Interaction of dietary sodium chloride and waterborne copper in rainbow trout (*Oncorhynchus mykiss*): copper toxicity and sodium and chloride homeostasis

Collins N. Kamunde, Soumya Niyogi, and Chris M. Wood

Abstract: Juvenile rainbow trout (*Oncorhynchus mykiss*) maintained on either low sodium chloride (LS (control), 1.4% NaCl) or high sodium chloride (HS, 11% NaCl) diet were exposed to 55 $\mu g \cdot L^{-1}$ waterborne copper (Cu) for 28 days. Cu-exposed fish maintained on the LS diet exhibited 26% mortality, more than double (11%) that in fish maintained on the HS diet. Waterborne Cu exposure inhibited growth by 56% in fish maintained on the LS diet and by 35% in those maintained on the HS diet. Whole-body and tissue Na^+ levels, measured 6 h after feeding, were increased by exposure to HS diet and reduced by waterborne Cu exposure. Exposure to elevated waterborne Cu increased whole-body and tissue Cu levels, whereas exposure to HS diet decreased these levels. Moreover, whole-body and tissue Cu concentrations were consistently lower in Cu-exposed fish maintained on HS diet relative to those maintained on LS diet. Plasma Na^+ and Cl^- levels were elevated by HS diet exposure and reduced by waterborne Cu exposure, whereas plasma Cu levels were decreased and increased by exposure to HS diet and waterborne Cu, respectively. These results demonstrate that elevated dietary NaCl modulates Na^+ and Cl^- homeostasis and reduces accumulation and toxicity of waterborne Cu.

Résumé : Des jeunes truites arc-en-ciel (*Oncorhynchus mykiss*), gardées à un régime faible (témoin) en chlorure de sodium (LS, 1,4 % NaCl) ou à un régime élevé (HS, 11 % NaCl), ont été exposés à 55 $\mu g \cdot L^{-1}$ de Cu en solution aqueuse pendant 28 jours. Les poissons gardés à un régime LS ont souffert une mortalité de 26 %, plus du double de celle (11 %) des poissons gardés au régime HS. L'exposition au Cu en solution aqueuse réduit la croissance des poissons gardés au régime LS de 56 % et celle des poissons gardés à HS de 35 %. Les concentrations de Na^+ dans le corps entier et les tissus, mesurées 6 h après l'alimentation, augmentent après un régime HS et diminuent après une exposition au Cu en solution aqueuse. Une exposition aux concentrations élevée de Cu en solution aqueuse augmente les concentrations de Cu dans le corps entier et les tissus, alors qu'un régime HS les diminue. De plus, les concentrations de Cu dans le corps entier et les tissus sont toujours plus faibles chez les poissons gardés à un régime HS et exposés au Cu que chez les poissons gardés au régime LS et exposés au cuivre. Les concentrations plasmatiques de Na^+ et de Cl^- augmentent avec un régime HS et diminuent après une exposition au Cu en solution aqueuse; en revanche, les concentrations plasmatiques de Cu diminuent à un régime HS et augmentent après une exposition au Cu en solution aqueuse. Ces résultats montrent qu'une augmentation de NaCl dans le régime module l'homéostasie de Na^+ et de Cl^- et réduit l'accumulation et la toxicité du Cu en solution aqueuse.

[Traduit par la Rédaction]

Introduction

The uptake of metal ions through biological membranes is strongly influenced by metal ion chemistry, but quite often ions that have very different chemical characteristics compete for uptake sites on biological surfaces. For example, despite a lower valence, a larger ionic radius, and a much higher concentration of Na^+ in natural waters compared with Cu^{2+}, these ions compete for uptake sites on the freshwater fish gill epithelium (Erickson et al. 1996; Santore et al. 2001; Grosell and Wood 2002). This is made possible in part by the involvement of three factors facilitating Cu^{2+} uptake. First, Cu^{2+} possesses several potential pathways of uptake and transport because it has the ability to form a wide range of coordination complexes with many cellular entities (Bury et al. 2003). Second, the hydrated (aquo) form of Cu^{2+}

Received 20 October 2003. Accepted 21 July 2004. Published on the NRC Research Press Web site at http://cjfas.nrc.ca on 26 March 2005.
J17805

C.N. Kamunde,[1,2] S. Niyogi,[3] and C.M. Wood. Department of Biology, McMaster University, Hamilton, ON L8S 4 K1, Canada.

[1]Corresponding author (e-mail: ckamunde@upei.ca).
[2]Present address: Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, PE CIA 4P3, Canada.
[3]Present address: Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, SK S7N 5E2, Canada.

 doi: 10.1139/F04-169

is large, such that in solution its mobility is similar to that of Na^+ (Handy et al. 2002) and it can therefore compete for diffusion sites on membranes. Third, the affinity of Cu^{2+} for binding (uptake) sites on the gill surface is more that four orders of magnitude higher than that of Na^+ (Santore et al. 2001), effectively compensating for the lower concentration of Cu^{2+} in natural waters.

Interactions between Na^+ and Cu in freshwater fish have therefore been the focus of several toxicological and physiological studies. Early studies showed that acute exposure of fish to waterborne Cu reduces plasma Na^+ and Cl^- concentrations (McKim et al. 1970; Schreck and Lortz 1978; Stagg and Shuttleworth 1982) as a consequence of inhibition of branchial Na^+ and Cl^- uptake processes (Laurén and McDonald 1985, 1986). Copper competitively inhibits (decreased affinity or increased K_m) and noncompetitively inhibits (reduced maximum uptake rate or J_{max}) the basolaterally located sodium pump, Na^+/K^+-adenosine triphosphatase (Na^+/K^+-ATPase; Laurén and McDonald 1987*b*; Sola et al. 1995; Li et al. 1998), leading to the impairment of branchial ionoregulatory mechanisms. However, very recent evidence now suggests that Na^+/K^+-ATPase impairment may not be the only potential mechanism for toxic action of Cu; other Na^+ (and Cl^-) uptake/transport processes are also impacted. There are now indications that Cu affects the epithelial Na^+ channel (Grosell and Wood 2002; Handy et al. 2002; Pyle et al. 2003), carbonic anhydrase and sodium-proton exchanger (Grosell et al. 2002), and ammonia excretion (Wilson and Taylor 1993; Taylor et al. 1996; Beaumont et al. 2003). All these mechanisms of toxicity are directly or indirectly associated with Na^+ metabolism and affirm that interference with Na^+ homeostasis is indeed the fundamental mechanism of acute waterborne Cu toxicity (Laurén and McDonald 1985, 1987*a*).

During prolonged sublethal exposure to waterborne Cu, partial or complete recovery of ionoregulatory function occurs, in part because of an increase in Na^+/K^+-ATPase activity (Stagg and Shuttleworth 1982; Laurén and McDonald 1987*a*; McGeer et al. 2000). Moreover, induction of metallothionein and increased sequestration of Cu in the gill (Dang et al. 1999, 2000) and liver (e.g., McCarter and Roch 1984) reduces the circulating levels of Cu. A possible additional strategy for fish to compensate for Cu-induced branchial Na^+ losses is to increase uptake and (or) utilization of dietary Na^+, because in addition to meeting their Na^+ requirement through branchial uptake (Lin and Randall 1995; Sullivan et al. 1995; Wood 2001), freshwater fish derive variable amounts of Na^+ from the diet depending on prevailing conditions. For example, in winter when foraging activity is low, trout principally meet their Na^+ requirement from water, whereas in summer, dietary and waterborne Na^+ uptakes are of the same magnitude (Smith et al. 1989). Several recent studies have advanced the concept that freshwater fish can take advantage of dietary ion surplus to mitigate the toxicity of waterborne toxicants that inhibit branchial ion uptake (Kamunde et al. 2003; Pyle et al. 2003; Baldisserotto et al. 2004). Specifically, Kamunde et al. (2003) and Pyle et al. (2003) demonstrated that elevated dietary Na^+ reduced gill binding and unidirectional uptake of waterborne Cu in rainbow trout while simultaneously decreasing branchial Na^+ uptake. Although the reduction in uptake and gill binding of Cu essentially implies reduction in Cu toxicity, protection against waterborne Cu toxicity by dietary NaCl is yet to be unambiguously demonstrated.

The purpose of the present study was therefore fourfold. First, because the concentrations of Cu used in our previous studies did not result in toxicity, we sought to expose juvenile rainbow trout to concentrations of waterborne Cu that would induce demonstrable toxicity, e.g., mortality/reduced survival and (or) growth inhibition, in order to characterize the potential protective effect of elevated dietary NaCl. Second, we evaluated the effects of both elevated waterborne Cu and dietary NaCl on Na^+ and Cl^- homeostasis by measuring tissue and plasma Na^+ and Cl^- concentrations soon after feeding Cu-exposed fish either a low or a high NaCl diet to capture the effect of Cu exposure when the fish are likely actively regulating an upsurge of plasma Na^+ from dietary uptake. Third, we examined changes in plasma Na:Cl ratios to assess the effect of waterborne Cu and dietary NaCl exposure on interdependency of Na^+ and Cl^- homeostatic mechanisms. Fourth, we assessed the potential protective effect of dietary NaCl on the bioaccumulation of Cu in whole-body and key target tissues. Overall, by evaluating growth, survival, and changes in tissue, plasma, and whole-body Na^+, Cl^-, and Cu during a long-term experiment, we sought to illuminate indicators of chronic Cu toxicity in freshwater fish.

Materials and methods

Experimental fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Hatchery, Mono Mills, Ontario, and acclimated to laboratory conditions in a single 500-L plastic tank for 3 weeks. Laboratory conditions included flow-through dechlorinated Hamilton municipal tap water (Na^+ 0.6, Ca^{2+} 1.0, Cl^- 0.7, Mg^{2+} 0.2, HCO_3^- 1.9, all in $mmol \cdot L^{-1}$; dissolved organic carbon 3.0 $mg \cdot L^{-1}$; hardness 120 $mg \cdot L^{-1}$ as $CaCO_3$; pH 7.8–8.0; temperature 12 ± 1 °C). Fish were maintained on 1.5% wet body weight daily ration of commercial granulated 1.0 grade dry trout pellet (Corey Feed Mills, Fredericton, New Brunswick). The commercial trout diet contained crude protein 54% (minimum), crude fat 19% (minimum), crude fiber 2% (maximum), Ca^{2+} 1.5% (actual), phosphorus 1.1% (actual), Na^+ 0.6% (actual), vitamin A 10 000 IU (international units)$\cdot kg^{-1}$ (minimum), vitamin D3 6000 IU$\cdot kg^{-1}$ (minimum), and vitamin E 400 IU$\cdot kg^{-1}$ (minimum). Measured concentrations of Cu in water and food were 2.81 ± 0.43 $\mu g \cdot L^{-1}$ (n = 28) and 9.6 ± 0.76 $\mu g \cdot g^{-1}$ (n = 6), respectively, and the mean fish weight obtained by bulk weighing of all the fish at the beginning of the experiment was 19.9 g (wet weight).

Experimental set-up

The experiment described here was part of a larger study comprising a complete 2 × 3 complete factorial design (two waterborne Cu levels and three dietary NaCl treatments) that also involved assessment of food consumption and preference during waterborne Cu exposure. Here, we focus on two treatments tailored to investigate the effect of dietary NaCl and chronic waterborne Cu exposure on sublethal toxicity, Cu accumulation, and Na^+ and Cl^- homeostasis. The results of the third treatment are the subject of a separate publication (S. Niyogi, C.N. Kamunde, and C.M. Wood, unpublished).

Following laboratory acclimatization, fish were equally (n = 33–35) and randomly distributed into six 150-L experimental tanks. Exposure to 55 $\mu g \cdot L^{-1}$ waterborne Cu was achieved via a constant drip of 2.5 $mL \cdot min^{-1}$ of a stock solution containing 68.75 $mg \cdot L^{-1}$ Cu (as $CuSO_4 \cdot 5H_2O$; Fisher Scientific, Toronto, Ontario) from a Marriott bottle into a head tank receiving 2.5 $L \cdot min^{-1}$ of dechlorinated Hamilton tap water. Uniform distribution of Cu in the head tank was achieved by constant vigorous aeration. The head tank supplied two experimental tanks (plus one for the diet selection study) at a flow rate of 800 $mL \cdot min^{-1}$. Actual Cu concentration in the experimental tanks was 52.34 ± 4.71 $\mu g \cdot L^{-1}$ (n = 28), and measured Cu concentration in the control tanks was 2.81 ± 0.76 $\mu g \cdot L^{-1}$ (n = 28). Fish in one of the tanks receiving Cu-contaminated water were maintained on a HS diet, whereas those in the other were maintained on a LS diet. The third Cu-treatment tank was used for a diet selection study. Two control tanks (plus one for a diet selection study) were maintained on regular dechlorinated tap water, with fish in one of the tanks receiving HS diet and the other receiving LS diet.

Diets, fish maintenance, and sampling

The HS diet was made in-house by supplementing commercial trout chow to a nominal level of 10% NaCl (by weight) as previously reported (Kamunde et al. 2003; Pyle et al. 2003). Control (i.e., LS) diet was taken through the same processing procedure except that no NaCl was added. Actual Na^+ concentrations were determined from samples (n = 6) of each diet: LS diet, 0.24 ± 0.02 $mmol \cdot g^{-1}$ (1.4% NaCl), HS diet, 1.89 ± 0.17 $mmol \cdot g^{-1}$ (11% NaCl). The diets were kept at –20 °C until offered to the experimental fish at 1.5% wet body weight ration twice per day (in the morning and evening for a total daily ration of 3.0%). One hour after each feeding, undigested food was siphoned off to minimize leaching of Na^+ into the water. All the fish were bulk-weighed weekly to determine growth and the ration for the ensuing week. Fish (n = 6–10) were sampled from each of the four treatments on days 4, 7, 14, and 28. An initial sample of 10 fish was obtained from the holding tank immediately before the random separation of the fish among the tanks, thereby providing the day 0 (baseline) data.

At each sampling time, fish were killed with an overdose of neutralized tricaine methanesulfonate; blood samples were taken immediately by caudal puncture and centrifuged for 4 min at 10 000g to separate plasma, which was decanted into separate plastic centrifuge tubes. The fish were then rinsed in running double-distilled water, blotted dry, and weighed. Gills, liver, gut, kidney, and the rest of the carcass were subsequently dissected into preweighed scintillation vials. Before placing them into scintillation vials, gills were rinsed in running double-distilled water for 30 s while the gut tissues were emptied of their contents, thoroughly washed in running double-distilled water, and blotted dry. All tissues, including plasma, were stored at –20 °C until analyzed for total Na^+, Cl^-, and Cu as described below.

Analysis

All tissues were digested overnight with six times their volume of 1 $mol \cdot L^{-1}$ HNO_3 (trace metal grade; Fisher Scientific, Nepean, Ontario), and subsamples were pipetted into 2-mL centrifuge tubes and centrifuged for 4 min at 10 000g. The supernatants were then diluted appropriately with 1% HNO_3 and analyzed for total Na^+ and Cu concentrations. Total Cu concentrations were measured by graphite furnace atomic absorption spectrophotometry (GFAAS; Varian SpectrAA-220, Mulgrave, Australia) with a graphite tube atomizer (GTA-110) while total Na^+ concentrations were measured by flame atomic absorption spectrophotometry (FAAS; Varian SpectrAA-220FS, Mulgrave, Australia). Water and plasma samples were similarly analyzed for total Na^+ and Cu concentrations after appropriate dilution. In addition, plasma was analyzed for Cl^- by the protocol of Zall et al. (1956) adapted for a microplate reader. Analytical quality assurance and control were maintained using method blanks and sample duplicates and were validated with certified reference materials (National Research Council of Canada, Ottawa, Ontario).

Calculations

Fish condition factor (k) was calculated using the following formula:

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

Whole-body total Na^+ and Cu concentrations were calculated by dividing the sum of Na^+ or Cu contents (concentrations multiplied by weight) of all the tissues sampled plus the carcass by the sum of weights of all the tissues plus the carcass.

Statistical analysis

All Na^+, Cl^-, and Cu accumulation data were statistically analyzed using three-way analysis of variance (ANOVA, Statistica version 6.0) with time, dietary NaCl, and level of waterborne Cu exposure as independent variables. A two-way ANOVA with dietary NaCl and waterborne Cu exposure levels as independent variables was used to analyze the condition factor and proportional distribution data (day 28 only). The proportional distribution data were arcsine transformed before statistical analysis. Tukey's honestly significant difference (HSD) test was used to delineate differences in mean values and to test for interactions among the main factors. Mean values were considered different at $P < 0.05$. The assumptions of ANOVA, i.e., homogeneity of variances and normality of distribution, were tested using Bartlett and χ^2 tests. All data met these assumptions. Differences in mortalities between control and Cu-exposed fish were delineated using Pearson's χ^2 test.

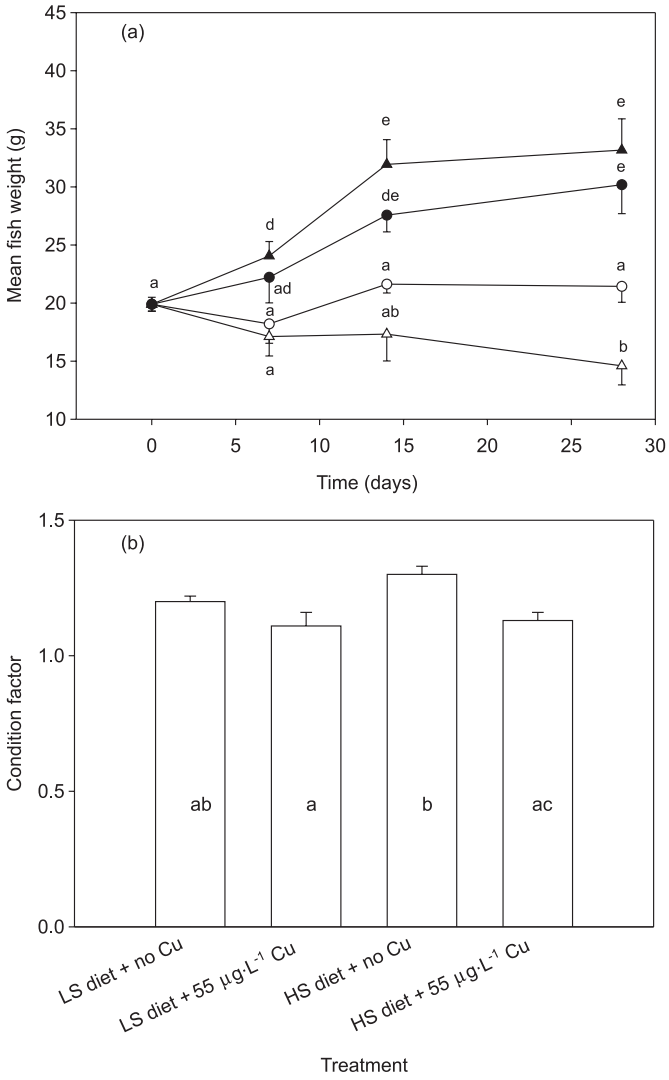
Results

Mortality and growth

Exposure of juvenile rainbow trout to 55 $\mu g \cdot L^{-1}$ waterborne Cu for 28 days caused 11% mortality in the fish maintained on HS diet, significantly lower ($P < 0.05$, Pearson's χ^2) than the 26% mortality in fish maintained on LS diets. This mortality occurred mainly during the first week of Cu exposure. Cu-unexposed fish on either diet showed no mortality. Growth was severely impacted by Cu exposure ($F_{[1,76]} = 81.03$, $P < 0.0001$), with the fish maintained on the LS diet showing net weight loss relative to the starting weight (Fig. 1a). At day 28, relative to control group, growth was not significantly altered (–9%, $P > 0.05$, not significant) in

Cu-unexposed fish maintained on HS diet but was inhibited by 35% ($P < 0.05$) in Cu-exposed fish maintained on HS diet and by 56% ($P < 0.05$) in Cu-exposed fish maintained on LS diet. Time had a significant effect on growth ($F_{[2,76]} = 7.01$, $P < 0.005$), and there was a strong interaction between dietary NaCl and waterborne Cu ($F_{[1,76]} = 10.73$, $P < 0.005$), indicating that elevated dietary NaCl partly protected against the Cu-induced growth inhibition. There was also a significant interaction between time and waterborne Cu ($F_{[2,76]} = 3.98$, $P < 0.05$). Fish condition factor ranged from 1.10 to 1.30 on day 28 (Fig. 1*b*) and was adversely impacted by exposure to elevated waterborne Cu ($F_{[1,32]} = 12.37$, $P < 0.005$) but not by exposure to elevated dietary NaCl.

Fig. 1. Effect of exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to elevated waterborne Cu and dietary NaCl on (*a*) mean body weight and (*b*) condition factor. Weight gain was significantly impaired by elevated waterborne Cu exposure ($F_{[1,76]} = 81.03$, $P < 0.0001$), whereas condition factor was reduced by waterborne Cu ($F_{[1,32]} = 12.37$, $P < 0.001$) and increased by dietary NaCl ($F_{[1,32]} = 3.13$, $P < 0.05$). Points and bars with different letters are significantly different ($P < 0.05$). ▲, LS diet + no Cu; ●, HS diet + no Cu; △, LS diet + 55 μg·L^{-1} waterborne Cu; ○, HS diet + 55 μg·L^{-1} waterborne Cu.



Whole-body and tissue Cu accumulation

Exposure to elevated waterborne Cu increased whole-body Cu concentration ($F_{[3,101]} = 62.95$, $P < 0.0001$) by up to 80% (Fig. 2*a*) in the fish maintained on the LS diet relative to their controls, whereas elevated dietary NaCl significantly impeded whole-body Cu accumulation ($F_{[1,101]} = 18.01$, $P < 0.0001$). Whole-body Cu concentration generally increased with time ($F_{[3,101]} = 5.51$, $P < 0.05$), and there was significant interaction between dietary NaCl and waterborne Cu ($F_{[1,101]} = 5.92$, $P < 0.05$).

Organ/tissue Cu concentrations generally followed the same trend as for whole-body (Figs. 2*b*–2*f*). In the gill (Fig. 2*b*), elevated waterborne Cu exposure greatly increased the total Cu concentration ($F_{[1,101]} = 145.18$, $P < 0.0001$). Gill Cu concentration increased 2.5-fold in the Cu-exposed fish maintained on the LS diet relative to controls and had achieved a steady state by day 28. In contrast, dietary NaCl substantially impeded Cu accumulation in the gills ($F_{[1,101]} = 19.66$, $P < 0.0001$); thus Cu-exposed fish maintained on the HS diet showed only a 1.8-fold increase in Cu concentration on day 7, declining to more or less the control value by day 28. Overall, gill Cu concentration increased significantly with time ($F_{[3,101]} = 12.54$, $P < 0.0001$). Three-way ANOVA revealed significant interactions between time and dietary NaCl ($F_{[3,101]} = 4.77$, $P < 0.01$), time and waterborne Cu ($F_{[3,101]} = 5.46$, $P < 0.01$), and dietary NaCl and waterborne Cu ($F_{[1,101]} = 16.69$, $P < 0.0001$), as well as a three-way interaction between time, dietary NaCl, and waterborne Cu ($F_{[3,101]} = 3.21$, $P < 0.05$).

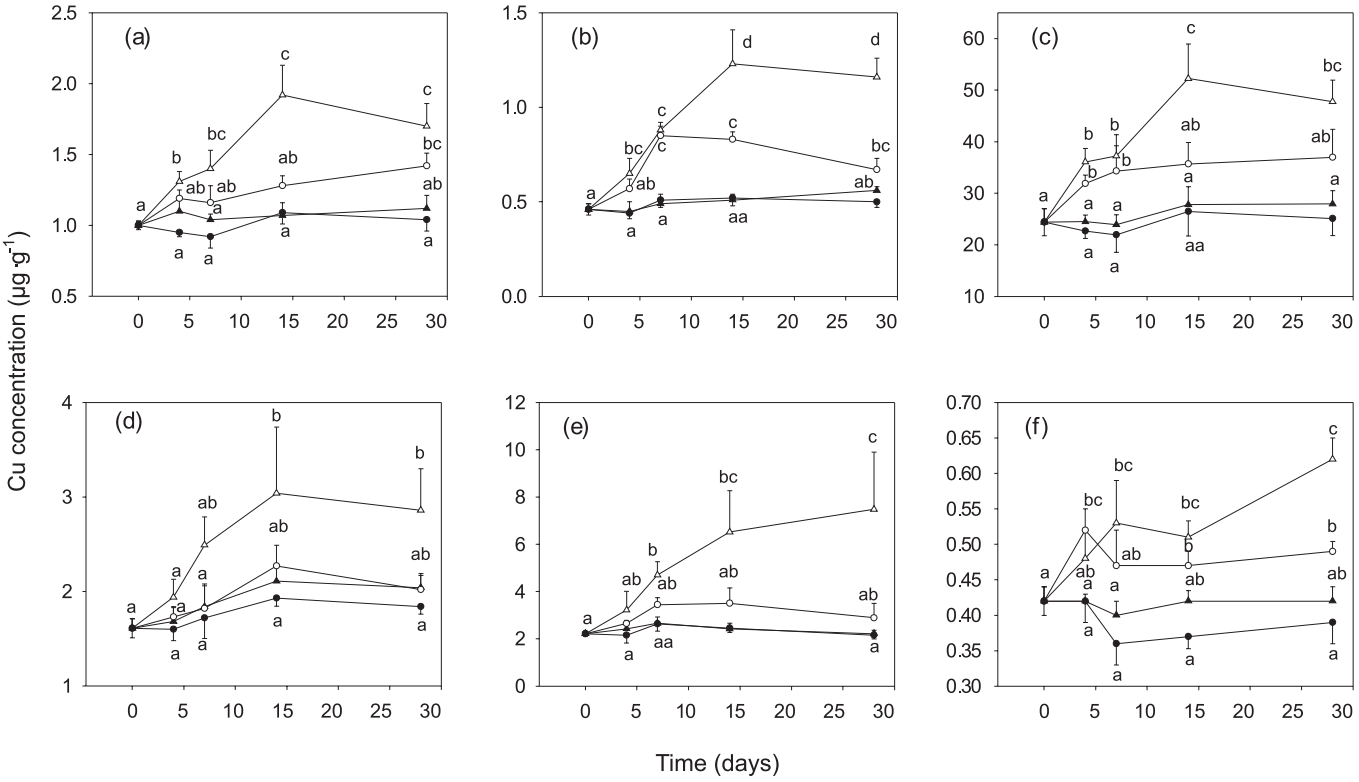
Of all tissues examined, the liver accumulated by far the greatest concentrations of Cu (Fig. 2*c*). Elevated waterborne Cu exposure significantly elevated the Cu concentrations ($F_{[1,101]} = 50.42$, $P < 0.0001$), whereas the HS diet reduced the accumulation of Cu ($F_{[1,101]} = 6.73$, $P < 0.05$) in the liver. A 40% reduction in Cu accumulation was observed in Cu-exposed fish maintained on HS diet relative to Cu-exposed fish maintained on LS diet (Fig. 2*c*). Overall liver Cu concentration had leveled off by day 14 of exposure in all Cu-exposed fish.

Kidney Cu accumulation (Fig. 2*d*) was only about 5% of that in the liver, and Cu concentrations were significantly increased by elevated waterborne Cu exposure ($F_{[1,101]} = 13.38$, $P < 0.005$). In addition, kidney Cu concentrations increased with time ($F_{[3,101]} = 4.93$, $P < 0.005$) but were significantly decreased by exposure to elevated dietary NaCl ($F_{[1,101]} = 10.49$, $P < 0.005$). There was significant interaction between dietary NaCl and waterborne Cu exposure ($F_{[1,101]} = 4.23$, $P < 0.05$) in determining Cu accumulation in the kidney.

The gut tissue (Fig. 2*e*) accumulated the next highest Cu concentrations relative to the liver, but overall these amounted to less than 15% of liver values. Elevated waterborne Cu exposure significantly elevated ($F_{[1,101]} = 29.23$, $P < 0.0001$), while dietary NaCl greatly impeded ($F_{[1,101]} = 11.87$, $P < 0.001$) the accumulation of Cu in gut tissues. There was significant interaction between dietary NaCl and waterborne Cu ($F_{[1,101]} = 10.31$, $P < 0.005$).

Overall, the carcass (Fig. 2*f*) contained the lowest concentrations of Cu of all of the tissues examined (0.35–

Fig. 2. Effect of elevated dietary NaCl and waterborne Cu exposure on accumulation of Cu in (*a*) whole body, (*b*) gill, (*c*) liver, (*d*) kidney, (*e*) gut, and (*f*) carcass. Elevated dietary NaCl impeded Cu accumulation, whereas elevated waterborne Cu increased Cu accumulation. Points on the same panel with different letters are significantly different ($P < 0.05$). ▲, LS diet + no Cu; ●, HS diet + no Cu; △, LS diet + 55 μg·L^{-1} waterborne Cu; ○, HS diet + 55 μg·L^{-1} waterborne Cu.



0.62 μg·g^{-1}). However, carcass Cu concentration was greatly increased by elevated waterborne Cu exposure ($F_{[1,101]}$ = 49.79, $P < 0.0001$), whereas elevated dietary NaCl significantly impeded accumulation of Cu ($F_{[1,101]} = 6.44$, $P < 0.05$).

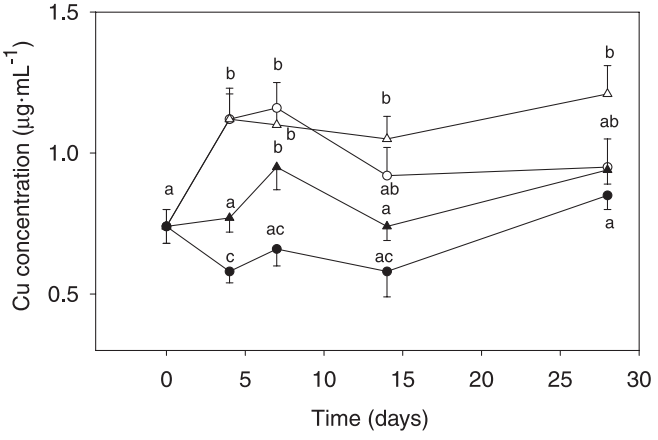
Plasma Cu concentrations

Plasma Cu concentrations (Fig. 3) were more tightly regulated than tissue Cu concentrations, varying only between 0.6 and 1.2 μg·mL^{-1}, but did respond to the experimental treatments. Plasma Cu concentrations were significantly elevated ($F_{[1,99]} = 23.42$, $P < 0.0001$) and depressed ($F_{[1,99]}$ = 19.18, $P < 0.0001$) following exposure to elevated waterborne Cu and dietary NaCl, respectively. There were interactions between time and dietary NaCl ($F_{[3,99]} = 4.73$, $P < 0.005$) and time and waterborne Cu ($F_{[3,99]} = 4.71$, $P < 0.005$), as well as between dietary NaCl and waterborne Cu ($F_{[1,99]} = 5.83$, $P < 0.05$).

Proportional distribution of Cu

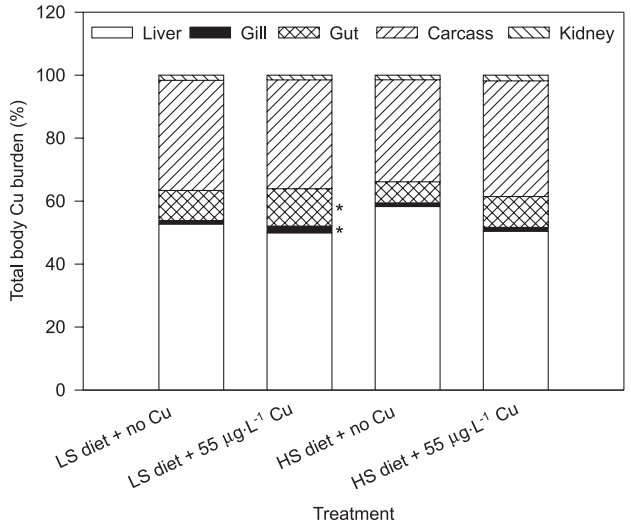
Mass-weighted Cu distributions in gill, liver, kidney, gut tissue, and carcass on day 28 are shown for the various treatment groups (Fig. 4). For all groups, the liver contained most of the Cu (49%–58%), followed in decreasing order by the carcass (32%–37%), gut tissues (7%–12%), gill (1%–2%), and kidney (1%–2%). There was a significant effect of exposure to both elevated waterborne Cu ($F_{[1,32]} = 7.30$, $P < 0.05$) and dietary NaCl ($F_{[1,32]} = 8.49$, $P < 0.01$) on the contribution of the gill to whole-body Cu burden, with a significant interaction ($F_{[1,32]} = 11.32$, $P < 0.01$) between the two factors. In addition, distribution of Cu in the gut tissues was

Fig. 3. Effect of elevated dietary NaCl and waterborne Cu exposure on plasma Cu concentrations. Elevated dietary NaCl decreased plasma Cu levels, whereas elevated waterborne Cu increased plasma Cu levels. Points with different letters are significantly different ($P < 0.05$). ▲, LS diet + no Cu; ●, HS diet + no Cu; △, LS diet + 55 μg·L^{-1} waterborne Cu; ○, HS diet + 55 μg·L^{-1} waterborne Cu.



significantly reduced by exposure to elevated dietary NaCl ($F_{[1,32]} = 4.33$, $P < 0.05$) but was unaffected by exposure to elevated waterborne Cu. There was a significant interaction ($F_{[1,32]} = 5.06$, $P < 0.05$) between waterborne Cu and dietary NaCl.

Fig. 4. Effect of elevated dietary NaCl and waterborne Cu exposure on proportional distribution of Cu in the main body compartments at day 28. Asterisk (*) indicates significantly different from controls ($P < 0.05$).



Whole-body and tissue Na^+ concentrations

Sodium concentrations were fairly uniform in all tissues examined, ranging from 35 to 90 $\mu mol \cdot g^{-1}$. Exposure to elevated dietary NaCl significantly increased whole-body Na^+ concentrations ($F_{[1,101]} = 100.43$, $P < 0.0001$) from 50–53 $\mu mol \cdot g^{-1}$ in control fish maintained on the LS diet to 63–72 $\mu mol \cdot g^{-1}$ in Cu-unexposed fish and 57–64 $\mu mol \cdot g^{-1}$ in Cu-exposed fish (Fig. 5*a*). In contrast, elevated waterborne Cu exposure caused significant reductions in whole-body Na^+ concentrations ($F_{[1,101]} = 10.83$, $P < 0.005$). These changes became attenuated with time such that by day 28, differences occurred based on dietary NaCl treatment but not waterborne Cu exposure level.

Gill Na^+ concentration (Fig. 5*b*) increased significantly ($F_{[1,101]} = 56.35$, $P < 0.0001$) in response to the HS diet from 40–50 to 64–75 $\mu mol \cdot g^{-1}$ in Cu-unexposed fish and 52–72 $\mu mol \cdot g^{-1}$ in Cu-exposed fish. In contrast, elevated waterborne Cu exposure caused a significant decline in the gill Na^+ concentrations ($F_{[1,101]} = 4.62$, $P < 0.05$). Generally, the greatest effect of waterborne Cu exposure on Na^+ levels occurred in the fish maintained on the HS diet.

Liver Na^+ concentrations increased dramatically in response to the HS diet exposure ($F_{[1,101]} = 153.63$, $P < 0.0001$), with values ranging from 42–50 $\mu mol \cdot g^{-1}$ in the controls to 70–88 $\mu mol \cdot g^{-1}$ in the group maintained on HS diet (Fig. 5*c*). In contrast, elevated waterborne Cu exposure significantly decreased liver Na^+ concentrations ($F_{[1,101]} = 4.60$, $P < 0.05$).

In the kidney (Fig. 5*d*), dietary NaCl increased ($F_{[1,101]} = 25.63$, $P < 0.0001$) and waterborne Cu exposure reduced ($F_{[1,101]} = 5.91$, $P < 0.05$) Na^+ concentrations. There was a significant interaction between time, dietary Na^+, and waterborne Cu ($F_{[3,101]} = 6.51$, $P < 0.001$). Kidney Na^+ concentrations showed a "cross-over" effect in the two groups maintained on HS diet whereby the Na^+ concentration decreased dramatically to 39 $\mu mol \cdot g^{-1}$ early in the exposure in Cu-exposed fish maintained on the HS diet but subsequently increased to a level of 61 $\mu mol \cdot g^{-1}$ on day 28, the highest kidney Na^+ concentration recorded in the experiment.

For the gut (Fig. 5*e*), exposure to the HS diet elevated ($F_{[1,101]} = 47.65$, $P < 0.0001$) while exposure to elevated waterborne Cu decreased ($F_{[1,101]} = 7.08$, $P < 0.01$) Na^+ concentrations. As with the kidney, there was a cross-over effect resulting in the gut tissues of Cu-exposed fish maintained on the HS diet having the highest Na^+ concentration at day 28.

Carcass Na^+ concentrations (Fig. 5*f*) were also significantly elevated by exposure to elevated dietary Na^+ ($F_{[1,101]} = 83.64$, $P < 0.0001$) and decreased by elevated waterborne Cu exposure ($F_{[1,101]} = 9.50$, $P < 0.005$). These effects were greatest early in the exposure and became attenuated with time.

Plasma Na^+ and Cl^- concentrations

Plasma Na^+ concentrations (Fig. 6*a*) were significantly reduced by exposure to elevated waterborne Cu ($F_{[1,99]} = 20.54$, $P < 0.0001$) but significantly increased by elevated dietary NaCl ($F_{[1,99]} = 38.13$, $P < 0.0001$) and by time ($F_{[3,99]} = 25.09$, $P < 0.0001$). There were interactions between dietary NaCl and waterborne Cu ($F_{[1,99]} = 3.94$, $P < 0.05$) and among the three factors ($F_{[3,99]} = 14.14$, $P < 0.0001$). Plasma Cl^- concentrations (Fig. 6*b*) were significantly increased ($F_{[1,99]} = 52.01$, $P < 0.0001$) and decreased ($F_{[1,99]} = 11.42$, $P < 0.005$) by elevated dietary NaCl and waterborne Cu exposure, respectively. Na:Cl ratios (Table 1) ranged from 0.90 to 1.30 and were significantly lower ($F_{[1,99]} = 15.97$, $P < 0.0005$) in fish maintained on HS diet. These ratios, however, increased with time ($F_{[1,99]} = 11.80$, $P < 0.0001$).

Discussion

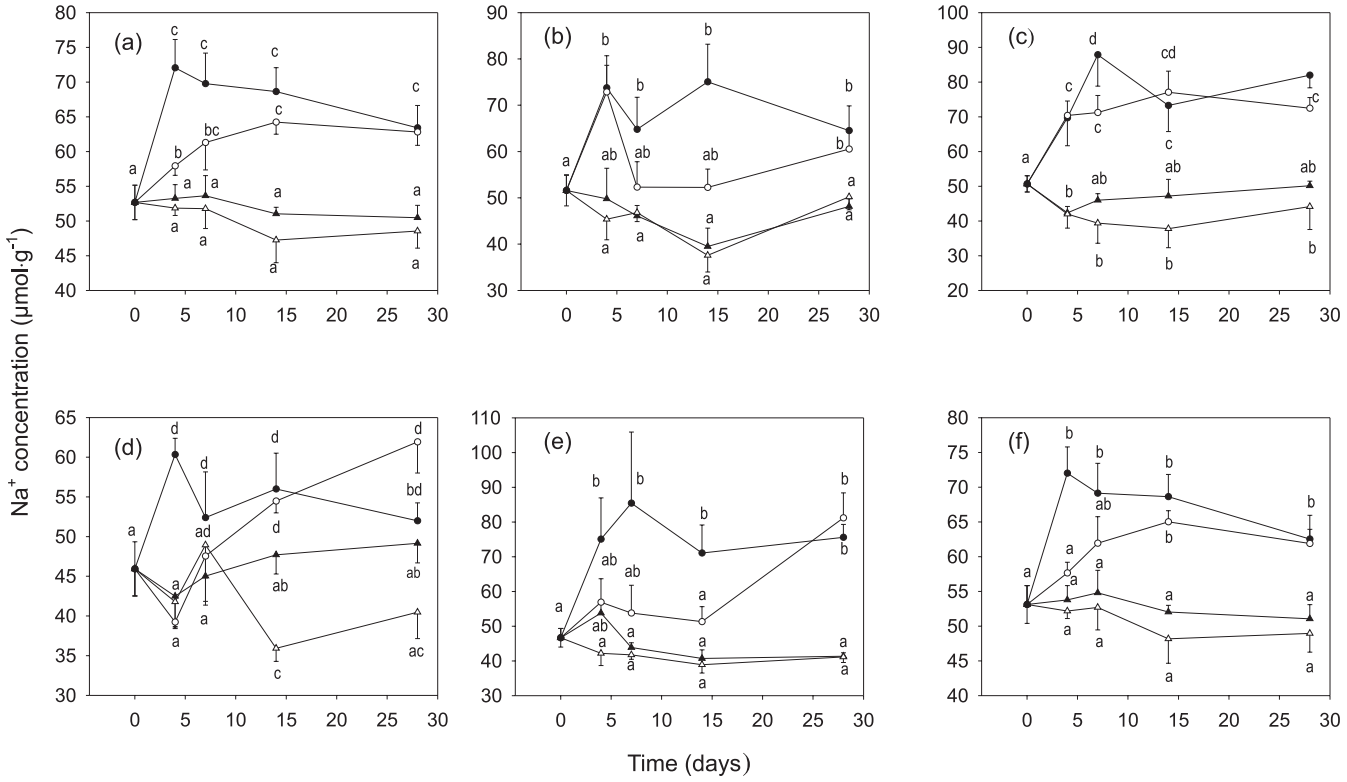
Mortality and growth

We achieved our objective of establishing quantifiable differences in toxicity whereby trout exposed to elevated waterborne Cu and maintained on the LS diet exhibited significantly higher mortality and greater growth inhibition relative to those maintained on the HS diet. Clearly, dietary NaCl protected fish against waterborne Cu toxicity. Although growth inhibition has been previously reported during waterborne Cu exposure in fish (e.g., Lett et al. 1976; Collvin 1985; Marr et al. 1996), ours is the first report of protection against the growth inhibitory effects of waterborne Cu exposure by elevated dietary NaCl in fish. This supports the proposition in our recent studies (Kamunde et al. 2003; Pyle et al. 2003) that reduction in the uptake rate and gill binding of Cu following elevated dietary NaCl exposure has implications for the toxicity of waterborne Cu in fish. The sustained growth retardation during chronic waterborne Cu exposure observed in the present study is in agreement with some studies (McKim and Benoit 1971; Buckley et al. 1982; Marr et al. 1996) but contrasts with others that reported recovery of weight gain to control levels with continued exposure (Drummond et al. 1973; Lett et al. 1976; Collvin 1985) or no effect of elevated waterborne Cu on growth (e.g., Taylor et al. 2000; McGeer et al. 2000, 2002).

Whole-body and tissue Cu and Na^+ concentrations

Cu-exposed fish maintained on HS diet had lower whole-body and tissue Cu concentrations relative to the Cu-exposed fish maintained on LS diet, consistent with the view that

Fig. 5. Effect of elevated dietary NaCl and waterborne Cu exposure on total Na^+ concentrations in (*a*) whole body, (*b*) gill, (*c*) liver, (*d*) kidney, (*e*) gut, and (*f*) carcass. Elevated dietary NaCl increased the Na^+ levels, whereas elevated waterborne Cu reduced the Na^+ levels. Points in the same panel with different letters are significantly different ($P < 0.05$). ▲, LS diet + no Cu; ●, HS diet + no Cu; △, LS diet + 55 $\mu g \cdot L^{-1}$ waterborne Cu; ○, HS diet + 55 $\mu g \cdot L^{-1}$ waterborne Cu.



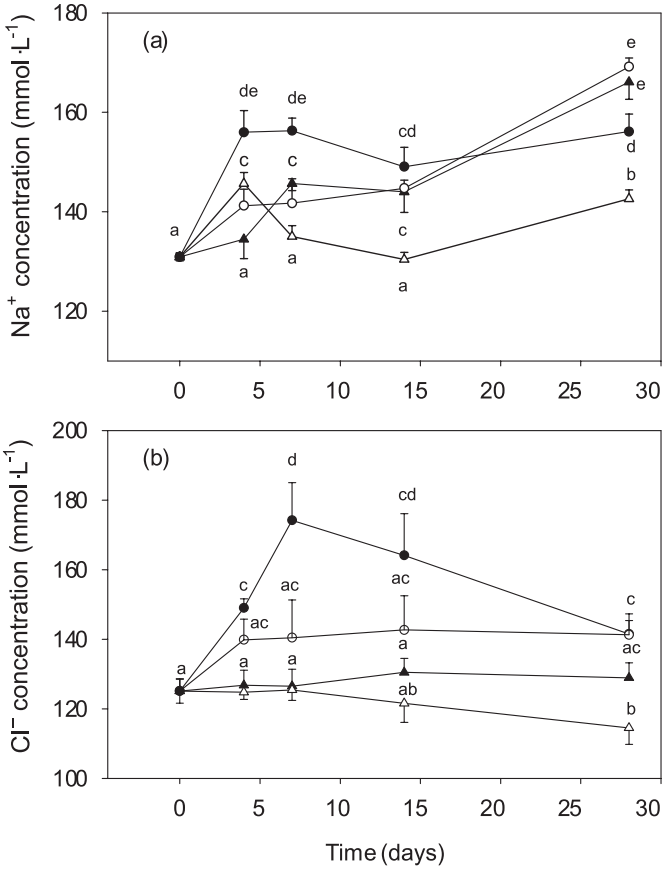
dietary NaCl impedes uptake and accumulation of Cu (Kamunde et al. 2003; Pyle et al. 2003). The efficacy of dietary NaCl in reducing Cu accumulation was extremely high in tissues such as the gill and gut in which Cu accumulation was reduced to concentrations equal to those of Cu-unexposed controls. The gill and gut tissues also showed interesting changes in proportional Cu distribution, which suggested that maintenance of fish on diets elevated in NaCl prevented imbalances in tissue Cu disposition. Whereas the contributions of the gill and gut tissues to whole-body Cu burden remained comparable to controls in Cu-exposed fish maintained on HS diet, both gill and gut tissues contributed significantly more to whole-body Cu burden in Cu-exposed fish maintained on LS diet. The significant accumulation of Cu in gut tissues of Cu-exposed fish maintained on LS diet was surprising because waterborne Cu rarely accumulates in gut tissues and gut Cu accumulation has been interpreted as diagnostic of dietary exposure (Handy 1992; Kamunde et al. 2001; Clearwater et al. 2002). It is possible that Cu accumulation in the gut was from stress-induced drinking, water ingested along with the food, and (or) from Cu-enriched bile via the hepatobiliary axis rather than directly from branchial uptake.

Unlike our recent study (Kamunde et al. 2003) in which minimal changes in tissue Na^+ concentrations occurred, the present study revealed highly significant elevations in whole-body and tissue Na^+ levels in response to elevated dietary NaCl exposure. This is probably because Na^+ analyses were performed on fish sacrificed 6 h after feeding, a period when fish tissues likely experienced an upsurge of tissue Na^+ concentration following Na^+ absorption from the gut. Moreover, this observation supports the notions that absorption of Na^+ from food occurs relatively rapidly (Smith et al. 1995) and that regulatory mechanisms promptly control the attendant upsurge in tissue Na^+ concentrations. Consequently, sampling after a substantial period of starvation (e.g., Kamunde et al. 2003) is unlikely to capture these apparently transient changes in tissue Na^+ levels.

Role of the kidney in Na^+ homeostasis during exposure to elevated dietary NaCl and waterborne Cu

The gill is the main organ for regulating Na^+ in freshwater fish, while the kidney contributes 10–30% to whole-body Na^+ balance depending on the prevailing conditions (McDonald and Wood 1981; Wood 1988, 1995). The role of the kidney is likely greater when branchial Na^+ regulating mechanisms are impaired or strained, for example, by exposure to waterborne ionoregulatory toxicants, or when freshwater fish are faced with higher Na^+ loads, for example, via dietary accretion. In the present study, exposure of rainbow trout to elevated waterborne Cu resulted in an initial decline in kidney Na^+ concentrations, which remained depressed in the fish maintained on LS diet. However, in fish maintained on HS diet, renal Na^+ concentrations recovered and surpassed values of the controls and the Cu-unexposed fish maintained on HS diet, suggesting that compensatory renal Na^+ retention occurs in animals faced with branchial Na^+ loss when Na^+ availability via the diet is maintained. These observations are in accord with Grosell et al. (1998) who reported that during acclimation to waterborne Cu, renal Na^+ efflux is reduced owing to increased tubular re-absorption, suggestive of renal

Fig. 6. Effect of elevated dietary NaCl and waterborne Cu exposure on levels of (*a*) plasma Na^+ and (*b*) Cl^-. Elevated dietary NaCl increased both the Na^+ and Cl^- levels, whereas elevated waterborne Cu reduced them. Points on the same panel with different letters are significantly different ($P < 0.05$). ▲, LS diet + no Cu; ●, HS diet + no Cu; △, LS diet + 55 $\mu g \cdot L^{-1}$ waterborne Cu; ○, HS diet + 55 $\mu g \cdot L^{-1}$ waterborne Cu.



Na^+ conservation as part of the mechanism of acclimation to waterborne Cu. The lack of Cu accumulation in kidneys of Cu-exposed fish maintained on HS diet likely indicates that the processes involved in renal Na^+ conservation concomitantly impede Cu accumulation. Moreover, although elevated dietary NaCl increases renal Na^+ excretion, in the absence of waterborne Cu exposure the gill remains the main site for Na^+ homeostasis in fish (Salman and Eddy 1987).

Role of the gut in Na^+ homeostasis during exposure to elevated NaCl and waterborne Cu

In addition to digestion and absorption of food, the fish gut has been shown to have an important role in ion regulation (Evans 1979; Smith et al. 1989). Specifically, exposure of freshwater fish to elevated dietary NaCl suppresses branchial Na^+ uptake (Smith et al. 1995; Kamunde et al. 2003; Pyle et al. 2003), increases diffusive Na^+ efflux via the gills (Smith et al. 1995; Pyle et al. 2003), and increases renal Na^+ excretion (Salman and Eddy 1987). Our data are consistent with these findings. Interestingly, little Na^+ is lost through fecal material even in fish fed high Na^+ diets (Salman and Eddy 1988), suggesting that reduction of gastrointestinal Na^+ uptake may not be important in Na^+ homeostasis in freshwater fish. However, during simultaneous exposure to elevated dietary NaCl and waterborne Cu, the gut appears to play a role in conserving Na^+. As with the kidney, gut tissue Na^+ concentration recovered from an initial depression and surpassed values of the controls and the Cu-unexposed fish maintained on HS diet, suggesting that compensatory gut Na^+ absorption occurs in the face of branchial loss when Na^+ availability via the diet is maintained. Because the amount of Na^+ absorbed from food is likely dependent on the animal's Na^+ balance at the time of measurement, future studies need to measure actual uptake rates and fecal losses of Na^+ during exposure to graded levels of both dietary NaCl and waterborne Cu.

Table 1. Effect of exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to elevated dietary NaCl and waterborne Cu on plasma Na:Cl ratios.

	Group			
Day	LS + no Cu	LS + Cu	HS + no Cu	HS + Cu
0	1.13±0.05	1.13±0.05	1.13±0.05	1.13±0.05
4	1.07±0.04	1.14±0.03	1.05±0.02	1.05±0.05
7	1.1 6±0.05	1.08±0.03	0.94±0.08*	1.04±0.07
14	1.11±0.05	1.08±0.05	0.94±0.07*	1.04±0.06
28	1.29±0.03	1.25±0.04	1.11±0.04*	1.22±0.05

Note: *, significantly different from LS + no Cu ($P < 0.05$); LS, low salt; HS, high salt.

Plasma Na^+ and Cl^- concentrations

Several studies have demonstrated that elevated waterborne Cu exposure decreases both plasma Na^+ and Cl^- levels in freshwater fish (McKim et al. 1970; Christensen et al. 1972; Wood 2001) and that the Na^+ and Cl^- losses occur in parallel (Stagg and Shuttleworth 1982; Wilson and Taylor 1993; Pelgrom et al. 1995). This indicates that the regulatory mechanisms of Na^+ and Cl^- are, at least in part, linked. In the present study, plasma Na:Cl ratios remain relatively constant in Cu-exposed fish compared with the unexposed fish, indicating that elevated waterborne Cu exposure affected Na^+ and Cl^- in a similar manner and that Na^+ and Cl^- homeostatic mechanisms are coupled. Indeed, in the current model of branchial ion transport in freshwater fish, Na^+ and Cl^- transport are indirectly coupled by carbonic anhydrase (Wood 2001; Grosell et al. 2002). Surprisingly, Na:Cl ratio analysis in the Cu-unexposed fish maintained on HS diet indicated that elevated dietary NaCl exposure increased plasma Cl^- to a greater extent than it did plasma Na^+ concentration, which is generally indicative of the condition of metabolic acidosis reflected in a decrease in plasma HCO_3^- concentration (Wood 2001). Interestingly, metabolic acidosis associated with a decrease in the strong ion difference (SID) between plasma Na^+ and Cl^- concentrations is a well-known effect of exposure to high waterborne NaCl levels (reviewed by Walker et al. 1989). The mechanism of this differential effect of elevated dietary NaCl exposure on the relative plasma concentrations of Na^+ and Cl^- is at present unknown but we speculate a greater inhibition of Na^+ influx and (or) greater stimulation of Na^+ efflux relative to Cl^-. Indeed, some mechanisms of red blood cell (rbc) volume regulation can cause differential transport of Na^+ and Cl^- between the plasma and rbc compartments. For example, exposure of whole blood to

elevated NaCl causes an initial rbc shrinkage followed by regulatory volume increase (RVI) with increased intracellular Na^+ (Brauner et al. 2002), which can cause preferential decrease in plasma Na^+ relative to Cl^-.

Cu homeostasis in freshwater fish: is plasma Cu concentration the homeostatic set point?

Based on constant plasma Cu concentrations during exposure of fish to elevated waterborne and (or) dietary Cu levels (Grosell et al. 1997, 1998; Kamunde et al. 2001), a Cu homeostatic system geared toward maintaining optimum plasma Cu concentration has been proposed (Kamunde and Wood 2004). Consistent with this proposal, overt toxicity is not observed when plasma concentrations are maintained in face of chronic sublethal Cu exposures (Grosell et al. 1997; Kamunde et al. 2002; Kamunde and Wood 2004). In the present study, we observed chronic toxicity (mortality and growth retardation) concomitant with elevated plasma Cu concentration, demonstrating that elevation of plasma Cu is associated with toxicity, a further indication that plasma Cu concentration is likely the set point in Cu homeostasis. Furthermore, because chronic elevation of plasma Cu was associated with chronic depression of Na^+ and Cl^- concentrations in plasma, whole body, and various tissues, loss of tissue Na^+ and Cl^- is likely a mechanism of chronic waterborne Cu toxicity, akin to acute toxicity, except that the magnitude of the depression is less in chronic toxicity.

In conclusion, elevated dietary NaCl protects juvenile rainbow trout from waterborne Cu-induced mortality, growth, and ionoregulatory impairment and impedes Cu accumulation in target tissues. We provide data suggesting that ionoregulatory disturbance (Na^+ and Cl^- loss) is the mechanism of chronic waterborne Cu toxicity, akin to acute Cu toxicity. Moreover, extrabranchial mechanisms of ion regulation in fish operating at the gut and the kidney appear to play greater roles in maintaining the Na^+ balance during Na^+ loading or Na^+ loss inducing challenges.

Acknowledgements

The Natural Sciences and Engineering Research Council of Canada (NSERC) MITE research network supported this research together with the Canada Research Chair Program (CMW) and EVS Environment Consultants (CNK). We thank Dr. Peter Chapman of EVS Environment Consultants for constructive comments on the manuscript.

References

Baldisserotto, B., Kamunde, C., Matsuo, A., and Wood, C.M. 2004. The effect of dietary calcium and acute waterborne cadmium exposure on cadmium, calcium and sodium uptake in rainbow trout. Aquat. Toxicol. **67**: 57–73.

Beaumont, M.W., Butler, P.J., and Taylor, E.W. 2003. Exposure of brown trout, *Salmo trutta*, to a sublethal concentration of copper in soft acidic water: effects upon gas exchange and ammonia accumulation. J. Exp. Biol. **206**: 153–162.

Brauner, C.J., Wang, T., and Jensen, F.B. 2002. Influence of hyperosmotic shrinkage and β-adrenergic stimulation on red blood cell volume regulation and oxygen binding properties in rainbow trout. J. Comp. Physiol. B, **172**: 251–262.

Buckley, J.T., Roch, M., McCarter, J.A., Rendell, C.W., and Matheson, A.T. 1982. Chronic exposure of coho salmon to sublethal concentrations of copper. 1. Effect on growth, on accumulation and distribution of copper, and on copper tolerance. Comp. Biochem. Physiol. C, **72**: 15–19.

Bury, N.R., Walker, P.A., and Glover, C.N. 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. **206**: 11–23.

Christensen, G.M., McKim, J.M., Brungs, W.A., and Hunt, E.P. 1972. Changes in the blood of the brown bullhead (*Ictalurus nebulosus* Lesueur) following short- and long-term exposure to copper (II). Toxicol. Appl. Pharmacol. **23**: 417–427.

Clearwater, S.J., Farag, A.M., and Meyer, J.S. 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp. Biochem. Physiol. C, **132**: 269–313.

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

Dang, Z.C., Flik, G., Lock, R.A.C., and Wendelaar Bonga, S.E. 1999. Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in freshwater. Am. J. Physiol. **277**: R320–R331.

Dang, Z.C., Flik, G., DuCouret B., Hogstrand, C., Wendelaar Bonga, S.E., and Lock, R.A.C. 2000. Effects of copper on cortisol receptor and metallothionein expression in gills of *Onchorhynchus mykiss.* Aquat. Toxicol. **51**: 45–54.

Drummond, R.A., Spoor, W.A., and Olson, G.F. 1973. Some short-term indicators of sublethal effects of copper on brook trout, *Salvelinus fontalis*. J. Fish Res. Board Can. **30**: 698–701.

Erickson, R.J., Benoit, D.A., Mattson, V.R., Nelson, H.P., Jr., and Leonard, E.N. 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. Environ. Toxicol. Chem. **15**: 181–193.

Evans, D.H. 1979. Fish. *In* Comparative physiology of osmoregulation in animals. Vol. l. *Edited by* G.M.O. Maloiy. Academic Press, Orlando, Florida. pp. 305–370.

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

Grosell, M., Nielsen, C., and Bianchini, A. 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp. Biochem. Physiol. C, **133**: 287–303.

Grosell, M.H., Hogstrand, C., and Wood, C.M. 1997. Copper uptake and turnover in both Cu acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. **38**: 257–276.

Grosell, M.H., Hogstrand, C., and Wood, C.M. 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.

Handy, R.D. 1992. The assessment of episodic metal pollution. II. The effects of cadmium and copper enriched diets on tissue contaminant analysis in rainbow trout. Arch. Environ. Contam. Toxicol. **22**: 82–87.

Handy, R.D., Eddy, F.B., and Baines, H. 2002. Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. Biochim. Biophys. Acta, **1566**: 104–115.

Kamunde, C.N., and Wood, C.M. 2004. Environmental chemistry, physiological homeostasis, toxicology, and environmental regulation of copper, an essential element in freshwater fish. Austral. J. Ecotoxicol. **10**: 1–20.

Kamunde, C.N., Pyle, G.G., McDonald, D.G., and Wood, C.M. 2003. Influence of dietary sodium on waterborne copper toxicity in rainbow trout, *Oncorhynchus mykiss*. Environ. Toxicol. Chem. **22**: 342–350.

Kamunde, C.N., Grosell, M., Lott, J.N.A., and Wood, C.M. 2001. Copper metabolism and gut morphology in rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal dietary copper exposure. Can. J. Fish. Aquat. Sci. **58**: 293–305.

Laurén, D.J., and McDonald, D.G. 1985. Effects of copper on branchial ionoregulation in rainbow trout, *Salmo gairdneri* Richardson: modulation by water hardness and pH. J. Comp. Physiol. B, **155**: 635–644.

Laurén, D.J., and McDonald, D.G. 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, D.J., and McDonald, D.G. 1987*a*. Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. Can. J. Fish. Aquat. Sci. **44**: 99–104.

Laurén, D.J., and McDonald, D.G. 1987*b*. Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. Can. J. Fish. Aquat. Sci. **44**: 105–111.

Lett, P.F., Farmer, G.J., and Beamish, F.W.H. 1976. Effect of copper on some aspects of bioenergetics of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board. Can. **33**: 1335–1342.

Li, J., Quabius, E.S., Wendelaar Bonga, S.E., Flik, G., and Lock, R.A.C. 1998. Effects of water-borne copper on branchial Na^+/K^+-ATPase activities in Mozambique tilapia *(Oreochromis mossambicus*). Aquat. Toxicol. **43**: 1–11.

Lin, H., and Randall, D.J. 1995. Proton pumps in fish gills. *In* Cellular and molecular approaches to fish ionic regulation. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, New York. pp. 229–225.

Marr, J.C.A., Lipton, J., Cacela, D., Hansen, J.A., Bergman, H.L., Meyer, J.S., and Hogstrand, C. 1996. Relationship between copper exposure duration, tissue copper concentration, and rainbow trout growth. Aquat. Toxicol. **36**: 17–30.

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

McDonald, D.G., and Wood, C.M. 1981. Branchial and renal net ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. J. Exp. Biol. **93**: 101–118.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., and Wood, C.M. 2000. Effects of sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Ionoregulatory disturbance and metabolic costs. Aquat. Toxicol. **50**: 231–243.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., and Wood, C.M. 2002. The role of dissolved organic carbon in moderating the bioavailability and toxicity of Cu to rainbow trout during chronic waterborne exposure. Comp. Biochem. Physiol. C, **133**: 147–160.

McKim, J.M., Christensen, G.M., and Hunt, E.P. 1970. Changes in the blood of brook trout (*Salvelinus fontinalis*) after short-term and long-term exposure to copper. J. Fish. Res. Board Can. **27**: 1883–1889.

McKim, J.M., and Benoit, D.A. 1971. Effects of long-term exposures to copper on survival, growth and reproduction of brook trout (*Salvelinus fontalis*). J. Fish. Res. Board Can. **28**: 655–662.

Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M., and Wendelaar Bonga, S.E. 1995. Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. Aquat. Toxicol. **32**: 303–320.

Pyle, G.G., Kamunde, C.N., McDonald, D.G., and Wood, C.M. 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout, *Oncorhynchus mykiss*. J. Exp. Biol. **206**: 609–618.

Salman, N.A., and Eddy, F.B. 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.

Salman, N.A., and Eddy, F.B. 1988. Kidney function in response to salt feeding in rainbow trout (*Salmo gaidneri* Richardson). Comp. Physiol. A, **89**: 535–539.

Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E., and Meyer, J.S. 2001. Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and daphnia. Environ. Toxicol. Chem. **20**: 2397–2402.

Schreck, C.B., and Lortz, H.W. 1978. Stress resistance of coho salmon (*Oncorhynchus kisutch*) elicited by cadmium and copper, and potential use of cortisol as an indicator of stress. J. Fish. Res. Board. Can. **35**: 1124–1129.

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

Smith, N.F., Eddy, F.B., and Talbot, C. 1995. Effect of dietary salt load on transepithelial Na^+ exchange in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. **198**: 2359–2364

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

Stagg, R.M., and Shuttleworth, T.J. 1982. The effects of copper on ionic regulation by the gills of the freshwater adapted flounder (*Platichthys flesus* L.). J. Comp. Physiol. B, **149**: 83–90.

Sullivan, G.V., Fryer, J.N., and Perry, S.F. 1995. Immunolocalization of proton pump (H^+-ATPase) in pavement cells of rainbow trout gill. J. Exp. Biol. **198**: 2619–2629.

Taylor, E.W., Beaumont, M.W., Butler, P.J., Mair, J., and Mujallid, M.S.I. 1996. Lethal and sub-lethal effects of copper upon fish: role for ammonia toxicity? *In* Toxicology of aquatic pollution physiological, cellular and molecular approaches. *Edited by* E.W. Taylor. Society for Experimental Biology Seminar Series No. 57. pp. 85–113.

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

Walker, R.L., Wilkes, P.R.H., and Wood, C.M. 1989. The effect of hypersaline exposure on the oxygen affinity of the blood of the freshwater teleost, *Catostamus commersoni*. J. Exp. Biol. **142**: 125–142.

Wilson, R.W., and Taylor, E.W. 1993. The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. J. Comp. Physiol. B, **163**: 38–47.

Wood, C.M. 1988. Acid–base and ionic exchanges at the gills and kidney after exhaustive exercise in rainbow trout. J. Exp. Biol. **136**: 461–481

Wood, C.M. 1995. Excretion. *In* Physiological ecology of Pacific salmon. *Edited by* C. Groot, C. Margolis, and W.C. Clarke. University of British Columbia Press, Vancouver. pp. 381–438.

Wood, C.M. 2001. Toxic responses of the gill. *In* Target organ toxicity in marine and freshwater teleosts. Vol. 1. Organs. *Edited by* D. Schlenk and W.H. Benson. Taylor and Francis, London, New York. pp. 1–89.

Zall, D.M., Fisher, M.D., and Garner, Q.M. 1956. Photometric determination of chlorides in water. Anal. Chem. **28**: 1665–1678.