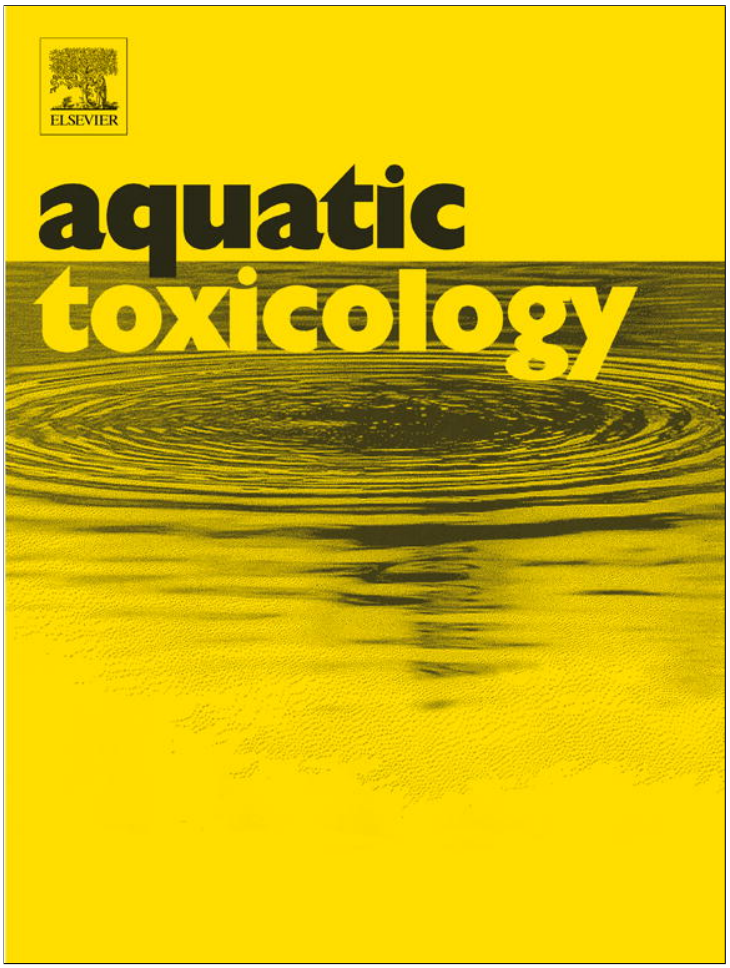

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# Metal and pharmaceutical mixtures: Is ion loss the mechanism underlying acute toxicity and widespread additive toxicity in zebrafish?

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ABSTRACT

The acute toxicities and mechanisms of action of a variety of environmental contaminants were examined using zebrafish larvae (*Danio rerio*; 4–8 days post fertilization). Toxic interactions were observed between metals. For example, the addition of a sublethal level of nickel (15% of the $LC_{50}$, one third of the $LC_{01}$) to all copper treatments decreased the copper 96 h $LC_{50}$ by 58%, while sublethal copper exposure (6% of the copper $LC_{50}$, 13% of the $LC_{01}$) decreased the cadmium 96 h $LC_{50}$ by 47%. Two predictive models were assessed, the concentration addition (CA) model, which assumes similar mechanisms of action, and the independent action (IA) model, which assumes different mechanisms of action. Quantitative comparisons indicated the CA model performed better than the IA model; the latter tended to underestimate combined toxicity to a greater extent. The effects of mixtures with nickel or ammonia were typically additive, while mixtures with copper or cadmium were typically greater than additive. Larvae exposed to cadmium, copper or nickel experienced whole body ion loss. Decreases were greatest for $Na^+$ followed by $K^+$ (as high as 19% and 9%, respectively, in 24 h). Additive toxicity between copper and other pharmaceutical compounds such as fluoxetine (Prozac™), β-naphthoflavone, estrogen and 17α-ethinylestradiol were also observed. Similar to metals, acutely toxic concentrations of fluoxetine, β-naphthoflavone and ammonia all decreased whole body $Na^+$ and $K^+$. Overall, whole body $Na^+$ loss showed the greatest correlation with mortality across a variety of toxicants. We theorize that a disruption of ion homeostasis may be a common mechanism underlying the acute additive toxicity of many contaminants in fish.



## 1. Introduction

Humans synthesize more than a hundred thousand chemicals for consumer products (Hartung and Rovida, 2009), many of which are dispersed into the environment. Indeed, the presence of multiple contaminants in polluted ecosystems is not only common, but is in fact the norm (Yang et al., 1998). For example, waters surrounding mining operations near Sudbury, ON, Canada contain a large number of metals that are above background levels, and some that reach concentrations that are acutely toxic to aquatic biota (Pyle et al., 2005). In addition, receiving areas for municipal, agricultural and industrial wastewater may contain a wide variety of organic contaminants (e.g. pesticides, polycyclic aromatic hydrocarbons, phthalates, phenolics, polychlorinated biphenyls, organotins, nutrients, hormones and pharmaceuticals (e.g. fluoxetine, 17α-ethinylestradiol)) and inorganic contaminants (e.g. metals, ammonia) (Parker et al., 1994; Fent, 1996; McBride, 2003; Basta et al., 2005). Despite the prevalence of multiple contaminants in polluted environments, there are relatively few data on how they interact in the toxic response of target organisms.

Nevertheless, there has been growing interest in the toxicity of mixtures in recent years as regulatory authorities have started to consider possible frameworks for the regulation of toxicant mixtures in aquatic ecosystems (ANZECC and ARMCANZ, 2000; USEPA, 2007; Scientific Committee on Health and Environmental Risks, 2012; European Commission, 2012). Unfortunately, there is some confusion in the literature as to terminology. The term "additive toxicity" has been used to describe the increase in toxicity that occurs in response to combinations of toxicants with similar modes of action, where the concept of "concentration addition" applies. However the same term is often also used to describe the increase in toxicity that occurs in response to combinations of toxicants with different modes of action, where the concept of "response addition" applies. Norwood et al. (2003) explain these two concepts in detail. To avoid confusion, we prefer the term "independent action" (IA), rather than "response addition" for the latter, and throughout this paper we use the term "addition" to refer only to "concentration addition" (CA).

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Zebrafish are a popular model vertebrate in many research areas including developmental biology (Lieschke and Currie, 2007), and consequently embryo and larval development have been extensively characterized (Kimmel et al., 1995; Parichy et al., 2009). In addition, many recent studies have utilized zebrafish embryos for examining chemical toxicity, either effects on embryo development, survival or gene expression (e.g. Nagel, 2002; Voelker et al., 2007; Lammer et al., 2009). Presently, we employ an assay with zebrafish larvae (Alsop and Wood, 2011) to examine the acute toxicities of a variety of environmental contaminants, as well as mixtures.

The focus of this study was on metals, which are some of the more acutely toxic substances of concern globally (Martins et al., 2007). We hypothesized additive toxicity may exist between compounds with similar mechanisms of action and ion homeostasis may be a target of many contaminants. For example, we previously showed that zebrafish exposed to a variety of metals experience whole body ion loss (primarily $Na^+$; Alsop and Wood, 2011), therefore, metals may display additive toxicity. In previous studies, the toxicities of metals such as cadmium, copper, nickel and zinc were additive in a variety of fish species (e.g. Lloyd, 1961; Finlayson and Verrue, 1982; Hamilton and Buhl, 1997). Acute ammonia toxicity is also associated with ion loss in fish (Hon et al., 2013), so the interactions of metals with this ubiquitous toxicant (Randall and Tsui, 2002; Ip and Chew, 2010) were also tested. In addition, some pharmaceuticals such as fluoxetine (Prozac™) are acutely toxic to fish (Brooks et al., 2003; Henry and Black, 2008). Therefore, we tested for toxic interactions between copper and a variety of organic compounds including some pharmaceuticals (e.g. fluoxetine, acetaminophen, 17α-ethinylestradiol, antibiotics), which are increasingly prevalent in aquatic environments (Mompelat et al., 2009; Corcoran et al., 2010). These compounds have been synthesized to target a variety of different cellular pathways. For example, 17α-ethinylestradiol is an estrogen receptor agonist (Notch and Mayer, 2011), while fluoxetine is a selective serotonin reuptake inhibitor (SSRI) (Mennigen et al., 2011). They would therefore be expected to act through cellular pathways distinct from those of metals and from each other, so additive toxicity would not be expected.

However, we observed widespread additive toxicity between acutely toxic contaminants. Furthermore, decreases in whole body $Na^+$ levels were observed with exposure to all toxic substances that were examined. We hypothesize that this effect on ion homeostasis may be a common mechanism of acute toxicity and the underlying mechanism of additive toxicity. The implications of these findings on tests for establishing water quality criteria are discussed.

## 2. Materials and methods

### 2.1. Animals and breeding

Animal care and breeding have been previously outlined (Alsop and Wood, 2011). Briefly, adult zebrafish (*Danio rerio*; 0.3–0.9 g) were held in three 40-L aquaria with 25 fish per tank, equipped with aeration and a recirculating charcoal filter. Photoperiod was maintained at 12 h light/12 h dark and temperature at 28 °C. Water was moderately hard, dechlorinated City of Hamilton tap water, from Lake Ontario (hardness = 141 mg $CaCO_3$/L, pH = 7.9, DOC = 3.5 mg/L, $Na^+$ = 0.7 mM, $K^+$ = 0.4 mM, $Ca^{2+}$ = 1.3 mM, $Mg^{2+}$ = 0.34 mM).

Fertilized eggs were acquired with egg-capturing trays that were removed 1.5 h after the start of the light period. Eggs were transferred to 200-mL beakers containing 50 mL water at 28.5 °C (30 eggs/beaker). Water in the beakers was changed every 48 h. Hatching typically occurred between 48 and 60 h post fertilization (hpf). Fertilization and hatching rates were both >95%.

### 2.2. Chemicals

Stock solutions of $Cd(NO_3)_2{\cdot}4H_2O$ (Fisher Scientific, Ottawa, ON), $CuSO_4{\cdot}5H_2O$, $LaCl_3{\cdot}7H_2O$, $Mg(NO_3)_2{\cdot}6H_2O$ and $NiSO_4{\cdot}6H_2O$ (all Sigma–Aldrich, Oakville, ON) were prepared in deionized water (acidified to 0.05% $HNO_3$) and stored at 4 °C. Stocks for ammonia ($NH_4NO_3$; Caledon, Georgetown, ON) and epinephrine-HCl (Sigma–Aldrich) were prepared fresh daily in deionized water.

Stock solutions (10 mM) for acetaminophen, dexamethasone, erythromycin, 17β-estradiol, 17α-ethinylestradiol, fluoxetine, β-naphthoflavone, pregnenolone-16α-carbonitrile, rifampicin and cortisol (Sigma–Aldrich) were all prepared in dimethyl sulfoxide (DMSO). For the exposures, 1 μL of stock solution was added to 10 mL of water that resulted in a final compound concentration of 1 μM, as well as a final DMSO concentration of 0.01%. Experimental concentrations of metals and ammonia were measured (see below) while the other chemicals are expressed as nominal levels.

### 2.3. Exposures

All exposures were performed in moderately hard, dechlorinated Hamilton tap water (composition as above). Experiments with larvae were performed in polystyrene 6-well tissue culture plates (Falcon™) with 10 mL of water and 10 larvae per well (9–11 larvae per well for whole body cation experiments). The 96 h $LC_{50}$ trials were initiated when larvae had reached 96 hpf [~2 days post hatch (dph)]. In these experiments, 6 concentrations of the toxicant were used, including a control treatment. Each LC estimate was based on one experiment. For experiments 24–48 h in duration, 5–7 dpf larvae were utilized. Exposures were conducted at 28 °C in the moderately hard water described above, and 99% of the water was changed every 24 h. The exception was the epinephrine exposure, where water was changed every 8 h due to the instability of this catecholamine. Static renewal exposures with adult zebrafish were conducted in 9-L plastic tanks at 27 °C with aeration, and 97% daily water replacement. All toxicity tests were 96 h. In preliminary experiments involving ion homeostasis, we found that $Na^+$ levels in larvae exposed to copper at 2.4 μM were significantly decreased at 24 h (13.0%) and 46 h (21.3%) of exposure (but not at 6 h or 12 h). Therefore to minimize the "survivor effect" (i.e. bias due to the sampling of only surviving, possibly more resistant individuals), tests for whole body ion levels were 24–46 h in duration.

We tested the acute toxicity of metals alone and in binary/ternary mixtures. In these experiments, 96 h $LC_{50}$ experiments were performed with a primary metal alone, and in the presence of a secondary metal that was added at a constant, sublethal concentration (i.e. < $LC_{01}$) to all primary metal exposures. Given that variation may occur between tests, LC experiments with the primary metal alone and in mixture with secondary metals were performed concurrently, with the same pool of larvae, in order to confidently compare resultant effects. When testing pharmaceuticals, we conducted experiments with a screening approach, where compounds were tested at one concentration (1 μM) alone and in combination with a sublethal concentration of copper (0.79 μM). If toxicity was observed in the screen, the compound was retested along with a series of dilutions.

Prior to water changes in the toxicity experiments, water samples were collected for metal and ammonia analyses. For metals, measurements were performed on water samples 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% $HNO_3$) after collection. The dissolved cadmium, copper and nickel concentrations were consistently 94–100% of the total waterborne metal concentrations, so all data are expressed as measured, dissolved concentrations.

### 2.4. Analyses

Waterborne copper and nickel 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 $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $K^+$ and cadmium levels were analyzed by flame atomic absorption spectroscopy (Spectra AA 220FS; Varian) after dilution with 1% $HNO_3$ (for $Na^+$ and cadmium measurements), 0.5% $HNO_3$ and 0.5% $LaCl_3$ (for $Ca^{2+}$ and $Mg^{2+}$ measurements), or 0.5% $CsCl_2$ (for $K^+$ measurements). Waterborne ammonia levels were determined based on the methods of Verdouw et al. (1978)

Whole body ion content of larvae was determined by terminally 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% $HNO_3$ for 48 h at 60 °C. $Na^+$, $Ca^{2+}$, $Mg^{2+}$ and $K^+$ contents were analyzed by flame atomic absorption spectroscopy, as above.

### 2.5. Gene expression

Larvae were exposed to 0.79 μM copper, 1.0 μM β-naphthoflavone (βNF) or both copper and βNF (plus a control treatment) to examine the effects on the expression of genes associated with the oxidative stress response in zebrafish (cytochrome c oxidase-17, catalase; Craig et al., 2007). After 36 h of exposure, larvae were frozen in liquid $N_2$ and stored at −80 °C until the RNA extraction procedure. Total RNA was extracted from 5 pools of 10 larvae per treatment with Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was quantified spectrophotometrically (Nanodrop ND-1000; Fisher Scientific, Wilmington, DE). First strand cDNA was synthesized from 1 μg of total RNA treated with DNase and reverse transcribed with SuperScript II RNase H-reverse transcriptase (Invitrogen). Expression of cytochrome c oxidase-17, catalase and elongation factor 1α (normalizing gene) was quantified in duplicate on a Stratagene MX3000P real-time PCR machine with SYBR Green and ROX reference dye (Bio-Rad, Mississauga, ON). For primer sequences and further details please see Craig et al. (2007).

### 2.6. Statistics

Environmental toxicity data analysis software, Tox Calc™ package (Tidepool Scientific Software), was used to estimate the 96 h LCs (e.g. $LC_{50}$, $LC_{10}$, $LC_{01}$) and 95% confidence intervals (CI) with the '96 h-survival acute fish test' and maximum likelihood probit analysis. The values were calculated from the survivorship and toxicant concentration data [measured for metals (dissolved fractions) and ammonia, otherwise nominal] from all treatments. There were no partial responses in the fluoxetine exposures, and therefore the maximum likelihood procedure in Tox Calc was unable to determine the LC and CI estimates. Instead, the LCs and CIs for fluoxetine and fluoxetine plus copper were estimated using the methods of Litchfield and Wilcoxon (1949).

Differences in whole body ions from single contaminant exposures were analyzed with a Student's *t*-test (SPSS). Sodium levels and gene expression data from combined contaminant exposures 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 were considered significant if $p < 0.05$.

### 2.7. Assessment of metal interactions with predictive models

Our previous study found that zebrafish exposed to cadmium, copper or nickel experienced similar patterns of whole body cation loss (primarily $Na^+$; Alsop and Wood, 2011). This suggests that metals may have a common mechanism of action, and the effects of multiple metals in an exposure may be additive. To test this hypothesis, observed effects of metal mixtures were compared to the predicted effects of a concentration addition (CA) model, which is calculated from the following equation (Faust et al., 2000, 2003; Belden and Lydy, 2006; Belden et al., 2007; Tollefsen et al., 2012):

$$ECx_{mix} = \left( \sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \quad (1)$$

where $ECx_{mix}$ is the total concentration of the mixture that causes *x* effect, $p_i$ represents the proportion of metal *i* in the mixture, and $ECx_i$ is the concentration of metal *i* that would cause *x* effect. This approach has been used in other studies examining mixtures of other contaminants in fish (Belden and Lydy, 2006; Belden et al., 2007; Tollefsen et al., 2012). There were often large differences between the concentrations of the primary and secondary metals in the mixture tests due to differences in toxicity between metals, and our approach that used low levels of the secondary metal. Therefore, metal concentrations were normalized to the toxic unit (TU) scale (Sprague, 1970; Norwood et al., 2003; Belden and Lydy, 2006; Belden et al., 2007), prior to the calculations for the CA model. For each metal, the $LC_{50}$ represented 1 TU, and each exposure concentration was divided by the $LC_{50}$ to generate the equivalent TUs.

Predictions based on dissimilar mechanisms of metal toxicity are also warranted, since it has also been suggested that copper impacts $Na^+$ homeostasis, cadmium targets $Ca^{2+}$ homeostasis, and nickel targets neither (see Alsop and Wood, 2011 for discussion and references therein). Predictions assuming different mechanisms of action are made using the independent action (IA) model (Faust et al., 2000, 2003; Belden and Lydy, 2006; Belden et al., 2007; Tollefsen et al., 2012):

$$E(C_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(C_i)] \quad (2)$$

where $E(C_{mix})$ is the total effect of the metal mixture and $E(C_i)$ represents the effect expected from metal *i*. Because we generally used very low concentrations of the secondary metal ($<LC_{01}$) that caused no mortality when tested by itself, the IA model predicts unaltered toxicity of the primary metal (i.e. identical LC estimates in the presence or absence of the secondary metal).

Quantitative comparisons between the observed responses and the two predictive models were performed using model deviation ratios (MDR; Belden and Lydy, 2006; Belden et al., 2007; Tollefsen et al., 2012). These were calculated as the predicted concentrations causing a certain effect divided by observed concentrations causing the same effect. The MDRs for the $LC_{10}$ and $LC_{50}$ estimations of the metal mixtures are reported. An MDR of 1 signifies agreement with the model, while an MDR > 1 indicates the model underestimates toxicity (i.e. the predicted LC is greater than the measured LC) and an MDR < 1 indicates the model overestimates toxicity (i.e. the predicted LC is less than the measured LC). However, MDRs within a factor of 2 (i.e. 0.5 < MDR < 2.0) can be considered to be in agreement with the model (Belden and Lydy, 2006; Tollefsen et al., 2012).

## 3. Results

### 3.1. Effects of metal mixtures on toxicity

Toxic interactions were observed between metals in 96 h $LC_{50}$ tests. For example, the copper $LC_{50}$ was 4.04 μM (95% CI = 3.16–4.85 μM), which was reduced by 43% to 2.31 μM (1.73–2.91 μM) with the addition of 1.78 μM cadmium to each copper exposure treatment (which was 8% of the cadmium $LC_{50}$

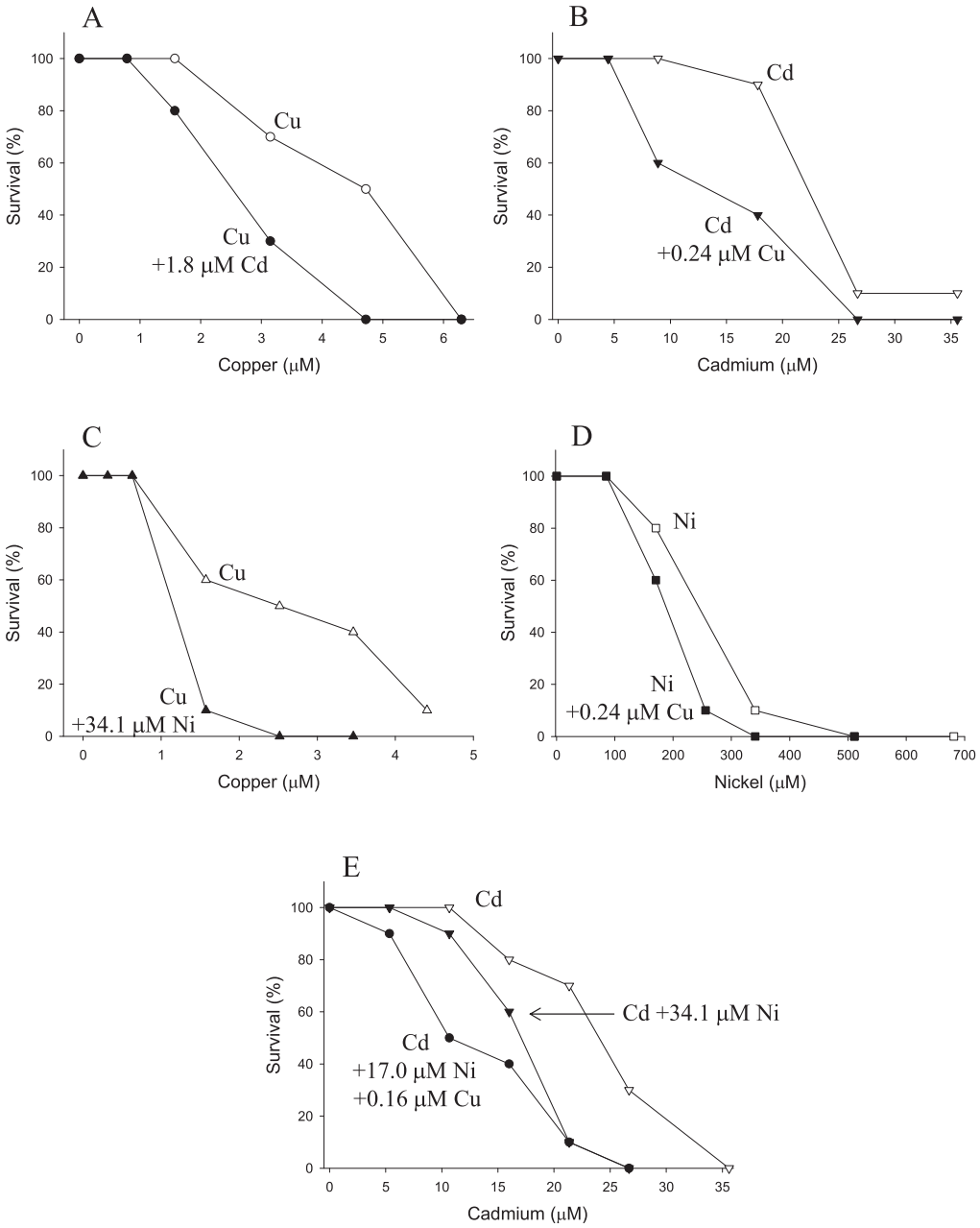


**Fig. 1.** Larval zebrafish survival after 96 h exposure (4–8 dpf) to different concentrations of (A) copper alone or with 1.78 μM cadmium added to all copper treatments, (B) cadmium alone or with 0.24 μM copper added to all cadmium treatments, (C) copper alone or with 34.1 μM nickel added to all copper treatments, (D) nickel alone or with 0.24 μM copper added to all nickel treatments, and (E) cadmium alone or with 34.1 μM nickel or with 17 μM nickel and 0.16 μM copper added to all cadmium treatments. The 96 h $LC_{01}$, $LC_{10}$ and $LC_{50}$ estimates are listed in Table 1. Each mixture exposure was conducted concurrently with an exposure involving the primary metal alone, to take into consideration the possibility of variation between tests.

or 14% of the $LC_{01}$, i.e. sublethal; Fig. 1A and Table 1). The $LC_{50}$s for the binary mixture predicted by the two models were 3.72 μM (CA model) and 4.04 μM (IA model), resulting in MDRs of 1.6 (CA) and 1.8 (IA), respectively (Table 2). The observed and predicted $LC_{10}$s and resulting MDRs for all metal mixtures are reported in Table 2.

The cadmium $LC_{50}$ was reduced by 47% from 22.7 μM (18.6–26.5 μM) to 12.1 μM (8.8–15.6 μM) with the addition of 0.24 μM copper (6% of the copper $LC_{50}$ or 13% of the $LC_{01}$) to each cadmium treatment (Fig. 1B and Table 1). The $LC_{50}$s predicted by the two models for this binary mixture were 21.4 μM (CA) and 22.7 μM (IA), resulting in MDRs of 1.8 (CA) and 1.9 (IA) respectively (Table 2).

Interactions between copper and nickel were also examined. The $LC_{50}$ for copper in this series of tests was 2.34 μM (1.64–3.16 μM), however, with the addition of 34.1 μM nickel to all treatments (sublethal; 15% of the nickel $LC_{50}$, 32% of the $LC_{01}$), the copper $LC_{50}$ was reduced by 51% to 1.14 μM (1.03–1.23 μM; Fig. 1C and Table 1). The $LC_{50}$s predicted by the two models for this binary mixture were 1.98 μM (CA) and 2.34 μM (IA), resulting in MDRs of 1.7 (CA) and 2.1 (IA) respectively (Table 2).

The 96 h $LC_{50}$ for nickel was decreased by 18% (223.5–182.6 μM) with the addition of 0.24 μM copper (10% of $LC_{50}$, 49% of $LC_{01}$) to all nickel treatments (Fig. 1D and Table 1). The $LC_{50}$s predicted by the two models for this binary mixture were 202.9 μM (CA) and 223.5 μM (IA), resulting in MDRs of 1.1 and 1.2, respectively (Table 2). This effect of copper on the nickel $LC_{50}$ was less than what was observed when 0.24 μM copper was included in the cadmium $LC_{50}$ test, which decreased by 47% (Fig. 1B and Table 1).

**Table 1**
The 96 h $LC_{01}$, $LC_{10}$ and $LC_{50}$ estimations for copper, cadmium, nickel and ammonia alone or in combination. The numbers in parentheses in the LC columns indicate the 95% confidence intervals for each estimation. Each mixture exposure was conducted concurrently with an exposure involving the primary metal (or ammonia) alone, to take into consideration the possibility of variation between tests. For exposures involving two or three metals, the first metal (or ammonia) listed is the primary metal, while the additional metal(s) was included in each of the primary metal treatments, although at a concentration below its/their $LC_{01}$(s).

| Exposure | $LC_{01}$ (μM) | $LC_{10}$ (μM) | $LC_{50}$ (μM) |
|---|---|---|---|
| Copper (test 1) | 1.90 (0.60–2.64) | 2.67 (1.31–3.33) | 4.04 (3.17–4.85) |
| Copper + cadmium (1.78 μM; 14% $LC_{01}$) | 0.94 (0.37–1.37) | 1.41 (0.76–1.85) | 2.31 (1.74–2.91) |
| Cadmium (test 1) | 12.8 (5.7–16.5) | 16.6 (10.0–19.8) | 22.7 (18.6–26.5) |
| Cadmium + copper (0.24 μM; 13% $LC_{01}$) | 3.8 (1.3–5.9) | 6.4 (3.2–8.8) | 12.1 (8.8–15.6) |
| Copper (test 2) | 0.49 (0.07–0.90) | 0.99 (0.31–1.46) | 2.34 (1.64–3.16) |
| Copper + nickel (34.1 μM; 32% $LC_{01}$) | 0.63 (0.50–0.74) | 0.82 (0.7–0.92) | 1.14 (1.03–1.23) |
| Nickel (test 1) | 108.2 (42.7–148.4) | 149.9 (83.6–189.5) | 223.5 (172.1–283.6) |
| Nickel + copper (0.24 μM; 49% $LC_{01}$) | 103.5 (30.6–137.3) | 133.6 (62.0–162.9) | 182.6 (138.1–214.5) |
| Nickel (test 2) | 119.1 (52.4–157.8) | 161.8 (97.2–197.0) | 235.6 (191.9–280.0) |
| Nickel + copper (0.47 μM; 97% $LC_{01}$) | 103.7 (37.7–140.5) | 141.1 (75.2–174.4) | 205.9 (162.1–245.7) |
| Cadmium (test 2) | 9.2 (2.0–12.9) | 13.8 (6.3–17.0) | 22.8 (19.0–32.9) |
| Cadmium + nickel (34.1 μM; 32% $LC_{01}$) | 8.8 (4.6–11.2) | 11.5 (7.6–13.6) | 15.9 (13.3–18.3) |
| Cadmium + nickel (17.0 μM; 16% $LC_{01}$) + copper (0.16 μM; 8% $LC_{01}$) | 3.3 (0.9–5.3) | 5.7 (2.5–8.0) | 11.3 (8.1–14.2) |
| Ammonia | 330 (249–398) | 458 (375–525) | 677 (602–745) |
| Ammonia + copper (0.79 μM; 42% $LC_{01}$) | 330 (268–384) | 426 (363–485) | 577 (509–684) |

Similarly, 0.47 μM copper (20% of $LC_{50}$, 97% of $LC_{01}$) decreased the nickel LC50 by 13% (235.6–205.9 μM; Table 1). The $LC_{50}$s predicted by the two models for this binary mixture were 196.2 μM (CA) and 223.5 μM (IA), resulting in MDRs of 1.0 and 1.1, respectively (Table 2). However, 0.79 μM copper appeared to have a greater effect on nickel toxicity; when larvae were exposed to 0, 120, 170 or 240 μM nickel there were 0%, 0%, 0% and 40% mortality rates, respectively. When exposed to an additional 0.79 μM copper in each of these nickel exposures, mortality rates increased to 0%, 10%, 60% and 80%, respectively (data not shown).

The toxicity of cadmium alone and cadmium in binary or ternary mixtures with nickel and copper were examined. The cadmium 96 h $LC_{50}$ for this series of tests was 22.8 μM (19.0–32.9 μM). When 34.1 μM nickel was added to each cadmium treatment (sublethal; 15% of the nickel $LC_{50}$, 32% of the $LC_{01}$), the cadmium $LC_{50}$ was decreased by 30% to 15.9 μM cadmium (13.3–18.3 μM; Fig. 1E and Table 1). The $LC_{50}$s predicted by the two models were 19.3 μM (CA) and 22.8 μM (IA), resulting in MDRs of 1.2 (CA) and 1.4 (IA), respectively (Table 2). Furthermore, when both 17 μM nickel (16% of the nickel $LC_{01}$) and 0.16 μM copper (8% of the copper $LC_{01}$) were added to each cadmium treatment, cadmium $LC_{50}$ was reduced by 50% to 11.3 μM cadmium (8.1–14.2 μM) (Fig. 1E and Table 1). The $LC_{50}$s predicted by the two models were 20.1 μM (CA) and 22.8 μM (IA), resulting in MDRs of 1.8 (CA) and 2.0 (IA), respectively (Table 2).

**Table 2**
The $LC_{10}$ and $LC_{50}$ estimates for binary and ternary metal exposures, including observed values and those predicted by the concentration addition (CA) and independent action (IA) model calculations. Data for ammonia are also shown. The toxic unit approach was used to calculate values for the CA model. The predicted $LC_{10}$ and $LC_{50}$ estimates from the CA and IA models were quantitatively compared through calculations of the model deviation ratio (MDR; predicted/observed). The asterisk (*) represents the primary metal (or ammonia) in the test, for which the LCs are reported. The secondary metal was added to each treatment in the 96 h LC test at a constant concentration (% of its respective $LC_{01}$ given in parentheses).

| | Observed $LC_{10}$ (μM) | CA $LC_{10}$ (μM) | IA $LC_{10}$ (μM) | Observed $LC_{50}$ (μM) | CA $LC_{50}$ (μM) | IA $LC_{50}$ (μM) |
|---|---|---|---|---|---|---|
| Cu* + Cd (14% $LC_{01}$) | 1.41 | 2.41 | 2.67 | 2.31 | 3.75 | 4.04 |
| **MDR** | | **1.7** | **1.9** | | **1.6** | **1.8** |
| Cd* + Cu (13% $LC_{01}$) | 6.4 | 15.3 | 16.6 | 12.1 | 21.5 | 22.7 |
| **MDR** | | **2.4** | **2.6** | | **1.8** | **1.9** |
| Cu* + Ni (32% $LC_{01}$) | 0.82 | 0.81 | 0.99 | 1.14 | 2.02 | 2.34 |
| **MDR** | | **1.0** | **1.2** | | **1.8** | **2.1** |
| Ni* + Cu (49% $LC_{01}$) | 133.6 | 120.9 | 149.9 | 182.6 | 202.9 | 223.5 |
| **MDR** | | **0.9** | **1.1** | | **1.1** | **1.2** |
| Ni* + Cu (97% $LC_{01}$) | 141.1 | 109.6 | 161.8 | 205.9 | 196.2 | 235.6 |
| **MDR** | | **0.8** | **1.2** | | **1.0** | **1.1** |
| Cd* + Ni (32% $LC_{01}$) | 11.5 | 11.3 | 13.8 | 15.9 | 19.8 | 22.8 |
| **MDR** | | **1.0** | **1.2** | | **1.3** | **1.4** |
| Cd* + Ni (16% $LC_{01}$) + Cu (8% $LC_{01}$) | 5.7 | 11.8 | 13.8 | 11.3 | 20.4 | 22.8 |
| **MDR** | | **2.1** | **2.4** | | **1.8** | **2.0** |
| Ammonia* + Cu (42% $LC_{01}$) | 426 | 353 | 458 | 577 | 566 | 677 |
| **MDR** | | **0.8** | **1.1** | | **1.0** | **1.2** |

**Table 3**
Larval mortalities with exposure to a variety of waterborne pharmaceutical compounds at 1 μM, unless otherwise stated. The 'Mix' treatment is composed of exposure to 0.1 or 0.01 μM each for acetaminophen, dexamethasone, erythromycin, 17β-estradiol, fluoxetine, β-naphthoflavone, pregnenolone-16α-carbonitrile and rifampincin. All listed concentrations are nominal values, while copper was measured and always within 94% of the nominal concentration (0.79 μM; 42% of $LC_{01}$, 20% of $LC_{50}$).

| Pharmaceutical | Control | Pharmaceutical alone | 0.79 μM Cu alone | Pharma + 0.79 μM Cu |
|---|---|---|---|---|
| Acetaminophen (1 μM) | 0 | 0 | 0 | 0 |
| Dexamethasone (1 μM) | 0 | 10% | 0 | 10% |
| Erythromycin (1 μM) | 0 | 0 | 0 | 0 |
| 17β-Estradiol (1 μM) | 0 | 0 | 0 | 20% |
| 17α-Ethinylestradiol (1 μM) | 0 | 0 | 0 | 10% |
| 17α-Ethinylestradiol (0.1 μM) | 0 | 0 | 0 | 0 |
| Fluoxetine (1.0 μM) | 0 | 100% | 0 | 100% |
| Fluoxetine (0.65 μM) | 0 | 0 | 0 | 30% |
| Fluoxetine (0.5 μM) | 0 | 0 | 0 | 25% |
| Fluoxetine (0.1 μM) | 0 | 0 | 0 | 10% |
| Fluoxetine (0.05 μM) | 0 | 0 | 0 | 0% |
| β-Naphthoflavone (1 μM) | 0 | 5% | 0 | 70% |
| β-Naphthoflavone (0.1 μM) | 0 | 0 | 0 | 20% |
| β-Naphthoflavone (0.01 μM) | 0 | 0 | 0 | 0 |
| Pregnenolone-16α-carbonitrile (1 μM) | 0 | 0 | 0 | 0 |
| Rifampicin (1 μM) | 0 | 0 | 0 | 0 |
| Mix (0.1 μM each) | 0 | 0 | 0 | 60% |
| Mix (0.01 μM each) | 0 | 0 | 0 | 0 |

The toxic interactions between ammonia and copper were also tested. Ammonia alone was acutely toxic, with a 96 h $LC_{50}$, expressed as total ammonia, of 676.7 μM (602.2–744.1 μM). The addition of 0.79 μM copper to all ammonia treatments decreased the ammonia $LC_{50}$ by 15% to 577.1 μM (CI 509.0–654.2 μM; Table 1). The ammonia $LC_{50}$s for this binary mixture predicted by the two models were 565.8 μM (CA) and 676.7 μM (IA), resulting in MDRs of 1.0 (CA) and 1.2 (IA), respectively (Table 2).

Mixture effects were not limited to larvae. In adults, 96 h survivorship was examined with exposure to 1.97 μM copper (59% of the adult 96 h Cu $LC_{50}$; Alsop and Wood, 2011), 22.2 μM cadmium (65% of the adult 96 h Cd $LC_{50}$; Alsop and Wood, 2011) or both 1.97 μM copper and 22.2 μM cadmium. There were no mortalities in the control or cadmium alone exposures, and 20% mortality in the copper alone exposure by 96 h (Fig. 2A). However, by 24 h of exposure, there was 100% mortality in the copper and cadmium co-exposure treatment (Fig. 2A).

The toxicities of a variety of organic compounds (1 μM) were tested alone and in combination with copper (0.79 μM; 20% of 96 h $LC_{50}$, 42% of $LC_{01}$) in zebrafish larvae. Exposures to dexamethasone, fluoxetine and β-naphthoflavone (βNF) alone for 96 h resulted in some mortalities. For dexamethasone, there was 10% mortality, which did not increase with the addition of 0.79 μM copper (Table 3). Fluoxetine exposure resulted in mortalities, with a 96 h $LC_{50}$ estimated at 0.81 μM (0.68–0.96 μM). The addition of copper to the fluoxetine treatments resulted in concentration-dependent mortalities (Table 3), which decreased the $LC_{50}$ by 24% to 0.62 μM (0.59–0.65 μM). The fluoxetine $LC_{50}$s for this binary mixture predicted by the two models were 0.68 μM (CA) and 0.81 μM (IA), resulting in MDRs of 1.1 (CA) and 1.3 (IA), respectively.

Exposure to 1 μM βNF resulted in 5% mortality, which increased to 70% with the addition of 0.79 μM copper. There were no mortalities with exposure to 0.1 μM βNF alone, however, with the addition of copper 20% mortality was observed (Table 3).

Exposure to a variety of other pharmaceuticals (acetaminophen, erythromycin, 17β-estradiol, 17α-ethinylestradiol, pregnenolone-16α-carbonitrile, rifampincin) at 1 μM for 96 h did not result in any mortality. The addition of 0.79 μM copper did not potentiate the toxicity of most of these chemicals, except for 17β-estradiol and 17α-ethinylestradiol, where 20% and 10% mortalities, respectively, were observed with co-exposures (Table 3). In addition, exposure to a mixture of acetaminophen, dexamethasone, erythromycin, 17β-estradiol, fluoxetine, βNF, pregnenolone-16α-carbonitrile and rifampincin (all at 0.1 μM) did not induce mortality. However, with the addition of 0.79 μM copper to the mixture, a 60% mortality rate was observed (Table 3).

### 3.2. Cation homeostasis

Control cation levels averaged 10.7 ± 0.23 nmol $Na^+$/larva, 14.4 ± 0.21 nmol $K^+$/larva, 6.9 ± 0.18 nmol $Ca^{2+}$/larva and

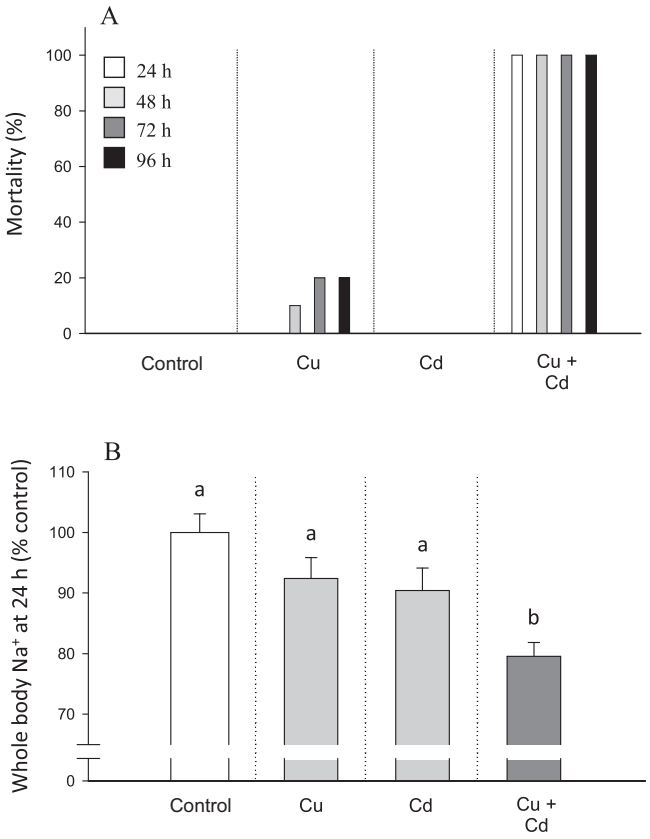


**Fig. 2.** (A) Adult zebrafish mortality at 24, 48, 72 and 96 h of exposure to 2.0 μM copper, 22.2 μM cadmium or both 2.0 μM copper and 22.2 μM cadmium. (B) Whole body $Na^+$ levels of larvae exposed to 0.79 μM copper, 5.34 μM cadmium or both 0.79 μM copper and 5.34 μM cadmium for 24 h. Bars with different letters are significantly different as determined by ANOVA followed by Tukey's honestly significant difference test to determine differences among groups ($p < 0.05$). $N = 9$ pools of two larvae.

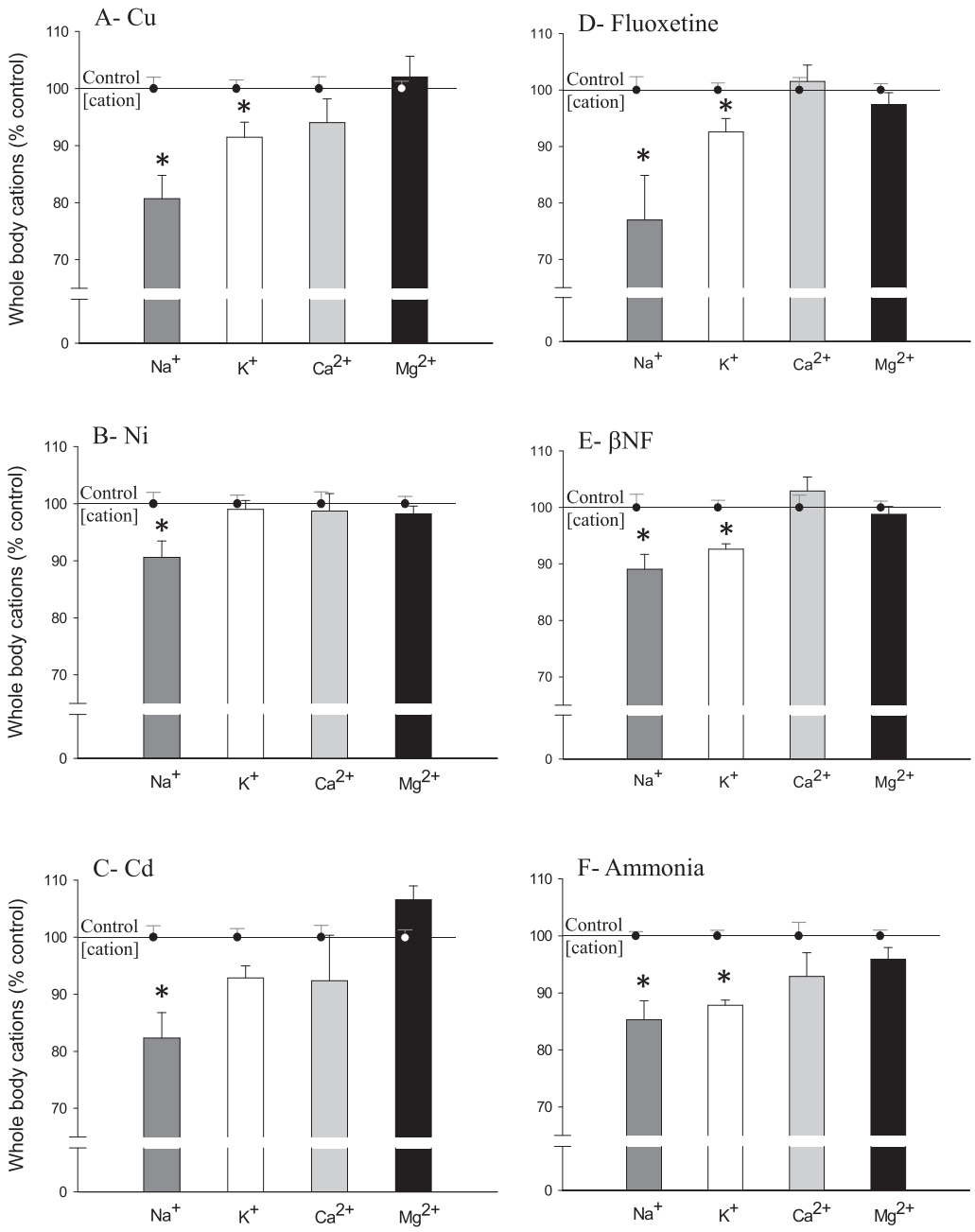


**Fig. 3.** Whole body contents of $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ in 6 dpf zebrafish larvae exposed to (A) 2.36 μM copper, (B) 162 μM nickel and (C) 15.6 μM cadmium for 24 h. Mortalities after 24 h were 26%, 0% and 49% in the copper, nickel and cadmium exposures, respectively. $N = 6$ pools of 10 larvae. In addition, 7 dpf zebrafish larvae were exposed to (D) 0.7 μM fluoxetine and (E) 1 μM β-naphthoflavone (βNF) for 40 h. Mortalities after 40 h were 15% and 2%, respectively, $N = 6$ pools of 12 larvae. (F) Whole body ion content in 7 dpf zebrafish larvae exposed to 500 μM ammonia for 46 h. There were no mortalities during the exposure. $N = 4$ pools of 10 larvae. An asterisk (*) indicates a significant difference from the control level ($p < 0.05$) determined with a Student's *t*-test. Data are means ± S.E.M.

$2.9 \pm 0.04$ nmol $Mg^{2+}$/larva. Copper, nickel and cadmium alone exposures all impacted cation levels. For example, individual 24 h exposures to 2.4 μM Cu (59% of $LC_{50}$), 162 μM Ni (73% of $LC_{50}$) and 15.6 μM Cd (69% of $LC_{50}$), resulted in 24-h mortality rates of 26.1%, 0% and 49.2%, and $Na^+$ losses of 19.3%, 9.4% and 17.7% in the survivors, respectively (Fig. 3A–C). Copper also reduced whole body $K^+$ levels, although to a lesser extent than $Na^+$, while $Ca^{2+}$ and $Mg^{2+}$ were not significantly altered in any metal exposure (Fig. 3A–C).

The effects of copper, cadmium and copper plus cadmium exposures on $Na^+$ loss was to be performed with adult zebrafish, however, there were no survivors in the binary mixture treatment (see Section 3.1 above; Fig. 2A) so a follow-up study with larvae was performed instead. Larvae were exposed separately to 0.79 μM copper (one-third of the larval 96 h $LC_{50}$) or 5.34 μM cadmium (one quarter of the larval 96 h $LC_{50}$) for 24 h and experienced no mortalities. This was accompanied by 7.6% and 9.6% decreases in whole body $Na^+$, respectively, although these changes were not significant (Fig. 2B). However, larvae exposed to both 0.79 μM copper and 5.34 μM cadmium experienced 6% mortality and a significant 20.4% decrease in whole body $Na^+$ after 24 h of exposure (Fig. 2B).

Effects of fluoxetine (0.7 μM) and βNF (1.0 μM) on whole body cations were tested with 40-h exposures that resulted in 15% and 2% mortality rates, respectively. Fluoxetine significantly decreased survivor whole body $Na^+$ (23%) and $K^+$ (7%), while βNF also decreased $Na^+$ (11%) and $K^+$ (7%) (Fig. 3D and E). There were no

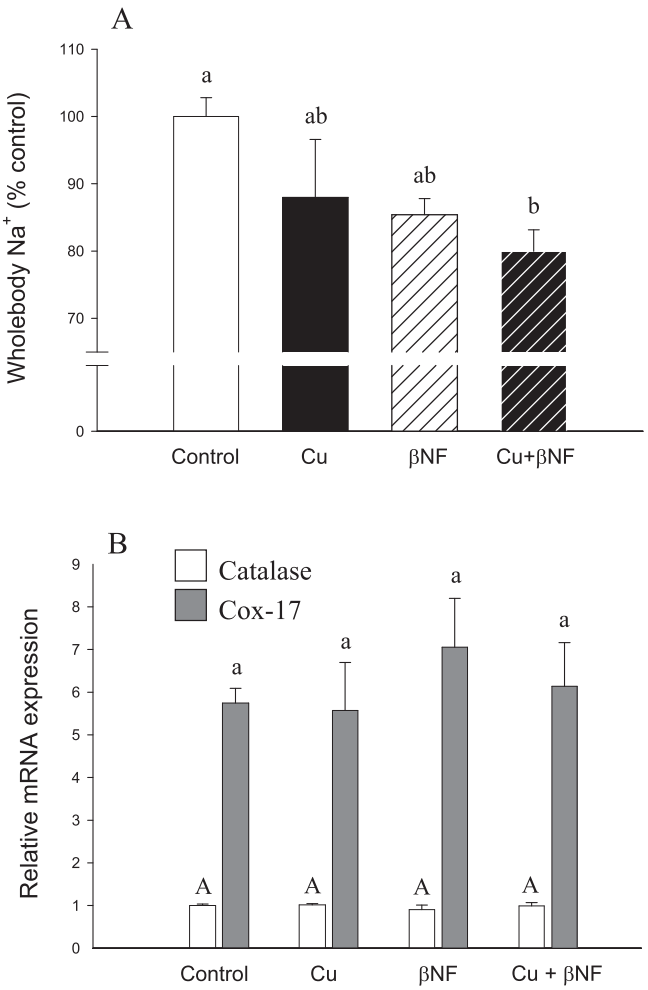


**Fig. 4.** The effects of exposure to 0.79 μM copper, 1 μM β-naphthoflavone (βNF) or both 0.79 μM copper and 1 μM βNF for 36 h on whole body (A) $Na^+$ and (B) the expression of catalase and cox-17 normalized to the expression of EF1α. Bars with different letters are significantly different as determined by ANOVA followed by Tukey's honestly significant difference test to determine differences among groups ($p < 0.05$). $N = 5$ (pools of 10 larvae). Data are means ± S.E.M.

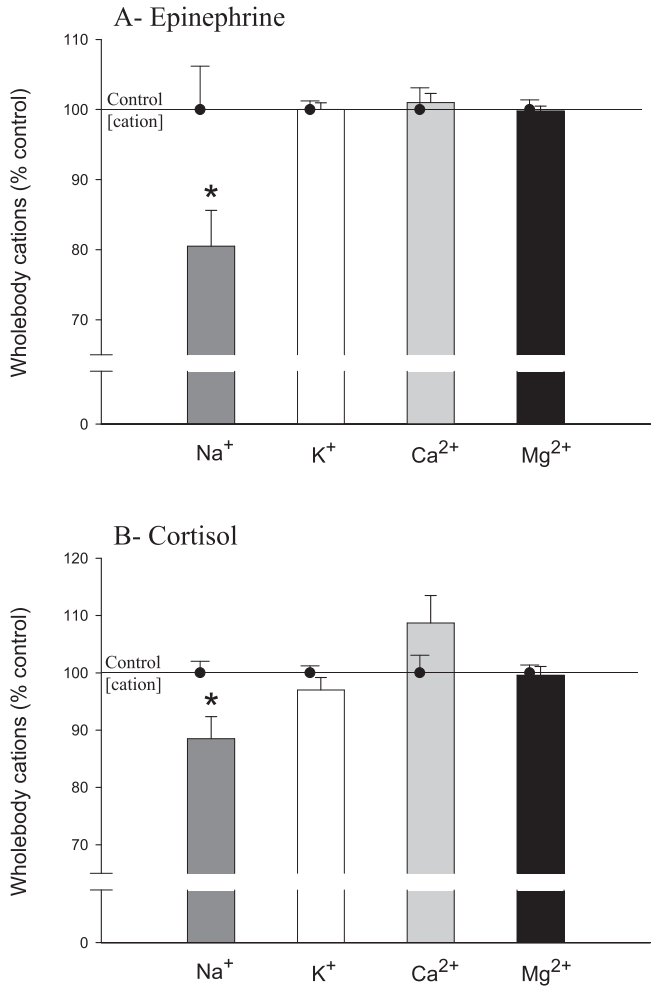


**Fig. 5.** Whole body content of $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ in 7 dpf zebrafish larvae exposed to (A) 1 μM epinephrine or (B) 1 μM cortisol for 32 h. An asterisk (*) indicates a significant difference from the control level ($p < 0.05$) determined with a Student's *t*-test. $N = 6$ pools of 11 larvae. Data are means ± S.E.M.

changes in $Ca^{2+}$ or $Mg^{2+}$ with exposure to either compound (Fig. 3D and E). Exposure to 500 μM ammonia for 46 h resulted in no mortalities and significant 15% and 12% decreases in whole body $Na^+$ and $K^+$, respectively, without significant changes in whole body $Ca^{2+}$ or $Mg^{2+}$ (Fig. 3F).

To test whether $Na^+$ loss with toxicant exposure was due to oxidative stress, larvae were exposed to 1 μM βNF, 0.79 μM copper or both for 36 h. While the effect of βNF or copper alone on whole body $Na^+$ was not significant, exposure to both βNF and copper caused a significant 20% decrease in whole body $Na^+$ (Fig. 4A). However, the expressions of catalase and cox-17 were not affected by exposure to βNF, copper or both simultaneously (Fig. 4B).

Finally, 1 μM epinephrine exposure for 32 h caused a significant 20% decrease in whole body $Na^+$ (Fig. 5A), while 1 μM cortisol decreased $Na^+$ by 12% (Fig. 5B). The other three cations were not affected.

## 4. Discussion

### 4.1. Larval assay

The simple ability to test this many contaminants alone and in combination was physically possible due to the availability and small size of the zebrafish larvae and assay. From 4 to 8 dpf (2–6 dph), the fish have matured to the point where they respond to contaminants with similar sensitivities and physiological mechanisms as adults (Alsop and Wood, 2011). However, larvae do not have to be fed (they are sustained by their yolk), and the larval stage is the most sensitive life stage compared to adults and embryos where the chorion can be protective (Alsop and Wood, 2011). The larvae are very robust; there were no mortalities in any control treatment during the 96 h toxicity experiments, so even a single mortality during an exposure would flag the chemical for further testing.

### 4.2. Mixture toxicity

Toxic interactions were observed between metals, where sublethal levels of one metal increased the toxicity of another. When comparing the CA and IA models, the CA MRD values were consistently lower (closer to 1), indicating that the CA model was a better predictor of mixture toxicity than was the IA model. The CA MDR values for the $LC_{50}$s were typically >1 and <2, and thus were within the range of acceptability for agreement with the CA concept according to previous studies (see Section 2.7; Belden and Lydy, 2006; Belden et al., 2007; Tollefsen et al., 2012). However, this range of CA MDRs also suggests that there is a consistent underestimation of metal mixture toxicity. In addition, the 95% CIs for the

observed metal mixture $LC_{50}$s with cadmium or copper as the primary metal (but not nickel) did not overlap the predicted CA $LC_{50}$s values (Tables 1 and 2), indicating a significant difference (Belden and Lydy, 2006). This suggests that the CA model does not always accurately predict metal mixture toxicity, and the effects of some metal mixtures are greater than additive.

Additive toxicity has been reported in previous studies on other species of fish. For example, copper, zinc and nickel were additive in rainbow trout (Lloyd, 1961; Marking, 1977), while the toxicities of copper and cadmium as well as zinc and cadmium were additive in chinook salmon (Finlayson and Verrue, 1982). Furthermore, the effects of a copper/zinc co-exposure were greater than additive, while a copper/manganese co-exposure was additive in the longfin dace (Lewis, 1978). Another study found the type of interaction was dependent on the specific metal mixtures, and observed additive, greater than additive and antagonistic effects in Colorado squawfish and razorback sucker larvae (Hamilton and Buhl, 1997). These findings across species are environmentally significant given that metal impacted waters are most often contaminated with more than one metal (Pyle et al., 2005).

Generally, the MDRs were lower in mixture experiments that included nickel (Table 2), indicating a lesser degree of additivity associated with this metal. This may be due to the much higher levels of nickel used in the experiments (due to its lower toxicity) competing with cadmium and copper for non-specific anionic sites on metal transport epithelia such as the gill (Alsop and Wood, 1999). For example, the nickel 96 h $LC_{50}$ was 224 μM, and in a binary mixture, copper was added at 0.24 μM as the secondary metal, a difference of almost 1000-fold. Similarly, although copper (0.79 μM) did potentiate the toxicity of ammonia, which also had a much higher absolute concentration in the exposures (96 h $LC_{50}$ = 677 μM), this was to a lesser extent than it did with other metals. In fact, the only MDRs from the present study that were <1 for the CA model were tests with nickel and ammonia. Previous studies have observed additive toxicity between ammonia and copper or zinc in rainbow trout (Herbert and Shurben, 1964; Herbert and Van Dyke, 1964).

The acute toxicities of a variety of pharmaceuticals were tested alone (1 μM) and in combination with sublethal levels of copper (0.79 μM). While pharmaceutical levels in the environment are generally in the pM to low nM range (e.g. 17α-ethinylestradiol; Combalbert and Hernandez-Raquet, 2010), for toxicity testing purposes exposures of 1 μM were performed and subsequently diluted if mortalities were observed (see Table 3). For copper, 0.79 μM Cu is environmentally realistic, as levels upwards of 3 μM have been reported in polluted waters (Taylor et al., 2003; Pyle et al., 2005).

Fluoxetine is an SSRI and prescribed as an antidepressant in drugs such as Prozac™ (Mennigen et al., 2011). Fluoxetine was toxic to zebrafish larvae, with a 96 h $LC_{50}$ of approximately 0.81 μM. This is similar to the 7 d $LC_{50}$ of 1.8 μM for mosquitofish larvae (Henry and Black, 2008) and the 48 h $LC_{50}$ of 2.3 μM for fathead minnows (Brooks et al., 2003). There were sublethal effects associated with fluoxetine; zebrafish larvae would float at the surface on their sides at levels ≥ 0.5 μM, similar to mosquitofish exposed to fluoxetine levels ≥ 1.7 μM (Henry and Black, 2008). Fluoxetine also exhibited additive toxicity with copper, which was an unexpected result given our assumption that the mechanism of fluoxetine toxicity would involve the serotonin pathway and would be unrelated to metal toxicity. Environmental levels of fluoxetine range from 0.04 to 1.8 nM (reviewed by Brooks et al., 2003), which is considerably less than the $LC_{50}$ of 0.81 μM for zebrafish larvae. However, the concern is that even at low concentrations, the presence of several contaminants could reach a toxic threshold due to additive effects.

### 4.3. Ion homeostasis

Given that fluoxetine showed additive toxicity with copper and the known effects of copper on whole body ion loss, we were prompted to examine whole body ions in larvae exposed to fluoxetine and other non-metal contaminants. Indeed, exposure to metals, fluoxetine, βNF or ammonia all resulted in decreased whole body $Na^+$ and $K^+$ levels in zebrafish larvae. Our previous study on metals concluded that ion loss appears to be the result of increased permeability and leakage from the fish to the more dilute aquatic environment (Alsop and Wood, 2011). Thus ion loss may represent the common mechanism of toxicity that explains the interactions between toxicants observed in the present study. Cadmium, copper and nickel were chosen for testing since they were originally thought to have different mechanisms of action based on data from rainbow trout (e.g. Niyogi and Wood, 2004). However, they were all subsequently shown to induce the same acute effect on ion loss in zebrafish (Alsop and Wood, 2011).

Ion loss in response to contaminant exposures from the present study and a previous study (Alsop and Wood, 2011) were plotted against mortality rates and show a relationship that is most apparent for $Na^+$ (Fig. 6A). This analysis illustrates that mortalities did not occur without some $Na^+$ loss in the survivors (Fig. 6A). There is less of an obvious relationship with $K^+$, although all exposures that resulted in mortalities also induced some degree of $K^+$ loss in the survivors (Fig. 6B). In contrast, some mortality occurred in the absence of an effect on whole body $Ca^{2+}$ (Fig. 6C). Decreased $Ca^{2+}$ levels did occur only in some metal exposures, most likely due to the shared transport pathways across epithelia (Alsop and Wood, 2011). In addition, while metals inhibit $^{45}Ca$ uptake in zebrafish (Alsop and Wood, 2011), fluoxetine and βNF do not (D. Alsop and C.M. Wood, unpublished results). However, these compounds do show additive toxicity with copper, further evidence that $Ca^{2+}$ disruption is not the mechanism underlying acute mortality. Overall, the effect of acutely toxic contaminants has the strongest relationship with whole body $Na^+$ levels. Although we cannot rule out $K^+$ loss as part of the acute toxic mechanism, $Na^+$ loss is more sensitive than $K^+$, in that $Na^+$ loss is sometimes observed with no associated $K^+$ loss (particularly if there were no mortalities associated with the exposure), and $Na^+$ levels were always decreased to a greater extent than $K^+$.

Initially, we thought contaminant-induced ion loss may be due to increased oxidative stress that could cause tissue damage and increased ion permeability. However, the results of two experiments argue against this hypothesis. First, the expression of catalase and cox17, two enzymes associated with an increase in oxidative stress (Craig et al., 2007), did not change in larvae with exposure to copper, βNF or both contaminants together (even though decreased whole body $Na^+$ levels were observed in the co-exposure treatment). Secondly, the antioxidant Trolox (a water soluble vitamin E analog) was not protective in larvae exposed to copper or nickel (Alsop and Wood, 2011).

Alternatively, ion loss may be due to epinephrine and cortisol signaling as part of the endocrine stress response. Previous studies have shown that epinephrine infusions induce rapid and substantial ion losses in rainbow trout through increased permeability (McDonald and Rogano, 1986; Vermette and Perry, 1987; Gonzalez and McDonald, 1992). Indeed, zebrafish larvae exposed to 1 μM epinephrine or cortisol experienced decreased whole body $Na^+$ levels. Copper, cadmium and zinc have been shown to stimulate cortisol levels in fish (Craig et al., 2009; Fırat and Kargın, 2010), although we could find no studies that measured catecholamine levels with metal exposure. Fluoxetine injections can increase circulating cortisol levels in fish and are toxic at higher doses (Morando et al., 2009), while fluoxetine was also a very potent modulator of specific cortisol-responsive genes in zebrafish

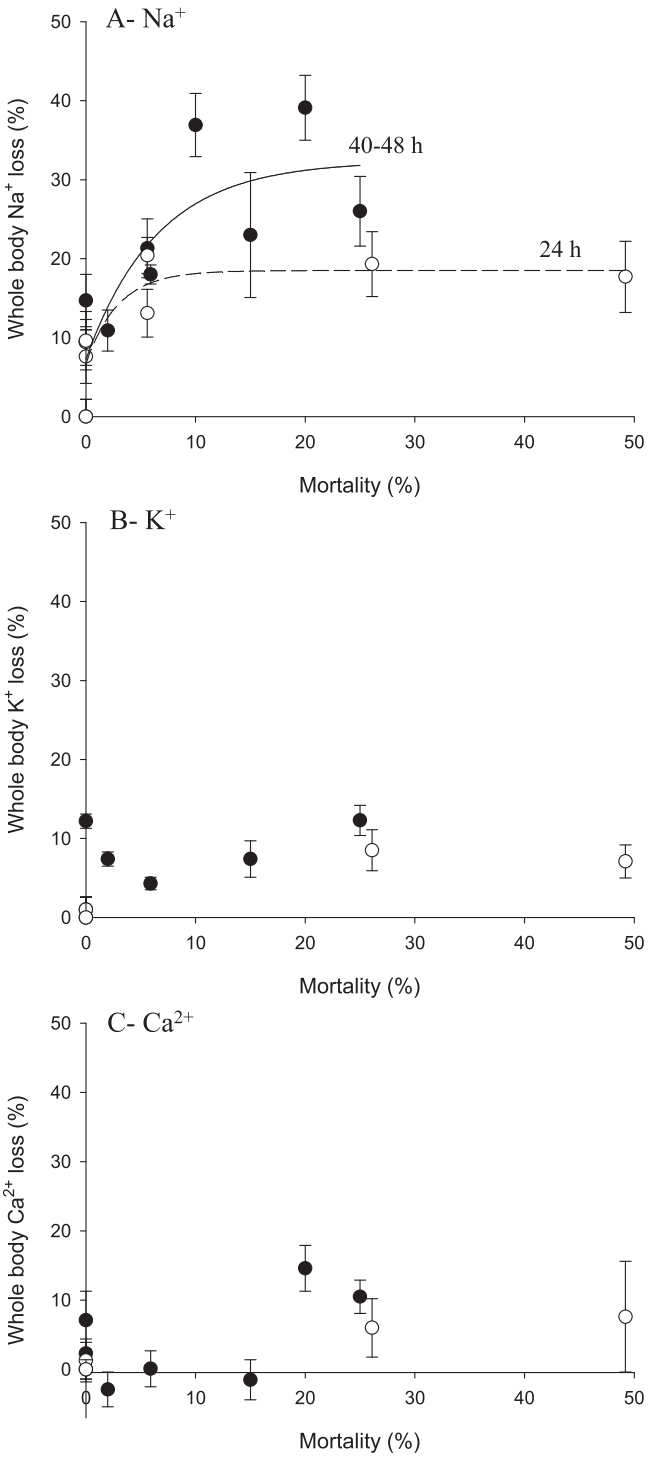


**Fig. 6.** Comparison of mortality rates to whole body losses of (A) $Na^+$, (B) $K^+$ and (C) $Ca^{2+}$ in surviving larvae from experiments in the present study and Alsop and Wood (2011). Exposures include multiple experiments with cadmium, copper and nickel (that varied in time and concentration), along with exposures to fluoxetine, β-naphthoflavone and ammonia. The relationship between $Na^+$ loss and mortality was significant from both 24 h and 40–48 h exposures ($p = 0.037$, $R^2 = 0.73$ and $p = 0.013$, $R^2 = 0.71$, respectively). Levels of $Mg^{2+}$ (data not shown) did not change in any experiment. The open circles and dashed line represent exposures of 24 h, while filled circles and solid line represent exposures of 40–48 h. All data were included, even if changes were not significant from the control treatment. Data are means ± S.E.M.

larvae at fluoxetine concentrations of 0.08 and 0.8 μM (Park et al., 2012). Zebrafish larvae have a functional cortisol stress response by 4 dpf (Alsop and Vijayan, 2008), and at 4 dpf β-adrenergic receptor expression is responsive to hypoxia, while heart rate is responsive to norepinephrine and propranolol (Steele et al., 2009).

### 4.4. Implications of additive toxicity to environmental regulations

There are mounting studies showing additive effects between similar classes of contaminants (pesticides, metals, estrogenic compounds) in fish, other animals (e.g. insects and crustaceans), and plants (Norwood et al., 2003; Belden et al., 2007; Kortenkamp, 2007). There are fewer data on the interactions between different classes of contaminants. However, the present study has shown many compounds that are acutely toxic on their own also have the potential to display additive toxicity with other acutely toxic contaminants, regardless of their chemical nature (e.g. an atom (copper), a simple amine (ammonia), a more complex organic molecule (fluoxetine)). The common underlying mechanism of toxicity appears to be ion loss. In order to adequately protect aquatic biota, acute water quality criteria should ultimately recognize the toxic interactions between contaminants, and the policies of environmental regulatory authorities are starting to move in this direction (ANZECC and ARMCANZ, 2000; USEPA, 2007; Scientific Committee on Health and Environmental Risks, 2012; European Commission, 2012). One approach could be to utilize the CA model as a straightforward and broadly applicable risk assessment tool, as has been suggested for pesticides (Belden et al., 2007). However, given the considerable potential for the CA model to underestimate metal mixture toxicities (see Section 4.2) further data are required to understand the degree of additivity across more metals. With the larval zebrafish assay, establishing a better data set of interactions across metals would be possible, and this would be useful for predicting mixture toxicity in aquatic environments that suffer from metal contamination, such as areas impacted by mining (Pyle et al., 2005).

However, it would be a daunting task to test the toxicities and toxic interactions amongst all contaminants of environmental concern. Screening individual chemicals for impacts on whole body $Na^+$ in zebrafish larvae would be a practical approach, as this appears to be a consistent response associated with acute toxicity and a potential biomarker for additive toxicity. $Na^+$ can be measured in a single larva, which would lend itself to screens and predictions of toxic interactions. Subsequent experiments could then determine whether compounds identified as $Na^+$ effectors exhibited additive toxicity with model toxicants such as copper.

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