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

COLLINS N. KAMUNDE,* GREG G. PYLE, D. GORDON MCDONALD, and CHRIS M. WOOD
Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

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Abstract—Juvenile rainbow trout were fed diets containing control (0.26 mmol/g) or elevated (1.3 mmol/g) dietary Na^+ in combination with either background (19 nmol/L) or moderately elevated levels (55 or 118 nmol/L) of waterborne Cu for 21 d. Unidirectional waterborne Na^+ uptake rates (measured with ^{22}Na) were up to four 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^+ preexposure 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 appears to modify gill Cu-binding characteristics and may be important considerations in future development of a chronic biotic ligand model for Cu.

Keywords—Na^+/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 [1]. The primary pathway for Na^+ uptake in freshwater fish gill is believed to be via an apical Na^+ channel [2,3]. However, absorption of dietary Na^+ can contribute to the maintenance of ionic balance [4] and may alter waterborne Na^+ flux rates [5,6], indicating that branchial and gastrointestinal mechanisms cooperate to maintain ionic balance in fish. This cooperative phenomenon has been exploited to enhance the capacity of freshwater salmonids to adapt to seawater by prefeeding them dietary NaCl, which mimics the salt load in a marine environment [7–10]. In addition, dietary Na^+ absorption may be important in situations where branchial Na^+ uptake is impaired. Several waterborne contaminants, including acid [11], Cu [12], and silver [13], 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 the Na^+ balance [1,6,14,15], with death resulting from cardiovascular collapse [15]. More recently, Grosell and Wood [16] demonstrated that pharmacological blockade of Na^+ uptake with phenamil and bafilomycin decreased Cu uptake. In addition, Wapnir and Stiel [17] and Wapnir [18] 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. [19] and D'Cruz et al. [20] showed that feeding protected against ionoregulatory disturbance associated with environmental acid exposure in trout, while D'Cruz and Wood [21] 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 [6].

Our recent investigation [6] 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

* To whom correspondence may be addressed (kamundcn@mcmaster.ca).

toxicity testing and derivation of water quality criteria [22–25], 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 to 7 g in weight were obtained from Humber Springs Trout Farm (ON, Canada) and were maintained for two weeks in laboratory conditions consisting of a constant flow of aerated Hamilton (ON, Canada) city tap water containing 0.6 mmol/L Na^+, 0.7 mmol/L Cl^-, and 1.0 mmol/L Ca^{2+}, with hardness 1.4 as $CaCO_3$, alkalinity 0.95, and dissolved organic carbon 3.0 mg/L. Water pH and temperature were 7.9 to 8.2 and 14°C, respectively, and photoperiod was 12:12 h light:dark. Background Cu concentration was 31.5 to 47.3 nmol/L. 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 two-week period. 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 0.11 mmol/L Na^+, 0.10 mmol/L Cl^-, and 0.13 mmol/L Ca^{2+}, with hardness 0.16 as $CaCO_3$, alkalinity 0.15 as $CaCO_3$, and dissolved organic carbon 0.3 mg/L. Water pH and temperature were 6.9 to 7.1 and 14 ± 1°C, respectively, and background Cu concentration was 19 nmol/L. Fish were maintained in this water for 2.5 months before initiation of the experiment because previous data [26] suggest that soft water acclimation, as evidenced by recovery of whole-body electrolytes, takes about 10 weeks in this species. During the preexperimental and soft water acclimation period, fish were fed once daily at 2% wet body weight on commercial trout chow (Corey Feed Mills, Fredericton, NB, Canada) containing 55% crude protein, 17% crude fat, 2% crude fiber, 1.5% Ca^{2+}, and 0.6% Na^+. The measured Cu concentration of the diet was 0.27 μmol/g.

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 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 (~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, Canada) was 1.27 mmol/g (~30 mg/g), in comparison with 0.25 mmol/g (~6 mg/g) 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 (0800–0900 h) and evening (1800–1900 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-d exposure and ration was adjusted accordingly.

Exposure set-up

The exposure set-up was a 2 × 3 factorial design (two diets [control and elevated] and three waterborne Cu treatments [nominally 0, 55, and 118 nmol/L]) and consisted of six 200-L plastics tanks partitioned in half by dividers, giving a total of 12 experimental chambers. The dividers were perforated to allow free movement of water, therefore providing 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 60 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, Canada) at concentrations of 31.5 and 47.2 μmol/L, maintained at drip rates of 1.8 and 2.6 ml/min, respectively, in two head tanks receiving soft water at flow rates of 1.05 L/min. 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, 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 (means ± standard error of the mean [SEM]) 19.2 ± 0.8 nmol/L (n = 18) for controls, 58.6 ± 1.4 nmol/L for the medium concentration (n = 18), and 113.5 ± 4.3 nmol/L (n = 18) for the high concentration.

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 experiments involving different feeding regimes and waterborne metal exposure levels. To check for random effects, all the treatments were duplicated. Moreover, keeping fish together allowed for social interactions and better reflected 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 were simultaneously exposed to waterborne ^{64}Cu (3.3 μCi/L; McMaster University Nuclear Reactor, Hamilton, ON, Canada) and ^{22}Na (0.025 μCi/L; Amersham Pharmacia Biotech, Piscataway, NJ, USA) 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 to 13.0 nmol/L, 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 min 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 and 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 preweighed 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, USA) 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 were 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 with six volumes of 1 N HNO_3 (trace metal grade; Fisher Scientific, Nepean, ON, Canada), and 1.5-ml aliquots were removed and centrifuged for 4 min at 13,000 *g*. A subsample of the supernatant was diluted appropriately with 0.5% HNO_3 and total tissue Cu concentration determined by graphite furnace atomic absorption spectroscopy (Varian AA-1275 with graphite tube furnace atomizer, Mississauga, ON, Canada) 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, Canada) 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 activities (cpm) in all tissues and carcass. Fish weights were determined by summing the weights of all tissues and the rest of the 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 of water, and c is the total Cu or Na^+ concentration in water in μmol/L. 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 milliliter 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. [26]. Control fish and fish chronically exposed to dietary Na^+ alone and to the high water Cu level (118 nmol/L) with or without high dietary Na^+ were tested using nominal Cu concentrations of 0, 0.08, 0.16, and 0.47 μmol/L waterborne Cu. Actual water Cu concentrations during the test, determined by atomic absorption spectroscopy, were (mean ± SEM) 0.10 ± 0.007 μmol/L (n = 12), 0.18 ± 0.008 μmol/L (n = 12), 0.31 ± 0.01 μmol/L (n = 12), and 0.48 ± 0.01 μmol/L (n = 12), respectively. For each concentration, 10 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 radiolabeled $CuNO_3$. 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 (as appropriate) 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-d 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 [27].

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) plus 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) had significantly lower Cu concentrations relative to the fish on elevated waterborne Cu level plus control dietary Na^+ (0.26 mmol/g) on both days 14 and 21. For the

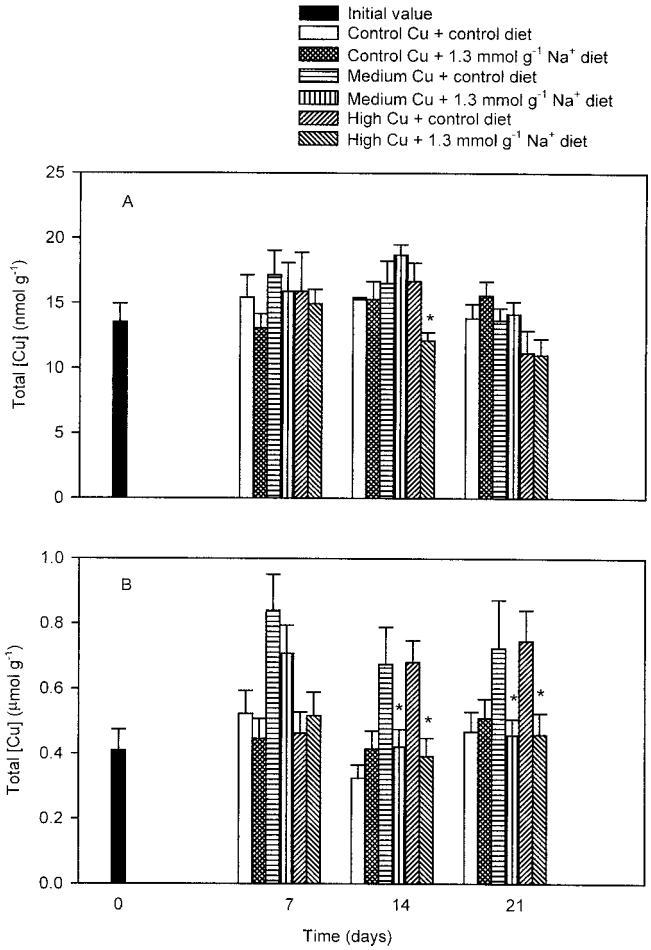

Fig. 1. Whole-body (**A**) and liver (**B**) Cu concentrations in juvenile rainbow trout exposed to 1.3 mmol/g dietary Na^+ in combination with background [19], 55, or 118 nmol/L waterborne Cu. Values are means ± standard error of the mean (n = 10 per bar except high Cu + control diet on day 21, when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

gills, Cu concentrations remained unchanged in all groups except on day 21, when the fish exposed to 55 nmol/L waterborne Cu plus high dietary Na^+ had lower Cu concentrations compared with the group exposed to 55 nmol/L waterborne Cu plus 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 the 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/h for fish on the control diet and between 0.03 and 0.05 nmol/g/h for fish exposed to high dietary Na^+. Unidirectional Na^+ uptake rates ranged from 0.35 to 0.43 µmol/g/h in fish exposed to the control dietary Na^+ level and 0.15 to 0.38 µmol/g/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^+ covaried. This covariation 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 5,800 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) 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 (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 [26,28,29]. According to Salman and Eddy [8], 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

Table 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 waterborne Cu. Values are means ± standard error of the mean ($n = 10$ for each value except for the group on Na^+ diet + 118 nmol/L water [Cu] on day 21, where $n = 5$). * = 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 water [Cu]	0.40 ± 0.03	0.48 ± 0.03	0.48 ± 0.05
Control diet + 118 nmol/L 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 water [Cu]	0.39 ± 0.03	0.42 ± 0.02	0.44 ± 0.03
Na^+-diet + 118 nmol/L 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 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 water [Cu]	0.48 ± 0.03	0.42 ± 0.02	0.43 ± 0.07
Na^+-diet + 118 nmol/L 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 water [Cu]	0.43 ± 0.04	0.40 ± 0.02	0.41 ± 0.03
Control diet + 118 nmol/L 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 water [Cu]	0.30 ± 0.02*	0.44 ± 0.02	0.42 ± 0.04
Na^+-diet + 118 nmol/L water [Cu]	0.32 ± 0.01	0.49 ± 0.03	0.40 ± 0.04

50% of test organisms) for fish of comparable size and age in water of similar chemistry [26,29]. 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 [30]). Furthermore, in acute toxicity, it is probably the gill, rather than whole-body, metal burden that is important for toxicity because the gill is the primary target organ for waterborne Cu toxicity [1,31]. 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 the 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 [5,6]. 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. [4] evaluated the relative contribution of branchial and gastrointestinal Na^+ uptake to whole-body Na^+ balance in laboratory and wild salmonids over a one-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 [5]. 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 to waterborne Cu is in agreement with previous studies using environmentally realistic levels of Cu exposure [26,32]. 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 [33]. To this end, Wapnir and Stiel [17] and Wapnir [18] 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 [5,6] have convincingly shown that dietary Na^+ reduces waterborne Na^+ uptake in trout

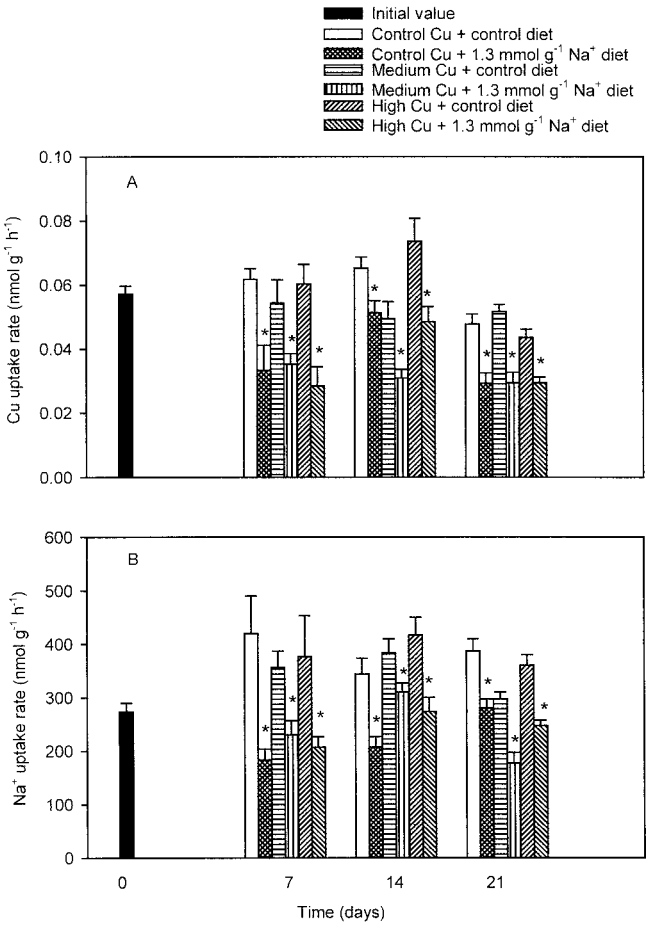

Fig. 2. Whole-body unidirectional Cu (**A**) and Na^+ (**B**) uptake rates in juvenile rainbow trout exposed to 1.3 mmol/g dietary Na^+ in combination with background [19], 55, or 118 nmol/L waterborne Cu. Flux measurements were performed at background Cu levels. Values are means ± standard error of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

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 and 55 mmol/kg, 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. Because part of Cu uptake occurs via the Na^+ uptake pathway [16], 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 [12,14] 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 [16] reported that waterborne Na^+ reduced waterborne Cu uptake but waterborne Cu at the levels used (less than 0.16 μmol/L) 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 ab-

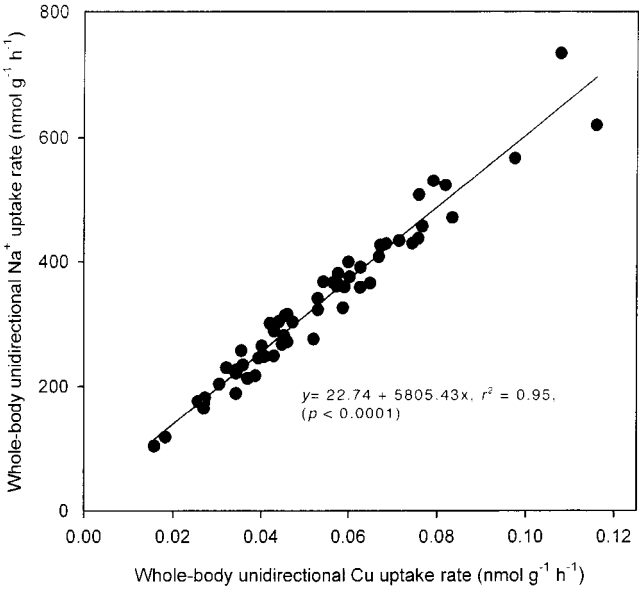

Fig. 3. Correlation between whole-body Na^+ and Cu uptake rates in juvenile rainbow trout exposed to 1.3 mmol/g dietary Na^+ in combination with background [19], 55, or 118 nmol/L waterborne Cu. Measurements of uptake rates were performed at background Cu levels. Each data point represents new whole-body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

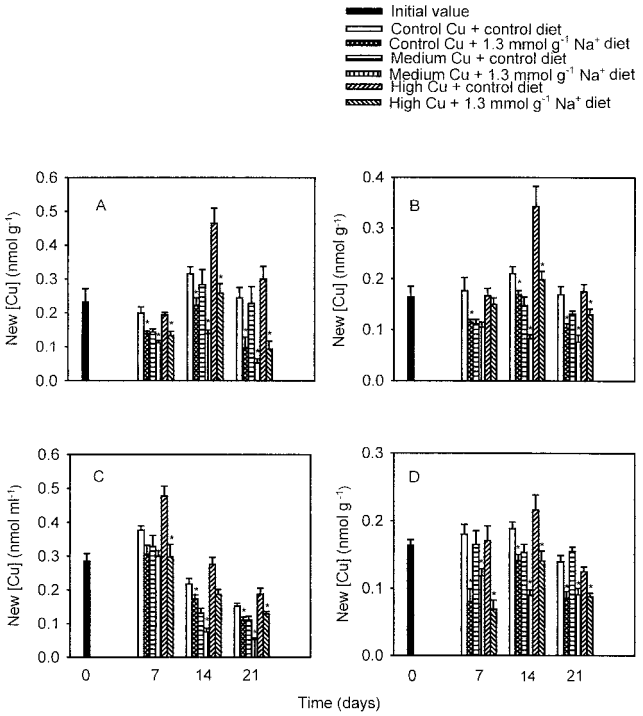

Fig. 4. Newly accumulated Cu levels in gill (**A**), liver (**B**), plasma (**C**), and carcass (**D**) of juvenile rainbow trout exposed to 1.3 mmol/g dietary Na^+ in combination with background [19], 55, or 118 nmol/L waterborne Cu. All measurements were performed at background Cu levels. Values are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

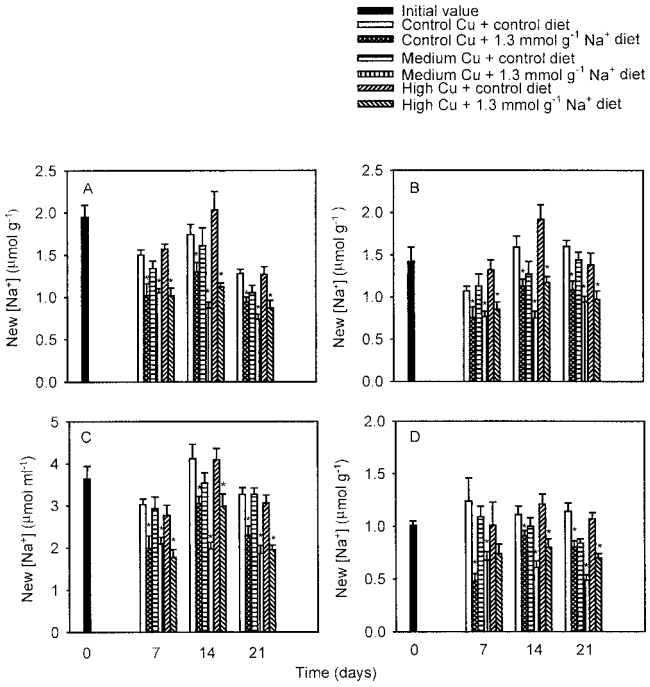

Fig. 5. Newly accumulated Na^+ levels in gills (**A**), livers (**B**), plasma (**C**), and carcasses (**D**) in juvenile rainbow trout exposed to 1.3 mmol/g dietary Na^+ in combination with background [19], 55, or 118 nmol/L waterborne Cu. All measurements were performed at background Cu levels. Values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

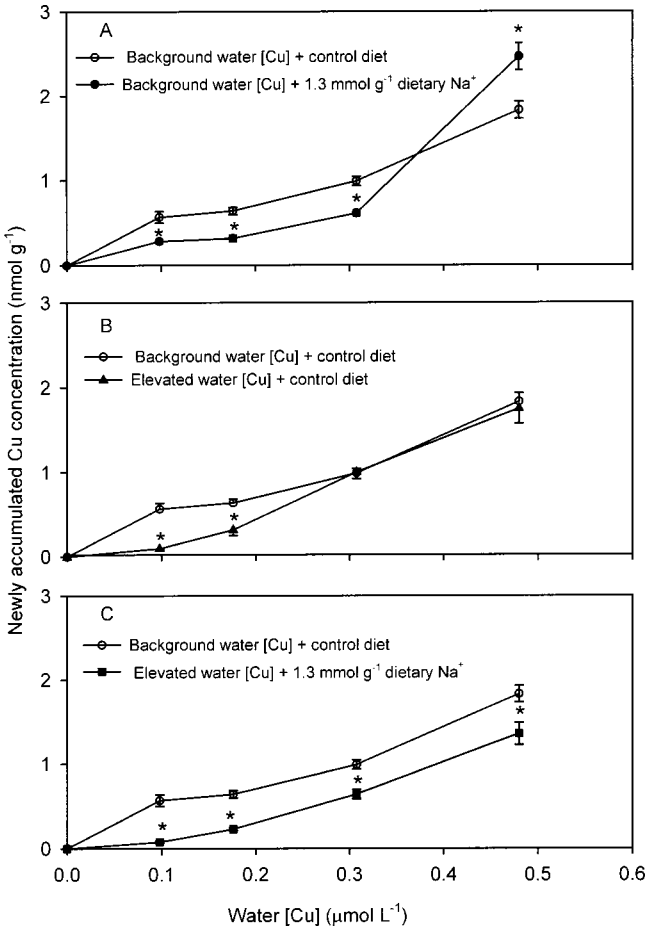

Fig. 6. Gill Cu binding in juvenile rainbow trout exposed to control conditions, elevated (1.3 mmol/g) dietary Na^+, and elevated (118 nmol/L) waterborne Cu with or without elevated dietary Na^+ for 21 d. Values are means ± standard error of the mean (n = 10 per data point). (**A**) Gill Cu binding in control and fish exposed to elevated dietary Na^+ at background levels of water Cu. (**B**) Gill binding between controls and fish exposed to elevated waterborne Cu together with control diet. (**C**) Binding between controls and fish exposed to a combination of elevated dietary Na^+ and elevated waterborne Cu. * = significant difference from control group, $p < 0.05$.

sence 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 covaried with Na^+ uptake during chronic exposure to elevated dietary Na^+ and waterborne Cu levels (Figs. 2 and 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 [14]. 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. [6], 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) causes significant reduction in unidirectional waterborne Cu uptake rate [32].

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 [16]. 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., Pyle et al. [6], Laurén et al. [14]). 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. [34] demonstrated a covariation 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. [35] 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 nonessential 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

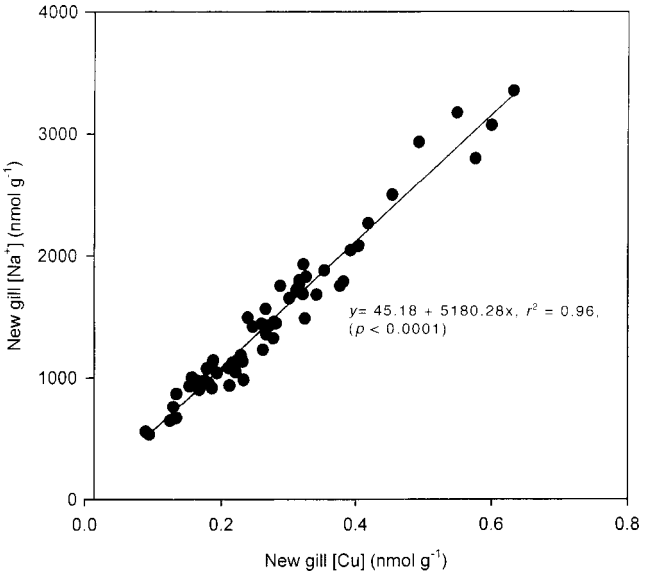

Fig. 7. Correlation between newly accumulated gill Na^+ and newly accumulated gill Cu in juvenile rainbow trout exposed to 1.3 mmol/g dietary Na^+ in combination with background [19], 55, or 118 nmol/L waterborne Cu. Flux measurements were performed at background Cu levels. Each data point represents newly accumulated gill Na^+ and Cu values for the same fish on day 14.

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 [36]. Recent data [32] 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 μmol/L. 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 [16,26,32]. 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. [26] and Grosell and Wood [16], 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 that has been proposed as a regulatory tool [22–25]. Presently, based on the protective effect of waterborne Na^+ on Cu toxicity [37], Na^+ binding to the gill is considered in the Cu BLM [25]. 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. Because 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 the 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) is environmentally realistic and the dietary Na^+ level was only about four times higher than some natural fish diets [4]. 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 [38]. These authors [38] 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 [4], 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 reduces Cu binding to the gill biotic ligand and is therefore likely to mitigate acute Cu toxicity. Because the BLM predicts the amount of metal bound to the gill that causes acute toxicity [22–25], 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; [6], this study), dietary Cu content [39,40], and acclimation to waterborne Cu ([32,41], this study) affect binding of Cu to the gills.

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