less than 15 μg l^{-1} Cu and low affinity-high capacity sites becoming important above this level of water Cu. More recently, Grosell and Wood (2002) identified a sodium-sensitive and a sodium-insensitive component of the high affinity gill Cu-binding sites. Here we demonstrate a diametrically opposite effect of waterborne Cu pre-exposure on gill Cu binding dependent on test concentration. The waterborne Cu concentration at which this reversal occurs is about 10 μg l^{-1} and suggests a different effect of waterborne Cu pre-exposure on the high affinity-low capacity sites and low affinity-high capacity sites as described by Taylor *et al.* (2000). In agreement with the proposal by Reid and McDonald (1991), the high affinity sites appear to be a small proportion of total sites. The present data also offer a credible explanation to the variable reports on the effects of waterborne Cu pre-exposure on subsequent uptake of Cu into the gills (Grosell *et al.,* 1996, 1997, 1998; McCarter and Roch ,1984; Taylor *et al.,* 2000). In these studies assessment of Cu uptake over a wide range of waterborne Cu levels (1–100 μg l^{-1}) produced different results possibly due to different effects of Cu pre-exposure on the two gill Cu-binding sites.

Copper uptake at the gills is thought to occur at least partly through Cu-ATPase based on vanadate sensitivity of branchial Cu uptake (Campbell *et al.,* 1999) and partial cloning of a putative Cu-ATPase in gill tissue (Grosell *et al.,* 2001). The recent identification of a phenamil-sensitive, bafilomycin-sensitive, high affinity Cu uptake pathway in trout gills (Grosell and Wood, 2002) suggests the involvement of the apical Na^+ channel/H^+-ATPase in part of the high affinity Cu uptake. Taking results from both Taylor *et al.* (2000) and Grosell and Wood (2002) into consideration, we interpret this

differential gill Cu-binding to mean that pre-exposure to waterborne Cu down-regulates high affinity Cu uptake sites (both sodium-sensitive and sodium-insensitive) but stimulates low affinity-high capacity Cu uptake sites in trout gills. The adaptive significance of the latter is not obvious at the present time, but clearly it did not translate into greater internal or whole-body uptake at the exposure concentration.

Perspectives

This study demonstrates greater uptake and turnover of dietary Cu relative to waterborne Cu in fish, and a marked reduction in the uptake rates of waterborne but not dietary Cu following chronic waterborne Cu exposure. Clearly, waterborne Cu pre-exposure modifies gill, but not gut, Cu uptake mechanisms. The binding and transport kinetics of Cu in the gut epithelium in fish and the effect of Cu pre-exposure on both dietary and waterborne Cu uptake kinetic parameters require further attention. Although recent studies suggest the presence of Cu transport proteins at the gill (Campbell, 1999; Grosell *et al.,* 2002) and gut (Clearwater *et al.,* 2000, Handy *et al.,* 2000) and the involvement of Na^+ uptake pathways (Grosell and Wood, 2002; Wapnir, 1991), characterization of these transport mechanisms using pharmacological and molecular biological tools remains incomplete. Furthermore, illumination of the relationship and interactions between Cu transport at the gill, gut, and hepatobiliary interface would greatly contribute to the understanding of Cu regulation and metabolism in fish.

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Table 4-1. Whole body Cu uptake rates in control and Cu-exposed juvenile rainbow trout after 48-h exposure to waterborne or dietary ^{64}Cu. Values are means ± SEM, ng g^{-1} h^{-1}, n = 10 per value. * Indicates significant difference from control waterborne fish at background Cu level at the same time and + indicates significant difference from control fish for the same exposure route and water Cu level.

Exposure	Waterborne				Dietary			
Water [Cu]	Background (2.8 μg l^{-1})		High (22 μg l^{-1})		Background (2.8 μg l^{-1})		High (22 μg l^{-1})	
Treatment	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed
Day 0	0.08±0.009	-	-	-	0.80±0.10*	-	-	-
Day 7	0.04±0.004	0.05±0.004	0.39±0.03*	0.38±0.04*	0.86±0.12*	0.70±0.20*	0.87±0.12*	0.65±0.12*
Day 14	0.06±0.004	0.05±0.008	0.58±0.04*	0.47±0.03*+	1.25±0.13*	1.31±0.16*	0.94±0.20*	1.11±0.20*
Day 28	0.05±0.007	0.03±0.001+	0.59±0.02*	0.32±0.02*+	1.62±0.48*	0.78±0.47*	1.84±0.26*	1.50±0.23*

Table 4-2. Exchangeable Cu pools (% of total Cu content exchangeable in 48 h) in tissues of control and Cu-exposed rainbow trout on day 28 following 48-h exposure to waterborne and dietary ^{64}Cu. Calculations were made using either the system specific activity (A) or previous compartment analysis (B). Values are means ± SEM, n = 10 per value. Percentage data were converted to arc sine for statistical analysis. * Indicates significant difference from control waterborne exposure pools at background Cu level and + indicates significant difference from control fish at the same water [Cu] and exposure route.

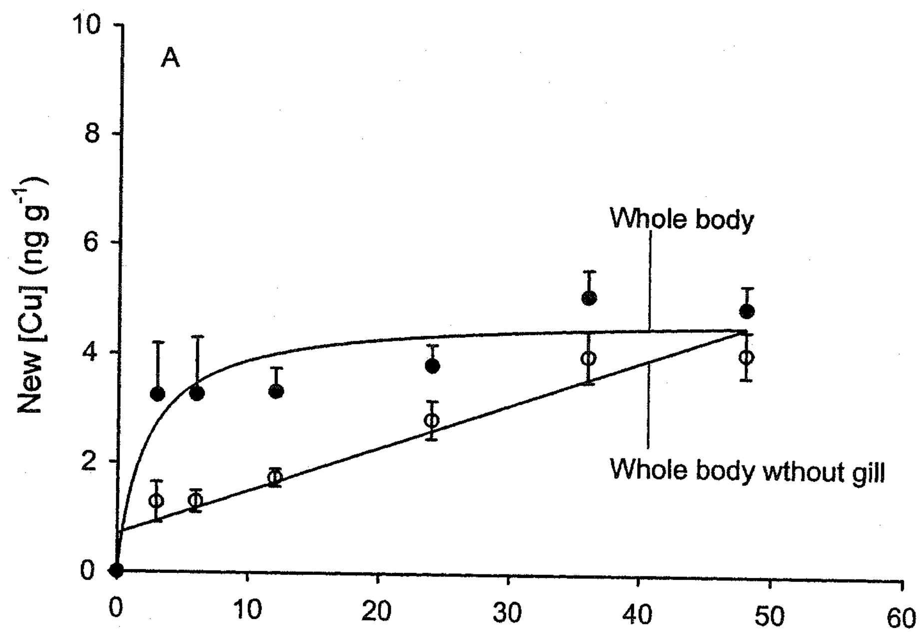
A: Exchangeable Cu using system (water or diet) specific activities after 48-h waterborne or dietary ^{64}Cu exposure

Tissue	Waterborne ^{64}Cu exposure				Dietary ^{64}Cu exposure			
Water [Cu]	Background (2.8 μg l^{-1})		High (22 μg l^{-1})		Background (2.8 μg l^{-1})		High (22 μg l^{-1})	
	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed
Gill	1.99±0.14	1.60±0.20	15.99±1.35*	11.58±1.90*+	4.48±1.00*	1.97±0.04	2.64±0.60	3.66±0.59*
Liver	0.034±0.003	0.020±0.002+	1.01±0.05*	0.34±0.04*+	2.27±0.53*	0.56±0.21*+	0.90±0.27*	1.05±0.21*
Plasma	0.78±0.10	1.03±0.11	10.72±1.86*	8.77±1.18*	8.40±1.55*	4.54±1.37*	5.46±1.72*	6.34±1.18*
Gut	0.18±0.03	0.22±0.05	2.55±0.42*	2.11±0.67*	42.92±9.96*	21.61±6.93*	36.50±7.62*	42.46±6.30*
Carcass	0.19±0.02	0.12±0.02+	1.70±0.19*	1.00±0.13*+	3.41±0.93*	1.77±0.52*	2.21±0.63*	3.63±0.63*

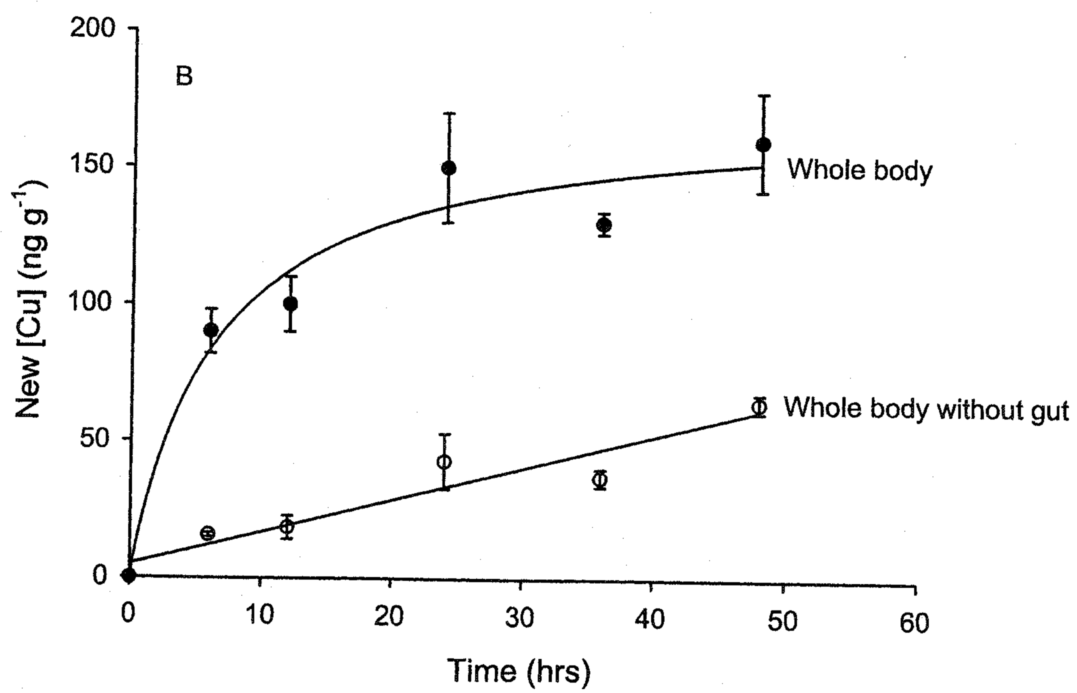
B: Exchangeable Cu using previous compartment analysis after 48-h waterborne or dietary ^{64}Cu exposure

Tissue	Waterborne ^{64}Cu exposure				Dietary ^{64}Cu exposure			
Water [Cu]	Background (2.8 μg l^{-1})		High (22 μg l^{-1})		Background (2.8 μg l^{-1})		High (22 μg l^{-1})	
	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed	Control	Cu-exposed
Gill	1.99±0.14	1.60±0.20	15.99±1.35*	11.58±1.90*	82.93±7.81*	91.02±12.03*	93.25±14.24*	62.50±6.12*
Liver	6.44±2.47	3.06±0.37	17.43±5.48*	10.39±2.98	29.57±4.11*	19.94±4.18*	38.45±5.76*	30.08±5.33*
Plasma	56.98±9.52	53.28±6.68	50.07±5.22	78.05±14.36	13.12±1.50*	15.18±2.95*	9.62±2.11*	12.33±2.31*
Gut	34.90±8.11	24.43±6.56	33.29±9.06	16.89±4.11	42.92±9.96	21.61±6.93	36.50±7.62	42.46±6.30
Carcass	27.65±3.64	16.75±3.02+	22.98±4.69	26.47±6.32	69.40±9.99*	52.52±8.19*	86.23±40.54*	61.10±11.09*

Figure 4-1. Time course of unidirectional waterborne (A) and dietary (B) Cu uptake traced with ^{64}Cu in juvenile rainbow trout during acute exposure. Values are means ± SEM, ng g^{-1}, n = 5 for each data point. Closed circles represent whole-body newly accumulated Cu for each route of uptake and open circles represent Cu accumulation in the whole-body without the gills for waterborne exposure and whole-body without the gut for the dietary exposure. Uptake into the whole-body approached a plateau for both routes of uptake while uptake into the whole-body without the gills or gut remained linear over time. The r^2 for all the regression lines were between 0.91 and 0.95.

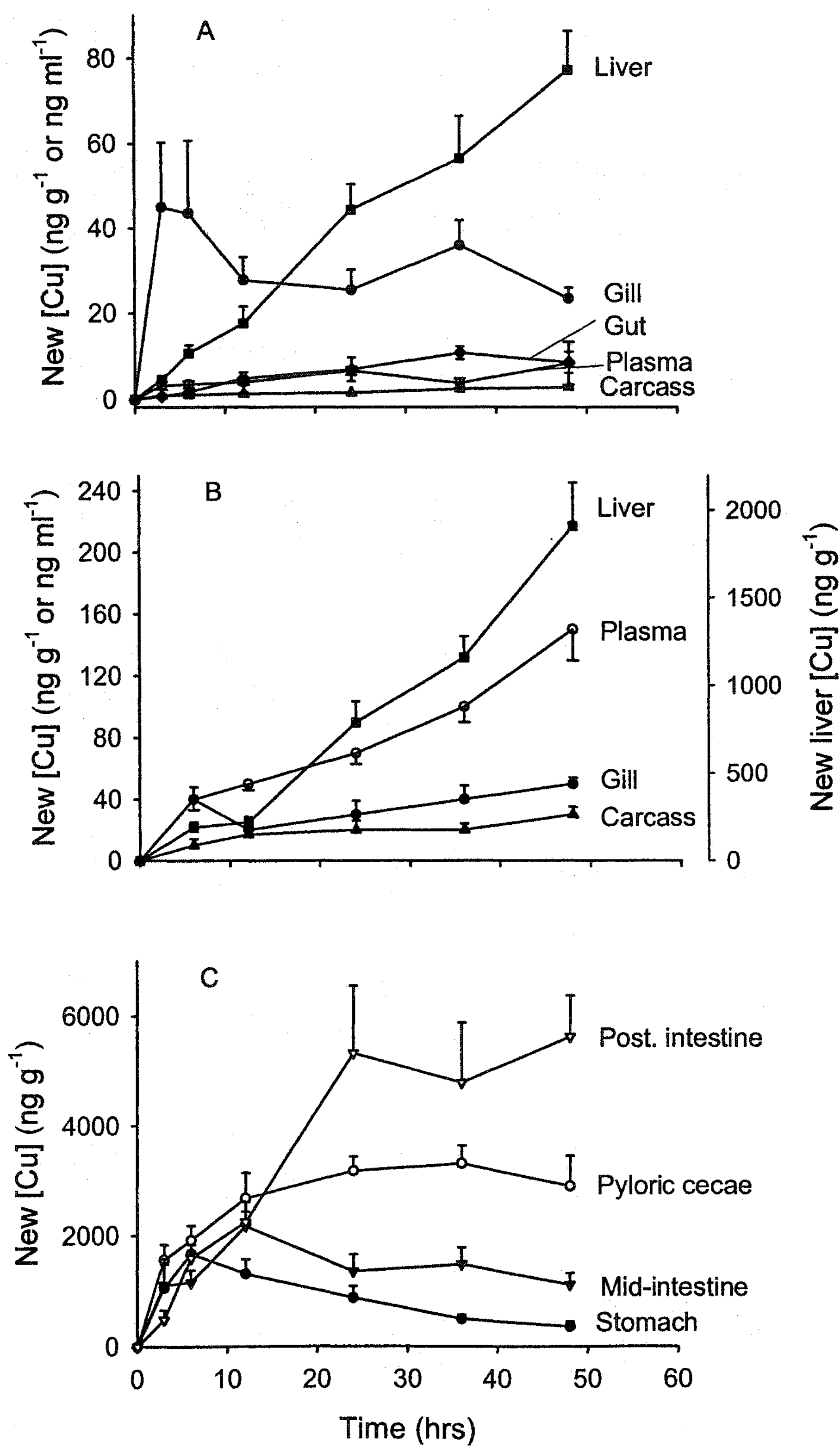


A
New [Cu] (ng g-1)
Whole body
Whole body wthout gill
0
2
4
6
8
10
0
10
20
30
40
50
60



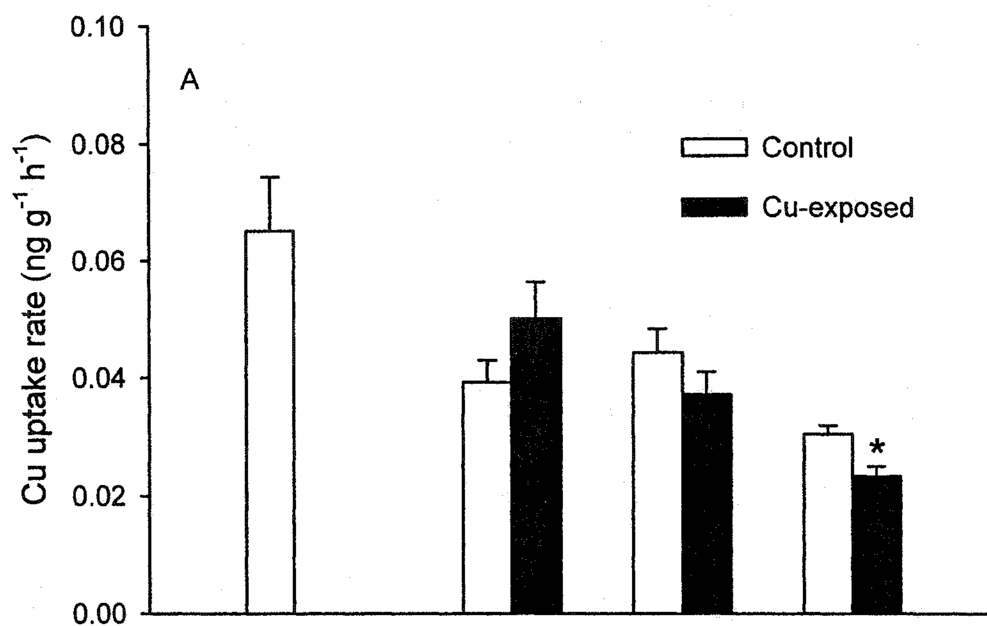
B
New [Cu] (ng g-1)
Whole body
Whole body without gut
Time (hrs)
0
50
100
150
200
0
10
20
30
40
50
60

Figure 4-2. Distribution of newly accumulated Cu in tissues of juvenile rainbow trout during acute exposure to waterborne (A) and dietary (B and C) ^{64}Cu for 48 h. Values are means ± SEM, ng g^{-1}, n = 5 for each data point. Note the difference in scale for the liver plot in panel B.

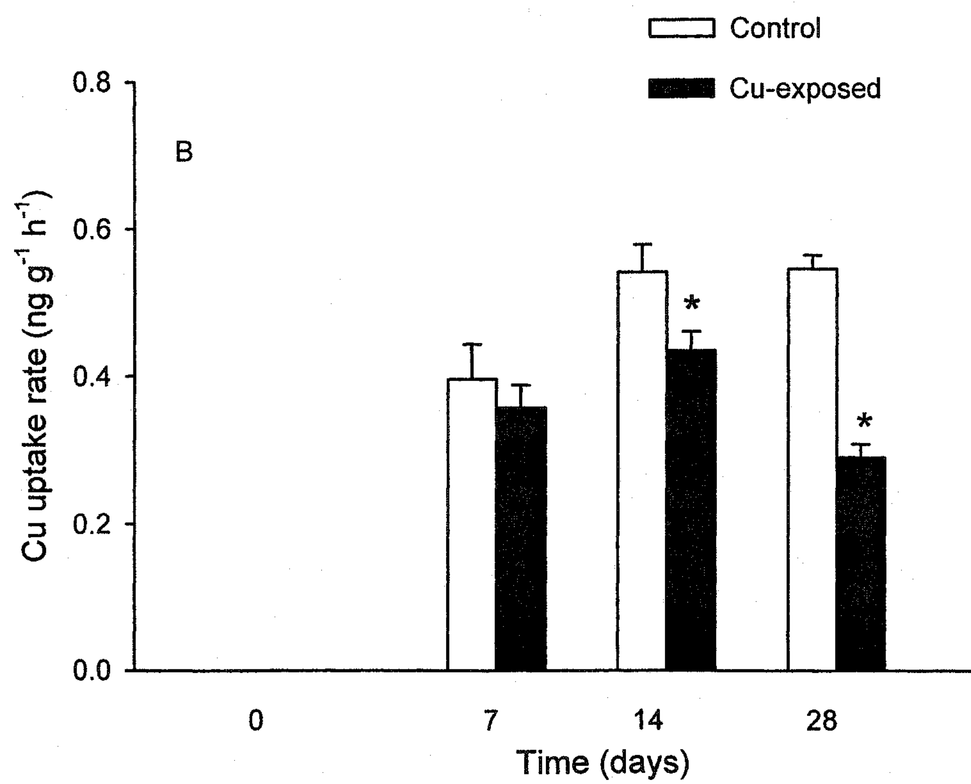


A
New [Cu] (ng g-1 or ng ml-1)
80
60
40
20
0
Liver
Gill
Gut
Plasma
Carcass
B
New [Cu] (ng g-1 or ng ml-1)
240
200
160
120
80
40
0
Liver
Plasma
Gill
Carcass
New liver [Cu] (ng g-1)
2000
1500
1000
500
0
C
New [Cu] (ng g-1)
6000
4000
2000
0
Post. intestine
Pyloric cecae
Mid-intestine
Stomach
0
10
20
30
40
50
60
Time (hrs)

Figure 4-3. Whole-body (without the gills) unidirectional Cu uptake rates traced with ^{64}Cu, from the water in control and chronic Cu-exposed rainbow trout. Values are means ± SEM, ng g^{-1} h^{-1}, n = 10 per bar. Panel A shows uptake rates at 2.8 μg l^{-1} waterborne [Cu] and panel B shows uptake rates at 22 μg l^{-1} waterborne Cu. * Indicates significant difference from respective control, $p < 0.05$.

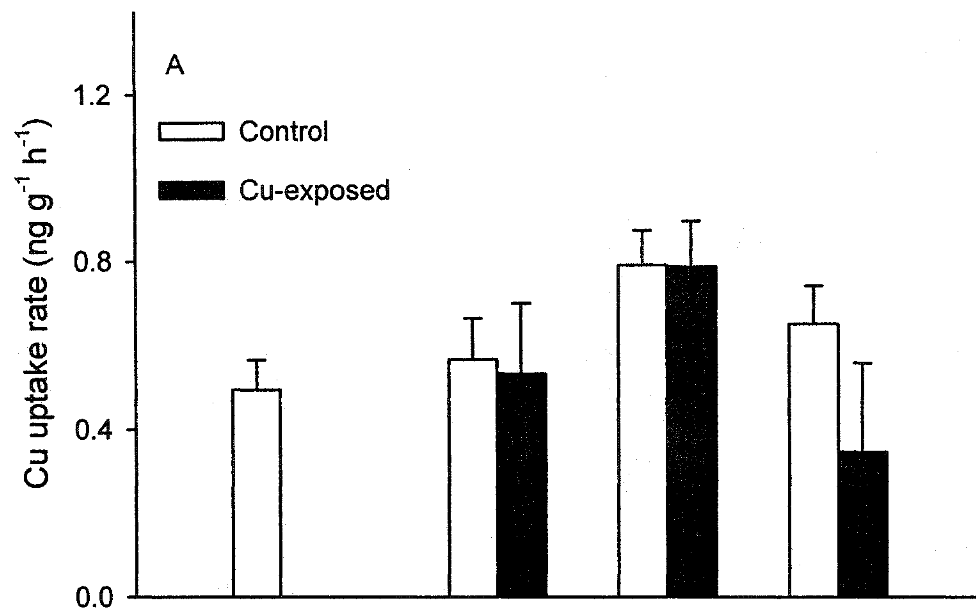


0.10
0.08
0.06
0.04
0.02
0.00
A
Control
Cu-exposed
Cu uptake rate (ng g^{-1} h^{-1})
*



Control
Cu-exposed
0.8
0.6
0.4
0.2
0.0
B
Cu uptake rate (ng g^{-1} h^{-1})
*
*
0
7
14
28
Time (days)

Figure 4-4. Whole-body (without the gut) unidirectional Cu uptake rates traced with ^{64}Cu, from the diet in control and chronic Cu-exposed rainbow trout. Values are means ± SEM, ng g^{-1} h^{-1}, n = 10 per data point. Panel A shows dietary Cu uptake rate at 2.8 μg l^{-1} waterborne [Cu] and panel B shows uptake rates at 22 μg l^{-1} waterborne [Cu].


A
Control
Cu-exposed
Cu uptake rate (ng g^{-1} h^{-1})
1.2
0.8
0.4
0.0

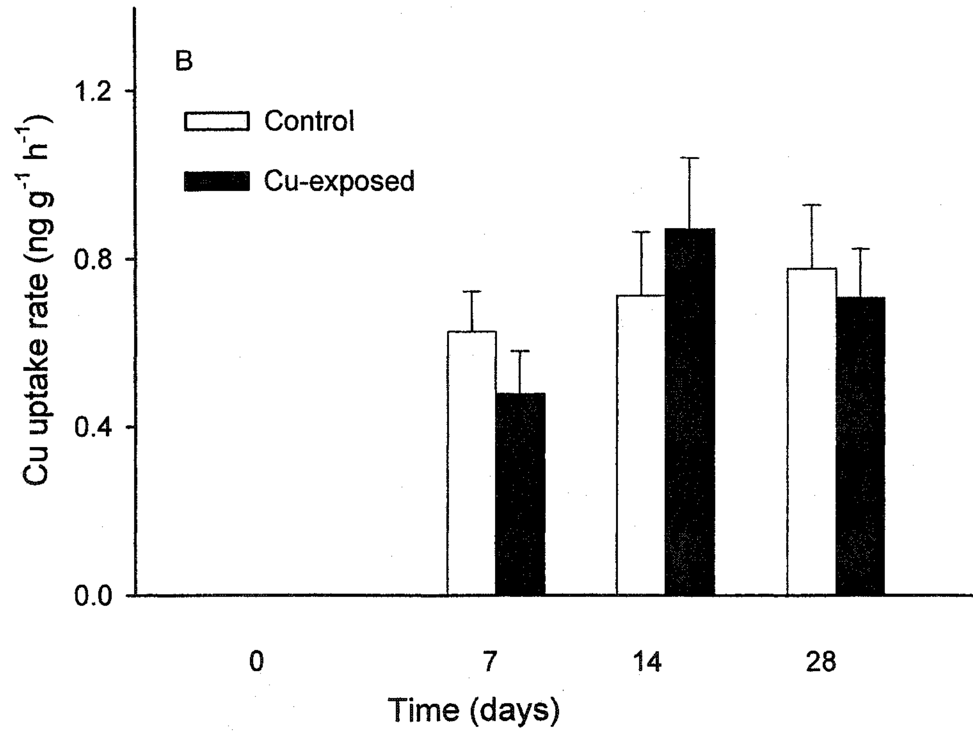

B
Control
Cu-exposed
Cu uptake rate (ng g^{-1} h^{-1})
1.2
0.8
0.4
0.0
0
7
14
28
Time (days)

Figure 4-5. Newly accumulated Cu calculated by reference to the specific activity of Cu in the exposure system (water or diet), in tissues of control and chronic Cu-exposed fish 48 h after waterborne and dietary ^{64}Cu exposures. Values are means ± SEM, ng g^{-1} or ng ml^{-1}, n = 10 per bar. Panels 1A to 5A are data for exposures performed at 2.8 μg l^{-1} waterborne [Cu] and panels 1B to 5B are data for exposures performed at 22 μg l^{-1} waterborne Cu. + Indicates significant difference from newly accumulated waterborne Cu at background Cu level and * indicates significant difference from control fish within an exposure condition at a particular sampling time, $p < 0.05$.

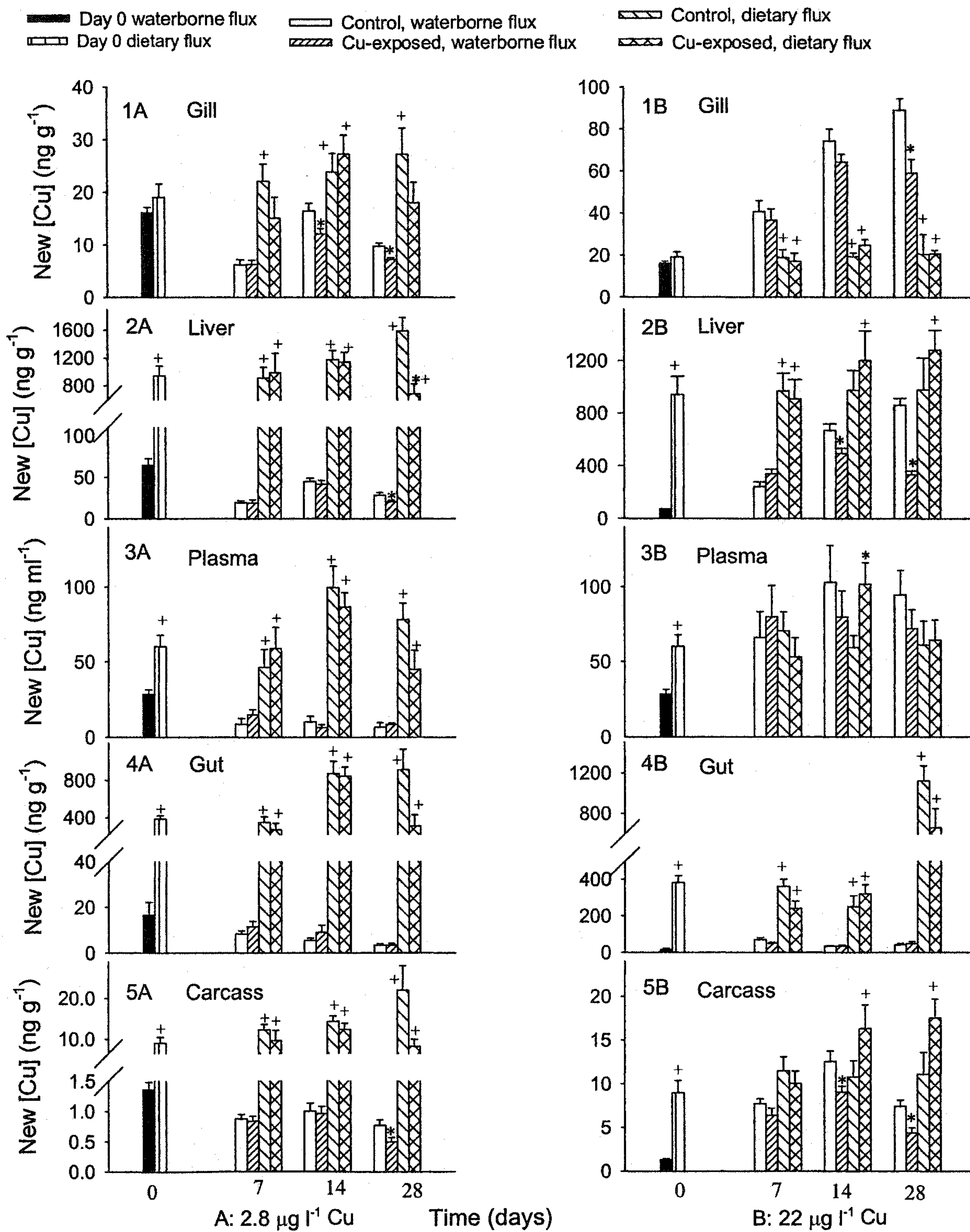
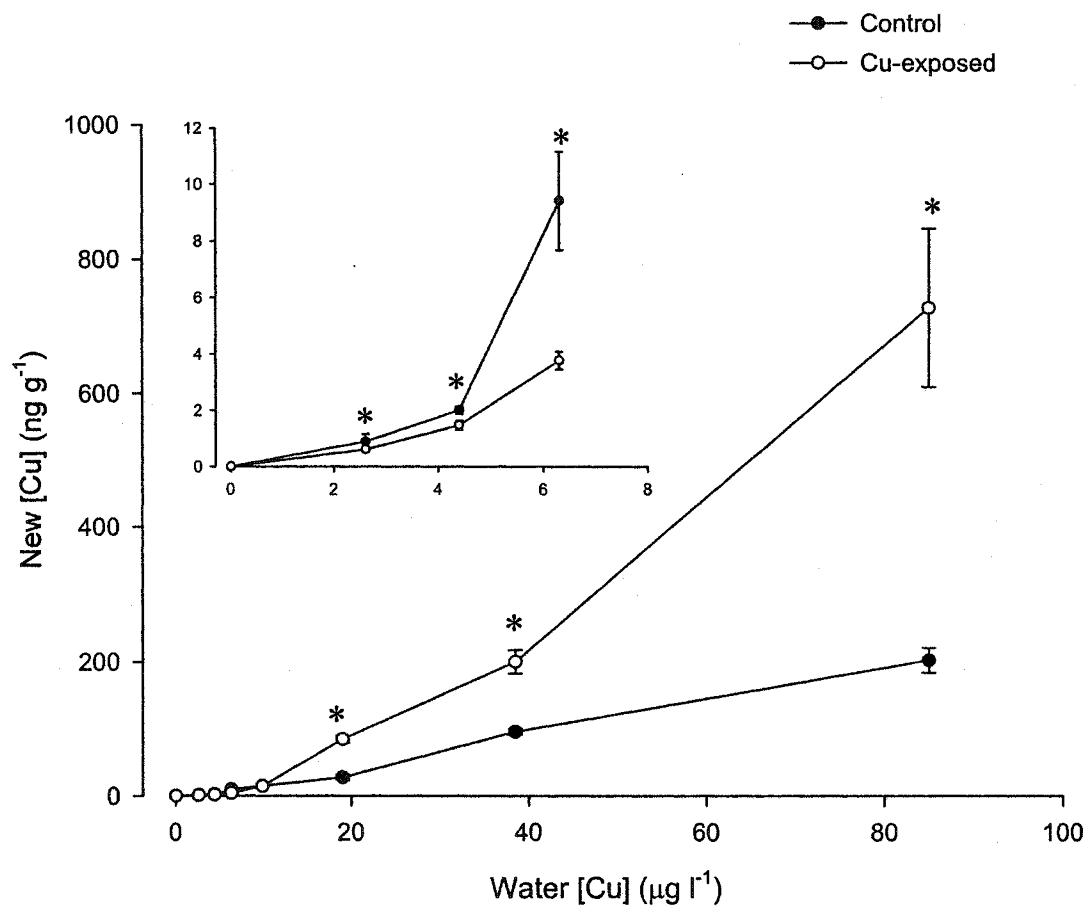

Day 0 waterborne flux
Day 0 dietary flux
Control, waterborne flux
Cu-exposed, waterborne flux
Control, dietary flux
Cu-exposed, dietary flux
1A Gill
2A Liver
3A Plasma
4A Gut
5A Carcass
1B Gill
2B Liver
3B Plasma
4B Gut
5B Carcass
New [Cu] (ng g-1)
New [Cu] (ng ml-1)
A: 2.8 μg l-1 Cu
Time (days)
B: 22 μg l-1 Cu

Figure 4-6. Gill Cu-binding in control and acclimated rainbow trout. Values are means ± SEM, ng g^{-1}, n = 8 per data point for both control and chronic Cu-exposed fish at day 28. Insert shows gill Cu-binding at water [Cu] below 10 μg l^{-1}. * Indicates significant difference between control and acclimated fish, $p < 0.05$.


Control
Cu-exposed
New [Cu] (ng g^{-1})
Water [Cu] (μg l^{-1})
1000
800
600
400
200
0
0
20
40
60
80
100
12
10
8
6
4
2
0
0
2
4
6
8
*

CHAPTER 5

THE INFLUENCE OF RATION SIZE ON COPPER HOMEOSTASIS DURING SUBLETHAL DIETARY COPPER EXPOSURE IN JUVENILE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

ABSTRACT

The influence of ration size on homeostasis and sublethal toxicity of copper (Cu) was assessed in rainbow trout (*Oncorhynchus mykiss*) during dietary Cu exposure in synthetic soft water. A constant dietary dose of 0.24 μmol Cu g fish^{-1} day^{-1} as $CuSO_4 \cdot 5H_2O$ was delivered via diets containing 15.75, 7.87, and 5.24 μmol g^{-1} fed at 1.5, 3.0, and 4.5% wet body weight daily ration, respectively. Juvenile rainbow trout showed clear effects of ration but not Cu on growth suggesting that growth is hardly a sensitive endpoint for detection of sublethal dietary Cu exposure. All Cu-exposed fish accumulated the same total metal load when expressed on a per fish basis. This suggests that differences in tissue and whole-body Cu concentrations among the treatments reflected the differences in the fish size rather than total Cu accumulation, and demonstrate that absorption and accumulation of Cu from the gut during dietary exposure are independent of the food quantity in which the Cu is delivered. Fish fed a high ration exhibited greater mass-specific unidirectional uptake of waterborne Cu than fish fed a low ration indicating an increased need for Cu for growth processes in rapidly growing fish. Stimulated excretion of Cu was indicated by greater Cu accumulation in

the bile of the Cu-exposed fish. Branchial Na^+, K^+-ATPase was not affected by dietary Cu exposure or ration but gut Na^+, K^+-ATPase activities showed stimulatory effects of increasing ration but not of Cu exposure. The 96-h LC50 for waterborne Cu (range 0.17 to 0.21 μmol l^{-1} (10.52 - 13.20 μg l^{-1}) was the same in all treatment groups indicating that ration size was unimportant and that dietary Cu did not induce an increase in tolerance to waterborne Cu. Taken together these results suggest that the nutritional status, fish size, and growth rates should be considered when comparing whole-body and tissue Cu concentration data for biomonitoring and risk assessment. Moreover, expressing the exposure as total metal dose rather than metal concentration in the diet is more appropriate.

Keywords: Cu homeostasis; dietary Cu; rainbow trout; ration; Na^+,K^+-ATPase; risk assessment

INTRODUCTION

Although dietary factors have marked effects on fish physiology and metabolism (Cowey and Sargent, 1979), the possible modifying effect of food-related variables on the toxicity and homeostatic regulation of metals has been largely neglected in aquatic toxicology (Lanno *et al.*, 1989). Most dietary studies carried out on fish have concentrated on growth-ration relationships geared toward establishing nutritional adequacy of diets for fish in aquaculture (Brett and Groves, 1979; Cho *et al.*, 1982; Cowey, 1992). These studies have established that growth of fish is strongly regulated by the quantity of food consumed. However, metal contaminants in the diet may influence fish health negatively by inducing toxicosis or by affecting food utilization. For example, some previous studies have reported that environmental pollutants affect appetite of the fish resulting in changes in the dynamics of metal/chemical uptake, metabolism, and depuration (Jiminez *et al.*, 1987; Lanno *et al.*, 1989; Wilson *et al.*, 1994; D'Cruz *et al.*, 1998). Several studies that have specifically assessed the effect of feeding/starvation (Buckley *et al.*, 1982; Collvin, 1985; Segner, 1987) on metal toxicity employed waterborne exposures and reported variable results. However, there are indirect indications that nutritional factors such as high ration may mitigate waterborne (Taylor *et al.*, 2000) and dietary (Kamunde *et al.*, 2001) Cu toxicity in fish. These data highlight the need for a detailed examination of the role of nutritional status on the responses of fish to metals exposure.

There is apparently no agreement on the level of dietary Cu toxic to fish. Although earlier work by Lanno *et al.* (1989) determined the toxic threshold to be

approximately 730 μg Cu g^{-1} diet, recent data have not concurred with this finding (Berntssen *et al.*, 1999; Kamunde *et al.*, 2001). Possible causes of these discrepancies include differences in exposure periods, feeding regimes, fish size, and species. Several studies have investigated the effects of varying dietary metal concentrations in fish at constant ration (see review by Handy, 1996) but none, to our knowledge, have investigated the influence of constant metal load presented in different ration levels.

Impairment of branchial Na^+,K^+-ATPase during acute waterborne Cu exposure has been unambiguously characterized in rainbow trout (Laurén and McDonald, 1987b; Li *et al.*, 1998). However, it remains to be determined whether dietary Cu imparts toxicity by affecting Na^+,K^+-ATPase activities in the gastrointestinal tract. Furthermore, since dietary Cu has been shown to accumulate in the gills in some studies (Miller *et al.*, 1993; Kamunde *et al.*, 2001, 2002c), it would be interesting to assess if Cu accumulated at the gill from the diet would have a similar inhibitory effect as waterborne Cu.

The aim of the present study was therefore to test the hypothesis that fish maintained on high ration have superior ability to regulate and arrest the deleterious effects of dietary Cu exposure. This was done by offering juvenile rainbow trout the same Cu load in three rations ranging from 1.5% to 4.5% wet body weight per day. We anticipated that sublethal endpoints of chronic metal toxicity such as growth would be influenced by the nutritional status of the animal and subsequently impact metal uptake, distribution, excretion, and accumulation. In addition, exposing fish to metal under different feeding regimes is environmentally realistic because food abundance and feeding indices vary with season in aquatic ecosystems (Segner, 1987; Smith and

Griffith, 1994). A second objective was to establish possible connections between tissue Cu accumulation, metal dose, and toxicity for purposes of risk assessment in aquatic toxicology. Previous studies (Miller *et al.*, 1992; Farag *et al.*, 1995; Marr *et al.*, 1996) have associated tissue metal residues with adverse effects and recently Bergman and Dorward-King (1997) proposed the use of tissue metal burdens for biomonitoring, risk assessment, and derivation of water quality criteria. Third, a waterborne Cu toxicity test was performed to determine if dietary Cu induced acclimation to waterborne Cu (McDonald and Wood, 1993) and whether ration size had any role in this process. Finally, we examined the possible effect of chronic sublethal dietary Cu exposure and ration size on branchial and gastrointestinal tract Na^+,K^+-ATPase activities.

MATERIALS AND METHODS

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) 9-10 g in weight were obtained from Humber Springs Trout Farm, Ontario, and acclimated to laboratory conditions for two weeks. Laboratory conditions consisted of a flow-through of aerated dechlorinated Hamilton tap water containing: Na^+ 0.6 mmol l^{-1}, Cl^- 0.7 mmol l^{-1}, Ca^{2+} 1 mmol l^{-1}, Mg^{2+} 0.21 mmol l^{-1}, hardness 1.4 mmol l^{-1} as $CaCO_3$, alkalinity 0.95 mmol l^{-1} as $CaCO_3$, and dissolved organic carbon (DOC) 3.0 mg l^{-1}. Water pH and temperature were 7.9-8.2, and 14 °C, respectively, and background Cu concentration was about 47.24 nmol l^{-1} (3 µg l^{-1}). Subsequently the fish were gradually acclimated to synthetic soft water. Soft water was generated from dechlorinated Hamilton tap water by reverse osmosis and

mixed gradually with regular dechlorinated tap water to achieve a final mixture of about 6:1 reverse osmosis:tap water, over a period of two weeks. Fish were then maintained in this synthetic water for 2 months before initiation of the experiment. Water composition at the end of acclimation was: Na^+ 0.12 mmol l^{-1}, Cl^- 0.10 mmol l^{-1}, Ca^{2+} 0.13 mmol l^{-1}, Mg^{2+} 0.04 mmol l^{-1}, and hardness 0.20 mmol l^{-1} as $CaCO_3$, alkalinity 0.15 mmol l^{-1} as $CaCO_3$, DOC 0.4 mg l^{-1}. Background Cu in soft water was 22.05 nmol l^{-1} (1.4 μg l^{-1}), pH was 7.0, and temperature was 14 °C. During the pre-experimental and acclimation period, fish were fed once daily at 2% wet body weight on commercial trout chow (Corey Feed Mills Ltd., Fredericton, New Brunswick) containing 55% crude protein, 17% crude fat, 2% crude fiber, 1.5% Ca^{2+}, 0.6% Na^+). Cu concentration of the diet was 0.31 μmol g^{-1} (20 μg g^{-1}).

Experimental diets and feeding

Experimental diets were made in-house by supplementing the commercial trout chow with the required amount of Cu calculated to deliver 0.24 μmol (15 μg) Cu g $fish^{-1}$ d^{-1} in 1.5%, 3.0%, and 4.5% daily ration. This was achieved by making diets containing 15.75 (1000), 7.87 (500), and 5.24 (333) μmol g^{-1} (μg g^{-1}) Cu for the 1.5, 3.0, and 4.5% rations respectively (Table 1). For all the diets the commercial trout chow was ground in a blender and the appropriate amount of Cu (as $CuSO_4 \cdot 5H_2O$) for each diet dissolved in 10% diet weight of double distilled water and mixed in a pasta maker for 45 minutes. This ensured homogenous distribution of the Cu throughout the food. Thereafter further 30% diet weight double distilled water was added (bringing total volume of water added

to 40% diet weight) and mixed for a further 15 minutes. The food was subsequently extruded via a pasta maker, air-dried, and broken into small pellets (approximately 3 mm^3) by hand. Control diet was treated in the same way except that no Cu was added. All diets were kept at –20 °C till use. The nominal and actual Cu concentrations of the diets are shown in Table 1. During the experiment fish were fed the designated diet at half the designated ration twice a day, once in the morning (08:00-09:00h) and again in the evening (18:00-19:00h). The fish were allowed to feed for 1 h, after which fecal material was siphoned and a water sample taken for Cu analysis. Visual observation revealed that all food was ingested. Bulk fish weights obtained weekly for each group were used to calculate the ration for the following week.

Exposure set-up

The exposure set-up was a complete factorial design composed of six 150-l tanks partitioned in half by dividers giving a total of 12 experimental chambers for 3 treatments and respective controls, in duplicates. Water flow rate into the tanks was 1.2 l min^{-1} providing 50% replacement of the water in 1.4 h. Each partition of the tank was provided with gentle aeration. For each ration the control and treatment group were kept together in one tank (but in separate partitions), so that any changes in waterborne Cu concentration would affect both the control and the treatment group in the same way. A previous study (Kamunde *et al.*, 2001) had reported elevated waterborne Cu concentration, probably from excretion, during static waterborne Cu flux experiments in fish exposed to dietary Cu. However, since the fish were maintained in a flow-through

system in the present study, measurement of water Cu concentration soon after feeding (range 18.9–25.2 nmol l^{-1} (1.2-1.6 μg l^{-1}) showed no significant changes.

Sampling

Fish were bulk weighed in a 20-l bucket lined with a plastic sieve and then starved for 36 hours before sampling on days 0, 9, 21, and 35. For each treatment and its associated control, 10 fish (5 from each replicate) were randomly removed, initially for determination of unidirectional waterborne Cu uptake as described below. Subsequently, the fish were killed with an overdose of neutralized tricaine methanesulfonate (MS-222; 1g l^{-1} containing 0.25 g l^{-1} $NaHCO_3$) and organs [gills, liver, muscle, gut (stomach, pyloric cecae + anterior intestine, mid intestine and posterior intestine), plasma, bile, and rest of carcass] were collected into separate pre-weighed scintillation vials or bullet tubes, weighed, and then frozen for subsequent analyses.

Unidirectional waterborne Cu uptake

The effect of the dietary exposure conditions on unidirectional waterborne Cu uptake via gills was assessed at each sampling time. For each feeding regime 10 control and 10 Cu-treated fish were moved into partitioned 20-l flux containers and exposed to waterborne ^{64}Cu at ambient water Cu concentration. The radioisotope ^{64}Cu (as $CuNO_3$) was prepared at the McMaster University Nuclear Reactor. The radioisotope dosage administered (0.7 μCi l^{-1}) added a total concentration of 3.15 nmol (0.2 μg) l^{-1} Cu into the water, and therefore did not substantially elevate the nominal

water Cu concentration or alter the body or tissue burdens accumulated in the preceding weeks. The fish were exposed to the ^{64}Cu for 12 h under static water conditions and continuous gentle aeration similar to the protocol of Kamunde *et al.* (2001, 2002c). The tanks used for the flux measurements were pre-incubated overnight with soft water of the same chemical characteristics (i.e., same Cu concentrations) as the water used during the flux at all sampling times. A 10-ml water sample was taken from each tank 15 minutes after introduction of ^{64}Cu and again after the12 h flux period. Water ^{64}Cu activity and total Cu concentration did not change by more than 5% during the 12 h period. Unidirectional Cu uptake was determined as outlined in Calculations (below).

Na^+,K^+-ATPase activities

Na^+,K^+-ATPase activities were determined for the entire gill baskets and specific sections of the gut (stomach, pyloric cecae [includes anterior intestine], mid intestine, and posterior intestine) after 35 days of exposure to dietary Cu employing the microplate UV detection method (McCormick, 1993). Six fish from each group were sacrificed as described above. Gut and gill samples were rinsed in double distilled water upon dissection, frozen immediately in liquid nitrogen, and subsequently stored at $^-70$ °C until they could be analyzed for Na^+,K^+-ATPase activity. The tissues were initially thawed and subsequently homogenized on ice in 10x volumes of 4:1 SEI:SEID buffer for 45 seconds; SEI = 250 mM sucrose, 10 mM Na_2EDTA 50 mM imidazole, pH 7.3; SEID = 0.5% sodium deoxycholic acid in SEI. The homogenized tissues were

then centrifuged at 5 000 x *g* and the supernatant immediately analyzed for Na^+,K^+-ATPase activity. For all the tissues10 μl of the supernatant was used in a reaction mixture containing 50 μl salt solution (50 mM imidazole, 189 mM NaCl, 10.5 mM $MgCl_2.5H2O$, 42 mM KCl, pH 7.5) and 150 μl assay solution A or B, where assay solution A contains 50 mM imidazole, 2.8 mM phosphoenolpyruvate, 0.22 nicotinamide adenosine dinucleotide, 0.7 mM adenosine triphosphate, 4 U ml^{-1} lactate dehydrogenase, 5 U ml^{-1} pyruvate kinase, pH 7.5, while assay solution B is solution A + 0.5 mM ouabain. Each sample was read in quadruplicates, two with solution A and two with solution B. In this assay the rate of hydrolysis of ATP to ADP in the presence and absence of ouabain is coupled to the oxidation of NADH to NAD^+. Changes in absorbance of the reaction mixture due to NADH oxidation were measured at 340 nM over 15-sec intervals for 10 minutes. Na^+,K^+-ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of oubain, and normalized to total protein of the respective sample as determined according to Bradford (1976).

Toxicity test

Tolerance to acute waterborne Cu exposure was assessed using a 96-h acute toxicity test carried out at the end of the 35-day experiment. To allow direct comparison between treatments and controls, the containers (30-l capacity) used for the test were partitioned in the same way as the experimental tanks to ensure exposure to the same Cu concentration for both treatment and control. Ten fish for each treatment and respective control were tested in five Cu concentrations (nominally 0, 0.08, 0.16,

0.32, and 0.63 μmol l^{-1}) according to the protocol of Taylor *et al.* (2000). The test was carried in a flow-through system at a flow rate of 150 ml min^{-1} allowing 50% replacement of water in about 2.3 h. The water Cu concentration was checked 3 times daily by atomic absorption analysis and maintained within ± 5% of the nominal value. Fish were not fed during the toxicity test. Mortality was monitored and dead fish were removed and treatment and time of death recorded. The 96 h LC50 was calculated by Probit analysis (SPSS version 10.0, SPSS Inc., Chicago IL) using the measured mean total water Cu concentrations.

Analysis

At all sampling times the tissues and water samples were first measured for ^{64}Cu activity by means of a Canberra-Packard MINAXI Gamma counter with an on-board program for decay correction. Subsequently, the tissues were re-weighed and digested overnight at 70°C with 6 volumes of 1N nitric acid (Fisher Scientific, trace metal grade), and then centrifuged for 4 min at 13 000 x *g*. A sub-sample of the supernatant was diluted appropriately with 0.5% nitric acid and total tissue Cu concentrations were determined by atomic absorption spectroscopy (AAS; Varian AA-1275 with GTA-95 atomizer) using a 10-μl injection volume and the operating conditions specified for Cu by the manufacturer (McKenzie, 1982). Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range. Water total Cu concentrations from the samples taken after feeding and from the waterborne Cu uptake and waterborne Cu toxicity tests were analyzed by

comparable furnace atomic absorption methods using acidified samples without digestion.

Calculations

Specific growth rate (SGR) was calculated on a per tank basis (n = 2 per treatment and respective control) for 3 growth periods using the formula:

$$SGR = 100[(\ln(wt2)-\ln(wt1))\bullet t^{-1}]$$

where *wt1* and *wt2* are tank biomass at the start and end of each growth period and *t* is the interval in days.

Food conversion efficiency (FCE) was calculated on a per tank basis (n = 2 per treatment and respective control) for 3 growth periods using the formula:

$$FCE\ (\%) = 100(\text{weight gain per tank} \bullet \text{food eaten}^{-1})$$

Whole-body total Cu concentration was calculated by dividing the sum of Cu contents (concentration multiplied by weight) of all the tissues plus the carcass by the sum of weights of all the tissues plus carcass.

Retention efficiency of Cu was calculated as:

$$RE\ (\%) = 100[(C2-C1)\bullet \text{Cu eaten}^{-1}]$$

where *C1* and *C2* are total Cu content per fish (μmol fish^{-1}) at the beginning and at the end of the experiment, respectively.

The unidirectional uptake of waterborne Cu by the whole fish over 12 h was calculated by adding up ^{64}Cu activities (cpm) in all tissues and carcass. Fish weights were determined by summing up the weights of all tissues and rest of carcass for each fish. Whole-body Cu uptake was then calculated using the equation:

$$a(bc^{-1})^{-1}$$

where *a* is the mean ^{64}Cu cpm per gram fish, *b* is the ^{64}Cu cpm per l of water and *c* is the mean total Cu concentration in water in μmol l^{-1}. The uptake was then expressed as a rate by dividing by the time of exposure (12 h).

Statistical analysis

Data are presented as means ± SEM. Treatment and replicate effects were analyzed using 3-way factorial analysis of variance (ANOVA) (Statistica 5.0, StatSoft Inc., Tulsa, OK) with time, ration, and diet Cu exposure as variables. Subsequently *a posteriori* Tukey's honestly significant difference (HSD) test was used to make comparisons between means of the measurements. Chi-square goodness of fit and Bartlett tests performed on all data sets revealed that assumptions of normality of distribution and homogeneity of variance were met. All data from replicate tanks did

not show any statistical difference when the means were tested against each other using Tukey's HSD test. Hence data from the replicates were pooled. Growth data (cumulative weight gains, SGR, and FCE were compared with a paired Student's *t*-test. LC50 values with 95% confidence limits (95% C.L.) were computed using Probit analysis on SPSS 10.0 (SPSS Inc. Chicago, IL). Proportional data were arcsine transformed before statistical analyses.

RESULTS

Growth

At the start of the experiment mean fish weight was between 9 and 10 g for all the treatments. Growth occurred in all the treatments as shown by the cumulative weight gain curves (Fig. 1). There was a clear, statistically significant, effect of ration but not Cu on growth for all the treatments and the growth-ration relationship was curvilinear (Fig. 1, insert). Fish on 1.5% ration with or without 0.24 μmol Cu g $fish^{-1}$ d^{-1} showed the lowest growth gaining only 5 g $fish^{-1}$ in 35 days to reach a final weight of 14-15 g. Fish on the 3.0 and 4.5% daily ration gained 14 g $fish^{-1}$ to 23-24 g, and 16 g $fish^{-1}$ to 25-26 g by day 35, respectively. The differences in weight gains were reflected in the per tank specific growth rates which were highest in fish on 4.5% ration (Fig. 2A). However, food conversion was highest in fish on low ration and decreased in the highest ration (Fig. 2B).

The weights of liver, gastrointestinal tract, and gill relative to whole-body weight (somatic indices) are shown in Fig. 3. Hepatosomatic index ranged from 1 to 2.3%,

being low in the fish on lower ration and higher in the fish on high ration, but was not affected by dietary Cu exposure. Gastrointestinosomatic index ranged between 3 and 5%, tended to increase with ration and was slightly decreased with dietary Cu exposure. Branchiosomatic index tended to be lower in fish on intermediate ration, though the effect was not consistent over time (Fig. 3C). Dietary Cu exposure had no effect on the branchiosomatic index.

Whole-body Cu status

Exposure of juvenile rainbow trout to the same Cu quantity resulted in the same total Cu content per fish (Fig. 4A) regardless of the ration in which the Cu was delivered. Whole fish Cu content increased linearly over time. For the control fish, Cu content increased from an initial value of about 75 to 180, 335, and 340 nmol per fish at day 35 in the groups on 1.5, 3.0, and 4.5% body weight ration d^{-1}, respectively. All Cu-exposed fish had the same Cu content per fish reaching a high of about 630 nmol per fish at day 35. However, when expressed as a concentration (per wet body weight) there were striking differences among the treatment groups. While all the controls had Cu concentrations of between 12 and 21 nmol g^{-1}, fish exposed to 0.24 μmol Cu g $fish^{-1}$ day^{-1} delivered in 1.5% body weight ration d^{-1} had 4-fold higher whole-body Cu concentration than the respective control group. For fish in which the Cu load was delivered in 3.0 or 4.5% body weight ration d^{-1}, only about 1.5-fold increase in whole body Cu concentration occurred relative to the respective controls (Fig. 4B).

Retention efficiency (RE) of Cu was decreased strongly by Cu-exposure, and to a lesser extent by ration (Table 2). For all the Cu-exposed treatments RE was the same regardless of ration, but much lower than for the control fish, and ranged from 0.7% to 0.8% for all the feeding regimes. Among the control groups RE decreased from about 9% in the fish on 1.5% body weight ration d^{-1} to 5.6% in fish on 4.5% body weight ration d^{-1}.

Tissue partitioning of Cu

Accumulated Cu partitioned into most body tissues sampled, with the liver showing the highest concentrations. For the control fish, Cu content in liver increased significantly from an initial value of about 30 to 80, 170, and 190 nmol per liver at day 35 in the group on 1.5, 3.0, and 4.5% body weight ration d^{-1}, respectively (Fig. 5A). Moreover, the liver Cu content was significantly lower at day 35 in the fish on 1.5% body weight ration d^{-1} compared to both 3.5 and 4.5 body weight ration d^{-1}. However, as with whole-body, the total Cu content (nmol) per liver was the same for all the Cu-exposed groups independent of ration, and rose linearly from about 30 nmol per liver at the beginning of the experiment to about 400 nmol Cu per liver at the end of the experiment. Liver Cu concentration also increased linearly over time in all the treatments (Fig. 5B). Livers of the group fed the 1.5% body weight ration d^{-1} (15.75 μmol g^{-1} Cu diet) showed the highest liver Cu concentration of 3.62 μmol g^{-1} followed by the group on 3.0% (7.87 μmol g^{-1} Cu diet) with 1.26 μmol g^{-1}, 4.5% body weight ration d^{-1} (5.24 μmol g^{-1} Cu diet) with 0.94 μmol g^{-1}, and controls (0.32 μmol g^{-1} Cu

diet) with 0.40 to 0.63 μmol g^{-1}. In direct correlation with liver Cu concentration, bile Cu concentration was highest in the group in which Cu was delivered in 1.5% body weight ration d^{-1} followed in descending order by the 3.0%, 4.5% body weight ration d^{-1}, and the control groups in decreasing order (Fig. 6A). In addition, bile concentration appeared to be more or less in steady state in all Cu-exposed fish from day 21 onwards.

Significant Cu accumulation occurred in the kidney, carcass, and to a very small extent in the muscle (day 35 only), in the groups fed high Cu diets (Fig. 6B-D). For all the organs and tissues, the group on 1.5% body weight ration d^{-1} + Cu had the highest Cu concentration. However, plasma and gill Cu concentration remained unchanged (Fig. 6E and F), although plasma values for day 35 were significantly higher than for the rest of the period in all the treatments.

Cu accumulation in segments of the gut is shown in Fig. 7A-D. In all the treatment groups, the posterior intestine had the highest Cu concentration followed in descending order by the pyloric cecae + anterior intestine, stomach, and mid-intestine. As with whole-body, fish on 1.5% body weight ration d^{-1} had higher Cu concentrations in the gut segments followed in decreasing order by the fish on 3.0 and 4.5% body weight ration d^{-1}. Cu concentration in the control group followed a similar pattern except that the concentration in the mid-intestine was slightly higher than in the stomach. Cu concentrations in all regions of the gut appeared to be more or less in steady state from day 9 onwards.

Proportional distribution of Cu in the major tissues and organs (liver, carcass, entire gut, gill, and kidney) in control and exposed animals at the end of the 35-day

exposure is shown in Fig. 8. About 50-59% of the Cu in control animals was contained in the liver and this proportion increased to 61-69% in Cu exposed fish. The carcass contained about 30-40% of the Cu in control animals and this proportion decreased to 15-26% in Cu-exposed animals. Gut contained 7-10% of the Cu in control fish and fish on the 3.0 and 4.5% body weight ration d^{-1} + Cu, and 22% in the 1.5% body weight ration d^{-1} Cu-exposed group. The gills and kidneys each contained less than 2% of the total body Cu burden and this proportion decreased to less than 1% in the Cu-exposed animals at all rations.

Waterborne Cu uptake

Whole-body unidirectional Cu uptake from the water was determined by ^{64}Cu appearance in the fish at various times during the 35-day experiment (Fig. 9). Waterborne Cu uptake decreased over time as the fish increased in size and was significantly lower at days 21 and 35 relative to day 0 for all the groups. High ration appeared to stimulate Cu uptake, with the uptake rates being significantly higher for the fish on 4.5% body weight ration d^{-1} on days 21 and 35. However there was no significant influence of dietary Cu pre-exposure on waterborne Cu uptake at any time. In the flux experiments, Cu speciation analysis by the MINEQL+ aquatic geochemical model (Schecher and McAvoy, 1994) indicated that Cu was present as 25% Cu^{2+}, 7.3% $CuOH^{+}$, 3% aqueous $CuCO_3$, and 64.4% Cu-humate. There was no $CuCl_2$ species indicating that under conditions of our exposure complexation of Cu with Cl was unimportant.

Acute toxicity test

A 96-h acute waterborne Cu toxicity test carried out at the end of the experiment showed no effect of ration or dietary Cu on Cu tolerance. The LC50s (means ± 95% C.L.) for the 3 control groups ranged from 0.17 ± 0.07 to 0.19 ± 0.08 $\mu mol\ l^{-1}$ while the LC50s in the Cu-fed groups ranged from 0.17 ± 0.07 to 0.21 ± 0.08 $\mu mol\ l^{-1}$. There were no significant differences amongst the groups.

Na^{+},K^{+}-ATPase activities

Na^{+},K^{+}-ATPase activities (Fig. 10) in various gastrointestinal tissues and the gill were determined at day 35. Except for the stomach in the fish on 1.5% body weight ration d^{-1}, chronic Cu exposure had no effect on the Na^{+},K^{+}-ATPase in the gut tissue. However, high ration significantly increased the Na^{+},K^{+}-ATPase activity in the mid-intestine, pyloric cecae (includes anterior intestine) and posterior intestine. For the stomach, though the activity increased with ration, this was not statistically significant. In the gill (Fig. 10E) neither dietary Cu exposure nor ration affected the Na^{+}, K^{+}-ATPase activity.

DISCUSSION

Growth

Over the range of ration sizes used in the present study, specific growth rate increased with ration size (Fig. 2A) in agreement with many previous studies (Brett and Groves, 1979). The curvilinear growth-ration relationship seen in the present study

(Fig. 1, insert) is fairly standard and results from the inability of fish to convert food materials into body tissues at high rations effectively so that excess undigested food is passed out in feces. The latter was reflected in the lower food conversion efficiencies in the fish on 4.5% body weight ration d^{-1} (Fig. 2B). Furthermore, for juvenile rainbow trout (9-26 g) maintenance ration must be less than 1.5%, since fish maintained on this ration showed positive growth characterized by high food conversion efficiency.

Contrary to our original hypothesis, dietary Cu (0.24 μmol Cu g $fish^{-1}$ d^{-1}) did not affect growth at the feeding regimes used. This suggests that even at the lowest ration of 1.5%, the possible energy requirement for Cu regulation or detoxification was accommodated within the normal energy budget, with excess energy used in growth. Several other studies have also reported lack of growth inhibition by dietary Cu (Berntssen *et al.,* 1999; Kamunde *et al.*, 2001 2002c) even in the face of mortality (Mount *et al.,* 1994). These previous observations and current data suggest that growth is not a sensitive endpoint for detection of sublethal dietary Cu exposure.

Whole-body Cu status

In the present study where total metal intake was kept constant while the ration was varied, whole-body Cu concentration varied inversely with ration (Fig. 4B). However, the absolute Cu content was the same in all the Cu-exposed groups irrespective of the ration size (Fig. 4A), indicating that differences in whole-body Cu concentrations were a reflection of differences in fish body size. Therefore growth dilution rather than variations in Cu uptake and retention explained the differences in

whole-body Cu concentrations. Recently, Kamunde *et al.* (2002c) similarly demonstrated that rapidly growing rainbow trout exhibited decreased whole body Cu concentration due to growth dilution. Thus in employing body metal residues for risk assessment (Bergman and Doward-King, 1997), nutritional status of the animal is an important consideration because it has direct bearing on metal concentrations in animal tissues. Furthermore, for dietary metal, the present study suggests that expressing the exposure as total metal dose rather than metal concentration in the diet is more appropriate.

That the absolute amount of Cu retained from a similar dose delivered in different rations was the same suggests that the absorption of Cu was independent of feeding regime and depended mainly on the amount of Cu offered. Studies on nutrient bioavailability have generally showed that the efficiency of absorption decreases with increasing food intake (Brett and Groves, 1979) due to increases in the gut evacuation time and passage of undigested food material. Our data on the retention efficiency of Cu in fish fed control diets, but not in fish fed Cu-loaded diets (Table 2), are consistent with this dogma. The control observations are consistent with the notion that absorption efficiency of Cu decreases with dose (Turnlund *et al.*, 1998; Linder, 1991; Clearwater *et al.*, 2000; Kamunde *et al.*, 2002c). Even small changes in total metal dose (Table 1) that occurred in fish fed control diet containing the same metal concentration (0.35 μmol g^{-1}) at variable ration were reflected in the Cu retention efficiency and Cu content of the fish (Table 1, Fig. 4A). Interestingly, although decreased food conversion efficiency at high ration was evident (Fig. 2B), Cu retention efficiency in fish exposed to the same total Cu

dose did not decrease (Table 2). This suggests that Cu absorption efficiency tends to increase as Cu concentration in the diet decreases.

Copper partitioning

Food ration size significantly influenced the concentration of Cu in the organs analyzed except gill and plasma (Figs. 5, 6, 7). Previous studies have reported highest Cu concentration in livers of starved *versus* fed fish exposed to waterborne Cu (Segner, 1987). In addition, Saari *et al.* (1993) found that food restriction improved the Cu status in rat livers during Cu deficiency and argued that the relatively slower growth of the liver due to food restriction left Cu more concentrated. Our observations following dietary Cu exposure at different rations show that a low feeding regime results in higher Cu concentrations in tissues. We attribute higher metal concentration in animals on lower ration to the smaller size of the organs in the face of the same gastrointestinal Cu uptake. Note also the hepatosomatic and gastrointestinosomatic indices were lower in fish on low ration (Fig. 3). The smaller size of these organs likely contributed to the higher concentrations of Cu in the fish on 1.5% body weight ration d^{-1}.

Proportional distribution analysis (Fig. 8) revealed that in control fish, the liver and the carcass were the main Cu reservoirs but following exposure to dietary Cu, the gut became an important tissue for Cu deposition. These observations are in agreement with Kamunde *et al.* (2001, 2002c). Moreover, the group on the lower ration retained a much higher proportion of Cu in the gut than the groups on higher ration suggesting that these animals were not as efficient in clearing Cu from the gut tissue to internal organs.

During waterborne Cu exposures, much less of the total body Cu burden accumulates in gut tissue (Miller *et al.*, 1993; Kamunde *et al.*, 2001). So clearly the proportion of total metal burden in the gut tissue is a useful risk assessment tool for diagnosing a dietary *versus* a waterborne route of uptake in contaminated fish collected from the wild.

Copper absorption and distribution in the gut tissue

All the segments of the gut showed significant accumulation of Cu following the exposure, suggesting that the whole gut participated in Cu absorption. However dietary Cu also induces metallothionein (MT) in the gut (Handy *et al.*, 1999) and most of the metal accumulation in the gut tissues may be MT-bound to limit cytotoxicity. Our data suggest that the posterior intestine is the most active site for Cu absorption and/or sequestration, followed in decreasing order by the pyloric cecae + anterior intestine, stomach, and mid-intestine. Proportionally, however, the majority of the Cu was in the pyloric cecae + anterior intestine (50%) followed in decreasing order by posterior intestine (20%), stomach (20%) and mid-intestine (10%). These findings are in close agreement with a recent study (Clearwater *et al.*, 2000) that reported, 44% in pyloric cecae, 23% in posterior intestine, and 12% in mid-intestine 24 h after administration of ^{64}Cu by an esophageal catheter. However, these authors reported that over 99% of a single ^{64}Cu dose had left the stomach in 24 h.

In humans it is generally thought that the acidic environment in the stomach contributes to the freeing of Cu bound to food and facilitates peptic digestion and release of Cu from organic complexes (Gollan, 1975). This is followed by partial

absorption of Cu in the stomach with most of the absorption occurring in the intestine. Copper is thought to enter the intestinal mucosal cells by simple diffusion (Linder and Hazegh-Azam, 1996) and/or via a high affinity uptake mechanism (Lee *et al.*, 2001) and to exit the basolateral membrane by a different mechanism (Linder, 1991), possibly a divalent cation transporter (Rolfs and Hediger, 1999). In addition, studies on Menke's disease suggest the presence of a basolateral Cu-ATPase which discharges Cu into the serosal blood capillaries where Cu binds to albumin and amino acids for transport to the liver (Camakaris *et al.*, 1995; Harrison and Dameron, 1999).

Although the situation in fish is less clear, similar mechanisms of Cu absorption are likely. The acidic fish stomach (Fange and Groves, 1979) may free Cu from the food with subsequent partial gastric absorption as in mammals. Indeed our Cu accumulation data suggest a significant role of fish stomach in Cu absorption. In addition, Q_{10} analysis (Clearwater *et al.*, 2000) suggested both simple diffusion (apical) and biologically mediated (active) transport (basolateral) components of gut Cu uptake in trout whereas Handy *et al.* (2000) reported a basolateral limiting step in Cu uptake in African walking catfish via a Cu/anion symport and possibly a Cu-ATPase.

Waterborne Cu uptake

Assessment of unidirectional uptake of waterborne Cu via gills following dietary Cu exposure at different rations revealed three interesting findings (Fig. 9). First, Cu uptake rates per unit body weight decreased over time with increasing fish size. This agrees with previous findings by Kamunde *et al.* (2001) that described a non-linear

decrease in Cu uptake with body weight in rainbow trout. Second, Cu uptake rates from the water were much higher than in several previous studies (Kamunde *et al.,* 2001, 2002c), possibly explained by the fact that the experiment was done in soft water in which rainbow trout generally exhibit higher Cu uptake rates due to fewer competing and complexing ions (Taylor *et al.*, 2000). Third, fish on high ration exhibited greater mass specific Cu uptake rate than fish on low ration probably because there is an increased need for Cu for growth processes in rapidly growing fish since Cu is essential for fish growth (Kamunde *et al.*, 2002c). The current data together with a previous study that showed greater uptake of Cu in fed fish compared to starved fish (Segner, 1987) suggest a modifying effect of an animal's nutritional status (energy balance) on Cu metabolism. The maintenance of Cu homeostasis thus involved changes in branchial Cu uptake rates related to ration and fish size but not dietary Cu exposure. The latter observation is contrary to our previous observation (Kamunde *et al.*, 2001) in which exposure to 15.75 μmol g^{-1} Cu diet at 4% wet body weight ration per day in hard water caused significant accumulation of Cu in the gills and reduced subsequent waterborne Cu uptake rate. However, under the conditions of the Kamunde *et al.* (2001) study, the fish received 0.63 μmol Cu g $fish^{-1}$ d^{-1}, almost threefold higher than in the present study.

Tolerance to waterborne Cu

Pre-exposure to sublethal waterborne Cu is generally associated with classical acclimation (McDonald and Wood, 1993), i.e., increased tolerance characterized by increased LC50 (e.g., Dixon and Sprague, 1981; Buckley *et al.*, 1982). However, few

studies have assessed the effect of dietary Cu on waterborne Cu tolerance. Miller *et al.* (1993) reported increased tolerance to waterborne Cu following exposure to 10.77 μmol g^{-1} Cu in the diet presented *ad libitum* for 42 days. In the present study a dietary dose of 0.24 μmol Cu g $fish^{-1}$ d^{-1} presented in various rations did not induce tolerance to waterborne Cu. Although the diet Cu concentrations used in this study (5.24 - 17.75 μmol g^{-1}) encompass the diet Cu concentration used by Miller *et al.* (1993), it is possible that fish in the latter study received more total Cu since they were fed *ad libitum*. Moreover, there was significant accumulation of Cu in the gills and elevation of waterborne Cu (from food-derived Cu) during the exposure by Miller *et al.* (1993) that did not occur in the present study. Other studies have reported that exposure to waterborne and dietary metal results in enhanced detoxification and excretory mechanisms and induction of resistance (Lanno *et al.*, 1987; Hogstrand and Haux, 1991; Marr *et al.*, 1995; Szebedinszky *et al.*, 2000). We did not find increased tolerance to acute waterborne Cu exposure despite increased biliary Cu concentration, which suggests augmented biliary excretory capacity (Grosell *et al.*, 2001). Furthermore, it is striking that in *Oncorhynchus kisutch* the increase in tolerance to Cu preceded induction of metallothionein, a metal binding protein in the liver (McCarter and Roch, 1984). Overall these results suggest that changes in gill physiology may be more important than internal detoxification or excretion mechanisms in the development of tolerance to waterborne Cu.

Na^+,K^+-ATPase

The higher Na^+,K^+-ATPase activity in the gut of fish on higher rations compared to fish on low ration, is probably a reflection of the overall metabolic demand put upon the gastrointestinal tissues. Indeed the higher gastrointestinosomatic index in fish on high ration (Fig. 3) is likely an indication of the greater need of gut tissues for digestive and absorptive purposes. Nutrient absorption (e.g., glucose and amino acids) in mammalian and piscine intestine is an active process coupled to Na^+ transport (Schultz *et al.*, 1966; Kimmich, 1973; Karasov and Diamond, 1983; Buddington *et al.*, 1986). Iturri and Wolf (1982) have provided evidence of the involvement of Na^+,K^+-ATPase (which generates the Na^+ gradient in intestinal cells) in nutrient absorption. Thus the greater need for nutrient absorption in fish on high ration may explain the higher Na^+,K^+-ATPase activities in the gut tissues. Compared to the gills, gut tissue Na^+,K^+-ATPase appears to be more than 100 times less sensitive to the inhibitory effects of Cu. While enzyme inhibition occurred in the trout gill following accumulation of less than 6.30 nmol g^{-1} in a waterborne exposure (Laurén and McDonald, 1987b), up to 0.63 μmol g^{-1} Cu above control levels in a dietary exposure had no effect on gut Na^+,K^+-ATPase (Fig. 7). The factors responsible for this apparent insensitivity of gut Na^+,K^+-ATPase to Cu remain unclear but we speculate greater induction of MT concurrent with greater sequestration of Cu in gut than in gill tissue. MT is induced in the gut tissue (Handy *et al.*, 1999) but not in gill tissue (Laurén and McDonald, 1987b; Grosell *et al.*, 1997). However, the recent demonstration of MT in fish gill tissue following Cu exposure (Dang *et al.*, 1999, 2000) casts doubts on MT being a major contributor to this

insensitivity. Other possible explanations are that the Cu species in the gastrointestinal environment are less toxic than those in a water column, or that there are real tissue-specific differences in Na^+,K^+-ATPase enzyme kinetics. In addition, the stimulatory effect of feeding could have masked the inhibitory effect of Cu.

The lack of effect of dietary Cu exposure on branchial Na^+,K^+-ATPase is consistent with absence of Cu accumulation in gill tissue (Fig. 6E). Moreover, during chronic waterborne Cu exposures, branchial Na^+,K^+-ATPase activities in Cu-exposed fish recover and even exceed the control levels (Laurén and McDonald, 1987b; McGeer *et al.*, 2000). Since the gill samples in our study were collected at day 35, enzyme activities are likely to have recovered from any impact incurred early in the exposure.

Concluding remarks

The nutritional status of the fish influences whole-body and tissue Cu concentration as well as the uptake and elimination rates of waterborne Cu during sublethal dietary Cu exposure. Absorption and accumulation of Cu from the gut during dietary exposure appear to be independent of the food quantity in which it is delivered. Thus using body metal burdens for biomonitoring and risk assessment is relevant only if the dietary metal intake and the feeding regimes are taken into consideration since regimes that cause higher growth rates can result in lower whole body and tissue metal concentrations, even when exposure dose is the same. Moreover, expressing exposure as a dose rather than concentration appears to be more appropriate for dietary metal toxicity assessment.

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Table 5-1: Ration size, dietary Cu concentrations, and total Cu dose delivered per g fish per day during the experiments. Actual dietary Cu concentrations are means ± SEM, n = 9.

Ration (% wet body weight)	Diet Cu concentration [μmol g^{-1}, nominal (actual)]		Total Cu dose (μmol g $fish^{-1}$ day^{-1})	
	Control	Treatment	Control	Treatment
1.5	0.31(0.35±0.03)	15.75 (15.94±0.54)	$4.72x10^{-3}$	0.24
3	0.31(0.35±0.03)	7.87 (8.0±0.35)	$11.03x10^{-3}$	0.24
4.5	0.31(0.35±0.03)	5.24 (5.32±0.30)	$15.74x10^{-3}$	0.24

Table 5-2: Copper retention efficiencies (%) in juvenile rainbow trout following exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% daily ration for 35 days.

Treatment	Retention efficiency (%)
1.5% ration control	8.92
1.5% ration + Cu	0.84
3.0% ration control	7.10
3.0% ration + Cu	0.77
4.5% ration control	5.68
4.5% ration + Cu	0.73

Figure 5-1. Effect of exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight d^{-1} ration on growth in juvenile rainbow trout. Values are means ± SEM for cumulative mean weight gains per tank, n = 2 per treatment. For all the groups solid lines represent control fish and broken lines represent fish exposed to dietary Cu. ○, 1.5 % body weight d^{-1} ration control; ●, 1.5% body weight d^{-1} + 0.24 μmol g^{-1} d^{-1} Cu; □, 3.0% body weight d^{-1} ration control; ■, 3.0% body weight d^{-1} ration + 0.24 μmol g^{-1} d^{-1} Cu; Δ, 4.5% body weight d^{-1} ration control; ▲, 4.5% body weight d^{-1} ration + 0.24 μmol g^{-1} d^{-1} Cu. # Indicates significant difference relative to 3.0% body weight d^{-1} ration and * indicates significant difference relative to 1.5% body weight d^{-1} ration (*t*-test, $p<0.05$). There was no effect of Cu exposure on growth at any ration. Inset shows the growth-ration relationship under the conditions of exposure.

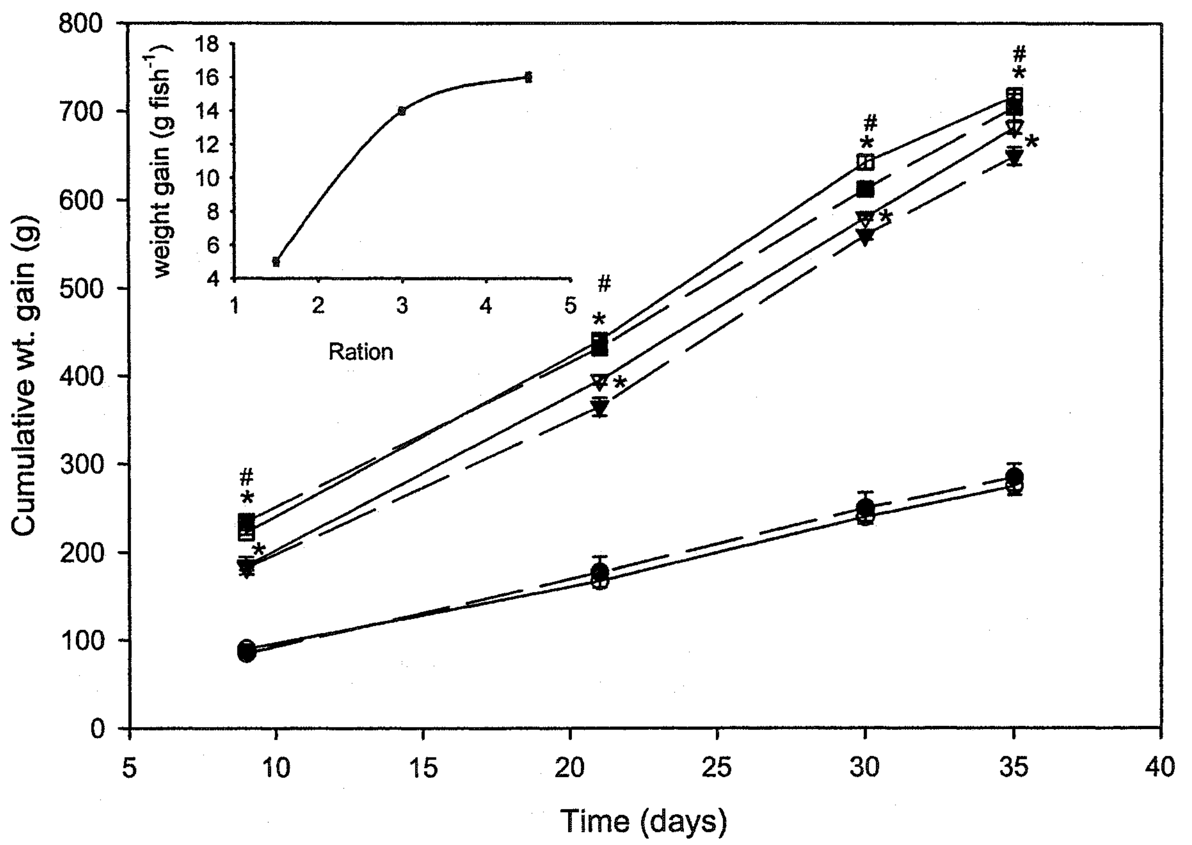

Cumulative wt. gain (g)
Time (days)
weight gain (g fish^{-1})
Ration
800
700
600
500
400
300
200
100
0
5
10
15
20
25
30
35
40
18
16
14
12
10
8
6
4
1
2
3
4
5

Figure 5-2. Effect of exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight per day ration on SGRs (A) and food conversion efficiencies (FCE) [B] in juvenile rainbow trout. Values are means ± SEM on per tank basis (n = 2). Bars with different letters within the same sampling time are significantly different (*t*-test, $p<0.05$).

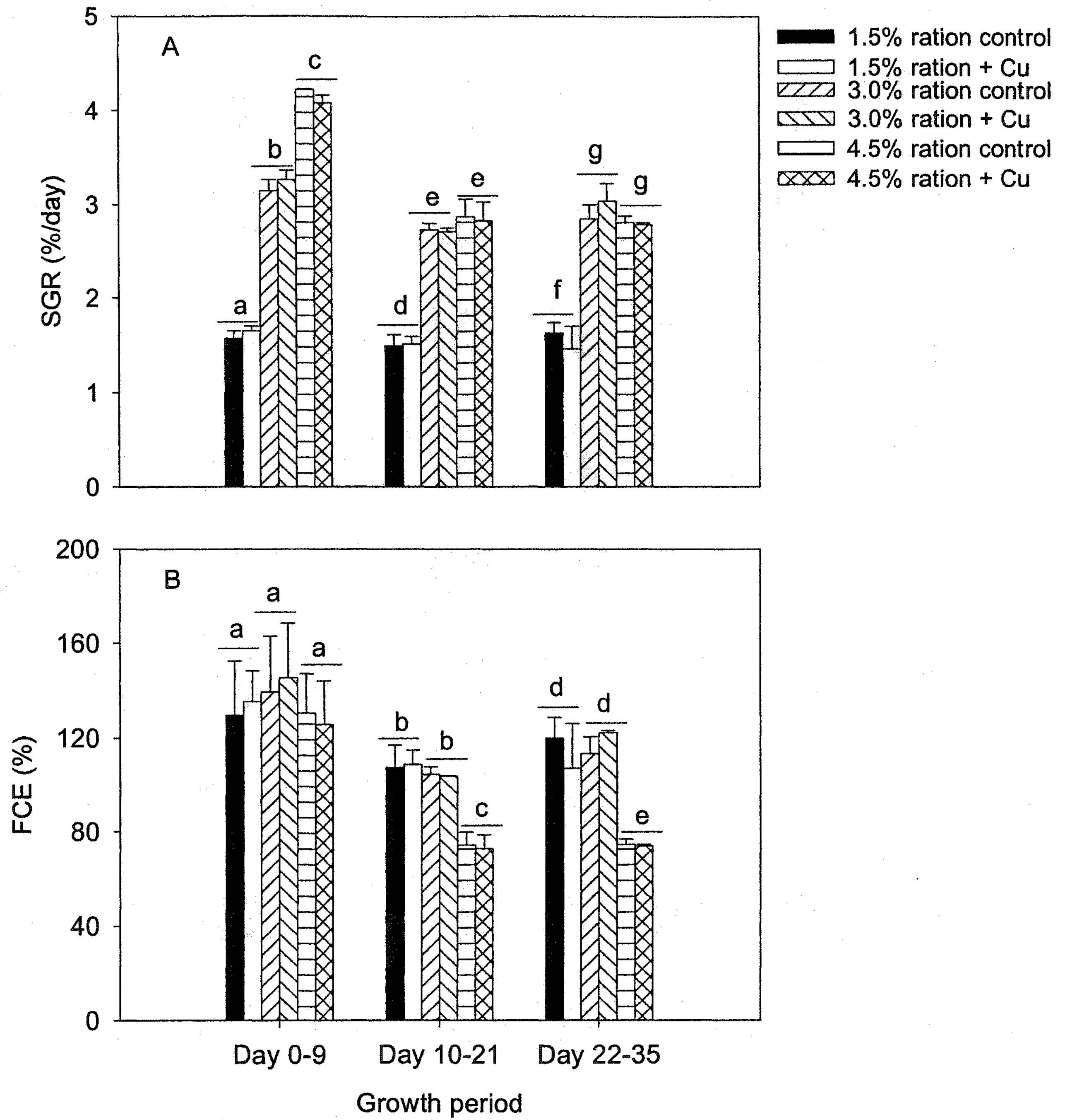

A
B
SGR (%/day)
FCE (%)
1.5% ration control
1.5% ration + Cu
3.0% ration control
3.0% ration + Cu
4.5% ration control
4.5% ration + Cu
Day 0-9
Day 10-21
Day 22-35
Growth period

Figure 5-3. Somatic indices for liver (A), gastrointestinal tract (B), and gill (C) in juvenile rainbow trout following exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight per day ration. HSI, hepatosomatic index; GSI, gastrointestinosomatic index; BSI, branchiosomatic index. Values are means ± SEM, n = 10 per data point. Bars with different letters within the same sampling time are significantly different, and # indicates significant difference from control for same ration at that sampling time, Tukey's HSD, $p<0.05$.

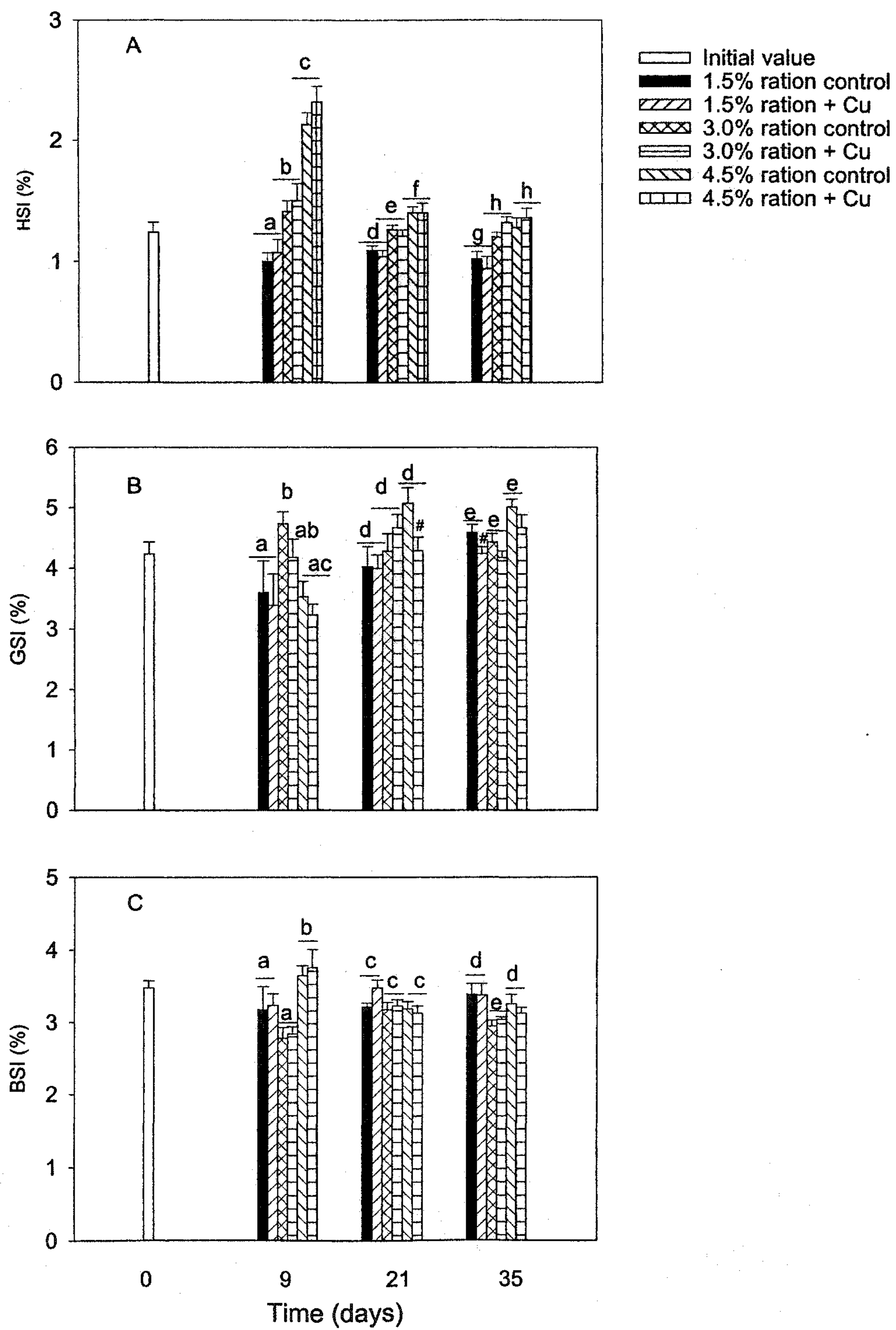
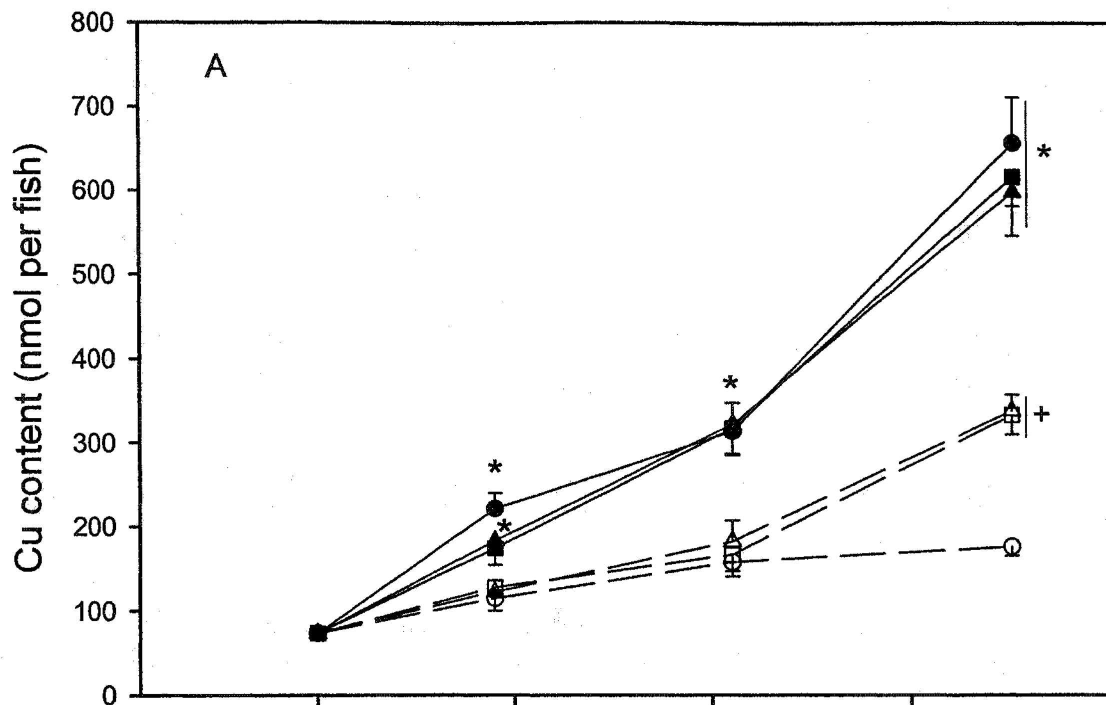

A
B
C
HSI (%)
GSI (%)
BSI (%)
Time (days)
0
9
21
35
Initial value
1.5% ration control
1.5% ration + Cu
3.0% ration control
3.0% ration + Cu
4.5% ration control
4.5% ration + Cu

Figure 5-4. Whole fish Cu content (A) and whole-body Cu concentration (B) in juvenile rainbow trout following exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, or 4.5% body weight d^{-1} ration. Values are means ± SEM, n = 10 per treatment per data point except day 0 controls where n = 24. ○, 1.5 % body weight d^{-1} ration control; ●, 1.5% body weight d^{-1} + 0.24 μmol g^{-1} d^{-1} Cu; □, 3.0% body weight d^{-1} ration control; ■, 3.0% body weight d^{-1} ration + 0.24 μmol g^{-1} d^{-1} Cu; Δ, 4.5% body weight d^{-1} ration control; ▲, 4.5% body weight d^{-1} ration + 0.24 μmol g^{-1} d^{-1} Cu. * Indicates significant difference from controls and + indicates significant difference from 1.5% body weight d^{-1} ration, Tukey's HSD, $p<0.05$. There were no significant differences amongst the experimental groups in A. In B all points for the 1.5% body weight d^{-1} ration were significantly higher than for the 3.0% and 4.5% body weight d^{-1} rations.


A
Cu content (nmol per fish)
0
100
200
300
400
500
600
700
800
*
+

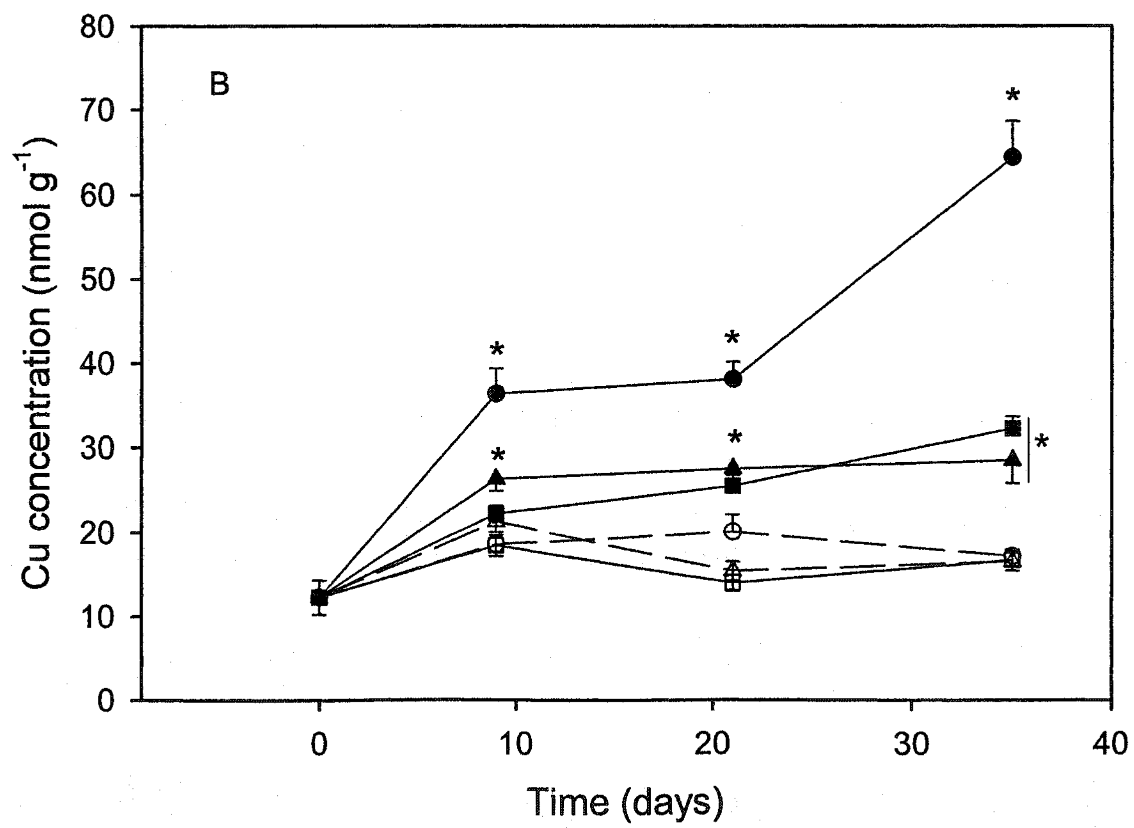
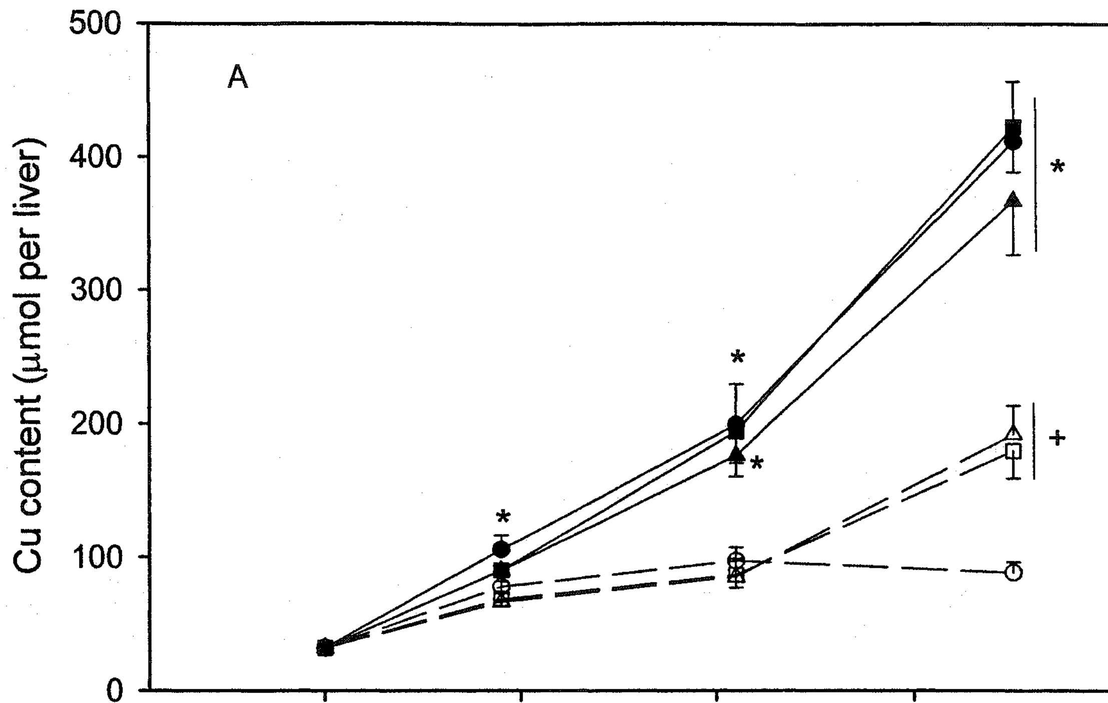
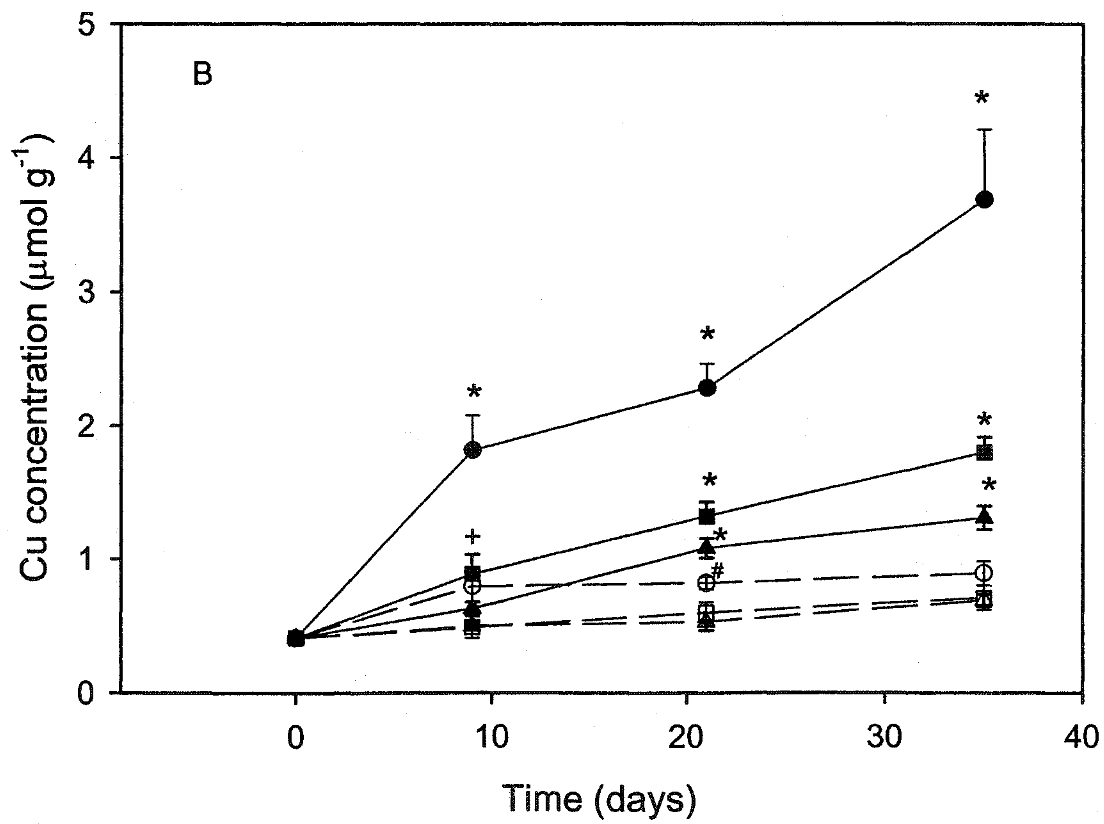

B
Cu concentration (nmol g^{-1})
0
10
20
30
40
50
60
70
80
0
10
20
30
40
Time (days)
*

Figure 5-5. Liver Cu content (A) and liver total Cu concentration (B) in juvenile rainbow trout following exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, or 4.5% ration. Values are means ± SEM, n = 10 per treatment per data point 0 controls where n = 24. ○, 1.5 % body weight d^{-1} ration control; ●, 1.5% body weight d^{-1} + 0.24 μmol g^{-1} d^{-1} Cu; □, 3.0% body weight d^{-1} ration control; ■, 3.0% body weight d^{-1} ration + 0.24 μmol g^{-1} d^{-1} Cu; ∇, 4.5% body weight d^{-1} ration control; ▼, 4.5% body weight d^{-1} ration + 0.24 μmol g^{-1} d^{-1} Cu. * Indicates significant difference from controls, and + indicates significant difference from 1.5% body weight d^{-1} ration, Tukey's HSD, $p<0.05$. There were no significant differences amongst the experimental groups in A. In B all points for the 1.5% body weight d^{-1} ration were significantly higher than for the 3.0% and 4.5% body weight d^{-1} rations.

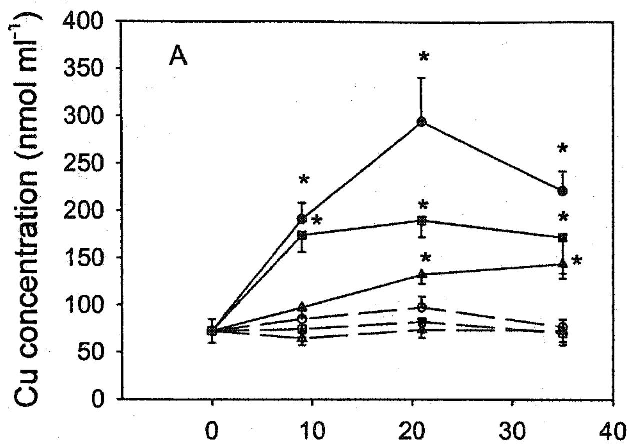


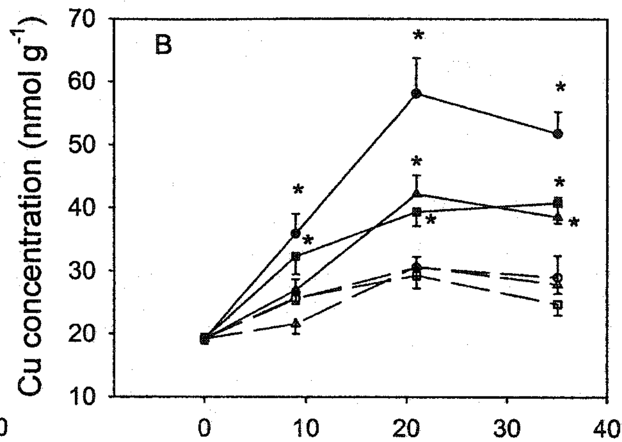
A
Cu content (μmol per liver)
500
400
300
200
100
0
*
+

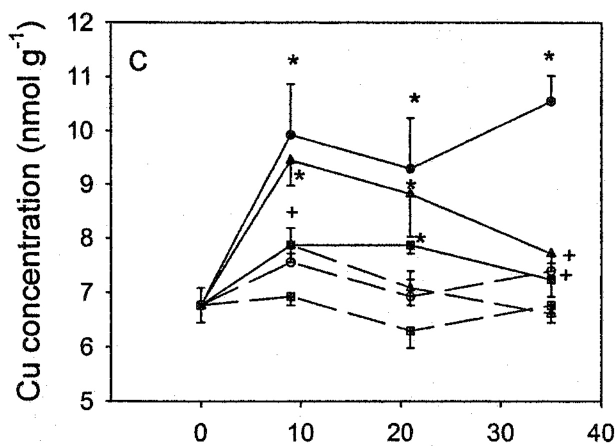


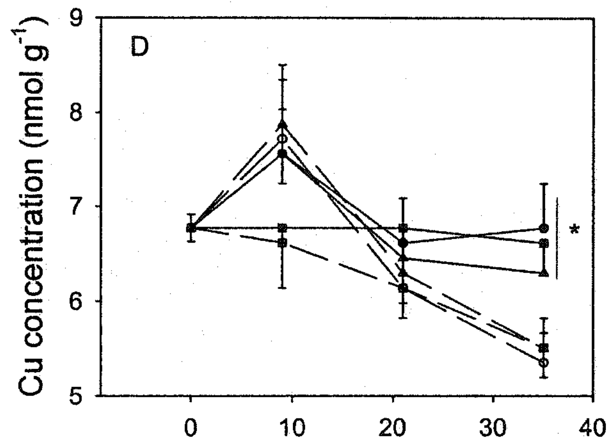
B
Cu concentration (μmol g-1)
5
4
3
2
1
0
0
10
20
30
40
Time (days)
*
+
#

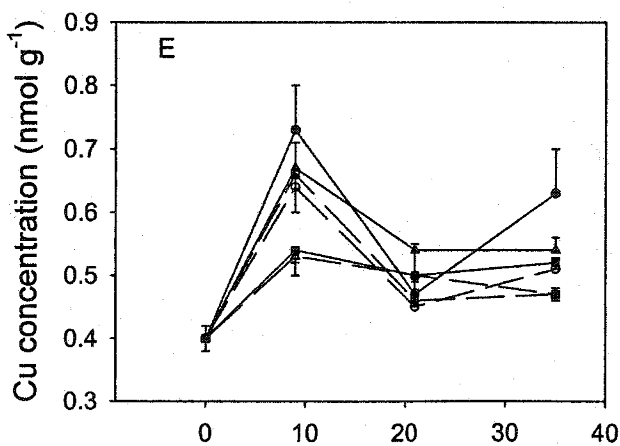
Figure 5-6. Cu concentration in tissues of juvenile rainbow trout following exposure to 0.24 µmol Cu per g fish per day delivered in 1.5, 3.0, or 4.5% body weight d^{-1} ration. A, bile; B, kidney; C, carcass; D, muscle; E, gill; F, plasma. Note difference in scale in different panels. Values are means ± SEM, n = 10 per treatment per data point for all the organs/tissues except day 0 controls where n = 24. ○, 1.5 % body weight d^{-1} ration control; ●, 1.5% body weight d^{-1} + 0.24 µmol g^{-1} d^{-1} Cu; □, 3.0% body weight d^{-1} ration control; ■, 3.0% body weight d^{-1} ration + 0.24 µmol g^{-1} d^{-1} Cu; Δ, 4.5% body weight d^{-1} ration control; ▲, 4.5% body weight d^{-1} ration + 0.24 µmol g^{-1} d^{-1} Cu. * Indicates significant difference from controls for each tissue, and + indicates significant difference from 3.0% body weight d^{-1} ration control, Tukey's HSD, $p<0.05$.

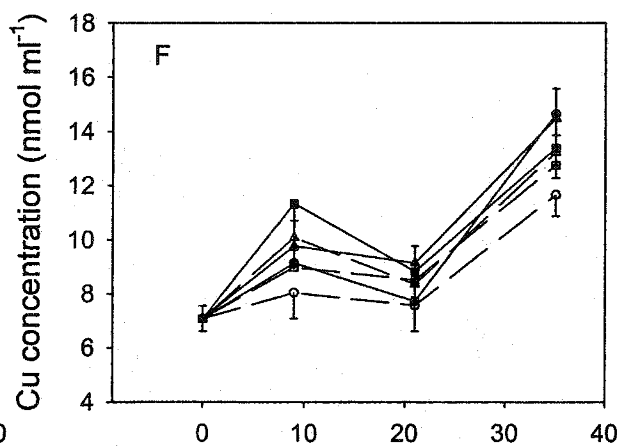






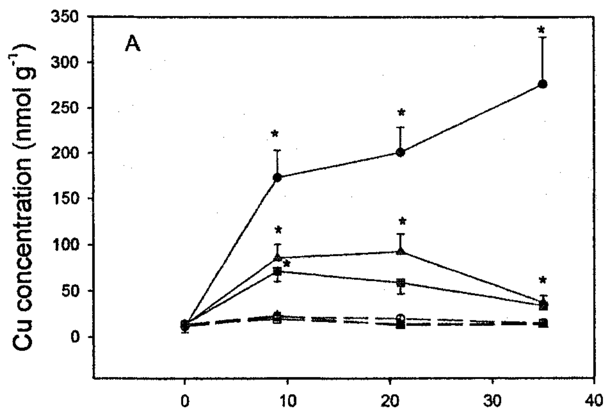


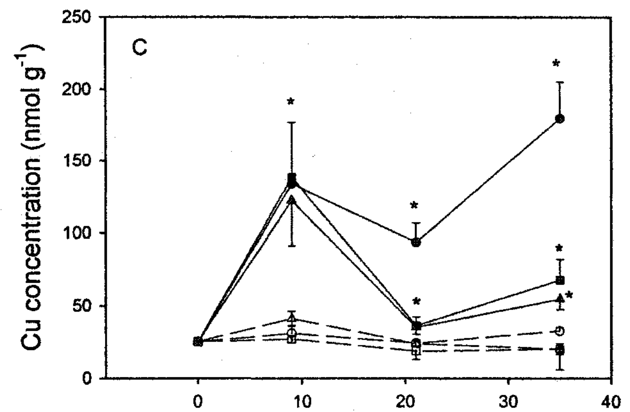


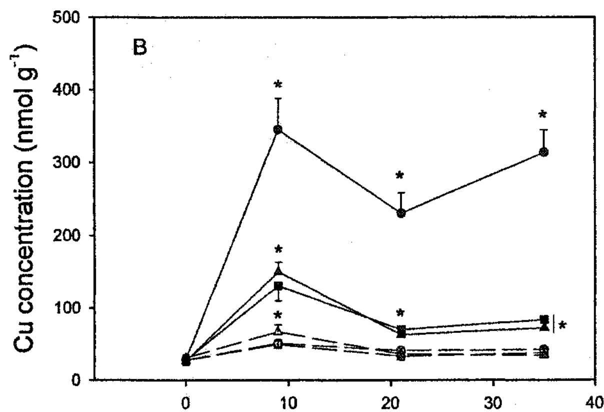


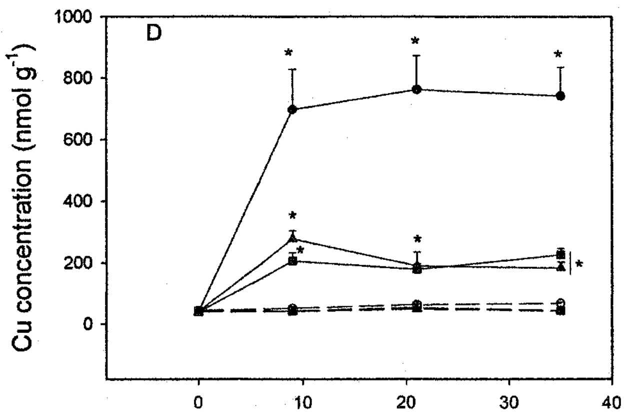
Time (days)

Figure 5-7. Cu concentration in various segments of the gut in juvenile rainbow trout following exposure to 0.24 µmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight d^{-1} rations. A, stomach; B, pyloric cecae + anterior intestine; C, mid-intestine; D, posterior intestine. Note difference in scale in different panels. Values are means ± SEM, n = 10 per treatment per data point for all the organs/tissues except day 0 controls where n = 24. ○, 1.5 % body weight ration control; ●, 1.5% body weight d^{-1} + 0.24 µmol g^{-1} d^{-1} Cu; □, 3.0% body weight d^{-1} ration control; ■, 3.0% body weight d^{-1} ration + 0.24 µmol g^{-1} d^{-1} Cu; Δ, 4.5% body weight d^{-1} ration control; ▲, 4.5% body weight d^{-1} ration + 0.24 µmol g^{-1} d^{-1} Cu. * Indicates significant difference from controls, Tukey's HSD, $p<0.05$.









Time (days)

Figure 5-8. Distribution of total body Cu in the main organs/tissues at the end of the exposure (35 days) in juvenile rainbow trout following exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight d^{-1} rations. Percentage values for liver, carcass, and gut are shown alongside the bars. Values for gill and kidney are 2.0% or less and are not shown. For all tissues at all rations, n = 10.

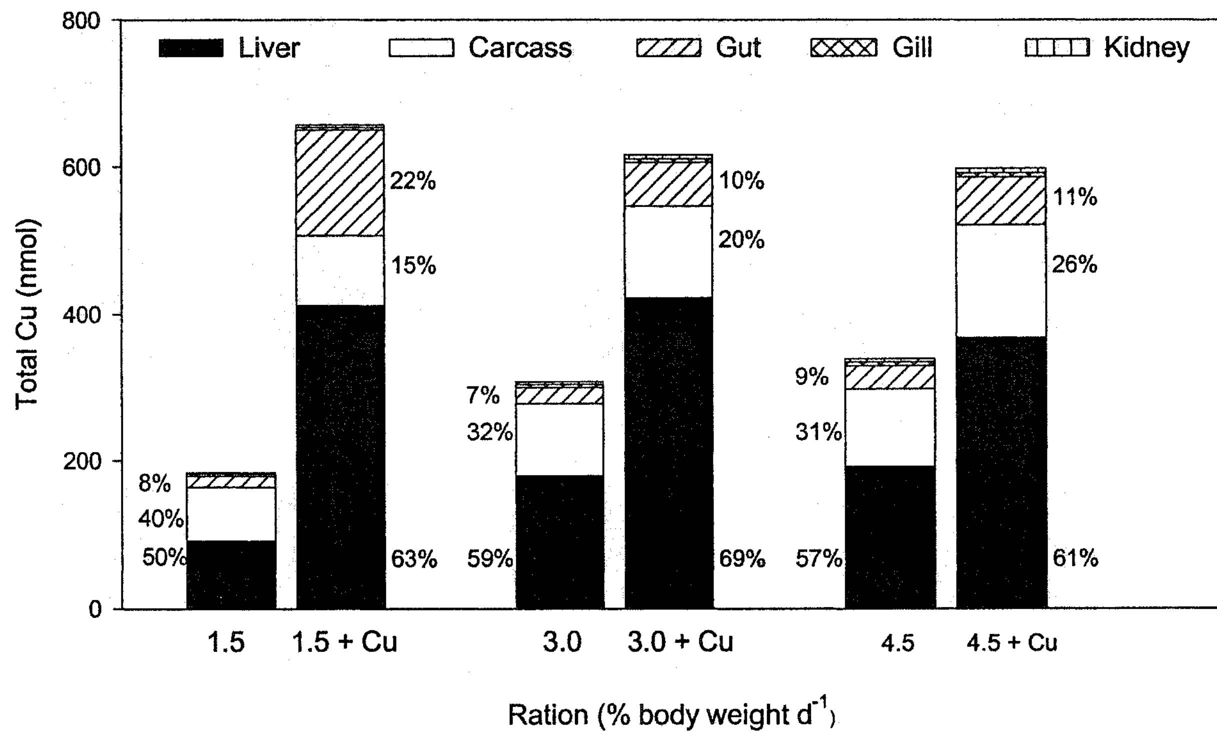
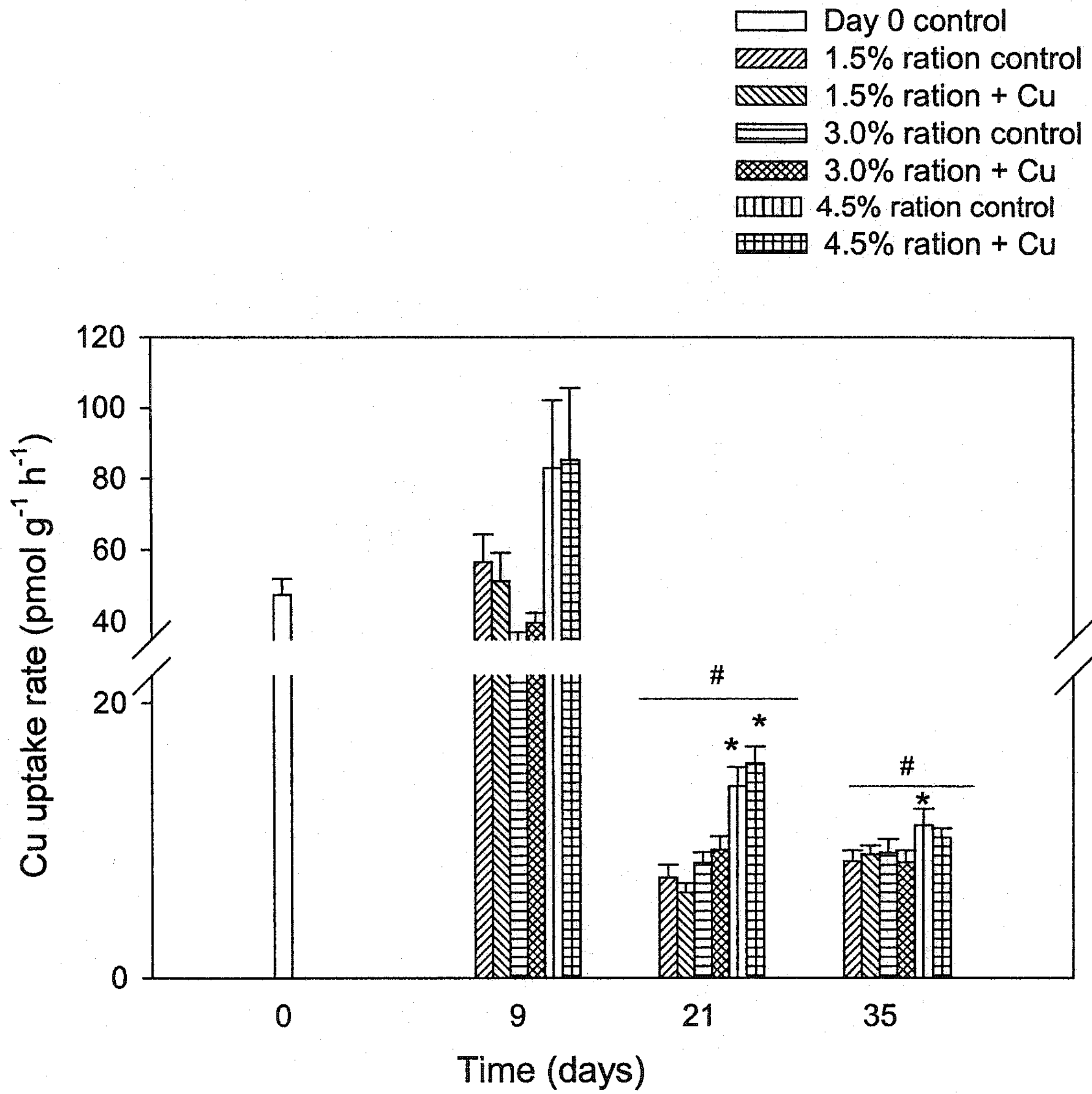
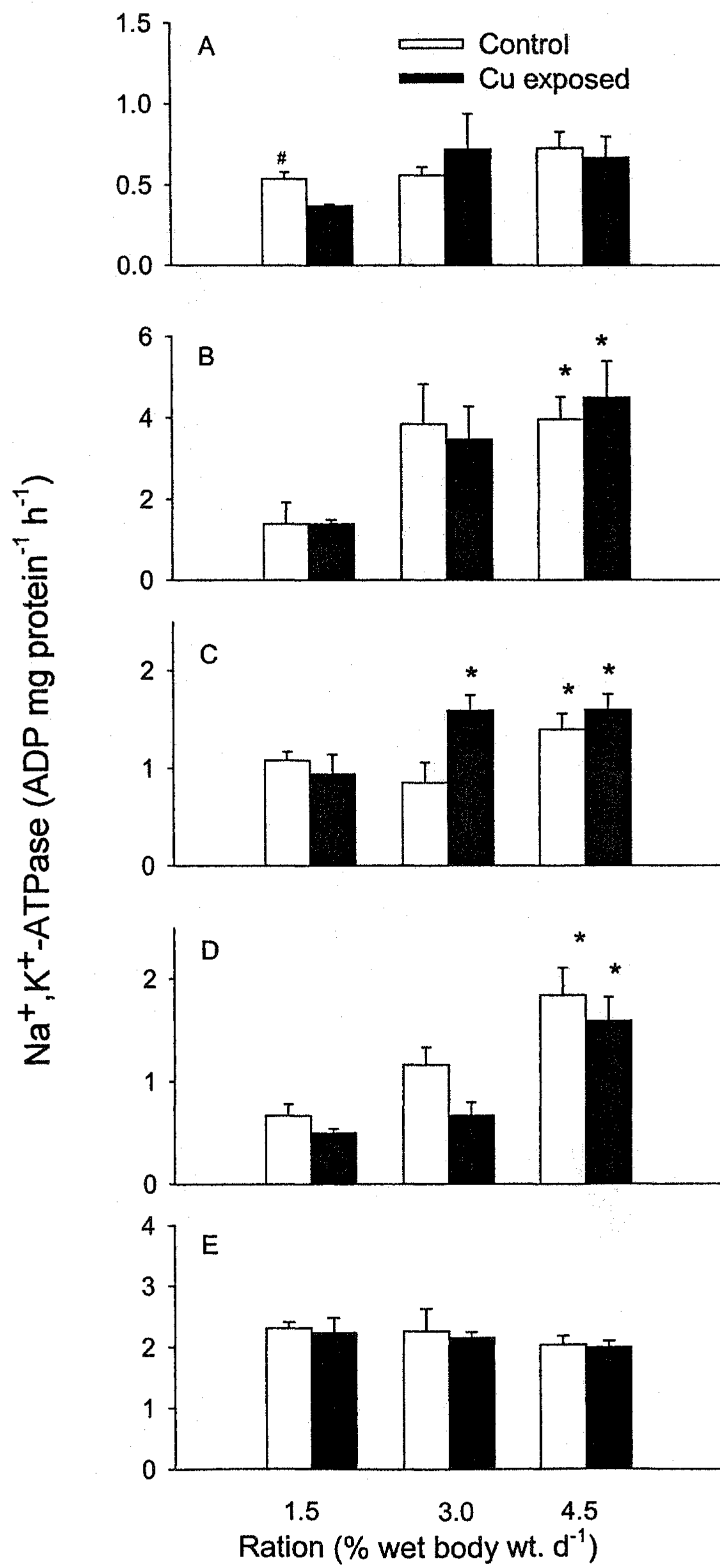

Liver
Carcass
Gut
Gill
Kidney
Total Cu (nmol)
800
600
400
200
0
8%
40%
50%
22%
15%
63%
7%
32%
59%
10%
20%
69%
9%
31%
57%
11%
26%
61%
1.5
1.5 + Cu
3.0
3.0 + Cu
4.5
4.5 + Cu
Ration (% body weight d-1)

Figure 5-9. Whole-body unidirectional waterborne Cu uptake rates measured using ^{64}Cu in juvenile rainbow trout following exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight d^{-1} rations. Values are means ± SEM, n = 10 per treatment per data point except day 0 control where n = 24. * Indicates significant difference from 1.5% body weight d^{-1} ration, and # indicates significant difference from day 0 value, Tukey's HSD, $p<0.05$.



Day 0 control
1.5% ration control
1.5% ration + Cu
3.0% ration control
3.0% ration + Cu
4.5% ration control
4.5% ration + Cu
Cu uptake rate (pmol g-1 h-1)
120
100
80
60
40
20
0
#
*
*
#
*
0
9
21
35
Time (days)

Figure 5-10. Na^+,K^+-ATPase activities in various segments of the gut in juvenile rainbow trout following 35 days of exposure to 0.24 μmol Cu per g fish per day delivered in 1.5, 3.0, and 4.5% body weight d^{-1} ration. A, stomach; B, pyloric cecae (includes anterior intestine); C, mid-intestine; D, posterior intestine; E, gill. Values are means ± SEM, n = 6 per treatment per organ/tissue. * Indicates significant difference from 1.5% body weight d^{-1} ration and # indicates significant difference from Cu-exposed fish at the same ration level, Tukey's HSD, $p<0.05$.


A
Control
Cu exposed
#
1.5
1.0
0.5
0.0
B
*
6
4
2
0
C
D
E
3
1
Na+,K+-ATPase (ADP mg protein-1 h-1)
1.5
3.0
4.5
Ration (% wet body wt. d-1)

CHAPTER 6

DIETARY SODIUM INHIBITS AQUEOUS COPPER UPTAKE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

ABSTRACT

Ours is the first study to demonstrate an influence of dietary sodium (Na^+) on waterborne copper (Cu) uptake in fish. We examined possible interactions between dietary Na^+ and the response of freshwater rainbow trout (*Oncorhynchus mykiss*) to waterborne Cu in light of recent evidence of interactions between Na^+ and Cu metabolism in the gills. Trout were maintained for 6 days on one of four diets of increasing Na^+ concentration (0.25, 0.51, 0.76, and 1.27 mmol g^{-1}, which corresponds to 0.6%, 1.2%, 1.8%, and 3.0% Na^+ by weight, respectively). At the end of 7 days, fish were exposed for 6 h to waterborne Cu spiked with ^{64}Cu to determine if the dietary Na^+ affected responses to a subsequent short-term waterborne Cu exposure. The radiotracer allowed us to distinguish between Cu occurring in fish tissues before the experiment, and 'newly accumulated' Cu arising from the experimental exposure. Dietary Na^+ concentrations of 1.8% or 3.0% reduced newly accumulated Cu concentrations in gill (from 93.9 ng g^{-1} in control to 38.9 and 20.0 ng g^{-1} in fish fed 1.8% or 3.0% Na^+-supplemented diets, respectively), liver (from 64.3 ng g^{-1} to 23.1 and 7.5 ng g^{-1}, respectively), kidney (from 29.3 ng g^{-1} to 11.7 and 7.8 ng g^{-1}, respectively), plasma (from 64.7 ng g^{-1} to 21.5 and 10.7 ng g^{-1}, respectively), and gut (from 6.8 ng g^{-1} to 3.4 and 2.2 ng g^{-1}, respectively) by 50.0% to 88.2%. The 3.0% Na^+-supplemented diets

also increased plasma and gut Na^+ concentrations by 38.1% (from 137.1 to 189.3 μmol g^{-1}) and 104.3% (from 189.3 to 115.4 μmol g^{-1}), respectively, relative to fish maintained on untreated diets. Whole-body uptake rates of both Na^+ and Cu were significantly reduced, and highly correlated ($r = 0.97$) with one another, in fish fed high-Na^+ diets relative to controls. Moreover, Na^+ efflux was 12% and 38% higher in fish fed 1.8% and 3.0% Na^+-enriched diets, respectively. Fish fed high-Na^+ diets also drank more water, but the contribution of drinking to waterborne Cu uptake was negligible. From these results, we speculate that, at least in part, aqueous Na^+ and Cu share a common branchial uptake route, probably through an apical Na^+ channel. According to this hypothesis, as the channel is down-regulated with increasing internal Na^+ concentrations, both Na^+ and Cu uptake from the water are inhibited.

INTRODUCTION

Although Cu is an essential nutrient for normal metabolic functioning (Mertz 1981; Watanabe *et al.,* 1997), it can be an important waterborne toxicant to aquatic organisms, particularly fish, when ambient concentrations exceed physiological thresholds (Wilson and Taylor, 1993; Taylor *et al.,* 2000). Many studies have demonstrated a modifying influence of water quality on Cu toxicity to fish. For example, pH affects Cu speciation which in turn affects bioavailability (Cusimano *et al.,* 1985); calcium (Ca^{2+}) associated with water hardness tends to reduce toxicity by competitively inhibiting Cu binding to fish gills (Pagenkopf, 1983; Laurén and McDonald, 1986; Playle *et al.,* 1992; Erickson *et al.,* 1996); and increasing concentrations of dissolved organic matter sequester waterborne Cu from biological uptake (Playle *et al.,* 1993; Hollis *et al.,* 1997). Although much is known about the modifying factors associated with water quality, almost nothing is known about the effects of diet quality on the response of fish to waterborne Cu.

The primary mechanism of Cu toxicity to fish results from the combined effects of a reduction in Na^+ influx and an increase in Na^+ efflux, giving rise to a net reduction of plasma and whole-body Na^+ (Laurén and McDonald ,1986; 1987a; Reid and McDonald, 1991). Reduced Na^+ influx is thought to be associated with non-competitive binding of Cu ions to the basolateral Na^+-pump, Na^+,K^+-ATPase, resulting in lower Na^+ uptake rates into the blood (Laurén and McDonald, 1987b; Pelgrom *et al.,* 1995; Li *et al.,* 1996; 1998). Massive Na^+ efflux is thought to be associated with Cu-induced damage to gill epithelia that results in a reduction of the integrity of

paracellular "tight-junctions," rendering the epithelium more permeable to internal Na^+ (Laurén and McDonald, 1986; Evans, 1987; McDonald and Wood, 1993). The result of this net loss of Na^+ is an increase in blood viscosity and blood pressure, a compensatory tachycardia, and, under acutely toxic conditions, cardiac failure (Wilson and Taylor, 1993).

The etiology of waterborne Cu toxicity to freshwater fish, as described above, is similar to that demonstrated in fish exposed to acidic water (Milligan and Wood, 1982; McDonald, 1983; McDonald and Prior, 1988; McDonald *et al.,* 1989a; 1989b). Under low pH conditions, fish demonstrate a similar ionoregulatory disturbance that leads to a net reduction in whole body Na^+. Sadler and Lynam (1987) first suggested that a pH induced ionoregulatory disturbance may be ameliorated by making use of dietary ions. Studies by Dockray *et al.* (1996), Wilson *et al.* (1996), and D'Cruz *et al.* (1998) implicated an ameliorative role of diet because satiation-fed fish exposed to acidic water demonstrated little or no stereotypical ionoregulatory disturbances when exposed to acidic water, contrary to findings in other studies where fish were maintained on limited (or no) rations. Moreover, acid-exposed fish had greater appetites relative to fish maintained under circumneutral conditions (Dockray *et al.,* 1996). These results prompted a subsequent study that identified dietary Na^+ content, rather than dietary energy content, as the key component that reduced ionoregulatory disturbances in acid-exposed fish (D'Cruz and Wood, 1998).

It seems from these studies that dietary Na^+ plays some role in reducing stereotypical ionoregulatory disturbances in fish exposed to acidic water. The purpose

of the present study was to determine if dietary Na^+ plays a similar type of protective role in rainbow trout (*Oncorhynchus mykiss*) exposed to waterborne Cu, and if so, to understand the mechanism(s) involved. This was achieved by feeding fish increasing concentrations of dietary Na^+ for one week, then challenging them with a short-term (6 h), sublethal exposure to waterborne Cu (20 μg l^{-1}), to study the effect of dietary Na^+ on Cu uptake, distribution, and effect on ionoregulatory processes, such as Na^+ flux, Na^+,K^+-ATPase activity, and drinking rates.

MATERIALS AND METHODS

Acclimation

Rainbow trout were purchased from Humber Springs Fish Hatchery (Orangeville, Ontario). Fish were held in a 600-l polypropylene tank supplied with aerated dechlorinated Hamilton, Ontario tap water ([Na^+] = 0.6 mmol l^{-1}, [Ca^{2+}] = 1.02 mmol l^{-1}, hardness = 120 mg l^{-1} as $CaCO_3$, pH = 7.6-8.0, background Cu concentration = 3 μg l^{-1}, temperature = 12-14 °C) at a rate of approximately 1 l min^{-1}. Fish were fed Corey Hatchery Feed (Corey Feed Mills, Ltd., Fredericton, New Brunswick, Canada: ionic composition given below) at a rate of 2% total fish weight daily. Bulk fish weights, composed of a sub-sample of approximately 30 fish, were monitored weekly and ration was adjusted accordingly. Photoperiod was maintained at 12 h light and 12 h dark. After two weeks under these conditions, fish were gradually acclimated to soft water by mixing reverse osmosis water with dechlorinated Hamilton tap water to achieve a final mixture of approximately 6:1 tap water: reverse osmosis water. The

final composition of the water at the end of the acclimation period was: $[Na^+] = 0.1$ mmol l^{-1}; $[Ca^{2+}] = 0.1$ mmol l^{-1}; hardness = 16 mg l^{-1} as $CaCO_3$; pH = 6.9-7.1, background Cu concentration = 1.2 μg l^{-1}, and temperature remained constant between 12 and 14 °C. Fish were held under these conditions for at least two months prior to experimentation. All subsequent experiments were conducted in this reconstituted soft water.

Experimental design

Four experiments were conducted during this study: (i) to determine the effect of dietary Na^+ on subsequent waterborne Cu uptake; (ii) to determine the effect of dietary Na^+ on whole-body Na^+ concentrations and subsequent aqueous Na^+ uptake; (iii) to determine the effect of dietary Na^+ on the simultaneous appearance of newly accumulated Cu and Na^+ in the gill and on Na^+,K^+-ATPase activity in gill tissue; and (iv) to determine the effect of feeding and dietary Na^+ on drinking rate.

In the first two experiments, five fish (wt. 9-12 g) were randomly assigned to each of four 20-l experimental tanks, where they were held for 7 days. Each of these tanks was supplied with approximately 100 ml min^{-1} of aerated, reconstituted soft water (see above). During the first 6 days of the 7-day exposure period, fish in each tank were fed at a rate of 3.0% total fish weight per day. Fish were not fed during the final 24 h. Fish in each tank received a single diet, where each diet ranged from 0.6% (control) to 3.0% Na^+ by weight (see *Diet preparation* below). Virtually all of the food provided was eaten within the first few minutes. Uneaten food and feces were

siphoned from each tank 20 minutes after feeding. This cleaning regimen, in addition to the flow-through experimental design, ensured that excess Na^+ from Na^+-supplemented diets did not accumulate in the water.

In these first two experiments, at the end of 7 days, fish were transferred for six hours to 6-l plastic flux chambers that contained either 20 μg Cu l^{-1} in vigorously-aerated, reconstituted soft water spiked with 1.5 mCi l^{-1} ^{64}Cu (first experiment), or reconstituted soft water spiked with 0.025 μCi l^{-1} ^{22}Na (second experiment; see *Copper and Na^+ fluxes*, below). In neither case did the addition of radiotracer significantly change the Cu or Na^+ concentrations in flux chambers. At the end of the 6-h flux in each experiment, fish were sacrificed in an overdose of MS-222 and dissected to separate tissues (see *Sampling and analysis*, below).

In the third experiment, 72 fish (wt. 60-105 g) were randomly assigned to two 150-l polypropylene tanks (i.e., 36 fish per tank). One tank received a normal diet (i.e., 3.0% total fish weight per day, untreated trout food), while the other tank received a 3.0% Na^+-supplemented diet at the same feeding rate. Fish were maintained under these conditions for seven days. On day eight (i.e., after a 24-h starvation period), 5-6 fish from each of the control and Na^+-diet exposed groups were moved into 20-l plastic containers to create four waterborne Cu and feeding treatments, namely: Fed, Fed + Cu, Na^+ Fed, and Na^+ Fed + Cu. Copper treatment comprised 20 μg l^{-1} labeled with ^{64}Cu (1.5 mCi l^{-1}, $CuNO_3$) for 6 h. In addition, water for all the groups was spiked with ^{22}Na (0.025 μCi l^{-1}). Simultaneous exposure to ^{64}Cu and ^{22}Na allowed for the measurement of newly accumulated Cu and Na^+ in the gill. Total Cu was also

measured in gills. For each treatment group, in addition to the samples for radioisotope counting, a sub-sample of the gill (2 middle gill arches) was dissected out and immediately frozen in liquid nitrogen for Na^+,K^+-ATPase activity analysis (see *Na^+,K^+-ATPase activities*, below).

In the fourth experiment, drinking rates were determined in three groups of fish (wt. 60-105 g) namely, unfed control, fed control, and Na^+-diet fed (n = 5-6) in the absence of waterborne Cu (see *Drinking rates*, below). For this experiment fish were moved into flux tanks a day before the experiment and feeding took place in the flux tanks in the presence of the drinking rate marker ([^{3}H]PEG-4000).

Diet preparation

All diets were prepared with granulated hatchery feed which had been ground to a powder (Corey Feed Mills, Ltd., Fredericton, New Brunswick, manufacturer's specifications: [Na^+] = 0.3 mmol g^{-1} (6 mg g^{-1}; i.e., 0.6%); [P] = 0.4 mmol g^{-1} (11 mg g^{-1}); [Cu] = 17.3 μg g^{-1}; crude protein = 55%; crude fat = 17%; crude fiber = 2%). Analytical grade NaCl was dissolved in 40% v/w distilled, deionized water and mixed into a pre-weighed sample of fish food to yield diets having 0.6% (control, no NaCl added but subjected to the same treatment as other diets), 1.2%, 1.8%, and 3.0% Na^+ by weight. The resulting paste was extruded through a pasta maker, air-dried, and broken into smaller pellets by hand. This method gave Na^+ concentrations very close to nominal values. Actual measured Na^+ concentrations in the four diets were: 0.25 (0.6%), 0.51 (1.2%), 0.76 (1.8%), and 1.27 (3.0%) mmol g^{-1}.

Copper and Na^+ fluxes

Fish were exposed to radioactive Na^+ or Cu, as ^{22}Na ($t_{1/2}$ = 31.2 months; Amersham Pharmacia Biotech, Inc., Piscataway, NJ) or ^{64}Cu (prepared at McMaster University Nuclear Reactor from $CuNO_3$, $t_{1/2}$ = 12.65 h), respectively. The use of radioisotopes allowed us to discriminate between newly accumulated Na^+ or Cu taken up by the fish during an experimental exposure from Na^+ or Cu already occurring in fish tissues before exposure to elevated (experimental) dietary Na^+ or waterborne Cu concentrations. Consequently, specific radioactivity corresponding to the Na^+ or Cu isotope in fish tissues after experimental exposures represents 'newly accumulated' Na^+ or Cu. Newly accumulated Cu or Na^+ was calculated by the following equation (Grosell *et al.,* 1997):

$$M_{New} = \frac{a}{\left(\frac{b}{c}\right)}, \qquad \text{(eq. 1)}$$

where M_{New} is the newly accumulated Cu or Na^+ concentration (ng g^{-1} or µmol g^{-1}, respectively), a is the number of γ-emissions per minute (i.e., counts per minute, or cpm) per gram of tissue or per liter as appropriate, b is the number of γ-emissions (cpm) per liter of water, and c is the total Cu or Na^+ concentration of the water. Recent studies in our laboratory have demonstrated that ^{64}Cu uptake into fish tissues is linear for up to 12 h (Kamunde *et al.,* 2001). Therefore, because Cu exposures in the present study were only 6 h in duration, uptake was linear with time. Vigorous aeration throughout the flux period ensured thorough mixing.

Unidirectional Na^+ and Cu uptake rates were determined by summing the uptake into all the individual tissues and dividing the result by the specific radioactivity in the environment, the fish's weight in kg, and the length of the exposure period (6 h) to convert to a rate. Net Na^+ flux rates were calculated from the changes in total water Na^+ over the flux period by analyzing water samples taken 15 minutes after the start of the flux and at the end of the 6 h flux period for Na^+ as described in *Sampling and analysis* below. Sodium efflux was calculated from the difference between net Na^+ flux and influx rates.

Sampling and analysis

In the first two experiments gills, liver, kidney, gut (esophagus to rectum, rinsed in deionized water [18 megaohm Nanopure II, Sybron/Barnstead, Boston, MA] to remove any partially digested food), plasma, and carcass were dissected from fish. Gill sampling involved the removal of entire gill baskets, because the volume of cartilaginous material was small, and it was impractical to separate it out in these juvenile fish. Whole blood was collected by caudal puncture using 1-ml heparinized syringes fitted with 23-gauge needles. Blood samples were immediately centrifuged at 10 000 × *g* for 5 minutes to separate cellular material from plasma. Separated plasma was decanted from the cellular material and used in subsequent analyses. Ten ml water samples were collected from each flux chamber (one at the beginning (15 minutes) and the other at the end of the flux (6 h) in all three experiments, and acidified with 100 μl concentrated HNO_3 (trace metal grade, Fisher Scientific, Nepean, Ontario).

Whole-body metal concentrations were calculated according to the following equation:

$$WB = \frac{\sum_{i=1}^{6} C_i m_i}{m_{WB}}, \qquad \text{(eq. 2)}$$

where, WB is the whole body Cu or Na^+ concentration in ng g^{-1} or μmol g^{-1}, respectively, $i=1$ to 6 represents individual tissues (gill, liver, kidney, plasma, gut, and carcass) within a single fish, C_i is the concentration of either Cu (ng g^{-1}) or Na^+ (μmol g^{-1}) in tissue i, m is the mass (g) of tissue i, and m_{WB} is the total mass (g) of the fish.

For Na^+,K^+-ATPase activities determined in the third experiment, only gill tissue was analyzed. Rather than entire gill baskets as in the previous two experiments, two middle gill arches per fish were used. Gill filaments were immediately frozen in liquid nitrogen for subsequent analyses.

Radioactivity in tissue and water samples containing ^{64}Cu and ^{22}Na was measured on a Canberra-Packard Minaxi Auto-Gamma 5000 series gamma counter with on-board automatic decay correction for ^{64}Cu (Canberra-Packard Instruments, Meriden, CT). In the third experiment where fish were exposed to ^{64}Cu and ^{22}Na simultaneously, samples were counted immediately after dissection, and were then stored for two weeks to allow the ^{64}Cu to decay to undetectable levels. Samples were then recounted for ^{22}Na, and decay-corrected accordingly. The difference between the first and second count provided a measure of ^{64}Cu activity. Tissue and water [^{3}H]PEG-

4000 activity was counted on a liquid scintillation counter (LKB Wallac 1217 Rackbeta, Pharmacia-LKB AB, Helsinki) using internal standardization.

After tissues (except plasma and water samples) were counted, they were digested in 5 volumes of 1 N HNO_3 (trace metals grade; Fisher Scientific, Nepean, Ontario) at 70°C for 24 h, and subsequently centrifuged for 5 minutes at 10 000 x *g*. A sub-sample of the supernatant (or whole plasma or water sample) was diluted appropriately in 0.5% HNO_3. Total Cu concentrations were measured by graphite furnace atomic absorption spectrophotometry (GFAAS; Varian 1275 AA with GTA-95 atomizer, Mississauga, Ontario) using a 10-μl injection volume and operating conditions as suggested by the manufacturer for Cu. Total Na^+ concentrations were measured using flame atomic absorption spectrophotometry (FAAS; Varian 1275). Certified analytical standards (National Research Council of Canada) analyzed simultaneously with experimental samples were within the specified range.

Na^+,K^+-ATPase activities

Na^+,K^+-ATPase activities were determined for two gill arches from fish after 7 days of exposure to dietary Na^+ followed by 6 h of acute exposure to waterborne Cu (20 μg l^{-1}) using a slightly modified version of the microplate UV detection method described in McCormick (1993). Samples were frozen immediately in liquid nitrogen and subsequently stored at –70°C until analyzed for Na^+,K^+-ATPase activity. In this assay, the rate of hydrolysis of ATP to ADP in the presence and absence of oubain (Sigma, St. Louis, MO) was coupled to the oxidation of NADH to NAD^+. Changes in

absorbance of the reaction mixture due to NADH oxidation were measured at 340 nM over 15-sec intervals for 10 minutes. Na^+,K^+-ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of oubain and normalized to total protein in each respective sample as determined by the Bradford (1976) method.

Drinking rates

Drinking rates were measured by the method of Wilson *et al.* (1996) in unfed controls, fed controls, and in fish fed dietary Na^+ for 7 days. Fish were exposed to $[^3H]$PEG-4000 at a concentration of 5.0 mCi l^{-1} in the water for 6 hours. This is less than the period of time (10 h) by which the tracer reaches the anus at this temperature (C.M. Wood, unpublished results). Water samples (10-ml) were taken 15 minutes after addition of $[^3H]$PEG-4000 and again after the 6-h exposure. Fish were then killed with an overdose of MS-222. The entire gastrointestinal tract was exposed by dissection, ligated at esophagus and rectum, removed, and homogenized in 5 volumes of 8% $HClO_3$. Homogenate was processed for scintillation counting according to Wilson *et al.* (1996), and 1 ml was counted. Plasma was also counted to ascertain that the $[^3H]$PEG-4000 was absorbed.

Drinking rate was calculated using the equation:

$$D = \frac{C}{MTW}, \qquad \text{(eq. 3)}$$

where D is the drinking rate in ml kg^{-1} h^{-1}, C is the number of counts (cpm) in the entire gut, M represents counts (cpm) ml^{-1} water, T is the time in hours, and W is the weight of the fish in kg.

Statistical treatment

All data are reported as means ± SEM and were compared using analysis of variance (ANOVA). In cases where data did not meet normality or homogeneity of variance assumptions for ANOVA, significant differences were determined using a nonparametric Kruskall-Wallis Rank Sum test. Mean Na^+ and Cu concentrations in tissues of fish exposed to Na^+-supplemented diets in the first two experiments were compared to those of fish fed the control diet (i.e., normal, untreated trout food) using Dunnett's test. Mean Na^+,K^+-ATPase activities, and mean drinking rates, were compared among experimental treatments using Tukey-Kramer's Honestly Significant Difference test. Mean differences were considered to be significant when $p<0.05$.

RESULTS

Copper uptake

Copper uptake rates were significantly lower in rainbow trout fed 1.8% and 3.0% Na^+-enriched diets relative to controls (Fig. 1). Fish fed the 1.8% or 3.0% Na^+-supplemented diet took up waterborne Cu at a 52.9% or 75.0% lower rate, respectively, than fish fed the control diet.

Fish fed Na^+-supplemented diets of 1.8% Na^+ accumulated significantly less new Cu (as defined by equation 1) in a 6-h Cu flux period at 20 μg Cu l^{-1} than fish fed the control diet (0.6% Na^+) in gill, liver, and gut tissues (Fig. 2). New Cu uptake into kidney and plasma was reduced, but not significantly different from fish fed the control diet. Similarly, fish fed the 3.0% Na^+ diet accumulated substantially and significantly less new Cu in gills, liver, kidney, plasma, and gut relative to fish fed the control diet. Fish fed the 1.2% Na^+ diet accumulated a similar amount of new Cu relative to those fed the control diet ($p>0.05$), showing that the threshold for effect lay between 1.2% and 1.8% dietary Na^+.

Based on GFAAS analysis of total tissue Cu concentrations, only gill and liver tissues showed significantly lower total Cu concentrations in fish fed diets supplemented with 1.8% or 3.0% Na^+ relative to those fed the control diet (Fig. 3). Over the 7-day exposure, fish fed the 3.0% Na^+ diet exhibited a 25.1% reduction of Cu in the gills and 44.5% reduction of Cu in the liver, relative to fish fed the control diet. In fish fed the 1.8% Na^+ diet, only the 36.6% reduction in total liver Cu burden was significant. Neither kidney, plasma, gut nor carcass showed elevated total Cu relative to controls in any of the Na^+-supplemented diet treatments ($p>0.05$).

Sodium uptake

After 7 days of the experimental feeding regime, total Na^+ concentrations were significantly higher in gut tissue and plasma of fish fed the 3.0% Na^+ diet relative to fish fed the control diet (Fig. 4). There were no significant differences in other tissues

or at lower dietary Na^+ concentrations. As with waterborne Cu uptake rates, fish fed either 1.8% or 3.0% Na^+-supplemented diets demonstrated a significantly lower waterborne Na^+ uptake rate relative to fish maintained on the control diet (Fig. 5). Fish fed the 1.8% or 3.0% Na^+-supplemented diets took up waterborne Na^+ 40.8% or 44.0% slower than fish fed the control diet. Waterborne Na^+ and Cu uptake rates were strongly and positively correlated with one another ($r = 0.97$, $p = 0.02$). Na^+ efflux rates were also elevated in proportion to dietary Na^+ load, although these differences could not be evaluated statistically because they were measured on whole treatment groups, not individuals. Branchial Na^+ efflux rates were -0.41, -0.45, -0.47, and -0.57 $\mu mol\ g^{-1}\ h^{-1}$ in fish fed control (0.6%), 1.2%, 1.8%, and 3.0% Na^+ diets, resulting in negative net Na^+ flux rates in the 1.8% and 3.0% Na^+ treatment groups.

Na^+,K^+-ATPase activities

Na^+,K^+-ATPase activities in gill filaments (Fig. 6) varied significantly among experimental treatments. Generally, gills of fish exposed to waterborne Cu had lower Na^+,K^+-ATPase activities than those that were not exposed to Cu. Moreover, fish fed Na^+-supplemented diets showed higher gill filament Na^+,K^+-ATPase activity than those fed regular diets. Consequently, fish that were fed a Na^+-supplemented diet and were exposed to waterborne Cu showed gill Na^+,K^+-ATPase activity that was not significantly different from control fish (i.e., normal diet and no waterborne Cu; i.e., 'Fed' in Fig. 6).

Newly accumulated Na^+ and Cu in the gills

Newly accumulated gill Na^+ and Cu varied among experimental treatments (Fig. 7). Gills of fish from the Na^+ Fed + Cu treatment accumulated less than one third the amount of new Na^+ than those from the Fed + Cu treatment ($p<0.05$). However, new gill Na^+ did not vary between fish from Fed and Fed + Cu treatments, nor between Fed and Na^+ Fed + Cu treatments. Gills of Fed fish accumulated most new Cu, which was not significantly different from those of Fed + Cu treatment ($p>0.05$), but was 59.6% higher than in gills of fish from the Na^+ Fed + Cu treatment ($p<0.05$).

Drinking rates

Mean drinking rates were low and never exceeded 2 ml kg^{-1} h^{-1}, but still varied by treatment (Fig. 8). Drinking rates were not significantly different between fed and unfed fish ($p>0.05$). However, they did significantly differ between fish fed a normal diet (i.e., Fed) and those fed a 3.0% Na^+-supplemented diet ($p<0.05$). Fish fed the Na^+-supplemented diet demonstrated the highest drinking rates, which were 58.8% greater than in unfed fish.

DISCUSSION

Ours is the first study to demonstrate an influence of dietary Na^+ on waterborne Cu uptake in fish. Results reported here demonstrate that fish exposed to elevated dietary Na^+ take up significantly less waterborne Cu into their tissues, and at a slower rate, than fish maintained on a control diet.

Previous studies have shown that a dietary source of Na^+ can be just as important as a waterborne source for meeting physiological requirements in rainbow trout (Smith *et al.,* 1989; 1995; D'Cruz and Wood, 1998). Almost 100% of the Na^+ taken up from the diet is absorbed though the gut and taken up into the plasma (Smith *et al.,* 1995). Fish can lose Na^+ through their gills, liver (via biliary excretion), and kidneys, although Na^+ loss through the gills is much more important than other routes (Smith *et al.,* 1989). Fish maintain Na^+ homeostasis by modulating influx and efflux, primarily at the gills, as appropriate. Once plasma concentrations are elevated beyond the needs of the fish, branchial Na^+ efflux is stimulated and influx is inhibited to ensure that electrolyte balance is maintained (Salman and Eddy, 1987).

Fish can modulate branchial Na^+ influx by changing the activities of Na^+,K^+-ATPase in the basolateral membrane, and the proton pump, H^+-ATPase, in the apical membrane (McCormick, 1995; Lin and Randall, 1995; Karnaky, 1997). Na^+,K^+-ATPase extrudes intracellular Na^+ from branchial epithelium into the blood, while H^+-ATPase in the apical membrane pumps protons out of the cell, which increases the electrochemical gradient between the external and internal environments, thereby creating conditions that favour waterborne Na^+ influx (Lin and Randall, 1995). Sodium efflux, on the other hand, is primarily diffusive, and is modulated by changes in internal concentration, in transepithelial potential (and therefore in electrochemical gradient), and most importantly by changes in gill permeability (McDonald and Prior, 1988); (McDonald *et al.,* 1989b). The latter may reflect changes in both transcellular and paracellular pathways.

Results from this study suggest that waterborne Cu uptake is strongly associated with waterborne Na^+ uptake, and is therefore influenced by the same ionoregulatory mechanisms that control Na^+ homeostasis. Our results reveal that branchial Na^+ uptake was inhibited with increasing dietary Na^+ concentrations. This was apparent in the low branchial Na^+ uptake rates in fish fed diets containing 1.8% or 3.0% Na^+ (Fig. 5), which corresponds well with other studies investigating the ionoregulatory effects of dietary Na^+ (Salman and Eddy, 1987; Smith *et al.,* 1995). Branchial Na^+ uptake was likely inhibited in these fish as a response to elevated plasma Na^+ concentrations (Fig. 4). At the same time, branchial Cu uptake was also inhibited in fish fed 1.8% or 3.0% Na^+ diets (Fig. 1). Moreover, branchial uptake rates for waterborne Na^+ and Cu were strongly and positively correlated with one another ($r = 0.97$, $p = 0.02$). Further evidence supporting a close relationship between aqueous Na^+ and Cu uptake is shown in Fig. 7, where Cu-exposed fish fed Na^+-supplemented diets accumulated significantly less branchial Na^+ and Cu than those fed normal diets. Therefore, the evidence collected in this study suggests that dietary Na^+ inhibits branchial Cu uptake. In fact, dietary Na^+ was such an effective branchial Cu uptake blocker that gills, livers, kidneys, plasma, and guts of fish fed Na^+-enriched diets accumulated 50.0% to 88.2% less new Cu than fish maintained on a normal diet (Fig. 2).

Our results were based on food treated with NaCl in order to increase dietary Na^+ concentrations. We cannot rule out the possibility that reductions in branchial Cu uptake could be linked to dietary Cl^-. One possible way to determine if dietary Cl^- plays a role in reducing brachial Cu uptake is to repeat our experiments by

supplementing food with a different salt, such as $NaSO_4$. However, given recent evidence reported by Grosell and Wood (2002) demonstrating a common branchial Na^+-Cu uptake channel, we strongly suspect dietary Na^+, not Cl^-, inhibits branchial Cu uptake.

Radio-labeled ^{64}Cu was used in this study to distinguish between Cu taken up by fish during the 6-h exposure to elevated aqueous Cu (i.e., new Cu) and background Cu that occurred in tissues even before fish were exposed to elevated waterborne Cu (i.e., total Cu minus new Cu). An interesting effect of dietary Na^+ in these fish was the significant reduction of total Cu in gills of fish fed 3.0% Na^+-supplemented diets for the preceding 6 days, and in livers of fish fed either 1.8% or 3.0% Na^+-supplemented diets (Fig. 3). Newly accumulated Cu in gills of fish fed the 3.0% Na^+ diet accounted for only 5.0% of total gill Cu, whereas new Cu accounted for 0.11% and 0.04% of total Cu in livers of fish fed 1.8% and 3.0% Na^+ diets, respectively. Therefore, new Cu accounted for only a small fraction of the total Cu load—especially in livers. The significantly lower total gill and liver Cu concentrations in fish fed high-Na^+ diets suggests not only that normal Cu uptake was inhibited throughout the duration of the 6 day Na^+-diet feeding period, even before fish were exposed to elevated waterborne Cu in the 6 h flux, but also that net Cu efflux may have been stimulated in the Na^+-fed fish.

In addition to a reduction in branchial Na^+ influx, fish fed Na^+-supplemented diets also demonstrated high Na^+ efflux in order to maintain electrolyte balance. Sodium efflux rates were 12% and 38% higher in fish maintained on 1.8% and 3.0% Na^+-supplemented diets, respectively, resulting in negative net flux rates relative to fish

maintained on the control diet. This result corroborates other studies that have demonstrated an increase in Na^+ efflux in fish maintained on Na^+-supplemented diets (Smith *et al.,* 1995).

One of the ways that waterborne Cu causes ionoregulatory disturbances in fish is via non-competitive inhibition of branchial Na^+,K^+-ATPase activity (Laurén and McDonald 1987a; Sola *et al.,* 1995; Pelgrom *et al.,* 1995; Li *et al.,* 1998). Intracellular Cu competes with Mg^{2+} for binding sites on the ATP molecule, and forms a physiologically inert ATP complex, Cu-ATP (Li *et al.,* 1996). Normal functioning of Na^+,K^+-ATPase requires Mg-ATP. Once the basolateral Na^+,K^+-ATPase is inhibited by Cu, the branchial cell can no longer extrude intracellular Na^+ into the blood (i.e., influx is inhibited). Results reported here show Cu inhibition of branchial Na^+,K^+-ATPase in both fed fish and Na^+-fed fish when exposed to waterborne Cu. However, in the Na^+-fed fish, the inhibition was not significant relative to the fed-fish control (Fig. 6). This observation suggests that in fish fed high-Na^+ diets, Cu was less available in branchial epithelium to inhibit Na^+,K^+-ATPase activity.

Other studies examining the ionoregulatory effects of dietary Na^+, albeit at much higher concentrations (i.e., 12% NaCl w/w) than those used here, have demonstrated an increase in branchial Na^+,K^+-ATPase activity (Salman and Eddy, 1987), for which there was a non-significant tendency in the present study. Increasing Na^+,K^+-ATPase activity is commonly seen in freshwater-adapted euryhaline fish after being transferred to salt water (McCormick, 1995). The increased Na^+,K^+-ATPase extrudes excess branchial Na^+ from the gills as a means of regulating Na^+ homeostasis.

Possibly, increased Na^+,K^+-ATPase activity in gills of fish fed high-Na^+ diets may serve to regulate branchial Na^+ in the same way.

Fish fed Na^+-supplemented diets showed significantly higher drinking rates than either fed or unfed fish (Fig. 8). Undoubtedly, these increased drinking rates represent another means by which Na^+-fed fish maintain osmoregulatory and ionoregulatory balance. Freshwater fish usually drink only small quantities of water because of the hypotonic nature of the dilute medium in which they reside, which causes a constant water influx across the gills and body surface (Karnaky, 1997). In order to compensate for this water influx, fish produce a copious amount of dilute urine and limit the amount of water they consume through drinking. However, as internal Na^+ concentrations increase with a high-Na^+ diet, freshwater fish will increase their drinking rates to dilute the extra Na^+ absorbed from the diet.

It could be argued that increased drinking rates in Na^+-fed fish might contribute to a higher waterborne Cu exposure through the gut, which could potentially offset any protection conferred by dietary Na^+ observed in the gills. On average, Na^+-fed fish drank 1.7 ml kg^{-1} h^{-1} (Fig. 8) of water that contained 20 μg Cu l^{-1}. Therefore, these fish would consume approximately 0.034 ng g^{-1} h^{-1}, at least tenfold lower than Cu uptake rate across the gills (Fig. 1), so the contribution would be minor.

Taken together, results from this study suggest a common branchial uptake route shared between waterborne Na^+ and Cu (Fig. 9). Recently, Grosell and Wood (2002) have presented evidence for two high affinity mechanisms for branchial Cu uptake in the gills of rainbow trout, one that directly competes for external Na^+, and a

second that is independent of external Na^+. In accord with this study, we speculate that this common route is likely in the form of an apical Na^+ channel, whose regulation depends on internal Na^+ concentrations, and a driving potential established by H^+-ATPase (Lin and Randall, 1995). Dietary Na^+ is taken up through the gut, causing an increase in plasma Na^+ concentrations. Plasma Na^+ can then be lost by passive diffusion to the water through the gills. Some of the excess Na^+ may be taken up by branchial cells via simple diffusion or by a Ca^{2+}/Na^+ exchanger on the basolateral membrane (Verbost *et al.,* 1994). Sodium is also being taken up actively from the water through a putative Na^+ channel energized by the proton pump that it shares with Cu. The putative Na^+ channel would be down-regulated in response to elevated intracellular Na^+ concentrations, resulting in a sharp reduction of aqueous Na^+ and Cu uptake. The net effect is an increase in branchial Na^+ efflux, and a decrease in Na^+ and Cu influx.

Of course, the model we have proposed here does not preclude the other (Na^+-independent) branchial uptake routes for Cu characterized by Grosell and Wood (2002) Indeed, given that some new Cu accumulated in all tissues examined upon exposure to waterborne Cu for 6 h, regardless of Na^+ content in the food, Cu was likely being taken up from the water by some other route in addition to the shared Na^+ channel as suggested here. In this regard, Kamunde *et al.* (2001; 2002c) have recently demonstrated that branchial Cu uptake is also responsive to internal Cu status of the fish. There is an interesting parallel here to cadmium metabolism, which is thought to be taken up across the gill via apical Ca^{2+} channels in the ionocyte (Verbost *et al.,*

1989). Recently, Zohouri *et al.* (2001) reported that elevated dietary Ca^{2+} reduced but did not eliminate branchial cadmium uptake in rainbow trout.

In conclusion, dietary Na^+ effectively blocks waterborne Cu uptake in rainbow trout. Aqueous Cu uptake inhibition was closely associated with an inhibition of aqueous Na^+ uptake. Consequently, we propose that waterborne Na^+ and Cu share a common apical channel that is regulated, at least in part, based on internal Na^+ requirements. Although this study demonstrates the influence of dietary Na^+ on waterborne Cu uptake during short-term Cu exposures, the same principles seem to apply under chronic exposure conditions (Kamunde *et al.,* 2002a). Possible implications to wild fish may include protective effects of high Na^+ diets against waterborne Cu toxicity in nature, active dietary choice of high-Na^+ food items by Cu-stressed fish, and perhaps even Cu deficiency in fish raised on high-Na^+ diets in aquaculture. All these potential consequences deserve further investigation.

Acknowledgements

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Figure 6-1: New Cu uptake rate (as defined by equation 1) into rainbow trout fed for 7 days on diets ranging in Na^+ concentration after a subsequent 6-h exposure to 20 μg l^{-1} of waterborne Cu. Bars represent means ± SEM n = 4-5, asterisks (*) represent significant difference from control (0.6%) diet ($p<0.05$).

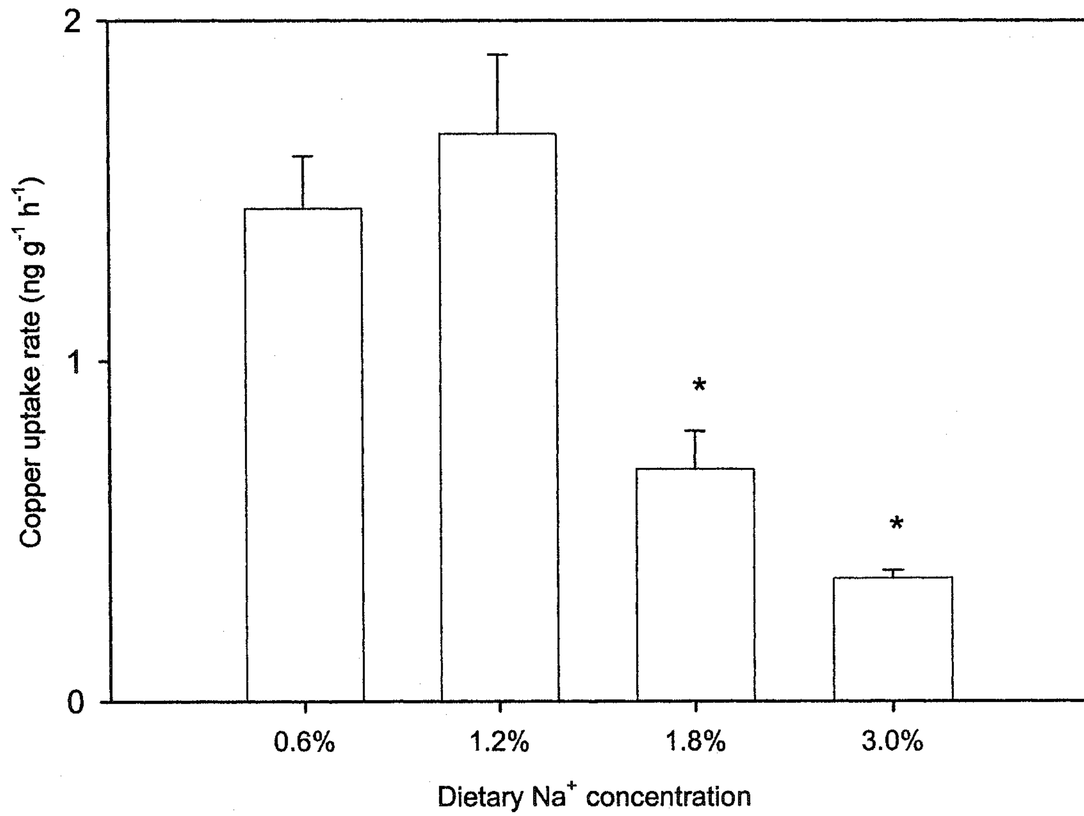

2
1
0
Copper uptake rate (ng g-1 h-1)
*
*
0.6%
1.2%
1.8%
3.0%
Dietary Na+ concentration

Figure 6-2: Newly accumulated Cu (as defined by equation 1) in gills, liver, kidney, blood plasma, and gut (inset) of rainbow trout fed for 7 days on diets ranging in sodium concentration after a subsequent 6-h exposure to 20 $\mu g\ l^{-1}$ of waterborne Cu. Points represent means ± SEM, n = 4-5, asterisks (*) represent significant difference from control (0.6%) diet ($p<0.05$).

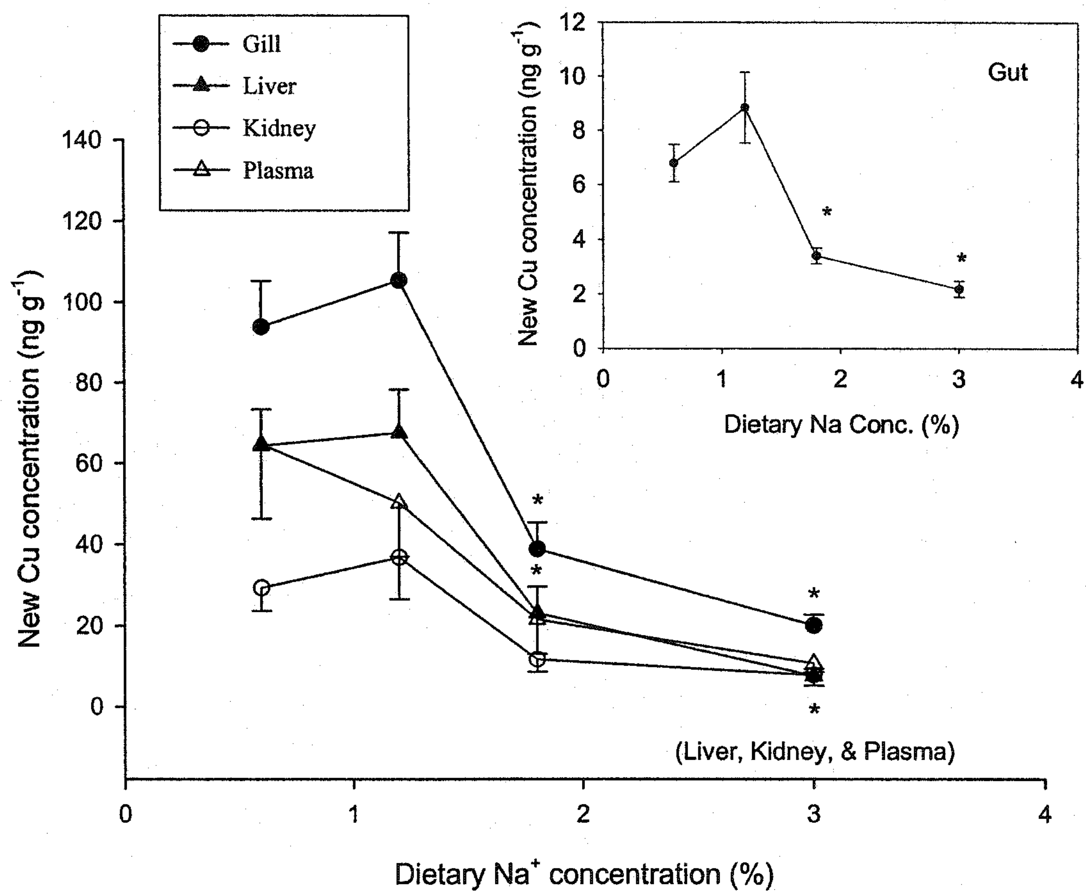
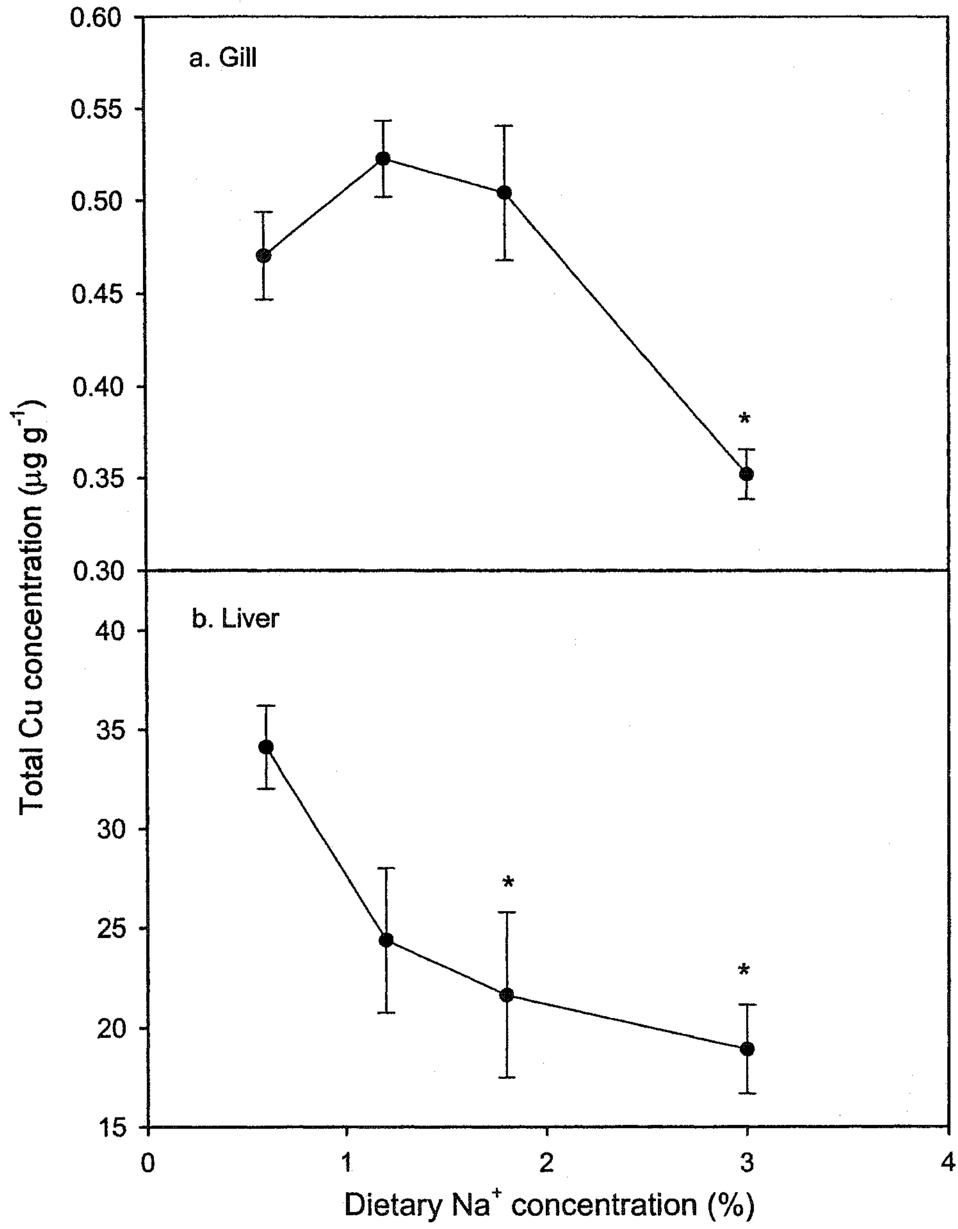

Gill
Liver
Kidney
Plasma
New Cu concentration (ng g-1)
140
120
100
80
60
40
20
0
0
1
2
3
4
Dietary Na+ concentration (%)
*
*
*
*
(Liver, Kidney, & Plasma)
Gut
12
10
8
6
4
2
0
Dietary Na Conc. (%)
*
*

Figure 6-3: Total Cu concentrations in gills (a) and livers (b) of rainbow trout fed for 7 days on diets ranging in Na^+ concentration after a subsequent 6-h exposure to 20 µg l^{-1} of waterborne Cu. Same format as Fig. 2.



a. Gill
b. Liver
Total Cu concentration (μg g-1)
Dietary Na+ concentration (%)
0.60
0.55
0.50
0.45
0.40
0.35
0.30
40
35
30
25
20
15
0
1
2
3
4
*
*
*

Figure 6-4: Total Na^+ concentrations in gut tissue and plasma of rainbow trout fed for 7 days on diets ranging in Na^+ concentration after a subsequent 6 hour exposure to 20 μg l^{-1} of waterborne copper. Same format as Fig. 2.

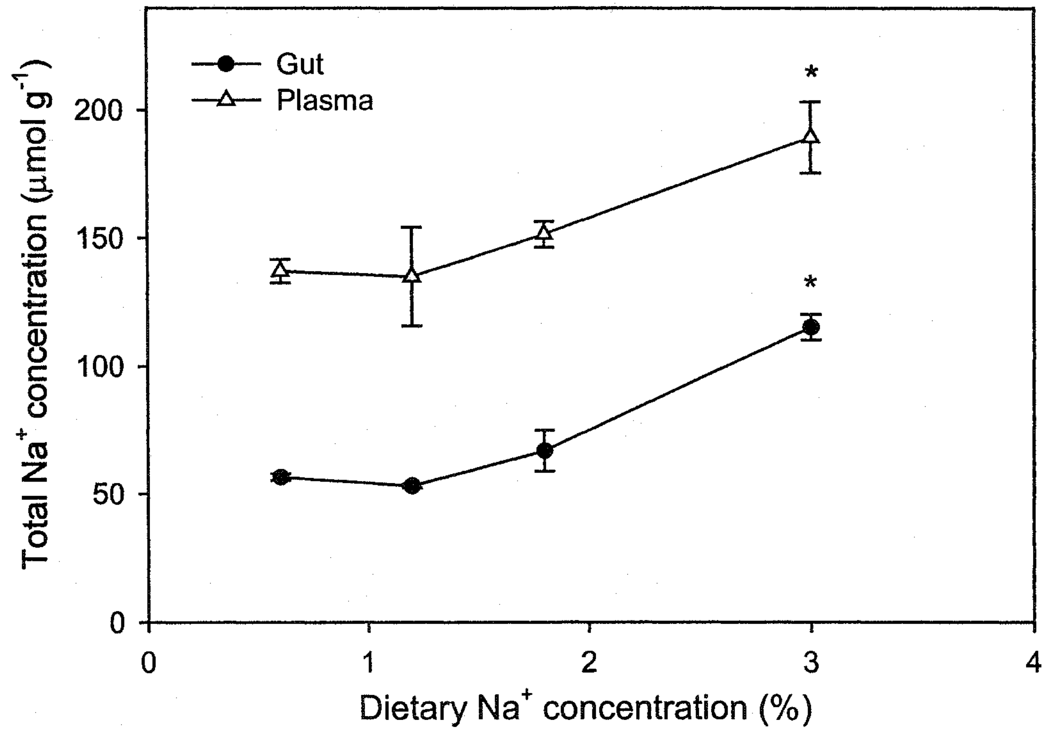
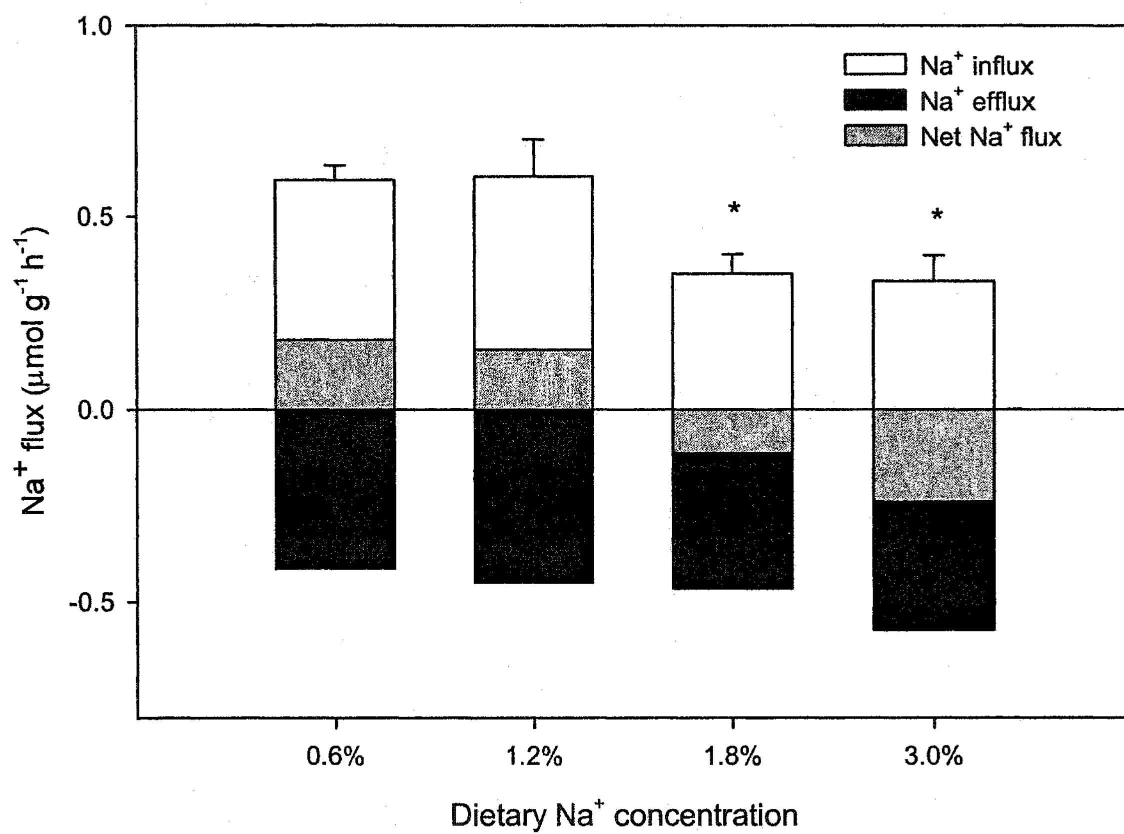

Gut
Plasma
Total Na+ concentration (µmol g-1)
Dietary Na+ concentration (%)
0
50
100
150
200
0
1
2
3
4
*
*

Figure 6-5: Na^+ influx, efflux, and net flux rates into juvenile rainbow trout fed for 7 days on diets ranging in Na^+ concentration after a subsequent 6 hour exposure to 20 μg l^{-1} of waterborne Cu. Na^+ influx rates were determined on individual fish, which facilitated the calculation of means and standard errors (bars; n = 4-5) and statistical comparisons (asterisks [*] indicate statistical significance). However, efflux rates were determined on groups of fish, which precluded statistical comparisons among treatment groups for efflux or net flux data.



Na+ influx
Na+ efflux
Net Na+ flux
1.0
0.5
0.0
-0.5
Na+ flux (µmol g-1 h-1)
*
*
0.6%
1.2%
1.8%
3.0%
Dietary Na+ concentration

Figure 6-6: Branchial Na^+,K^+-ATPase activity in rainbow trout exposed to different feeding regimes and waterborne Cu concentrations. Experimental treatments included feeding fish 3.0% of their total body weight per day on untreated food ('Fed'), or feeding fish 3.0% of their total body weight per day on food that was supplemented with 3.0% Na^+ by weight ('Na^+ Fed'). Fish were then exposed to either no Cu in the water, or to 20 $\mu g\ l^{-1}$ of dissolved Cu for 6 h. Bars represent means ± SEM, n = 5-6, bars sharing the same letter are not significantly different from one another ($p>0.05$).

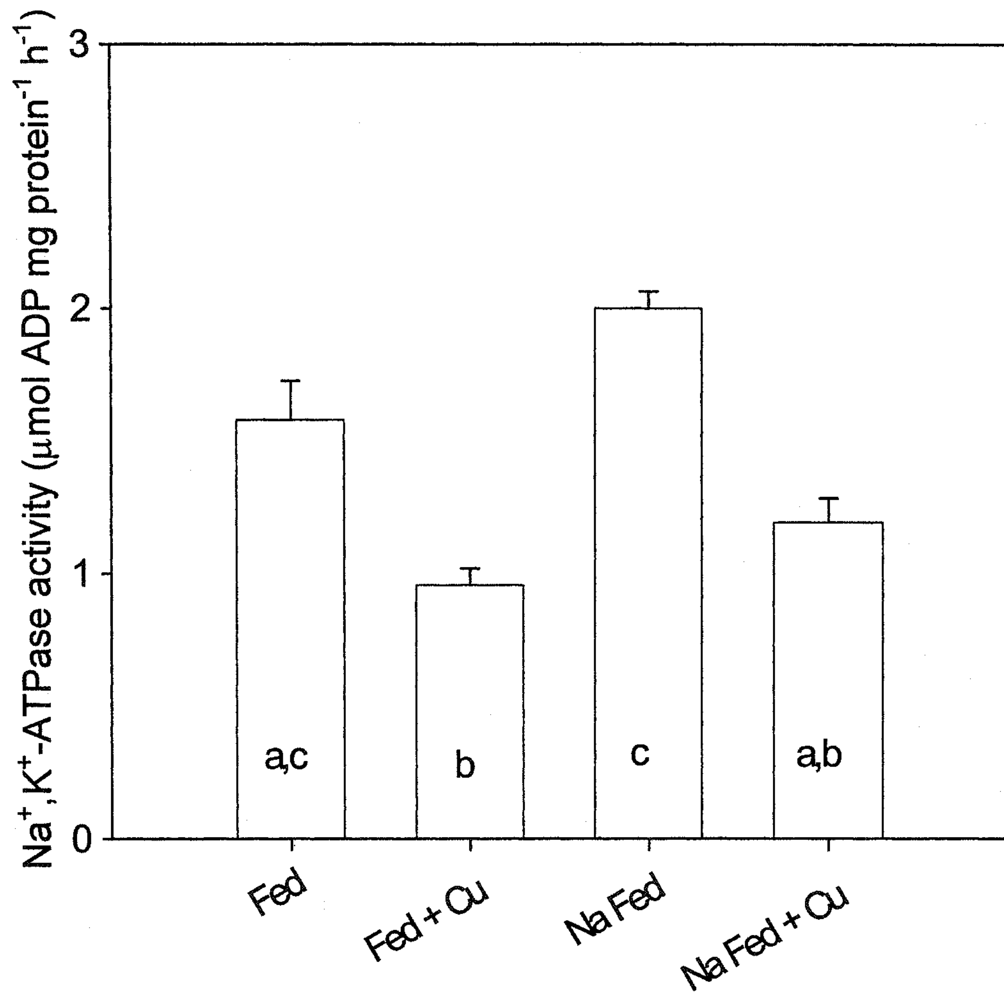

Na+,K+-ATPase activity (µmol ADP mg protein-1 h-1)
3
2
1
0
a,c
b
c
a,b
Fed
Fed + Cu
Na Fed
Na Fed + Cu

Figure 6-7: New Na^+ (a) and Cu (b) accumulation (as defined by equation 1) in gills of rainbow trout exposed to a normal diet and no waterborne Cu ('Fed'), a normal diet and 20 $\mu g\ l^{-1}$ waterborne Cu ('Fed + Cu'), or a diet supplemented with 3.0% Na^+ by weight and 20 $\mu g\ l^{-1}$ waterborne Cu ('Na^+ Fed + Cu'). Same format as Fig. 6.

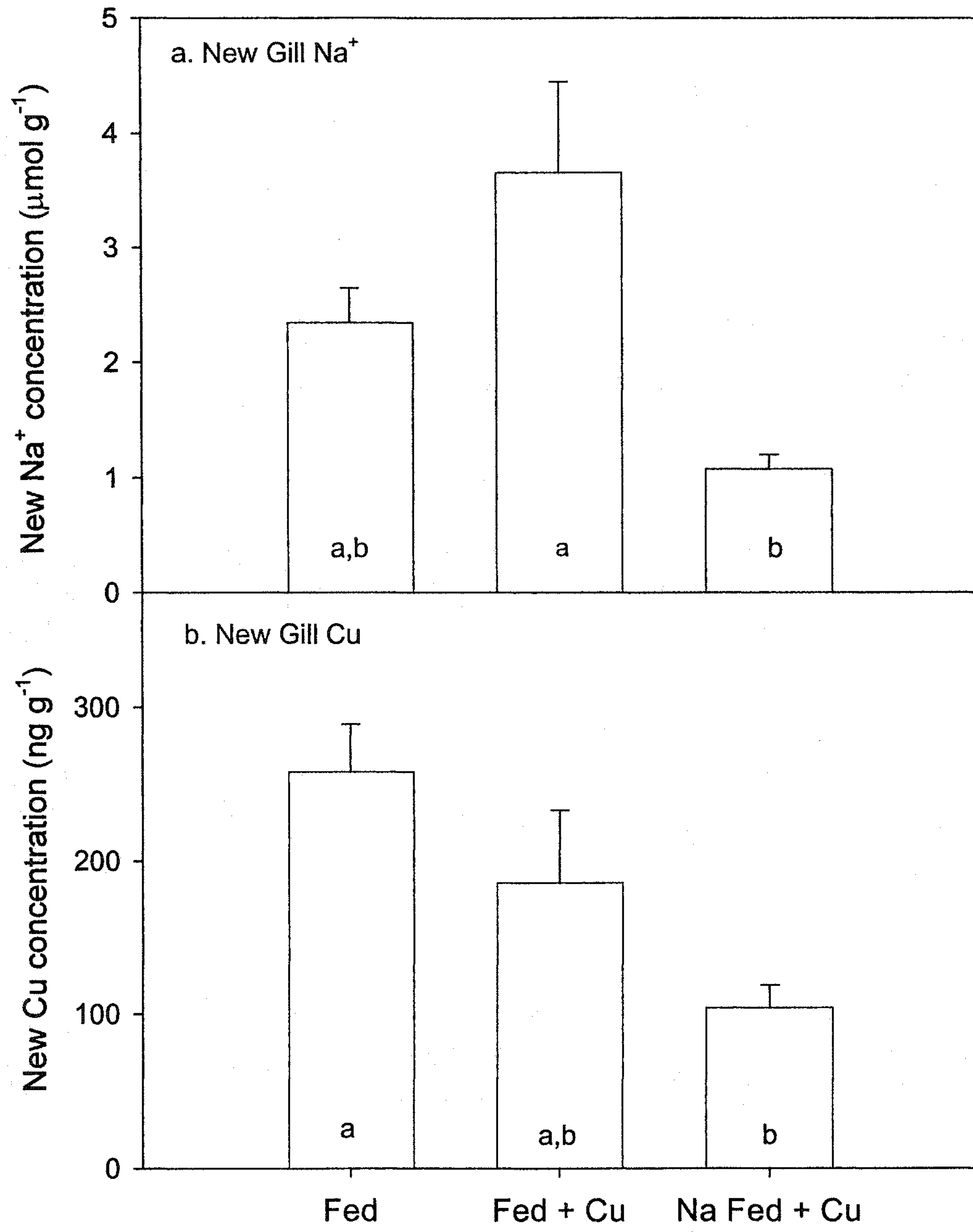

a. New Gill Na^+
New Na^+ concentration (µmol g^{-1})
5
4
3
2
1
0
a,b
a
b
b. New Gill Cu
New Cu concentration (ng g^{-1})
300
200
100
0
a
a,b
b
Fed
Fed + Cu
Na Fed + Cu

Figure 6-8: Drinking rates in rainbow trout that were either starved ('Unfed'), fed 3.0% of their total weight on a normal diet ('Fed'), or fed 3.0% of their total weight on a diet supplemented with 3.0% Na^+ by weight ('Na^+ Fed') for 7 days. Bars represent means ± SEM, n = 5, bars sharing the same letter are not significantly different from one another ($p>0.05$).

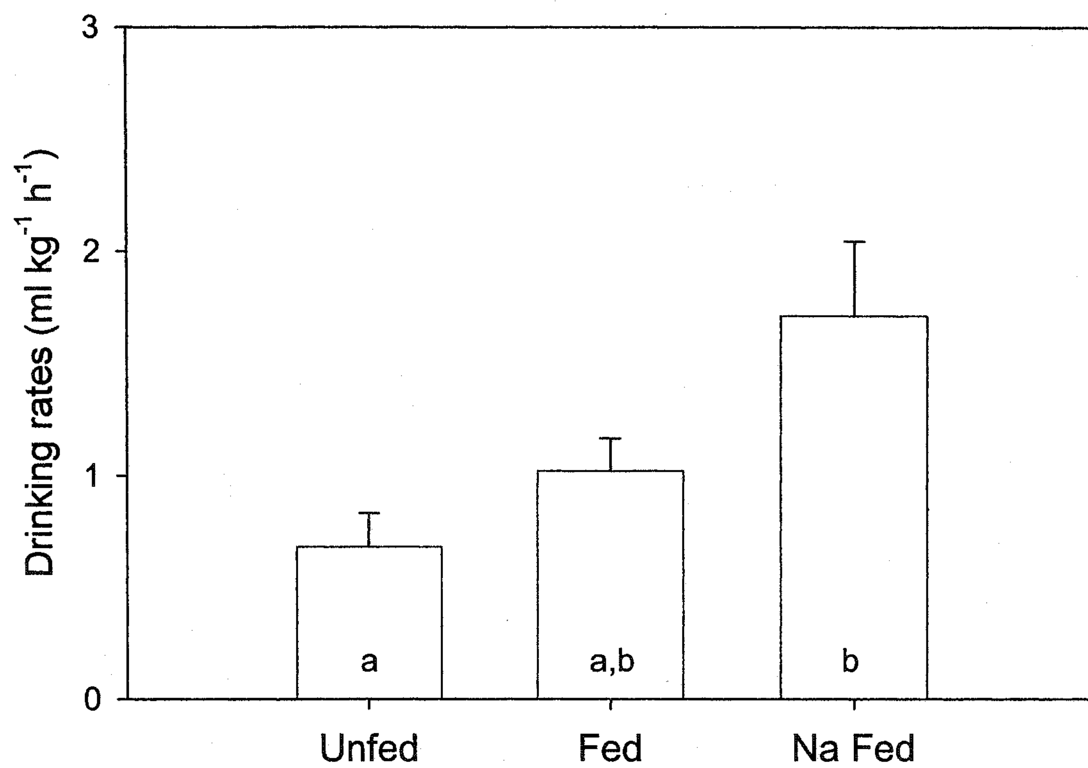
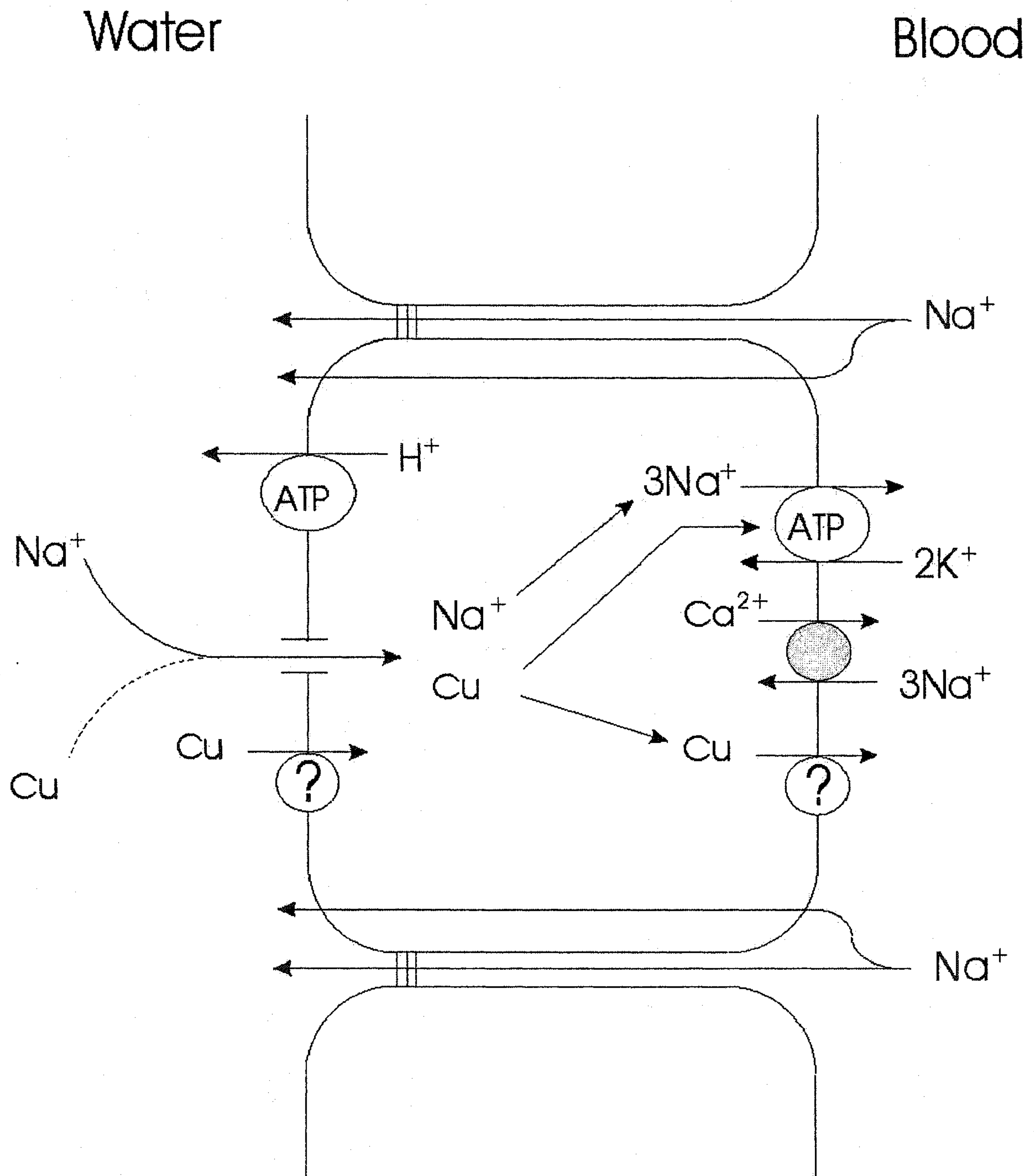

Drinking rates (ml kg-1 h-1)
3
2
1
0
a
a,b
b
Unfed
Fed
Na Fed

Figure 6-9: Conceptual model of Na^+ and copper regulation in chloride cells of rainbow trout gills, including a common apical channel shared between Na^+ and Cu (dotted line). As Na^+ absorbed from the diet accumulates in these cells, the apical channel is down regulated. This results in reduced Na^+ and Cu uptake, which is thought to be the mechanism by which dietary Na^+ protects against waterborne Cu exposure. Details are given in the text.


Water
Blood
Na+
H+
ATP
3Na+
ATP
2K+
Na+
Na+
Ca2+
Cu
3Na+
Cu
Cu
?
?
Cu
Na+

CHAPTER 7

INFLUENCE OF DIETARY SODIUM ON WATERBORNE COPPER TOXICITY IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

ABSTRACT

Juvenile rainbow trout were fed diets containing control (0.26 mmol g^{-1}) or elevated (1.3 mmol g^{-1}) dietary Na^+ in combination with either background (19 nmol l^{-1}) or moderately elevated levels (55 or 118 nmol l^{-1}) of waterborne Cu for 21 days. Unidirectional waterborne Na^+ uptake rates (measured with ^{22}Na) were up to 4 orders of magnitude higher than those of Cu (measured with ^{64}Cu). Chronic exposure to elevated dietary Na^+ alone or in combination with elevated waterborne Cu decreased whole-body uptake rates of waterborne Na^+ and Cu. Accumulation of new Cu and Na^+ at the gills was positively and highly significantly correlated and responded to the experimental treatments in a similar fashion, suggesting that Na^+ and Cu have common branchial uptake pathways, and that dietary Na^+ pre-exposure modifies these pathways. Chronic exposure to elevated waterborne Cu significantly increased Cu concentrations in the liver but caused only modest increases in total Cu concentrations in the whole-body and gill. Chronic exposure to elevated dietary Na^+ slightly decreased whole-body Cu concentration on day 14 and greatly reduced liver Cu concentration on days 14 and 21; new Cu accumulation in whole-body, gill, and internal organs was reduced on all days. Chronic exposure to elevated waterborne Cu or dietary Na^+ alone reduced short-term

gill Cu-binding at low waterborne Cu concentrations. At high waterborne Cu concentrations, chronic exposure to elevated waterborne Cu had no effect, while elevated dietary Na^+ increased Cu-binding to the gills. Combined chronic exposure to elevated dietary Na^+ and waterborne Cu decreased gill Cu-binding over the entire range of Cu concentrations tested. Clearly, chronic exposure to elevated dietary Na^+ and waterborne Cu appear to modify gill Cu-binding characteristics and may be important considerations in future development of a chronic Biotic Ligand Model (BLM) for Cu.

Keywords: Na^+ and Cu uptake, Dietary sodium, Gill Cu-binding, Rainbow trout, Soft water

INTRODUCTION

Freshwater fish thrive in dilute external media and constantly lose body ions such as Na^+ to the environment primarily via the gills and to some extent via the kidney and gut. To maintain ionic balance they actively take up ions from the environment via the gills (Wood, 2001). The primary pathway for Na^+ uptake in freshwater fish gill is believed to be via an apical Na^+ channel (Lin and Randall, 1995; Sullivan *et al.*, 1995). However, absorption of dietary Na^+ can contribute to the maintenance of ionic balance (Smith *et al.*, 1989) and may alter waterborne Na^+ flux rates (Smith *et al.*, 1995; Pyle *et al.*, 2002) indicating that branchial and gastrointestinal mechanisms cooperate to maintain ionic balance in fish. This co-operative phenomenon has been exploited to enhance the capacity of freshwater salmonids to adapt to seawater by pre-feeding them dietary NaCl, which mimics the salt load in a marine environment (Salman and Eddy, 1987, 1988, 1990; Pelletier and Besner, 1992). In addition, dietary Na^+ absorption may be important in situations where branchial Na^+ uptake is impaired. Several waterborne contaminants including exposure to acid (McDonald, 1983), Cu (Laurén and McDonald, 1987a), and silver (Morgan *et al.*, 1997) impair Na^+ homeostasis in fish. However, not much is known about the possible use of dietary salt to alleviate waterborne metal toxicity.

It has been suggested that Cu and Na^+ share, at least partially, the uptake pathways in gill as well as gut epithelia. For example, waterborne Cu exerts toxicity at the gills by disturbing Na^+ balance (Laurén and McDonald, 1987a and b; Wilson and Taylor, 1993; Wood, 2001; Pyle *et al.*, 2002) with death resulting from cardiovascular

collapse (Wilson and Taylor, 1993). More recently Grosell and Wood (2002) demonstrated that pharmacological blockade of Na^+ uptake with phenamil and bafilomycin decreased Cu uptake. In addition, Wapnir and Stiel (1987) and Wapnir (1991) reported parallel Cu and Na^+ transport in rat jejunum and proposed that this interaction occurs at the apical Na^+ channel in mammals.

There are therefore undoubtedly marked interactions between branchial and gastrointestinal Na^+ uptake mechanisms in the maintenance of Na^+ homeostasis, and a linkage between Na^+ and Cu uptake mechanisms. The question of whether dietary salt protects against the toxic effects of waterborne xenobiotics that disrupt Na^+ homeostasis in fish is therefore of interest in aquatic toxicology. To this end, Dockray *et al.* (1996) and D'Cruz *et al.* (1998) showed that feeding protected against ionoregulatory disturbance associated with environmental acid exposure in trout, while D'Cruz and Wood (1998) demonstrated that the salt content of the diet, rather than its energy, was the key protective agent. More recently, we have demonstrated that dietary Na^+ reduces waterborne Cu uptake during short-term exposure to waterborne Cu (Pyle *et al.*, 2002).

Our recent investigation (Pyle *et al.*, 2002) focused primarily on unveiling the physiological mechanisms of interactions between waterborne Cu, dietary Na^+, and waterborne Na^+ uptakes using short-term exposures. In the current study, we investigated the long-term effects of simultaneous dietary Na^+ and waterborne Cu exposure on Na^+ and Cu homeostasis and sublethal toxicity in juvenile rainbow trout. We hypothesized that changing whole-body Na^+ status or requirement via increased dietary salt intake would protect against waterborne Cu uptake and sublethal toxicity.

Second, we tested the hypothesis that dietary Na^+ intake reduces the metabolic cost associated with branchial ion regulation and/or Cu homeostasis leading to improved growth performance. Third, given the recent development of the biotic ligand model (BLM) for site-specific toxicity testing and derivation of water quality criteria (Bergman and Dorward-King, 1997; MacRae *et al.,* 1999; Di Toro *et al.*, 2001; Santore *et al.*, 2001), we assessed the possible effects of dietary Na^+ and chronic exposure to environmentally realistic waterborne Cu levels on gill biotic ligand Cu-binding.

MATERIALS AND METHODS

Fish and soft water acclimation

Juvenile rainbow trout (*Oncorhynchus mykiss*) 6-7 g in weight were obtained from Humber Springs Trout Farm, Ontario, and maintained for two weeks in laboratory conditions consisting of a constant flow of aerated Hamilton City tap water containing in mmol l^{-1}: Na^+ 0.6, Cl^- 0.7, Ca^{2+} 1.0, hardness 1.4 as $CaCO_3$, alkalinity 0.95 as $CaCO_3$, and dissolved organic carbon 3.0 mg l^{-1}. Water pH and temperature were 7.9-8.2, and 14 °C, respectively, and photoperiod was 12 h light and 12 h dark. Background Cu concentration was 31.5-47.3 nmol l^{-1}. Subsequently, the fish were acclimated to soft water. Soft water acclimation entailed gradual exposure of fish to water of increasingly lower ionic content over a period of two weeks. This was achieved by mixing soft water generated from dechlorinated Hamilton City tap water by reverse osmosis with reducing proportions of regular dechlorinated tap water to achieve a final mixture of about 6:1 reverse osmosis:tap water over two weeks. The composition of the water at the end of

acclimation was in mmol l^{-1}: Na^+ 0.11, Cl^- 0.10; Ca^{2+} 0.13, hardness 0.16 as $CaCO_3$, alkalinity 0.15 as $CaCO_3$, and dissolved organic carbon 0.3 mg l^{-1}. Water pH and temperature were 6.9-7.1 and 14 ± 1 °C, respectively, and background Cu concentration was 19 nmol l^{-1}. Fish were maintained in this water for two and a half months before initiation of the experiment since previous data (Taylor *et al.*, 2000) suggest that soft water acclimation, as evidenced by recovery of whole-body electrolytes, takes about 10 weeks in this species. During the pre-experimental and soft water acclimation period, fish were fed once daily at 2% wet body weight on commercial trout chow (Corey Feed Mills, Fredericton, New Brunswick) containing in %: crude protein 55, crude fat 17, crude fiber 2, Ca^{2+} 1.5, Na^+ 0.6. The measured Cu concentration of the diet was 0.27 μmol g^{-1}.

Experimental diets, feeding, and growth

Experimental diets were made in-house by supplementing the commercial trout chow with NaCl to achieve a concentration of 1.3 mmol g^{-1} Na^+. Essentially, the appropriate amount of NaCl was dissolved in 40% (v/w) diet weight of double-distilled water, added to commercial trout chow that had first been finely ground, and then mixed thoroughly for 45 min in a pasta maker. The resulting food paste was then extruded, air-dried, and broken into small pellets (approximately 3 mm^3) by hand. Control diet was treated in the same way except that no NaCl was added. Both control and Na^+-supplemented diets were kept at –20 °C until they were used. The actual Na^+ content of the salt-loaded diet, determined by flame atomic absorption spectroscopy (Varian

Spectra AA220, Mississauga, ON) was 1.27 mmol g^{-1} (~30 mg g^{-1}), in comparison to 0.25 mmol g^{-1} (~6 mg g^{-1}) in the control diet. During the experiment, fish were fed the designated diet at 3.0% wet body weight per day at half the total ration (1.5%) twice a day, in the morning (08:00-09:00 h) and evening (18:00-19:00 h). Visual observation revealed that all the food was ingested. Growth was assessed weekly, and the bulk fish weights that were obtained each week for each group were used to calculate the ration for the following week. Mortalities were recorded daily during the 21-day exposure and ration was adjusted accordingly.

Exposure set-up

The exposure set-up was a 2x3 factorial design (2 diets: control and elevated, and 3 waterborne Cu treatments: nominally 0, 55, and 118 nmol l^{-1}) and consisted of six 200-l plastic tanks partitioned in half by dividers, giving a total of 12 experimental chambers. The dividers were perforated to allow free movement of water, and therefore identical water composition, between the two compartments while retaining the fish within the designated compartment. Each half of the tank was provided with gentle aeration and contained 60 fish (120 for the entire tank) providing a loading density of sixty 13 g fish per 100 l of water. For all the tanks, fish on one side of the partition were fed the high salt diet while those on the other received the control diet.

The Cu dosing system consisted of two Marriotte bottles containing stock solutions of Cu ($CuSO_4 \cdot 5H_2O$, Fisher Scientific, Toronto, ON) at concentrations of 31.5 and 47.2 μmol l^{-1} maintained at drip rates of 1.8 and 2.6 ml min^{-1}, respectively,

into two head tanks receiving soft water at flow rates of 1.05 l min^{-1}. Water in the head tanks was continuously mixed by aeration to ensure even distribution of Cu. All the experimental tanks were supplied with the designated water at flow rates of 0.5 l min^{-1} providing half replacement of the water in 4.6 h. Actual water Cu concentrations based on daily samples taken in the exposure tanks and determined by atomic absorption spectrophotometry were in means ± SEM, nmol l^{-1} (n): control 19.2 ± 0.8 (18), medium 58.6 ± 1.4 (18), and high 113.5 ± 4.3 (18).

For all measurements, individual fish, rather than the tank, was our experimental unit because it is technically very difficult to use one fish per tank in chronic toxicological experiment involving different feeding regimes and waterborne metal exposure levels. To check for random effects, all the treatments were duplicated. Moreover, keeping fish together allows for social interactions, and better reflects the natural conditions.

Unidirectional waterborne Cu and Na^+ uptake

The effect of the experimental exposure conditions on unidirectional waterborne Cu and Na^+ uptake via the gills was assessed on days 0, 7, 14, and 21 following bulk weighing on these days. The fish were starved for 24 h prior to the flux measurements. For each combination of dietary Na^+ and waterborne Cu exposure, five fish from each replicate (n = 10 per treatment) were moved into plastic bags containing 5 l of soft water and simultaneously exposed to waterborne ^{64}Cu (3.3 μCi l^{-1}, McMaster University Nuclear Reactor, Hamilton, ON) and ^{22}Na (0.025 μCi l^{-1}, Amersham

Pharmacia Biotech., Piscataway, NJ) at background water Cu concentration for 3 h under static water conditions and continuous gentle aeration. The short and long half-lives of ^{64}Cu (12.65 h) and ^{22}Na (31.2 months) facilitated dual-labeled experiments allowing for direct measurements of unidirectional Cu and Na^+ uptake rates on the same set of animals. Addition of ^{64}Cu raised the total Cu concentration by 9.5-13.0 nmol l^{-1}, but addition of ^{22}Na did not significantly elevate the Na^+ concentrations in the water. A 10-ml water sample was taken from each bag 15 minutes after introduction of the radioisotopes and again after the 3-h flux period. Radioactivity of the water changed by no more than 18% during the 3 h. Fish were then killed with an overdose of neutralized methanesulfonate (MS-222), and samples were collected as described below.

Sampling

Blood was immediately collected by caudal puncture with 1-ml heparinized syringes, centrifuged at 13 000 × *g*, and plasma collected into centrifuge tubes. Gills, liver, and the rest of the carcass were then dissected out, rinsed in double-distilled water, placed into separate pre-weighed plastic scintillation vials or centrifuge tubes, and weighed. All samples were then analyzed for ^{64}Cu and ^{22}Na radioactivity and total Cu and Na^+ as described below.

Analysis

At all sampling times, tissue and water samples were immediately counted for total γ emission on a Canberra-Packard MINAXI Gamma counter (Canberra-Packard Instruments, Meriden, CT) in order to capture the short half-life ^{64}Cu activity using the ^{22}Na window. Tissues were then stored at –20°C for at least two weeks to allow for ^{64}Cu decay and counted again on the same window. The difference between the first and second (^{22}Na) counts for each sample represented the ^{64}Cu activity. All ^{64}Cu counts and ^{22}Na counts were manually corrected for decay. Subsequently, the tissues were digested overnight at 70 °C in 6 volumes of 1N HNO_3 (trace metal grade, Fisher Scientific, Nepean, ON), and 1.5-ml aliquots were removed and centrifuged for 4 min at 13 000 × *g*. A sub-sample of the supernatant was diluted appropriately with 0.5% HNO_3 and total tissue Cu concentration determined by graphite furnace atomic absorption spectroscopy (AAS; Varian AA-1275 with graphite tube furnace atomizer, Mississauga, ON) using the operating conditions for Cu specified by the manufacturer. Plasma samples were analyzed similarly after appropriate dilution with 0.5% HNO_3 without digestion, while water samples were only acidified with concentrated HNO_3 before analysis. Certified reference materials (estuarine water and dogfish liver, National Research Council of Canada, Ottawa, ON) analyzed along with the samples were within the specified range with typical recovery rates of 95%. Total Na^+ in tissue and water samples was measured by flame atomic absorption spectroscopy. Tissue digests and plasma samples were first diluted as appropriate with 0.5% HNO_3 and acidified water samples were analyzed without dilution.

Calculations

Whole-body total Cu concentration was calculated by dividing the sum of Cu contents (concentration multiplied by weight) of all the tissues plus the carcass by the sum of weights of all the tissues plus carcass.

Whole-body unidirectional uptake of waterborne Cu and Na^+ was calculated by adding up ^{64}Cu and ^{22}Na activities (cpm) in all tissues and carcass. Fish weights were determined by summing the weights of all tissues and the rest of carcass for each fish. Whole-body Cu or Na^+ uptake was then calculated using the equation:

$$a(bc^{-1})^{-1},$$

where a is the ^{64}Cu or ^{22}Na cpm per gram of fish, b is the ^{64}Cu or ^{22}Na cpm l^{-1} of water and c is the total Cu or Na^+ concentration in water in $\mu mol\ l^{-1}$. The uptake was then divided by the time of exposure (3 h) to convert to a rate.

Accumulation of new Cu and Na^+ in various body tissues (gill, liver, plasma, and the rest of the carcass) was determined based on the water specific activity using an analogous equation, with a being the ^{64}Cu or ^{22}Na cpm per gram of tissue or ml of plasma.

Gill Cu-binding

To characterize the effects of chronic exposure to elevated dietary Na^+ and waterborne Cu on gill surface Cu binding, a 3-h gill Cu-binding assay was carried out

on day 21 according to the protocol of Taylor *et al.* (2000). Control fish and fish chronically exposed to dietary Na^+ alone and to the high water Cu level (118 nmol l^{-1}) with or without high dietary Na^+ were tested using nominal Cu concentrations of 0, 0.08, 0.16, and 0.47 μmol l^{-1} waterborne Cu. Actual water Cu concentrations during the test, determined by AAS were, means ± SEM (n): 0.10 ± 0.007 (12), 0.18 ± 0.008 (12), 0.31 ± 0.01 (12), and 0.48 ± 0.01 (12) μmol l^{-1}. For each concentration ten fish were used. The exposure was carried out as for the unidirectional flux measurements except that only ^{64}Cu was used and the total Cu dose was delivered as radio-labeled $CuNO_3$. Water samples (10-ml each) were taken from each bag 15 min after introduction of ^{64}Cu and again after 3 h. Subsequently, fish were killed with an overdose of neutralized MS-222 and gills excised, rinsed in double distilled water, and weighed. All the water and gill samples were then counted for ^{64}Cu γ radioactivity on the Cu window using a Canberra-Packard MINAXI Gamma counter with onboard decay correction.

Statistical analysis

Effects of experimental treatments on growth, tissue Cu concentration, gill Cu-binding, and waterborne Cu and Na^+ uptake at each sampling point were assessed using analysis of variance (ANOVA) with time, dietary Na^+, and waterborne Cu concentrations as independent variables. Student-Newman-Keuls or Student *t*-tests were used to detect significant differences among treatments as appropriate. In all cases differences were considered significant at $p < 0.05$.

RESULTS

Growth, mortality, and Cu and Na^+ accumulation

Mean fish weight increased from 13 to 20 g in all groups during the 21-day experimental period, and neither chronic exposure to dietary Na^+ nor waterborne Cu had a significant effect on growth. All groups, including the control, showed some mortality ranging from 1.7 to 4.6%. However, this mortality was neither related to dietary Na^+ nor waterborne Cu exposure and was within the limit (<10%) for data acceptability (ASTM, 2000).

The exposure conditions had modest effects on whole-body Cu concentration (Fig. 1A). Fish exposed to the high waterborne Cu level in combination with control diet had significantly higher whole-body Cu levels relative to the fish on elevated waterborne Cu (118 nmol l^{-1}) + high dietary Na^+ on day 14. However, the liver showed a distinct pattern whereby chronic exposure to elevated dietary Na^+ reduced liver Cu concentration (Fig. 1B). Livers of fish exposed to the elevated waterborne Cu levels in combination with the elevated Na^+ diet (1.3 mmol g^{-1}) had significantly lower Cu concentration relative to the fish on elevated waterborne Cu level + control dietary Na^+ (0.26 mmol g^{-1}) on both days 14 and 21. For the gills, Cu concentrations remained unchanged in all groups except on day 21 when the fish exposed to 55 nmol l^{-1} waterborne Cu + high dietary Na^+ had lower Cu concentrations compared to the group exposed to 55 nmol l^{-1} waterborne Cu + control Na^+ diet (Table 1). Neither chronic exposure to elevated dietary Na^+ nor waterborne Cu had any effect on plasma and carcass Cu concentrations (Table 1).

Whole-body and plasma Na^+ concentrations remained within narrow margins of the controls throughout the exposure (data not shown) indicating that Na^+ homeostasis was maintained under the exposure conditions.

Unidirectional uptake and tissue distribution of newly accumulated Cu and Na^+

Whole-body unidirectional Cu and Na^+ uptake rates (Fig. 2) responded in a parallel fashion to the exposure conditions. At all sampling times, fish chronically exposed to elevated dietary Na^+ levels with or without elevated waterborne Cu exhibited reduced whole-body unidirectional uptake rates of both Cu and Na^+. Unidirectional Cu uptake rates were between 0.04 and 0.07 nmol g^{-1} h^{-1} for fish on control diet and between 0.03 and 0.05 $nmol^{-1}$ h^{-1} for fish exposed to high dietary Na^+. Unidirectional Na^+ uptake rates ranged from 0.35 to 0.43 μmol g^{-1} h^{-1} in fish exposed to control dietary Na^+ level and 0.15 to 0.38 μmol g^{-1} h^{-1} in fish exposed to high dietary Na^+. Thus Na^+ uptake rates were up to four orders of magnitude higher than Cu uptake rates. Clearly the unidirectional uptake rates of Cu and Na^+ co-varied. This co-variation between Cu and Na^+ uptake was best seen on day 14 when over 95% of the variation in Cu uptake could be explained by the change in Na^+ uptake (Fig. 3). The slope of the regression line indicates that for every mole of Cu, the fish took up 5800 moles of Na^+. For the other sampling days, Na^+ and Cu uptake rates were significantly correlated, although the r^2 values were lower and ranged from 0.35 to 0.72.

Uptake of new Cu into the gill, liver, plasma, and the rest of the carcass was reduced in fish chronically exposed to high dietary Na^+ at all levels of waterborne Cu

(Fig. 4). Similarly, uptake of new Na^+ into gill, liver, plasma, and carcass was reduced by chronic exposure to high dietary Na^+ (Fig. 5). However, chronic waterborne Cu exposure did not have any effect on the uptake of waterborne Na^+ or Cu. Note, however, that all of these flux measurements were made at background levels of Cu in the water, not at the elevated levels to which the Cu treated groups had been exposed.

Gill Cu-binding

The 3-h gill Cu-binding assay revealed three interesting effects of chronic exposure to elevated dietary Na^+ and waterborne Cu (Fig. 6). First, chronic exposure to elevated dietary Na^+ alone decreased the binding of Cu to the gills for all but the highest (0.47 μmol l^{-1}) waterborne Cu concentration tested (Fig. 6A). Second, chronic waterborne Cu exposure alone decreased the gill Cu-binding at water Cu concentrations less than 0.32 μmol l^{-1} (Fig. 6B). Third, chronic exposure to a combination of elevated waterborne Cu and elevated dietary Na^+ decreased gill Cu-binding over the entire range of waterborne Cu tested (Fig. 6C).

DISCUSSION

Bioenergetics of dietary Na^+ and waterborne Cu exposure

The present study is the first to assess effects of simultaneous chronic exposure to elevated dietary Na^+ and waterborne Cu in fish. Our results revealed no effect on juvenile rainbow trout growth of chronic waterborne Cu and dietary Na^+ exposure, alone or in combination, at the concentrations used. Clearly, growth is not a sensitive

indicator of sublethal effects of Cu exposure at environmentally realistic levels, which is in agreement with several previous studies (Collvin, 1985; Taylor *et al.*, 2000; Kamunde *et al.*, 2002b). According to Salman and Eddy (1988) the relationship between dietary Na^+ added to commercial trout food pellets and specific growth rate is linear and negative. These authors reported that dietary Na^+ adversely impacted growth at 9.2 and 11.6% levels of NaCl supplementation, due to interference with dietary content of other components especially protein and energy. Therefore, the level of salt supplementation of 3.0% Na^+ (7.5% NaCl) used in the present study may not have been high enough to significantly alter the content of other key dietary components.

Toxicity and regulation of whole-body Cu and Na^+

Generally, the exposure conditions did not alter whole-body total Cu concentration (except for the high waterborne Cu + control diet treatment on day 14, Fig. 1A), or cause significant treatment related mortality although the high Cu concentration was about half of the 96-h LC50 (concentration lethal to 50% of test organisms) for fish of comparable size and age in water of similar chemistry (Taylor *et al.*, 2000; Kamunde and Wood, 2002). This is consistent with concentration-response curves for metals that are characterized by low mortality at about 0.5×LC50 and almost 100% mortality at 2×LC50. However, feeding may also have mitigated Cu toxicity as previously postulated (see Introduction; also Lanno *et al.*, 1989). Furthermore, in acute toxicity, it is probably the gill, rather than whole-body, metal burden that is important for toxicity since the gill is the primary target organ for waterborne Cu toxicity

(McDonald and Wood, 1993; Wood, 2001). In this study, the Cu exposure levels were probably insufficient to significantly elevate total gill Cu burden (Table 1). The unchanged gill and whole-body Cu concentrations in face of chronic Cu exposure suggest these measures are not sensitive indicators of sublethal Cu exposure. However, the liver Cu burden (Fig. 1B) may be useful in this regard, as discussed below.

Plasma and whole-body Na^+ concentrations were unchanged by the experimental treatments, indicating that Na^+ balance was maintained. Rainbow trout thus appear to have a strong regulatory capacity for Na^+ such that any changes in internal Na^+ concentrations following exposure to high dietary Na^+ were likely transient, triggering the necessary regulatory mechanisms (such as increased efflux and decreased influx rates) important in maintaining Na^+ homeostasis. Increased efflux and decreased influx of Na^+ have previously been associated with elevated plasma Na^+ caused by dietary Na^+ loading (Smith *et al.*, 1995; Pyle *et al.*, 2002). However, it is also likely that the effects of elevated dietary Na^+ and waterborne Cu exposure balanced each other because they have opposite consequences on internal Na^+ levels. Whereas exposure to elevated dietary Na^+ would potentially increase plasma and whole-body Na^+ concentrations, waterborne Cu exposure would tend to induce Na^+ loss.

Smith *et al.* (1989) evaluated the relative contribution of branchial and gastrointestinal Na^+ uptake to whole-body Na^+ balance in laboratory and wild salmonids over a 1-year period and demonstrated that branchial Na^+ influx in winter was greater than dietary Na^+ intake, whereas in summer, dietary Na^+ uptake matched the branchial influx for wild fish and by far exceeded it in laboratory fish. This led the authors to

suggest that dietary Na^+ is surplus to requirements and is therefore lost via excretion. Moreover, the gill is the main site for Na^+ excretion, with gut playing a minor role in this process (Smith *et al.*, 1995). Thus the gill is more important than the gut in Na^+ homeostasis.

Total Cu concentration in tissues

Consistent with the modest changes in whole-body Cu concentration, the tissues analyzed showed minimal or no change in total Cu concentration. The lack of change in tissue Cu concentration in fish chronically exposed waterborne Cu is in agreement with previous studies using environmentally realistic levels of Cu exposure (Taylor *et al.*, 2000; Kamunde *et al.*, 2002b). Interestingly, however, the liver did display markedly decreased Cu accumulation in the fish chronically exposed to both elevated waterborne Cu and dietary Na^+ levels (Fig. 1B). Thus, exposure to high dietary Na^+ inhibits Cu accumulation in the liver during waterborne Cu exposure. This highlights the sensitivity of the liver to waterborne Cu and dietary Na^+ exposure, and its importance in Cu homeostasis in fish. In addition to its implications in aquatic toxicology, reduction of Cu accumulation in the liver by dietary Na^+ raises the interesting possibility of a human health implication. Possibly, dietary Na^+ loading may offer a potential preventive treatment of Wilson's disease, a disorder of Cu metabolism in humans characterized by massive accumulation of Cu in the liver (Schaefer and Gatlin, 1999). To this end, Wapnir and Stiel (1987) and Wapnir (1991) have proposed that there may be a Cu-Na^+ linkage during intestinal absorption in mammals.

Whole-body Na^+ and Cu uptake rates

Two earlier studies that evaluated waterborne Na^+ uptake following dietary Na^+ exposures (Smith *et al.*, 1995; Pyle *et al.*, 2002) convincingly shown that dietary Na^+ reduces waterborne Na^+ uptake in trout tissues and whole-body. Our study fully corroborates these studies to the extent that unidirectional Na^+ uptake rate is reduced by chronic dietary Na^+ exposure. In freshwater fish, plasma and body Na^+ levels are tightly regulated at about 150 mmol l^{-1} and 55 mmol kg^{-1}, respectively. An increase in plasma and/or internal Na^+ concentrations following dietary Na^+ exposure would trigger a feedback mechanism resulting in a reduction in branchial Na^+ influx concurrent with an increased Na^+ efflux, resulting in the return to normal of Na^+ levels. Since part of Cu uptake occurs via Na^+ uptake pathways (Grosell and Wood, 2002), the decrease in Na^+ uptake would be associated with a decrease in waterborne Cu uptake.

Chronic waterborne Cu exposure, however, had no effect on unidirectional Na^+ uptake in the present study. Previous studies (Laurén and McDonald, 1987a and b) reported that waterborne Cu reduced Na^+ influx, concurrent with stimulation of Na^+ efflux, processes that can potentially cause death if prolonged. However, Grosell and Wood (2002) reported that waterborne Na^+ reduced waterborne Cu uptake but waterborne Cu at the levels used (less than 0.16 μmol l^{-1}) had no effect on Na^+ uptake. In the present study, Na^+ uptake rates were not affected by chronic exposure to elevated waterborne Cu. Note that in contrast with previous studies, the Na^+ uptake measurements were made in the absence of elevated waterborne Cu concentration. This therefore is likely the explanation for the discrepancy with earlier studies and indicates

that competition for uptake sites rather than modification of the uptake sites by prior exposure is the most important factor defining the Na^+/Cu interaction at the trout gill epithelium.

Whole-body unidirectional uptake rate of Cu co-varied with Na^+ uptake during chronic exposure to elevated dietary Na^+ and waterborne Cu levels (Fig. 2, 3). Although Cu uptake rate is up to four orders of magnitude lower than Na^+ uptake rate, the Na^+ uptake pathway plays a significant role in Cu uptake and toxicity (Laurén and McDonald, 1987b). The big difference in the uptake rates of Na^+ and Cu is probably a reflection of the macronutrient status of Na^+, and therefore its greater requirements for physiological functions. Clearly Na^+ uptake pathways are important in Cu metabolism consistent with Pyle *et al.* (2002), the only other study that assessed Cu uptake following dietary Na^+ exposure. However, no effects of chronic waterborne Cu exposure on subsequent uptake of waterborne Cu were seen. Possibly the waterborne Cu level used for the chronic exposure was below the threshold necessary to induce changes in unidirectional Cu uptake rates. In this regard, we have recently shown that chronic exposure to higher levels of waterborne Cu (0.35 μmol l^{-1}) causes significant reduction in unidirectional waterborne Cu uptake rate (Kamunde *et al.*, 2002b).

Tissue distribution of newly accumulated Cu and Na^+

Newly accumulated Cu and Na^+ in the gill were both reduced by chronic exposure to elevated dietary Na^+. For example, on day 14 over 95% of the new gill Cu accumulation could be explained by new gill Na^+ accumulation (Fig. 7). This

parallelism between newly accumulated gill Cu and Na^+ supports the notion that, in part, these elements share common transport pathways at the gills. Recent pharmacological data indicate that the shared pathway is the apical Na^+ channel (Grosell and Wood, 2002). This is, of course, separate from the well-recognized inhibitory effect of higher levels of waterborne Cu on the basolateral Na^+,K^+-ATPase (e.g., Laurén and McDonald, 1987a; Pyle *et al.*, 2002). Interestingly, similar observations and conclusions have been drawn for zinc and cadmium on the one hand, and calcium on the other. For example, Hogstrand *et al.* (1998) demonstrated a co-variation between the uptake of waterborne Zn and Ca and concluded that these elements enter the gill via the apical Ca channel. More recently Zohouri *et al.* (2001) showed that dietary Ca reduces waterborne Cd uptake, suggesting that Cd also enters the fish gill epithelium via Ca pathways. Thus, there do seem to be some clear trends in the manner in which potentially toxic essential and non-essential metals interact with sites of uptake of macronutrients at the gill.

Chronic dietary Na^+ exposure decreased newly accumulated Cu and Na^+ in internal organs/tissues including plasma, liver, and the rest of the carcass. Thus, the pattern of new Cu and Na^+ accumulation at the primary target organ (gill) reflected the uptake pattern of these elements into internal organs. This not only suggests a reduction in the rate of apical entry but also a reduction in basolateral exit of Cu at the gill epithelium. Determining which of these two processes plays the dominant role offers an interesting topic for future research.

Gill Cu-binding

The short-term (typically 3 h) gill Cu-binding assay is designed to evaluate binding of Cu to the gill surface at equilibrium (Playle *et al.*, 1992). Recent data (Kamunde *et al.*, 2002b) suggest that significant internalization does occur during this period and that 3 h represents a peak of Cu binding at or in the gill. Thus the system may be more realistically described as kinetic rather than in equilibrium. Nonetheless, our data reveal that combined chronic exposure to elevated waterborne Cu and dietary Na^+ decreased Cu-binding to the gills throughout the entire range of Cu concentrations tested, while chronic exposure to elevated waterborne Cu alone decreased gill Cu-binding only at water Cu concentrations below 0.32 $\mu mol\ l^{-1}$. However, chronic exposure to dietary Na^+ alone decreased gill Cu-binding, but to a lesser extent. This occurred at all Cu concentrations except at the highest, where gill Cu-binding was elevated. We explain these observations on the basis of different Cu transport mechanisms at the gill that respond differently to chronic exposure to elevated waterborne Cu and dietary Na^+. Previous studies have reported the existence of several Cu transport mechanisms at the gills (Taylor *et al.*, 2000; Grosell and Wood, 2002; Kamunde *et al.*, 2002b). In the present study the Cu-binding sites markedly down-regulated by chronic waterborne Cu exposure at the low ambient Cu concentrations are likely high affinity Cu-sensitive sites, while the others may be the Na^+-sensitive sites as described by Taylor *et al.* (2000) and Grosell and Wood (2002), respectively.

The changes in the gill Cu-binding properties following chronic exposure to waterborne Cu and dietary Na^+ may have important implications for the Cu BLM which

has been proposed as a regulatory tool (Bergman and Dorward-King, 1997; MacRae *et al.,* 1999; Di Toro *et al.*, 2001; Santore *et al.*, 2001). Presently, based on the protective effect of waterborne Na^+ on Cu toxicity (Erickson *et al.*, 1996), Na^+ binding to the gill is considered in the Cu BLM (Santore *et al.*, 2001). Our data clearly show modifying effects of chronic dietary Na^+ exposure on Cu binding to gills. Although no acute or chronic effects of Cu were seen under the conditions of exposure in the present study, some important physiological effects of chronic dietary Na^+ exposure on Cu-gill interactions did occur. Since these effects include reduction in metal uptake, consideration of dietary factors such as quality in the Cu BLM may be warranted because metal binding to the gill has direct bearing on toxicity.

Likewise, effects of chronic exposure to elevated waterborne Cu might be important considerations in the refinement of BLM because modifications of the gill metal-binding characteristics due to acclimation likely occur in Cu-contaminated aquatic environments where fish are chronically exposed to Cu over extended periods of time. Our data are significant from a regulatory perspective because the chronic waterborne Cu exposure level used (118 nmol l^{-1}) is environmentally realistic, and the dietary Na^+ level was only about 4x higher than some natural fish diets (Smith *et al.*, 1989). The relevance of dietary quality factors arises from the observation that in natural settings, predaceous fish exhibit diet shifts from zooplankton, through benthic invertebrates, to predominantly fish in reference lakes (low to moderate contamination), whereas in highly contaminated lakes, these shifts do not occur and the fish feed predominantly on zooplankton (Sherwood *et al.*, 2000). These authors (Sherwood *et al.*, 2000) argued that

the lack of dietary shifts in contaminated lakes is due to lack of diet options. Whether, given the choice, fish in contaminated waters can select prey items based on higher mineral (e.g., Na^+ or Ca^{2+}) content remains an interesting hypothesis to be tested. However, based on the wide differences in Na^+ contents of various natural diets (Smith *et al.*, 1989), diet selection by feral fish can result in large differences in dietary Na^+ intake.

CONCLUSIONS

The present study indicates that chronic exposure to waterborne Cu concentrations as high as one half the 96-h LC50 in combination with high dietary Na^+ does not have significant effects on mortality and growth, although important changes in the rates of metal uptake occur. Chronic exposure to elevated dietary Na^+ reduces whole-body Cu uptake rates while chronic exposure to either or both dietary Na^+ or waterborne Cu reduce Cu-binding to the gill biotic ligand and are therefore likely to mitigate acute Cu toxicity. Because the BLM predicts the amount of metal bound to the gill that causes acute toxicity (Bergman and Dorward-King, 1997; MacRae *et al.,* 1999; Di Toro *et al.*, 2001; Santore *et al.*, 2001), consideration of factors other than water quality characteristics that influence metal binding to the gill is likely important. There is now a convincing body of evidence that dietary quality (e.g., Na^+ content [Pyle *et al.*, 2002, this study] and dietary Cu content [Kamunde *et al.*, 2001; 2002c]) and acclimation to waterborne Cu (Grosell *et al.*, 1997; Kamunde *et al.*, 2002b; this study) affect binding of Cu to the gills.

Acknowledgements

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Table 7-1. Total Cu concentration in gill, plasma, and carcass of juvenile rainbow trout following exposure to control and elevated dietary Na^+ in combination with either background (19), 55, or 118 nmol l^{-1} waterborne Cu. Values are means ± SEM, n = 10 for each value except for the group on Na^+-diet + 118 nmol l^{-1} water [Cu] on day 21 where n = 5. * Indicates significant difference from the value for fish on control diet at a particular day and waterborne Cu level.

Day and exposure conditions	Gill	Plasma	Carcass
Day 0			
Control	0.44 ± 0.01	0.51 ± 0.04	0.46 ± 0.04
Day 7			
Control diet + background water [Cu]	0.34 ± 0.02	0.45 ± 0.02	0.42 ± 0.04
Control diet + 55 nmol l^{-1} water [Cu]	0.40 ± 0.03	0.48 ± 0.03	0.48 ± 0.05
Control diet + 118 nmol l^{-1} water [Cu]	0.35 ± 0.02	0.45 ± 0.03	0.35 ± 0.04
Na^+-diet + background water [Cu]	0.34 ± 0.01	0.48 ± 0.06	0.45 ± 0.04
Na^+-diet + 55 nmol l^{-1} water [Cu]	0.39 ± 0.03	0.42 ± 0.02	0.44 ± 0.03
Na^+-diet + 118 nmol l^{-1} water [Cu]	0.32 ± 0.02	0.41 ± 0.05	0.35 ± 0.03
Day 14			
Control diet + background water [Cu]	0.42 ± 0.02	0.46 ± 0.04	0.47 ± 0.06
Control diet + 55 nmol water [Cu]	0.50 ± 0.04	0.43 ± 0.03	0.41 ± 0.06
Control diet + 118 nmol l^{-1} water [Cu]	0.49 ± 0.06	0.48 ± 0.04	0.34 ± 0.03
Na^+-diet + background water [Cu]	0.46 ± 0.03	0.43 ± 0.03	0.49 ± 0.05
Na^+-diet + 55 nmol l^{-1} water [Cu]	0.48 ± 0.03	0.42 ± 0.02	0.43 ± 0.07
Na^+-diet + 118 nmol l^{-1} water [Cu]	0.42 ± 0.02	0.53 ± 0.02	0.38 ± 0.02
Day 21			
Control diet + background water [Cu]	0.37 ± 0.01	0.53 ± 0.02	0.42 ± 0.03
Control diet + 55 nmol l^{-1} water [Cu]	0.43 ± 0.04	0.40 ± 0.02	0.41 ± 0.03
Control diet + 118 nmol l^{-1} water [Cu]	0.28 ± 0.01	0.51 ± 0.05	0.37 ± 0.03
Na^+-diet + background water [Cu]	0.36 ± 0.01	0.44 ± 0.03	0.47 ± 0.02
Na^+-diet + 55 nmol l^{-1} water [Cu]	0.30 ± 0.02*	0.44 ± 0.02	0.42 ± 0.04
Na^+-diet + 118 nmol l^{-1} water [Cu]	0.32 ± 0.01	0.49 ± 0.03	0.40 ± 0.04

Figure 7-1. Whole-body (A) and liver (B) Cu concentrations in juvenile rainbow trout exposed to 1.3 mmol g^{-1} dietary Na^{+} in combination with background (19), 55, or 118 nmol l^{-1} waterborne Cu. Values are means ± SEM, n = 10 per bar except high Cu + control diet on day 21 when n = 5. * Indicates significant difference from fish on control diet at the same level of waterborne Cu exposure, $p < 0.05$.

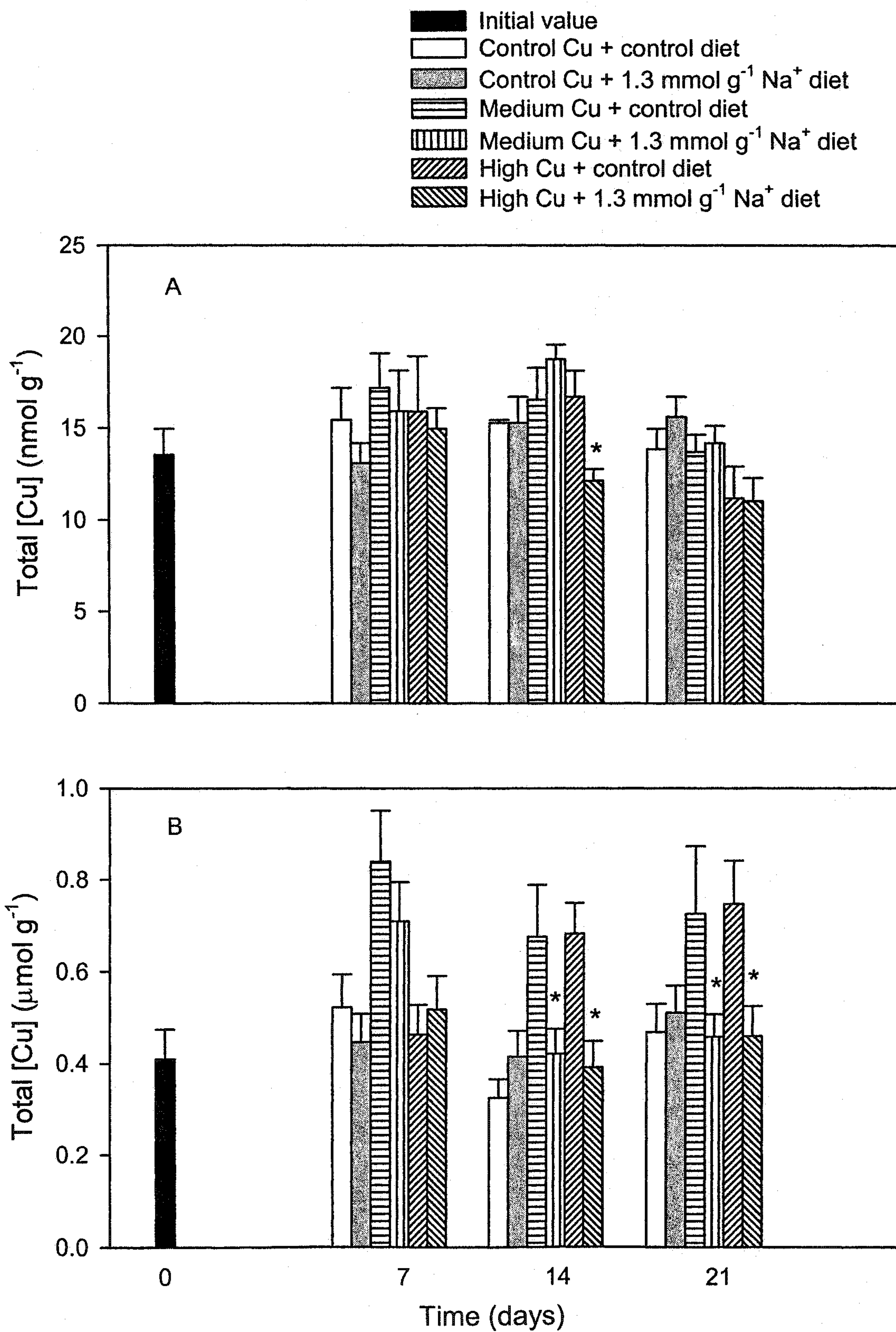

Initial value
Control Cu + control diet
Control Cu + 1.3 mmol g-1 Na+ diet
Medium Cu + control diet
Medium Cu + 1.3 mmol g-1 Na+ diet
High Cu + control diet
High Cu + 1.3 mmol g-1 Na+ diet
A
Total [Cu] (nmol g-1)
B
Total [Cu] (µmol g-1)
Time (days)
0
7
14
21

Figure 7-2. Whole-body unidirectional Cu (A) and Na^+ (B) uptake rates in juvenile rainbow trout exposed to 1.3 mmol g^{-1} dietary Na^+ in combination with background (19), 55, or 118 nmol l^{-1} waterborne Cu. Flux measurements were performed at background Cu levels. Values are means ± SEM, n = 10 per bar except high Cu + control diet on day 21 where n = 5. * Indicates significant difference from fish on control diet at the same level of waterborne Cu exposure, $p < 0.05$.

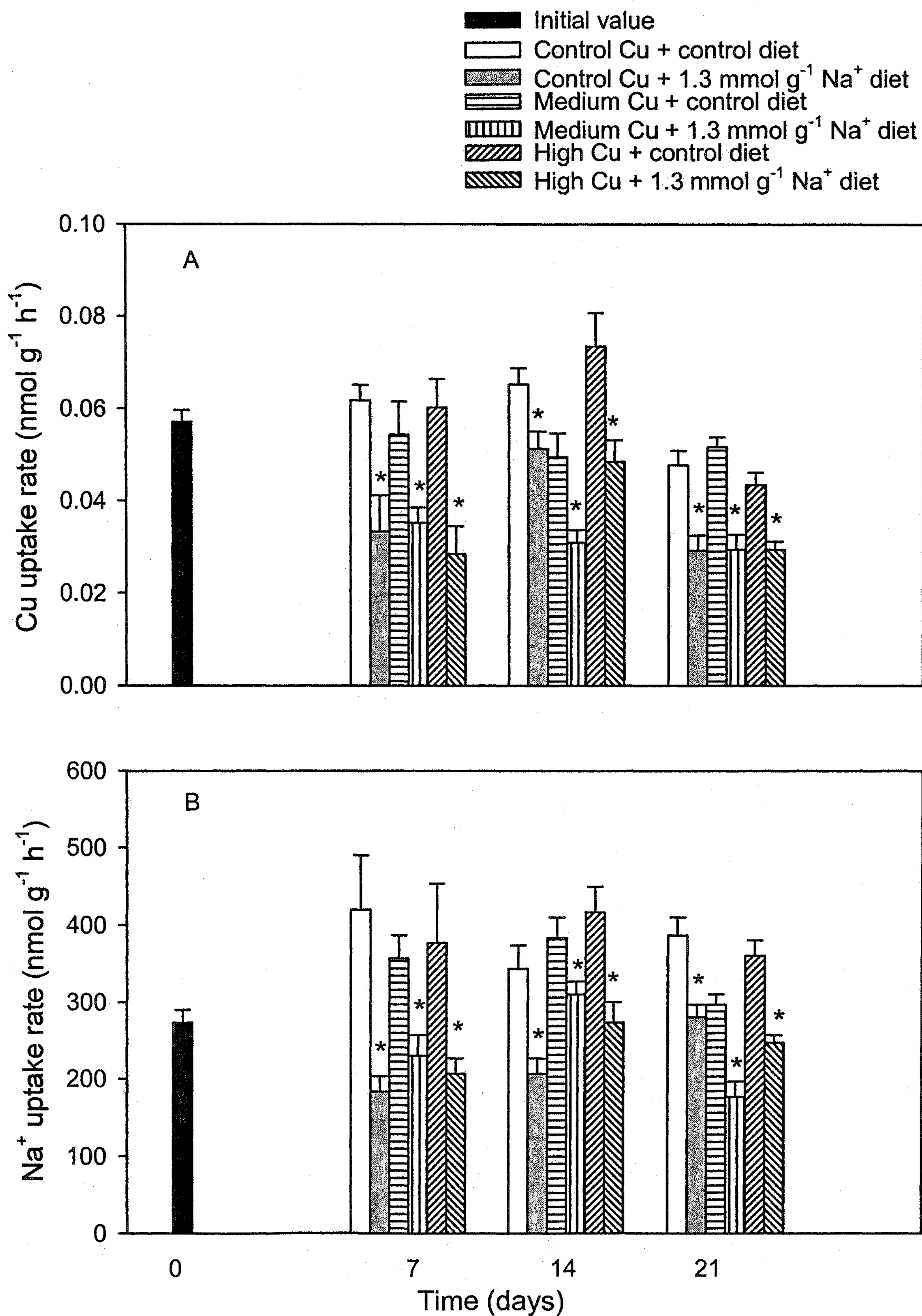

Initial value
Control Cu + control diet
Control Cu + 1.3 mmol g-1 Na+ diet
Medium Cu + control diet
Medium Cu + 1.3 mmol g-1 Na+ diet
High Cu + control diet
High Cu + 1.3 mmol g-1 Na+ diet
A
Cu uptake rate (nmol g-1 h-1)
0.10
0.08
0.06
0.04
0.02
0.00
B
Na+ uptake rate (nmol g-1 h-1)
600
500
400
300
200
100
0
0
7
14
21
Time (days)

Figure 7-3. Correlation between whole-body Na^+ and Cu uptake rates in juvenile rainbow trout exposed to 1.3 mmol g^{-1} dietary Na^+ in combination with background (19), 55, or 118 nmol l^{-1} waterborne Cu. Measurements of uptake rates were performed at background Cu levels. Each data point represents new whole-body Na^+ and Cu uptake rates for the same fish on day 14.

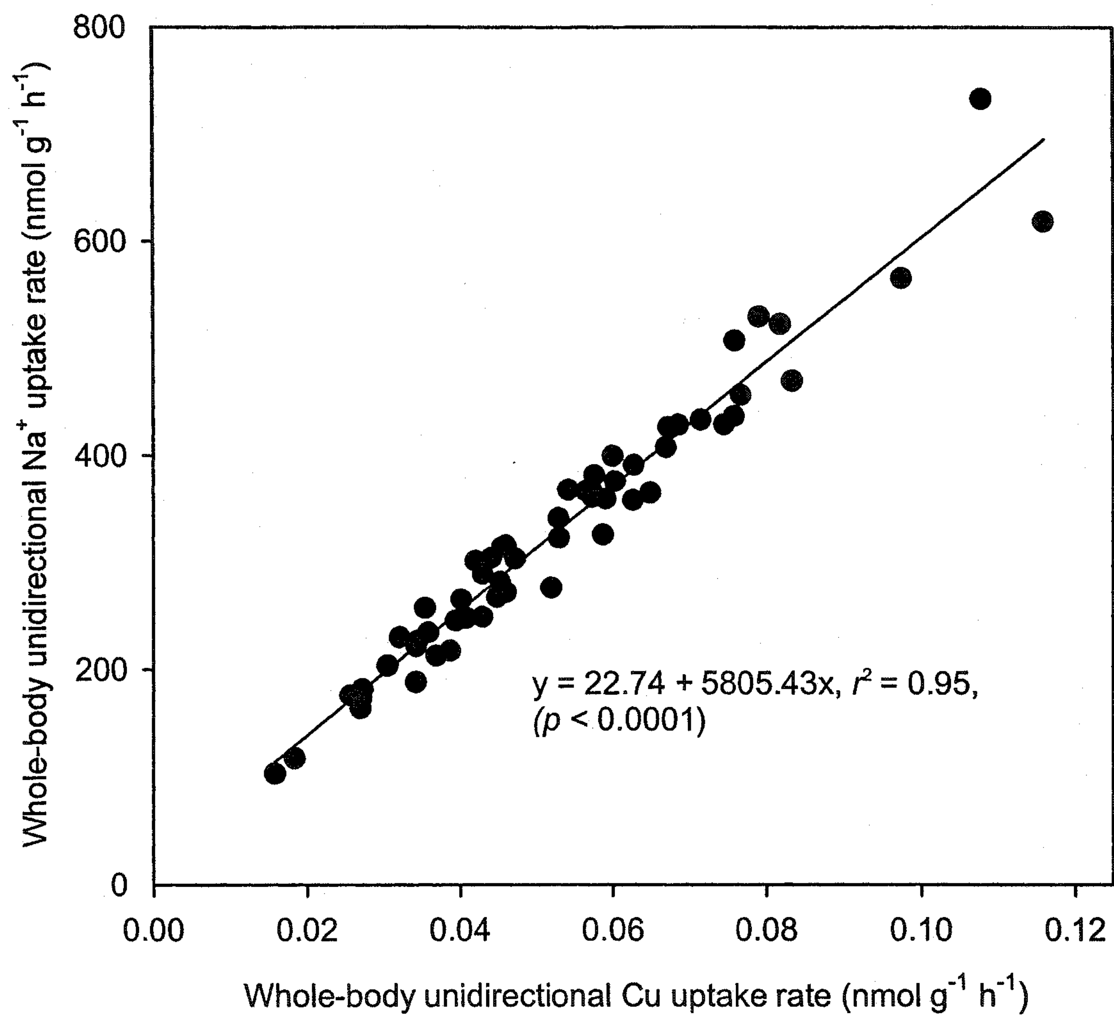
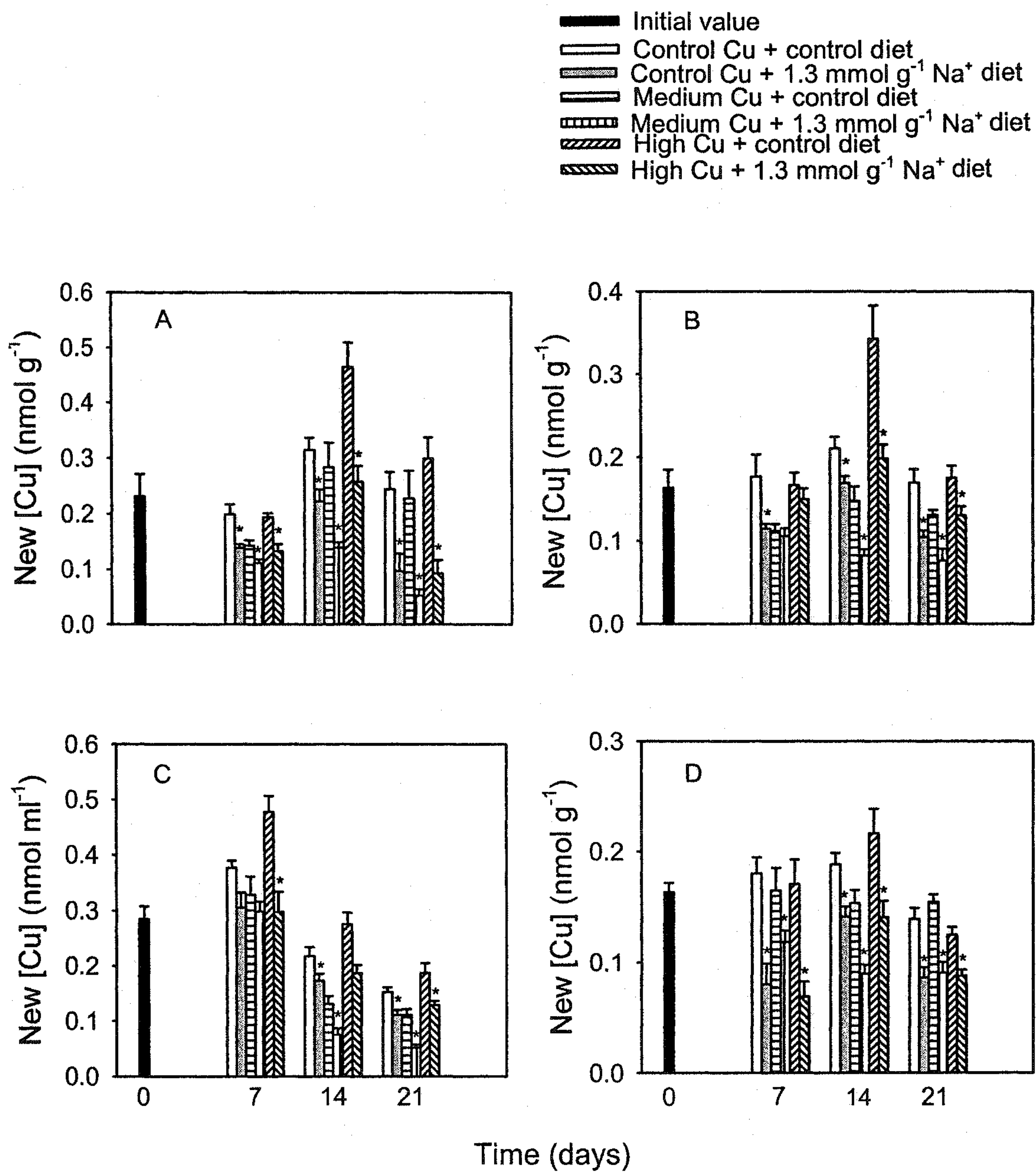

800
600
400
200
0
Whole-body unidirectional Na+ uptake rate (nmol g-1 h-1)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Whole-body unidirectional Cu uptake rate (nmol g-1 h-1)
y = 22.74 + 5805.43x, r2 = 0.95,
(p < 0.0001)

Figure 7-4. Newly accumulated Cu levels in gill (A), liver (B), plasma (C), and carcass (D) of juvenile rainbow trout exposed to 1.3 mmol g^{-1} dietary Na^+ in combination with background (19), 55, or 118 nmol l^{-1} waterborne Cu. All measurements were performed at background Cu levels. Values are means ± SEM, n = 10 per bar except high Cu + control diet on day 21 where n = 5. * Indicates significant difference from fish on control diet at the same level of waterborne Cu exposure, $p < 0.05$.



Initial value
Control Cu + control diet
Control Cu + 1.3 mmol g-1 Na+ diet
Medium Cu + control diet
Medium Cu + 1.3 mmol g-1 Na+ diet
High Cu + control diet
High Cu + 1.3 mmol g-1 Na+ diet
A
B
C
D
New [Cu] (nmol g-1)
New [Cu] (nmol ml-1)
Time (days)

Figure 7-5. Newly accumulated Na^+ levels in gills (A), livers (B), plasma (C), and carcasses (D) in juvenile rainbow trout exposed to 1.3 mmol g^{-1} dietary Na^+ in combination with background (19), 55, or 118 nmol l^{-1} waterborne Cu. All measurements were performed at background Cu levels. Values are means ± SEM, n = 10 per bar except high Cu + control diet on day 21 where n = 5. * Indicates significant difference from fish on control diet at the same level of waterborne Cu exposure, $p < 0.05$.

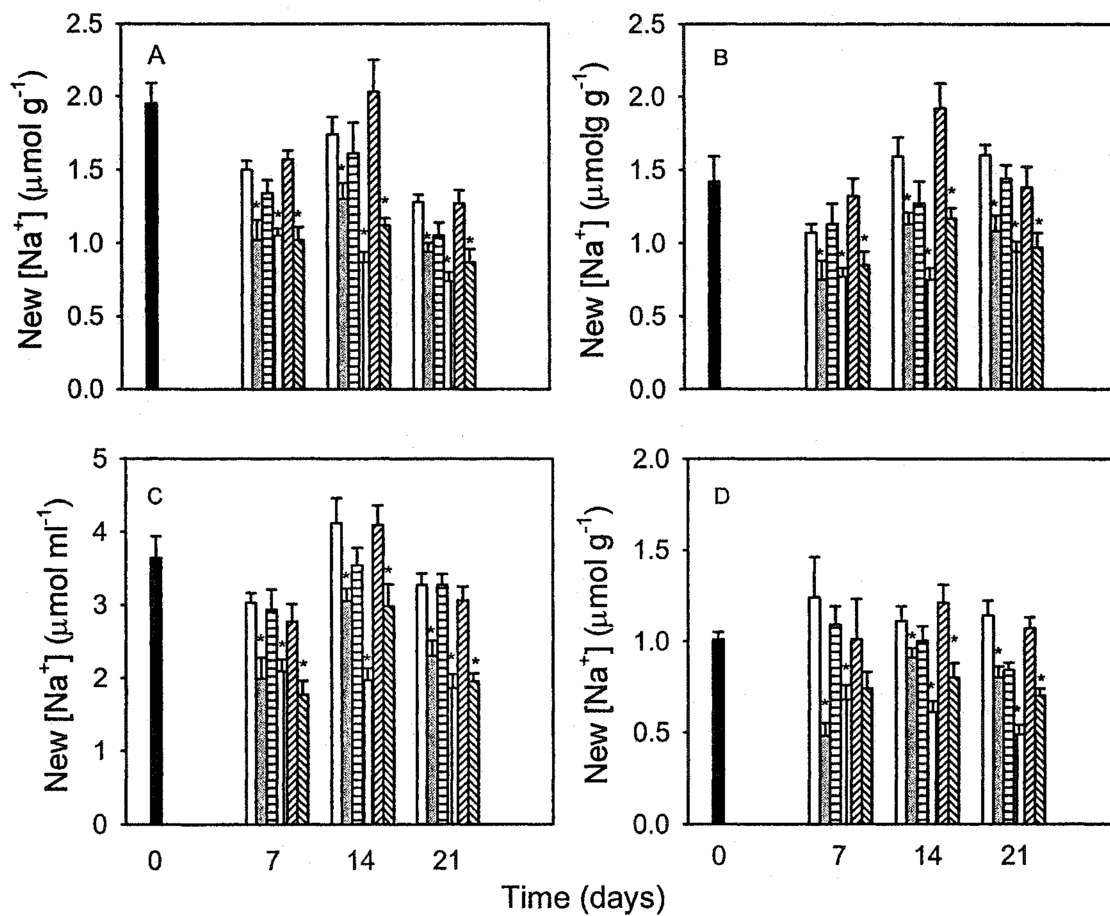

Initial value
Control Cu + control diet
Control Cu + 1.3 mmol g-1 Na+ diet
Medium Cu + control diet
Medium Cu + 1.3 mmol g-1 Na+ diet
High Cu + control diet
High Cu + 1.3 mmol g-1 Na+ diet
A
B
C
D
New [Na+] (μmol g-1)
New [Na+] (μmolg g-1)
New [Na+] (μmol ml-1)
New [Na+] (μmol g-1)
Time (days)
0
7
14
21

Figure 7-6. Gill Cu binding in juvenile rainbow trout exposed to control conditions, elevated (1.3 mmol g^{-1}) dietary Na^+, and elevated (118 nmol l^{-1}) waterborne Cu with or without elevated dietary Na^+ for 21 days. Values are means ± SEM, n = 10 per data point. Panel A compares gill Cu binding in control and fish exposed to elevated dietary Na^+ at background levels of water Cu, panel B compares gill binding between controls and fish exposed to elevated waterborne Cu together with control diet, and panel C compares the binding between controls and fish exposed to a combination of elevated dietary Na^+ and elevated waterborne Cu. * Indicates significant difference from control group, $p < 0.05$.

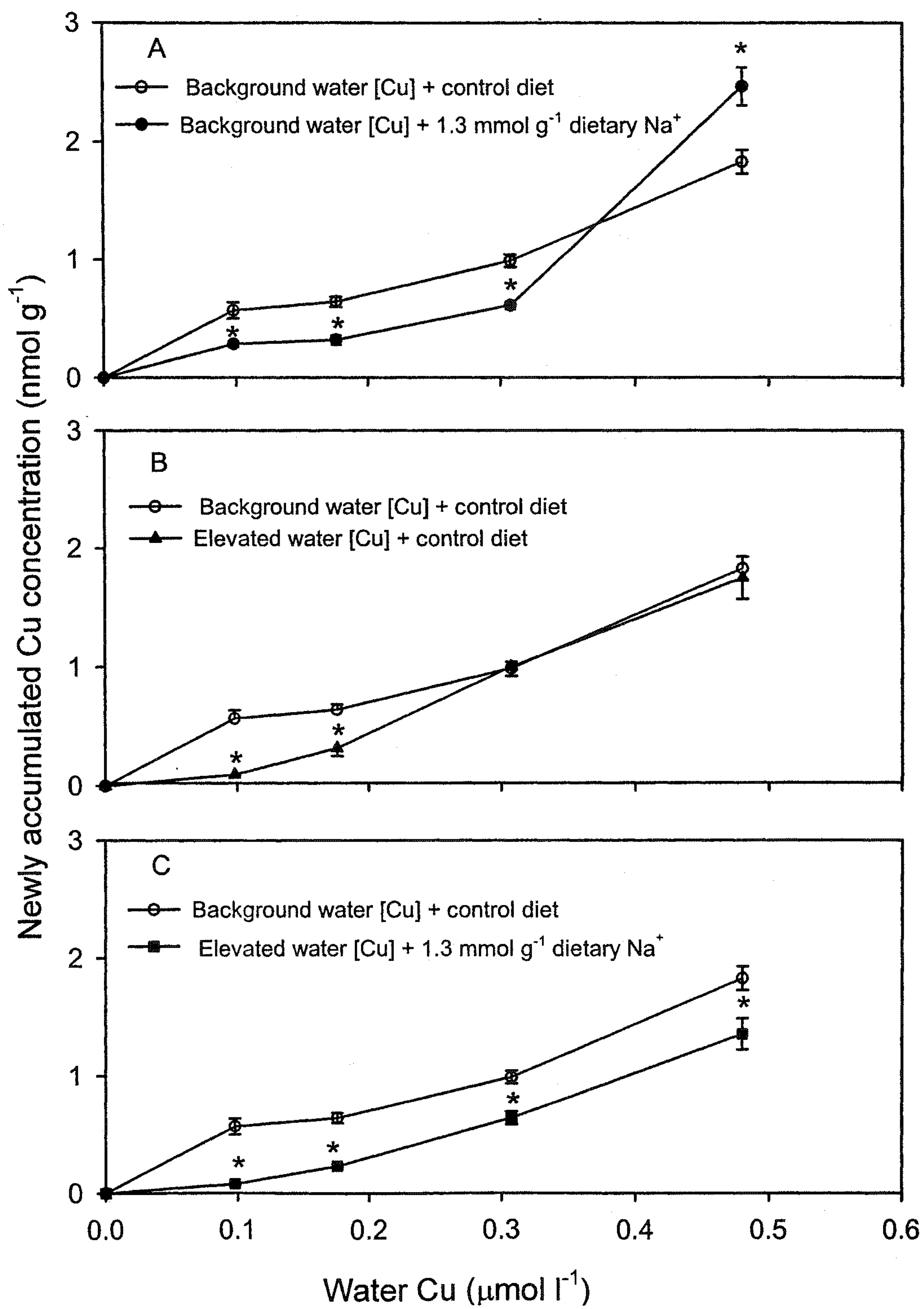
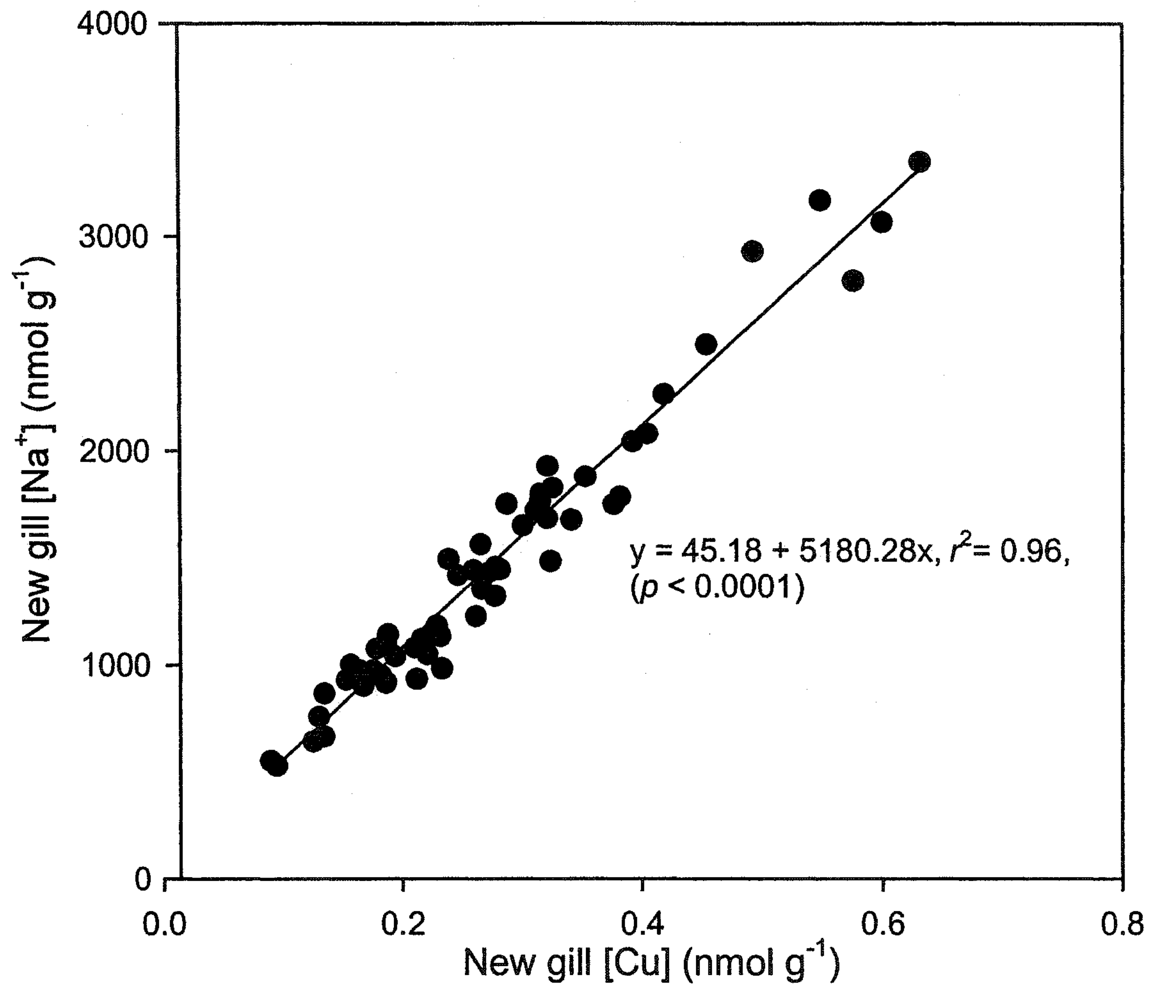

A
Background water [Cu] + control diet
Background water [Cu] + 1.3 mmol g-1 dietary Na+
B
Background water [Cu] + control diet
Elevated water [Cu] + control diet
C
Background water [Cu] + control diet
Elevated water [Cu] + 1.3 mmol g-1 dietary Na+
Newly accumulated Cu concentration (nmol g-1)
Water Cu (μmol l-1)
0
1
2
3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*

Figure 7-7. Correlation between newly accumulated gill Na^+ and newly accumulated gill Cu in juvenile rainbow trout exposed to 1.3 mmol g^{-1} dietary Na^+ in combination with background (19), 55, or 118 nmol l^{-1} waterborne Cu. Flux measurements were performed at background Cu levels. Each data point represents newly accumulated gill Na^+ and Cu values for the same fish on day 14.



4000
3000
2000
1000
0
0.0
0.2
0.4
0.6
0.8
New gill [Na+] (nmol g-1)
New gill [Cu] (nmol g-1)
y = 45.18 + 5180.28x, r2= 0.96,
(p < 0.0001)

CHAPTER 8

SUMMARY

In complex organisms such as fish, Cu homeostasis is achieved not only at the cellular level but also at the tissue and organismal level. This chapter summarizes and models Cu metabolism and homeostasis in fish based on data generated in this research, and relevant data from literature. Several areas deficient in data have been identified for future research.

Fish whole-body Cu metabolism

Whole-body Cu metabolism in fish entails regulated uptake, distribution, and excretion and occurs by coordinated interactions of several organs (Fig. 1). The two primary sites of uptake, i.e., the gut and gills, absorb Cu from the diet and/or the water, and deliver it primarily via blood plasma in the primary phase of distribution to the liver, which occupies a central locus in whole fish Cu metabolism. Plasma Cu transport in fish is not well characterized but preliminary electrophoretic analyses (Kamunde and Wood, unpublished) suggest involvement of at least three proteins, putatively albumin, ceruloplasmin, and transcuprien, as in mammals. Cu from dietary uptake is channeled directly to the liver via the portal circulation whereas Cu from branchial uptake may accumulate in other organs before reaching the liver. In the liver Cu may follow one of three fates: i) incorporation into transport proteins such as ceruloplasmin for secondary

transport into other tissues, ii) incorporation into cupro-proteins, e.g., metallothionein for biological function or storage, or iii) excretion via bile. Evidence generated from the present and previous studies indicates that Cu exposure via water and diet increases liver Cu burden and stimulates hepatobiliary excretion. A proportion of the Cu transported to the kidney, gill, and probably also skin may be excreted depending on requirements for Cu, whilst the gastrointestinal tract provides a conduit for eventual extrusion of biliary Cu, and may participate directly in Cu excretion via intestinal fluids or exfoliation of Cu-impacted intestinal epithelial cells. Whole-body Cu levels and uptake rates in fish vary depending on the prevailing waterborne and dietary Cu levels, body requirements, and growth rates. However, plasma Cu levels appear to be maintained constant over a wide range of waterborne and dietary Cu exposure levels suggesting that plasma Cu may be the homeostatic endpoint in fish. I therefore speculate a whole-body Cu homeostatic system in fish (Fig. 2) that is geared toward maintaining constant plasma Cu concentration. This system likely encompasses central sensors, possibly within the central nervous system or blood circulation system, and local sensors within target cells. Moreover, because of a demonstrated linkage between Na^+ and Cu metabolism (e.g., Grosell and Wood, 2002; Wapnir, 1991) a partial linkage with the Na^+ sensing mechanisms cannot be ruled out. In this speculative homeostatic system, changes in plasma Cu are detected by sensors in the central nervous system (CNS) or the circulatory system from where messages (possibly neural and/or hormonal) are sent to the regulatory organs, which for fish are the gills, gut, and the liver. Depending on the nature of the change in plasma Cu concentration, increased or decreased uptake occurs

at the gill and gut concurrent with increased or decreased hepatobiliary excretion. Cu translocating proteins, such as the Cu-ATPases, within these organs likely mediate these changes (see Fig. 2 legend). The net effect is the return to normal plasma Cu levels which is 0.6-0.8 μg ml^{-1} in rainbow trout.

Homeostatic regulation of cellular Cu

The goal of maintaining plasma (extracellular) Cu concentration is to satisfy the changing Cu requirements of the cells. Although the mechanisms of Cu regulation in vertebrate cells have not been well defined, physiologically essential levels of cellular Cu can be achieved via a coordinated series of interactions between transport proteins, vesicles, and soluble peptides (Harris, 2001). These transport molecules are part of a Cu homeostatic system that regulates cellular Cu uptake, transport, storage, and efflux. Several aspects of cellular Cu homeostasis in fish remain unclear. For example: what are the specific proteins involved?, what is regulated?, what are the sensing mechanisms?, where are the sensors located?, and how does the mechanism function? Most of the studies addressing cellular Cu homeostasis have been carried out using simple systems like yeast and bacteria, and thus fail to address the complexity of multicellular organisms. In simple organisms like yeast, regulator proteins act as Cu sensors that trigger shut-down of uptake proteins when intracellular Cu levels rise (Harris, 2001). Alternatively, vesicles with Cu-transporting ATPases work in conjunction with mobile Cu carriers (e.g., chaperones) to get rid of excess cytoplasmic

Cu or activate enzymes such as Cu/Zn-superoxide dismutase that protect the cell from oxidative damage.

Although the scientific literature often gives reference to cellular Cu homeostasis, it is not clear at what level cellular Cu is maintained. However, due to a great variability in Cu concentrations in various tissues, cell-type specific Cu-levels are likely. This raises the speculation that possibly only Cu levels within some sensitive cell compartment is regulated. To this end, free cytosolic Cu is low (O'Halloran and Culotta, 2000) and it is therefore likely that Cu homeostasis is geared toward satisfying cellular Cu requirements while maintaining safe levels of cytosolic freely dissociable Cu. In vertebrates no system for sensing Cu has been identified (Harris, 2001) although metal binding sites on the Cu transporting P-type proteins have been proposed to sense the cellular Cu levels (Strausak *et al.,* 1999). Evidence for this comes from the observation that metal binding sites at the N-terminus of Cu-ATPase bind Cu(1) with high affinity and are involved in ligand exchange with Cu chaperones.

Future research on Cu metabolism and homeostasis in fish (and indeed other vertebrates), therefore, needs to focus on characterizing the regulatory endpoint (both at organismal and cellular levels) and identifying the nature and location of the sensing mechanisms, and the mode of integration of the various systems involved in Cu metabolism and regulation. More research is required to illuminate the kinetics of Cu transporting and regulating proteins and factors that influence their function and expression. Moreover, the Na^+-Cu linkage need to be explored in depth using molecular tools to fully understand the relative contributions of Na^+ and Cu-specific pathways in

Cu metabolism. Efforts on such future research will eventually elucidate an all-inclusive picture of Cu metabolism in which whole animal and tissue Cu levels can be linked and explained by specific changes at cellular and molecular levels.

Figure 8-1. Composite scheme of whole-body Cu metabolism in fish. Whole-body Cu metabolism in fish involves coordinated interactions of several organ systems. The gut and the gills are the principal sites of Cu uptake and at background levels of Cu, gastrointestinal absorption accounts for the majority of Cu uptake. Cu taken up from these sites is transported via blood plasma in the primary phase of transport to the liver that occupies a central locus in Cu metabolism. In the liver Cu is incorporated into various proteins for biological function, detoxification, and storage. Protein bound Cu (primarily ceruloplasmin-bound Cu) enters the secondary phase of transport to the rest of the body. Copper uptake is balanced by excretory losses via bile, the gut, and gills, and other less important losses via the kidney and the rest of the body (miscellaneous losses).

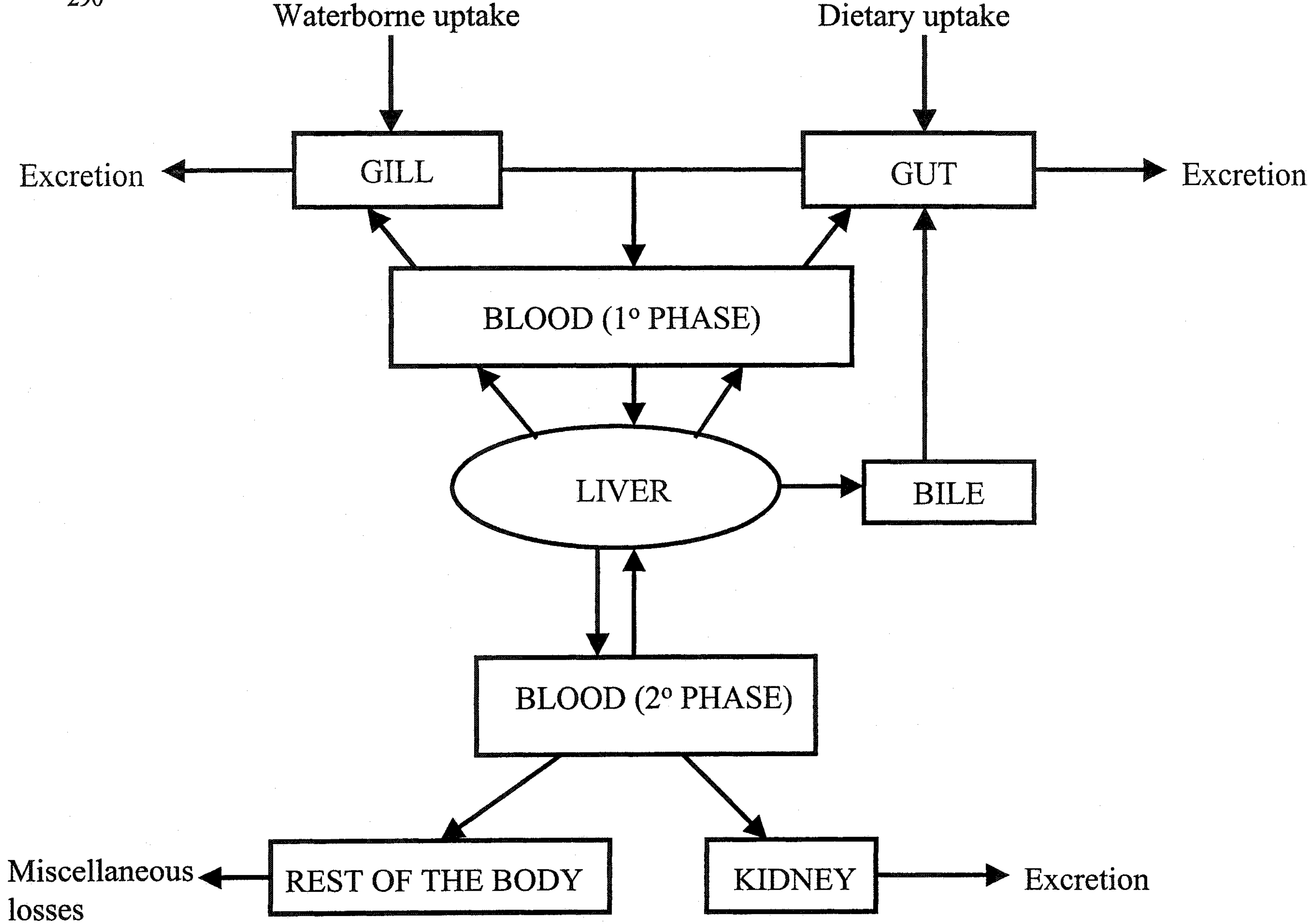
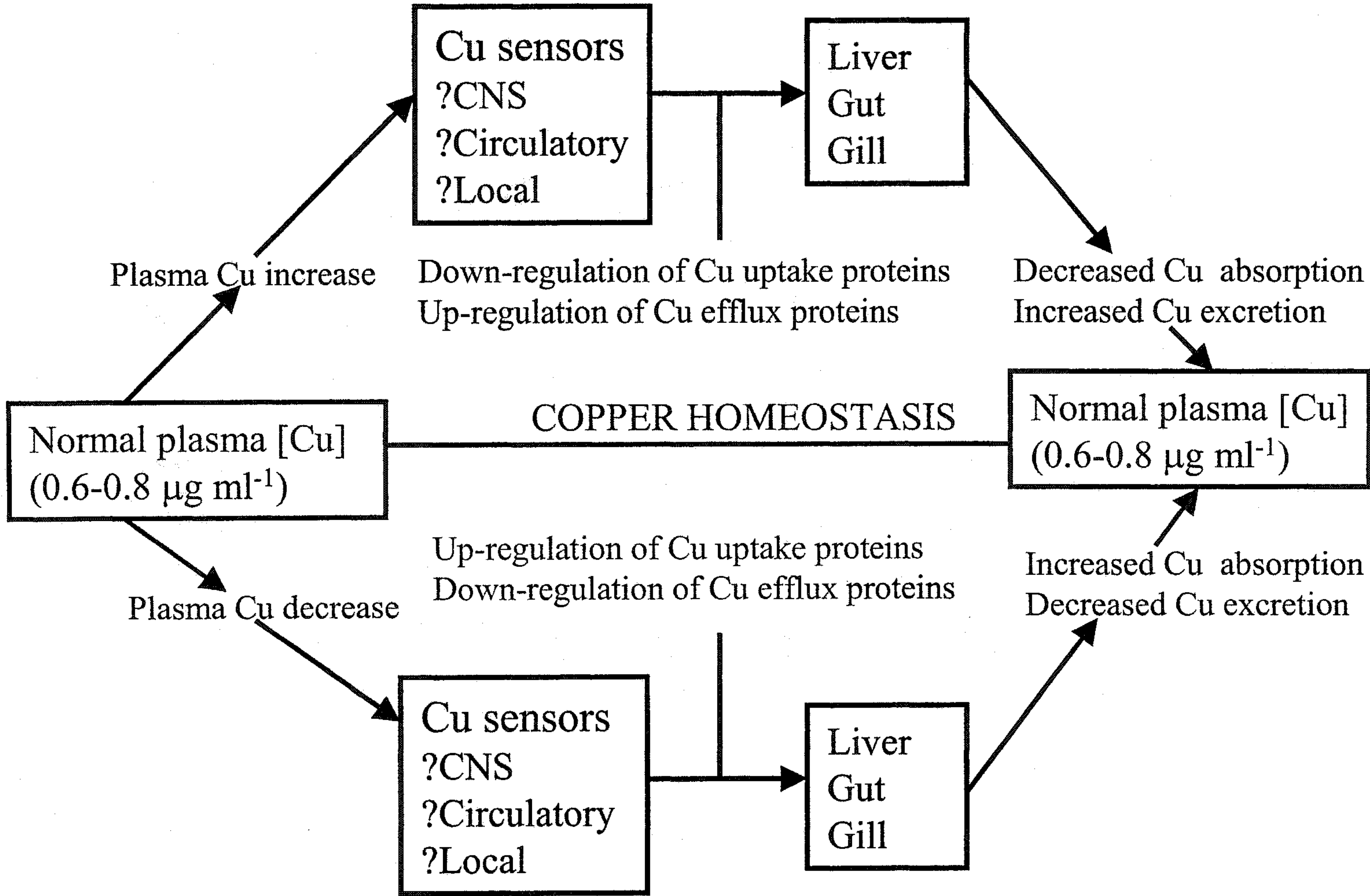

Waterborne uptake
Dietary uptake
Excretion
GILL
GUT
Excretion
BLOOD (1° PHASE)
LIVER
BILE
BLOOD (2° PHASE)
Miscellaneous losses
REST OF THE BODY
KIDNEY
Excretion

Figure 8-2. Schematic diagram of Cu homeostasis in rainbow trout. Cu homeostatic mechanisms in trout maintain plasma Cu levels of 0.6-0.8 $\mu g\ ml^{-1}$. Putative Cu sensors locally located or within the central nervous system or the circulatory system are responsible for monitoring Cu levels in blood/extracellular fluid since Cu is both essential and toxic. Messages (hormonal, chemical or neural), are transmitted from these receptors to the effector organs primarily the liver, gut, and gill. Other organs including the kidney may also play some less important roles. These organs possess Cu translocating proteins (e.g., Cu-ATPases) that mediate Cu uptake, transport, and efflux. In the event that increased plasma Cu level results in elevated cellular Cu concentration, there ensues a down-regulation of the uptake proteins (e.g., Menke's protein) concurrent with migration of this protein from the *trans*-Golgi network (TGN) to the plasma membrane where it enhances Cu efflux. At the same time there is an up-regulation of proteins involved in biliary excretion (e.g., Wilson's protein) to rid the animal of the excess Cu. In the event of decreased plasma/cellular Cu concentration the reverse occurs: the Menke's protein is translocated to the basolateral membrane of branchial (and gastrointestinal) cells to boost Cu absorption. At the same time biliary and branchial Cu excretion are reduced and the Wilson's protein moves to the TGN to enhance delivery of Cu to ceruloplasmin for onward transport to other tissues. The net effect is that plasma or extracellular Cu concentration is maintained at physiological levels, and Cu is delivered at the required amount to the various cupro-proteins. Concomitant with constant extracellular Cu, cellular Cu levels are maintained at safe tissue-specific levels.


COPPER HOMEOSTASIS IN TROUT
Normal plasma [Cu] (0.6-0.8 μg ml-1)
Plasma Cu increase
Cu sensors
?CNS
?Circulatory
?Local
Liver
Gut
Gill
Down-regulation of Cu uptake proteins
Up-regulation of Cu efflux proteins
Decreased Cu absorption
Increased Cu excretion
COPPER HOMEOSTASIS
Normal plasma [Cu] (0.6-0.8 μg ml-1)
Plasma Cu decrease
Up-regulation of Cu uptake proteins
Down-regulation of Cu efflux proteins
Cu sensors
?CNS
?Circulatory
?Local
Liver
Gut
Gill
Increased Cu absorption
Decreased Cu excretion

APPENDIX

Chapter 2 F values.

Measurement		
Copper load	Time ($F_{2,81}$)	Diet Cu ($F_{2,81}$)
Whole-body [Cu]	65.9 ($p<0.0001$)	60.7 ($p<0.0001$)
Whole-body Cu content	112.5 ($p<0.0001$)	80.9 ($p<0.0001$)
Liver [Cu]	33.5 ($p<0.0001$)	21.1 ($p<0.0001$)
Gut [Cu]	46.6 ($p<0.0001$)	127.7 ($p<0.0001$)
Carcass [Cu]	0.6 ($p>0.05$)	4.0 ($p<0.05$)
Gill [Cu]	18.4 ($p<0.001$)	7.7 ($p<0.001$)
Kidney [Cu]	5.1 ($p<0.001$)	7.7 ($p<0.001$)
Bile [Cu]	4.1 ($p<0.05$)	19.1 ($p<0.0001$)
Plasma [Cu]	7.3 ($p<0.05$)	1.8 ($p>0.05$)
Muscle [Cu]	3.7 ($p<0.05$)	0.8 ($p>0.05$)
Proportional distribution		
Liver	41.5 ($p<0.0001$)	3.2 ($p<0.05$)
Gut	12.3 ($p<0.0001$)	52.1 ($p<0.0001$)
Carcass	115.2 (<0.0001)	12.9 ($p<0.0001$)
Gill	93.2 ($p<0.0001$)	11.6 ($p<0.0001$)
Kidney	3.7 ($p<0.05$)	0.6 ($p>0.05$)

Chapter 3 F values.

Measurement		**Main Effect**	
Copper load	Time ($F_{3,266}$)	Diet Cu ($F_{2,267}$)	Water Cu ($F_{1,268}$)
Whole-body [Cu]	6.8 ($p<0.001$)	112.2 ($p<0.0001$)	25.2 ($p<0.0001$)
Whole-body Cu content	21.9 ($p<0.0001$)	38.1 ($p<0.0001$)	11.2 ($p<0.001$)
Liver [Cu]	7.5 ($p<0.0001$)	98.0 ($p<0.0001$)	22.6 ($p<0.0001$)
Gut [Cu]	2.5 ($p>0.05$)	127.5 ($p<0.0001$)	27.0 ($p<0.0001$)
Carcass [Cu]	9.5 ($p<0.0001$)	55.8 ($p<0.0001$)	15.22 ($p<0.0001$)
Gill [Cu]	15.6 ($p<0.0001$)	3.8 ($p<0.05$)	6.2 ($p<0.05$)
Proportional distribution			
Liver	15.6 ($p<0.0001$)	84.3 ($p<0.0001$)	29.7 ($p<0.0001$)
Gut	10.2 ($p<0.001$)	31.2 ($p<0.0001$)	11.6 ($p<0.001$)
Carcass	6.7 ($p<0.001$)	122.3 ($p<0.0001$)	45.1 ($p<0.0001$)
Gill	10.1 ($p<0.0001$)	20.3 ($p<0.0001$)	7.3 ($p<0.01$)
Somatic indices			
Liver (HSI)	21.9 ($p<0.0001$)	0.9 ($p>0.05$)	2.0 ($p>0.05$)
Gill (BSI)	15.5 ($p<0.0001$)	1.0 ($p>0.05$)	2.0 ($p>0.05$)
Gut (GSI)	29.7 ($p<0.0001$)	1.937 ($p>0.05$)	0.5 (>0.05)
Growth and FCE			
	Time ($F_{6,84}$)	Diet Cu ($F_{2,84}$)	Water Cu ($F_{1,91}$)
Wt gain	263.7 ($p<0.0001$)	24.6 ($p<0.0001$)	55.6 ($p<0.0001$)
	Time ($F_{2,42}$)	Diet Cu ($F_{2,42}$)	Water Cu ($F_{1,43}$)
FCE	4.3 ($p<0.05$)	5.4 ($p<0.01$)	8.5 ($p<0.01$)
Waterborne Cu uptake kinetics			
	Exposure Cu ($F_{4,220}$)	Diet Cu ($F_{2,222}$)	Water Cu ($F_{1,223}$)
Whole-body Cu uptake	22.3 ($p<0.0001$)	10.9 ($p<0.0001$)	25.7 ($p<0.0001$)

Chapter 4 F values.

Measurement		**Main Effect**	
Waterborne Cu uptake	Time ($F_{3,144}$)	Acclimation ($F_{1,146}$)	Water Cu ($F_{1,146}$)
Whole-body	85.4 ($p<0.0001$)	18.6 ($p<0.0001$)	978.3 ($p<0.0001$)
Whole-body less gill	58.9 ($p<0.0001$)	15.8 ($p<0.0001$)	620.7 ($p<0.0001$)
Liver	94.1 ($p<0.0001$)	4.3 ($p<0.05$)	757.6 ($p<0.0001$)
Gill	64.6 ($p<0.0001$)	5.1 ($p<0.01$)	548.8 ($p<0.0001$)
Carcass	380.4 ($p<0.0001$)	10.6 ($p<0.001$)	343.3 ($p<0.0001$)
Plasma	1.6 ($p>0.05$)	0.5 ($p>0.05$)	66.5 ($p<0.0001$)
Gut	7.6 ($p<0.0001$)	1.0 ($p>0.05$)	132.5 ($p<0.0001$)
Dietary Cu uptake			
Whole-body	24.6 ($p<0.0001$)	0.1 ($p>0.05$)	6.5 ($p<0.01$)
Whole-body less gut	8.9 ($p<0.001$)	0.1 ($p>0.05$)	1.0 ($p>0.05$)
Liver	1.7 ($p>0.05$)	0.4 ($p>0.05$)	0.1 ($p>0.86$)
Gill	2.6 (>0.05)	0.1 ($p>0.05$)	0.1 ($p>0.05$)
Carcass	6.3 ($p<0.001$)	0.3 ($p>0.05$)	0.4 ($p>0.05$)
Plasma	6.5 ($p<0.001$)	0.2 ($p>0.05$)	0.4 ($p>0.05$)
Gut	69.6 ($p<0.0001$)	0.7 ($p>0.05$)	1.5 ($p>0.05$)
Exchangeable Cu pools	Exposure route	Acclimation	Water Cu
Liver	41.1 ($p<0.0001$)	5.6 ($p<0.05$)	6.0 ($p<0.05$)
Gill	89.7 ($p<0.0001$)	3.8 ($p<0.05$)	11.6 (<0.001)
Carcass	47.2 ($p<0.0001$)	4.7 ($p<0.05$)	1.0 (>0.05)
Plasma	63.2 ($p<0.0001$)	4.1 ($p<0.05$)	7.3 ($p<0.01$)
Gut	3.4 (>0.05)	4.7 ($p<0.05$)	0.1 (>0.05)

Chapter 4 F values for waterborne *versus* dietary Cu uptake interaction.

Tissue	Exposure route ($F_{1,288}$)
Whole-body	652.9 ($p<0.0001$)
Whole-body less gill or gut	567.0 ($p<0.0001$)
Liver	460.0 ($p<0.0001$)
Gill	70.1 ($p<0.0001$)
Carcass	57.3 ($p<0.0001$)
Gut	863.4 ($p<0.0001$)
Plasma	32.2 ($p<0.0001$)

Chapter 5 F values.

Measurement	Main effect		
Copper load	Time ($F_{3,300}$)	Diet Cu ($F_{1,300}$)	Ration ($F_{2,300}$)
Whole-body [Cu]	201.2 ($p<0.0001$)	380.8 ($p<0.0001$)	79.0 (<0.0001)
Whole-body Cu content	441.4($p<0.0001$)	257.6 ($p<0.0001$)	0.9 ($p>0.05$)
Liver [Cu]	34.6 ($p<0.001$)	59.3 ($p<0.001$)	35.0 ($p<0.001$)
Liver Cu content	320.0 ($p<0.001$)	221.6 ($p<0.001$)	0.2 ($p>0.05$)
Gill [Cu]	25.7 ($p<0.001$)	0.7 ($p>0.05$)	1.2 ($p>0.05$)
Kidney [Cu]	155.6 ($p<0.001$)	152.6 ($p<0.001$)	16.3 ($p<0.001$)
Plasma [Cu]	79.2 ($p<0.001$)	3.7 ($p>0.05$)	3.1 ($p<0.05$
Bile [Cu]	34.8 ($p<0.001$)	156.5 ($p<0.001$)	22.3 ($p<0.05$)
Carcass [Cu]	7.13 ($p<0.01$)	9.2 ($p<0.01$)	5.9 ($p<0.01$)
Stomach [Cu]	39.8 ($p<0.001$)	197.5 ($p<0.001$)	61.8 ($p<0.001$)
Pyloric cecae [Cu]	111.6 ($p<0.001$)	315.1 ($p<0.001$)	107.7 ($p<0.001$)
Mid-intestine [Cu]	16.2 ($p<0.001$)	37.6 ($p<0.001$)	3.6 ($p<0.05$)
Posterior intestine [Cu]	46.5 (<0.001)	253.1 ($p<0.001$)	73.6 ($p<0.001$)
Cu uptake			
Whole-body	76.6 ($p<0.001$)	0.1 ($p>0.05$)	7.4 ($p<0.001$)
Somatic indices			
Liver (HSI)	15.0 ($p<0.0001$)	0.3 ($p>0.05$)	27.18 ($p<0.0001$)
Gill (BSI)	8.3 ($p<0.001$)	0.6 ($p>0.05$)	7.6 ($p<0.001$)
Gut (GSI)	13.6 ($p<0.0001$)	1.0 ($p>0.05$)	1.2 ($p>0.05$)
Na^+,K^+-ATPase		Diet Cu ($F_{1,24}$)	Ration ($F_{2,24}$)
Stomach	-	0.2 ($p>0.05$)	5.5 ($p<0.05$)
Pyloric cecae	-	0.1 ($p>0.05$)	6.6 ($p<0.01$)
Mid-intestine	-	2.7 ($p>0.05$)	4.3 ($p<0.05$)
Posterior intestine	-	1.2 ($p>0.05$)	6.0 ($p<0.01$)
Gill	-	0.2 ($p>0.05$)	3.3 ($p>0.05$)

Chapter 6 F values.

Measurement	**Main effect**
Cu and Na^+ uptake	Dietary Na^+ ($F_{3,15}$)
Gill	23.0 ($p<0.0001$)
Liver	16.1 ($p<0.0001$)
Gut	19.1 ($p<0.0001$)
Whole-body Cu	28.8 ($p<0.0001$)
Whole-body Na^+	15.3 ($p<0.0001$)
Total Cu	
Gill	9.1 ($p<0.001$)
Liver	4.1 ($p<0.03$)
Total Na^+	
Gut	33.3 ($p<0.0001$)
Plasma	3.9 ($p<0.03$)
Na^+,K^+-ATPase experiment	
	Dietary Na^+ ($F_{4,21}$)
Gill Na^+,K^+-ATPase	17.5 ($p<0.0001$)
Gill new Cu	6.0 ($p<0.02$)
Gill new Na^+	8.1 ($p<0.005$)
Drinking experiment	
	Dietary Na^+ ($F_{2,12}$)
Drinking rate	5.3 ($p<0.02$)

Chapter 7 F values.

Measurement	**Main effect**		
Cu uptake	Time ($F_{3,211}$)	Dietary Na^+ ($F_{1,213}$)	Water Cu ($F_{2,212}$)
Whole-body	218.9 ($p<0.0001$)	41.6 ($p<0.0001$)	14.0 ($p<0.0001$)
Gill	78.1 ($p<0.0001$)	34.5 ($p<0.0001$)	1.8 ($p>0.05$)
Liver	147.0 ($p<0.0001$)	23.2 ($p<0.0001$)	0.9 (p>0.0.05)
Carcass	262.5 ($p<0.0001$)	92.4 ($p<0.0001$)	1.8 ($p>0.05$)
Plasma	75.3 ($p<0.0001$)	60.9 ($p<0.0001$)	43.4 ($p<0.0001$)
Na^+ uptake			
Whole-body	104.7 ($p<0.0001$)	43.2 ($p<0.0001$)	2.8 ($p>0.05$)
Gill	458.7 ($p<0.0001$)	8.3 ($p<0.01$)	0.9 ($p>0.05$)
Liver	238.7 ($p>0.0001$)	8.4 ($p<0.01$)	0.5 ($p>0.05$)
Carcass	133.2 ($p<0.0001$)	5.1 ($p<0.05$)	0.5 ($p>0.05$)
Plasma	393.6 ($p<0.0001$)	8.2 ($p<0.01$)	0.6 ($p>0.05$)
Total [Cu]			
Liver	7.2 ($p<0.001$)	6.5 ($p<0.05$)	9.3 ($p<0.001$)
Gill	25.1 ($p<0.0001$)	3.9 ($p<0.05$)	3.4 ($p<0.05$)
Plasma	4.1 ($p<0.05$)	0.5 ($p>0.05$)	1.1 ($p>0.05$)
Carcass	2.93 ($p>0.05$)	2.6 ($p>0.05$)	2.1 ($p>0.05$)
Whole-body	4.3 ($p<0.01$)	0.1 ($p>0.05$)	3.5 ($p<0.03$)

REFERENCES

Andrews NC. 1999. The iron transporter DMTI. *Int J Biochem Cell Biol* **31**, 9991-9994.

ASTM. American Society of Testing Materials. 2000. Standard guide for conducting toxicity testing with fishes, micro invertebrates, and amphibians. E 729-796. In *Annual Book of ASTM Standards*, Vol. 11.05. Philadelphia, PA, pp 213-233.

Baker DH. 1986. Problems in animal experiments designed to establish dietary requirements for essential nutrients. *J Nutr* **116**, 2339-2349.

Baker JPT. 1969. Histological and electron microscopical observations on copper poisoning in the winter flounder (*Pseudopleronectes americanus*). *J Fish Res Board Can* **26**, 2785-2793.

Bergman HL and Dorward-King EJ (Eds). 1997. *Reassessment of Metals Criteria for Aquatic Life Protection: Priorities for Research and Implementation*. Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.

Berntssen MHG, Lundebye A-K, and Maage A. 1999. Effects of elevated dietary copper concentrations on growth, feed utilization and nutritional status of Atlantic salmon (*Salmo salar* L.) fry. *Aquaculture* **174**, 167-181.

Bingham MJ, Ong T-J, Summer KH, Middleton RB, and McArdle HJ. 1998. Physiologic function of the Wilson disease gene product, ATP7B. *Am J Clin Nutr* **67**(suppl.), 982S-987S.

Bradford MM. 1976. A refined and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248.

Bradley R, Duquensnay W, and Sprague JB. 1985. Acclimation of rainbow trout *Salmo gairdneri* Richardson to zinc: kinetics and mechanism of enhanced tolerance induction. *J Fish Biol* **27**, 367-379.

Bremner I. 1998. Manifestations of copper excess. *Am J Clin Nutr* **67**(suppl.), 1069S-1073S.

Brett JR. 1979. Environmental factors and growth. In *Bioenergetics and Growth*, Fish Physiology vol. viii. WS Hoar, DJ Randall, and JR Brett (eds.), pp. 599-975. Academic Press, New York, San Francisco, London.

Brett JR and Groves TDD. 1979. Physiological Enegertics. In *Bioenergetics and Growth*, Fish Physiology vol. viii. WS Hoar, DJ Randall, and JR Brett (eds.), pp. 280-344. Academic Press, New York, San Francisco, London.

Buckley JA, Roch M, McCarter JM, Rendell CA, and Matheson AT. 1982. Chronic exposure of coho salmon to sublethal concentrations of copper. I. Effect on growth, accumulation and distribution of copper, and on copper tolerance induction. *Comp Biochem Physiol* **72C**, 367-379.

Buddington RK, Chen JN, and Diamond J. 1986. Genetic and phenotypic adaptation of intestinal nutrient transport to diet in fish. *J Physiol* **39**, 261-281.

Bury NR, Grosell M, Wood CM, Hogstrand C, Wilson RW, Rankin JC, Busk M, Lecklin T, and Jensen FB. 2001. Intestinal iron uptake in the European flounder (*Platichthys flesus*). *J Exp Biol* **204**, 3779-3787.

Bury NR, Grosell M, Grover AK, and Wood CM. 1999. ATP-dependent silver transport across the basolateral membrane of rainbow trout gills. *Toxicol Appl Pharmacol* **159**, 1-8.

Camakaris J, Voskoboinik I, and Mercer JF. 1999. Molecular mechanisms of copper homeostasis. *Biochem Biophys Res Comm* **261**, 225-232.

Camakaris J, Petris M, Baily L, Shen P, Lockhart P, Glover TW, Barcroft C, Patton J, and Mercer JF. 1995. Gene amplification of the Menkes (MNK;

ATP7A) P-type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. *Hum Mol Genet* **4**, 2117-2123.

Campbell HA, Handy RD, and Nimmo M. 1999. Copper uptake kinetics across the gills of rainbow trout (*Oncorhynchus mykiss*) measured using an improved isolated perfused head technique. *Aquat Toxicol* **46**, 177-190.

Canonne-Hergaux F, Gruenheid S, Ponka P, and Gros P. 1999. Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brushborder and regulation by dietary iron. *Blood* **93**, 4406-4417.

CCME (Canadian Council of Ministers of the Environment). 1995. Canadian Water Quality Guidelines. Ottawa, Ontario, Canada

Cho CY, Slinger SJ, and Bayley HS. 1982. Bioenergetics of salmonid Fishes: Energy intake, expenditure and productivity. *Comp Biochem Physiol* **73B**, 25-41.

Clearwater SJ, Baskin SJ, Wood CM, and McDonald DG. 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. *J Exp Biol* **203**, 2455-2466.

Colman JR and Young LB. 1979. Metal binding by intestinal mucus. *Scanning Electron Microsc* **11**, 801-806.

Collvin L. 1985. The effects of copper on growth, food consumption and conversion of perch, *Perca fluviatilis* L offered maximal food rations. *Aquat Toxicol* **6**, 105-113.

Cousins RJ. 1985. Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiol Rev* **65**, 238-309.

Cusimano RF, Brakke DF, and Chapman GA. 1985. Effects of pH on the toxicities of cadmium, copper, and zinc to steelhead trout (*Salmo gairdneri)*. *Can J Fish Aquat Sci* **43**, 1497-1503.

Cowey, CB. 1992. Nutrition: estimating requirements of rainbow trout. *Aquaculture* **100**, 177-189.

Cowey CB and Sargent JR 1979. Nutrition. In *Bioenergetics and Growth*, Fish Physiology vol. viii. WS Hoar, DJ Randall, and JR Brett (eds.), pp. 1-58. Academic Press, New York, San Francisco, London.

Crespo S, Nonnotte G, Colin A, Leray LC, Nonnotte L, and Aubrée A. 1986. Morphological and functional alterations induced in trout intestine by dietary cadmium and lead. *J Fish Biol* **28**, 68-80.

Dallinger R, Segner PH, and Back H. 1987. Contaminated food and uptake of heavy metals by fish: a review and a proposal for further research. *Oecologia* **73**, 91-98.

Dallinger R, and Kautzky H. 1985. The importance of contaminated food for the uptake of heavy metals by rainbow trout (*Salmo gairdneri*): a field study. *Oecologia* **67**, 82-89.

Dang Z, Lock RAC, Flik G, and Wendelaar Bonga SE. 1999. Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water. *Am J Physiol* **277**, R320-R331.

D'Cruz LM and Wood CM. 1998. The influence of dietary salt and energy on the response to low pH in juvenile rainbow trout. *Physiol Zool* **71**, 642-657.

D'Cruz LM, Dockray JJ, Morgan IJ, and Wood CM. 1998. Physiological effects of sublethal acid exposure in juvenile rainbow trout on a limited or unlimited ration during a simulated global warming scenario. *Physiol Zool* **71**, 359-376.

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

DiDonato M and Sakar B. 1997. Copper transport and alterations in Menkes and Wilson diseases. *Biochim Biophys Acta* **1360**, 3-16.

Di Toro DM, Allen HE, Bergman HL, Meyer JS, Panquin PR, and Santore RC. 2001. Biotic ligand model of acute toxicity of metals. 1. Technical basis. *Environ Toxicol Chem* **20**, 2383-2396.

Dixon DG and Sprague JB. 1981. Acclimation to copper by rainbow trout (*Salmo gairdneri*) – a modifying factor in toxicity. *Can J Fish Aquat Sci* **38**, 880-888.

Dockray JJ, Reid SD, and Wood CM. 1996. Effects of elevated summer temperatures and reduced pH on metabolism and growth of juvenile rainbow trout (*Oncorhynchus mykiss*) on unlimited ration. *Can J Fish Aquat Sci* **53**, 2752-2763.

Erickson RJ, Benoit DA, Mattson VR, Nelson HP Jr, and Leonard EN. 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. *Environ Toxicol Chem* **15**, 181-193.

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

Ezeasor DN and Stokoe WM. 1980. Scanning electron microscopic study of the gut mucosa of rainbow trout *Salmo gairdneri* R. *J Fish Biol* **17**, 529-539.

Fairweather-Tait SJ. 1997. Bioavailability of copper. *Eur J Clinical Nutr* **51** (Suppl.), S24-S26.

Fange R and Groves D. 1979. Digestion. In *Bioenergetics and Growth*, Fish Physiology vol. viii. WS Hoar, DJ Randall, and JR Brett (eds.), pp. 161-260.

Farag AM, Stansbury MA, Hogstrand C, MacConnell E, and Bergman HL. 1995. The physiological impairment of free-ranging brown trout exposed to metals in the Clark Fork River, Montana. *Can J Fish Aquat Sci* **52**, 2038-2050.

Farag AM, Boese CJ, Woodward DF, and Bergman HL. 1994. Physiological changes and metal tissue accumulation in rainbow trout exposed to food-borne and waterborne metals. *Environ Toxicol Chem* **13**, 2021-2029.

Gatlin DM and Wilson RP. 1986. Dietary copper requirements of fingerling channel catfish. *Aquaculture* **54**, 277-285.

Gollan JL. 1975. Studies on the nature of complexes formed by copper with human alimentary canal secretions and their influence on copper absorption. *Clin Sci Mol Med* **49**, 237-245.

Goode CA, Dinh CT, and Linder MC. 1989. Mechanisms of copper transport and delivery in mammals: review and recent findings. In *Copper Bioavailability and Metabolism*. C Kies (Ed.) pp. 131-144. Plenum Press, New York.

Grosell M and Wood CM. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. *J Exp Biol* **205**, 1179-1188.

Grosell M, Kamunde C, Wood CM, and Walsh PJ. 2001a. Copper transport across fish gills. *Society for Experimental Biology, Annual Meeting,* University of Kent, Canterbury, England, 2-6 April pp. 89.

Grosell M, McGeer JC, and Wood CM. 2001b. Plasma copper clearance and biliary Copper excretion are stimulated in copper-acclimated trout. *Am J Physiol* **280**, R796-R806.

Grosell M, Hansen HJM, and Rosenkilde P. 1998. Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne copper exposure. *Comp Biochem Physiol* **120C**, 295-305.

Grosell MH, Hogstrand C, and Wood CM. 1998. Renal Cu and Na excretion and hepatic Cu metabolism in both Cu acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* **40**, 275-291.

Grosell MH, Hogstrand C, and Wood CM. 1997. Copper uptake and turnover in both Cu acclimated and non acclimated rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicol* **38**, 257-276.

Grosell M, Boetius I, HJM, and Rosenkilde P. 1996. Influence of pre-exposure to sublethal levels of copper on ^{64}Cu uptake and distribution among tissues of European eel (*Anguilla anguilla*). *Comp Biochem Physiol* **114C**, 229-235.

Guastadisegni C, Balduzzi M, Mancuso MT, and Consiglio ED. 1999. Liver mitochondrial alterations in chloroform-treated Sprague-Dawley rats. *J Toxicol Environ Health* **57**, 415-429.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, and Hediger MA. 1997. Cloning and characterization of a mammalian protein coupled metal-ion transporter. *Nature* **388**: 482-488.

Halliwell B and Gutteridge JMC. 1984. Oxygen toxicity, oxygen radicals, transition metals and diseases. *Biochem J* **219**, 1-4.

Hamilton SJ and Mehrle PM. 1986. Metallothionein in fish: review of its importance in assessing stress from metal contaminants. *Trans Am Fish Soc* **115**, 596-609.

Handy RD. 1996. Dietary exposure to toxic metals in fish. In *Toxicology of Aquatic Pollution. Physiological, Cellular and Molecular Approaches*. EW Taylor (ed.), pp. 29-60. Cambridge University Press.

Handy RD. 1993. Assessment of episodic metal pollution. II. The effects of cadmium and copper enriched diets on tissue contamination analysis in rainbow trout (*Oncorhynchus mykiss*). *Arch Environ Contam Toxicol* **22**, 82-87.

Handy RD. 1992. The effect of acute exposure to dietary Cd and Cu on organ toxicant concentrations in rainbow trout, *Oncorhynchus mykiss*. *Aquat Toxicol* **27**, 1-14.

Handy RD, Musonda MM, Philips C, and Fella SJ. 2000. Mechanisms of gastrointestinal copper absorption in the African walking catfish: copper dose-

effects and a novel anion-dependent pathway in the intestine. *J Exp Biol* **203**, 2365-2377.

Handy RD, Sims DW, Giles A, Campbell HA, and Musonda MM. 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. *Aquat Toxicol* **38**, 257-276.

Harris ED. 2001. Copper homeostasis: the role of cellular transporters. *Nutr Rev* **59**, 281.

Harris ED. 1991. Copper transport: an overview. *Pro Soc Exp Med* **192**, 130-140.

Harrison MD and Dameron CT. Molecular mechanisms of copper metabolism and the role of Menkes disease protein. *J Biochem Mol Toxicol* **13**, 93-106, 1998.

Hogstrand C and Haux C. 1991. Binding and detoxification of heavy metals in lower vertebrates with reference to metallothionein. *Comp Physiol* **100C**, 137-141.

Hogstrand C, Reid SD, and Wood CM. 1995. Calcium *versus* zinc transport in gill of freshwater rainbow trout, and cost of adaptation to waterborne zinc. *J Exp Biol* **186**, 55-73.

Hogstrand C, Webb N, and Wood CM. 1998. Covariation in regulation of affinity for branchial zinc and calcium uptake in freshwater rainbow trout. *J Exp Biol* **201**, 1809-1815.

Hollis L, Muench L, and Playle RC. 1997. Influence of dissolved organic matter on copper binding, and calcium on cadmium binding, by gills of rainbow trout. *J Fish Biol* **50**, 703-720.

Hughes GM. 1984. General anatomy of the gills In *Fish Physiology* vol. 10. WS Hoar and DJ Randall (eds.), Academic Press, pp 1-72.

Iturri SJ and Wolf D. 1982. Inhibition of the active transport of D-glucose and L-tyrosine by DDT and DDE in the rat small intestine. *Comp Biochem Physiol* **71C**, 131-134.

Jimnez BD, Crimo CP, and McCarthy JF. 1987. Effects of feeding and temperature on uptake, elimination and metabolism of benzo(a)pyrene in bluegill sunfish (*Lepomis macrochirus*). *Aquat Toxicol* **10**, 41-57.

Julshamn K, Andersen KJ, Ringdal O, and Brenna J. 1988. Effect of dietary copper on hepatic concentration and subcellular distribution of copper and zinc in rainbow trout *(Salmo gairdneri)*. *Aquaculture* **73,** 143-155.

Kamunde CN and Wood CM. 2002. The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquat Toxicol* (in press).

Kamunde CN, Pyle GG, McDonald DG, and Wood CM. 2002a. Influence of dietary sodium on waterborne copper toxicity in rainbow trout, *Oncorhynchus mykiss*. *Environ Toxicol Chem* (in press).

Kamunde CN, Clayton C, and Wood CM. 2002b. Waterborne *versus* dietary copper uptake in trout and the effects of waterborne copper acclimation. *Am J Physiol* **383**, R69-R78.

Kamunde CN, Grosell M, Higgs D, and Wood CM. 2002c. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. *J Exp Biol* **205**, 279-290.

Kamunde CN, Grosell M, Lott JNA, and Wood CM. 2001. Effects of dietary copper exposure on copper metabolism and gut morphology in rainbow trout Oncorhynchus mykiss. *Can J Fish Aquat Sci* **58**, 293-305.

Karasov WH and Diamond JD. 1983. Adaptive regulation of sugar and amino acid transport by vertebrate intestine. *Am J Physiol* **245**, G443-G462.

Karnaky KJ Jr. 1997. Osmotic and ionic regulation In *The Physiology of Fishes* DH Evans (Ed.), Boca Raton, FL, CRC Press, pp. 157-176.

Kimmich GA. 1973. Coupling between Na^+ and sugar transport in small intestine. *Biochim Biophys* **300**, 31-78.

Lanno RP, Hicks BE, and Dixon DG. 1989. Feeding and nutritional considerations in aquatic toxicology. *Hydrobiologia* **188/189**, 525-531.

Lanno RP, Hicks B, and Hilton JW. 1987. Histological observations on intrahepatocytic copper-containing granules in rainbow trout reared on diets containing elevated copper levels. *Aquat Toxicol* **10**, 251-263.

Lanno RP, Hicks B, and Hilton JW. 1985. Maximum tolerable and toxic levels of dietary copper in rainbow trout (*Salmo gairdneri* Richardson). *Aquaculture* **49**, 257-268.

Laurén DJ and McDonald DG. 1987a. Acclimation to copper by rainbow trout, *Salmo gairdneri*: Physiology. *Can J Fish Aquat Sci* **44**, 99-104.

Laurén DJ and McDonald DG. 1987b. Acclimation to copper by rainbow trout, *Salmo gairdneri*: Biochemistry. *Can J Fish Aquat Sci* **44**, 105-111.

Laurén DJ and McDonald DG. 1986. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. *Can J Fish Aquat Sci* **43**, 1488-1496.

Laurén DJ and McDonald DG. 1985. Effect of copper and branchial ion regulation in rainbow trout, *Salmo gairdneri*: *J Comp Physiol* **155B**, 635-644.

Lee J, Pena MMO, Nose Y, and Thiele DJ. 2002. Biochemical characterization of the human copper transporter Ctr1. J Biol Chem **277**, 4380-4387.

Lee J, Prohaska JR, and Thiele DJ. 2001. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. *Proc Natl Acad Sci USA* **98**, 6842-6847.

Lee J, Prohaska JR, Dagenais SL, Glover TW, Thiele DJ. 2000. Isolation of a Murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. *Gene* **254**. 87-96.

Li J, Quabius ES, Wendelaar Bonga SE, Flik, G, and Lock RAC. 1998. Effects of water-borne copper on branchial Na/H-ATPase activities in Mozambique tilapia *(Oreochromis mossambicus)*. *Aquat Toxicol* **43**, 1-11.

Li J, Lock RAC, Klaren PHM, Swarts HGP, Schuurmans Stekhoven FMAH, Wendelaar Bonga SE, and Flik G. 1996. Kinetics of Cu^{2+} inhibition of Na^{+}/K^{+}-ATPase. *Toxicol Lett* **87**, 31-38.

Lin H and Randall DJ. 1995. Proton pumps in fish gills. In *Cellular and Molecular Approaches to Fish Ionic Regulation*, pp. 229-225. CM Wood and TJ Shuttleworth (eds.). Academic Press, New York.

Linder MC. 1991. *Biochemistry of copper*. pp. 15-161. Plenum Press, New York.

Linder MC and Hazegh-Azam, M. 1996. Copper biochemistry and molecular biology. *Am J Clin Nutr* **63**, 797S-811S.

Lorentzen M, Maage A, and Julshamn K. 1998. Supplementing copper to fishmeal based diet fed to Atlantic salmon parr affects liver copper and selenium concentrations. *Aquacult Nutr* **4**, 67-72.

Lüllmann-Rauch R. 1979. Drug -induced lysosomal storage disorders In *Applied Biology and Therapeutics* vol 6. JT Dingle, PJJ Jacques and IH Shaw (Eds.). North-Holland Publishing Company, Amsterdam, New York, Oxford, pp. 49-130.

MacRae RK, Smith DE, Swoboda-Colberg N, Meyer JS, and Bergman HL. 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontalis*) gills: implications for assessing bioavailable metal. *Environ Toxicol Chem* **18**, 1180-1189.

Mallatt J. 1985. Fish gill structural changes induced by toxicants and other irritants: A statistical review. *Can J Aquat Sci* **42**, 630-648.

Marr JCA, Lipton J, Cacela D, Hansen JA, Bergman HL, Meyer JS, and Hogstrand C. 1996. Relationship between copper exposure duration, tissue copper concentration, and rainbow trout growth. *Aquat Toxicol* **36**, 17-30.

Marr JCA, Bergman HL, Lipton J, and Hogstrand C. 1995. Differences in relative sensitivity of naïve and metals-acclimated brown trout and rainbow trout exposed to representative of the Clark Fork River, Montana. *Can J Fish Aquat Sci* **52**, 2016-2030.

McCarter JA and Roch M. 1984. Chronic exposure of coho salmon to sublethal concentrations of copper. II. Kinetics of metabolism of metallothionein. *Comp Biochem Physiol* **77C**, 83-87.

McCormick SD. 1995. Hormonal control of gill Na^+,K^+-ATPase and chloride cell function. In *Cellular and Molecular Approaches to Fish Ionic Regulation*, pp. 285-315. CM Wood and TJ Shuttleworth (eds.). Academic Press, New York, NY.

McCormick SD. 1993. Methods for non-lethal gill biopsy and measurement of Na^+,K^+,-ATPase activity. *Can J Fish Aquat Sci* **50**, 656-658.

McDonald DG and Wood CM. 1993. Branchial mechanism of acclimation to metals in freshwater fish. In *Fish Ecophysiology*, pp. 297-32. JC Rankin and FB Jensen (eds.). Chapman and Hall, London.

McDonald DG, Wood CM, Rhem RG, Mueller ME, Mount DR, and Bergman HL. 1991. Nature and time course of acclimation to aluminum in juvenile brook trout (*Salvelinus fontinalis*), I. Physiology. *Can J Fish Aquat Sci* **48**, 2006-2015.

McDonald DG, Reader JP, and Dalziel TRK. (1989a). The combined effects of pH and trace metals on fish ionoregulation. In *Acid Toxicity and Aquatic Animals.* Cambridge University Press Cambridge, NY, pp. 221-241.

McDonald DG, Tang Y, and Boutilier RG. 1989b. Acid and ion transfer across

the gills of fish: mechanisms and regulation. *Can J Zool* **67**, 3046-3054.

McDonald DG and Prior ET. 1988. Branchial mechanisms of ion and acid-base regulation in the freshwater rainbow trout, *Salmo gairdneri*. *Can J Zool* **66**, 2699-2708.

McDonald DG. 1983. The effects of H^+ upon the gills of freshwater fish. *Can J Zool* **61**, 691-703.

McKenzie T. 1982. Analytical data for the GTA-95 In *Analytical Methods for Graphite Tube Atomizers*. E Rothery (Ed.), Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia, pp. 33.

Mertz W. 1981. The essential trace elements. *Science* **213**, 1332-1338.

Miller P A, Lanno RP, McMaster ME, and Dixon DG. 1993. Relative contributions of dietary and waterborne copper to tissue copper burdens and waterborne-copper tolerance in rainbow trout (*Oncorhynchus mykisss*). *Can J Fish Aquat Sci* **50**, 1683-1689.

Miller PA, Munkitrrick KR, and Dixon DG. 1992. Relationship between concentrations of copper and zinc in water, sediment, benthic invertebrates, and tissues of white sucker (*Catostomus commersoni*) at metal-contaminated sites. *Can J Fish Aquat Sci* **49**, 978-984.

Milligan CL and Wood CM. 1982. Disturbances in hematology, fluid volume, distribution, and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. *J Exp Biol* **99**, 397-415.

Milne DB. 1998. Copper intake and assessment of copper status. *Am J Clin Nutr* **67**, 1041S-1045S.

Morgan IJ, Henry RP, and Wood CM. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. *Aquat Toxicol* **38**, 145-163.

Mount DR, Barth AK, Garrison TD. 1994. Dietary and waterborne exposure of rainbow trout (*Oncorhynchus mykiss*) to copper, cadmium, lead, and zinc using live diet. *Environ Toxicol Chem* **13**, 2031-2041.

Murai T, Andrews JW, and Smith RG. 1981. Effects of dietary copper on channel catfish. *Aquaculture* **22**, 353-357.

Nonnotte L, Nonnotte G, and Leray C. 1986. Morphological changes in the middle intestine of rainbow trout, *Salmo gairdneri*, induced by hyperosmotic environment. *Cell Tiss Cult Res* **243**, 619-628.

NRC. 1993. Nutrient requirement of fish. National Academy of Sciences, Washington, DC, pp. 103

O'Halloran TV and Culotta VC. 2000. Metallochaperones: an intracellular shuttle service for metal ions. *J Biol Chem* **275**, 25057-25060.

Ogino C and Yang GY. 1980. Requirements of carp and rainbow trout for dietary manganese and copper. *Bull Jap Soc Sci Fish* **46**, 455-458.

Owen CA Jr and Hazelrig JB. 1968. Copper deficiency and copper toxicity in the rat. *Am J Physiol* **215**, 334-338.

Pagenkopf GK. 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. *Environ Sci Technol* **17**, 342-347.

Paris-Palacios S, Biagianti-Risbourg S, and Vernet G. 2000. Biochemical and (ultra)structural hepatic perturbations of *Brachydanio rerio* (Teleostei, Cyprinidae) exposed to two sublethal concentrations of copper sulphate. *Aquat toxicol* **50**, 109-124.

Pelgrom SMGJ, Lock RAC, Balm PHM, and Wendelaar Bonga SE. 1995. Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. *Aquat Toxicol* **32**, 303-320.

Pelletier E and Besner M. 1992. The effect of salty diets and gradual transfer to sea water on osmotic adaptation, gill Na^+,K^+-ATPase activation and survival of brook charr, *Salvelinus fontalis*, Mitchill. *J Fish Biol* **41**, 791-803.

Pena MM, Lee J, and Thiele D. 1999. A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr* **129**, 1251-1260.

Petris MJ, Mercer JFB, Culvenor JG, Lockhart, PA, and Camakaris J. 1996. Ligand regulated transport of Menkes copper P-type ATPase efflux pump from the Golgi apparatus to plasma: a novel mechanism of regulated trafficking. *EMBO J* **15**, 6084-6095.

Playle RC and Dixon DG. 1993. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modeling of metal accumulation. *Can J Fish Aquat Sci* **50**, 2678-2687.

Playle RC, Dixon DG, and Burnison K. 1993. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. *Can J Fish Aquat Sci* **50**, 2667-2677.

Playle RC, Gensemer RW, and Dixon DG. 1992. Copper accumulation on gills of fathead minnows: influence of water hardness, complexation, and pH of the gill microenvironment. *Environ Toxicol Chem* **11**, 381-391.

Pyle G, Kamunde CN, McDonald DG, and Wood CM. 2002. Dietary sodium inhibits aqueous copper uptake in rainbow trout, *Oncorhynchus mykiss*. *J Exp Biol* (in press).

Reid SD and McDonald DG. Metal binding activity of the gills of rainbow trout *Oncorhynchus mykiss*). *Can J Fish Aquat Sci* **48**, 1061-1068, 1991.

Rolfs A and Hediger MA. 1999. Metal ion transporters in mammals: structure, function and pathological implications. *J Physiol* **518**, 1-12.

Saari JT, Johnson WT, Reeves PG, and Johnson L. 1993. Amelioration of effects of severe dietary copper deficiency by food restriction in rats. *Am J Clin Nutr* **58**, 891-896.

Sadler K and Lynam S. 1987. Some effects on the growth of brown trout from exposure to aluminium at different pH levels. *J. Fish Biol* **31**, 209-219.

Salman NA and Eddy FB. 1990. Increased seawater adaptability in non-smolting rainbow trout by salt feeding. *Aquaculture* **86**, 259–270.

Salman NA and Eddy FB. 1988. Effect of dietary sodium chloride on growth, food intake and conversion efficiency in rainbow trout (*Salmo gairdneri* Richardson). *Aquaculture* **70**, 132-144.

Salman NA and Eddy FB. 1987. Response of chloride cell numbers and gill Na^+, K^+-ATPase activity of freshwater rainbow trout (*Salmo gairdneri*) to salt feeding. *Aquaculture* **61**, 41-48.

Santore RC, Di Toro DM, Panquin PR, Allen HE, and Meyer S. 2001. Biotic ligand model for acute copper toxicity in freshwater fish and Daphnia. *Environ Toxicol Chem* **20**, 2397-23402.

Satoh S, Yamamoto H, and Takeuchi T. 1983. Effects on growth and mineral composition of carp of deletion of trace elements or magnesium from fish meal diet. *Bull Jap Soc Sci Fish* **49**, 431-435.

Schaefer M and Gatlin JD. 1999. Genetic disorders of membrane transport IV. Wilson's disease and Menkes disease. *Am J Physiol* **276**, G311-G314.

Schecher WD and McAvoy DC. 1994. MINEQL+, User's Manual. Environmental Research Software, Hallowell, ME.

Schultz SG, Fuisz RE, and Curran PF. 1966. Amino acid and sugar transport in rabbit ileum. *J Gen Comp Physiol* **49**, 849-855.

Segner H. 1987. Response of fed and starved roach, *Rutilus rutilus*, to sublethal copper contamination. *J Fish Biol* **30**, 423-437.

Shearer KD. 1984. Changes in elemental composition of hatchery-reared rainbow trout, *Salmo gairdneri,* associated with growth and reproduction. *Can J Fish Aquat Sci* **41**, 1592-1600.

Sherwood GD, Rasmussen JB, Rowan DJ, Brodeur J, and Hontela A. 2000. Bioenergetic costs of heavy metal exposure in yellow perch (*Perca flavescens*): in situ estimates with a radiotracer (^{137}Cs) technique. *Can J Fish Aquat Sci* **57**, 441-450.

Smith N, Eddy F, and Talbot C. 1995. Effect of dietary salt load on transepithelial Na exchange in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* **198**, 2359-64.

Smith RW, and Griffith JS. 1994. Survival of rainbow trout during their first winter in Henry's Fork of the Snake River, Idaho. *Trans Am Fish Soc* **123**, 747-756.

Smith N, Talbot C, and Eddy F. 1989. Dietary salt intake and its relevance to ionic regulation in freshwater salmonids. *J Fish Biol* **35**, 749-753.

Sola F, Isaia J, and Masoni A. 1995. Effects of copper on gill structure and transport function in the rainbow trout, *Oncorhynchus mykiss*. *J Appl Toxicol* **15**, 391-398.

Solioz M and Odermatt A. 1995. Copper and silver transport by CopB-ATPases in membrane vesicles of *Enterococcus hirae*. *J Biol Chem* **270**, 9217-9224.

Spry DJ, Hodson PV, and Wood CM. 1988. Relative contributions of dietary and waterborne zinc in the rainbow trout, *Salmo gairdneri*. *Can J Fish Aquat Sci* **45**, 32-41.

Stagg RM and Shuttleworth TJ. 1982. The effects of copper on ionic regulation by the gills of the freshwater adapted flounder (*Platichthys flesus* L). *J Comp Physiol* **149**, 83-90.

Strausak D, La Fontaine S, Hill J, Firth SD, Lockhart PJ, and Mercer JF. 1999. The role of GMXCXXC metal binding sites in the copper-induced redistribution of the Menkes protein. *J Biol Chem* **274**, 11170-11177.

Sullivan GV, Fryer JN, and Perry SF. 1995. Immunolocalization of proton pump (H^+-ATPase) in pavement cells of rainbow trout gill. *J Exp Biol* **198**, 2619-2629.

Szebedinszky C, McGeer CJ, McDonald DG, and Wood CM. 2000. Effects of chronic Cd exposure via the diet or water on internal organ-specific distribution and subsequent gill Cd uptake kinetics in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* **20**, 597-607.

Taylor EW, Baeumont MW, Butler PJ, Mair J, and Mujallid MSI. 1995. Lethal and sub-lethal effects of copper upon fish: role for ammonia toxicity? In *Toxicology of Aquatic Pollution Physiological, Cellular and Molecular Approaches*. EW Taylor (ed) pp. 85-113.

Taylor LN, McGeer JC, Wood CM, and McDonald DG. 2000. The physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: an evaluation of chronic indicators. *Environ Toxicol Chem* **19**, 2298-2308.

Turnlund JR, Keyes WR, Peiffer GL, and Scott KC. 1998. Copper absorption, excretion, and retention by young men consuming low dietary Cu determined by using the stable isotope ^{65}Cu. *Am J Clin Nutr* **67** (suppl.), 1219S-1225S.

Turnlund JR., Scott KC, Peiffer GL, Jang WR, Keye WR, and Sakanashi TM. 1997. Copper status of young men consuming a low copper diet. *Am J Clin Nutr* **65**, 72-78.

Turnlund JR, Keyes WR, Anderson HL, and Acord LL. 1989. Copper absorption and retention in young men at three levels of dietary copper by use of stable isotope ^{65}Cu. *Am J Clin Nutr* **49**, 870-878.

Uauy R, Olivares M, and Gonzalez M. 1998. Essentiality of copper in humans. *Am J Clin Nutr* **67** (suppl.) 952S-959S.

USEPA (United States Environmental Protection Agency). 1986. Quality criteria for water. Report no. USEPA-440/5-86-001. U. S. Environmental Protection Agency, Washington, DC

Verbost PM, Schoenmakers Th JM, Flik G, and Wendelaar Bonga SE. 1994. Kinetics of ATP- and Na^+-gradient driven Ca^{2+} transport in basolateral membranes from gills of freshwater- and seawater-adapted tilapia. *J Exp Biol* **186**, 95-108.

Verbost PM, Van Rooij J, Flik G, Lock RAC, and Wendelaar Bonga SE. 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *J Exp Biol* **145**, 185-198.

Vulpe CD and Packman S. 1995. Cellular copper transport. *Ann Rev Nutr* **15**, 293-322.

Wapnir RA. 1998. Copper absorption and bioavailability. *Am J Clin Nutr* **67** (suppl.), 1054S-1068S.

Wapnir RA. 1991. Copper-sodium linkage during intestinal absorption: inhibition by amiloride. *Proc Exp Biol Med* **196**, 410-414.

Wapnir RA and Stiel L. 1987. Intestinal absorption of copper: effect of sodium. *Proc Soc Exp Biol Med* **185**, 277-282.

Watanabe T, Kiron V, and Satoh S. 1997. T race minerals in fish nutrition. *Aquaculture* **151**, 185-207.

Weiss P, Bogden RT, and Enslee EC. 1986. Hg-and Cu-induced hepatocellular changes in the mummichog, *Fundulus heteroclitus*. *Environ Health Perspect* **65**, 167-173.

Wilson RW, Bergman HL, and Wood CM. 1994. Metabolic costs and physiological consequences of acclimation to aluminum in juvenile rainbow trout (*Oncorhynchus mykiss*). 1. Acclimation specificity, resting physiology, feeding, and growth. *Can J Fish Aquat Sci* **51**, 527-535.

Wilson R, Gilmour K, Henry R, and Wood C. 1996. Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid-base balance? *J Exp Biol* **199**, 2331-2343.

Wilson RW and Taylor EW. 1993. The physiological response of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. *J Comp Physiol* **163B**, 38-47.

Wolf K. 1963. Physiological salines for freshwater teleosts. *Progr Fish Cultur* **25**, 135-140.

Wood CM. 2001. Toxic responses of the gill. In *Target Organ Toxicity in Marine and Freshwater Teleosts* vol. 1 – Organs, pp. 1-89. D Schlenk and WH Benson (eds). Taylor and Francis, London, New York.

Wood CM. 1992. Flux measurements as indices of H^+ and metal effects on freshwater fish. *Aquat Toxicol* **22**, 239-264.

Woodward DF, Farag AM, Bergman HL, Delonay AJ, Little EE, Smith CE, and Barrows, FT. 1995. Metals-contaminated benthic invertebrates in the Clark Fork River, Montana: effects on age-0 brown trout and rainbow trout. *Can J Fish Aquat Sci* **52**, 1994-2004.

Woodward DF, Brumbaugh WG, Delonay AJ, Little EE, and Smith CE. 1994. Effects on rainbow trout fry of a metals-contaminated diet of benthic invertebrates from the Clark Fork River, Montana. *Trans Am Fish Soc* **123**, 51-62.

Zhou B and Gitschier J. 1997. hCTR1: a human gene for copper uptake identified by complementation in yeast. *Proc Natl Acad Sci* USA **94**, 7481-7486.

Zohouri MA, Pyle GG, and Wood CM. 2001. Dietary Ca inhibits waterborne Cd uptake in Cd-exposed rainbow trout, *Oncorhynchus mykiss*. *Comp Biochem Physiol* **130C**, 347-356.