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Interactive effects of waterborne metals in binary mixtures on short-term gill–metal binding and ion uptake in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Metal binding to fish gills forms the basis of the biotic ligand model (BLM) approach, which has emerged as a useful tool for conducting site-specific water quality assessments for metals. The current BLMs are designed to assess the toxicity of individual metals, and cannot account for the interactive effects of metal mixtures to aquatic organisms including fish. The present study was designed mainly to examine the interactive effects of waterborne metals (Cd, Zn, Cu, Ag, and Ni) in specific binary combinations on short-term (3 h) gill–metal binding and essential ion (Ca^{2+} and Na^{+}) uptake (a physiological index of toxicity) in fish, using juvenile freshwater rainbow trout (*Oncorhynchus mykiss*) as the model species. We hypothesized that binary mixtures of metals that share a common mode of uptake and toxicity (*e.g.*, Cd and Zn – Ca^{2+} antagonists, Cu and Ag – Na^{+} antagonists) would reduce the gill binding of each other *via* competitive interactions and induce less than additive effects on ion transport. In addition, the mixture of metals that have different modes of uptake and toxicity (*e.g.*, Cd and Cu, or Cd and Ni) would not exhibit any interactive effects either on gill–metal binding or ion transport. We found that both Zn and Cu reduced gill–Cd binding and *vice versa*, however, Ni did not influence gill–Cd binding in fish. Surprisingly, Ag was found to stimulate gill–Cu binding especially at high exposure concentrations, whereas, Cu had no effect on gill–Ag binding. The inhibitory effect of Cd and Zn in mixture on branchial Ca^{2+} uptake was significantly greater than that of Cd or Zn alone. Similarly, the inhibitory effect of Cu and Ag in mixture on branchial Na^{+} uptake was significantly greater than that of Cu or Ag alone. The inhibitory effects of Cd and Zn mixture on Ca^{2+} uptake as well as Cu and Ag mixture on Na^{+} uptake were found to follow the principles of simple additivity. In contrast, no significant additive effect on either Ca^{2+} or Na^{+} uptake was recorded in fish exposed to the mixture of Cd and Cu. Overall, we found that although the effects of metal mixture interactions on gill–metal binding did not always match with our original assumptions, the effects of metal mixtures on toxicity in fish were generally consistent with our predictions. The findings of the present study have important implications for improving the BLM approach to assess metal mixture toxicity in fish.

1. Introduction

Current water quality regulations for metals are primarily designed to protect aquatic organisms against the toxic effects of single metals, and do not account for the interactive effects of metals in mixtures. However, in the real world, organisms inhabiting metal-contaminated natural waters are almost always exposed to the elevated levels of metals in mixtures (Borgmann et al., 2008; Vijver et al., 2011). To date, the pathophysiological and toxicological implications of exposure to individual metals have been studied extensively in aquatic organisms including fish. However, until recently, very few studies have been carried out to examine the toxic implications of metal mixtures on aquatic organisms (Norwood et al., 2003; Borgmann et al., 2008; Vijver et al., 2011; Balistrieri and Mebane, 2014; Tipping and Lofts, 2015 for review). In particular, very little is known about the interactive effects of metals in mixtures on their uptake and accumulation in the fish gill, which is known to be the primary target organ for metal toxicity in fish during acute exposures (Niyogi and Wood, 2004a).

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The Biotic Ligand Model (BLM) has emerged as a practical tool for the generation of site-specific water quality guidelines and criteria in North America, Europe, and other jurisdictions (Di Toro et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004a; Bodar et al., 2005). The BLM is a computational approach that integrates physiology, toxicology, and geochemistry into a modeling framework that relates ambient water chemistry to metal accumulation on the biotic ligand (*e.g.*, fish gill). The BLM predicts toxicity in fish based on the short-term (*e.g.*, 3 h) critical accumulation of metal(s) on the fish gill. In freshwater, the BLM approach has been a success, since it has significantly improved our ability to regulate individual metals in freshwater ecosystems. One of the primary reasons behind the success of single metal BLMs is the fact that there is a sound physiological basis for understanding the mechanisms underlying observed toxicity. For example, gill accumulation of Cu^{2+} or Ag^{+} in fish has been linked to interference with Na^{+} uptake mechanisms, thereby disrupting ionic balance in the organism and leading to toxicity (Grosell et al., 2002; Morgan et al., 2004). Similar interactions have also been described for Zn^{2+} and Cd^{2+} interfering with Ca^{2+} uptake (Hogstrand et al., 1996; Niyogi and Wood 2004b). In contrast, Ni^{2+} has been found to be different, at least in fish, acting primarily as a respiratory toxicant rather than an ionoregulatory toxicant (Pane et al., 2003). Based on this knowledge, predictions can be made on how the interactions of these metals in mixtures are expected to influence gill-metal binding and toxicity in fish. These predictions can then be experimentally tested.

Although the current BLMs are mainly designed to assess the toxicity of single metals in aquatic organisms, a few recent studies have indicated that the present BLM approach has the potential to be used for evaluating the toxicity of metal mixtures (Playle, 2004; Kamo and Nagai, 2008; Iwasaki et al., 2014; Balistrieri et al., 2015; Farley et al., 2015; Farley and Meyer, 2015; Meyer et al., 2015a,b; Santore and Ryan, 2015). However, one of the major limitations for developing a sound metal mixture BLM approach for fish is the paucity of experimental data on short-term metal accumulation on fish gills following exposures to metal mixtures. Previous studies suggest that metal accumulation in fish exposed to metal mixtures often runs counter to predictions based on our understanding of single metal mechanisms of uptake and/or toxicity. For example, waterborne Cu (a Na^{+} antagonist) was found to reduce waterborne Cd (a Ca^{2+} antagonist) uptake in fish gills during Cu and Cd co-exposure, however, no such effect of Cd was recorded on Cu uptake (Pelgrom et al., 1995; Komjarova and Blust, 2009). Furthermore, a co-exposure of waterborne Cu and Pb (a Ca^{2+} antagonist; Rogers et al., 2003; Rogers and Wood, 2004) was reported to produce a synergistic effect on both Cu and Pb uptake in the fish gill (Tao et al., 1999; Komjarova and Blust, 2009). As expected, long-term Cd- and Cu-acclimated rainbow trout exhibited different changes in gill log K values for Cd, but Cu-acclimated trout exhibited cross-acclimation to Cd whereas Zn-acclimated trout did not (McGeer et al., 2007), which was unexpected. Also in trout, Cd and Pb, both of which are Ca^{2+} antagonists, exhibited less than additive gill binding as might be predicted, yet they exerted more than additive ionoregulatory toxicity, which was unexpected (Birceanu et al., 2008). These observations indicate that more in-depth research is required to develop our understanding of how metal mixtures interact at the biotic ligand, and how these interactions influence toxicity.

Important questions that need to be addressed in this context are: (i) Which metals compete for the same uptake sites? (ii) Are these interactions consistent as a function of metal concentration? (iii) Is toxicity additive for metals with the same physiological mechanism of action? (iv) What are the toxic consequences when metals with different mechanisms of action interact?

The present study was designed to examine the interactive effects of waterborne metals (Ag, Cu, Cd, Ni, and Zn) in binary mixtures on short-term (3 h) acute gill–metal binding, using rainbow trout (*Oncorhynchus mykiss*) as the representative species. In addition, as an index of toxicity, we evaluated the interactive effects of binary metal mixtures on the short-term (3 h) branchial influx of essential ions (Na^{+} and Ca^{2+}). We hypothesized that binary mixtures of metals sharing common binding sites at the fish gill (*e.g.*, Cd and Zn, or Cu and Ag) would produce competitive interaction on the gill-binding of each other, and less than additive inhibition of essential ion (Ca^{2+} or Na^{+}) transport. In contrast, binary mixtures of metals binding to different sites at the fish gill (*e.g.*, Cu and Cd, or Cd and Ni) would not exhibit any interactive effects either on the gill–metal binding or essential ion transport, and so toxic effects would be additive or possibly synergistic. Finally, in order to understand whether the metal mixture interactions at the gill are indicative of lethality, we also evaluated the interactive effects of metal mixtures (for Cd and Zn only) on 96 h mortality.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (*O. mykiss*) weighing 6–12 g were obtained from Humber Springs Fish Hatchery (Orangeville, Ontario). Fish were held for at least 2 weeks prior to their use in experiments in 200-L polyethylene tanks supplied with continuous aeration and flowing moderately hard dechlorinated Hamilton tap water [Lake Ontario water: Ca = 1 mmol L^{-1}, Na = 0.6 mmol L^{-1}, Cl = 0.7 mmol L^{-1}, dissolved organic carbon (DOC) = 0.2 mmol L^{-1}; hardness = 140 mg L^{-1} as $CaCO_3$, alkalinity = 95 mg L^{-1} as $CaCO_3$, pH 8.0, 12–14 °C]. Fish were fed 1% body weight every other day during the acclimation period, and then fed 1% body weight every day thereafter with Silver Cup Fish Feed for salmon fry [Manufacturer's specifications: 52% crude protein, 14% crude fat, 3% crude fiber, 12% ash, 1% sodium].

2.2. Interactive effects of binary metal mixtures on short-term gill–metal binding

A short-term (3 h) acute gill–metal binding assay, as described in Niyogi et al. (2008), was employed in dechlorinated Hamilton tap water using live fish transferred from the holding tanks. The present study examined the interactive effects of five metals (Cd, Cu, Zn, Ag, and Ni) on gill–metal binding in specific binary combinations (see below for details). The gill binding for any of these metals was measured using radio-labeled metal in the exposure water presented at the approximate 96 h LC_{50} concentration for juvenile trout in Hamilton water (see Fig. 2 of Niyogi and Wood, 2004a for a summary of values), whereas, the interactive effect of the competing metal (cold) was tested over a wide range of concentrations from below to slightly above its 96 h LC_{50} concentration. The 96 h LC_{50} concentration was chosen for evaluating the gill binding of each of the metals tested because it yields the LA_{50} (lethal accumulation 50 – the short-term (3 h) critical gill metal accumulation that leads to 50% mortality at 96 h). Six fish per treatment were used for each gill-binding assay performed in this study. Fish were placed in a 5-L clear polyethylene bag (containing 3 L of the exposure water) inside a dark supporting chamber. The bags were placed in a wet table to maintain the temperature between 12–14 °C, and each bag was supplied with its own airline. Water samples (5 mL) were taken in duplicate at the beginning and end of the 3 h exposure for the analysis of radioactivity and total metal concentrations (see below for details). Following 3 h of exposure, fish were euthanized with an overdose of anesthetic (50 mg L^{-1} buffered MS-222; Syndel Laboratories Ltd., Canada). The gill baskets were dissected out and rinsed, first in a concentrated solution of respective cold

metal (1 mM) for 15 s followed by ambient water for another 15 s, to remove any loosely bound radio-labeled metal, and blotted dry. The gills and water samples were then weighed before analysis, to determine their exact masses and volumes.

For the gill–metal binding assays, we examined the following combinations of waterborne metals in binary mixtures: Cd and Zn, Cu and Cd, Cu and Ag, and Cd and Ni. While nominal exposure concentrations are stated here, measured concentrations are reported throughout the Section 3.

To examine the effects of waterborne Zn, Cu or Ni on Cd gill binding, fish were exposed to 0.20 μmol L^{-1} radiolabeled Cd concentration (nominal) in combination with a competing metal (Zn, Cu, or Ni) at 6 different nominal concentrations [Zn added as $ZnSO_4 \cdot 7H_2O$ (Fisher Scientific, ON, Canada): control (no added Zn), 1, 2, 5, 10, and 20 μmol L^{-1}; Cu (added as $CuSO_4 \cdot 5H_2O$, Fisher Scientific, ON, Canada): control (no added Cu), 0.08, 0.25, 0.40, 0.80, and 1.40 μmol L^{-1}; Ni (added as $NiCl_2 \cdot 6H_2O$, Fisher Scientific, ON, Canada): control (no added Ni), 20, 40, 100, 250, and 420 μmol L^{-1}]. The total Cd concentration was achieved by spiking the exposure water with cold Cd (added as $CdCl_2$, Fisher Scientific, ON, Canada) and 3 μCi L^{-1} of ^{109}Cd (as $CdCl_2$, PerkinElmer, ON, Canada).

To examine the effects of Cd on Zn gill binding, fish were exposed to 18 μmol L^{-1} radiolabeled Zn (nominal) in combination with Cd at 6 different nominal concentrations [control (no added Cd), 0.02, 0.05, 0.10, 0.20, and 0.30 μmol L^{-1}]. The total Zn exposure concentration was achieved by spiking the exposure water with cold Zn (as $ZnSO_4 \cdot 7H_2O$) and 3 μCi L^{-1} of ^{65}Zn (as $ZnCl_2$, PerkinElmer, ON, Canada), and Cd was added as $CdCl_2$.

The effects of Cd on Cu gill binding were also examined similarly, except that fish were exposed to a radio-labeled Cu concentration of 1.25 μmol L^{-1} (nominal) in combination with the identical range of nominal Cd exposure levels mentioned above. The total Cu exposure concentration was achieved by spiking the exposure water with cold Cu (as $CuSO_4 \cdot 5H_2O$) and 3 μCi L^{-1} of ^{64}Cu (as $Cu(NO_3)_2$, McMaster University Nuclear Reactor, ON, Canada).

To study the interactions of Cu and Ag, Cu gill binding was evaluated as described above in combination with 6 different nominal concentrations of Ag [control (no added Ag), 0.01, 0.025, 0.05, 0.10, 0.20 μmol L^{-1}; added as $AgNO_3$, Fisher Scientific, ON, Canada]. In contrast, the effects of Cu on Ag gill binding were evaluated in fish exposed to 0.10 μmol L^{-1} radiolabeled Ag in combination with only control (no Cu added) and 1.25 μmol L^{-1} of nominal Cu concentrations. The total Ag exposure concentration was achieved by spiking the exposure water with cold Ag (as $AgNO_3$) and 5 μCi L^{-1} of ^{110}Ag (as AgCl, Eckert & Ziegler Isotope Products, GA, USA), and Cu was added as $CuSO_4 \cdot 5H_2O$.

2.3. Interactive effects of binary metal mixtures on short-term branchial essential ion (Na^+ and Ca^{2+}) uptake

To examine the interactive effects of binary metal mixtures on short-term (3 h) branchial Na^+ and Ca^{2+} uptake, fish were exposed to specific metals, both singly and in binary mixtures. We selected binary combinations of metals that were found to have interactive effects on gill–metal binding (see below for details). We evaluated the effects of Cu (1.25 μmol L^{-1}), Ag (0.10 μmol L^{-1}), Cd (0.20 μmol L^{-1}) along with Cu plus Ag (1.25 and 0.10 μmol L^{-1}, respectively), and Cu plus Cd (1.25 and 0.20 μmol L^{-1}, respectively), in addition to the control (no added metals in the exposure water), on branchial Na^+ uptake. Similarly, we also evaluated the effects of Cd (0.20 μmol L^{-1}), Zn (18 μmol L^{-1}), Cu (1.25 μmol L^{-1}) along with Cd plus Zn (0.20 and 18 μmol L^{-1}, respectively), and Cd plus Cu (0.20 and 1.25 μmol L^{-1}, respectively), in addition to the control (no added metals in the exposure water), on branchial Ca^{2+} uptake. The uptake of Na^+ and Ca^{2+} was evaluated at the ambient concentration of Na^+ and Ca^{2+} in dechlorinated Hamilton tap water.

The short-term (3 h) branchial Na^+ and Ca^{2+} influx was evaluated as described in Niyogi and Wood (2004b), using the same experimental approach and set-up employed for gill-binding assays in the present study. Briefly, fish (6 per treatment) were placed into 5-L clear polyethylene bags containing 3 L of appropriate exposure water under constant aeration and temperature (12–14 °C). Five minutes before the fish were introduced to the bags, 3 μCi L^{-1} of ^{22}Na (as NaCl, Amersham Pharmacia Biotech, QC, Canada) or ^{45}Ca as $CaCl_2$ (PerkinElmer, ON, Canada) was added. After 3 h of exposure, fish were sacrificed, and the gill baskets and carcass were collected and rinsed first in 10 mM NaCl or $Ca(NO_3)_2$ solution for 30 s followed by ambient water for another 30 s, to remove any loosely bound radioactivity, and blotted dry and weighed individually. Duplicate water samples (5 mL) were taken at the beginning and end of the 3 h exposure for the analysis of radioactivity and total ion and metal concentrations (see below for details).

2.4. Interactive effects of binary metal mixtures on fish mortality

In the present study, we only examined the interactive effects of Cd and Zn in mixtures on the mortality of fish, employing two different sets of experiments. In one set, fish were exposed for 96 h to an increasing range of waterborne Cd levels (control (no added Cd), 0.02, 0.04, 0.10, 0.20, and 0.40 μmol L^{-1} (nominal values); added as $CdCl_2$), alone and in combination with waterborne Zn (3 and 6 μmol L^{-1} (nominal values); added as $ZnSO_4 \cdot 7H_2O$). In the second set, fish were exposed for 96 h to an increasing range of waterborne Zn levels (control (no added Zn), 0.75, 1.50, 3, and 6 μmol L^{-1} (nominal values); added as $ZnSO_4 \cdot 7H_2O$), alone and in combination with waterborne Cd (0.10 and 0.20 μmol L^{-1} (nominal values); added as $CdCl_2$). In both set of experiments, fish ($n = 10$ per treatment) were exposed in polyethylene buckets containing 20 L of appropriate exposure water. All exposure chambers were fitted with an airline and kept in a wet table to maintain a constant temperature (12–14 °C). The exposures were conducted under static renewal system with 90% water exchange every 24 h. Water samples (5 mL) were collected for the analysis of metal levels, at the beginning whenever fresh exposure water was introduced into the exposure chambers. Fish mortality was recorded every 12 h during the entire exposure period.

2.5. Analytical techniques and calculations

Tissue (gill and carcass) and the first set of the duplicate water samples were counted for radioactivity (^{109}Cd, ^{64}Cu, ^{65}Zn, ^{110}Ag, and ^{22}Na) within 12 h of sampling in a gamma counter (1480 Automatic Gamma Counter, PerkinElmer, ON, Canada); tests demonstrated that counting efficiency for each radioisotope was constant. To record ^{45}Ca activity in tissue, tissue samples were first digested in 5 volumes of 1 N HNO_3 (ACS grade, Fisher Scientific, ON, Canada) for 48 h at 60 °C. Later, 0.5 mL of tissue digests were mixed with 5 mL of acid/organic compatible scintillation cocktail (Ultima Gold, PerkinElmer, ON, Canada). To count ^{45}Ca activity in the water, aliquots of water samples (1 mL each) were mixed with 10 mL of aqueous scintillation fluor (Optiphase, PerkinElmer, ON, Canada). Both tissue and water samples were counted for beta-radioactivity using a scintillation counter (Tricarb 2900TR, PerkinElmer, ON, Canada). Counting efficiencies for ^{45}Ca were determined by internal standardization, *i.e.* by addition/recovery of known amounts of ^{45}Ca to non-radioactive samples.

To measure total dissolved metal and ion concentrations, the second set of the duplicate water samples were filtered through 45-μm nylon syringe filters (Nalgene, NY, USA) and acidified with HNO_3 (1%; ACS Grade, Fisher Scientific, ON, Canada). The concentrations of Cd, Cu, Ag, and Ni were measured using a graphite

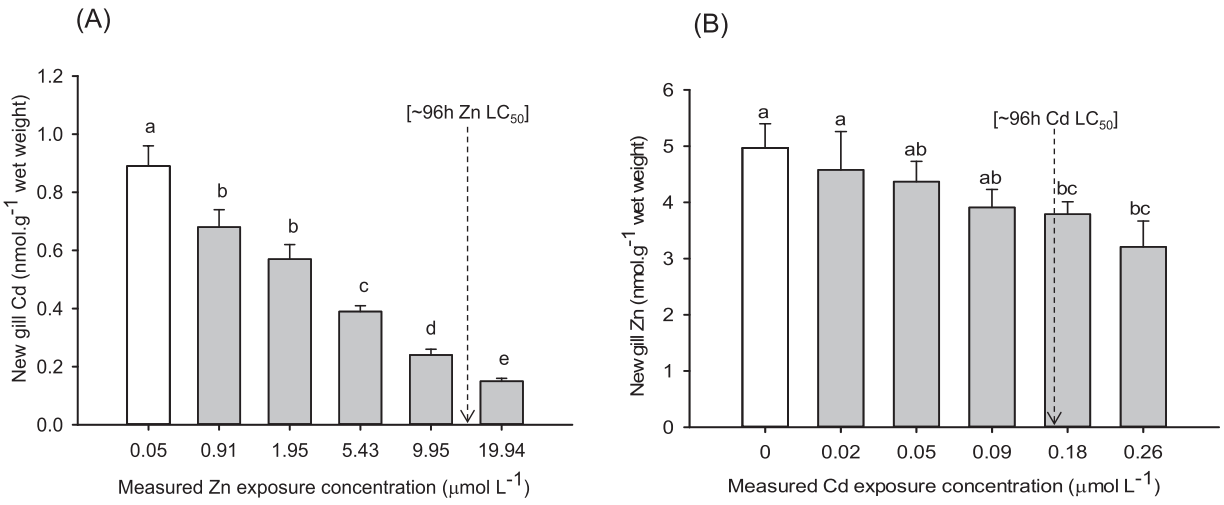

Fig. 1. (A) Effects of waterborne Zn on short-term (3 h) gill–Cd binding in rainbow trout exposed to 0.22 μmol L^{-1} waterborne Cd; and (B) effects of waterborne Cd on 3 h gill–Zn binding in rainbow trout exposed to 16.14 μmol L^{-1} waterborne Zn. The white bar represents control (no added competing metal in the exposure water) with measured background concentrations of Zn or Cd. The arrow indicates the approximate 96 h LC_{50} value of the competing metal (Zn or Cd) in rainbow trout under ambient water chemistry. Gill–Cd and gill–Zn binding data are presented as mean ± SEM ($n = 6$). Significant differences among treatments are indicated by different letters (one way ANOVA; $p < 0.05$).

furnace atomic absorption spectrophotometer (220GTA, Varian Instruments, CA, USA). Cation (Na^+ and Ca^{2+}) and Zn concentrations were estimated using a flame atomic absorption spectrophotometer (220FS, Varian Instruments, CA, USA). For the analysis of Ca^{2+}, $LaCl_3$ (0.02%) was added to each water sample prior to the analysis. The quality control and quality assurance for the ion and metal analysis were maintained by using appropriate method blanks, and certified standards (CPI International, Santa Rosa, Ca, USA) and reference materials (TM-25.4 and TMDA-54.5, Environment Canada, Burlington, ON, Canada). The recovery percentage of all the metals and ions analyzed were within the range of 93–105%. Final metal concentrations in samples were estimated by subtracting the method blank values.

The concentrations of free metals in the exposure waters were estimated by using Visual MINTEQ, version 3 (Gustafsson, 2010), which was supplied with the measured dissolved metal concentrations and the water chemistry data described above (major ions, pH, alkalinity and DOC). To model complexation of free metal ions and DOC in Visual MINTEQ, we used NICA-Donnan option with humic acid and fulvic acid inputs of 50% each.

The accumulation of New Metal(s) in the gills was calculated based on the accumulation of radioactivity (cpm) in the gill and the specific activity (bc^{-1}) of the water:

$$\text{NewMetalinthegill} = a \times (bc^{-1})^{-1}$$

where a is the cpm g^{-1} of tissue (wet weight) for any specific radioisotope; b is the measured counts in the water (cpm L^{-1}) for the same isotope; and c is the measured total metal concentration in the water (μmol L^{-1}). Final values were expressed as nmol New Metal per gram of tissue (wet weight).

The uptake of Na^+ and Ca^{2+} in the gill and carcass was also estimated using the same approach described above, except the final values were divided by the exposure time ($t = 3$ h) to derive the hourly rate of uptake. The rate of uptake of Na^+ and Ca^{2+} in tissues was expressed as μmol Na^+ or Ca^{2+} per kg of tissue (wet weight) per hour.

2.6. Statistical analysis

All data have been expressed as mean ± SEM (n) except 96 h mortality results, which were expressed as % values. The 96 h LC_{50} in fish exposed to waterborne Cd, with or without added Zn in the exposure, was calculated by the software, ToxCalc (1996). Significant differences among comparable treatments for both gill metal-binding and branchial influx of ions were tested with a one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons (SPSS 10.0 for Windows). Mean values were considered different at $p < 0.05$. The assumptions of ANOVA, *i.e.*, normality of distribution and homogeneity of variances were examined by Shapiro–Wilk's test and Levene's test, respectively (both at $\alpha = 0.05$). All of the data met these assumptions.

3. Results

3.1. Interactive effects of binary metal mixtures on gill–metal binding

Gill–Cd binding in fish exposed to a measured waterborne concentration of 0.22 μmol L^{-1} radiolabelled Cd (the approximate LC_{50}) was significantly influenced by Zn in the exposure. Waterborne Zn decreased short-term gill–Cd binding in a concentration-dependent manner (Fig. 1A). There was an ~80% reduction in gill-Cd binding at the highest Zn exposure concentration tested (19.94 μmol L^{-1}) relative to that in the control. The threshold for this effect was at a Zn concentration of only 0.91 μmol L^{-1}, far below the LC_{50} of Zn. In the reciprocal experiment (Fig. 1B), the measured radiolabelled Zn concentration was 16.14 μmol L^{-1}, reflecting the much higher LC_{50} of Zn. In contrast to Fig. 1A, the effect of waterborne Cd on gill–Zn binding was relatively modest, since only about a 35% decrease relative to the control was recorded at the highest waterborne Cd concentration tested (0.26 μmol L^{-1}). The first significant decrease in gill–Zn binding (by ~25%) occurred only at a Cd exposure concentration of 0.18 μmol L^{-1} (Fig. 1B), *i.e.* the Cd LC_{50}. Based on the Visual MINTEQ derived estimations of free Cd^{2+} and Zn^{2+} concentrations in the exposure, the increase in dissolved Zn levels did not alter the Cd^{2+} concentrations in exposure, and *vice versa* (data not shown).

Waterborne Cu also decreased gill–Cd binding, but the concentration-dependency was less clear (Fig. 2A). In this experiment the measured radiolabelled Cd concentration was 0.22 umol L^{-1}. There was no change in gill–Cd binding up to 0.24 μmol L^{-1} Cu in the exposure water, followed by a significant

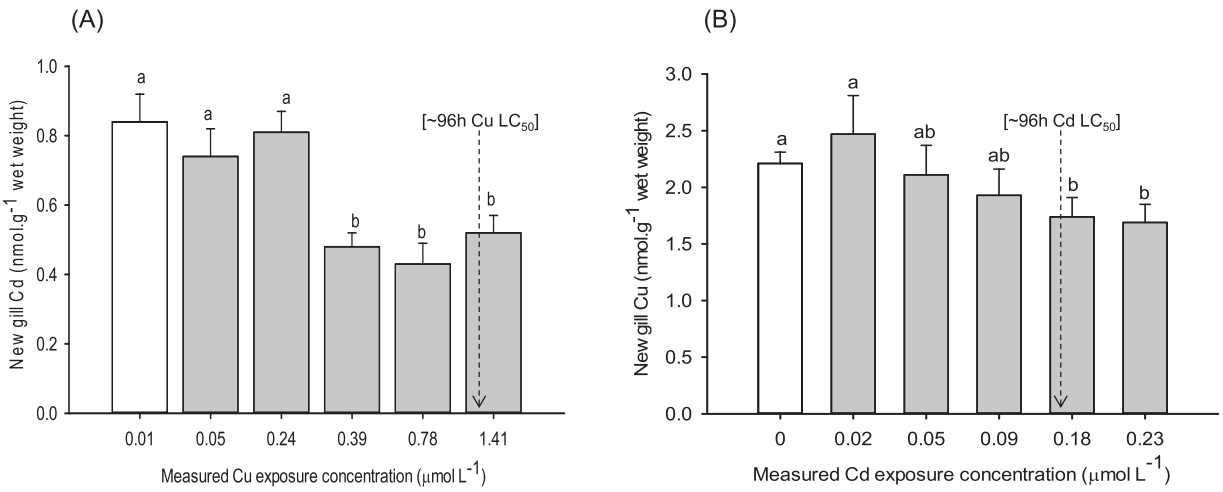

Fig. 2. (A) Effects of waterborne Cu on short-term (3 h) gill–Cd binding in rainbow trout exposed to 0.20 μmol L^{-1} waterborne Cd; and (B) effects of waterborne Cd on 3 h gill–Cu binding in rainbow trout exposed to 1.30 μmol L^{-1} waterborne Cu. The white bar represents control (no added competing metal in the exposure water) with measured background concentrations of Cu or Cd. The arrow indicates the approximate 96 h LC_{50} value of the competing metal (Cu or Cd) in rainbow trout under ambient water chemistry. Gill–Cd and gill–Cu binding data are presented as mean ± SEM ($n = 6$). Significant differences among treatments are indicated by different letters (one way ANOVA; $p < 0.05$). Note that gill–Ag binding was not significantly affected by waterborne Cu (B).

decrease (~40%) in gill–Cd binding at a Cu exposure concentration range of ≥0.39 μmol L^{-1} (Fig. 2A). This threshold is well below the Cu LC_{50}, but the inhibition stayed constant up to the approximate Cu LC50 concentration (1.41 μmol L^{-1}). In the reciprocal experiment, trout were exposed to a measured radiolabelled Cu concentration of 1.30 μmol L^{-1}; waterborne Cd reduced gill–Cu binding, with no change up to 0.09 μmol L^{-1} Cd exposure concentrations, followed by a small (~22%) but significant decrease in gill–Cu binding at Cd exposure levels of 0.18–0.23 μmol L^{-1} (Fig. 2B), which approximate the Cd LC_{50}. Free Cd^{2+} concentrations in the exposure was not influenced by the increase in dissolved Cu level, and *vice versa* (data not shown).

The influence of waterborne Ni on Cd-binding was also examined, again using a measured radiolabelled Cd concentration of 0.20 μmol L^{-1}. Note that very high levels of Ni were employed, in view of the low toxicity of this metal. Gill–Cd binding was not influenced by 20–410 μmol L^{-1} of waterborne Ni, the latter value surpassing the Ni LC_{50} (Fig. 3).

In tests with Ag as an antagonist, trout were again exposed to a measured radiolabelled Cu concentration of 1.30 μmol L^{-1}. Interestingly, waterborne Ag significantly increased gill–Cu binding, but only at exposure concentrations ≥0.09 μmol L^{-1} (Fig. 4A), which approximate the Ag LC_{50} concentration. Gill–Cu binding increased by almost 90% at the highest Ag exposure concentration tested (0.17 μmol L^{-1}) compared to that in the control. In contrast, gill–Ag binding in fish exposed to the LC_{50} concentration (0.09 μmol L^{-1}) of waterborne Ag was found to be not affected by exposure to the LC_{50} concentration (1.31 μmol L^{-1}) of waterborne Cu (Fig. 4B). Again, the concentrations of free Cu^{2+} in the exposure were not influenced by the elevated levels of dissolved Ag, and *vice versa* (data not shown).

3.2. Interactive effects of binary metal mixtures on branchial essential ion uptake

Since there was no difference in the pattern of effects of all single metal and binary metal mixture treatments on the influx of Ca^{2+} and Na^{+} in the gills *vs.* whole body, we only present the branchial influx data here. The rate of short-term (3 h) branchial Ca^{2+} influx was significantly decreased by both waterborne Cd (0.17 μmol L^{-1}) and Zn (16.95 μmo L^{-1}) individually at their approximate LC_{50} concentrations (Fig. 5). However, Cd inhibited Ca^{2+} influx rate by 37% relative to the control, whereas the inhibitory effect of Zn was >2-fold higher (79%) than that of Cd. The mixture of Cd and Zn at their approximate LC_{50} concentrations (0.18 and 16.05 μmol L^{-1}, respectively) produced significantly greater inhibition (86% relative to the control) of Ca^{2+} influx rate than either Cd or Zn alone exposure (Table 1A). Waterborne Cu at its LC_{50} concentration (1.20 μmol L^{-1}) alone also inhibited branchial Ca^{2+} influx, although the effect was smaller (24% decrease relative to the control) relative to that of Cd or Zn alone (Fig. 5). Similarly, the mixture of Cd and Cu (0.18 and 1.19 μmol L^{-1}, respectively) reduced Ca^{2+} influx rate by approximately 31% relative to the control, which was not statistically different from the inhibitory effect of Cu alone (Fig. 5; Table 1A).

The rate of short-term (3 h) branchial Na^{+} influx was significantly decreased by LC_{50} concentrations of both waterborne Cu (1.17 μmol L^{-1}) and Ag (0.08 μmol L^{-1}) individually (Fig. 6). However, Ag inhibited Na^{+} influx rate by 52% relative to the control, which was significantly higher than the inhibitory effect (30%) of

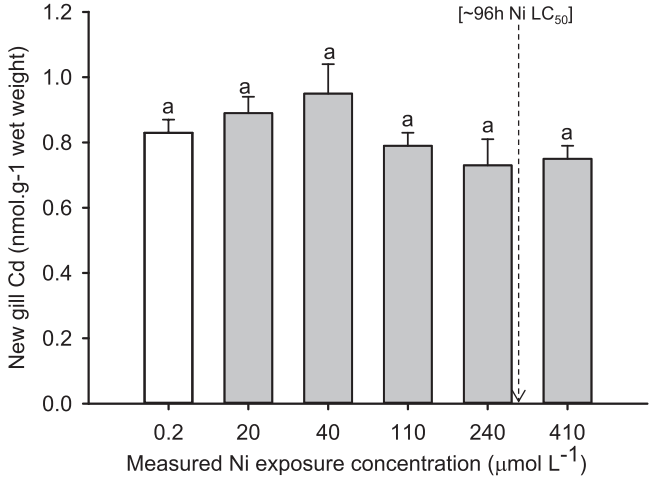

Fig. 3. Effects of waterborne Ni on short-term (3 h) gill–Cd binding in rainbow trout exposed to 0.20 μmol L^{-1} waterborne Cd. The white bar represents control (no added Ni in the exposure water) with measured background concentration of Ni. The arrow indicates the approximate 96 h LC_{50} value of Ni in rainbow trout under ambient water chemistry. Gill-Cd binding data are presented as mean ± SEM ($n = 6$). No significant differences were observed among any of the treatments (one way ANOVA; $p < 0.05$).

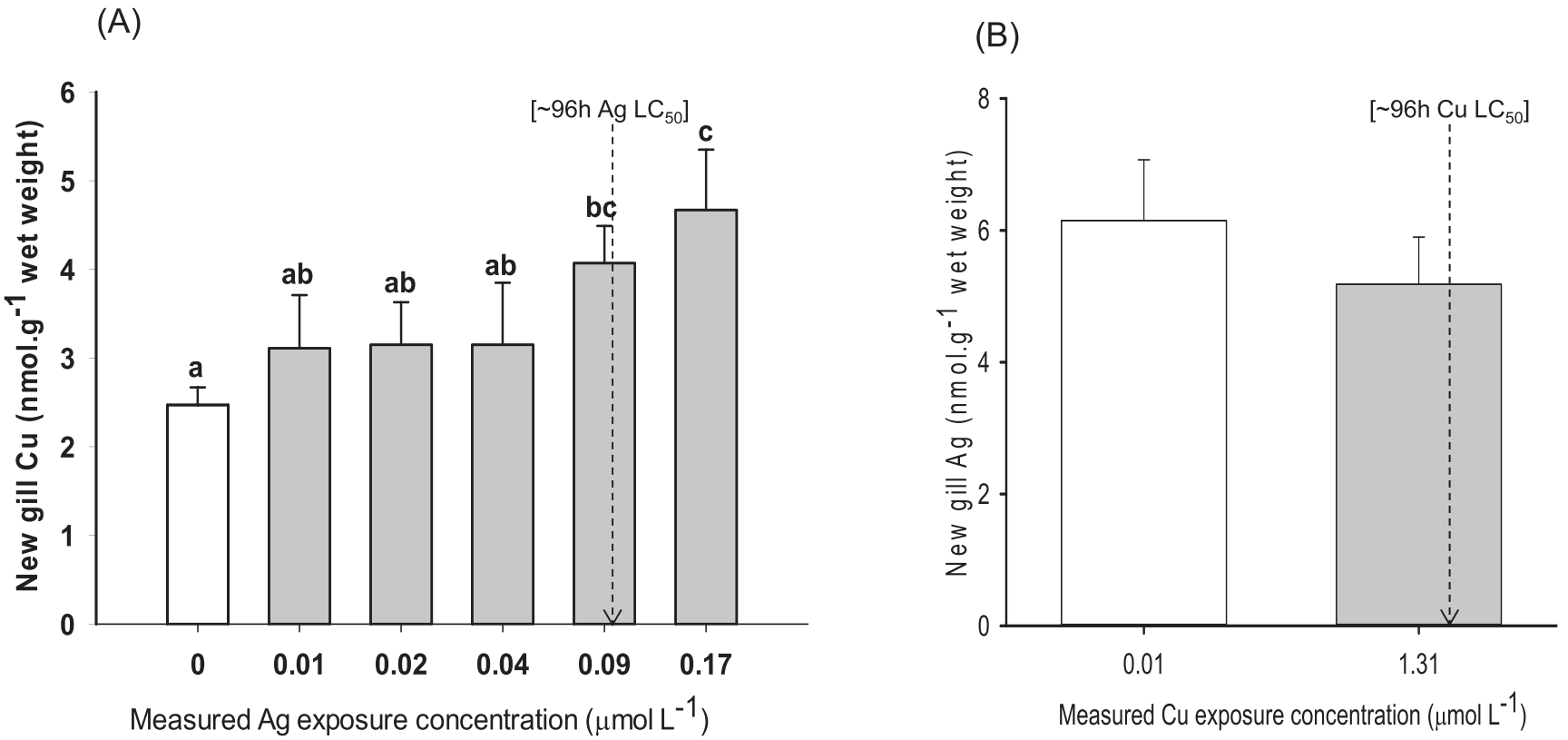

Fig. 4. (A) Effects of waterborne Ag on short-term (3 h) gill–Cu binding in rainbow trout exposed to 1.30 μmol L^{-1} waterborne Cu; and (B) effects of waterborne Cu on 3 h gill–Ag binding in rainbow trout exposed to 0.09 μmol L^{-1} waterborne Ag. The white bar represents control (no added competing metal in the exposure water) with measured background concentrations of Ag or Cu. The arrow indicates the approximate 96 h LC_{50} value of the competing metal (Ag or Cu) in rainbow trout under ambient water chemistry. Gill–Cu and gill–Ag binding data are presented as mean ± SEM ($n = 6$). Significant differences among treatments are indicated by different letters (one way ANOVA; $p < 0.05$).

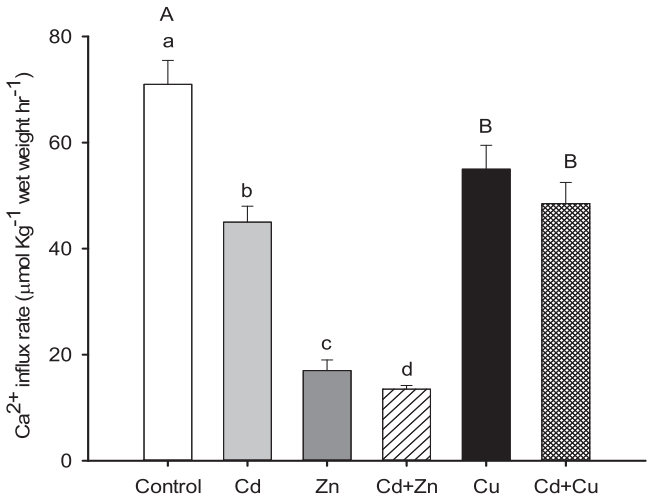

Fig. 5. Effects of waterborne Cd and Zn, and Cu and Cd (singly and in mixture) on branchial Ca^{2+} influx rate in rainbow trout. The white bar represents control (no added metals in the exposure water). The measured Cd, Zn and Cu concentrations in respective single metal exposures were 0.17, 16.95, and 1.20 μmol L^{-1}, respectively. In Cd and Zn mixture treatment, the measured Cd and Zn exposure concentrations were 0.18 and 16.05 μmol L^{-1}, respectively. In Cd and Cu mixture treatment, the measured Cd and Cu exposure concentrations were 0.18 and 1.19 μmol L^{-1}, respectively. Branchial Ca^{2+} influx data are presented as mean ± SEM ($n = 6$). Significant differences among comparable treatments are indicated by different letters (one way ANOVA; $p < 0.05$).

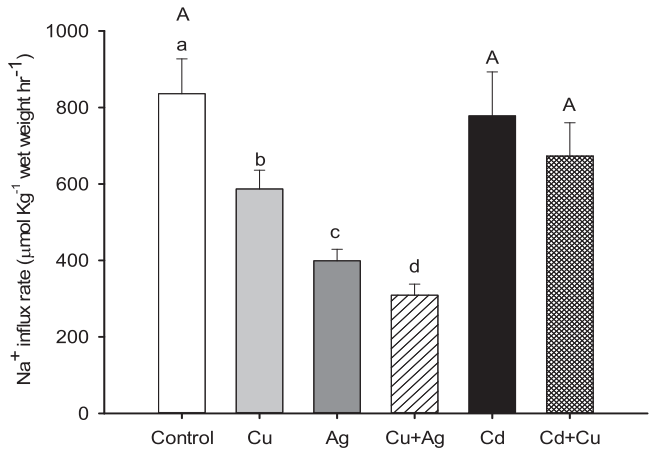

Fig. 6. Effects of waterborne Cu and Ag, and Cd and Cu (singly and in mixture) on branchial Na^+ influx rate in rainbow trout. The white bar represents control (no added metals in the exposure water). The measured Cu, Ag and Cd concentrations in respective single metal exposures were 1.17, 0.08, and 0.18 μmol L^{-1}, respectively. In Cu and Ag mixture treatment, the measured Cu and Ag exposure concentrations were 1.18 and 0.09 μmol L^{-1}, respectively. In Cd and Cu mixture treatment, the measured Cd and Cu exposure concentrations were 0.17 and 1.18 μmol L^{-1}, respectively. Branchial Na^+ influx data are presented as mean ± SEM ($n = 6$). Significant differences among comparable treatments are indicated by different letters (one way ANOVA; $p < 0.05$).

Cu. The mixture of Cu and Ag (1.18 and 0.09 μmol L^{-1}, respectively) produced significantly greater inhibition (63% decrease relative to the control) of Na^+ influx rate than either Cu or Ag alone exposure (Table 1B). In contrast, waterborne Cd alone at its LC_{50} concentration (0.18 μmol L^{-1}) and in mixture with Cu (0.17 and 1.18 μmol L^{-1}, respectively) did not have any significant effects on the branchial Na^+ influx, as the inhibitory effects were marginal (7% and 19% decrease, respectively, relative to the control) (Fig. 6; Table 1B).

We also evaluated whether the combined effects of binary metal mixtures on branchial ion uptake (an index of toxicity) measured in the present study can be explained by predictions of simple additivity. The simple additive response of binary metal mixtures on influx of ions (Ca^{2+} or Na^+) was predicted using the following equation (based on the approach outlined by Norwood et al., 2003):

$$\text{SimpleAdditivity}(\%\text{inhibitionof}Ca^{2+}\text{or}Na^{+}\text{influxrate}) = [(a+b)-f] \times 100$$

where a and b are the measured inhibition of ion influx rates by Metal 1 and 2 relative to the control (as % fraction), and f is the interaction factor which is estimated by multiplying a and b.

Based on the evaluations described above, we found that the measured effects of waterborne Cd and Zn mixture on short-term branchial Ca^{2+} influx in fish exhibited simple additive response, whereas, the measured effects of Cd and Cu mixture did not match the predicted response of simple additivity (Table 1). Similarly, we also observed that the measured effects of waterborne Cu and Ag mixture on short-term branchial Na^+ influx in fish followed the principle of simple additivity, but the same did not occur for waterborne Cd and Cu mixture (Table 1).

Table 1
Measured vs. predicted effects of different binary metal mixtures on the short-term (3 h) branchial influx of Ca^{2+} (A) or Na^+ (B) in rainbow trout. The predicted effects were estimated based on the principle of simple additivity (see text for details). Values are expressed as % inhibition of influx rate relative to the control.

(A)

Treatments	Measured inhibition of Ca^{2+} influx (%)	Predicted inhibition of Ca^{2+} influx (%)
Cd	37	–
Zn	79	–
Cu	24	–
Cd + Zn	86	87
Cd + Cu	31	52

(B)

Treatments	Measured inhibition of Na^+ influx (%)	Predicted inhibition of Na^+ influx (%)
Cu	30	–
Ag	52	–
Cd	7	–
Cu + Ag	63	66
Cu + Cd	19	35

– means not applicable.

3.3. *Interactive effects of binary metal mixtures (Cd and Zn) on 96 h fish mortality*

A similar concentration-dependent increase in 96 h mortality was recorded in juvenile rainbow trout exposed to waterborne Cd (0.02–0.36 μmol L^{-1}) both in the absence and presence of added waterborne Zn (3.15–6.21 μmol L^{-1}) (Table 2A). The 96 h LC_{50} values of Cd for rainbow trout did not change significantly among the treatments, averaging 0.18 ± 0.03 μmol L^{-1} overall (data not shown). In contrast, we did not observe any mortality in fish exposed to waterborne Zn (0.8–3.2 μmol L^{-1}), followed by only 20% mortality at 6.5 μmol L^{-1} Zn exposure (Table 2B). However, the presence of Cd increased the toxicity of waterborne Zn (0.78–6.46 μmol L^{-1}), and the fish mortality rate almost doubled as the Cd exposure concentration increased from 0.08 to 0.19 μmol L^{-1} (Table 2B).

Table 2
Mortality (%) in rainbow trout exposed for 96 h to: (A) different concentrations of waterborne Cd in the absence and presence of added Zn, and (B) different concentrations of waterborne Zn in the absence and presence of added Cd. The measured Zn and Cd concentrations in the exposure water are presented as the mean value (n = 12).

(A)			
Cd Concentration (μmol L^{-1})	% Mortality		
	No added Zn (Control) (0.05 μmol L^{-1})	Zn (3.15 μmol L^{-1})	Zn (6.21 μmol L^{-1})
0	0	0	0
0.02	0	0	0
0.05	20	10	10
0.09	70	50	70
0.17	90	100	80
0.36	100	100	100
(B)			
Zn Concentration (μmol L^{-1})	% Mortality		
	No added Cd (Control) (0 μmol L^{-1})	Cd (0.08 μmol L^{-1})	Cd (0.19 μmol L^{-1})
0.05	0	60	90
0.78	0	40	100
1.67	0	50	100
3.16	0	60	80
6.46	20	50	100

4. Discussion

4.1. Interactive effects of binary metal mixtures on gill-metal binding

The present study investigated the interactions of binary mixtures of metals with apparently common (Cd *vs*. Zn, and Cu *vs*. Ag) as well as different (Cd *vs*. Cu, Cd *vs*. Ni) branchial uptake pathways in fish. To date, there is a great paucity of published data on how metal mixture interactions influence metal uptake in fish gills, and our study provides important new insights to address that knowledge gap. In general, our findings indicate that the effects of metal mixtures interactions on gill–metal binding are complex, and cannot always be explained based on our current understanding of the uptake mechanisms of waterborne metals in fish. In our analysis, we explicitly assume that acclimatory changes in the gills are not occurring in our short-term exposures (a few hours) and toxicity-testing (up to 96 h), as for most metals, these appear to take at least a week or more (*e.g.* McDonald and Wood, 1993).

In the current study, we observed that waterborne Zn decreased gill–Cd binding and *vice versa* in rainbow trout (Fig. 1A and B). Both Cd and Zn are Ca^{2+} analogs, which are believed to compete with Ca^{2+} for uptake *via* apical voltage independent Ca^{2+} channels on the gill epithelium (Hogstrand et al., 1996; Niyogi and Wood, 2004b), and thus, our results are consistent with that understanding. Reductions in short-term branchial Cd uptake by waterborne Zn have also been reported in zebrafish (*Danio rerio*) (Wicklund-Glynn, 2001; Komjarova and Blust, 2009). Cd is known to bind to rainbow trout gills with approximately 100 fold greater affinity than Zn ($\log K_{Cd}$ = 7.5 *vs*. $\log K_{Zn}$ = 5.6; Niyogi et al., 2008; Alsop and Wood, 2000). However, in the present study we found that the competitive effect of waterborne Zn (a nutrient metal) on gill–Cd binding was greater than the effect of waterborne Cd (a non-nutrient metal) on gill–Zn binding when both were present in their acute effect ranges. We recorded a significant decrease (~20%) in gill–Cd binding relative to the control (equivalent to the Cd LA_{50}) even at the lowest Zn exposure concentration used in the current study (0.9 μmol L^{-1}), which was far below the 96 h LC_{50}. This was followed by a 70–80% reduction in gill–Cd binding at Zn exposure range (10–20 μmol L^{-1}) that corresponds with the 96 h LC_{50} of Zn in rainbow trout in the ambient water (Alsop et al., 1999). In contrast, only about a 25% decrease in gill–Zn binding relative to the control (equivalent to the Zn LA_{50}) was observed at 0.18 μmol L^{-1} Cd (96 h LC_{50} of Cd in rainbow trout in the ambient water (Niyogi et al., 2008)), with no effect on gill–Zn binding at lower Cd exposure concentrations.

The likely reason behind this observation is that the pool of Cd binding sites (Cd–B_{max}) in the trout gill is much smaller than the pool of Zn binding sites (Zn–B_{max}) (Niyogi and Wood, 2004a) – which include sites that are specific to Zn only, in addition to sites where both Cd and Zn compete for binding (Ca^{2+} uptake sites). This conclusion is also supported by the approximately 5-fold higher absolute LA_{50} level for Zn than for Cd (Fig. 1A *vs*. B). The existence of high affinity Zn transporters (*e.g.*, ZIP transporters) for uptake of this essential metal has been reported in the fish gill (Qiu and Hogstrand, 2005; Qiu et al., 2005). Moreover, it has been suggested that Zn has a lower affinity for Ca^{2+} uptake sites relative to the ZIP transporters (Hogstrand, 2012), which might also have been a factor behind the lack of effect on gill–Zn binding at lower Cd exposure concentrations.

Interestingly, waterborne Cd and Cu also reduced the gill binding of each other (Fig. 2A and B). Similar to our observations with Cd and Zn interactions, the essential nutrient metal Cu, at concentrations well below its 96 h LC_{50} (~1.3 μmol L^{-1}; Taylor et al., 2000) was found to be protective against gill–Cd binding, but the non-nutrient metal Cd only started to reduce gill–Cu binding once Cd exposure concentration reached its 96 h LC_{50} of 0.18 μmol L^{-1}. These findings are consistent with Komjarova and Blust (2009), who also reported a significant reduction in short-term (24 h) branchial uptake of Cd in zebrafish with elevated Cu level in the water, but no effect on branchial Cu uptake with increased Cd level in the exposure water. Competitive interaction of waterborne Cd and Cu has also been reported with tissue-specific metal accumulation in tilapia (*Oreochromis mosambicus*) (Pelgrom et al., 1995). Traditionally, Cd is considered to be a Ca^{2+} antagonist (Niyogi and Wood, 2004b), whereas, Cu is thought to be a Na^{+} antagonist and its uptake is believed to occur mainly *via* an apical Na^{+} channel in the gill epithelium (Laurén and McDonald, 1987a,b; Grosell and Wood, 2002; Grosell, 2012). However, recent studies have provided evidence of shared uptake of Cd and Cu in the fish gill, *via* multiple transporters such as the apical voltage insensitive calcium channel (ECaC), divalent metal transporter 1 (DMT1), and ZIP-8 (Cooper et al., 2007; Alsop and Wood, 2011; Komjarova and Bury, 2014). It is also possible that the Cd and Cu interaction observed in the present study might be due to the non-specific competition for the binding sites in trout gill, similar to the competitive effect of other natural cations (*e.g.*, Ca^{2+}, Mg^{2+}, Na^{+}, or H^{+}) on gill–metal binding. It is

important to note here that the LA_{50} of Cu in rainbow trout is more than 2-fold higher than that of Cd (Fig. 2; Niyogi and Wood 2004a), and this might have resulted in a greater competitive effect of Cu on gill–Cd binding compared to the effect of Cd on gill–Cu binding.

In the present study, we did not observe any competitive effect of Ni on gill–Cd binding (Fig. 3), even at a Ni exposure level of 410 μmol L^{-1}, which was about 2-fold higher than its 96 h LC_{50} in rainbow trout in the ambient water (Pane et al., 2003). In contrast to our study, Komjarova and Blust (2009) reported reduced short-term Cd uptake in the gill of zebrafish with elevated Ni concentrations in the water, however they used much lower exposure concentrations of Cd (0.025 μmol L^{-1}) and Ni (0.1–1.6 μmol L^{-1}) relative to the present study. The mechanism of Ni uptake in the fish gill remains unknown to date, and at present there is no evidence of a shared transport of Ni and Cd in the trout gill. Moreover, Ni is known to be a respiratory toxicant to fish during acute exposure (Pane et al., 2003; Pane et al., 2003), whereas, acute Cd exposure is known to cause toxicity by disrupting branchial Ca^{2+} uptake (Niyogi and Wood, 2004b). Thus, the lack of Ni and Cd interaction observed in the present study was consistent with our original prediction.

In the current study, we observed no competitive interaction between waterborne Cu and Ag on gill metal-binding (Fig. 4), but rather surprisingly gill–Cu binding was found to be stimulated at higher Ag exposure concentrations used (0.09–0.17 μmol L^{-1}), which is equal to or higher than the 96 h LC_{50} concentration of Ag in rainbow trout under ambient water chemistry (Galvez and Wood, 1997). Similar to Cu, Ag is also known to be a Na^+ antagonist in fish, and its uptake in the gill is believed to occur mainly *via* the apical Na^+ channels (Bury and Wood, 1999). Thus, our results are not in synchrony with the expected competitive interactions of Cu and Ag for the binding sites in fish gills. Nevertheless, it is noteworthy that Nadella et al. (2007) reported that Ag stimulated both Cu and Na^+ uptake across the intestine of trout, albeit at much higher concentrations of the three metals than used here. A portion of Cu uptake in the gut appears to be linked to Na^+ transport; Nadella et al. (2007) suggested that Ag may activate some sort of Na^+-coupled Cu transport mechanism by increasing the apical conductance to Na^+. However, the Na^+ influx measurements of the present study showing that both Ag alone, and Ag plus Cu in combination, inhibited branchial Na^+ uptake (Fig. 6) argue against this interpretation at the gills. The effect remains mysterious, and further investigation is required.

4.2. Interactive effects of binary metal mixtures on branchial ion flux

In the present study, both waterborne Cd and Zn alone, at their respective 96 h LC_{50}s, significantly reduced short-term branchial Ca^{2+} influx in rainbow trout (Fig. 5). However, the inhibitory effect of an LC_{50} level of Zn on Ca^{2+} influx was about 2-fold higher than that of Cd at its LC_{50} level. Interestingly, the inhibitory effect of Zn on gill–Cd binding was also 2-fold higher relative to the effect of Cd on gill–Zn binding, when the two metals were present at concentrations that matched their respective LC_{50}s. These observations further indicate that the competitive interactions of Cd and Zn occurred primarily for binding to the Ca^{2+} transport sites. Both Cd and Zn are known to inhibit branchial Ca^{2+} influx in fish during acute exposures (Hogstrand et al., 1996; Niyogi and Wood, 2004b), thus, our results are consistent with previous observations. Interestingly, we also found that Cd and Zn in mixture caused significantly greater inhibition of Ca^{2+} influx relative to Cd or Zn alone, and this interactive effect seemed to occur in a simple additive manner (Table 1). By direct competition for the same site, we might expect less than additive effects. However, our current understanding of the interactive effects of metal mixtures on branchial influx of ions is extremely limited, and to the best our knowledge only one previous study has investigated the effects of binary metal mixtures on short-term branchial influx of ions. Birceanu et al. (2008) reported that acute exposure to a binary mixture of high levels of waterborne Cd and Pb (another Ca^{2+} antagonist in fish gill; Rogers et al., 2003; Rogers and Wood, 2004) resulted in less than additive effects in terms of gill binding, but more than additive effects on the branchial influx of both Ca^{2+} and Na^+ in rainbow trout.

Our study also revealed that acute Cu exposure that corresponds with its 96 h LC_{50} significantly reduced branchial Ca^{2+} influx in trout (Fig. 5). Alsop and Wood (2011) also reported reduced Ca^{2+} uptake in zebrafish during acute exposure to waterborne Cu. The decrease in Ca^{2+} influx by Cu recorded in the current study is consistent with the reduced gill Cd-binding at the corresponding Cu exposure level. Collectively, these findings suggest that the competitive interaction of Cd and Cu recorded in our study occurs mainly at the Ca^{2+} transport sites. However, unlike Cd and Zn, Cd, and Cu did not seem to cause any significant additive effect on branchial Ca^{2+} influx in trout (Fig. 5; Table 1A).

The short-term branchial influx of Na^+ was significantly reduced by both Cu and Ag individually, at exposure concentrations that matched their respective 96 h LC_{50}s, with a greater effect for Ag than Cu (Fig. 6). Previous studies also found that both Cu and Ag decrease branchial Na^+ uptake during acute exposure (Grosell and Wood, 2002; Morgan et al., 2004). The binary mixture of Cu and Ag produced a significantly greater inhibition of Na^+ compared to that in either Cu alone or Ag alone treatment, and the interactive effect of Cu and Ag appeared to follow the principle of simple additivity (Table 1B). It is important to note that although the interactive effect of Cu and Ag on gill–metal binding was antithetical to our prediction, the effect of Cu and Ag mixture on branchial Na^+ influx indicated that metals that are known to compete for the same binding sites at the gill cause additive inhibition of essential ion uptake, similar to the Zn *vs.* Cd interaction on Ca^{2+} influx.

In the present study, acute Cd exposure did not have any significant effect on branchial Na^+ influx in rainbow trout, which is expected since Cd is not known to compete with Na^+. Interestingly though, Cd appeared to ameliorate the inhibition of Na^+ influx induced by Cu only exposure, as no significant difference in the rate of Na^+ uptake rate was recorded between the control and mixture (Cd and Cu) treatment. Collectively, the interactive effects of Cd and Cu in mixtures on both Ca^{2+} and Na^+ influx suggest that the toxicity of binary mixtures of metals with apparently different modes of toxic action may not be predicted on the basis of simple additivity.

4.3. Interactive effects of binary metal mixtures (Cd and Zn) on acute toxicity

In the present study, sub-lethal levels of waterborne Zn (3–6 μmol L^{-1}) did not elicit any protective effect against the acute toxicity of Cd in rainbow trout (Table 2A). In addition, the toxicity in fish exposed to sub-lethal waterborne Zn was found to increase as a function of Cd levels in the exposure (Table 2B). Thus, in seeming opposition to the competitive interactions of Zn and Cd on the gill binding of each other (Fig. 1A and B), this did not result into reduced mortality in fish. Mebane et al. (2012) previously reported an antagonistic effect of waterborne Cd and Zn interaction on fish mortality, but only when the toxicity was predominantly caused by Zn. In contrast, Meyer et al. (2015a,b); Meyer et al. (2015a,b) recently reported that sub-lethal levels of waterborne Zn ameliorated Cd induced acute mortality in *Daphnia magna*, however sub-lethal to lethal levels of waterborne Cd did not have much influence on Zn-induced acute mortality. Nevertheless, these observations suggest that the interactions of Cd and Zn may elicit less than additive toxicity depending on the ratio of Cd and Zn exposure concentrations in the mixture. We conclude that further acute toxicity testing involving a broader range of exposure concentrations and metal-mixture

combinations is needed to understand how the metal-mixture interactions on the gill–metal binding influence the acute toxicity (lethality) in fish.

5. Conclusions

The findings of the current study indicated that the short-term gill binding interactions of metals are not always predictable, based on our current knowledge of branchial metal transport in fish. Among the binary metal mixture combinations examined in our study, only Cd *vs*. Zn, and Cd *vs*. Ni interactions were found to be consistent with our original assumptions that metals that bind to the common sites on fish gill (Cd and Zn) would interact in a competitive manner, whereas metals that bind to different binding sites (Cd and Ni) would not interact with each other. Surprisingly, no competitive interaction was recorded between Cu and Ag, although both are Na^+ antagonists and have a common transport pathway in the fish gill. Moreover, Cd and Cu were found to reduce gill binding of each other, indicating that they compete for binding to the same sites in fish gill. Nonetheless, our study revealed that the physiological indices of toxicity (the inhibition of essential ion transport) measured in our study were more consistent with original assumptions on metal mixture interactions in fish. For example, the mixture of metals with a common mode of toxic action produced a simple additive inhibition on branchial ion transport (Cd and Zn mixture on Ca^{2+} uptake, and Cu and Ag mixture on Na^+ uptake). In contrast, the mixture of metals with different modes of toxic action (Cd and Cu) did not appear to have any significant interactive effects of either branchial Ca^{2+} or Na^+ uptake. Overall, these findings suggest that the effects of the metal mixture interactions on gill-metal binding and toxicity in fish are complex, and the current BLM framework will need to be modified to integrate these interactions for its future application in metal mixture toxicity assessment in aquatic ecosystems.

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References

Alsop, D., Wood, C.M., 2011. Metal uptake and acute toxicity in zebrafish: common mechanisms across multiple metals. Aquat. Toxicol. 105, 385–393.
Alsop, D.H., McGeer, J.C., McDonald, D.G., Wood, C.M., 1999. Costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water. Environ. Toxicol. Chem. 18, 1014–1025.
Alsop, D.H., Wood, C.M., 2000. Kinetic analysis of zinc accumulation in the gills of juvenile rainbow trout: effects of zinc acclimation and implications for biotic ligand modeling. Environ. Toxicol. Chem. 19, 1911–1918.
Balistrieri, L.S., Mebane, C.A., Schmidt, T.S., Keller, W., 2015. Expanding metal mixture toxicity models to natural stream and lake invertebrate communities. Environ. Toxicol. Chem., http://dx.doi.org/10.1002/etc.2824
Balistrieri, L.S., Mebane, C.A., 2014. Predicting the toxicity of metal mixtures. Sci. Total Environ. 466–467, 788–799.
Birceanu, O., Chowdhury, M.J., Gillis, P.L., McGeer, J.C., Wood, C.M., Wilkie, M.P., 2008. Modes of metal toxicity and impaired branchial ionoregulation in rainbow trout exposed to mixtures of Pb and Cd in soft water. Aquat. Toxicol. 89, 222–231.
Bodar, C.W.M., Pronk, M.E.J., Sijm, D.T.H.M., 2005. The European Union risk assessment on zinc and zinc compounds: the process and the facts. Int. Environ. Assess. Manage. 1, 301–319.
Borgmann, U., Norwood, W.P., Dixon, D.G., 2008. Modelling bioaccumulation and toxicity of metal mixtures. Hum. Ecol. Risk Assess. 14, 266–289.
Bury, N.R., Wood, C.M., 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na+ channel. Am. J. Physiol. 277, R1385–R1391.
Cooper, C., Shayeghi, M., Techau, M., Capdevila, D., MacKenzie, S., Durrant, C., Bury, N., 2007. Analysis of the rainbow trout solute carrier 11 family reveals iron import >pH 7.4 and a functional isoform lacking transmembrane domains 11 and 12. FEBS Lett. 581, 2599–2604.
Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383–2396.
Farley, K.J., Meyer, J.S., 2015. Metal mixture modeling evaluation project: 3. Lessons learned and steps forward. Environ.l Toxicol. Chem., http://dx.doi.org/10.1002/etc.2837
Farley, K.J., Meyer, J.S., Balistrieri, L.S., De Schamphelaere, K.A.C., Iwasaki, Y., Janssen, C.R., Kamo, M., Lofts, S., Mebane, C.A., Naito, W., Ryan, A.C., Santore, R.C., Tipping, E., 2015. Metal mixture modeling evaluation project: 2. Comparison of four modeling approaches. Environ. Toxicol. Chem., http://dx.doi.org/10.1002/etc.2820
Galvez, F., Wood, C.M., 1997. The relative importance of water hardness and chloride levels in modifying the acute toxicity of silver to rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 16, 2363–2368.
Grosell, M., Nielsen, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comput. Biochem. Physiol. 133C, 287–303.
Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.
Grosell, M., 2012. Copper. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Essential Metals: Homeostasis and Toxicology of Essential Metals,Volume 31 A Fish Physiology. Elsevier, New York, pp. 53–133.
Gustafsson, J.P., 2010. Visual MINTEQ. Land Water Research Engineering, Stockholm, Sweden.
Hogstrand, C., 2012. Zinc. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Essential Metals: Homeostasis and Toxicology of Essential Metals, Volume 31A Fish Physiology. Elsevier, New York, pp. 135–200.
Hogstrand, C., Verbost, P.M., Bonga, S., Wood, C.M., 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am. J. Physiol. 270, R1141–R1147.
Iwasaki, Y., Kamo, M., Naito, W., 2014. Testing an application of a biotic ligand model to predict acute toxicity of metal mixtures to rainbow trout. Environ. Toxicol. Chem., http://dx.doi.org/10.1002/etc.2780
Kamo, M., Nagai, T., 2008. An application of the biotic ligand model to predict the toxic effects of metal mixtures. Environ. Toxicol. Chem. 7, 1479–1487.
Komjarova, I., Blust, R., 2009. Multimetal interactions between Cd Cu, Ni, Pb, and Zn uptake from water in the zebrafish *Danio rerio*. Environ. Sci. Technol. 43, 7225–72290.
Komjarova, I., Bury, N.R., 2014. Evidence of common cadmium and copper uptake routes in zebrafish *Danio rerio*. Environ. Sci. Technol. 48, 12946–12951.
Laurén, D.J., McDonald, D., 1987a. Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. Can. J. Fish. Aquat. Sci. 44, 105–111.
Laurén, D.J., McDonald, D., 1987b. Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. Can. J. Fish. Aquat. Sci. 44, 99–104.
McDonald, D.G., Wood, C.M., 1993. Branchial acclimation to metals. In: Rankin, J.C., Jensen, F.B. (Eds.), Fish Ecophysiology. Chapman & Hall, London, pp. 297–321.
McGeer, J.C., Nadella, S.R., Alsop, D.H., Hollis, L., Taylor, L.N., McDonald, D.G., Wood, C.M., 2007. Influence of acclimation and cross-acclimation of metals on acute Cd toxicity and Cd uptake and distribution in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 84, 190–197.
Mebane, C.A., Dillon, F.S., Hennessy, D.P., 2012. Acute toxicity of cadmium lead, zinc, and their mixtures to stream-resident fish and invertebrates. Environ. Toxicol. Chem. 31, 1334–1348.
Meyer, J.S., Farley, K.J., Garman, E.R., 2015a. Metal mixtures modeling evaluation: 1. Background. Environ. Toxicol. Chem., http://dx.doi.org/10.1002/etc.2792
Meyer, J.S., Ranville, J.F., Pontasch, M., Gorsuch, J.W., Adams, W.J., 2015b. Acute toxicity of binary and ternary mixtures of Cd, Cu, and Zn to *Daphnia magna*. Environ. Toxicol. Chem., http://dx.doi.org/10.1002/etc.2787
Morgan, T.P., Grosell, M., Gilmour, K.M., Playle, R.C., Wood, C.M., 2004. Time course analysis of the mechanism by which silver inhibits active Na^+ and Cl^- uptake in gills of rainbow trout. Am. J. Physiol. 287, R234–R242.
Nadella, S.R., Grosell, M., Wood, C.M., 2007. Mechanisms of dietary Cu uptake in freshwater rainbow trout: evidence for Na-assisted Cu transport and a specific metal carrier in the intestine. J. Comp. Physiol. B 177, 433–446.
Niyogi, S., Kent, R., Wood, C.M., 2008. Effects of water chemistry variables on gill binding and acute toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*): a biotic ligand model (BLM) approach. Comput. Biochem. Physiol. 148C, 305–314.
Niyogi, S., Wood, C.M., 2004a. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.
Niyogi, S., Wood, C., 2004b. Kinetic analyses of waterborne Ca and Cd transport and their interactions in the gills of rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*), two species differing greatly in acute waterborne Cd sensitivity. J. Comput. Physiol. 174B, 243–253.
Norwood, W.P., Borgmann, U., Dixon, D.G., Wallace, A., 2003. Effects of metal mixtures on aquatic biota: a review of observations and methods. Hum. Ecol. Risk Assess. 9, 795–811.

Pane, E., Richards, J., Wood, C., 2003. Acute waterborne nickel toxicity in the rainbow trout (*Oncorhynchus mykiss*) occurs by a respiratory rather than ionoregulatory mechanism. Aquat. Toxicol. 63, 65–82.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comput. Biochem. Physiol. 133C, 3–35.

Pelgrom, S., Lamers, L., Lock, R., Balm, P., Bonga, S.W., 1995. Interactions between copper and cadmium modify metal organ distribution in mature tilapia, *Oreochromis mossambicus*. Environ. Pollut. 90, 415–423.

Playle, R.C., 2004. Using multiple metal–gill binding models and the toxic unit concept to help reconcile multiple-metal toxicity results. Aquat. Toxicol. 67, 359–370.

Qiu, A., Hogstrand, C., 2005. Functional expression of a low-affinity zinc uptake transporter (FrZIP2) from pufferfish (*Takifugu rubripes*) in MDCK cells. Biochem. J. 390, 777–786.

Qiu, A., Shayeghi, M., Hogstrand, C., 2005. Molecular cloning and functional characterization of a high-affinity zinc importer (DrZIP1) from zebrafish (*Danio rerio*). Biochem. J. 388, 745–754.

Rogers, J., Richards, J., Wood, C., 2003. Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 64, 215–234.

Rogers, J.T., Wood, C.M., 2004. Characterization of branchial lead–calcium interaction in the freshwater rainbow trout *Oncorhynchus mykiss*. J. Exp. Biol. 207, 813–825.

Santore, R.C., Ryan, A.C., 2015. Development and application of a multi-metal multi-biotic ligand model for assessing aquatic toxicity of metal mixtures. Environ. Toxicol. Chem., http://dx.doi.org/10.1002/etc.2869

Tao, S., Liang, T., Cao, J., Dawson, R.W., Liu, C., 1999. Synergistic effect of copper and lead uptake by fish. Ecotoxicol. Environ. Saf. 44, 190–195.

Taylor, L.N., McGeer, J.C., Wood, C.M., McDonald, D.G., 2000. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators. Environ. Toxicol. Chem. 19, 2298–2308.

Tipping, E., Lofts, S., 2015. Testing WHAM-F_{Tox} with laboratory toxicity data for mixtures of metals (Cu, Zn, Cd, Ag, Pb). Environ. Toxicol. Chem., http://dx.doi.org/10.1002/etc.2773

ToxCalc, 1996. ToxCalc. Tidepool Scientific Software, McKinleyville, California.

Vijver, M.G., Elliott, E.G., Peijnenburg, W.J., de Snoo, G.R., 2011. Response predictions for organisms water-exposed to metal mixtures: a meta-analysis. Environ. Toxicol. Chem. 30, 1482–1487.

Wicklund-Glynn, A., 2001. The influence of zinc on apical uptake of cadmium in the gills and cadmium influx to the circulatory system in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. 128C, 165–172.