Aquatic Toxicology 177 (2016) 51–62

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Revisiting the mechanisms of copper toxicity to rainbow trout: Time course, influence of calcium, unidirectional Na^+ fluxes, and branchial Na^+, K^+ ATPase and V-type H^+ ATPase activities

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ARTICLE INFO

Article history:
Received 25 March 2016
Received in revised form 11 May 2016
Accepted 15 May 2016
Available online 17 May 2016

Keywords:
Water hardness
Alkalinity
Biotic ligand model
Gill Cu burden
Carbonic anhydrase

ABSTRACT

In order to resolve uncertainties as to the mechanisms of toxic action of Cu and the protective effects of water [Ca], juvenile rainbow trout were acclimated to baseline soft water (SW, $[Na^+] = 0.07$, $[Ca^{2+}] = 0.15$, $[Mg^{2+}] = 0.05$ mmol L^{-1}) and then exposed to Cu with or without elevated [Ca] but at constant titratable alkalinity (0.27 mmol L^{-1}). The 96-h LC50 was 7-fold higher (63.8 versus 9.2 μg Cu L^{-1}; 1.00 versus 0.14 μmol Cu L^{-1}) at [Ca] = 3.0 versus 0.15 mmol L^{-1}. Gill Cu burden increased with exposure concentration, and higher [Ca] attenuated this accumulation. At 24 h, the gill Cu load (LA50 ≈ 0.58 μg Cu g^{-1}; 9.13 nmol Cu g^{-1}) predictive of 50% mortality by 96 h was independent of [Ca], in accord with Biotic Ligand Model (BLM) theory. Cu exposure induced net Na^+ losses (J^{Na}_{net}) by increasing unidirectional Na^+ efflux rates (J^{Na}_{out}) and inhibiting unidirectional Na^+ uptake rates (J^{Na}_{in}). The effect on J^{Na}_{out} was virtually immediate, whereas the effect on J^{Na}_{in} developed progressively over 24 h and was associated with an inhibition of branchial Na^+, K^+ ATPase activity. The J^{Na}_{in} inhibition was eventually significant at a lower Cu threshold concentration (15 μg Cu L^{-1}) than the J^{Na}_{out} stimulation (100 μg Cu L^{-1}). Elevated Ca protected against both effects, as well as against the inhibition of Na^+, K^+ ATPase activity. Branchial V-type H^+ ATPase activity was also inhibited by Cu exposure (100 μg Cu L^{-1}), but only after 24 h at high [Ca] (3.0 mmol L^{-1}). These novel results therefore reinforce the applicability of BLM theory to Cu, clarify that whether Na^+ influx or efflux is more sensitive depends on the duration of Cu exposure, show that elevated water [Ca], independent of alkalinity, is protective against both mechanisms of Cu toxicity, and identify V-type H^+ATPase as a new Cu target for future investigation.

1. Introduction

Copper (Cu) is an essential nutrient, but at high concentrations it becomes toxic and potentially lethal to aquatic animals (Wood, 2001; Grosell, 2012). Water calcium (Ca) concentration is usually the major component of water "hardness", and the protective effects of hardness against acute Cu toxicity to freshwater fish have been known for many years (e.g. Lloyd and Herbert, 1962; Howarth and Sprague, 1978; Chakoumakos et al., 1979; Miller and MacKay, 1980; Pagenkopf, 1983; Spry and Wiener, 1991). Hardness was incorporated as a modifying factor into early environmental regulations for Cu (e.g. US EPA, 1985; CCREM, 1987), some of which are still used in many jurisdictions today. More recently, water Ca concentration was manipulated extensively in the data sets (Playle et al., 1992, 1993; Erickson et al., 1996, 1997; Welsh et al., 1996, 2000; Bury et al., 1999; Meyer et al., 1999) which led to the Biotic Ligand Model (BLM; Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004). Today, Ca is a key variable in the modern BLM used to derive ambient water quality criteria for Cu in many jurisdictions (e.g. US EPA, 2007; ECHA, 2008; Lathouri and Korre, 2015).

The physiological basis for the protective action of Ca against Cu toxicity has not been studied as thoroughly. Most investigations have been symptom-based, rather than mechanism-based, showing that decrements of plasma and/or whole body Na^+ (and Cl^-) concentrations are less severe in Cu-exposed fish when water Ca or hardness is high (reviewed by Wood, 2001; Grosell, 2012). As water Ca is well known to regulate the permeability of the gills to

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http://dx.doi.org/10.1016/j.aquatox.2016.05.009
0166-445X/

ions (Potts and Fleming, 1971; Cuthbert and Maetz, 1972; Hunn, 1985), it is commonly believed that Cu displaces Ca ions from key binding sites that normally help to limit diffusive Na^+ permeability through these pathways, and that Ca can compete with Cu for uptake pathways, thereby reducing inhibitory effects of Cu on Na^+ uptake mechanisms. Elevations in water Ca concentration would therefore oppose deleterious actions of Cu in both elevating Na^+ efflux and reducing Na^+ influx at the gills.

However, the fundamental mechanistic work remains that of Laurén and McDonald (1985, 1986, 1987a,b), and it does not support this interpretation with respect to Ca. In this classic series of papers on juvenile rainbow trout, these workers showed that sublethal and lethal waterborne Cu exposures caused inhibition of unidirectional Na^+ influx rates (J^{Na}_{in}) and increases in unidirectional Na^+ efflux rates (J^{Na}_{out}) across the gills, leading to pronounced Na^+ net loss rates (J^{Na}_{net}), which, when high enough, resulted in death. The threshold Cu concentrations for inhibition of J^{Na}_{in} were considerably lower than those needed to increase J^{Na}_{out}, suggesting that J^{Na}_{in} blockade was normally the most important toxic mechanism. This reduction of J^{Na}_{in} was attributed to an inhibition by Cu of branchial Na^+, K^+ATPase activity on the basolateral membranes of gill ionocytes (Lorz and McPherson, 1976; Laurén and McDonald, 1987a,b), and the increase in J^{Na}_{out} was attributed to an elevation in both paracellular and transcellular permeability of the gills (Laurén and McDonald, 1985, 1986). However, these workers detected no clear protective effects of Ca on either the influx or efflux components of Na^+ balance, whereas in contrast, increased alkalinity was highly protective against both effects (Laurén and McDonald, 1985, 1986). These investigators therefore concluded that most of the protective effects of "hardness" seen with natural waters were actually due to the accompanying higher alkalinity (HCO_3^- and CO_3^{2-}) as a co-variate (Shaw and Brown, 1974) which would complex Cu, reducing its bioavailability and in turn its capacity to affect either Na^+ influx or efflux.

In the course of physiological work in support of a Cu BLM specific to rainbow trout, in which the background medium was ion-poor, low alkalinity soft water (Ng et al., 2010), we made initial discoveries which challenged some of these conclusions, particularly with respect to the role of Ca and the threshold Cu concentrations for effects on J^{Na}_{in} versus J^{Na}_{out}. We therefore expanded this work to look at these topics in detail. Since the studies of Laurén and McDonald, more recent evidence has emerged that some of the uptake of Na^+ may occur through apical channels (Avella and Bornancin, 1989) which have recently been identified as a putative acid-sensing ion channels (ASICs) (Dymowska et al., 2014). This Na^+ transport is thought to be driven electrochemically by a proton gradient established by another branchial ATPase, an apically located V-type (vacuolar) H^+ATPase (Lin and Randall, 1991, 1993). The channel/proton pump mechanism is thought to play a particularly important role in Na^+ uptake from ion-poor soft water (Kirschner, 2004; Parks et al., 2008). There is evidence that Cu may compete with Na^+ for uptake through this channel (Grosell and Wood, 2002; Goss et al., 2011). Indeed, it has been suggested, but never proven, that Cu may also directly inhibit the apical V-type H^+ATPase as another potential site of toxic action (Grosell, 2012). We therefore employed time course studies as well as direct measurements of branchial Na^+,K^+ATPase and V-type H^+ATPase activities to investigate these possibilities.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*; ~2 g) were obtained from Humber Springs Trout Farm (Orangeville, ON, Canada) and held under laboratory conditions for at least 14 days in a flow-through system in aerated 500-L polyethylene tanks. The tanks were supplied at approximately 500 ml min^{-1} with dechlorinated hard water (HW) from the City of Hamilton which originated from Lake Ontario. Water composition was (in μmol L^{-1}) $[Na^+]$ = 600; $[Cl^-]$ = 700; $[K^+]$ = 50; $[Ca^{2+}]$ = 1000; $[Mg^{2+}]$ = 150, [titratable alkalinity] = 1800, with dissolved organic carbon (DOC) = 2 mg C L^{-1}. The background [Cu] in the water was less than 1 μg Cu L^{-1}, pH was 7.7–7.9; and temperature was 12–13 °C. Fish were fed with commercial trout pellets (Martin Feed Mills, Elmira, ON, Canada) at 2% body weight per day. A 12-h light/12-h dark photoperiod was maintained throughout the study. Procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (AUP 06-01-05).

2.2. Soft water acclimation

Acclimation to soft water (SW) involved step-wise exposure to lower ion concentrations. During this process, the flow rate of dechlorinated HW was gradually reduced in the holding tank every two days and at the same time the flow rate of ion-poor water was gradually increased. The latter was produced using a reverse osmosis system (Anderson Water Systems, Dundas, ON, Canada). Water composition was progressively adjusted over 8 days until it consisted of 85% reverse osmosis water and 15% dechlorinated HW. The resultant SW mixture had the following composition (in μmol L^{-1}): $[Na^+]$ = 70; $[Cl^-]$ = 90 $[Ca^{2+}]$ = 150; $[Mg^{2+}]$ = 50; [titratable alkalinity] = 270; [Cu] ≈ 1; DOC = 0.8 mg C L^{-1}, pH = 7.0, temperature = 13 °C. This SW was the basis for the water used in all tests; only $CaCl_2$ was added, so concentrations of Na^+, Mg^{2+}, titratable alkalinity, and DOC remained at these values in all experiments. Fish were kept in these conditions in a flow-through system with 2% ration daily feeding for at least 22 days for acclimation before Cu exposure experiments began. Feeding was suspended 24 h before beginning the Cu exposure and no food was given to fish during the exposure.

2.3. 96-h LC50 assay with sampling for Na^+ and Cu status

Following SW acclimation, 15 fish (~7 g) were transferred to each of fourteen 20-L dark polyethylene tanks containing either baseline SW (0.15 mmol L^{-1} Ca) or the SW with added Ca (3.0 mmol L^{-1}), which was added as $CaCl_2$ (General Chemical Canada Ltd., Mississauga, ON, Canada). Each tank was served with aeration, and received a flow of water (250 ml min^{-1}) from a head tank via a separate mixing chamber. The experimental tanks were duplicated for each Cu treatment. A stock solution of $CuSO_4 \cdot 5H_2O$ in 0.05% HNO_3 was introduced to the mixing chambers using separate Mariotte bottles in order to achieve the desired Cu concentrations. The nominal Cu concentrations were 0, 2, 4, 7, 10, 15 and 25 μg Cu L^{-1} (actual measured dissolved concentrations (after 0.45-μm filtration) were 0.2, 1.4, 2.5, 4.0, 12.7, 17.5 and 20.2 μg Cu L^{-1} respectively) for the baseline SW and 0, 5, 10, 25, 50, 100 and 200 μg Cu L^{-1} (actual 2.3, 6.6, 12.2, 26.2, 42.2, 99.0 and 203.0 μg Cu L^{-1} respectively) for SW with 3 mmol Ca L^{-1}. Fish were fasted during the exposure. At 24 h, water samples from each tank were passed through a 45-μm Acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI, USA) and acidified for Cu, Ca^{2+}, Mg^{2+}, Na^+ and dissolved organic carbon measurements (DOC). At the same time, 7 fish from each Cu treatment were euthanized with an overdose (0.6 g L^{-1}) of neutralized tricaine methanesulfonate (MS-222; Syndel Laboratories, Qualicum Beach, BC, Canada), rinsed in clean water, and dissected for gills, liver and remaining carcass. The tissue samples were blotted dry and stored at −20 °C for later analyses of tissue and carcass Cu and Na^+ concentrations. No tissue samples were

collected from a Cu exposure treatment at 24 h if mortality had occurred within this period.

In addition, the fish in each tank were monitored every 3–5 h over 96 h and dying fish (overturned) were removed immediately, euthanized in MS-222 as above, and stored at −20 °C for whole body Cu and Na^+ analyses. At 96 h, water was sampled as at 24 h. The 96 h LC50 (concentration of Cu to kill 50% of the fish) was estimated using the logistic equation: y = 100/(1 + (LC50/x)n), where y = % mortality, x = Cu concentration, and n = slope (Meyer and Adams, 2010).

2.4. Cu exposures for Na^+ fluxes and branchial ATPase activities

Three experiments were conducted to test the impact of waterborne Cu on unidirectional Na^+ flux rates and/or the activities of both Na^+, K^+ ATPase and V-type H^+ATPase in the gills.

In the first experiment, fish (6.0 ± 0.4 g) were transferred into four 20-L dark polyethylene tanks (N = 25 fish per tank), each with a different Cu exposure concentration. Each experimental tank received a flow of SW (250 ml min^{-1}) from a head tank via a separate mixing chamber, and was served with individual aeration. A stock solution of $CuSO_4 \cdot 5H_2O$ in 0.05% HNO_3 was introduced to the mixing chambers using separate Mariotte bottles in order to achieve the following nominal Cu concentrations: 0, 4, 7 and 10 μg Cu L^{-1}. These were intended to cover the range up to the measured 96-h LC50 in SW (see Results). Actual measured dissolved Cu concentrations (after 0.45-μm filtration) were: 1.1 ± 0.3, 4.5 ± 0.4, 7.3 ± 0.9 and 8.7 ± 0.6 μg Cu L^{-1}, respectively. At the end of 21 h of exposure, conditions were adjusted for a unidirectional Na^+ influx measurement (J^{Na}_{in}) using ^{24}Na (prepared at McMaster University Nuclear Reactor from Na_2CO_3, $t_{1/2}$ = 14.96 h) as a radiotracer. The water flow into tanks was terminated, and water volume was reduced to 10 L (using a siphon tube). Each tank was then spiked with ^{24}Na (2 μCi L^{-1}), yielding a specific activity of approximately 0.03 μCi μmol Na^{-1}. The Na^+ flux test lasted for 3 h. Water samples (10 ml) were taken at the beginning and the end of the flux test to monitor changes in total Na^+ and ^{24}Na radioactivity in the water, which were small. J^{Na}_{in} was calculated from the appearance of ^{24}Na in the fish.

In contrast to expectations, the above experiment did not show any significant influence of Cu on J^{Na}_{in}. Therefore, a second experiment was carried out exposing the SW-acclimated fish (7.0 ± 0.3 g) to a wider range of Cu concentrations (nominal: 0, 15, 100 and 300 μg Cu L^{-1}; actual measured, after 0.45-μm filtration (i.e. dissolved): 2.3 ± 0.4, 15.7 ± 0.7, 103.0 ± 1.9 and 306.6 ± 3.8 μg Cu L^{-1} respectively) in the baseline SW, and SW with the addition of either 1.2 or 3.0 mmol L^{-1} Ca (added as $CaCl_2$). The goals were to examine the potential protective effects of Ca against Cu toxicity by monitoring the unidirectional Na^+ efflux (J^{Na}_{out}) and net flux rates (J^{Na}_{net}) as well as J^{Na}_{in}. All the experimental Cu concentrations exceeded the measured 96-h LC50 in baseline SW (see Results) so eventual mortality was expected. Therefore, in contrast to the first experiment, Na^+ flux rates were measured over the first 3 h of exposure to avoid the problem of subsequent mortality that would arise with more prolonged exposures to these high Cu concentrations. Furthermore, the fish were placed individually into dark, well-aerated 1-L plastic flux containers, each holding 300 ml of solution (n = 6 fish per treatment). This allowed the measurement of J^{Na}_{in}, J^{Na}_{out}, and J^{Na}_{net} on an individual basis. The exposure water with ^{24}Na (1 μCi L^{-1}) and the appropriate Cu concentration was allowed to equilibrate in the test chambers for 24 h, and then the fish was gently added. A water sample (10 ml) was taken at times 0 h and 3 h for later analysis of changes in total Na^+ and ^{24}Na radioactivity in the water. After water sampling at 3 h, fish were euthanized as above. The gills from the 100 and 300 μg Cu L^{-1} treatments were immediately dissected out, rinsed with clean water, blotted dry, plunged into liquid nitrogen, and later stored at −80 °C until assay for Na^+, K^+ ATPase and V-type H^+ ATPase activities

The third experiment was designed to characterize the time course over which the effects of Cu and Ca developed. A similar flux measurement procedure to that in the second experiment was employed, except that J^{Na}_{in}, J^{Na}_{out}, and J^{Na}_{net} were measured over sequential 6-h periods for 24 h. The fish (12.3 ± 0.5 g; n = 6 per treatment) were individually exposed to 0, 15, 35, and 100 μg Cu L^{-1} in baseline SW (nominal Ca = 0.15 mmol L^{-1}, measured Ca = 0.13 mmol L^{-1}, Mg = 0.05 mmol L^{-1}) or in SW with added Ca (nominal Ca 3.0 mmol L^{-1}, measured Ca = 2.87 mmol L^{-1}, Mg = 0.05 mmol L^{-1}). The measured Cu concentrations (0.45-μm filtration) for the treatment series were 3.7 ± 0.1, 17.3 ± 0.7, 36.6 ± 1.3 and 120.0 ± 2.4 μg Cu L^{-1} (baseline SW) and 3.2 ± 0.2, 17.6 ± 0.4, 36.7 ± 0.7 and 114.6 ± 1.6 μg Cu L^{-1} (SW + 3.0 mmol L^{-1} Ca). The flux medium (exposure solution spiked with Cu and ^{24}Na) in each chamber was entirely renewed every 6 h with the same medium that had been pre-prepared in bulk for all renewals over the 24 h and adjusted for temperature (13 °C). A water sample (10 ml) was taken at times 0 and 6 h for measuring flux rates. At the end of the total flux period (24 h), the fish were euthanized, and gills from all treatments were collected and frozen as above to determine branchial ATPase activities.

2.5. Tissue and water analyses

Concentrations of Cu, Na^+, Ca^{2+}, and Mg^{2+} in water and tissue digests were measured using atomic absorption spectroscopy (Varian AA-220, Mississauga, ON, Canada) in furnace mode (for Cu) and flame mode (for Na^+, Ca^{2+}, and Mg^{2+}). Water samples (5–10 ml) were filtered using a 45 μm Acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI, USA) and acidified to 1% HNO_3 (Trace Metal Grade, Fisher Scientific, Toronto, ON, Canada) before analysis. DOC was analysed using a total organic carbon (TOC) analyzer (Shimadzu TOC-VCPH, Mandel Scientific Company Inc., Guelph, ON, Canada). Tissue and carcass samples were digested in five volumes of 1 N HNO_3 (trace metal grade) using sealed vials incubated in a laboratory oven (Fisher Scientific) at 60 °C for 72 h. ^{24}Na counts were measured using a gamma counter (1480 Wallac Wizard 3 inch crystal Automatic Gamma Counter, Perkin Elmer, Canada). Absolute counts per minute (cpm) were calculated from measured cpm values after background and decay correction.

Measured ^{24}Na radioactivity and total [Na^+] in the water samples were used for flux calculations. Water [Cu], [Ca^{2+}], and [Mg^{2+}] were measured to ensure that actual concentrations were reflective of the nominal concentrations.

Theoretical speciation analysis of Cu solution chemistry under selected exposure conditions was performed using WHAM VII (Windermere Humic Acid Model; Tipping et al., 2011), and an assumption of 10% humic acid, 90% fulvic acid in the DOC component, as in the Biotic Ligand Model. After critical review of the WHAM VII thermodynamic database, we exchanged the default formation constant for CO_3^{2-} complexation in WHAM VII for that recommended by the International Union for Pure and Applied Chemistry (IUPAC; Powell et al., 2007).

2.6. Calculations for unidirectional and net Na^+ fluxes

Unidirectional Na^+ uptake (J^{Na}_{in}) for the first experiment was calculated from the measured whole body ^{24}Na radioactivity of fish (R_F in cpm) and mean measured specific activity (SA in cpm μmol^{-1}) of the radioisotope during the flux time (T) using the following equations:

$$SA = 0.5\left(\frac{[R1]}{[ion1]} + \frac{[R2]}{[ion2]}\right) \quad (1)$$

$$J^{Na}in = \frac{R_F}{W \cdot T \cdot SA} \quad (2)$$

where *ion1* and *ion2* are the initial and final total Na^+ concentrations in the water ($\mu mol\ L^{-1}$), W the weight of the fish (g), and *R1* and *R2* are the water ^{24}Na radioactivity concentrations ($cpm\ L^{-1}$) at the beginning and at the end of the flux period, respectively.

In the second and third experiments, influx (J^{Na}_{in}) was calculated based on disappearance of ^{24}Na radioactivity from the external water – i.e. differences in measured radioactivity concentrations between water samples at the beginning and at the end of the flux test, while the net flux (J^{Na}_{net}) was calculated from differences in the total Na^+ concentrations of the water over the same period. Efflux (J^{Na}_{out}) was calculated as the difference between J^{Na}_{in} and J^{Na}_{net}. The following equations were used to calculate Na^+ fluxes (Wood, 1992):

$$J^{Na}net = \frac{([ion1]- [ion2]).\ V}{W.\ T} \quad (3)$$

$$J^{Na}in = \frac{([R1]- [R2]) \cdot V}{W \cdot T \cdot SA} \quad (4)$$

$$J^{Na}_{out} = J^{Na}_{net} - J^{Na}_{in} \quad (5)$$

where V is the volume of the exposure solution in the flux chamber (L). SA was calculated using Eq. (1).

2.7. ATPase assays

The ATPase activities were measured in crude gill homogenate following a method adapted from Lin and Randall (1993) and Hawkings et al. (2004). In particular, ouabain and *N*-ethylmaleimide (NEM) were employed as Na^+, K^+ATPase and H^+ATPase inhibitors respectively, and sodium azide was used to remove background activity of mitochondrial ATPase. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

On the day of assay, a working stock solution was prepared from: 50 mmol L^{-1} imidazole, 2.8 mmol L^{-1} phosphoenol pyruvate (PEP), 0.23 mmol L^{-1} NADH, 3.5 mmol L^{-1} ATP, 4 U ml^{-1} lactate dehydrogenase (LDH), and 5 U ml^{-1} pyruvate kinase (PK). Then the solution was divided into four portions to prepare four different reagents (A: control; B: ouabain; C: ouabain + sodium azide; D: ouabain + sodium azide + NEM).

Ouabain (500 $\mu mol\ L^{-1}$) was added to solutions B-D, sodium azide (5 mmol L^{-1}) to solutions C and D, and NEM (1 mmol L^{-1}) to solution D. Then a salt solution (50 mmol L^{-1} imidazole, 189 mmol L^{-1} NaCl, 10.5 mmol L^{-1} $MgCl_2$, 42 mmol L^{-1} KCl), was added to each of solutions A,B,C, and D, so that the volumes of salt solution to stock working solution had a ratio of 1:3. All solutions were well vortexed and kept on ice.

In the second experiment (see Section 2.4) one or two gill arches (cartilage plus soft tissues) were thawed and homogenized for 1–2 s in 250 µl SEI-EGTA buffer solution (250 mmol L^{-1} sucrose, 10 mmol L^{-1} EGTA, 50 mmol L^{-1} imidazole; pH 7.3) using a Tissue-Tearor™ homogenizer (Biospec Products, Bartlesville, OK, USA) set at a speed of 15 000 rpm. In order to increase sensitivity in the third experiment (see Section 2.4), soft gill tissues were dissected from the cartilaginous tissues while still frozen, and only these were homogenized. Subsequently, 250 µl of Nanopure® water (Thermo Fisher Scientific, Waltham, MA, USA) were added and tissue was further homogenized for 30 s, or until it was well-ground. Gill homogenization was performed on ice, and the homogenate was immediately centrifuged at 2000*g* for 7 min at 4 °C. The supernatant (thick and red in color) was discarded and the pellet was further homogenized for 15 s in 500 µl of SEID-EGTA buffer solution (SEI-EGTA plus 0.5% sodium deoxycholic acid) and centrifuged again as before. On an ice-cold surface, replicates of 10 µl of the supernatant, now clearer and less red in color, were pipetted into 12 separate wells of a 96-well microplate. Next, 200 µl of solution A were added to the first three wells, 200 µl of solution B to the next three wells, and so on. The plate was then read at 25 °C in a temperature-controlled microplate reader (Thermomax, Molecular Devices, Menlo Park, CA, USA) at a wavelength of 340 nm for 20 min at 10-s intervals. To standardize the ATPase assay, a standard curve was run from 0 to 20 nmol ADP $well^{-1}$.

Activities of the ATPase enzymes (ADP mg $protein^{-1}$ h^{-1}) were calculated using the slope of linear disappearance of NADH over time for reaction treatments, the slope of the standard curve, the volume of the reaction mixtures, and the protein content in the crude homogenate (Lin and Randall, 1993; McCormick, 1993). Na^+, K^+ATPase activity was determined by subtracting the difference in ATP hydrolysis rate between the control wells and the ouabain-treated ones. V-type H^+ATPase activity was obtained by calculating the difference in ATP hydrolysis between wells treated with ouabain + sodium azide and those treated with ouabain + sodium azide + NEM. The method of Bradford (1976) was used to determine protein concentrations in the crude homogenates at 595 nm on the same microplate reader.

2.8. Statistical analysis

Data are presented as means ± SE (n). We used one-way or two-way analysis of variance (ANOVA) as appropriate to test whether statistically significant effects occurred between treatments. When the F values were significant, the post hoc Tukey-Kramer multiple comparison test was used to make comparisons between means of the measurements. Dunnett's test was conducted to determine significant differences between treatment means and control. All statistical tests and non-linear estimations, and estimates of 95% CI, were run using GraphPad Instat 3 (GraphPad Software Inc., La Jolla, CA, USA) or Statistica 9.1 (StatSoft Inc., Tulsa, OK, USA) at $P < 0.05$.

3. Results

3.1. 96-h LC50 and tissue Na^+ and Cu status

The estimated 96-h LC50 for Cu in the baseline SW was 9.2 (CI: 5.7–12.7) µg Cu L^{-1} (0.14 µmol Cu L^{-1}). The addition of 3.0 mmol Ca L^{-1} to the SW resulted in a 7-fold decrease in Cu toxicity, raising the 96-h LC50 to 63.8 (CI: 32.3–95.3) µg Cu L^{-1} (1.00 µmol Cu L^{-1}).

When fish were sampled at 24 h, before any mortality had occurred, two clear trends were evident. Firstly, the gill Cu burden increased with the exposure concentration (Fig. 1). Secondly, the elevation in water Ca from 0.15 to 3.0 mmol Ca L^{-1} reduced both the background gill Cu burden by 50% and its rate of rise in relation to the Cu exposure concentration. Notably however, at the eventual 96-h LC50 concentrations (9.2 µg Cu L^{-1} in baseline SW, 63.8 µg Cu L^{-1} in high Ca), the increments in gill Cu burden above background at 24 h were virtually identical (about 0.58 µg Cu g^{-1} in both) at the two Ca concentrations. Cu burdens in liver and carcass, as well as Na^+ concentrations in gill, liver, and carcass were also measured at 24 h (Table 1). Liver Cu rose about 3–5-fold relative to control levels in the fish exposed in the baseline SW (0.15 mmol Ca L^{-1}), but was very variable, so only some of the increases were significant. There were no significant changes in tissue Na^+ concentrations or in Cu burdens in the carcass in these fish. At 3.0 mmol Ca L^{-1}, there were no significant changes in any of the parameters (Table 1).

Fish were also sampled shortly prior to death throughout the 96-h exposures for measurements of whole-body Cu burdens and Na^+ concentrations. There was a slight trend for increasing whole-body Cu concentrations with exposure concentration, but the increase was not significant in either the baseline SW or the SW + 3 mmol

Table 1
Tissue Cu and Na^+ levels (mean ± SE, n = 7) measured at 24 h of exposure, in juvenile rainbow trout exposed to various concentrations of Cu in either baseline SW (0.15 mmol Ca L^{-1}) or SW with added Ca (3. 0 mmol Ca L^{-1}) in the initial tests used for determining 96-h LC50.

Baseline SW (0.15 mmol Ca L^{-1})						
Waterborne Cu (μg L^{-1})		**Tissue Cu (μg g^{-1})**		**Tissue Na (μmol g^{-1})**		
Nominal	**Measured**	**Liver**	**Carcass**	**Gill**	**Liver**	**Carcass**
0	0.20	10.4 ± 1.1	0.69 ± 0.06	75.5 ± 12.7	46.2 ± 2.0	44.5 ± 1.0
2	1.36	38.9 ± 13.4	0.51 ± 0.04	62.0 ± 1.9	51.4 ± 4.5	38.5 ± 0.5
4	2.50	35.3 ± 9.0	0.58 ± 0.04	62.9 ± 1.8	52.0 ± 2.3	44.0 ± 1.1
7	4.10	50.9 ± 15.4*	0.56 ± 0.05	63.7 ± 2.3	53.0 ± 4.5	40.2 ± 2.0
10	12.7	46.4 ± 10.8*	0.52 ± 0.06	55.2 ± 3.6	49.8 ± 6.4	35.0 ± 2.8
15	17.5	46.5 ± 7.5*	0.55 ± 0.04	62.1 ± 6.6	48.8 ± 5.4	37.3 ± 4.1
25	20.2	46.9 ± 7.4*	0.62 ± 0.01	60.0 ± 5.0	51.7 ± 5.4	40.4 ± 2.5
SW with added Ca (3.0 mmol Ca L^{-1})						
0	2.30	28.6 ± 7.6	0.48 ± 0.06	60.6 ± 1.4	30.5 ± 0.9	48.2 ± 1.6
5	6.55	16.4 ± 2.0	0.57 ± 0.08	63.1 ± 1.6	30.2 ± 0.6	47.4 ± 1.5
10	12.2	33.3 ± 7.8	0.75 ± 0.10	59.7 ± 3.1	29.9 ± 1.6	48.5 ± 2.3
25	26.2	22.7 ± 5.7	0.67 ± 0.05	63.0 ± 0.9	31.8 ± 1.1	50.3 ± 1.1
50	42.2	29.1 ± 9.3	0.69 ± 0.02	65.5 ± 1.1	31.7 ± 0.8	49.3 ± 1.3
100	99.2	25.5 ± 3.5	0.70 ± 0.06	60.4 ± 2.3	31.0 ± 0.5	45.7 ± 1.6
200[a]	203	–	–	–	–	–

[a] Tissue Cu and Na were not measured as the mortality of fish started to occur within 24 h of the start exposure at this concentration.
* Indicates significant difference ($p < 0.05$) from the control for the same tissue.

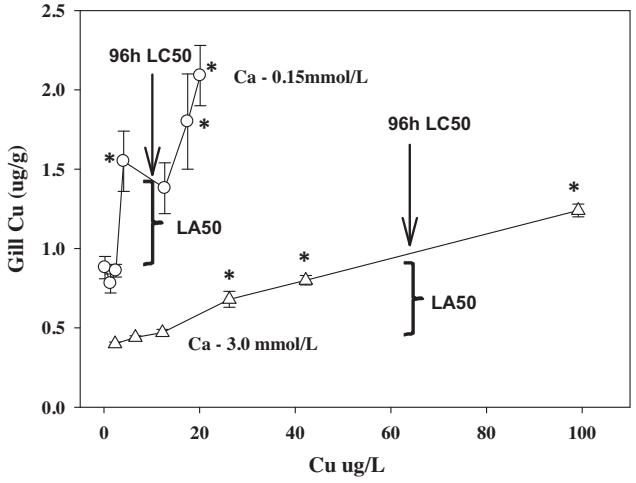

Fig. 1. Gill Cu burdens (means ± SE, n = 7), measured at 24 h of exposure, in juvenile rainbow trout exposed to various concentrations of Cu in either baseline SW (0.15 mmol Ca L^{-1}) or SW with added Ca (3. 0 mmol Ca L^{-1}) in the initial tests used for determining 96-h LC50. Where SEMs cannot be seen, they are smaller than the symbol for the mean. The vertical arrows indicate the eventual 96-h LC50 concentrations, and the brackets indicate the increments in gill Cu burden above background at 24 h (LA50) at these respective Cu concentrations. The increments were virtually identical (about 0.58 μg Cu g^{-1} in both) at the two different Ca levels. Asterisks represent significant differences from the respective control values ($P < 0.05$).

Ca L^{-1} tests (Fig. 2A,B). However, the whole-body Na^+ concentrations decreased significantly with increasing Cu concentrations. The decreases at the 96-h LC50 concentration were between 10% and 12% in the baseline SW (Fig. 2A) and between 17% and 26% in the SW with added Ca (Fig. 2B). In both test series, the whole-body Na^+ losses were close to 50% at the highest Cu concentrations tested (20.2 and 203 μg Cu L^{-1} respectively).

3.2. Cu effects on unidirectional and net Na^+ flux rates

The goal of the first flux experiment was to determine whether the decrements in whole body Na^+ seen in Cu-exposed trout (Fig. 2) could be explained by an inhibition of J^{Na}_{in} at 21–24 h, so a Cu exposure range up to approximately the measured 96-h LC50 in SW was tested. There was clearly no inhibition of J^{Na}_{in} (Fig. 3).

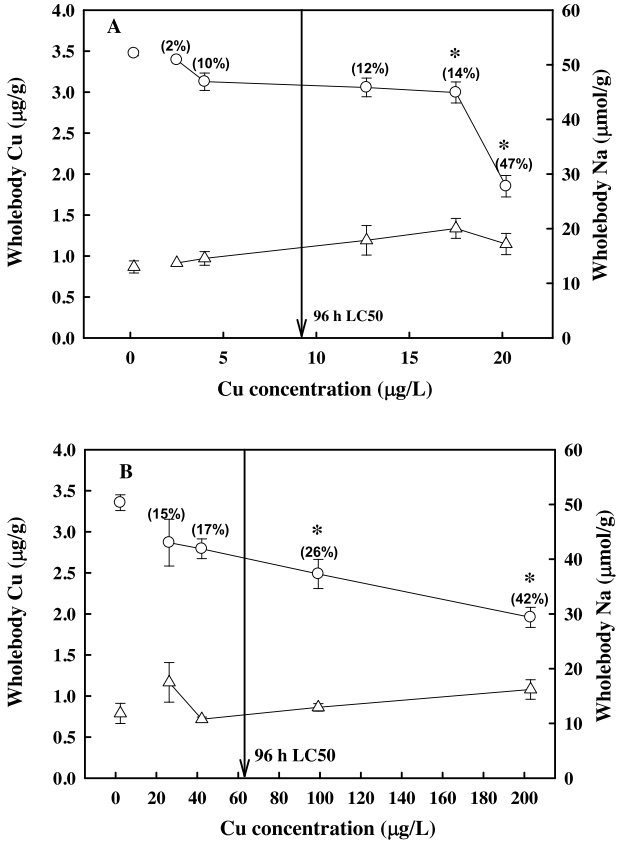

Fig. 2. Whole-body Na and Cu levels (means ± SE) in juvenile rainbow trout sampled shortly prior to death due to Cu exposure in either (A) the baseline SW (0.15 mmol Ca L^{-1}) or (B) SW with added Ca (3.0 mmol Ca L^{-1}) in the initial tests used for determining 96-h LC50. From left to right, the n numbers in panel (A) are 7, 1, 6, 6, 7, 4 and in panel (B) are 7, 2, 5, 5, 8. The circles and triangles represent Na^+ and Cu levels respectively in dying fish. The percentages represent whole-body Na^+ loss in dying fish relative to the control fish. The vertical arrow shows the respective 96-h LC50s. Where SEMs cannot be seen, they are smaller than the symbol for the mean. Asterisks represent significant differences from the respective control values ($P < 0.05$).

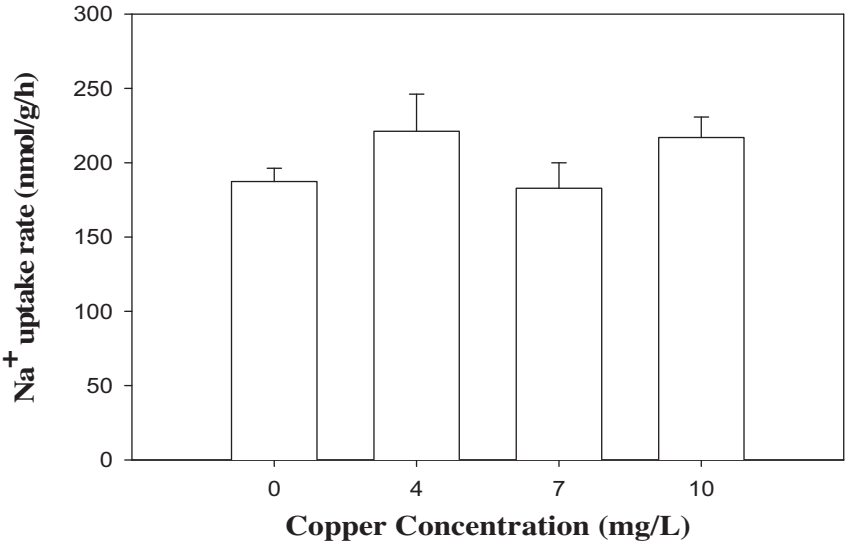

Fig. 3. Unidirectional Na^+ uptake rates (J^{Na}_{in}; means ± SE, n = 8) measured in the first flux experiment in juvenile rainbow trout exposed to various concentrations of Cu in baseline SW (0.15 mmol Ca L^{-1}) for 24 h. J^{Na}_{in} was measured over a 3-h period between 21 and 24 h of exposure. There were no significant differences ($P > 0.05$).

The goals of the second flux experiment were to examine both the effects of Cu, and the potential protective effects of Ca, on J^{Na}_{out} and J^{Na}_{net} as well as on J^{Na}_{in}. Much greater Cu concentrations (up to 30-fold higher) were tested, so Na^+ flux rates were measured over the first 3 h to avoid any problems with subsequent mortality that would arise with more prolonged exposures to these high Cu concentrations. This also allowed assessment of the immediate effects of Cu challenge.

Similar to the first flux experiment, fish exposed for 3 h to these much higher Cu concentrations (that would eventually prove lethal) did not exhibit any reductions in J^{Na}_{in}, regardless of the water Ca concentration (0.15, 1.2, or 3.0 mmol Ca L^{-1}; Fig. 4). However, at the two highest Cu concentrations, J^{Na}_{out} increased significantly, resulting in significantly elevated J^{Na}_{net}. Thus J^{Na}_{out} was more sensitive than J^{Na}_{in} to waterborne Cu during the first 3 h of exposure. This occurred only in the baseline SW (Fig. 4A), and not in the two media with elevated Ca concentrations (Fig. 4B,C). Therefore high Ca clearly protected against the immediate increase in J^{Na}_{out} caused by high waterborne Cu. In the two-way ANOVA, the effects of Ca, the effects of Cu, and interaction effects were all significant for both J^{Na}_{net} and J^{Na}_{out}.

The objectives of the third experiment were to characterize the time courses of development for the effects of Cu and Ca on unidirectional and net Na^+ fluxes. Over 24 h of exposure, the two-way ANOVA demonstrated that both exposure time and Cu levels strongly affected Na^+ fluxes (J^{Na}_{in} and J^{Na}_{net}) in SW at the baseline Ca level (0.15 mmol Ca L^{-1}; Fig. 5A); interaction effects were also significant. In the first 6 h, J^{Na}_{in} was reduced by approximately 35% and 45% at 35 and 100 μg Cu L^{-1} respectively, relative to the control influx rate that remained the same over time. However, with the progression of exposure time (6–12 h, 12–18 h, and 18–24 h), Cu inhibited J^{Na}_{in} more strongly with further decreases of 66–99% at 35 and 100 μg Cu L^{-1}. Cu effects on J^{Na}_{in} were not pronounced at 15 μg Cu L^{-1} and a significant inhibition (37%) was only observed at 12–18 h.

The pattern of effects of Cu on J^{Na}_{out} were very different from those on J^{Na}_{in} in baseline SW (Fig. 5A). J^{Na}_{out} was elevated (by 1.2–1.8 fold) only at the highest concentration tested (100 μg Cu L^{-1}), resulting in more negative J^{Na}_{net} (by 3.2–6.2 fold), and the effect was more or less stable over time. In the two-way ANOVA, only the influence of Cu level was significant for J^{Na}_{out},. Thus J^{Na}_{out} was less sensitive than J^{Na}_{in} to waterborne Cu under the conditions of these tests, and effects were immediate rather than time-dependent.

As observed in the second experiment (Fig. 4), the addition of Ca (3 mmol L^{-1}) to SW ameliorated the effects of Cu on Na^+ fluxes over 24 h (Fig. 5B). However here, the protective effects were exerted on J^{Na}_{in} as well as on J^{Na}_{out}. The inhibitory effect of Cu (at 35 and 100 μg Cu L^{-1}) on J^{Na}_{in} was both delayed and reduced in magnitude, whereas the stimulatory effect of Cu (at 100 μg Cu L^{-1}) on J^{Na}_{out} was eliminated entirely (Fig. 5B). In the two-way ANOVAs, only the influence of Cu on J^{Na}_{in} was significant. Thus again, the threshold for Cu effects on J^{Na}_{out} was higher than for effects on J^{Na}_{in}, and elevated Ca protected against both.

3.3. Cu effects on branchial ATPase activities

In trout sampled from the second flux experiment, the activities of both Na^+, K^+ATPase (Fig. 6A) and V-type H^+ATPase enzymes (Fig. 6B) were relatively similar to each other in the baseline SW. However, there was a significant decrease in the activity of V-type H^+ATPase enzyme in all treatment conditions in the presence of 3.0 mmol Ca L^{-1} (Fig. 6B). Exposure of the fish to high levels of Cu (100 and 300 μg Cu L^{-1}) for 3 h had no effect on the activities of either Na^+, K^+ ATPase (Fig. 6A) or V-type H^+ ATPase enzymes (Fig. 6B) in either baseline SW or 3.0 mmol L^{-1} Ca. In the two-way ANOVAs, only the influence of Ca on V-type H^+ATPase enzyme activity was significant.

In trout from the third flux experiment sampled after 24 h, the activities of both Na^+, K^+ ATPase and V-type H^+ATPase enzymes in baseline SW were about twice as high as in the second experiment (compare Figs. Fig. 77A,B and Fig. 66 A). This is likely explained by the fact that only soft gill tissues rather than whole gills were assayed in the third experiment. Exposure of fish to Cu for 24 h resulted in a decrease of Na^+, K^+ ATPase activity in the baseline SW (0.15 mmol Ca L^{-1}) but the effect was only significant at 100 μg Cu L^{-1}. The presence of 3.0 mmol Ca L^{-1} protected against this effect; significant inhibition of this enzyme was not observed at any Cu level. In the two-way ANOVA, only the influence of Cu was significant.

On the other hand, effects of 24 h of Cu exposure on V-type H^+ATPase activity were quite the opposite (Fig. 7): no inhibition in the baseline SW but a significant inhibition at 100 μg Cu L^{-1} in

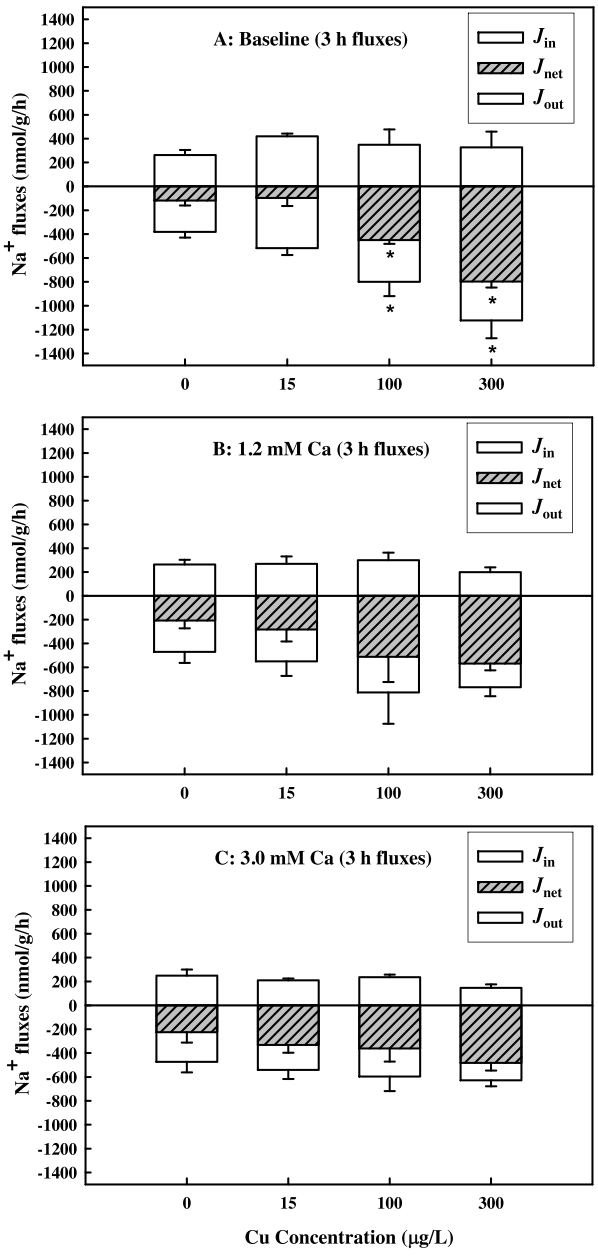

Fig. 4. Unidirectional and net Na^+ flux rates (means ± SE, n = 6) measured in the second flux experiment in juvenile rainbow trout over the first 3 h of exposure to various concentrations of Cu in either (A) the baseline SW (0.15 mmol Ca L^{-1}) or (B) SW with added Ca (1.2 mmol Ca L^{-1}) or (C) SW with more added Ca (3.0 mmol Ca L^{-1}). The open bars above the x-axis indicate Na^+ influx rates (J^{Na}_{in}), while open bars below it indicate Na^+ efflux rates (J^{Na}_{out}) and the grey hatched bars represent Na^+ net flux rates (J^{Na}_{net}). Asterisks represent significant differences from control values ($P < 0.05$).

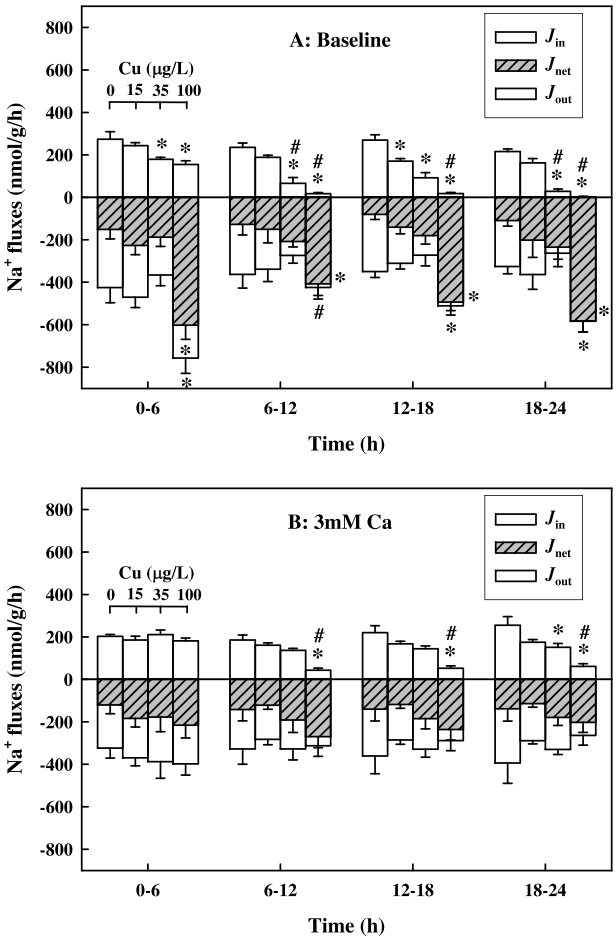

Fig. 5. Unidirectional and net Na^+ flux rates (means ± SE, n = 6) measured in the third flux experiment in juvenile rainbow trout during successive 6-h periods over 24 h of exposure to various concentrations of Cu in either (A) the baseline SW (0.15 mmol Ca L^{-1}) or (B) SW with added Ca (3.0 mmol Ca L^{-1}). The open bars above the x-axis indicate Na^+ influx rates (J^{Na}_{in}), while open bars below it indicate Na^+ efflux rates (J^{Na}_{out}) and the grey hatched bars represent Na^+ net flux rates (J^{Na}_{net}). Asterisks represent significant differences from control values within the same time point ($P < 0.05$). The number signs represent the effects of time with significant differences from $t_{0\text{-}6\,h}$ for the same Cu treatment ($P < 0.05$).

the presence of added Ca (3.0 mmol L^{-1}). The effects of Cu, Ca, and their interactions were all significant in the two-way ANOVA.

4. Discussion

4.1. Overview

Our results are novel in resolving the issues raised in the Introduction with respect to the classic mechanistic work of Laurén and McDonald (1985, 1986, 1987a,b) on the physiological effects of Cu on trout, and confirm that Cu is a powerful ionoregulatory toxicant with actions on both Na^+ uptake and efflux pathways. In particular, we demonstrate that whether Na^+ influx or efflux is more sensitive depends on the duration of Cu exposure, and that Ca alone (i.e. independent of alkalinity) is protective against Cu effects on both pathways, and reduces gill Cu accumulation. The results re-inforce the applicability of Biotic Ligand Model (BLM) theory to Cu toxicity, and identify V-type H^+ATPase as a new enzymatic target for Cu.

4.2. Na^+ loss, gill Cu accumulation, and mortality

In agreement with an extensive literature (reviewed by Wood, 2001; Grosell et al., 2002; Grosell, 2012), trout dying from Cu toxicity suffered decreases in whole-body Na^+ concentrations (Fig. 2). Interestingly, these effects were not evident in the tissue, gill, and carcass measurements made at 24 h (Table 1), indicating that they take some time to develop. Na^+ loss gradually sets in motion a chain of fluid shifts, vasoconstriction, and blood viscosity increases that kill the fish by causing cardiovascular collapse (Milligan and Wood, 1982; Wilson and Taylor, 1993). However the threshold for this phenomenon is usually around a 20% loss of whole-body or plasma [Na^+], and it is difficult to believe that losses of 2–12%, in fish dying at Cu concentrations generally below the 96-h LC50 (Fig. 2), could in themselves induce mortality. These small losses were for the more sensitive fish in the population, suggesting that a second mech-

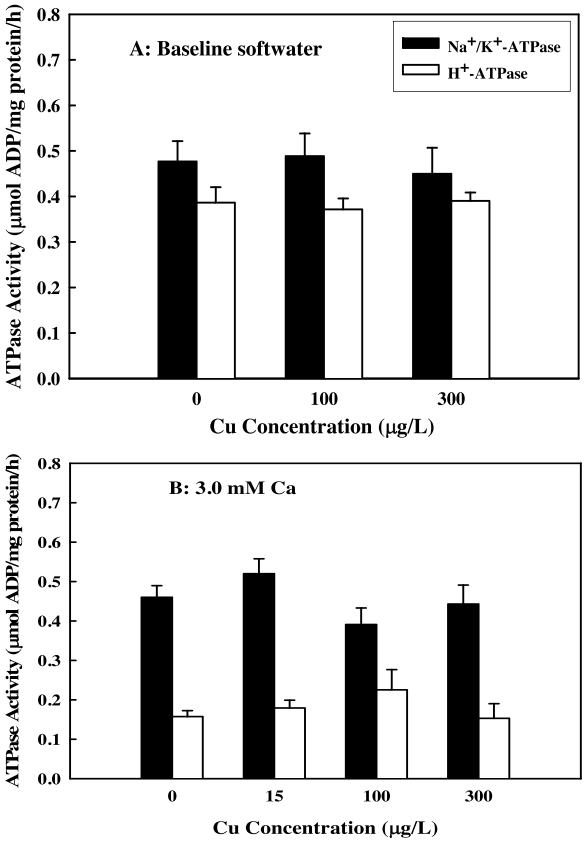

Fig. 6. Gill enzyme activities (means ± SE, n = 6) of (A) Na^+, K^+ ATPase and (B) V-type H^+ ATPase in juvenile rainbow trout of the second flux experiment exposed to various concentrations of Cu for 3 h inthe baseline SW (0.15 mmol Ca L^{-1}) or SW with added Ca (3.0 mmol Ca L^{-1}). There were no significant differences between control and Cu treatments for either enzyme. However, in all treatments, V-type H^+-ATPase activity at 3.0 mmol Ca L^{-1} was significantly lower than in baseline SW ($P < 0.05$).

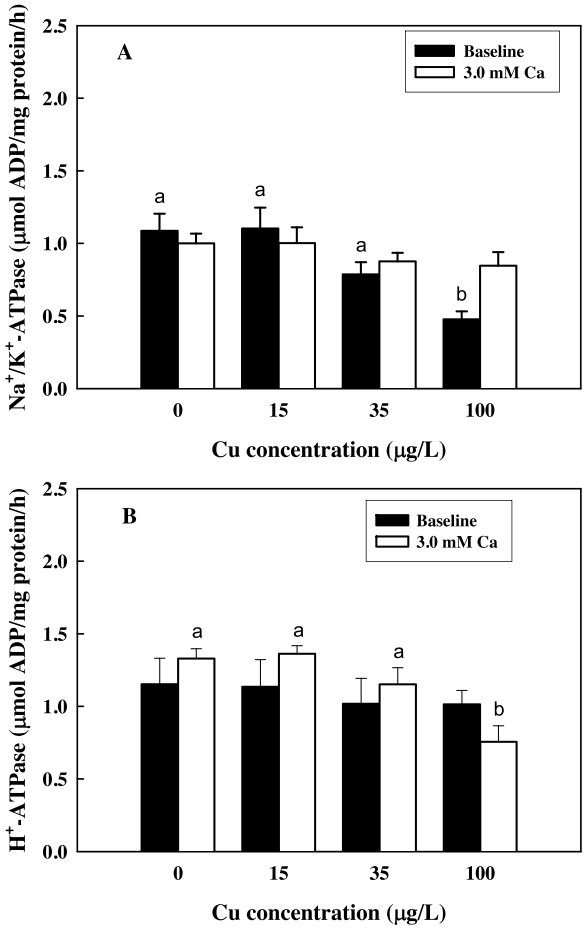

Fig. 7. Gill enzyme activities (means ± SE, n = 6) of (A) Na^+, K^+ ATPase and (B) V-type H^+ ATPase in juvenile rainbow trout of the third flux experiment exposed to various concentrations of Cu for 24 h in the baseline SW (0.15 mmol Ca L^{-1}) or SW with added Ca (3.0 mmol Ca L^{-1}). Dissimilar letters shows significant differences ($P < 0.05$) between Cu treatments for the same Ca concentration.

anism of toxicity may also contribute. For silver (Ag), which is a similar ionoregulatory toxicant, Janes and Playle (2000) found that respiratory toxicity occurred as a compounding toxic mechanism in trout exposed in very ion-poor water comparable to the baseline SW used in the present study. De Boeck et al. (1995, 2007) have reported that Cu can also act as a respiratory toxicant in other species of fish, but not in rainbow trout, albeit in tests done in harder water.

Gill Cu accumulation at 24 h, long prior to death, increased with exposure concentration and correlated extremely well with eventual mortality (Fig. 1), thereby fulfilling key tenets of the BLM. This agrees well with earlier findings of Playle et al. (1992, 1993) and Meyer et al. (1999) on fathead minnows. Indeed, at both Ca concentrations in the exposure water, the elevation above background at 24 h, predictive of 50% lethality at 96 h (LA50), was about 0.58 μg Cu g^{-1}, or about 9.3 nmol Cu g^{-1}. This agrees very well with earlier work establishing 5.2–9.7 nmol g^{-1} as the LA50 at 24 h for trout exposed to Cu in similar SW at pH's ranging from 5.0 to 8.0 (Ng et al., 2010). As with variations in pH, elevated water Ca exerted clear protective action by reducing gill Cu uptake, such that a much higher exposure Cu concentration (63.8 μg Cu L^{-1} versus 9.2 μg Cu L^{-1}, representing the respective 96-h LC50 values) was required to reach the same LA50 at 3.0 mmol Ca L^{-1} than at 0.15 mmol Ca L^{-1}, in perfect accord with BLM theory (Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004). Note however that Grosell and Wood (2002) reported that even higher Ca concentrations (up to 20 mmol L^{-1}) had no effect on branchial Cu uptake in juvenile rainbow trout. The explanation for this apparent discrepancy is likely the difference in time; our measurements were made after 24-h exposure, whereas those of Grosell and Wood were made after only 2-h exposure.

4.3. The protective effects of calcium

Higher water Ca clearly protected not only against Cu accumulation in the gills (Fig. 1), but also against the actions of Cu in causing elevations in J^{Na}_{out} (Fig. 4), decreases in J^{Na}_{in} (Fig. 5), and therefore increases in J^{Na}_{net} (Figs. 4 and 5). As alkalinity was the same in the low and high Ca trials, these protective actions cannot be explained by effects on Cu bioavailability, but must rather reflect competition of Ca with Cu for binding sites on the gills which are involved in Cu toxicity.

The theoretical speciation calculations of Table 2 based on WHAM VII are instructive in this regard. They suggest that the protection by elevated water Ca that increased the 96-h LC50 from 9.2 to 63.8 μg Cu L^{-1} (a 7-fold increase) occurred despite the fact that the concentration of free Cu^{2+} ion increased by 240-fold. The latest version of the BLM (3.1.2.37; accessed May 11, 2016; http://www.windwardenv.com/biotic-ligand-model/) run in speciation mode yields a similar 150-fold higher free Cu^{2+} ion concentration at the higher LC50. Therefore protection by Ca competition is highly effective. Another interesting result from this modeling exercise is the role of Ca in displacing Cu from DOC, thereby contributing to the much higher free Cu^{2+} ion concentration (Table 2).

This idea of protection by competition was first suggested by Pagenkopf (1983). This does not preclude the possibility that ele-

Table 2
The speciation of Cu in selected exposures as calculated by WHAM VII. The LC50 concentrations in low Ca (0.15 mmol Ca L^{-1}) and high Ca (3.0 mmol Ca L^{-1}) water are highlighted in bold. See text for details.

	Total Cu (nmol L^{-1})	Cu^{2+} (nmol L^{-1})	$CuOH^+$ (nmol L^{-1})	$Cu(OH)_2$ (nmol L^{-1})	$Cu(HCO_3)^+$ (nmol L^{-1})	$CuCl^+$ (nmol L^{-1})	$CuCO_3$ (nmol L^{-1})	$Cu(CO_3)_2^{2-}$ (nmol L^{-1})	Cu-DOC (nmol L^{-1})
0.15 mmol Ca L^{-1}, 9.2 μg Cu L^{-1} (LC50)	144.8	1.3	0.2	0.001	0.015	0.0002	0.7	0.0001	142.6
3.0 mmol Ca L^{-1}, 9.2 μg Cu L^{-1}	144.8	11.1	1.1	0.007	0.097	0.099	3.9	0.001	128.6
0.15 mmol Ca L^{-1}, 63.8 μg Cu L^{-1}	1003.9	102.4	11.8	0.1	1.2	0.01	50.1	0.014	838.3
3.0 mmol Ca L^{-1}, 63.8 μg Cu L^{-1} (LC50)	**1003.9**	**315.9**	**30.1**	**0.2**	**2.8**	**2.8**	**110.2**	**0.03**	**540.1**
0.15 mmol Ca L^{-1}, 300 μg Cu L^{-1}	4720.6	1719.5	197.5	1.5	19.8	0.3	840.4	0.2	1941.0
3.0 mmol Ca L^{-1}, 300 μg Cu L^{-1}	4720.6	2424.9	230.8	1.6	21.4	21.8	846.0	0.3	1173.6

vated water Ca had other, physiologically-based, protective effects (see below). In future studies this possibility could be illuminated by looking directly at the relationship between gill Cu burden and simultaneously measured changes in gill unidirectional and net Na fluxes, though unfortunately such data were not collected in the present study.

In this respect, our conclusions differ fundamentally from those of Laurén and McDonald (1985, 1986) who reported no clear protective actions of Ca against these same effects. The most probable cause for this discrepancy is the difference in experimental protocol (Erickson et al., 1997). Laurén and McDonald (1985, 1986) acclimated their trout for 10–14 days to the different Ca levels used in their studies, whereas in the current investigation, fish were acclimated to the low Ca level (0.15 mmol Ca L^{-1}) in the baseline SW for 22 days, and then acutely exposed to higher Ca levels. Other studies have shown that acclimation of fish to different water Ca levels results in complex changes in the "tightness" of the gills, binding affinity of gill sites for Ca, the kinetics of Na^+ transport, and therefore in J^{Na}_{out} and J^{Na}_{in} (Oduleye, 1975; McDonald et al., 1980, 1983; McDonald and Rogano, 1986; Wood et al., 1990; Hollis et al., 1997; Boisen et al., 2003; Craig et al., 2007). Very likely, this also results in changes in the ability of Ca to compete with Cu for binding sites which are involved in affecting these processes. In contrast, in our studies, these acclimation effects would not confound interpretation as the protective actions of high Ca were seen after acute transfer, but the conclusions of Laurén and McDonald (1985, 1986) may be more relevant to real world situations.

In nature, Mg is the other component of water hardness, but this was not investigated in the present study as our focus was on clarifying the results of Laurén and McDonald (1985, 1986) who manipulated only water Ca concentration in their studies. However, in BLM studies performed in parallel to the present investigation (M.J. Chowdhury, A. Cremazy, T.Y.-T. Ng, and C.M. Wood, unpublished results), we found that the protective efficiency of Mg was only about 8% of that of Ca on a molar basis with respect to acute Cu toxicity to rainbow trout.

An additional *caveat* with respect to environmental relevance is that a substantial portion of the data in the present study and associated interpretations are based on Cu concentrations well above the 96-h LC50 (range = 9.2–63.8 μg Cu L^{-1}). This approach was required to detect thresholds for effects in the first 24 h of exposure before substantial gill damage occurred, but it raises the question whether the same qualitative trends would have occurred at later times at lower Cu exposure levels. In future, it will be informative to follow unidirectional Na^+ fluxes throughout a much longer time course during sublethal Cu exposures.

The fact that high waterborne Ca decreased Na^+ loss (Fig. 5) despite still elevated levels of gill-bound Cu (Fig. 1) at 24 h (e.g. at 100 μg Cu L^{-1}) might indicate that not all Cu at the gill is bound to sites involved in causing toxicity. Indeed there is now some evidence that points to the presence of several different Cu binding sites at the gill (Taylor et al., 2002).

While we have attributed all protective effects caused by the addition of $CaCl_2$ to Ca, we cannot absolutely eliminate the possibility that elevated Cl concentrations may also have contributed to protection. However, inasmuch as Cu speciation calculations showed a negligible effect of elevated Cl concentration on Cu speciation (Table 2), it is unlikely that Cl played an important role.

4.4. Time course of effects and relative sensitivity of Na^+ uptake versus Na^+ efflux to Cu

The conclusion of Laurén and McDonald (1985, 1986) that the Cu concentration threshold for inhibition of J^{Na}_{in} was considerably lower than that for elevation of J^{Na}_{out}

was based on flux measurements over 0–12 h and 12–24 h (Laurén and McDonald, 1985) or 0–24 h (Laurén and McDonald, 1986) of Cu exposure. The 3-fold higher water Na concentration used by these workers may have contributed to this difference, However, more importantly, their experiments did not have the same time resolution as our tests. The present results demonstrate that their conclusion must be qualified because the time courses were very different for the effects of Cu on J^{Na}_{out} (virtually immediate) and J^{Na}_{in} (slowly developing). However, it should be pointed out that in one test (at 50 μg Cu L^{-1} in low alkalinity, high Ca water) reported by Laurén and McDonald (1986), where only J^{Na}_{in} and not J^{Na}_{out} was measured, a significant decrease in J^{Na}_{in} was seen after a delay of 2 h. Our measurements over the first 3 h of Cu exposure in baseline SW revealed clear increases in J^{Na}_{out} at 100 and 300 μg Cu L^{-1}, without any disturbance of J^{Na}_{in} (Fig. 4A) – i.e. J^{Na}_{out} was more sensitive initially, in opposition to the conclusion of Laurén and McDonald (1985, 1986). At 100 μg Cu L^{-1}, this stimulation of J^{Na}_{out} persisted more or less unchanged at 0–6 h, 6–12 h, 12–18 h, and 18–24 h (Fig. 5). In contrast, while there was no inhibition of J^{Na}_{in} at Cu concentrations up to 300 μg Cu L^{-1} in the first 3 h (Fig. 4), at 0–6 h, there was a slight inhibition at 35 and 100 μg Cu L^{-1}, which developed progressively with time such that inhibition became significant at 15 μg Cu L^{-1} by 12–18 h, and J^{Na}_{in} was almost totally abolished by 35 and 100 μg Cu L^{-1} by 18–24 h (Fig. 5A). Throughout the four periods, the only effects on J^{Na}_{out} occurred at 100 μg Cu L^{-1}. Therefore J^{Na}_{in} was much more sensitive over the longer term, in agreement with the conclusion of Laurén and McDonald (1985, 1986). Our study is the first to show the difference in the timing of such outcomes of Cu toxicity.

The fast time course and constancy of the J^{Na}_{out} response, in combination with the protective effect of elevated water Ca, suggests that it results from a simple displacement of Ca^{2+} ions from key binding sites (intercellular tight junctions, channels) on the gills by Cu, thereby increasing paracellular and/or transcellular permeability. The same explanation was given by Laurén and McDonald (1985, 1986). The slow, progressive time course for the inhibition of J^{Na}_{in} suggests that it results not from apical competition processes, but rather from penetration of Cu through to the basolateral membranes of branchial ionocytes, with associated inhibition of Na^+,K^+ATPase activity (see Section 4.4). This enzyme is located at the basolateral side of the cell, and therefore is not immediately available for Cu inhibition. A similar time course effect has been seen very clearly with Ag, which is also an ionoregulatory toxicant with specific inhibitory action on Na^+, K^+ ATPase activity; inhibition of J^{Na}_{in} and enzyme activity developed in parallel over 24 h (Morgan et al., 1997; Morgan et al., 2004). Notably, in the present study, in fish exposed to 100 μg Cu L^{-1} in background SW (0.15 mmol Ca L^{-1}), there was no inhibition of Na^+, K^+ ATPase activity after 3 h (Fig. 6), but a significant inhibition after 24 h, and high waterborne Ca (3.0 mmol L^{-1}) protected against both gill Cu accumulation (Fig. 1) and inhibition of Na^+, K^+ ATPase activity at this time (Fig. 6), as well as partially against inhibition of J^{Na}_{in} (Fig. 5). The fact that protection of J^{Na}_{in} was only partial suggests that other enzymatic mechanisms may have also been involved (see Section 4.4).

4.5. The effects of Cu on branchial transport enzymes

Our finding that 24 h of Cu exposure decreased branchial Na^+, K^+ ATPase activity (Fig. 6A) is in agreement with many previous reports (e.g. Lorz and McPherson, 1976; Laurén and McDonald, 1987b; Pelgrom et al., 1995; Beckman and Zaugg, 1988; Sola et al., 1995; Li et al., 1998). The fact that J^{Na}_{in} was inhibited at 12–18 h at a lower Cu threshold (15 μg Cu L^{-1}; Fig. 5A) than that associated with inhibition of Na^+, K^+ ATPase activity (100 μg Cu L^{-1}; Fig. 6A) at 24 h could be because of dissociation of the Cu from the enzyme due to dilution during the assay, and/or because another enzymatic target is also involved. One such possibility is cytoplasmic carbonic anhydrase (CA), as suggested by Grosell (2012). CA is thought to supply the H^+ ions needed for the apical V-type H^+ATPase/Na^+ channel system and/or the apical Na^+/H^+ exchanger, as well as HCO_3^- ions for apical Cl^-/HCO_3^- exchange (Kirschner, 2004; Parks et al., 2008). In studies on Ag toxicity, CA has been identified as an additional target enzyme in the gills, whose inhibition contributes to the more rapid reduction of both Na^+ and Cl^- uptake (Morgan et al., 1997; Morgan et al., 2004). Recently, CA inhibition has been demonstrated in guppies exposed to a low level of Cu (20 μg L^{-1}) in fresh water (0.33 mmol Ca L^{-1}) for 12 h (Zimmer et al., 2012).

The mechanism by which Cu inhibits the Na^+, K^+ ATPase enzyme appears to be through its high affinity for thiol groups on cysteine residues, resulting in interference with Mg^{2+} binding to an activation site which is critical for phosphorylation and thus transport activity (Li et al., 1996). In the V-type H^+ATPase, there is a cysteine residue at a catalytic site (Nishi and Forgac, 2002), so it is possible that Cu could similarly inhibit this enzyme, contributing to a reduction in J^{Na}_{in}. Indeed, Grosell (2012) suggested that the apical V-type H^+ATPase could be yet another potential target of Cu toxicity. Our finding that significant inhibition occurred after 24 h of exposure to 100 μg Cu L^{-1} in the presence of added Ca (3.0 mmol Ca L^{-1}), but not in the baseline SW (0.15 mmol Ca L^{-1}), is the first concrete demonstration of this idea. In two recent studies, there were non-significant trends for inhibition of branchial V-type H^+ATPase activity by Cu exposure in fish exposed to Cu at intermediate Ca levels (guppies, 12 h at 20 μg Cu L^{-1}, Ca = 0.33 mmol L^{-1}, Zimmer et al., 2012; trout, 6–9 h at 50 μg Cu L^{-1}, Ca = 0.90 mmol L^{-1}; Lim et al., 2015). It is not clear why higher Ca levels should increase the susceptibility of V-type H^+ATPase to Cu inhibition, but at least short-term exposure to Ca alone caused a down-regulation of activity (Fig. 6). As mentioned earlier, the high Ca treatment raised Cl levels by almost 6 mmol L^{-1} but it is unlikely this would have reduced V-type H^+ ATPase activity. If anything, this would be expected to increase Cl/HCO_3 exchange activity at the gills and/or induce a reduction in the internal Strong Ion Difference, both of which could induce acidosis; a compensatory increase in branchial V-type H^+-ATPase activity rather than the observed decrease would be anticipated.

Perhaps it was the combination of Ca and Cu effects that produced the significant inhibition at 24 h.

Future studies should systematically examine the time-dependent, concentration- dependent, and Ca-dependent sensitivity of V-type H^+ ATPase, Na^+, K^+ ATPase,and carbonic anhydrase activities in trout gills.

Acknowledgements

This study was supported by a Collaborative Research and Development Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada, and the International Copper Association (ICA), the Copper Development Association (CDA), the International Lead Zinc Research Organization (ILZRO, now the International Lead Association), the Nickel Producers Environmental Research Association (NiPERA), Noranda-Falconbridge (now Glencore), Teck-Cominco (now Teck Resources), and Inco (now Vale). CMW was supported by the Canada Research Chair Program. We thank Dr. Anne Crémazy for help with speciation analysis and Sunita Nadella for excellent technical assistance.

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