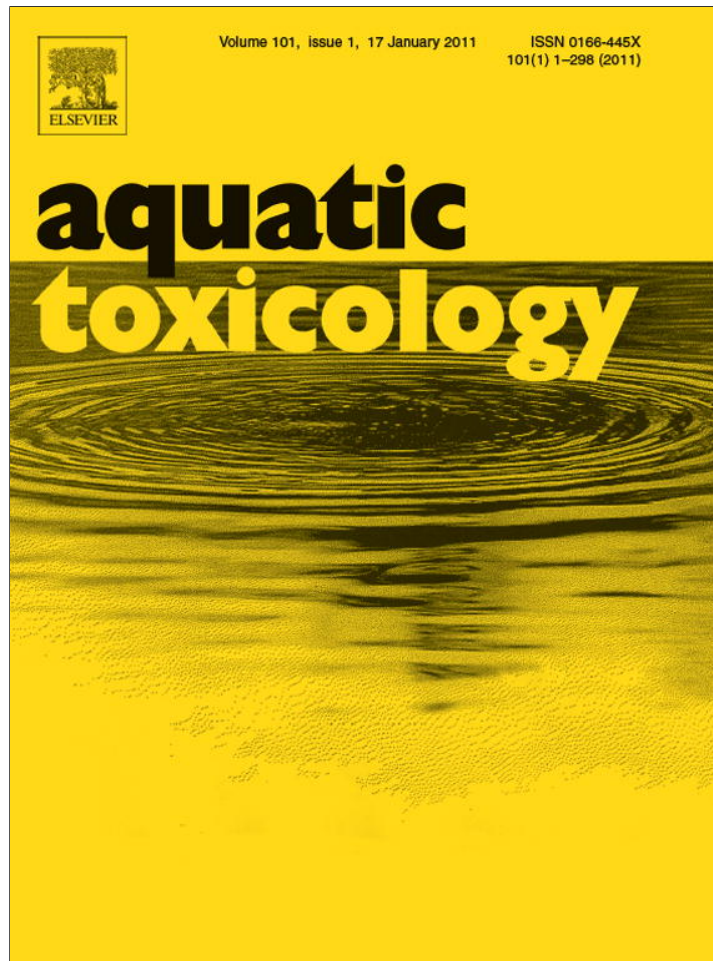


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# Aquatic Toxicology

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## Metal uptake and acute toxicity in zebrafish: Common mechanisms across multiple metals

Derek Alsop\*, Chris M. Wood

Department of Biology, McMaster University, 1280 Main St. W., Hamilton, ON L8S 4K1, Canada

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### ABSTRACT

Zebrafish larvae (*Danio rerio*) were used to examine the mechanisms of action and acute toxicities of metals. Larvae had similar physiological responses and sensitivities to waterborne metals as adults. While cadmium and zinc have previously been shown to reduce  $\text{Ca}^{2+}$  uptake, copper and nickel also decreased  $\text{Ca}^{2+}$  uptake, suggesting that the epithelial transport of all these metals is through  $\text{Ca}^{2+}$  pathways. However, exposure to cadmium, copper or nickel for up to 48 h had little or no effect on total whole body  $\text{Ca}^{2+}$  levels, indicating that the reduction of  $\text{Ca}^{2+}$  uptake is not the acute toxic mechanism of these metals. Instead, mortalities were effectively related to whole body  $\text{Na}^+$ , which decreased up to 39% after 48 h exposures to different metals around their respective 96 h LC50s. Decreases in whole body  $\text{K}^+$  were also observed, although they were not as pronounced or frequent as  $\text{Na}^+$  losses. None of the metals tested inhibited  $\text{Na}^+$  uptake in zebrafish ( $\text{Na}^+$  uptake was in fact increased with exposure) and the observed losses of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were proportional to the ionic gradients between the plasma and water, indicating diffusive ion loss with metal exposure. This study has shown that there is a common pathway for metal uptake and a common mechanism of acute toxicity across groups of metals in zebrafish. The disruption of ion uptake accompanying metal exposure does not appear to be responsible for the acute toxicity of metals, as has been previously suggested, but rather the toxicity is instead due to total ion loss (predominantly  $\text{Na}^+$ ).

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### 1. Introduction

Metals are some of the more toxic substances of concern in the environment (Martins et al., 2007), and due to their wide spread dispersal from mining, coal burning, sewage, as well as agricultural, industrial and domestic runoff, they are of global concern. For fish in the aquatic environment, the acute toxic target of metals is primarily ionoregulation. In salmonids for example, cadmium and zinc disrupt  $\text{Ca}^{2+}$  uptake and homeostasis (Hogstrand et al., 1996; Niyogi and Wood, 2004) while copper impacts  $\text{Na}^+$  uptake and homeostasis (Grosell and Wood, 2002; Handy et al., 2002; Taylor et al., 2003). Metals such as nickel may also have effects on respiration (Pane et al., 2003).

Zebrafish are a popular model vertebrate in many areas of research including developmental biology (Lieschke and Currie, 2007). As a result, embryo and larval development are well characterized (Kimmel et al., 1995; Parichy et al., 2009). A number of recent studies have utilized zebrafish embryos for evaluating chemical toxicity, either effects on embryonic development (ter-

atogenicity) and survivorship (e.g. Nagel, 2002; Lammer et al., 2009; Selderslaghs et al., 2009; Brannen et al., 2010), or molecular endpoints (gene transcript abundance; Voelker et al., 2007; Liedtke et al., 2008).

For many contaminants, the sensitivities of zebrafish embryos and larvae are similar (Lammer et al., 2009). However, the egg chorion can act as a barrier to protect fish embryos from various contaminants including metals, some polymers, polychlorinated biphenyls, antibiotics and high molecular weight non-ionic surfactants (Guadagnolo et al., 2001; Finn, 2007; Lammer et al., 2009), resulting in lower sensitivities to waterborne exposures of these contaminants. Indeed, metal sensitivity is highest during the larval stage, compared to other times in the life history of a fish (Chapman, 1978; Dave and Xiu, 1991). Therefore, a larval assay would provide metal toxicity data for the stage requiring the greatest level of protection.

Presently, we have developed an acute toxicity assay using zebrafish larvae that has a number of advantages. The small size of the larvae (length = 3–4 mm) and the assay (6 well culture plates) saves space, time and resources. At the start of the exposures [4 d post fertilization (dpf) or 1–2 d post hatch (dph)], the fish have differentiated and functional tissues and organs (Kimmel et al., 1995; Parichy et al., 2009), and are sustained by their yolk reserve.

\* Corresponding author. Tel.: +1 905 525 9140x23550; fax: +1 905 522 6066.  
 E-mail addresses: [alsopde@mcmaster.ca](mailto:alsopde@mcmaster.ca), [dalsop@uwaterloo.ca](mailto:dalsop@uwaterloo.ca) (D. Alsop).

We hypothesize larvae will have similar physiological responses to metals as adults. With this assay, the uptake and the acute toxicity of metals were examined, with a focus on ion homeostasis. New mechanisms were discovered, and these were similar across groups of metals. Differences between zebrafish and rainbow trout were also observed, and are discussed.

## 2. Materials and methods

### 2.1. Animals

Adult zebrafish (*Danio rerio*; 0.3–0.9 g) were purchased from a commercial supplier and held in three 40-L aquaria with 25 fish per tank. Each tank was equipped with aeration and a recirculating charcoal filter while photoperiod was maintained at 12 h light/12 h dark and temperature at 28 °C. Water was hard, dechlorinated City of Hamilton tap water, from Lake Ontario (hardness = 141 mg CaCO<sub>3</sub>/L, see Section 2.3. for more details). Fish were fed to satiation two times daily with commercial pellets (New Life Spectrum; Homestead, Florida).

Fertilized eggs were acquired with egg-capturing trays placed in each tank prior to the end of the light period and allowed to develop at 28.5 °C, similar to Alsop and Vijayan (2008). Hatching typically occurred between 48 and 72 hpf. Fertilization and hatching rates were both >95%.

Juvenile rainbow trout (approximately 1 g each) were purchased from Humber Springs Trout Hatchery (Orangeville, ON). These were held in a 20-L tank with aeration, a fresh water flow of 250 mL/min at 12 °C (dechlorinated tap water), 12 h light/12 h dark and fed commercial trout pellets once daily.

### 2.2. Chemicals

The metal salts AgNO<sub>3</sub>, Ca(NO<sub>3</sub>)<sub>2</sub>, Cd(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O and CsCl<sub>2</sub> were obtained from Fisher Scientific (Ottawa, ON). Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, LaCl<sub>3</sub>·7H<sub>2</sub>O, Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, NaNO<sub>3</sub>, NiSO<sub>4</sub>·6H<sub>2</sub>O, Pb(NO<sub>3</sub>)<sub>2</sub> and ZnSO<sub>4</sub>·7H<sub>2</sub>O were purchased from Sigma–Aldrich (Oakville, ON). Stock metal solutions for exposures were prepared in deionized water (acidified to 0.05% HNO<sub>3</sub>) and stored at 4 °C. The radioisotopes <sup>45</sup>Ca (as <sup>45</sup>CaCl<sub>2</sub>), <sup>22</sup>Na (as <sup>22</sup>NaCl) and <sup>63</sup>Ni (as <sup>63</sup>NiCl<sub>2</sub>) were purchased from PerkinElmer (Boston, MA).

### 2.3. Exposures

All larval experiments were performed in polystyrene 6-well tissue culture plates (Falcon™) with 10 mL of water and 10 larvae per well (10–12 larvae in experiments examining radiolabeled cation uptake or whole body cation levels). Exposures were conducted at 28 °C and 99% of the water was changed every 24 h when exposures exceeded 1 d (e.g. 96 h LC50s). Exposures with adult zebrafish were conducted in 9-L plastic tanks at 26.5 °C with aeration.

Exposures were conducted in one of three water types: (1) hard water from Lake Ontario (hardness = 141 mg CaCO<sub>3</sub>/L, pH 7.8, Na<sup>+</sup> = 700 μM, K<sup>+</sup> = 38 μM, Ca<sup>2+</sup> = 1350 μM, Mg<sup>2+</sup> = 336 μM, dissolved organic carbon (DOC) = 3.5 mg/L), (2) soft water (hardness = 7.8 mg CaCO<sub>3</sub>/L, pH 7.34, Na<sup>+</sup> = 70 μM, K<sup>+</sup> = 3 μM, Ca<sup>2+</sup> = 61 μM, Mg<sup>2+</sup> = 17 μM, DOC = 0.9 mg/L), and (3) soft water for lead exposures only (hardness = 11.7 mg CaCO<sub>3</sub>/L, pH 7.48, Na<sup>+</sup> = 220 μM, K<sup>+</sup> = 14 μM, Ca<sup>2+</sup> = 75 μM, Mg<sup>2+</sup> = 42 μM, DOC = 0.9 mg/L).

Water samples were collected for metal measurements before and after passage through an Acrodisc 0.45 μm Supor Membrane filter (Pall Life Sciences, Ville St. Laurent, QC) to determine total and dissolved concentrations, respectively. Water samples were acidified (to 0.5% HNO<sub>3</sub>) after collection.

Experiments examining the uptake of <sup>45</sup>Ca or <sup>22</sup>Na in zebrafish were 5 h in duration, at a concentration of 0.2 μCi of radionuclide per well (10 mL) for larval experiments, and 1.5 μCi/L for adult experiments, conducted in 800-mL plastic tanks with aeration. In the <sup>45</sup>Ca uptake experiments, larvae were exposed to 30, 300 and 3000 μg Cd/L, 150 μg Cu/L and 15 mg Ni/L, while adults were exposed to 50, 150 and 250 μg Cu/L (all in hard water). In the <sup>22</sup>Na uptake experiments, larvae were exposed to 3, 30 and 300 μg Cu/L, 300 and 3000 μg Cd/L, while adults were exposed to 30 and 300 μg Cu/L (all in hard water).

Effects of metal exposures on whole body cation levels were examined in zebrafish larvae and adults in hard water. Larvae were exposed to 100 μg Cu/L, 9000 μg Ni/L or 1200 μg Cd/L for 40 h, while adults were exposed to 20 and 200 μg Cu/L or 400 and 4000 μg Cd/L for 42 h.

Uptakes of <sup>22</sup>Na and <sup>45</sup>Ca (separately) by juvenile rainbow trout were tested over 7-h exposures in 3 L plastic tanks with aeration at 12 °C. Trout were exposed to the radionuclides in control hard water or hard water plus 150 μg Cu/L.

The uptake of <sup>63</sup>Ni (0.5 μCi/well, 5 h) and acute nickel toxicity (96 h LC50) were examined with zebrafish larvae in soft water and soft water with the addition of 0.5 mM Na<sup>+</sup>, Mg<sup>2+</sup> or Ca<sup>2+</sup> (as nitrate salts).

For experiments involving <sup>45</sup>Ca, <sup>22</sup>Na and <sup>63</sup>Ni, the internal (fish) specific activity was always less than 1% of the external (water) specific activity, eliminating the need to correct for backflux (Maetz, 1956).

### 2.4. Analyses

Waterborne copper, nickel, lead and silver were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian, Palo Alto, CA), along with the certified reference material TM-15 (National Water Research Institute, Environment Canada, Burlington, ON). Water Na<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, cadmium and zinc levels were analyzed by flame atomic absorption spectroscopy (Spectra AA 220FS; Varian) after dilution with 1% HNO<sub>3</sub> (for Na<sup>+</sup>, cadmium and zinc measurements), 0.5% HNO<sub>3</sub> and 0.5% LaCl<sub>3</sub> (for Ca<sup>2+</sup> and Mg<sup>2+</sup> measurements) or 0.5% CsCl<sub>2</sub> (for K<sup>+</sup> measurements). Nominal concentrations only are reported for cobalt.

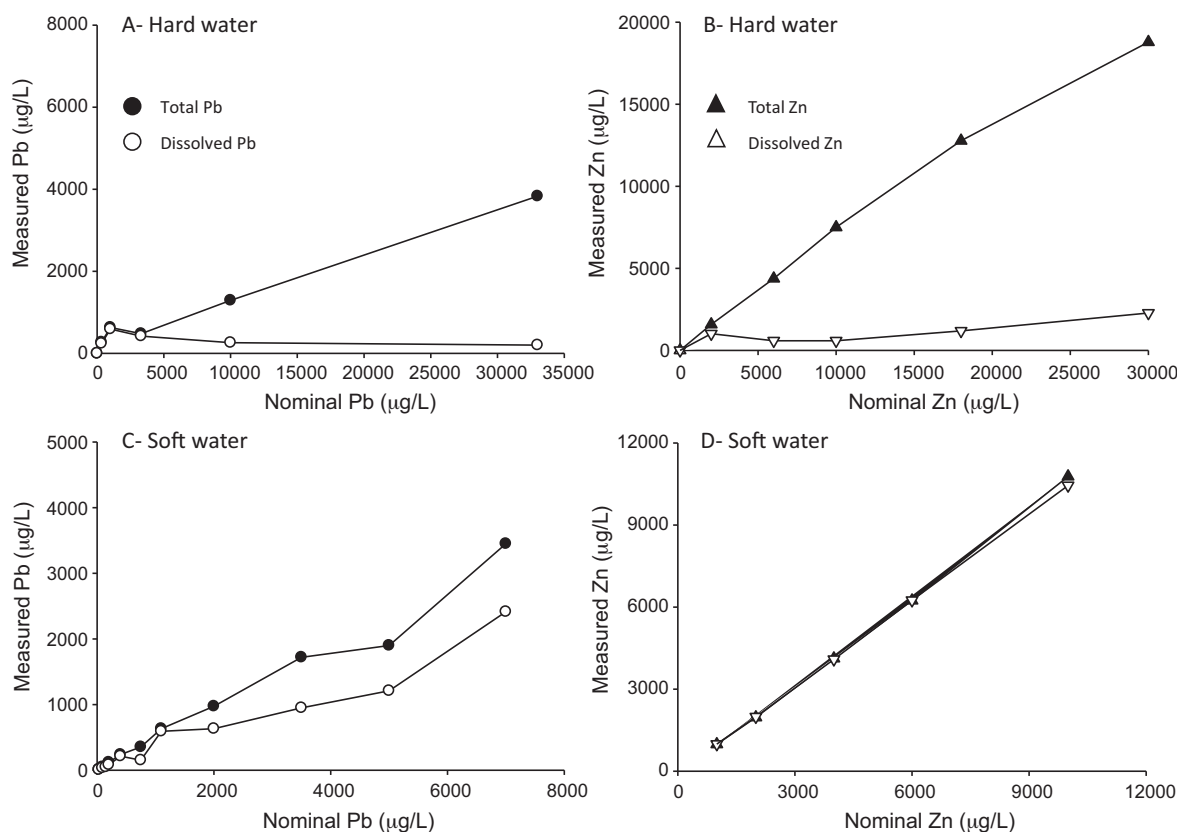
Whole body ion content or radioactive counting of larvae were determined by first anesthetizing fish with an overdose of MS222 (0.25 g/L), followed by three rinses with deionized water. Larvae were then digested in 1 mL 25% HNO<sub>3</sub> for 48 h at 60 °C. Adults were processed in a similar manner, except fish were digested in eight volumes of 25% HNO<sub>3</sub>. Na<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and K<sup>+</sup> in the digests were analyzed by flame atomic absorption spectroscopy, as above.

<sup>22</sup>Na and <sup>45</sup>Ca radioactivities were measured by a gamma counter (Wallac 1480 Wizard 3") and liquid scintillation counter (LKBWallac 1217 Rackbeta LSC), respectively. The counts were corrected for background and quenching.

### 2.5. Statistics

Ion uptake were first screened for normality and homogeneity of variance prior to a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test for multiple comparisons (SPSS) to determine significant differences among groups. Differences in whole body ions were analyzed with a Student's *t*-test (SPSS). Differences were considered significant if *p* < 0.05.

The environmental toxicity data analysis software, Tox Calc™ package (Tidepool Scientific Software), was used to estimate the 96 h LC50s with 95% confidence intervals (CI). The values were calculated from the survivorship and metal concentration data (measurement of the dissolved fraction when possible, otherwise



**Fig. 1.** Total and dissolved concentrations of lead and zinc in hard water [(A) and (B), respectively, 141 mg CaCO<sub>3</sub>/L] and soft water [(C) (11.7 mg CaCO<sub>3</sub>/L) and (D) (7.8 mg CaCO<sub>3</sub>/L), respectively] over a range of nominal concentrations. See Section 2.3 for a more complete description of water chemistries.

nominal) from all treatments. LC50 values were considered significantly different when the 95% CI did not overlap (Environment Canada, 2005).

### 3. Results

#### 3.1. Waterborne metal concentrations

The dissolved cadmium, copper, nickel and silver concentrations (i.e. measured after water samples were passed through a 0.45 µm filter) were consistently 94–100% of the total waterborne metal concentrations. In contrast, the dissolved lead and zinc levels in hard water were far less than the total levels, especially at the higher concentrations (Fig. 1A and B). In addition, total levels of lead or zinc in hard water were well below nominal concentrations (Fig. 1A and B), with visible formation of precipitates at higher concentrations. For example, lead at nominal concentrations of 1000 and 10,000 µg/L had total levels of 630 and 1290 µg/L and dissolved levels of only 590 and 200 µg/L, respectively (Fig. 1A). For zinc, nominal concentrations of 2000 and 10,000 µg/L resulted in total measured levels of 1581 and 7502 µg/L and dissolved levels of only 1014 and 587 µg/L, respectively. This loss of dissolved lead and zinc in hard water clearly explains the absence of mortalities at any nominal concentration of these metals (Table 1). In soft water, the recoveries of total and dissolved lead and zinc levels were much higher (Fig. 1C and D), which translated to increased toxicity (Table 1). The loss of lead in hard water, with high alkalinity, is similar to the findings of Besser et al. (2005) who found low recovery of total and dissolved waterborne lead at concentrations between 1000 and 16,000 µg Pb/L, and higher recovery at levels ≤ 120 µg Pb/L.

**Table 1**

Larval 96 h LC50s for a variety of metals in hard and soft water. Experiments were conducted in 6 well culture plates with 10 mL water and 10 larvae per well. Water was changed every 24 h. Cadmium and copper experiments were also performed with adults. LC50s for silver, cadmium, copper, nickel, lead and zinc are estimated using the measured levels of the dissolved waterborne metal fractions, while cobalt is based on nominal values. The measured total and dissolved values were typically within 10% of the nominal values, with the exceptions of lead and zinc in hard water (see footnotes and text for more solubility information). In all cases metals were significantly more toxic in soft water than in hard water (no overlap of the 95% CIs).

Metal	LC50 – hard water (µg/L)	LC50 – soft water (µg/L)
Cadmium	Larva – 1730 (CI 1107–2363) (other LC50 trials – 1417, 2251.7 and 2552) Adult – 3822 (CI 2643–5301)	121.8 (CI 77.7–168.9)
Cobalt	32,922 (CI 20,522–45,884)	4262 (CI 2635–6229)
Copper	Larva – 148.4 (CI 136.4–174.8) (other LC50 trials – 143.3, 144.4, 147.2, 256.8) Adult – 212.1 (CI 178.8–249.5)	11.66 (CI 8.87–20.13)
Lead	> 590 µg/L <sup>a</sup> No mortalities	52.9 (CI 26.2–105.3)
Nickel	13,120 (CI 10,098–16,647) (other LC50 trials – 9068, 18919)	589.9 (CI 281.6–814.6)
Silver	12.9 (CI 7.9–19.3)	3.11 (CI 2.06–4.46)
Zinc	>2280 µg Zn/L <sup>b</sup> No mortalities	2535 (CI 1620.8–3441.6)

<sup>a</sup> 590 µg Pb/L was the highest concentration of dissolved lead that was measured in hard water at a total lead concentration of 630 µg/L (nominal level of 1000 µg Pb/L). The highest lead level tested was 33,300 µg/L (nominal), although the total concentration measured was only 3830 µg/L while the dissolved fraction was 200 µg/L (Fig. 1A).

<sup>b</sup> 2280 µg Zn/L was the highest concentration of dissolved zinc that was measured in hard water at a total zinc concentration of 18,788 µg/L (nominal level of 30,000 µg Zn/L) (Fig. 1B).

### 3.2. Metal toxicity

Zebrafish sensitivity was dependent on the metal (Table 1). Larvae and adults were much more sensitive to the group 11 metals (copper and silver: larval LC50s of 148  $\mu\text{g Cu/L}$  and 13  $\mu\text{g Ag/L}$  in hard water) than the group 12 metals (zinc and cadmium: LC50s of  $\gg 2280 \mu\text{g Zn/L}$  and 1730  $\mu\text{g Cd/L}$ ) (Table 1), or other metals such as nickel (group 10; LC50s of 9068–18,919  $\mu\text{g/L}$  in different tests) or cobalt [group 9; LC50 = 32,922  $\mu\text{g/L}$  (nominal)] (Table 1). Similar to zinc, the 96 h LC50 for lead (group 14) in hard water could not be determined due to 100% larval survival in all exposure concentrations, most likely stemming from low solubility (see Section 3.1). However, lead and zinc solubility increased in soft water (Fig. 1C and D), which resulted in mortalities (96 h LC50s = 53  $\mu\text{g Pb/L}$  and 2535  $\mu\text{g Zn/L}$ ) (Table 1). Larvae were approximately 40% more sensitive to copper and greater than 2-fold more sensitive to cadmium than adults (Table 1).

All metals were more toxic in soft water (8 or 12 mg  $\text{CaCO}_3/\text{L}$ ) than hard water (141 mg  $\text{CaCO}_3/\text{L}$ ), with increased sensitivities ranging from 4.2-fold (silver) to 22.2-fold (nickel) (Table 1).

### 3.3. Cation homeostasis

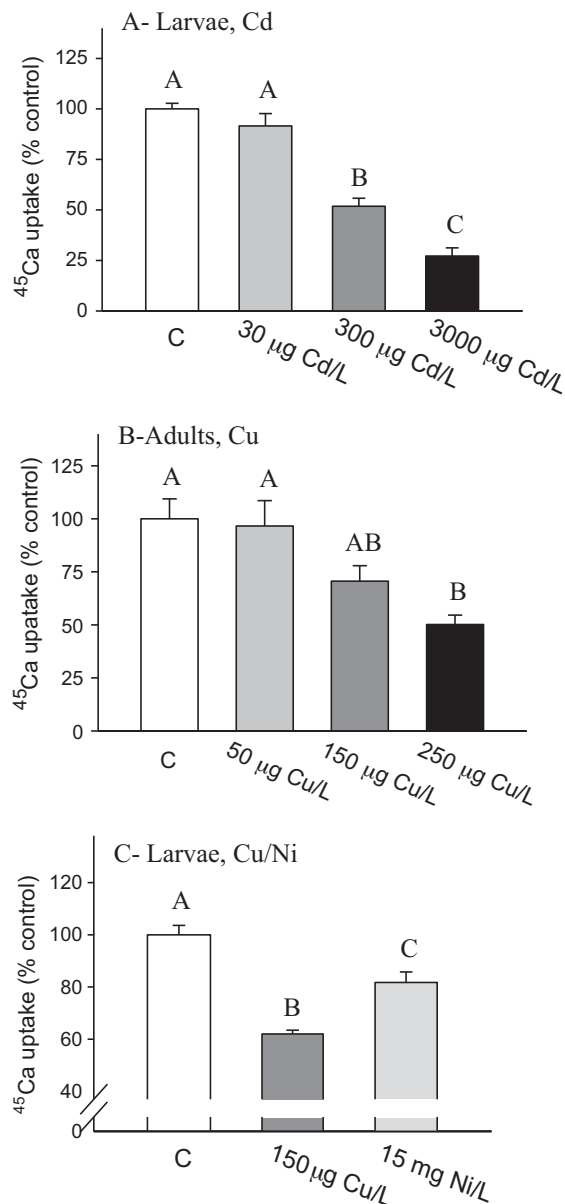
Cadmium exposures of 300 and 3000  $\mu\text{g/L}$  (below and above the cadmium LC50 of 1730  $\mu\text{g/L}$ ) reduced  $^{45}\text{Ca}$  uptake by 48% and 73%, respectively (Fig. 2A). In addition, adult zebrafish exposed to 50, 150 and 250  $\mu\text{g Cu/L}$  experienced a dose-dependent decrease in  $^{45}\text{Ca}$  uptake, to a maximum of 50% at 250  $\mu\text{g Cu/L}$  (Fig. 2B). Larvae exposed to 150  $\mu\text{g Cu/L}$  or 15,000  $\mu\text{g Ni/L}$  also experienced significant 38% and 18% decreases in  $^{45}\text{Ca}$  uptake (Fig. 2C).

Control cation levels averaged 10.7 nmol  $\text{Na}^+/\text{larva}$ , 14.4 nmol  $\text{K}^+/\text{larva}$ , 6.9 nmol  $\text{Ca}^{2+}/\text{larva}$  and 2.9 nmol  $\text{Mg}^{2+}/\text{larva}$ . Copper, nickel and cadmium exposures all impacted cation levels. For example, a 40 h exposure in hard water to 100  $\mu\text{g Cu/L}$  (67% of the copper LC50; Table 1) resulted in 25% mortality and significant decreases in survivor whole body  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  by 26%, 12.3% and 10.5%, respectively (Fig. 3A). There was no change in the level of  $\text{Mg}^{2+}$  (Fig. 3A). Exposure to 9000  $\mu\text{g Ni/L}$  (~50% of the nickel LC50; Table 1) did not result in any mortalities by 40 h, and induced a 9.5% decrease in whole body  $\text{Na}^+$ , with no changes in  $\text{K}^+$ ,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  (Fig. 3B). In addition, a 48-h exposure to 22,000  $\mu\text{g Ni/L}$  resulted in 10% mortality and a significant 36.9% reduction in whole body  $\text{Na}^+$  (with no change in  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , while  $\text{K}^+$  was not measured) (data not shown). Exposure to 1200  $\mu\text{g Cd/L}$  (69% of the cadmium LC50; Table 1) for 40 h resulted in 5.9% mortality and a significant 18% reduction in whole body  $\text{Na}^+$ , with no changes in  $\text{K}^+$ ,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  (Fig. 3C). Another cadmium exposure (1750  $\mu\text{g/L}$ , 48 h) resulted in 20% mortality, and significant decreases in  $\text{Na}^+$  (39.1%) and  $\text{Ca}^{2+}$  (14.6%), while there was no change in  $\text{Mg}^{2+}$  ( $\text{K}^+$  was not measured) (data not shown).

Adult zebrafish exposed for 48 h to 200  $\mu\text{g Cu/L}$  and 4000  $\mu\text{g Cd/L}$  in hard water experienced significant reductions in whole body  $\text{Na}^+$  of 30% and 18.4%, respectively (Fig. 4A). There were no effects of copper or cadmium on whole body  $\text{Ca}^{2+}$  (Fig. 4B).

Experiments were performed to examine the mechanism(s) underlying the decrease in whole body  $\text{Na}^+$  with metal exposure. First, a range of copper concentrations did not inhibit radiolabeled  $^{22}\text{Na}$  uptake from the water in larvae (Fig. 5A). Instead,  $^{22}\text{Na}$  uptake was significantly increased by 60% in larvae exposed to 300  $\mu\text{g Cu/L}$  (Fig. 5A). Similar results were observed in adults (Fig. 5B). Like copper, cadmium increased  $^{22}\text{Na}$  uptake in larvae (Fig. 5C), although total whole body  $\text{Na}^+$  levels were significantly decreased with exposure to both metals (Fig. 3A and C).

Similar to zebrafish, juvenile rainbow trout exposed to 150  $\mu\text{g Cu/L}$  experienced a 22.5% decrease in  $^{45}\text{Ca}$  uptake (Fig. 6).

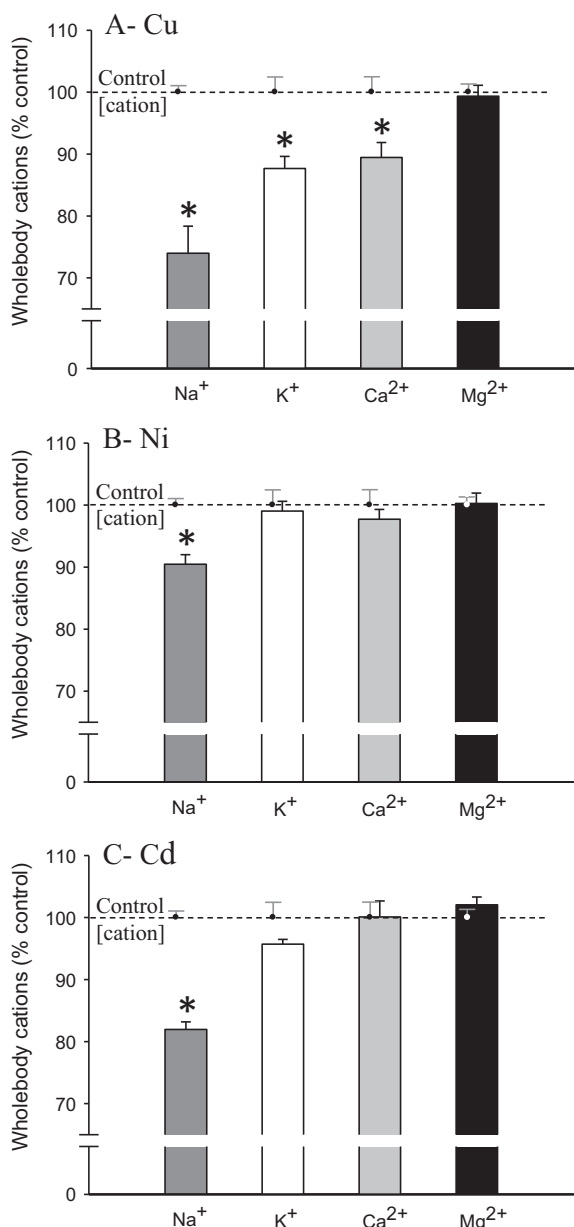


**Fig. 2.** Effects on  $^{45}\text{Ca}$  uptake (over 5 h) in hard water by (A) a range of cadmium (Cd) levels with 6 dpf larvae ( $N=6$  pools of 10 larvae), (B) a range of copper (Cu) levels with adult zebrafish ( $N=7$ ), (C) copper and nickel (Ni) in 7 dpf larvae ( $N=4$  pools of 11 larvae). Bars with different letters are significantly different as determined by ANOVA followed by Tukey's honestly significant difference test to determine differences among groups ( $p < 0.05$ ).

However, in contrast to zebrafish, 150  $\mu\text{g Cu/L}$  also resulted in a 65.2% decrease of  $^{22}\text{Na}$  uptake by trout (Fig. 6).

The concentrations of waterborne  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  were manipulated to test the impacts on nickel uptake and toxicity. Experiments were conducted in soft water and soft water plus 0.5 mM  $\text{Na}^+$ , 0.5 mM  $\text{Mg}^{2+}$  or 0.5 mM  $\text{Ca}^{2+}$ . Increased waterborne  $\text{Na}^+$  had no effect on  $^{63}\text{Ni}$  uptake, while  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  decreased  $^{63}\text{Ni}$  uptake by 29% and 53%, respectively (Fig. 7A). In comparison to nickel toxicity in control soft water [96 h LC50 = 590  $\mu\text{g Ni/L}$  (95% CI = 281.6–814.6  $\mu\text{g/L}$ ); Table 1], only  $\text{Ca}^{2+}$  supplemented waters resulted in an increase in the LC50 by 4.8-fold to 2823  $\mu\text{g Ni/L}$  (95% CI = 1922–4238  $\mu\text{g/L}$ ; Fig. 7B).

Trolox (100  $\mu\text{M}$ ; a water soluble antioxidant and vitamin E analog) did not reduce copper or nickel induced mortalities at a variety of metal concentrations in zebrafish larvae (data not shown).

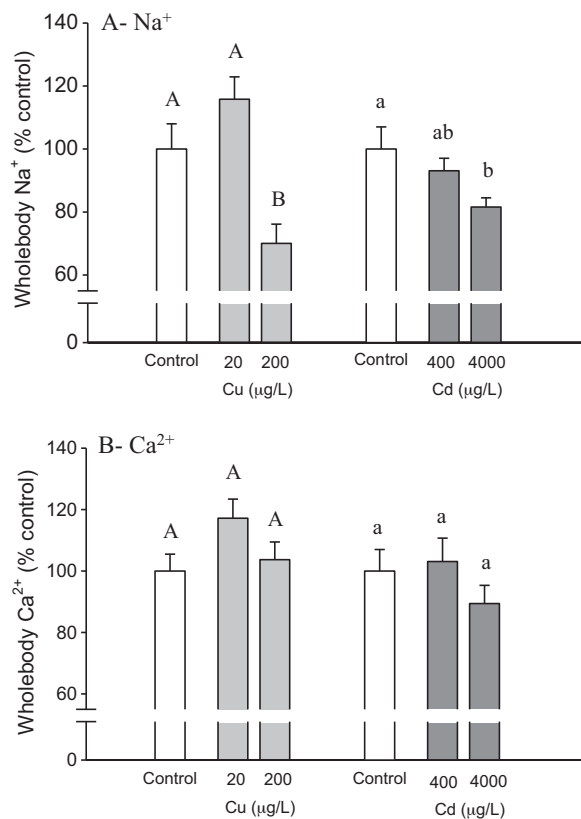


**Fig. 3.** Whole body contents of Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> in 7 dpf zebrafish larvae exposed to (A) 100 µg Cu/L, (B) 9000 µg Ni/L and (C) 1200 µg Cd/L in hard water for 40 h. Mortalities after 40 h were 25%, 0% and 5.9% in the copper, nickel and cadmium exposures, respectively. Absolute control levels of cations averaged 10.7 nmol Na<sup>+</sup>/larva, 14.4 nmol K<sup>+</sup>/larva, 6.88 nmol Ca<sup>2+</sup>/larva and 2.94 nmol Mg<sup>2+</sup>/larva. An asterisk (\*) indicates a significant difference from the control level ( $p < 0.05$ ) determined with a Student's *t*-test.  $N = 6$  pools of 10 larvae.

## 4. Discussion

### 4.1. Larval assay and metal toxicity

Larval zebrafish are well suited for toxicology studies, due in part to the straightforward and year-round availability of numerous fertilized eggs from adult breeding colonies. In addition, the diminutive size of the larva (3–4 mm; Parichy et al., 2009) has allowed for a very small assay footprint that saves time, resources and space. During the time frame of the assay (4–8 dpf or ~2–6 dph) the larvae are sustained by their yolk reserve and do not have to be fed exogenously. This is significant given that uneaten food and feces can impact water chemistry that in turn alters metal bioavailability and toxicity. From a water



**Fig. 4.** Whole body (A) Na<sup>+</sup> and (B) Ca<sup>2+</sup> levels in adult zebrafish after exposure to 20 or 200 µg Cu/L and 400 or 4000 µg Cd/L in hard water for 42 h. Bars with different letters are significantly different as determined by ANOVA followed by Tukey's honestly significant difference test to determine differences among groups ( $p < 0.05$ ),  $N = 8$ .

quality criteria standpoint, larvae lack a chorion that can be protective, and data derived from tests with larvae generally represent the most sensitive stage in the life history of a fish to contaminants such as metals (Chapman, 1978; Grosell et al., 2007).

The initial expectation for utilizing larvae in toxicity testing was that they would have developed to the point where they respond to contaminants with the same physiological mechanisms as adults to maintain homeostasis. Indeed, the effect of metals on Ca<sup>2+</sup> and Na<sup>+</sup> uptake and whole body ion concentrations were similar for larval and adult zebrafish. These life stages also had similar patterns of sensitivity to different metals (Table 1).

A literature search has brought to light the observation that salmonids, such as trout and salmon, are acutely very sensitive to metals from both group 11 (copper and silver) and group 12 (zinc and cadmium), while most other fish including cyprinids (like zebrafish) are sensitive to group 11 metals but insensitive to group 12 metals (Table 2). For example, zebrafish are most sensitive to copper and silver, with LC50s of 148 µg Cu/L and 12.9 µg Ag/L in hard water, which are similar values as rainbow trout (91 µg Cu/L and 8–15 µg Ag/L; Table 2). However, zebrafish are far less sensitive to cadmium and zinc, with LC50s of 1730 µg Cd/L and >>2280 µg Zn/L in hard water, compared to rainbow trout LC50s of 19 µg Cd/L and 869 µg Zn/L in similar water chemistries (Table 2). While the mechanism(s) underlying the different sensitivities between species is unknown, it may involve differences in the regulation and affinity of metal uptake, such as at the epithelial calcium channel (ECaC; see Section 4.2).

For other metals, salmonids and cyprinids have similar acute sensitivities. For example, the 96 h LC50 for nickel (group

**Table 2**  
The sensitivities of salmonids, cyprinids and other teleosts to silver, cadmium, copper and zinc. Values are 96 h LC50s in moderate or hard water (40–250 mg CaCO<sub>3</sub>/L). The different sensitivities between salmonids (e.g. trout) and cyprinids (e.g. zebrafish) are most apparent for the group 12 metals (cadmium, zinc).

Metal	Salmonids	Cyprinids	Other
Cadmium	Rainbow trout – 19.0 µg/L <sup>1</sup> Coho salmon – 10.4 µg/L <sup>2</sup>	Zebrafish larvae – 1730 µg/L <sup>3</sup> Zebrafish adults – 3822 µg/L <sup>3</sup> Common carp – 17,100 µg/L <sup>4</sup> Fathead minnow – 11,200–12,000 µg/L <sup>5</sup> Squawfish – 108 µg/L <sup>6</sup>	Perch – 8141 µg/L <sup>1</sup> Red shiner – 6620 µg/L <sup>7</sup> Stickelback – 6500–23,000 µg/L <sup>8</sup> Green sunfish – 11,520 µg/L <sup>7</sup>
Copper	Rainbow trout – 91 µg/L <sup>9</sup> Chinook Salmon – 58 µg/L <sup>10</sup>	Zebrafish larvae – 148 µg/L <sup>3</sup> Zebrafish adults – 212 µg/L <sup>3</sup> Common carp – 118–530 µg/L <sup>11</sup> Razorback sucker – 269,331 µg/L <sup>12,13</sup>	Bonytail – 231 µg/L <sup>12</sup> Colorado Squawfish – 305 µg/L <sup>13</sup>
Silver	Rainbow trout – 7.6–15.1 µg/L <sup>14</sup>	Zebrafish larvae – 12.9 µg/L <sup>3</sup> Fathead minnow – 6.7 µg/L <sup>15</sup>	Channel catfish – 17.3 µg/L <sup>15</sup>
Zinc	Rainbow trout – 869 µg/L <sup>16</sup>	Zebrafish larvae – >>2280 µg/L <sup>3</sup>	Guppy – 30,826 µg/L <sup>17</sup>

<sup>a</sup> 2280 µg Zn/L was the highest concentration of dissolved Zn that was recorded at a total Zn concentration of 18,788 µg/L.

<sup>1</sup>Niyogi et al. (2004); <sup>2</sup>Lorz et al. (1978); <sup>3</sup>Present study; <sup>4</sup>Suresh et al. (1993); <sup>5</sup>Pickering and Gast (1972); <sup>6</sup>Buhl (1997); <sup>7</sup>Carrier and Beitinger (1988); <sup>8</sup>Pascoe and Matthey (1977); <sup>9</sup>Taylor et al. (2000); <sup>10</sup>Hamilton and Buhl (1990); <sup>11</sup>Deshmukh and Marathe (1980); <sup>12</sup>Buhl and Hamilton (1996); <sup>13</sup>Hamilton and Buhl (1997); <sup>14</sup>Galvez and Wood (2002); <sup>15</sup>Holcombe et al. (1983); <sup>16</sup>Alsop et al. (1999); <sup>17</sup>Gul et al. (2009).

10) in hard water was 13,120 µg Ni/L for zebrafish larva and 15,300 µg Ni/L for rainbow trout (Pane et al., 2003)]. In addition, the acute toxicity of lead (group 14) is similar between juvenile rainbow trout (96 h LC50 = 100 µg Pb/L in soft water; Birceanu et al., 2008) and zebrafish larvae (53 µg Pb/L in soft water).

#### 4.2. Pathways of metal transport

Copper and nickel decreased Ca<sup>2+</sup> uptake in zebrafish larvae and adults. These two metals were not previously known to disrupt Ca<sup>2+</sup> homeostasis in freshwater fish, an effect that has been well established for other metals such as cadmium, lead and zinc (Hogstrand et al., 1996; Niyogi and Wood, 2004; Rogers and Wood, 2004). However, nickel has been used to block voltage gated calcium channels in mammalian studies for some time (e.g. Poulsen et al., 2011). The inhibition of waterborne Ca<sup>2+</sup> uptake by all these metals in zebrafish suggests their uptake is mediated by Ca<sup>2+</sup> transport pathways, perhaps similar to zinc and iron that are transported by ECaC in pufferfish (Qiu and Hogstrand, 2004).

Although metals inhibit Ca<sup>2+</sup> uptake, there was little effect on total whole body Ca<sup>2+</sup> levels after exposures of 48 h or less. Therefore, the decrease in Ca<sup>2+</sup> uptake does not appear to be the acute mechanism underlying mortality (which was instead effectively related to Na<sup>+</sup> loss, see Section 4.3). Shutting down Ca<sup>2+</sup> uptake pathways during metal exposures may instead be a purposeful physiological response in order to reduce metal accumulation and thereby protect against chronic toxicity. Interestingly, decreased whole body Ca<sup>2+</sup> levels are a long term effect of cadmium and copper exposure in rainbow trout (Pilgaard et al., 1994; McGeer et al., 2000).

Further evidence suggesting that metals are transported through Ca<sup>2+</sup> pathways is that elevated waterborne Ca<sup>2+</sup> decreases metal uptake. For example, increased Ca<sup>2+</sup> decreased nickel uptake and also decreased nickel toxicity in zebrafish. In contrast, elevated Na<sup>+</sup> did not decrease nickel uptake nor did it protect against nickel toxicity. Previous studies have also shown Ca<sup>2+</sup> decreases the uptake and toxicity of zinc and the toxicity of copper (Playle et al., 1993; Alsop and Wood, 1999; Taylor et al., 2000; Deleebeeck et al., 2007).

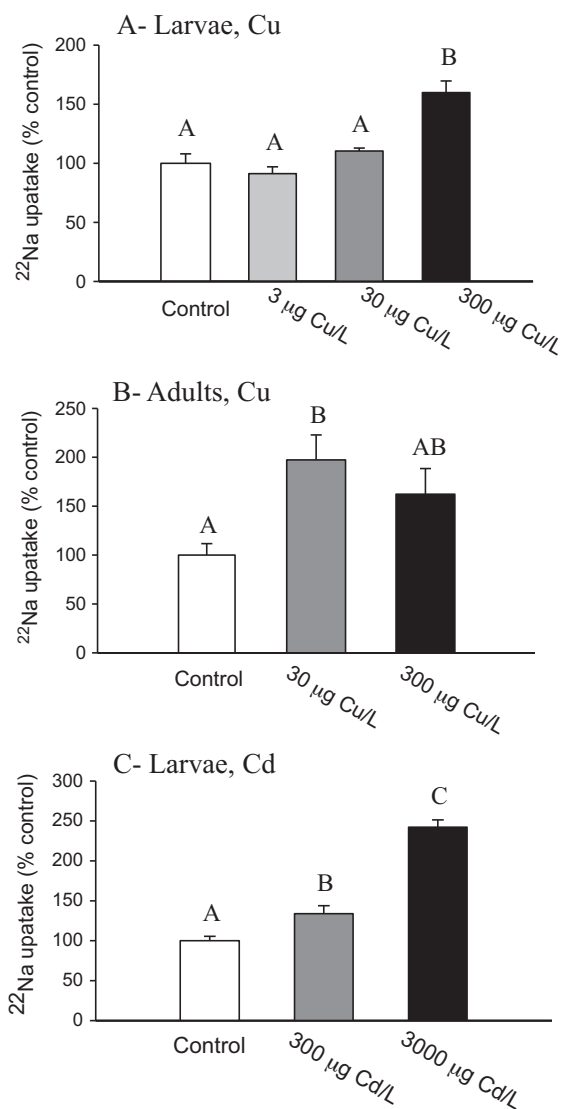
Metals, including copper, did not inhibit Na<sup>+</sup> uptake in zebrafish. However, earlier studies with rainbow trout showed that copper exposure decreased Na<sup>+</sup> uptake, suggesting it is transported across epithelia, such as gills, through Na<sup>+</sup> transport pathways (Grosell and Wood, 2002; Handy et al., 2002). Presently, copper did indeed

inhibit Na<sup>+</sup> uptake in rainbow trout, and it also decreased Ca<sup>2+</sup> uptake (although to a lesser extent than Na<sup>+</sup>). Inhibitory effects on both Na<sup>+</sup> and Ca<sup>2+</sup> uptake were also previously observed for lead in rainbow trout (Rogers and Wood, 2004; Rogers et al., 2005), although in this case lead had a greater impact on Ca<sup>2+</sup> than Na<sup>+</sup>. Effects on both Na<sup>+</sup> and Ca<sup>2+</sup> uptake in trout suggests that copper and lead may move through Na<sup>+</sup> and Ca<sup>2+</sup> transport pathways in this species. The reasons underlying the different effects of metals on Na<sup>+</sup> uptake between trout and zebrafish is unclear, although it is most likely due to fundamental differences in the mechanisms of ion uptake.

#### 4.3. Metals and ion homeostasis

Exposure to copper, cadmium and nickel alone all decreased whole body Na<sup>+</sup> levels. Ion loss with metal exposure may be the result of increased permeability and leakage from the fish to the more dilute aquatic environment. Fish plasma ion concentrations greatly exceed those in the water. For example, Na<sup>+</sup> has a 221-fold concentration gradient [zebrafish plasma Na<sup>+</sup> = 155 mM (Boisen et al., 2003); hard water Na<sup>+</sup> = 0.7 mM]. Other ions (based on rainbow trout plasma levels: K<sup>+</sup> = 2.8 mM, Ca<sup>2+</sup> = 4.3 mM and Mg<sup>2+</sup> = 0.8 mM; McDonald and Rogano, 1986; Pane et al., 2003) have smaller concentration gradients, such as K<sup>+</sup> (74-fold), Mg<sup>2+</sup> (2.4-fold) and Ca<sup>2+</sup> (3.2-fold, although free plasma Ca<sup>2+</sup> levels are likely much lower; Patel et al., 2009). The range of ion gradients fit well with the observed ion losses from zebrafish: the greatest effects of metals were on Na<sup>+</sup> followed by K<sup>+</sup>, while no decreases in Mg<sup>2+</sup> were observed. The two instances of decreased whole body Ca<sup>2+</sup> occurred with a 40 h copper exposure and 48 h cadmium exposure, and the magnitude of the loss was less than Na<sup>+</sup>. These effects on Ca<sup>2+</sup> may be related to inhibition of Ca<sup>2+</sup> uptake, given that there is a very little gradient for diffusive Ca<sup>2+</sup> loss.

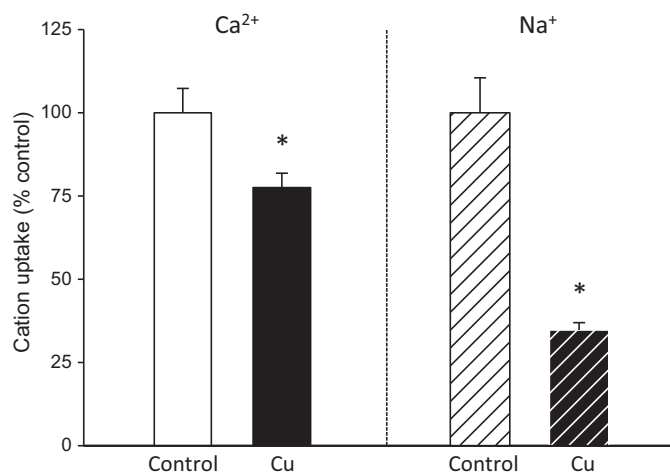
In support of the ion loss hypothesis, a study on killifish found copper was most toxic in freshwater where Na<sup>+</sup> gradients were highest, and least toxic at 10 ppt seawater (>50-fold increase in the 96 h LC50) where Na<sup>+</sup> concentrations were similar between the water and the fish (Grosell et al., 2007). In fathead minnow larvae, acutely lethal copper exposures induced an approximate 30% loss of whole body Na<sup>+</sup> by 12 h of exposure, which remained at this level to 48 h (Zahner et al., 2006). Na<sup>+</sup> loss is also the acute toxic mechanism in rainbow trout (Taylor et al., 2003). In addition, trout experienced decreases in both Na<sup>+</sup> and Ca<sup>2+</sup> with copper and cadmium exposure, where Na<sup>+</sup> recovered first (<5 d) and Ca<sup>2+</sup>



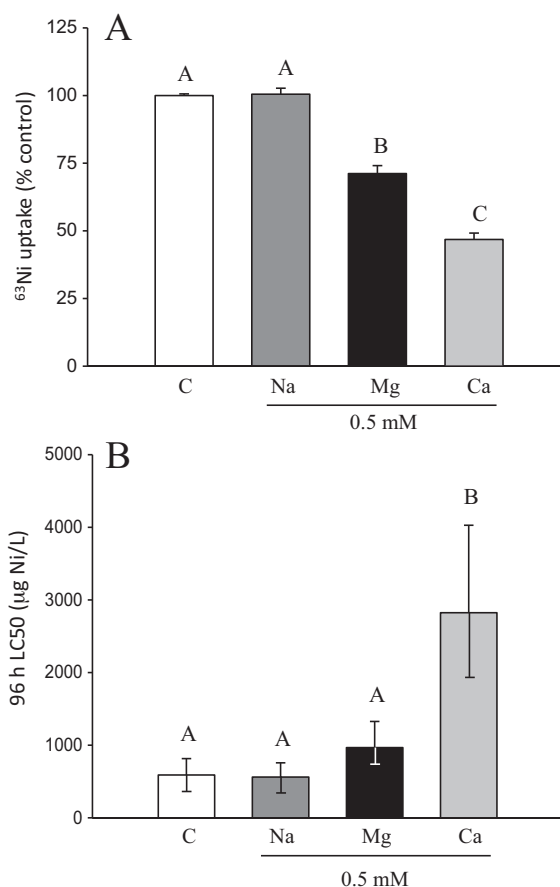
**Fig. 5.** Radiolabeled <sup>22</sup>Na uptake from the water (over 5 h) by (A) 7 dpf zebrafish larvae (N=4 pools of 11 larvae) or (B) adult zebrafish (N=8) in hard water or hard water with the addition of different concentrations of copper. (C) Effects of cadmium on <sup>22</sup>Na uptake by 7 dpf larvae. Bars with different letters are significantly different as determined by ANOVA followed by Tukey's honestly significant difference test to determine differences among groups ( $p < 0.05$ ). N=6 pools of 12 larvae.

levels remained depressed for longer periods of time (up to 20 d) (Pilgaard et al., 1994; McGeer et al., 2000). Presently with zebrafish, <sup>22</sup>Na uptake was increased with exposure to cadmium and copper. This may be a physiological response in an effort to restore Na<sup>+</sup> levels after metal-induced depletion.

Initially, we thought metal-induced ion loss might be due to increased oxidative stress and subsequent damage and leakage at transport epithelia. However, the antioxidant Trolox (a water soluble vitamin E analog) was not protective for larvae exposed to copper or nickel. Previously, Trolox did protect the oligochaete worm (*Lumbriculus variegates*) in copper toxicity tests (O'Gara et al., 2006). In addition, the expression of catalase and cox17, two enzymes associated with an increase in copper-induced oxidative stress (Craig et al., 2007), did not change in zebrafish larvae with exposure to copper (Alsop and Wood, unpublished data). Our current hypothesis is that ion loss is due to the endocrine stress response, as previous studies have shown epinephrine infusions induce rapid and substantial ion losses in rainbow



**Fig. 6.** Effect of copper (150 µg/L) on radiolabeled <sup>45</sup>Ca (solid bars) and <sup>22</sup>Na (hatched bars) uptake in hard water over 7 h by juvenile rainbow trout. An asterisk (\*) indicates a significant difference from the control level ( $p < 0.05$ ) determined with a Student's *t*-test. N=10 for <sup>45</sup>Ca and N=9 for <sup>22</sup>Na experiments.



**Fig. 7.** (A) Effects of 0.5 mM Na<sup>+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup> on <sup>63</sup>Ni uptake at 590 µg total Ni/L over 4 h in soft water. Bars with different letters are significantly different as determined by ANOVA followed by Tukey's honestly significant difference test to determine differences among groups ( $p < 0.05$ ). N=4 (pools of 12 larvae). (B) 96 h LC50s for nickel in soft water and soft water plus 0.5 mM NaNO<sub>3</sub>, Mg(NO<sub>3</sub>)<sub>2</sub> or Ca(NO<sub>3</sub>)<sub>2</sub>. Different letters indicate a significant difference as determined by a lack or overlap of the 95% CIs.



trout (McDonald and Rogano, 1986; Gonzalez and McDonald, 1992).

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