The Influence of Dietary Na on Cu Accumulation in Juvenile Rainbow Trout Exposed to Combined Dietary and Waterborne Cu in Soft Water

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Abstract. Fish inhabiting metal-contaminated environments can take up metals such as Cu via the gills as well as via the gut. Previous research on rainbow trout (*Oncorhynchus mykiss*) has indicated that dietary Na can reduce the accumulation of waterborne Cu; however, in hard water, dietary Na does not reduce the accumulation of dietary Cu. In this study, we exposed juvenile rainbow trout in soft water with slightly elevated [Cu] for 28 days to control or high levels of dietary Cu (6 and 580 µg Cu/g food, respectively) at low (1.5%), intermediate (3%), or high (4.5%) levels of dietary Na, for a total of six experimental groups. A separate gastrointestinal sampling experiment demonstrated that these levels resulted in moderately elevated Na concentrations in the gastrointestinal fluid, which declined between 6 h and 12 h post-feeding. Growth and condition indices were not affected by the dietary Cu and Na exposure. Among the control dietary Cu groups, those that received the highest amount of dietary Na had significantly higher whole-body [Cu] on days 18 and 28. In contrast, among the high-Cu groups, fish that were fed the highest amount of Na tended to have significantly lower whole-body [Cu] on days 9 and 18. Tissue Na concentrations did not differ among any of the groups, and unidirectional Na flux measurements demonstrated that Na homeostasis was not impaired by dietary or waterborne Cu. Our results suggest that elevated dietary Na stimulates Cu uptake via the gut under low-Cu conditions, thereby increasing whole-body [Cu], whereas under Cu-loaded conditions, downregulation of Cu uptake at the gills, and/or competitive inhibition of gut Cu uptake as a result of increased dietary Na, leads to decreased whole-body [Cu].

While a number of metals, such as Cu, are essential at low concentrations to maintain biological processes, at high concentrations they can interfere with organism function, potentially leading to death. Contamination by Cd, Zn, Ni, and Cu have been linked to declining fish populations (e.g., in soft-water Canadian Shield lakes, see Campbell and Stokes 1984; Yan *et al.* 1996; Eastwood and Couture 2002; Couture and Rajotte 2003). It is, therefore, of interest to determine ways in which the effects of contaminants may be mitigated, either by natural factors or anthropogenic manipulations.

Some naturally occurring cations, including Na, compete with metals such as Cu for binding sites in the organism; this competition may lead to reduced metal uptake (Niyogi and Wood 2003). For example, in rainbow trout (*Oncorhynchus mykiss*), waterborne Na and Cu appear to enter gill cells at least in part via the same channel, and increased water [Na] can inhibit the uptake of Cu via the gills (Grosell and Wood 2002). Conversely, high [Cu] in the water can severely reduce the amount of Na transferred across the gills (Laurén and McDonald 1985, 1987). In addition, elevated dietary Na, which tends to downregulate active Na uptake at the gills, can also reduce the amount of Cu taken up via the gills (Pyle *et al.* 2003; Kamunde *et al.* 2003). However, despite these recent advances in understanding the interactions between Cu and Na at the gills, much less is currently known about how Na and Cu interact in the gut following dietary exposures or simultaneous dietary and waterborne exposures, and the available evidence seems to be contradictory. Handy *et al.* (2000) reported that low [Na] in the intestinal contents tended to reduce Cu-uptake in an intestinal preparation of the walking catfish, but this was not confirmed by a recent study on isolated trout enterocytes (Burke and Handy 2005), where Na-removal had either no effect on Cu-uptake (at low intestinal [Cu]) or accelerated it (at high intestinal [Cu]).

In an earlier study, we examined these interactions *in vivo* by exposing groups of fish to either control or high levels of dietary Cu and simultaneously feeding low, medium, or high [Na] food (Kjoss *et al.* 2005). That study was conducted in relatively hard, Na-rich water with very low levels of background Cu (<1 µg L^{-1}), and we found that dietary Na did not reduce the Cu burden in fish exposed to control or relatively high dietary [Cu]. However, the situation may well be different in the relatively soft, acidic, Na-poor, Cu-rich waters that characterize some impacted lakes, e.g., in the Canadian Shield. A recent workshop (Meyer *et al.* 2005) has highlighted the need to study how environmental factors affect metal uptake and toxicity under more environmentally realistic conditions,

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i.e., where metal levels are elevated in both the diet and the water, and the water chemistry is typical of natural metal-impacted waters.

Therefore, we performed an experiment in ion-poor soft water with lower pH and slightly elevated background water-borne Cu concentration, conditions that were similar to those in mildly contaminated Canadian Shield lakes. Specifically, we investigated whether elevated [Na] in the diet of juvenile rainbow trout would lead to reduced whole-body and/or organ-specific dietary Cu accumulation in slightly Cu-contaminated soft water.

Methods

Acclimation

Approximately 450 trout fry (weight <1.0 g, total length <50 mm) were obtained from Humber Springs Trout Farm (Orangeville, ON, Canada) and initially held in one 200-L tank supplied with aerated flow-through, dechlorinated Hamilton tap water from Lake Ontario (moderate hardness: Na^+ = 500-600 µmol L^{-1}, Cl^- = 800 µmol L^{-1}, Ca^{2+} = 1000 µmol L^{-1}, hardness ~140 ppm as $CaCO_3$, background Cu <1.0 µg L^{-1}, pH ~7.9, temperature = 10–12°C). During the ensuing four-week acclimation period, we altered the water composition of the tank by gradually introducing reverse-osmosis (RO) water (Anderson Water Systems, Dundas, ON, Canada) and adjusting the ratio of tap water to RO water by 10% every day until the final proportion of nominally 80:20 (RO water:tap water) was attained. This was the final water composition used in this experiment, and will hereafter be referred to as "soft water" (Na^+ = ~150-200 µmol L^{-1}, Cl^- = 50–100 µmol L^{-1}, Ca^{2+} = 115–145 µmol L^{-1}, Cu = 3–6 µg L^{-1}, pH ~5.9–6.1). The chemical composition of this experimental water differed slightly from the target nominal 20% tap water, as some ions were 30% of hardwater values while others were only 15%. The moderately elevated Cu level was the result of a copper pipe placed in the outflow line of the reverse osmosis unit. Fish were allowed to acclimate to this composition for two weeks prior to the start of the experiment. During the acclimation period, fish were fed commercial fish feed (Silver Cup for salmon fry, Nelson & Sons, Inc., Murray, UT, USA; 15 mg Na/g, 6 µg Cu/g; see Kjoss et al. [2005] for other components of this feed) twice a day at a ration of 2% wet body mass per feeding, and a subset of fish (n = 20–30) was removed weekly and bulk weighed to adjust the food ration; ration was also adjusted to account for mortalities. By the time the experiment commenced, mean body mass was 1.2 ± 0.14 g.

Experimental Design and Diet Preparation

Fish were divided into six 11.3-L tanks (37–38 fish/tank), and flow rate and temperature were held constant at ~200 mL/min and 10 ± 2°C. All tanks were flow-through and were individually aerated.

We prepared six nominal diet formulations by adding $CuSO_4 \cdot 5H_2O$ (Fisher Scientific, Toronto, ON, Canada) and/or analytical grade NaCl (BioShop Canada, Burlington, ON, Canada) to commercial fish feed (Silver Cup for salmon fry, Nelson & Sons, Inc., Murray, UT, USA; 15 mg Na/g [i.e., 1.5% Na], 6 µg Cu/g). The food was ground, mixed with the dissolved salts, repelleted with a commercial pasta maker, and air-dried as described in detail by Kjoss et al. (2005). Diet treatments were as follows: Cu control groups (6 µg Cu/g; with no additional Na, with 3% Na by weight, or with 4.5% Na by weight), and high Cu groups (nominally 500 µg Cu/g; with no additional Na, with 3% Na, or with 4.5% Na). All diets were kept frozen at −20°C

until use. Actual mean measured [Na] (± S.E., n = 5 samples of each food group), determined by flame atomic absorption spectroscopy (FAAS; Varian SpectrAA−220FS, Mulgrave, Australia), was 15.2 ± 0.3 mg Na/g food (1.52%) for control food, 27.9 ± 0.4 mg Na/g food (2.8%), and 47.2 ± 0.6 mg Na/g food (4.7%). Actual mean [Cu] (± S.E.), determined by graphite furnace atomic absorption spectroscopy (GFAAS; Varian SpectrAA−220 with graphite tube atomizer, Mulgrave, Australia) was 579.1 ± 9.0 (high Cu groups) and 6.1 ± 0.1 µg Cu/g food (control Cu groups). Mean moisture content of the prepared diet was 8.5 ± 0.4%. Fish were fed twice daily at a total daily ration of 4% body weight, and approximate mean daily doses of Cu (sensu Clearwater et al. 2002) were 23.2 µg Cu/g fish/day in Cu-exposed groups and 0.24 µg Cu/g fish/day in control Cu groups. Fish were weighed in bulk on sampling days to adjust food rations. Faecal matter and any leftover food were siphoned two to three times daily to reduce waste build-up at the bottom of each tank.

Fish were sampled on days 0, 9, 18, and 28 of the experiment. Prior to a sampling day, fish were not fed for 36 h to allow the gut to clear; tanks were regularly siphoned during this period. On day 0 of the experiment, before the fish were separated into the experimental tanks, ten individuals were sacrificed with an overdose of neutralized tricaine methanesulfonate (MS-222, 1 g/L; Fisher Scientific). Fish were blotted dry and individually weighed, and body lengths were recorded. The whole gill basket, liver, and gut (esophagus, stomach, caecae, anterior, mid-, and posterior intestine, and rectum) were removed. Faeces and undigested food were carefully removed from the gut and discarded, and fat was removed and placed with the carcass. Guts were then rinsed in deionized NANOpure II (Sybron/Barnstead, Boston, MA, USA) water and blotted dry. On days 9 and 18 of the experiment, nine fish were removed from each tank and sampled in the same manner. On day 28, eight fish were sampled from each tank but were first subjected to a measurement of unidirectional Na fluxes (see below).

Tissues and carcasses were placed individually into pre-weighed vials and frozen at −20°C until use. They were then weighed and subsequently digested in appropriate volumes of 1N HNO_3 at 70°C for at least 24 h. Once digested, samples were centrifuged, and a sub-sample of the supernatant was diluted in an appropriate volume of 1% HNO_3. [Cu] and [Na] of individual tissues and carcasses, as well as of tank water samples collected approximately every second day, were determined by GFAAS and FAAS, respectively.

Unidirectional Na Flux Rates

On day 28 of the experiment, eight fish from each tank were moved into plastic flux chambers (two fish per 60-mL chamber), which were kept at the experimental temperature and were individually aerated. ^{22}Na (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) was then added to each chamber (~0.1 uCi [3.7 kBq]/chamber), and a 2-mL water sample was collected after 15 min. At the end of the flux period (3–5 h, depending on group), another 2-mL water sample was taken from each chamber. Fish were then sampled as described above, and the ^{22}Na activity of tissues, carcasses, and water samples was measured on a gamma counter (Canberra-Packard Minaxi Auto Gamma 5000 series, Meriden, CT, USA). Following the gamma-counting, tissues, carcasses, and water samples were analyzed for [Cu] and [Na] as described above.

Calculations

Whole-body [Cu] and [Na] were calculated for each fish as the sum of the Cu (or Na) content of all tissues (including carcass) divided by the sum of all tissue weights. Indices of body condition (hepatosomatic index, HSI; branchiosomatic index, BSI; gastrointestinosomatic index, GSI) were calculated as (tissue mass)/(wet mass of whole fish). An

Table 1. Mean tissue condition indices (HSI = hepatosomatic index, BSI = branchiosomatic index, GSI = gastrointestinosomatic index, CF = condition factor; see text for calculations of these ratios) of eight juvenile rainbow trout from each treatment group sampled on Day 28, as well as Cu retention (ret; %) over 28 days

		Group					
		Control Cu			High Cu		
Index	Day 0	1.5% Na	3% Na	4.5% Na	1.5% Na	3% Na	4.5% Na
HIS	0.020 ± 0.001^a	0.013 ± 0.001^b	0.012 ± 0.001^b	0.014 ± 0.001^b	0.013 ± 0.001^b	0.013 ± 0.001^b	0.012 ± 0.001^b
BSI	0.041 ± 0.003^a	0.028 ± 0.001^b	0.029 ± 0.002^b	0.029 ± 0.001^b	0.029 ± 0.001^b	0.029 ± 0.002^b	0.031 ± 0.001^b
GSI	0.067 ± 0.006^a	0.047 ± 0.002^b	0.056 ± 0.002^{ab}	0.053 ± 0.002^{ab}	0.048 ± 0.007^b	0.049 ± 0.005^{ab}	0.050 ± 0.004^{ab}
CF	0.952 ± 0.020^{ab}	1.009 ± 0.039^{ab}	0.937 ± 0.015^b	0.902 ± 0.030^b	1.056 ± 0.062^a	0.880 ± 0.034^b	0.961 ± 0.040^{ab}
Ret (%)		26.9	20.8	32.1	0.59	0.66	0.94

Day 0 data calculated from ten juvenile trout are provided for comparison. Superscripts indicate groups that differed from one another by ANOVA with Tukey's HSD test ($p < 0.05$). Note that retention was calculated based on mean data and were therefore not compared statistically.

overall condition factor (CF) was calculated as (wet mass of whole fish/[body length]^3) × 100. Mean dietary Cu retention (%) on a per-fish basis was calculated as ([C_f – C_i]/total Cu fed) × 100, where C_i and C_f are initial and final whole-body Cu content, respectively. Specific growth rate (SGR; %/day) and food conversion efficiency (FCE; %) were calculated as described by Kamunde *et al.* (2002a). Because of the mortalities that occurred among fish during the first half of the experiment (see Results), we only calculated SGR and FCE for the last experimental increment, i.e., from day 18 to day 28. Sodium influx (appearance of Na in the fish; J_{in}^{Na}), efflux (J_{out}^{Na}), and net flux (J_{net}^{Na}) were calculated as described by Kjoss *et al.* (2005), using the summated ^{22}Na cpm of all sampled tissues to represent the whole-body accumulation of ^{22}Na.

Intestinal Na and Cu Levels

To help interpret the results of this experiment (see below) as well as concurrent *in vitro* work conducted in our laboratory (see Discussion), we conducted an additional experimental series to determine what levels of Na are actually achieved in the intestinal fluid and tissues as a result of the dietary Na levels used in this study. In the first of these experiments, 52 juvenile rainbow trout (approximately 1.5 g) were obtained from the same source, and upon arrival in the laboratory were divided among three tanks. At the end of a ten-day acclimation period, fish were starved for three days, and then fed a one-time 4% ration of either high Cu, 1.5% Na food; high Cu, 3% Na food; or high Cu, 4.5% Na food. Twelve hours after feeding, ten fish from each group were sacrificed as described, and the gut was dissected out. In the second experiment, the protocol was repeated with juvenile rainbow trout ($n = 43$, approximately 3 g) from the same supplier, but with gastro-intestinal sampling at 6 h rather than 12 h.

At sampling, the gut was divided into the stomach, anterior intestine (defined as the portion of the intestine to which the caecae attached), and posterior intestine (remainder of intestine). Gut contents were carefully removed from each compartment and scraped into bullet tubes. Gut tissues were then rinsed and placed into separate bullet tubes, and the remaining carcass was also collected. Tissues were processed and analyzed for [Cu] and [Na] as described above. Gut contents were centrifuged for 10 min at 14,000 rpm, and 5 μl of the intestinal fluid phase of each compartment were diluted 1000× in 1% HNO$_3$ for analysis of [Na] and [Cu]. Gut fluid Cu data were unfortunately lost in the 12-h post-feeding set of experiments.

Statistics

For each sampling day, comparisons were made within treatments, i.e., control Cu and high Cu groups. One-way analysis of variance

(ANOVA) followed by Tukey's HSD test were used to make the comparisons, except when the data did not meet the statistical requirements of equal variance; in these cases, comparisons were made with a Kruskal-Wallis test followed by a non-parametric comparison of mean ranks. Percentage data were arcsine-transformed prior to analysis. Where noted, we made pairwise comparisons (two-sample *t*-tests) between control and high Cu groups that received comparable amounts of Na in the diet, and between gastrointestinal samples from fish receiving the same food but sampled at 6 h versus 12 h. Data are reported as means ± S.E.M., and differences were considered significant at $p \leq 0.05$.

Results

Mean tank water [Na] over the course of the experiment was 181.6 ± 3.12 μmol/L and did not differ among the six tanks. Mean water [Cu] of all six tanks was 4.30 ± 0.22 μg/L and only differed significantly between the control Cu, high Na group (3.60 ± 0.16 μg/L) and the high Cu, low Na group (5.19 ± 0.46 μg/L). This suggested that Na and Cu leaching out of supplemented diets was of minor concern.

Mortality over 28 days was <10% (20/225) and occurred predominantly during the first two weeks of the experiment. All groups experienced some mortality, ranging from two dead fish (high Cu, 3% Na and high Cu, 4.5% Na groups) to seven dead fish (low Cu, 4.5% Na group). Fish grew steadily over time from 1.20 ± 0.14 g on Day 0 to 2.10 ± 0.12 g on Day 28, and we found no significant differences in lengths, weights, SGR (2.47 ± 0.48 %/day), or FCE (60.1 ± 12.8%) among groups. In addition, tissue weights did not differ on any given day (data not shown), and indices of body condition (HSI, BSI, GSI, CF) showed few differences, although there were marked changes within groups from Day 0 values (Table 1). Cu retention was much higher in control groups than in high Cu groups, and within each of these two treatments, retention was highest in the groups that received the highest percent dietary Na (Table 1).

The results of the gastrointestinal sampling experiments are summarized in Table 2. We found substantial differences in gastrointestinal fluid Na concentrations among the different compartments, with absolute levels being generally lowest in the stomach fluid and highest in the posterior intestinal fluid. All were less than standard plasma Na$^+$ levels (130–160 mmol/L) in trout, although the posterior intestinal fluid Na approached these values. In general, there were also substantial reductions in absolute Na concentrations between 6 h and 12 h post-feeding,

Table 2. Gastro-intestinal fluid [Na] (µM), gut tissue [Na] (µM), gut tissue [Cu] (µg/g wet weight), and gut fluid [Cu] (mg/L; 6 h only) of ten juvenile rainbow trout per group sampled 6 h or 12 h after a one-time exposure (at 4% body weight ration) to the respective diet

	1.5% Na + highCu	3% Na + high Cu	4.5% Na + high Cu
Gastro-intestinal Fluid [Na]			
Stomach (6 h)	$62.12 \pm 4.43^{ab,X}*$	$53.24 \pm 11.12^{a,X}*$	$85.63 \pm 9.68^{b,X}*$
Anterior intestine (6 h)	$80.80 \pm 2.99^{a,Y}*$	$110.63 \pm 6.22^{b,Y}*$	$122.80 \pm 6.73^{b,Y}*$
Posterior intestine (6 h)	$108.88 \pm 7.11^{ab,z}*$	$93.60 \pm 5.41^{a,Y}$	$118.47 \pm 6.00^{b,Y}*$
Stomach (12 h)	$23.87 \pm 1.87^{a,A}*$	$24.59 \pm 1.24^{a,A}*$	$46.55 \pm 4.66^{b,A}*$
Anterior intestine (12 h)	$63.49 \pm 5.88^{a,B}*$	$58.81 \pm 7.26^{a,B}*$	$89.89 \pm 4.85^{b,B}*$
Posterior intestine (12 h)	$79.82 \pm 2.91^{C}*$	89.16 ± 5.30^{C}	$95.73 \pm 6.32^{B}*$
Gut tissue [Na]			
Stomach (6 h)	$38.62 \pm 1.07^{XY}*$	$36.29 \pm 1.88^{X}*$	$38.40 \pm 1.63^{X}*$
Anterior intestine (6 h)	$36.61 \pm 1.37^{X}*$	$37.56 \pm 2.13^{X}*$	$35.44 \pm 1.06^{X}*$
Posterior intestine (6 h)	$43.83 \pm 2.38^{Y}*$	$47.27 \pm 3.63^{Y}*$	$49.57 \pm 5.01^{Y}*$
Stomach (12 h)	$22.04 \pm 1.79*$	$21.09 \pm 1.71*$	$19.35 \pm 1.68^{A}*$
Anterior intestine (12 h)	$26.84 \pm 2.52*$	$24.02 \pm 2.06*$	$24.48 \pm 2.73^{AB}*$
Posterior intestine (12 h)	$24.55 \pm 2.91*$	$21.99 \pm 2.46*$	$29.96 \pm 1.83^{B}*$
Gut tissue [Cu]			
Stomach (6 h)	6.67 ± 0.92^{a}	$4.01 \pm 0.50^{b,X}*$	$4.39 \pm 0.44^{ab,X}*$
Anterior intestine (6 h)	7.27 ± 0.92	8.33 ± 1.18^{Y}	8.10 ± 0.72^{Y}
Posterior intestine (6 h)	5.22 ± 0.61	5.23 ± 0.75^{X}	4.88 ± 0.69^{X}
Stomach (12 h)	8.01 ± 1.89^{AB}	$5.91 \pm 0.56*$	$7.06 \pm 0.75^{AB}*$
Anterior intestine (12 h)	9.99 ± 1.10^{A}	7.63 ± 1.42	8.25 ± 1.25^{A}
Posterior intestine (12 h)	4.55 ± 0.81^{B}	4.78 ± 1.02	4.20 ± 0.63^{B}
Gut fluid [Cu]			
Stomach (6 h)	$26.16 \pm 1.06^{a,X}$	$22.65 \pm 2.47^{ab,X}$	$16.73 \pm 1.53^{b,X}$
Anterior intestine (6 h)	65.85 ± 5.47^{Y}	75.72 ± 9.19^{Y}	89.14 ± 10.72^{Y}
Posterior intestine (6 h)	54.63 ± 5.45^{Y}	56.73 ± 6.22^{Y}	70.52 ± 6.60^{Y}

Within rows, different lowercase letters indicate significant differences among groups within tissue types. Within columns, different capital letters indicate significant differences among tissue types within groups at either 6 h (XYZ) or 12 h (ABC) post-feeding. Asterisks indicate significant differences between comparable tissues at 6 h versus 12 h. Gut fluid [Cu] data at 12 h were not available.

most of which were statistically significant. On a relative basis, the biggest decreases were seen in the stomach fluid (approximately 50%). The same general patterns, with respect to both differences among compartments and differences between 6 h and 12 h samples, were seen in gut tissue Na concentrations, although the absolute concentrations and differences both tended to be much smaller than in the actual gastrointestinal fluids.

Gut fluid Na concentrations rose only modestly in response to large differences in Na concentrations in the diet, with the largest increases (up to 100%, but generally less) seen in the stomach fluid, especially at the 12-h sampling point. Surprisingly, there was no influence of the Na concentration of the diet on the Na concentrations within the various gut tissues.

Gut fluid Cu concentrations, measured only at 6 h post-feeding, were two- to three-fold higher in the anterior intestinal fluid than in the stomach fluid, and were only slightly (non-significantly) lower in the posterior intestinal fluids. The only significant difference attributable to dietary Na levels was a lower Cu concentration in the stomach fluid in the high Na-diet treatment. Cu concentrations in the gut tissue were generally higher in the anterior intestine than in the stomach at 6 h post-feeding, but tended to rise slightly over time in the wall of the stomach.

In the 28-day experiment, liver, gut, and carcass showed very similar trends of individual tissue [Cu], although at markedly different scales (Fig. 1a, b). In all three compartments, Cu-exposed groups had significantly higher [Cu] on days 9, 18, and 28. A notable exception to these general patterns was observed in the gills. Here, unexposed groups

actually had higher [Cu] than Cu-exposed groups on day 9 (Fig. 1c), whereas gill Cu levels were similar in exposed and non-exposed treatments on subsequent days. Overall, gill [Cu] was around 100-fold lower than liver [Cu]. Across all groups, when relative tissue masses were taken into account, the liver consistently accounted for the largest percentage of the whole-body Cu burden (~40–60%), followed by the carcass (~20–40%), gut (~8–12%), and gills (~2–7%).

Whole-body [Cu] increased over time in all groups, although significantly more so in the high Cu treatments. On days 9 and 18, the high Cu, 4.5% Na group had a lower whole-body [Cu] than the other two groups in the high-Cu treatment, although this difference was no longer apparent by Day 28 (Fig. 1d). The opposite was true in the control Cu groups, with the 4.5% Na treatment exhibiting higher whole-body [Cu] on days 18 and 28. Within each treatment (i.e., high Cu and control Cu groups), we conducted a two-way ANOVA with whole-body [Cu] as the dependent variable and time and dietary [Na] as the independent variables. In each case, we found a significant interaction between time and dietary [Na] (low Cu, $p = 0.0011$; high Cu, $p = 0.0201$).

In contrast to Cu, we found no significant differences in tissue [Na] among groups on any sampling day (data not shown). Liver and gills showed the highest [Na], followed by carcass and gut. Whole-body [Na] remained fairly stable, and the low-Na group tended to have lower concentrations in the control treatments (data not shown). Mean tissue Na as a percent of whole-body Na was very similar between treatments over time. Across all groups, when relative tissue masses were
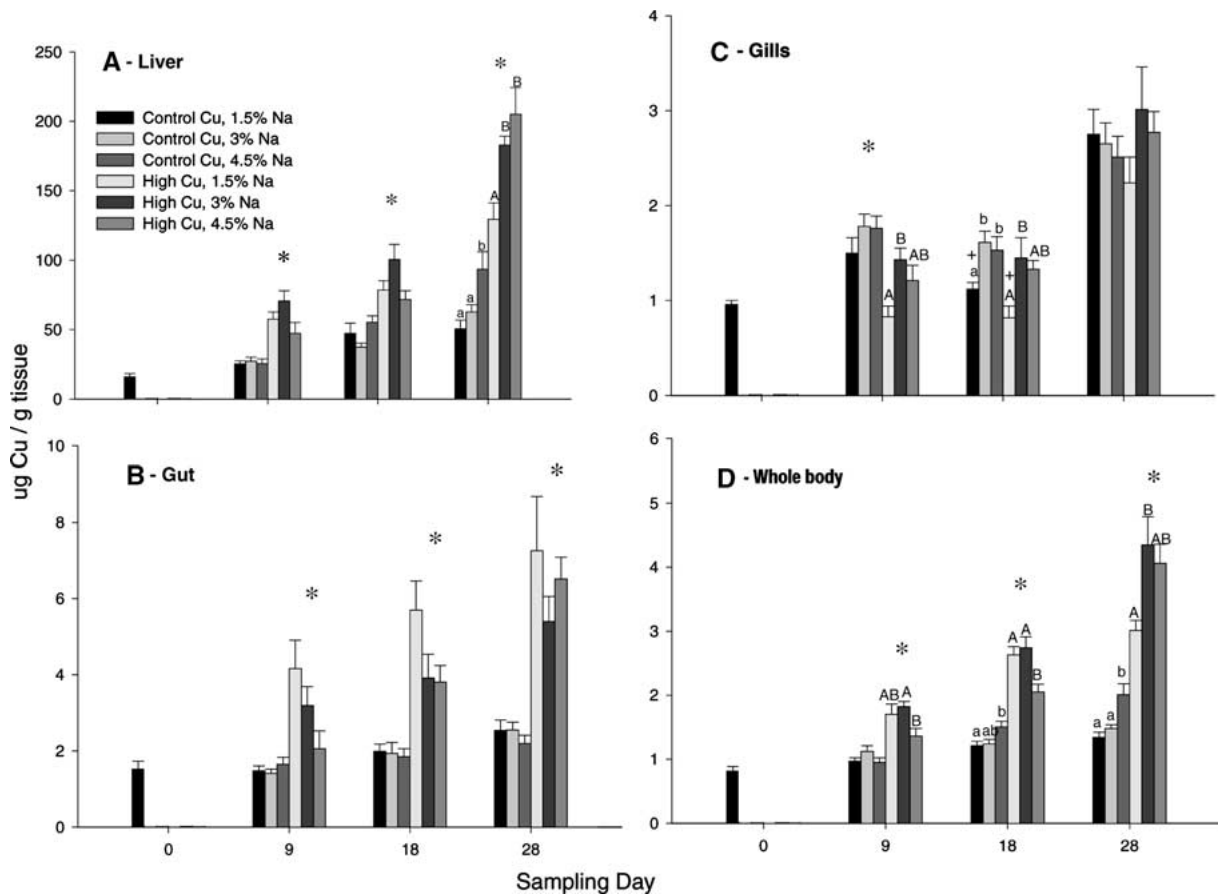
Fig. 1. Mean (± 1 S.E.M.) liver (A), gut (B), gill (C), and whole-body (D) [Cu] (μg Cu/g tissue wet weight) of eight or nine juvenile rainbow trout (ten on day 0) per treatment group on four sampling days. Asterisks denote significant differences between Cu-exposed and unexposed fish, and different letters above bars (lowercase for unexposed groups, uppercase for exposed) indicate groups that differed significantly within treatments (both comparisons: ANOVA or Kruskal-Wallis, $p < 0.05$). Crosses mark groups that received the same amount of Na but were fed either control or high amounts of Cu and differed from each other (two-sample *t*-test, $p < 0.05$).

taken into account, the liver consistently accounted for approximately 2–4% of the whole-body Na burden, the gill accounted for approximately 3–6%, and the gut accounted for approximately 3–5%. By far, the carcass accounted for the highest percentage of Na (~87–94%); notably in the carcass, the high Cu, high Na group had a significantly higher percentage than the other five groups on days 9, 18, and 28 (Fig. 2).

Unidirectional Na fluxes were very similar with no significant differences among groups, and we observed the same trends between treatments (Cu-exposed vs. control Cu). However, we found no differences between treatments in J_{in}, J_{net}, or J_{out}. In all groups, influx approximately equaled efflux (Fig. 3).

Discussion

In this study, we attempted to mimic ambient water and Cu-exposure conditions resembling those found in Canadian Shield lakes. Our experimental water [Cu] of 3.6–5.2 μg/L fell well within the ranges reported for natural reference lakes (*e.g.*, Campbell and Stokes 1984, Couture and Rajotte 2003).

Our measured experimental high Cu content in the diet was 579 μg/g wet weight, or ~633 μg/g dry weight, and was thus at the high end of the naturally occurring spectrum. For example, Miller *et al.* (1992) sampled eight Cu-contaminated lakes in the Manitouwage chain of northern Ontario, and reported invertebrate Cu concentrations of 57–189 μg/g wet weight, or about 285–945 μg/g dry weight. In contrast, Audet and Couture (2003) sampled the gut contents of benthivorous yellow perch (*Perca flavescens*) in lakes near Sudbury, Ontario, and found a [Cu] of ~40 μg/g (reference lake) and ~125 μg/g dry weight (contaminated lake).

Gastrointestinal Na and Cu Levels

The gastrointestinal sampling experiment was conducted to ensure that the elevated dietary Na treatments actually resulted in elevated levels of Na in the gastrointestinal fluids. The results show that indeed they did, but the differences were surprisingly modest and did not influence tissue Na concentrations in the wall of the tract (Table 2). It is noteworthy that Na levels in the fluids of all parts of the tract (even in the stomach fluid) appeared to fall quickly between 6 h and 12 h
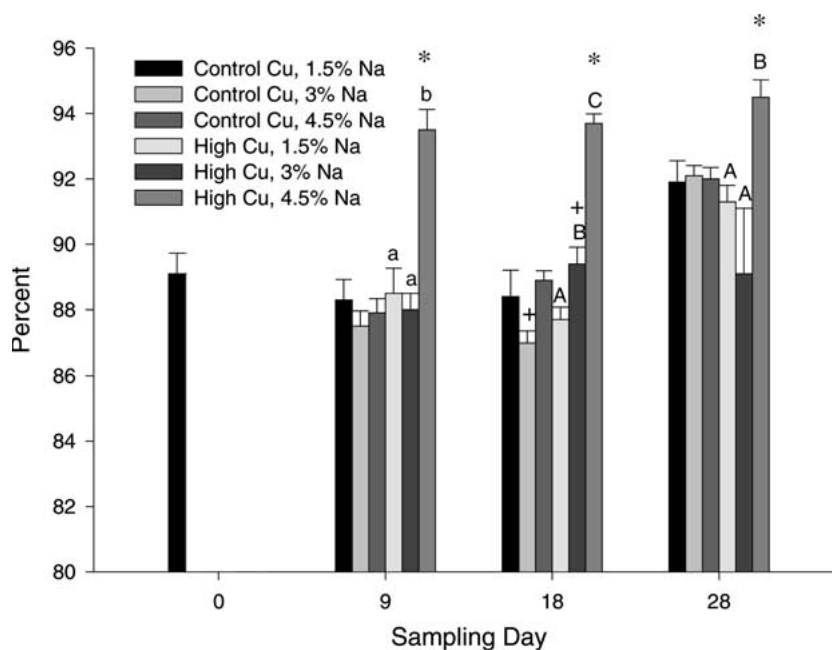
Fig. 2. Mean (± 1 S.E.M.) percent of whole-body Na burden contained in the carcasses of eight to ten juvenile rainbow trout per group on four sampling days. Asterisks indicate significant differences (ANOVA, $p < 0.001$) between the high Cu, 4.5% Na group and all other groups, and different letters above bars (lowercase for unexposed groups, uppercase for exposed) indicate groups that differed significantly within treatments (ANOVA or Kruskal-Wallis, $p < 0.05$). Crosses mark groups that received the same amount of Na but were fed either control or high amounts of Cu and differed from each other (two-sample t-test, $p < 0.05$). Percentages were arcsine-transformed prior to analyses.

post-feeding, and were below plasma levels at both times. Given that the concentration of Na in the ingested air-dried food was as high as ~ 2000 mmol.kg^{-1} (4.5% Na diet), it is remarkable that gastrointestinal fluid Na levels stayed so low. In general, these results are in agreement with the report of Smith *et al.* (1995) that trout fed even higher dietary NaCl loads than used here absorb almost all of the NaCl within 7 h and initiate rapid branchial compensation mechanisms to keep internal NaCl levels within tight limits. Without measurements of water ingestion and intestinal fluid absorption/production (which may be influenced by the high [Cu] of the diets), it is impossible to interpret these observations in detail. However, they do suggest a rapid absorption of Na in all parts of the tract, and that it is the transport mechanisms in the posterior part of the tract that are normally exposed to the highest lumenal Na concentrations. As with Na, Cu concentrations were much higher in the intestinal fluids than in the stomach fluid, but did not appear to change much between anterior and posterior segments (Table 2), perhaps reflecting biliary excretion of Cu (Grosell *et al.* 2001). As for Na, intestinal fluid Cu levels (50–90 mg/L) remained very low relative to the levels in the air-dried food (579 mg/kg), so the molar ratio of Na (~ 75 mmol/L) to Cu (~ 1 mmol/L) in the gastrointestinal fluids remained high, even under Cu-loading conditions.

The Effects of Dietary Na on Cu Accumulation

Under the conditions applied in this study, we observed only modest effects of dietary Na on Cu accumulation in juvenile trout. The addition of dietary Na did not lead to an overall

sustained decrease in the amount of Cu taken up from both water and diet. In fact, on several days, whole-body [Cu] among the control-Cu groups was higher in fish fed high levels of Na$^+$ (Fig. 1d). Interestingly, Handy *et al.* (2000) reported that Na-removal from the lumenal fluid tended to reduce Cu-uptake in an intestinal preparation of the African walking catfish. More recently, *in vitro* experiments conducted in our laboratory (Nadella *et al.*, unpublished results) have indicated that Cu-uptake in the trout gut may be similarly Na-dependent, being reduced by low lumenal [Na] and stimulated by high lumenal [Na]. Thus, Na and Cu uptake may actually be linked in the trout gut in a co-operative rather than an antagonistic fashion, possibly through a co-transporter or other facilitative mechanism, an idea for which there has also been some evidence in mammals (Wapnir and Stiel 1987; Wapnir 1991). Note, however, that the recent work of Burke and Handy (2005) using isolated trout enterocytes does not confirm this co-operative effect, but rather indicates that Na and Cu do not interact at low lumenal [Cu], and indeed may compete for an uptake mechanism when lumenal [Cu] is unusually high. The latter situation appears similar to Cu transport at the gills, where Na and Cu compete for a common transport pathway (Grosell and Wood 2002), and where increased dietary [Na] thereby causes a general downregulation of this branchial transport pathway, which in turn results in decreased uptake of both Na and Cu at the gills (Kamunde *et al.* 2003; Pyle *et al.* 2003). Furthermore, increased dietary [Cu] causes a downregulation of branchial Cu uptake (Kamunde *et al.* 2001, 2002a), although at present, it is unclear whether this occurs through the Na-transport pathway or through a Na-insensitive pathway (Grosell and Wood 2002). We noted a tendency for
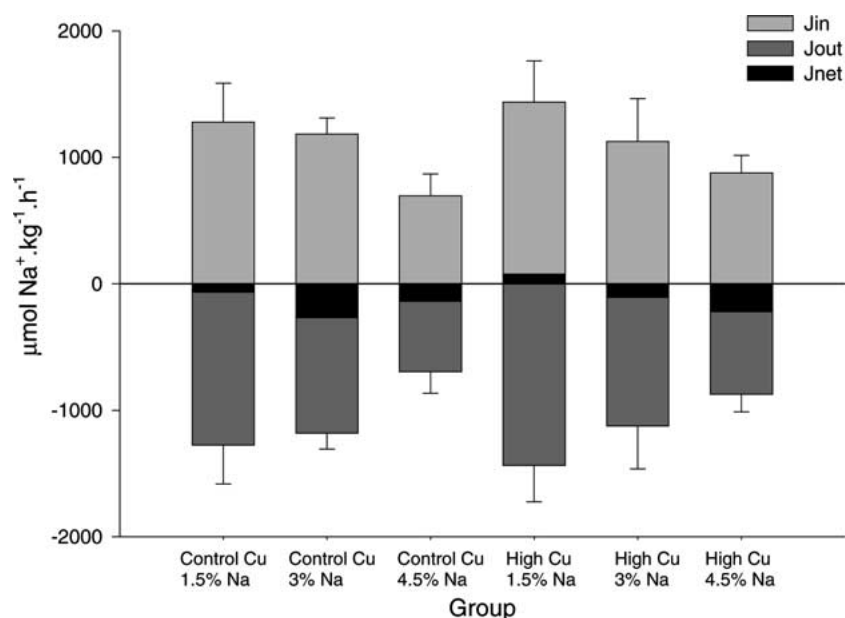
Fig. 3. Mean (± 1 S.E.M.) unidirectional Na influx (J_{in}; light gray bars), efflux (J_{out}; dark gray bars), and net flux rates (J_{net}; black bars) of eight juvenile rainbow trout per treatment group sampled on Day 28. We found no significant differences among groups.

branchial Na influx to be reduced at high dietary Na levels in fish on both low and high Cu diets, but it was not significant (Fig. 3), in contrast to the results of Smith *et al.* (1995), Pyle *et al.* (2003), and Kamunde *et al.* (2003), all of whom reported a significant inhibition of Na influx in trout fed high NaCl diets. Likely, this difference was because the present measurements were taken 36 h after the last feeding.

Based on this knowledge, we might predict that under conditions of low dietary [Cu], increased dietary [Na] would either stimulate or have no effect on Cu uptake via the gut. The higher whole-body [Cu] of the control-Cu groups fed high levels of Na on several days (Fig. 1d) suggests that the former effect occurred. Interestingly, in our previous hard water study without waterborne Cu exposure, dietary Na again appeared to slightly stimulate whole-body Cu accumulation and retention (Kjoss *et al.* 2005). We might also predict that under Cu-loaded conditions in the diet, Cu uptake at the gills would be downregulated (Kamunde *et al.* 2001, 2002a), and increased dietary [Na] would result in even greater downregulation of branchial Cu- uptake (Kamunde *et al.* 2003; Pyle *et al.* 2003), and also might competitively inhibit intestinal Cu-uptake (Burke and Handy 2005), thereby explaining the lower whole-body [Cu] on several days in the high-Cu groups fed high levels of Na (Fig. 1d). This would also explain why branchial Cu burden was not elevated by dietary Cu-loading, and indeed was lower on one sampling day (day 9).

The overall conclusion to be drawn from these complex interactions is that in situations such as in some Canadian Shield lakes, where [Cu] is elevated in both the diet and the water, it is likely that branchial Cu uptake is already downregulated, and any switchover a fish makes to a more Na-rich diet will help further reduce branchial Cu uptake. However, additional dietary Na may either enhance or inhibit Cu accumulation through the gut, depending on specific circumstances.

Tissue Cu and Na

In control Cu diet groups, Cu retention was greatest in the group that was fed the highest percentage of Na (Table 1); this group also had the highest whole-body [Cu] among the control groups on Day 28, reflecting the positive interaction in intestinal transport between Cu and Na discussed above. Among the high Cu diet groups, percent Cu retention was much lower than among controls, though still higher than in hard water. Again, the group that received the most dietary Na had the highest retention value, although in this case, it did not have the highest whole-body [Cu] among the high-Cu groups.

In our earlier hard water study (Kjoss *et al.* 2005), control tissue [Cu] tended to remain approximately the same over time for all tissues; in contrast, soft water controls in the current study tended to show steadily increasing [Cu] in the whole body, liver, and gut, and all fish in the soft water experiment had overall higher tissue [Cu] in whole body, liver, and gill. We attribute these differences directly to the higher waterborne [Cu] in the soft water exposure, which could explain the steady accretion of Cu in tissues of control fish. One notable exception was the gut, where the [Cu] of fish exposed to high dietary Cu in soft water was about half that of fish exposed to high dietary Cu in the hard water exposure. This is contrary to the findings by Kamunde *et al.* (2002a), who suggested that absorptive mechanisms for gastrointestinal Cu may require some threshold waterborne Cu levels for optimal performance and are therefore less effective when water [Cu] is low. These authors found that waterborne Cu had an apparent stimulatory effect on the bioavailability of dietary Cu and vice versa. However, their study did not consider interactions with Na, which may interfere with how dietary and waterborne Cu typically interact.

Tissue [Na] was similar between the current soft water study and our previous hard water study (Kjoss *et al.* 2005). In both

cases, we observed very few differences among groups, and [Na] tended to remain fairly stable over time. As noted earlier, this in accord with the observations of Smith *et al.* (1995) on rainbow trout that even higher dietary NaCl loads than used here result in negligible changes in tissue [Na] because of rapid homeostatic decreases and increases in branchial Na influx and efflux rates, respectively. Interestingly, the high Cu, 4.5% Na group showed a significantly higher percent of the whole-body burden in the carcass than the other five groups, a phenomenon observed in the previous hard water study as well. We speculate that under a high-Na diet, movement of Na into muscle tissue may be facilitated by the presence of Cu, possibly because of the partial depolarization of the muscle membrane potential reported by Beaumont *et al.* (2000) in trout exposed to sublethal Cu in soft acidic water. However, further studies are needed to verify this hypothesis.

In summary, at control levels of Cu in the diet, elevated dietary Na resulted in significantly greater long-term Cu accumulation. While the explanation for this phenomenon was not immediately apparent, the situation has been clarified by *in vitro* studies conducted concurrently in our laboratory (S. Nadella, unpublished results), which indicate that Cu uptake in the gut is actually stimulated rather than inhibited by dietary Na, in accord with earlier data on both mammals (Wapnir and Stiel 1987; Wapnir 1991) and fish (Handy *et al.* 2000). In contrast, at elevated Cu levels in the diet, elevated dietary Na resulted in lower long-term Cu accumulation. The result is attributable to downregulation of branchial Cu transport from the moderately Cu-enriched, low-Na soft water, because elevated dietary Na and elevated dietary Cu would both be expected to cause this phenomenon, in accord with earlier studies (Kamunde *et al.* 2002b, 2003; Pyle *et al.* 2003). In addition, competitive inhibition of intestinal Cu uptake by dietary Na may occur when Cu is abnormally high in the diet (Burke and Handy 2005).

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