Comparative Biochemistry and Physiology, Part C 168 (2015) 48–54

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Acute exposure to waterborne copper inhibits both the excretion and uptake of ammonia in freshwater rainbow trout (*Oncorhynchus mykiss*)

Michael Yu-Ting Lim [a], Alex M. Zimmer [a,*], Chris M. Wood [a,b]

[a] Department of Biology, McMaster University, Hamilton L8S 4K1, ON, Canada
[b] Department of Zoology, University of British Columbia, Vancouver V6T 1Z4, BC, Canada

ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form 24 November 2014
Accepted 2 December 2014
Available online 9 December 2014

Keywords:
Ammonia excretion (J_{amm})
Carbonic anhydrase (CA)
Copper
H^+-ATPase
High environmental ammonia (HEA)
Na^+/K^+-ATPase
Rhesus (Rh) glycoproteins
Sodium uptake (J^{Na}_{in})

ABSTRACT

In freshwater fish, exposure to sub-lethal concentrations of waterborne copper (Cu) results in inhibitions of ammonia excretion (J_{amm}) and Na^+ uptake (J^{Na}_{in}), yet the mechanisms by which these occur are not fully understood. In the present study, rainbow trout (*Oncorhynchus mykiss*) fry exposed to 50 μg/l Cu for 24 h displayed a sustained 40% decrease in J_{amm} and a transient 60% decrease in J^{Na}_{in}. Previously, these effects have been attributed to inhibitions of gill Na^+/K^+-ATPase and/or carbonic anhydrase (CA) activities by Cu. Trout fry did not display significant reductions in the branchial activities of these enzymes or H^+-ATPase over 24 h Cu exposure. Recently, Rhesus (Rh) glycoproteins, bi-directional NH_3 gas channels, have been implicated in the mechanism of Cu toxicity. Juvenile trout were exposed to nominal 0, 50, and 200 μg/l Cu for 3–6 h under control conditions (ammonia-free water) followed by 6 h exposure to high environmental ammonia (HEA; 1.5 mmol/l NH_4HCO_3). HEA led to significant ammonia uptake in control fish (0 μg/l Cu), and exposure to 50 and 200 μg/l Cu resulted in significant reductions of ammonia uptake during HEA exposure. This is the first evidence that Cu inhibits both the excretion and uptake of ammonia, implicating bi-directional Rh glycoproteins as a target for Cu toxicity. We propose a model whereby Rh blockade by Cu causes the sustained inhibition of J_{amm} and transient inhibition of J^{Na}_{in}, with H^+-ATPase potentially aiding in J^{Na}_{in} recovery. More work is needed to elucidate the role of Rh proteins in sub-lethal Cu toxicity.

1. Introduction

Copper (Cu) is a trace metal found in aquatic environments that can lead to physiological disturbances and potential death of aquatic organisms when present at elevated levels. Natural levels of Cu in the aquatic environment tend to be relatively low, ranging from 0.2 to 30 μg/l (USEPA, 2007), but systems affected by anthropogenic activities, such as industrial and mining sites, can reach levels up to 100 μg/l or even 200 mg/l (USEPA, 2007). Although some contaminated sites may not possess concentrations of Cu which could be considered lethal, there are a number of toxic effects at sub-lethal levels of exposure which are also of concern. These effects are numerous and variable, including behavioral changes, oxidative damage, decreases in swimming performance, acid–base disturbances, and many other adverse effects (Grosell, 2012). However, in freshwater fish, arguably the two most commonly observed effects of Cu exposure are the inhibitions of ammonia excretion and Na^+ uptake (see Grosell et al., 2002; Grosell, 2012 for reviews).

When measured simultaneously in the same study, the disruptions of ammonia and Na^+ balance by Cu in freshwater fish have been demonstrated to occur both independently (Blanchard and Grosell, 2006) and concomitantly (Wilson and Taylor, 1993; Zimmer et al., 2014). The simultaneous inhibition of the two processes is consistent with the current model for ammonia excretion in freshwater fish, the "Na^+/NH_4^+-exchange complex", where ammonia excretion and Na^+ uptake are loosely coupled to one another (Wright and Wood, 2009). In this model, the transport of ammonia as NH_3 across the gill epithelium is facilitated by Rhesus (Rh) glycoproteins (Nakada et al., 2007; Nawata et al., 2007) which are bi-directional transporters of NH_3 (Nawata et al., 2010). Upon reaching the apical environment, NH_3 is protonated to NH_4^+, effectively trapping ammonia and maintaining an outwardly directed partial pressure gradient for NH_3. Protons for this acid-trapping mechanism are supplied by cytosolic and membrane-bound carbonic anhydrase (CA), and apical Na^+/H^+-exchanger (NHE) and H^+-ATPase (see Wright and Wood, 2009 for review). In the latter two mechanisms, apical boundary layer acidification occurs in conjunction with Na^+ uptake *via* direct exchange in the case of NHE (energized by basolateral Na^+/K^+-ATPase) and by the electrogenic uptake of Na^+ *via* a putative epithelial channel in the case of H^+-ATPase, thereby providing a "Na^+/NH_4^+ exchange complex" (Wright and Wood, 2009, 2012).

Cu may act to disrupt the enzymes and/or transporters involved in the Na^+/NH_4^+ exchange mechanism, thereby inhibiting ammonia

* Corresponding author. Tel.: +1 905 525 9140x23237; fax: +1 905 522 6066.
E-mail addresses: limmy@mcmaster.ca (M.Y.-T. Lim), zimmea@mcmaster.ca (A.M. Zimmer), woodcm@mcmaster.ca (C.M. Wood).

http://dx.doi.org/10.1016/j.cbpc.2014.12.002
1532-0456/

excretion and Na^+ uptake. Studies have pointed to the inhibition of CA (Vitale et al., 1999; Zimmer et al., 2012) and Na^+/K^+-ATPase (Lauren and McDonald, 1987a; Pelgrom et al., 1995; Sola et al., 1995), as targets for Cu in aquatic organisms. There are, however, a number of conflicting studies reporting absences of inhibition of either of these enzymes (Grosell et al., 2003, 2004; Blanchard and Grosell, 2006; Zimmer et al., 2012), suggesting that the overall mechanism of action of Cu is still not fully understood. As such, the aim of the present study was to gain a better understanding of the mechanism of ammonia excretion (J_{amm}) and Na^+ uptake (J^{Na}_{in}) inhibition by waterborne Cu exposure. Freshwater rainbow trout (*Oncorhynchus mykiss*), which are relatively sensitive to Cu exposure (Ng et al., 2010), were studied. J_{amm}, J^{Na}_{in}, and gill Na^+/K^+-ATPase, H^+-ATPase, and CA activities were assessed in rainbow trout fry exposed to 50 μg/l Cu over 24 h in order to identify one or more of these enzymes as targets for Cu-induced disturbances in J_{amm} and J^{Na}_{in}. This level of exposure was chosen based on its environmental relevance and on some initial experiments showing consistent inhibitions of J_{amm} and J^{Na}_{in}.

In light of results from these 24 h exposures, another set of experiments was designed to determine if the bi-directional transport of ammonia is disrupted by Cu, potentially suggesting the blockade of Rh glycoproteins by Cu. In these experiments, juvenile rainbow trout were pre-exposed to Cu (50 or 200 μg/l) and subsequently exposed to high environmental ammonia (HEA; 1.5 mmol/l NH_4HCO_3). J_{amm} was assessed in these fish in order to determine if exposure to Cu can also reduce ammonia uptake during HEA exposure. Overall, our results confirm that Cu inhibits both J_{amm} and J^{Na}_{in} in freshwater fish and, for the first time, demonstrate that Cu can inhibit the bi-directional transport of ammonia, suggesting Rh glycoproteins as potential targets for Cu toxicity.

2. Material and methods

All procedures were approved by the McMaster University Animal Research Ethics Board (AUP 12-12-45) and were in accordance with the Guidelines of the Canadian Council on Animal Care.

2.1. Series 1 — 24 h Cu exposures in rainbow trout fry

2.1.1. Animals

Trout fry were chosen for this series as their smaller size allowed for conducting a large set of experiments simultaneously. Rainbow trout (*O. mykiss*) were acquired in the eyed embryonic stage from Rainbow Springs Hatchery in Thamesford, ON and raised to the fry stage (0.3–0.7 g) at McMaster University in Hamilton, ON. Embryos were kept in hatching trays supplied with well-aerated, flow-through, dechlorinated Hamilton tap water (moderately hard: $[Na^+]$ = 0.6 mequiv/l, $[Cl^-]$ = 0.8 mequiv/l, $[Ca^{2+}]$ = 1.8 equiv/l, $[Mg^{2+}]$ = 0.3 mequiv/l, $[K^+]$ = 0.05 mequiv/l; titration alkalinity 2.1 mequiv/l; pH ~8.0; hardness ~140 mg/l as $CaCO_3$ equivalents, 12 °C). Embryos hatched after 3–4 days, and remained in the yolk sac larval stage for approximately 30 days. After complete yolk sac absorption, fry were fed a daily 5% body mass ratio of commercial trout pellets and kept on this diet for a minimum of 1 month prior to experimentation. Trout fry were fasted for 24 h prior to study and experiments were conducted at 12 °C.

2.1.2. Experimental design

This experimental series was designed to assess J_{amm}, J^{Na}_{in}, and gill Na^+/K^+-ATPase, H^+-ATPase, and CA activities in response to acute 24-h exposure to 50 μg/l Cu, a concentration chosen based on consistent effects observed at 50 μg/l Cu in an initial pilot experiment employing 4 h exposures to nominal 0, 30, 50, and 80 μg/l Cu (data not shown). Overall, 42 fish in total were exposed individually to either control conditions (nominal 0 μg/l Cu) or to nominal 50 μg/l Cu (as $CuSO_4$) in 100-ml beakers containing 50 ml of constantly aerated water.

For control exposures, 12 fish were placed individually into beakers containing 50 ml control media in order to assess J_{amm} over the 24 h control period, J^{Na}_{in} over 0–3 h and 21–24 h, and gill enzymatic activity following 3 h and 24 h of control exposure. Fish were allowed to adjust to the beakers for 10 min, after which ^{22}Na was added to a final concentration of 0.01 μCi/ml to 6 of the beakers in order to measure J^{Na}_{in} over the first 0–3 h. Following 5 min of mixing by aeration, an initial 1.5-ml water sample was taken from all 12 beakers, representing t = 0 h for the control exposure. Following 3 h, an additional 1.5-ml water sample was taken from all 12 beakers (t = 3 h). In those beakers which had been spiked with ^{22}Na, fish were then removed and sacrificed *via* neutralized MS-222 overdose. Fish were rinsed twice with 5 mM NaCl and once with distilled water to remove surface bound isotope, weighed, and the entire gill basket was excised and cut in half laterally. One half of the gill basket was flash-frozen and stored at −80 °C for later enzymatic analyses and the other half, along with the whole body of the fish, was used to determine ^{22}Na gamma radioactivity. In the remaining 6 control beakers, additional 1.5-ml water samples were taken at 6, 9, and 12 h, after which fish were moved to beakers containing 50 ml of fresh control media and a water sample was taken immediately. At 21 h, ^{22}Na was added to the remaining 6 beakers, another water sample was taken, and the same protocol described above was followed in order to determine J^{Na}_{in} (and J_{amm}) over the final 21–24 h of control exposure. A 0.25-ml aliquot of each water sample was used for determining both gamma radioactivity and $[Na^+]$ (in radioactive samples only), and the remaining sample (1.25 ml) was frozen at −20 °C for later total ammonia concentration (T_{amm}) analysis. Fish samples (whole-body + half gill basket) were immediately counted for gamma radioactivity.

For Cu exposures, 30 fry were placed individually into beakers containing 50 ml of 50 μg/l Cu media in order to assess J_{amm} over the entire 24 h Cu exposure, J^{Na}_{in} at 0–3, 3–6, 6–9, 9–12, and 21–24 h, and gill enzymatic activity following 3, 6, 9, 12, and 24 h of Cu exposure. Cu exposures were run in parallel with control exposures and followed identical protocols except that J^{Na}_{in} (and J_{amm}) was also assessed at t = 3–6, 6–9, and 9–12 h following the methods described above for the t = 0–3 h control period. Note that for both control and Cu exposures, T_{amm} was measured in all samples, such that in some instances where 12 fish were undergoing flux measurements simultaneously (*e.g.*, t = 0–3 h), a sample size of n = 12 for J_{amm} was obtained. Water samples (5 ml) for dissolved Cu concentrations were taken at 0, 12, and 24 h in both the control and Cu-exposed treatments. Samples were filtered through a 0.45 μm filter, acidified to 1% HNO_3 using trace metal grade HNO_3 (Fisher Scientific, Ottawa, ON, Canada), and stored at 4 °C until further analysis.

2.2. Series 2 — Cu and high environmental ammonia (HEA) co-exposures in juvenile trout

2.2.1. Animals

Juvenile trout were chosen for this series as their larger size allowed for a better resolution for measuring ammonia uptake against a high ammonia background. Rainbow trout (3–6 g) were purchased from Humber Springs Trout Hatchery in Orangeville, ON. Fish were kept in flow-through, well-aerated, dechlorinated Hamilton tap water and fed commercial trout pellets at a ratio of 1% body mass once every 2 days. Fish were acclimated to the lab setting for at least 2 weeks and were fasted for 48 h prior to experimentation.

2.2.2. Experimental design

This series was conducted in order to determine if Cu exposure inhibits both the excretion and uptake of ammonia. A HEA concentration of 1.5 mmol/l NH_4HCO_3 was sufficient to elicit ammonia uptake from the environment based on observations from an initial pilot experiment wherein fish were exposed to 0, 0.5, 1.0, and 1.5 mmol/l NH_4HCO_3 for 3 h (data not shown).

Fish were placed individually in plastic containers containing 100 ml of either control (nominal 0 μg/l Cu) or nominal 50 μg/l media (constantly aerated dechlorinated Hamilton tap water). Following an initial 10-min adjustment period, a 1.5-ml water sample was removed. After 3 h, an additional water sample was removed and fish were transferred to containers which held 100 ml of HEA water and the appropriate Cu concentration. The NH_4HCO_3 stock used to make the HEA water was corrected to pH 8 with 1 M KOH in order to maintain pH between pre-exposures and HEA exposures in this series. Water samples were immediately collected, marking the beginning of the HEA exposure. Additional water samples were taken 3 and 6 h following the transfer to HEA, where thereafter fish were removed and weighed. Samples for the measurement of dissolved Cu concentrations were taken following the initial pre-exposure period and following HEA exposure using the protocol described in Series 1. An additional experiment following the same protocol was conducted except that the pre-HEA exposure was increased to 6 h and the Cu concentration was increased to 200 μg/l. The rationale for this additional experiment is explained in the Results section. All water samples were stored at −20 °C until later T_{amm} analysis.

2.3. Analytical procedures and calculations

T_{amm} (μmol/l) in water samples obtained in Series 1 and 2 was determined using the colorimetric method of Verdouw et al. (1978). Whole-body ammonia flux rates (J_{amm}; μmol/g/h) in both series were then calculated using the following formula:

$$J_{amm} = [(T_{amm}i - T_{amm}f) * V]/(w * t) \quad (1)$$

where $T_{amm}i$ and $T_{amm}f$ are the initial and final water T_{amm} (μmol/l), respectively, V the is volume (l; corrected for sample removal), w the is fish weight (g), and t is the duration of flux (h). Note that negative flux rates indicate ammonia excretion whereas positive flux rates indicate ammonia uptake.

Gamma radioactivity of ^{22}Na (counts per minute; cpm) in body, gill, and water samples from Series 1 was determined *via* gamma counting (Wizard 1480 3+ Auto Gamma Counter, Perkin Elmer, Woodbridge, ON, Canada) and water total [Na^+] (μmol/l) was determined by atomic absorption spectrophotometry (SpectrAA 220FS Atomic Absorption Spectrophotometer, Varian, Australia). Na^+ uptake rates (J^{Na}_{in}; μmol/g/h) were then calculated using the following formula:

$$J^{Na}_{in} = \left(R_{body} + 2 * R_{gill}\right) / \left(SA_{avg} * w * t\right) \quad (2)$$

where R_{body} and R_{gill} are gamma radioactivity (cpm) of the body and half gill basket, respectively, and SA_{avg} is the average of the specific activities (cpm/μmol) of the initial and final water samples.

Dissolved Cu concentrations in water samples from Series 1 and 2 were determined using a graphite furnace atomic absorption spectrophotometer (SpectroAA220, Varian, Mulgrave, Australia). National Research Council of Canada reference standards (TM-15.2; Ottawa, ON, Canada) were run in parallel with samples in order to determine the percentage recovery of Cu, which was 102.6 ± 3.3% overall.

2.4. Enzymatic analyses

Gill samples obtained in Series 1 were homogenized in an ice-cold Na-deoxycholate buffer. Gill homogenates were then assayed for Na^+/K^+-ATPase, H^+-ATPase, and CA activities using methods described previously (*e.g.*, McCormick, 1993; Nawata et al., 2007; Zimmer et al., 2012). Briefly, Na^+/K^+-ATPase and H^+-ATPase activities were determined indirectly by the disappearance of NADH (measured at 340 μm) on a plate reader (SpectraMAX Plus, Molecular Devices, Menlo Park, CA, USA) every 15 s for 30 min in the presence and absence of specific inhibitors: ouabain for Na^+/K^+-ATPase, and N-ethylmaleimide (NEM) with sodium azide for H^+-ATPase. Activity was determined as the difference between the rates of the non-inhibited and inhibited reactions. CA activity was determined *via* the delta-pH protocol of Henry (1991). Samples (10 μl) were assayed in 10 ml of pH 7.4 reaction buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris buffer, 10 mM NaH_2PO_4) kept at 2–4 °C. The reaction buffer was mixed constantly *via* a magnetic stirrer. The reaction was initiated by the addition of 1 ml CO_2-saturated distilled water and reaction rate was determined by measuring the pH of the solution every 5 s for 30 s using an Accumet AccTupH electrode connected to an Accumet AB15 meter (Fisher Scientific, Ottawa, ON, Canada). CA activity in homogenates was calculated as the rate of reaction divided by the rate of the non-catalyzed reaction (no sample added). All enzyme activities are presented as activity per mg protein and protein concentration in gill homogenates was determined using the Bradford reagent and a bovine serum albumin (BSA) standard curve (Sigma, Burlington, ON, Canada).

2.5. Statistical analyses

All data are presented as means ± SEM (n = sample size) and the significance level was accepted at $P < 0.05$. In general, comparisons between two independent individual means were conducted using a Student's t-test, single factor comparisons among multiple means were conducted using a one-way ANOVA analysis with a Holm–Sidak post-hoc test, and two factor (*e.g.*, time and Cu concentration) comparisons among multiple means were conducted using a two-way ANOVA analysis with a Holm–Sidak post-hoc test. Detailed descriptions of specific statistical tests are included in corresponding figure captions.

3. Results

3.1. Series 1 — 24 h Cu exposure in trout fry

No mortalities were observed in Series 1. The mean measured dissolved Cu concentration in the nominal 50 Cu μg/l exposures was 42.1 ± 1.5 μg/l, significantly greater than that of 3.6 ± 0.7 μg/l measured in control exposures (Table 1A). Mean initial water T_{amm} in this series has also been included in Table 1B.

In response to exposure to 50 μg/l Cu, J_{amm} was initially unaffected over the first 3 h relative to the simultaneous controls, but was inhibited by over 40% by 3–6 h of exposure (Fig. 1). Aside from the 0–3 h and 9–12 h time points, inhibition of J_{amm} by Cu was sustained throughout the duration of the 24 h exposure such that at 21–24 h J_{amm} remained reduced by 40% (Fig. 1). Note that in control fish, there were significant temporal changes in J_{amm}, which were absent in the Cu-exposed animals (Fig. 1).

Control J^{Na}_{in} values at 0–3 h and 21–24 h were 0.55 ± 0.05 and 0.59 ± 0.16 μmol/g/h, respectively; these means were not significantly different from one another and thus control data were pooled together for analysis. Similar to J_{amm}, J^{Na}_{in} was not significantly inhibited by Cu in the first 0–3 h of exposure, however, over 3–9 h of Cu exposure, J^{Na}_{in} was inhibited by nearly 60% relative to the mean control value (Fig. 2). However, unlike the results observed for J_{amm}, J^{Na}_{in} was not significantly different from control rates at 9–12 h and by 21–24 h there was no significant effect of Cu exposure on J^{Na}_{in} (Fig. 2).

Control means for gill Na^+/K^+-ATPase, H^+-ATPase, and CA activities (Fig. 3), similar to the J^{Na}_{in} data, are representative of pooled values from fish following 3 h and 24 h control exposure as activities of all three enzymes were not significantly different between these time points of control exposure (Na^+/K^+-ATPase: 3.45 ± 0.72 and 3.06 ± 0.27 μmol ADP/mg protein/h; H^+-ATPase: 1.57 ± 0.31 and 2.02 ± 0.09 μmol ADP/mg protein/h; CA: 0.14 ± 0.02 and 0.17 ± 0.02 mg protein^{-1} at 3 h and 24 h, respectively). Exposure to 50 μg/l had no significant inhibitory effects on the branchial activity of any of the enzymes measured (Fig. 3). However, after an initial non-significant drop in activity, there was a significant increase in gill H^+-ATPase between 6 and 24 h of Cu exposure (Fig. 3B).

Table 1
(A) Mean measured dissolved Cu concentrations (μg/l) in water samples from Series 1 and Series 2 and (B) mean measured initial total ammonia levels (T_{amm}; μmol/l) in water samples from Series 1 and 2.

A.

Nominal concentration	Control (0 μg/l)	50 μg/l	200 μg/l
Series 1	3.6 ± 0.7^{a}	42.1 ± 1.5^{b}	–
Series 2	3.2 ± 1.5^{a}	49.8 ± 3.6^{b}	160.7 ± 13.6^{c}

Means not sharing the same letter in a given series are significantly different from one another as determined by a one-way ANOVA on ranks with a Dunn's post-hoc test as normality could not be achieved by data transformation. ($P < 0.05$; $n = 6–10$)

B.

Nominal concentration	Control/pre-exposure	High external ammonia
Series 1	$7.0 \pm 0.5_{a}$	–
Series 2	9.6 ± 0.3^{a}	1613.2 ± 22.8^{b}

Means not sharing the same letter in either series are significantly different from one another as determined by a one-way ANOVA on ranks with a Dunn's post-hoc test as normality could not be achieved by data transformation. ($P < 0.05$; $n = 6–18$).

3.2. Series 2 — Cu and HEA co-exposures in juvenile trout

No mortalities were observed in response to exposure to nominal Cu concentrations of 50 or 200 μg/l or to 1.5 mmol/l NH_4HCO_3 (HEA) in the presence or absence of Cu. The mean measured dissolved Cu concentrations in HEA exposures were 3.2 ± 1.5, 49.8 ± 3.6, and 160.7 ± 13.6 μg/l, for control, 50 μg/l, and 200 μg/l treatments, respectively (Table 1A). Mean initial water T_{amm} in this series for both the pre-exposure and high external ammonia periods has also been included in Table 1B.

Juvenile rainbow trout exposed to 50 μg/l Cu displayed no significant changes in J_{amm} over the first 3 h of exposure (Fig. 4), similar to the results observed in fry. Upon transfer to HEA, both groups demonstrated a reversal of J_{amm} over the first 3 h of exposure, such that ammonia was being taken up from the environment, though this escaped statistical significance ($P = 0.061$) in the control group (Fig. 4). Over the second 3 h of HEA exposure ($t = 6–9$ h) there was a further, significant increase in ammonia uptake by the control group, whereas Cu exposure appeared to attenuate this increase such that ammonia uptake (*i.e.*, positive J_{amm}) was significantly lower in the Cu-exposed fish compared to control fish (Fig. 4).

Based on these initial results, a second experiment (Fig. 5) was conducted with a 6-h pre-HEA exposure to nominal 200 μg/l Cu in an attempt to determine if Cu could completely block ammonia uptake during HEA. In this experiment, ammonia excretion was significantly inhibited over the 3–6 h time point, prior to HEA exposure (Fig. 5). Following 6 h, fish were transferred to HEA where, in control fish, J_{amm} decreased significantly such that it was reversed, representing an uptake of ammonia from the environment. In fish exposed to 200 μg/l Cu, however, no significant difference in J_{amm} was observed over the first 3 h of HEA exposure compared to pre-HEA values (Fig. 5), indicating that Cu exposure inhibited ammonia uptake. Similar to the data obtained in the 50 μg/l Cu exposure, by the last 3 h of HEA exposure ($t = 9–12$) ammonia uptake was significantly lower in Cu-exposed fish compared to control fish (Fig. 5). Thus, in the same experiment, exposure to 200 μg/l Cu significantly inhibited both the efflux of ammonia under control conditions (at 3–6 h) and the influx of ammonia under HEA (at 9–12 h; Fig. 5).

4. Discussion

4.1. Overview

In rainbow trout fry, exposure to 50 μg/l Cu caused a significant and sustained inhibition of J_{amm} up to 24 h of exposure (Fig. 1), while J^{Na}_{in} appeared to be only transiently inhibited and was recovered by 12 h of Cu exposure (Fig. 2), though variability in J^{Na}_{in} in Cu-exposed

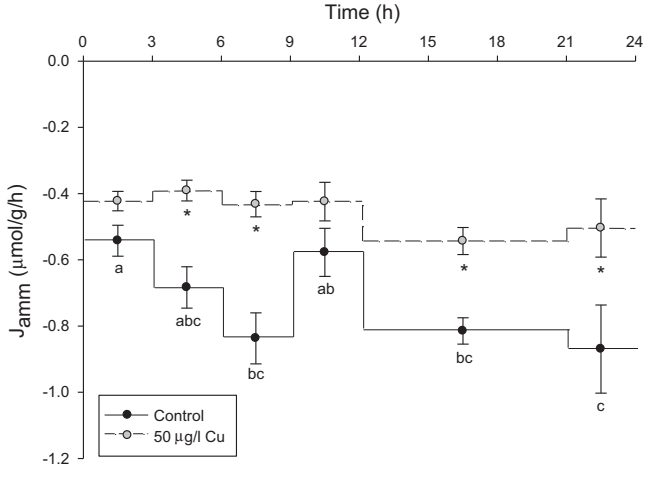

Fig. 1. Ammonia excretion rates (J_{amm}) during exposure to control conditions (nominal 0 μg/l Cu) and nominal 50 μg/l Cu over 24 h. Means which do not share the same letter within the control or Cu-exposed groups are significantly different from one another and asterisks represent significant differences between control and Cu-exposed groups at a given time point as determined by a two-way ANOVA with a Holm–Sidak post-hoc test; there was not a significant interaction between factors ($P < 0.05$; $n = 6–12$).

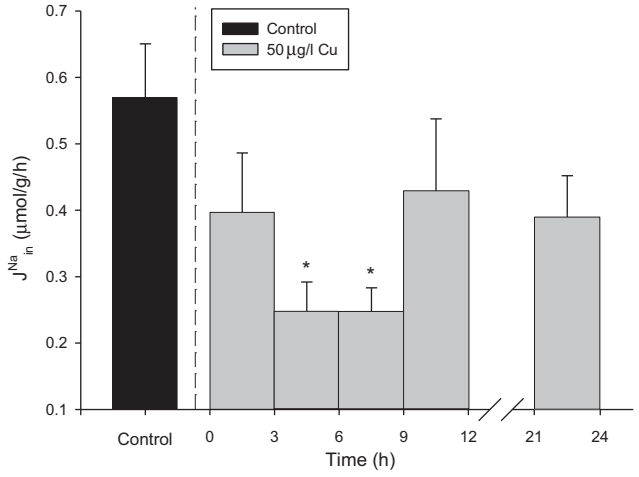

Fig. 2. Unidirectional Na^+ uptake (J^{Na}_{in}) in a control (nominal 0 μg/l Cu) group of fish, representing an average of uptake from fish at 0–3 h and 21–24 h, and in fish exposed to nominal 50 μg/l Cu over 24 h. Asterisks indicate significant differences between fish exposed to Cu and the control group as determined by a one-way ANOVA with a Holm–Sidak post-hoc test ($P < 0.05$; $n = 6$).

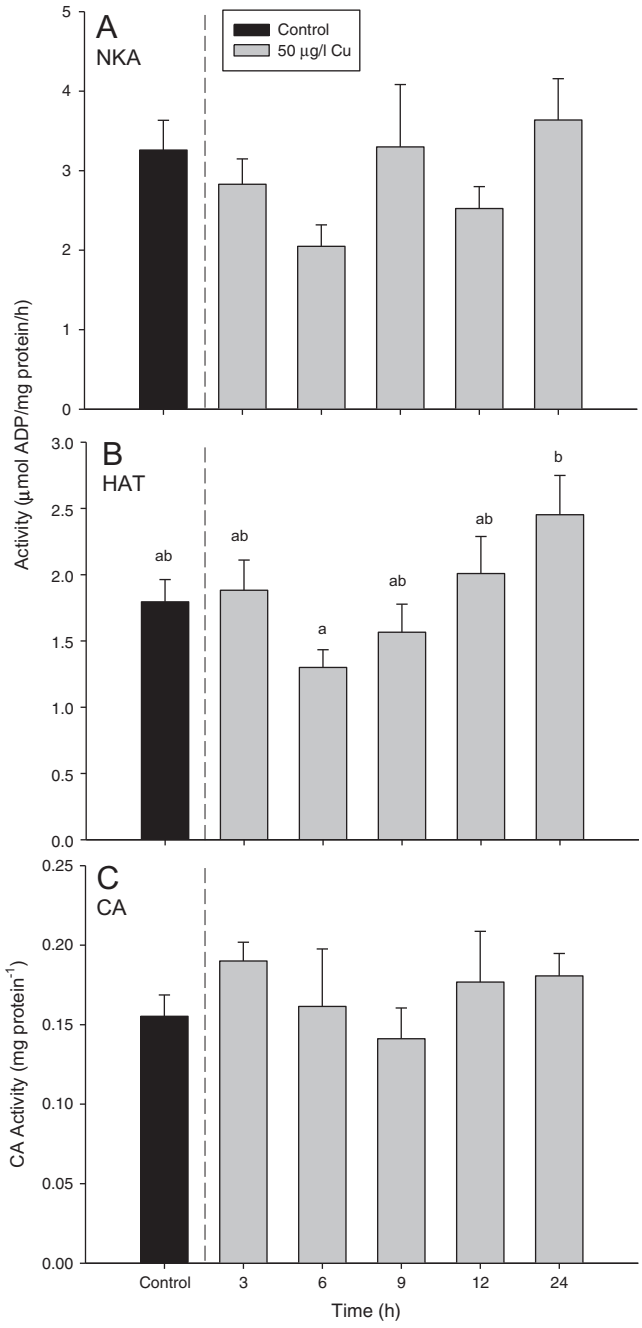

Fig. 3. Enzymatic activities of (A) Na^+/K^+-ATPase (NKA), (B) H^+-ATPase (HAT), and (C) carbonic anhydrase (CA) in the gills of a control (nominal 0 µg/l Cu) group of fish, representing an average of activities from fish at 3 h and 24 h, and in the gills of fish exposed to nominal 50 µg/l Cu over 24 h. Means which do not share the same letter are significantly different from one another as determined by a one-way ANOVA with a Holm–Sidak post-hoc test ($P < 0.05$; $n = 6$).

fish may have masked potential inhibition at 21–24 h of Cu exposure ($P = 0.099$). Although J_{amm} and J^{Na}_{in} are believed to be coupled in freshwater fish (see Wright and Wood, 2009, 2012), it appears possible from this work that exposure to Cu may uncouple these processes from one another. The inhibition of J^{Na}_{in} is believed to be a function of branchial Na^+ channel blockade and/or inhibition of branchial Na^+/K^+-ATPase activity (see Grosell, 2012), while the mechanism for the inhibition of J_{amm} is not clear. Despite inhibitions of both J_{amm} and J^{Na}_{in}, there was no evidence for the inhibition of gill Na^+/K^+-ATPase activity in rainbow trout fry in the present experiments (Fig. 3A), nor were there significant effects on H^+-ATPase or CA (Fig. 3B, C), two other important

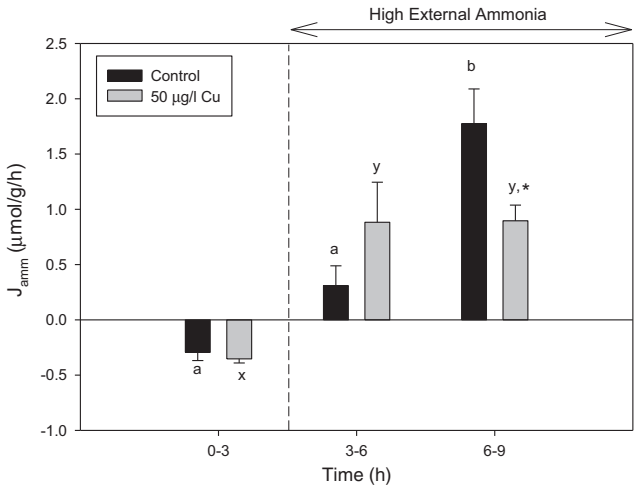

Fig. 4. Ammonia excretion rates (J_{amm}) in fish exposed to control (nominal 0 µg/l Cu) conditions and to nominal 50 µg/l Cu in ammonia-free water for 3 h followed by a 6 h exposure to high environmental ammonia (1.5 mmol/l NH_4HCO_3). Means which do not share the same letter within the control or Cu-exposed groups are significantly different from one another and asterisks represent significant differences between control and Cu-exposed groups at a given time point as determined by a two-way ANOVA with a Holm–Sidak post-hoc test; there was a significant interaction between factors ($P < 0.05$; $n = 6$).

components of the Na^+/NH_4^+ exchange mechanism (see Wright and Wood, 2009, 2012) and potential targets of Cu toxicity (Grosell, 2012; Zimmer et al., 2012). However, Grosell (2012) also noted that there was a need to investigate the possible effects of Cu on gill Rh glycoproteins, which could represent another potential target, one which has never been examined prior to the present study.

A property unique to the Rh glycoproteins, in contrast to other potential targets of Cu in the gill, is that they are bi-directional transporters (Nawata et al., 2010). In accord with this property, juvenile trout exposed acutely to 200 µg/l Cu exhibited an inhibition of ammonia excretion prior to HEA exposure but at both Cu concentrations there was an inhibition of ammonia uptake during HEA exposure (Figs. 4 and 5). In the 200 µg/l Cu exposure, the inhibition of ammonia transport

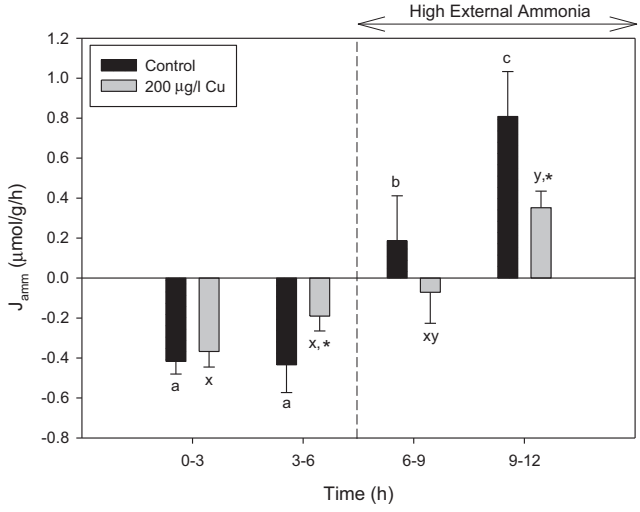

Fig. 5. Ammonia excretion rates (J_{amm}) in fish exposed to control (nominal 0 µg/l Cu) conditions and to nominal 200 µg/l Cu in ammonia-free water for 6 h followed by a 6 h exposure to high environmental ammonia (1.5 mmol/l NH_4HCO_3). Means which do not share the same letter within the control or Cu-exposed groups are significantly different from one another and asterisks represent significant differences between control and Cu-exposed groups at a given time point as determined by a two-way ANOVA with a Holm–Sidak post-hoc test; there was a significant interaction between factors ($P < 0.05$; $n = 7–9$).

might be attributable to an increase in pre-exposure time (3 h to 6 h) and/or concentration (50 μg/l to 200 μg/l).

HEA in these experiments was achieved using NH_4HCO_3 in order to minimize acid–base disturbances in the fish. However, the presence of additional carbonate ions may have influenced the speciation of Cu in solution. Using Visual MINTEQ (Visual MINTEQ ver. 3.0, KTH, Dept. of Land and Water Resources Engineering, Stockholm, Sweden), the speciation of Cu under control and HEA conditions was determined and the presence of Cu^{2+} was 1.5% and 1.0% of total in control and HEA, respectively, which was the function of increased HCO_3^- and not NH_4^+, as the proportion of Cu^{2+} was identical when substituting 1.5 mmol/l $NaHCO_3$ in place of NH_4HCO_3 in the MINTEQ input. Regardless, this difference in speciation did not seem to affect the capacity of Cu to block ammonia transport as in the 200 μg/l series, Cu inhibited the bi-directional transport of ammonia in the presence or absence of additional carbonate ions from NH_4HCO_3.

Overall, to our knowledge, this is the first demonstration of the inhibition of bi-directional ammonia transport by Cu. We propose a model by which Cu acts on Rh proteins to cause the sustained reduction of J_{amm} and the transient reduction of J^{Na}_{in}.

4.2. Toxic mechanism of action of Cu

The final lethal mechanism of Cu toxicity is likely a cardiovascular dysfunction resulting from a loss of osmotic balance (see Wilson and Taylor, 1993; Grosell, 2012), but the mechanism(s) by which Cu causes various sub-lethal effects leading to this remain incompletely understood. Disturbances in ammonia balance, indicated by increases in plasma ammonia and/or reductions in J_{amm} have been observed repeatedly in freshwater fish (Lauren and McDonald, 1985; Wilson and Taylor, 1993; Wang et al., 1998; Zimmer et al., 2012, 2014). Grosell et al. (2002) first proposed the inhibition of branchial CA activity as the underlying mechanism of Cu-induced inhibition of ammonia excretion. Despite many studies demonstrating the *in vitro* inhibition of CA activity by Cu (Magid, 1967; Vitale et al, 1999; DiTusa et al., 2001), to date only one study (on the guppy, *Poecilia vivipara*) has been able to demonstrate *in vivo* inhibition of gill CA activity in Cu-exposed fish (Zimmer et al., 2012). Indeed, in the present study, rainbow trout fry demonstrated no adverse effect of Cu exposure on gill CA activity (Fig. 3B), despite the significant inhibition of J_{amm} (Fig. 1). Possibly, this may be due to the nature of the CA activity assay which requires a large dilution of tissue homogenates, causing a dilution of Cu and a potential loss of inhibition (see Grosell, 2012). Regardless, there is insufficient evidence at hand to conclude that the reduction of J_{amm} by Cu is caused by effects on CA activity alone.

As in the present study (Fig. 3A, B), the branchial activities of other key components of the ammonia excretion mechanism in fish, such as Na^+/K^+-ATPase and H^+-ATPase, are also unresponsive to Cu exposure in many cases where ammonia balance is disturbed (Grosell et al., 2003; Blanchard and Grosell, 2006; Zimmer et al., 2012). Interestingly, H^+-ATPase activity increased significantly from 6 to 24 h (Fig. 3B) in the present study, potentially acting to restore J^{Na}_{in} by increasing electrogenic uptake of Na^+. A similar result was also observed in *P. vivipara*, where the eventual restoration of J_{amm} occurred in parallel with an increase in H^+-ATPase activity over the course of 96 h of Cu exposure (Zimmer et al., 2012). No study to date has addressed the effects of Cu on Rh proteins, arguably the most critical component of the ammonia excretion mechanism.

The effects of Cu on J^{Na}_{in} in freshwater fish have been studied extensively (Lauren and McDonald, 1987a, 1987b; Grosell and Wood, 2002; Blanchard and Grosell, 2006; Zimmer et al., 2014) and the mechanism of action has been generally well-characterized with gill Na^+/K^+-ATPase and putative Na^+ channels acting as primary targets (see Grosell, 2012 for review). Recently, however, it was demonstrated that in larval zebrafish (*Danio rerio*) acclimated to low pH, morpholino knockdown of Rh proteins resulted in a decrease in both J_{amm} and J^{Na}_{in} (Kumai and Perry, 2011). Thus, the blockade of Rh proteins by Cu could also account for the reduction in J^{Na}_{in} commonly observed in freshwater fish exposed to Cu. Indeed, in the present study, inhibition of J^{Na}_{in} occurred in the absence of observable reductions in gill Na^+/K^+-ATPase, H^+-ATPase, or CA activities (Fig. 3). Nevertheless, in the case of all of these enzymes, we cannot eliminate the possibility that this may be an artifact of sample dilution required by the assay (see Grosell, 2012).

4.3. Cu inhibits the bi-directional transport of ammonia

Ammonia uptake can be elicited *via* acute exposure to HEA, leading to increases in plasma ammonia levels (Wilson et al., 1994; Nawata et al., 2007; Zimmer et al., 2012, 2014; Liew et al., 2013). In the present study, both the inhibition of ammonia uptake by Cu under HEA (Fig. 4, 5), and the inhibition of ammonia excretion under control conditions (Figs. 1 and 5) may be explained by Rh protein blockade. Rh proteins are known to function bi-directionally (Nawata et al., 2010) and would thus facilitate both the excretion and uptake of ammonia. In general, Cu binds to peptide residues containing histidine, methionine, and cysteine (*e.g.*, She et al., 2003). In both human Rh and in the related bacterial ammonia transporter (AmtB), the pore of the protein channel consists of two consecutive histidine residues which act to conduct NH_3 (Khademi et al., 2004; Conroy et al., 2005). Thus, it is possible that Cu might interact with these residues, potentially leading to the observed bi-directional inhibition of ammonia transport. In future studies, *in vitro* methods will be useful in assessing the affinity of Cu for Rh channels. Assessing the transport of ammonia by Rh-expressing *Xenopus* oocytes (*e.g.*, Nawata et al., 2010) in the presence of Cu could also shed light on the specific role of Rh proteins in the toxic mechanism of action of Cu.

Nevertheless, we cannot eliminate another potential explanation of the bi-directional inhibition of ammonia transport: non-specific gill damage such as lamellar thickening, fusion of lamellae, lamellar blebbing, cellular hypertrophy, and many other structural aberrations which might increase the water-to-blood diffusion distance (Baker, 1969; Wilson and Taylor, 1993; Sola et al., 1995; De Boeck et al., 2001; van Heerden et al., 2004; Al-Bairuty et al., 2013). In one study, these changes were demonstrated to occur in as little as 4 h of exposure to Cu levels relevant to the present study (100 μg/l) in water of similar hardness to that used in the present study (DeBoeck et al., 2001). Similarly, van Heerden et al. (2004) demonstrated that the arithmetic mean thickness of the gill epithelium of rainbow trout increased significantly following 4 h of exposure to approximately 100 μg/l Cu. Therefore, we cannot discount the possibility that Cu-induced increases in gill epithelial diffusion distance caused a decrease in ammonia uptake in the present study (Figs. 4 and 5). In future studies, it will be useful to define the time course and threshold concentration of these possible morphological responses with respect to Cu-induced effects on ammonia transport.

4.4. Conclusions and perspectives

Cu exposure in rainbow trout fry led to a sustained inhibition of J_{amm} and an apparent transient inhibition of J^{Na}_{in}. Moreover, we demonstrated, for the first time, the bi-directional inhibition of ammonia transport by Cu in juvenile rainbow trout. Based on these observations, we propose a model whereby Cu acts upon Rh proteins, potentially in addition to other targets such as Na^+/K^+-ATPase, epithelial Na^+ channels, and/or CA, leading to a reduction in both J_{amm} and J^{Na}_{in}. The eventual restoration of J^{Na}_{in} may be attributed to increased H^+-ATPase activity over time, similar to the restoration of J_{amm} by 96 h of exposure in a previous study (Zimmer et al., 2012). This increase in H^+-ATPase activity, however, did not result in the restoration of J_{amm} over 24 h of Cu exposure in the present study, possibly due to continued Rh blockade by Cu. Overall, the data presented here warrant further investigation into the

involvement of Rh proteins, integral to both J_{amm} and J^{Na}_{in} freshwater fish, in the mechanism of sub-lethal Cu toxicity.

Acknowledgments

Special thanks to Dr. Scott Smith, Wilfrid Laurier University, for helpful information regarding the chemical speciation of Cu and ammonia. Thanks also to two anonymous referees for constructive comments which improved the manuscript. This research was supported by an NSERC (Natural Sciences and Engineering Research Council of Canada) (RGPIN473-12) Discovery Grant to CMW, who was also supported by the Canada Research Chairs. AMZ is supported by an NSERC Canada Graduate Scholarship.

References

Al-Bairuty, G.A., Shaw, B.J., Handy, R.D., Henry, T.B., 2013. Hisopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 126, 104–115.

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

Blanchard, J., Grosell, M., 2006. Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: is copper an ionoregulatory toxicant in high salinities? Aquat. Toxicol. 80, 131–139.

Conroy, M.J., Bullough, P.A., Merrick, M., Avent, N.D., 2005. Modelling the human rhesus proteins: implications for structure and function. Brit. J. Haematol. 131, 543–551.

De Boeck, G., Vlaeminck, A., Balm, P.H.M., Lock, R.A.C., De Wachter, B., Blust, R., 2001. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: interactions between Cu^{2+} and plasma cortisol elevation. Environ. Toxicol. Chem. 20, 374–381.

DiTusa, C.A., Christensen, T., McCall, K.A., Fierke, C.A., Toone, E.J., 2001. Thermodynamics of metal binding. 1. Metal ion binding by wild-type carbonic anhydrase. Biogeosciences 40, 5338–5344.

Grosell, M., 2012. Copper. In: Farrell, A.P., Wood, C.M., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Essential Metals. Fish Physiology 31A. Academic Press, San Diego, pp. 54–110.

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

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

Grosell, M., Wood, C.M., Walsh, P.J., 2003. Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxecephalus octodecemspinosus* during exposure to elevated water-borne copper. Comp. Biochem. Physiol. C 135, 179–190.

Grosell, M., McDonald, M.D., Walsh, P.J., Wood, C.M., 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Ospanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. Aquat. Toxicol. 68, 263–275.

Henry, R.P., 1991. Techniques for measuring carbonic anhydrase activity in vitro: the electrometric delta pH and pH stat assay. In: Dodgson, S.J., Tashian, R.E., Gros, G., Carter, N.D. (Eds.), The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics. Plenum, New York, pp. 119–126.

Khademi, S., O'Connell III, J., Remis, J., Robles-Colmenares, Y., Miercke, L.J.W., Stroud, R.M., 2004. Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 Å. Science 305, 1587–1594.

Kumai, Y., Perry, S.F., 2011. Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R1517–R1528.

Lauren, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. J. Comp. Physiol. B. 155, 635–644.

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

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

Liew, H.J., Sinha, A.K., Nawata, C.M., Blust, R., Wood, C.M., De Boeck, G., 2013. Differential responses in ammonia excretion, sodium fluxes and gill permeability explain different sensitivities to acute high environmental ammonia in three freshwater teleosts. Aquat. Toxicol. 126, 63–76.

Magid, E., 1967. The activity of carbonic anhydrases B and C from human erythrocytes and the inhibition of the enzymes by copper. Scand. J. Haemat. 4, 257–270.

McCormick, S.D., 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can. J. Fish. Aquat. Sci. 50, 656–658.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K., Hirose, S., 2007. Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R1743–R1753.

Nawata, C.M., Hung, C.Y., Tsui, T.K.N., Wilson, J.M., Wright, P.A., Wood, C.M., 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol. Genomics 31, 463–474.

Nawata, C.M., Wood, C.M., O'Donnell, M.J., 2010. Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. J. Exp. Biol. 213, 1049–1059.

Ng, T.Y.-T., Chowdhury, M.J., Wood, C.M., 2010. Can the biotic ligand model predict Cu toxicity across a range of pHs in softwater-acclimated rainbow trout? Environ. Sci. Technol. 44, 6263–6268.

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

She, Y.-M., Narindrasorasak, S., Yang, S., Spitale, N., Roberts, E.A., Sarkar, B., 2003. Identification of metal-binding proteins in human hepatoma lines by immobilized metal affinity chromatography and mass spectrometry. Mol. Cell. Proteomics 2, 1306–1318.

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

USEPA, 2007. Aquatic Life Ambient Freshwater Quality Criteria — Copper, EPA-822-07-001, 2007 Revision. US Environmental Protection Agency, Washington, DC.

Van Heerden, D., Vosloo, A., Nikinmaa, M., 2004. Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor-1α (HIF-1α) levels in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 69, 271–280.

Verdouw, H., van Echteld, C.J.A., Dekkers, E.M.J., 1978. Ammonia determination based on indophenols formation with sodium salicylate. Water Res. 12, 399–402.

Vitale, A.M., Monserrat, J.M., Castilho, P., Rodriguez, E.M., 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulate* (Decapoda, Grapsidae). Comp. Biochem. Physiol. C 122, 121–129.

Wang, T., Knudsen, P.K., Brauner, C.J., Busk, M., Vijayan, M.M., Jensen, F.B., 1998. Copper exposure impairs intra- and extracellular acid-base regulation during hypercapnia in the fresh water rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. B. 1698, 591–599.

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

Wilson, R.W., Wright, P.M., Munger, S., Wood, C.M., 1994. Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na^+/NH_4^+ exchange. J. Exp. Biol. 191, 37–58.

Wright, P.A., Wood, C.M., 2009. A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J. Exp. Biol. 212, 2303–2312.

Wright, P.A., Wood, C.M., 2012. Seven things fish know about ammonia and we don't. Respir. Physiol. Neurobiol. 184, 231–240.

Zimmer, A.M., Barcarolli, I.F., Wood, C.M., Bianchini, A., 2012. Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. Aquat. Toxicol. 122–123, 172–180.

Zimmer, A.M., Brauner, C.J., Wood, C.M., 2014. Exposure to waterborne Cu inhibits cutaneous Na^+ uptake in post-hatch larval rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 150, 151–158.