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Characterization of the effects of binary metal mixtures on short-term uptake of Cd, Pb, and Zn by rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Biotic Ligand Models (BLMs) for individual metals improve our ability to regulate metals in the aquatic environment by considering the effects of water quality parameters (ionic composition, pH, DOC) on metal bioavailability. However, in natural aquatic systems, organisms are often simultaneously exposed to multiple metals and these interactions are not currently considered in BLMs or most environmental regulations. Recently, several different mixture BLMs (mBLMs) have been developed to begin assessing this issue. Some of these models assume competitive interactions between all metals, while others assume only metals with similar modes of action (e.g., Na^+ or Ca^{2+} antagonists) will competitively interact. In this study, we used standard *in vivo* 3-h gill metal binding assays to characterize the uptake of Cd, Pb, and Zn individually and in binary mixtures with Ag, Cd, Cu, Pb, Ni, and Zn across a range of concentrations that encompassed the 96-h LC50 for each metal. Inhibition of Cd, Pb, and Zn uptake at the gill by introduction of a second metal was consistent with mode of action in some cases, but not others. Further, contrary to expectations, inhibition was always either non-competitive or could not be defined statistically. We also observed one example of stimulated metal uptake (Ni stimulated Zn uptake). Consistent with our previous experiments on Ag, Cu, and Ni, these studies suggest that current mBLM frameworks will need revision to better reflect the mechanisms underlying metal mixture interactions.

1. Introduction

Metals have historically been regulated on an individual basis in aquatic environments, typically based on dissolved waterborne concentrations and as a function of water hardness. The recognition that other water quality parameters besides hardness influence metal bioavailability led to the development of Biotic Ligand Models (BLMs) that can predict metal toxicity to a wide range of organisms as a function of multiple water quality parameters (ionic composition, pH, and dissolved organic carbon (DOC)) (Di Toro et al., 2001). Biotic Ligand Models integrate physiology, toxicology, and geochemistry into a modeling framework that relates local water chemistry to metal accumulation in target tissues (e.g., gills) of aquatic animals and in turn relates this accumulation to toxicity. Accumulated metals can interact with and inhibit proteins that normally facilitate essential ion transport. Metal binding to these proteins can be represented by a complexation reaction and an associated conditional binding constant (log K), with competing reactions decreasing metal accumulation and the resultant toxic response. These competing reactions include competition at the biotic ligand with other cations (e.g., Na^+, H^+, Ca^{2+}) and competing complexation reactions for the metal by other ligands in solution including inorganic anions (e.g., chloride, hydroxide, sulfide) as well as organic ligands such as dissolved organic carbon (DOC).

Playle and coworkers first characterized the effect of various water quality parameters (pH, major ions, and dissolved organic carbon) on short-term (3 h) metal accumulation at the fish gill (Playle and Dixon, 1993; Playle et al., 1993; Playle et al., 1992). Using these data, they then developed conditional binding constants (log K's) that could be used in geochemical equilibrium models. In these models, the gill is treated as another ligand in the system, allowing for predictions of metal accumulation at the gill as a function of water chemistry (Playle and Dixon, 1993; Playle et al., 1993; Playle et al., 1992). Short-term metal accumulation at the gill was also demonstrated to be a good predictor of whole animal metal toxicity, with a strong correlation between the 96-h LC50 and metal concentrations at the gill after 3 or

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24 h of exposure (termed the median lethal accumulation or LA50) (MacRae et al., 1999; Meyer et al., 1999; Morgan and Wood, 2004).

The physiological mechanisms underlying observed acute toxicity in fish is relatively well understood for some metals. For example, accumulation of Cu or Ag at the fish gill interferes with Na^+ uptake, thereby disrupting ionic balance in the organism and leading to toxicity (Grosell and Wood, 2002; Morgan et al., 2004). Similarly Cd and Zn interfere with Ca^{2+} uptake at the fish gill, leading to hypocalcemia and toxicity (Hogstrand et al., 1994; Niyogi and Wood, 2004). Lead also appears to primarily disrupt Ca^{2+} homeostasis, but can also interfere with Na^+ homeostasis at high concentrations (Rogers et al., 2005; Rogers and Wood, 2004). In contrast to these other metals, at least in fish, Ni^{2+} appears to be acting primarily as a respiratory toxicant although other mechanisms may also contribute to toxicity (Brix et al., 2017; Pane et al., 2004).

While development of single metal BLMs has greatly advanced our understanding of how metals affect aquatic organisms, it is widely recognized that metals typically occur in the environment as mixtures. Consequently, there is increasing regulatory interest in developing models that consider metal mixtures in setting water quality guidelines. The single metal BLM framework has now been modified to develop initial formulations of three metal mixture BLMs (mBLMs) and an alternative modeling approach, WHAM-F_{Tox} (Balistrieri and Mebane, 2014; Iwasaki et al., 2015; Santore and Ryan, 2015; Tipping and Lofts, 2015; Van Genderen et al., 2015). These mBLMs have been tested and their ability to predict the toxicity of metal mixtures has been demonstrated in cases where toxicity is additive. However, for some scenarios where measured metal mixture toxicity was less than additive the models did not perform well, while more than additive toxicity scenarios were not evaluated (Farley et al., 2015).

While it is encouraging that these initial mBLMs can make reasonably accurate predictions of metal mixture toxicity under many scenarios, it is important to note that there were significant differences in model assumptions. The most important differences include assumptions regarding the number of metal binding site types (e.g., one for all metals, separate binding sites for Ca- and Na-antagonists, or separate binding sites for each metal) and whether a concentration addition or independent action model was used to estimate toxicity (Balistrieri and Mebane, 2014; Iwasaki et al., 2015; Santore and Ryan, 2015; Tipping and Lofts, 2015). Additionally, the models were calibrated to initial data sets by adjusting log K's and LA50s in different ways such that, although two models might make a similar toxicity prediction for a mixture, the mechanisms underlying that prediction might be quite different between the models.

Conceptually, a mBLM should characterize interactions between metals for uptake by the organism and interactions between metals at the proximate site(s) of toxic action within the organism. Currently, mBLM frameworks are not constructed in this way due to data limitations. However, generating data to support such a framework may be necessary in order to successfully make toxicity predictions across a wide range of metal mixtures, particularly for scenarios where toxicity is more or less than additive. The objective of this study was to provide data on one component of such a model, the effects of binary metal mixtures on metal uptake. Specifically, this study characterized the short-term (3 h gill-binding) concentration-dependent uptake relationships for three metals (Cd, Pb, and Zn − primary metals) in juvenile rainbow trout over concentration ranges bracketing the 96-h LC50. These concentration-dependent uptake relationships were measured in the presence and absence of simultaneous exposure to the 96-h LC50 concentration of one of 5 other metals (Ag, Cd, Cu, Ni, Pb, or Zn − secondary metals) in binary combinations. This study complements our previous work evaluating Ag, Cu, and Ni as the primary metals using the same experimental design (Brix et al., 2016).

2. Materials and methods

2.1. Experimental design overview

The same experimental design used in Brix et al. (2016) was used in the current study along with the same analytical methods with some metal-specific modifications described below. Briefly, uptake of a single (primary) metal was first characterized over a range of concentrations that encompassed the 96-h LC50 for that metal. For Cd and Zn, radiolabeled metal (^{109}Cd and ^{65}Zn) was used, while for Pb unlabeled metal was used as a suitable radioisotope is not available. Using unlabeled metal for Pb uptake experiments is feasible because background Pb concentrations in fish gills are very low. The experiment was then repeated in the presence of a potentially interacting (secondary) metal at a single concentration (the LC50 for the secondary metal) across the range of exposure concentrations of the primary metal. Assuming Michaelis-Menten uptake kinetics, this approach allowed us to estimate changes in both binding capacity (B_{max}) and affinity (K_m) for the primary metal at toxicologically relevant concentrations. For uptake by a single-transporter, changes in K_m can be associated with competitive inhibition while changes in B_{max} can be associated with non-competitive inhibition (e.g., a change in the number of transporters or inhibitor-induced conformational changes in the protein that inactivate transport).

Because fish growth during holding has the potential to alter metal uptake kinetics, all experiments for a given metal were performed in a one-week period. To check whether any changes had occurred during this period, we characterized single metal uptake at the beginning and end of the study period for each metal.

2.2. Experimental animals

Juvenile rainbow trout (~3–6 g ww) were obtained from Humber Springs Trout Hatchery, Ontario, Canada. The fish were held in dechlorinated City of Hamilton tapwater ($[Na^+]$ = 0.6, $[Ca^{2+}]$ = 0.9, $[Mg^{2+}]$ = 0.15, $[K^+]$ = 0.05, $[Cl^-]$ = 0.8, $[SO_4^{2-}]$ = 0.25, [DOC] = 0.25 mM, alkalinity = 95 mg l^{-1}, pH = 7.9) at 12 °C under a photoperiod of 16 h light: 8 h dark. Preliminary metal uptake experiments with Cd revealed very little Cd uptake in this water due to competition with Ca as has been observed in previous studies (Hollis et al., 1997; Niyogi et al., 2008). To address this issue, all Cd uptake experiments were conducted in a synthetic low Ca water ($[Na^+]$ = 0.6, $[Ca^{2+}]$ = 0.1, $[Mg^{2+}]$ = 0.2, $[K^+]$ = 0.1, $[Cl^-]$ = 0.6, $[SO_4^{2-}]$ = 0.05, [DOC] = 0.08 mM, alkalinity = 95 mg l^{-1}, pH = 7.8), while Pb and Zn uptake experiments were conducted in dechlorinated City of Hamilton tapwater. Fish were acclimated to these conditions for at least one week prior to testing. During holding, fish were fed ~1% of their weight daily with commercial trout pellets, but were fasted for 72 h prior to use in testing.

2.3. Metal uptake experiments

Metal uptake at the rainbow trout gill was characterized using the general experimental protocol developed by Playle and Dixon (1993), although we modified it by radiolabeling the primary metal (Cd and Zn only). Fish were exposed to a range of 8–12 metal concentrations (n = 6 per treatment) with each treatment consisting of a single 800 ml polypropylene container with 650 ml of test solution (i.e., fish were treated as the unit of replication) that was gently aerated and maintained in a temperature controlled water bath. Test solutions were prepared and allowed to equilibrate for 0.5 h prior to fish exposure and were made from reagent grade metal salts of the appropriate secondary metal ($AgNO_3$, $Cd(NO_3)_2$•$4H_2O$, $CuSO_4$•$5H_20$, $NiSO_4$•$6H_2O$, $Pb(NO_3)_2$, or $ZnSO_4$) along with various concentrations of the primary metal (Cd $(NO_3)_2$•$4H_2O$, $Pb(NO_3)_2$, or $ZnSO_4$). For Cd and Zn, the primary metal solutions were labeled with 3–4 μCi l^{-1} of the metal radioisotope

(^{109}Cd, ^{65}Zn) while for Pb, unlabeled metal was used. Radioisotopes were obtained from commercial and government suppliers (^{109}Cd from Stuart Hunt, Mississauga, ON, Canada; ^{65}Zn from Oak Ridge National Laboratory, TN, USA).

The intent of these studies was to evaluate metal mixture interactions at concentrations associated with acute toxicity. Consequently, the primary metal concentrations evaluated in each experiment bracketed the 96-h LC50 based on previous studies conducted in our lab using the same dilution water and conditions. The exception was Cd, as the LC50 was determined in dechlorinated Hamilton tapwater while the Cd uptake experiments were conducted in the low Ca water described above. Similarly, the concentration of the secondary metal was the LC50 determined under the same conditions − Ag 96-h LC50: 0.11 μM (Hogstrand et al., 1996a), Cd 96-h LC50: 0.18 μM (Hollis et al., 1999), Cu 96-h LC50: 1.1 μM (Taylor et al., 2003), Ni 96-h LC50: 260 μM (Pane et al., 2003), Pb 96-h LC50: 4.8 μM (Rogers et al., 2003), Zn 96-h LC50: 17 μM (Alsop and Wood, 1999).

Exposure containers were randomly placed in the water bath and fish were randomly introduced to the exposure containers to minimize tank effects. A water sample was then collected at the beginning and end of the 3-h flux period for measuring radioactivity and dissolved (< 0.45 μm, Acrodisk Versapor® membrane filters, Pall Corporation, Ann Arbor, Michigan, USA) metal concentrations. At the end of the exposure, fish were euthanized by an overdose of MS-222 (tricaine methanesulfonate) and the gills excised. For Cd and Zn, gills were then rinsed in a 1 mM solution of "cold" (i.e. non-radioactive) metal to displace any loosely bound radioactive metal and then placed in plastic vials for direct counting using a gamma counter. For Pb, gill samples were first rinsed in 1 mM EDTA to remove loosely bound Pb and then digested in 1 ml of 1N HNO_3 at 70 °C overnight in sealed vials. Digests were then centrifuged and the supernatant analyzed by graphite furnace atomic absorption spectroscopy as described below. A sample of dilution water was also collected for analysis of ionic composition.

2.4. Analytical chemistry

Dissolved metal and Pb gill concentrations were measured by graphite furnace atomic absorption spectroscopy (SpectraAA220, Varian, Mulgrave, Australia) except for zinc which was measured using flame atomic absorption spectroscopy (SpectraAA220FS, Varian, Mulgrave, Australia). Environment Canada certified standards TM24 and TM25 were used along with appropriate method blanks to ensure quality control. Recovery of certified standards was 90–95% in all cases and method blanks were always less than the practical quantitation limit. Practical quantitation limits were as follows − Ag: 9 nM; Cd: 9 nM; Cu: 16 nM; Ni: 2 nM; Pb: 1.2 nM; Zn: 45 nM. Concentrations of major cations (Na, Ca, K, Mg) were determined by flame atomic absorption spectroscopy, while Cl concentrations were determined using the colorimetric mercuric thiocyanate method (Zall et al., 1956). Dissolved organic carbon concentration in the filtered exposure water was measured by high-temperature catalytic oxidation using a total organic carbon analyzer (Shimadzu TOC-VCSH, Kyoto, Japan).

Gamma radioactivity for ^{109}Cd and ^{65}Zn was analyzed using an automated gamma counter (Perkin Elmer Wizard 1480 3" Auto Gamma Counter). Lead accumulation at the gill (nmol g^{-1} ww) was calculated directly by taking into account the gill weight and volume of acid digest with a detection limit of 0.25 nmol g^{-1}. For Cd and Zn, metal accumulation at the gill (nmol g^{-1} ww) was calculated based on the accumulation of radioactivity in the gill and the specific activity of the radioisotope in the water:

$$\text{Metal accumulation} = a \times (bc^{-1})^{-1} \quad (1)$$

where a = the cpm g^{-1} of gill tissue (wet weight), b = the cpm ml^{-1} in the water, and c = the measured dissolved metal concentration in the water (nmol ml^{-1}).

2.5. Data analysis

All data and statistical analyses are presented as means ± SEM using measured rather than nominal test concentrations. Gill metal accumulation for the 3-h exposure period was evaluated as a function of waterborne metal concentration using the mean (initial and final sample) measured dissolved metal concentrations and estimated free metal ion concentrations. The latter were estimated with the speciation software Visual Minteq (Version 3.0; courtesy of J.P. Gustaffson, Royal Institute of Technology, Sweden) with the embedded NICA-Donnan model for organic complexation. The program was run using measured dissolved metal and ion concentrations. We used standard assumptions that 65% of DOM was chemically active and corresponded to fulvic acid containing 50% carbon by weight (Bryan et al., 2002).

Based on our previous experiments (Brix et al., 2016), we assumed metal accumulation at the fish gill would approximate a Michaelis-Menten type model:

$$Y = \frac{B_{max} * X}{(K_m + X)} \quad (2)$$

where, X is the free metal ion concentration, B_{max} is the maximum estimated concentration on the fish gill, and K_m is the half saturation constant. Alternatively, metal accumulation could be linear over the exposure concentration range tested, as we previously observed for Ni (Brix et al., 2016):

$$Y = mX + b \quad (3)$$

where, b = 0 and m is the slope of the line. Note, even in the case of apparent linear uptake, it is possible that uptake still conforms to Michaelis-Menten kinetics, when $X << K_m$ and $m = B_{max}/K_m$.

We used Aikake Information Criterion (AIC) (Akaike, 1974) corrected for sample size (AICc) in GraphPad Prism (v. 7.0) to test for differences in primary metal uptake in the presence and absence of a secondary metal. This analysis evaluated whether metal uptake kinetics in the presence and absence of a second metal are best described by a single model (i.e., no effect of the secondary metal on uptake of the primary metal uptake) or two separate models (i.e., effect of the secondary metal on uptake of the primary metal). The analysis provides a probability of whether a single model or two separate models is more appropriate. Note that these probabilities are not directly analogous to *p*-values and should not be treated as such. The analysis was used to evaluate the entire model as well as specific model parameters (B_{max}, K_m). We also used an F-test (Snedecor and Cochran, 1989) to compare models (and model parameters) in the absence and presence of a secondary metal.

3. Results

3.1. Cadmium

Cadmium concentrations were stable during the 3 h exposure period with initial and final concentrations generally within 10–20% of each other. Secondary metal concentrations were similarly stable except for Ag, where the final Ag concentration was only ~25% of the initial concentration. In the experiments with Ag, Cu, and Pb as the secondary metals, the mean dissolved Ag, Cu, and Pb concentrations were 0.016, 0.35, and 2.3 μM, which are significantly less than the intended concentrations equivalent to the LC50s of 0.11. 1.1, and 4.8 μM, respectively. Cadmium speciation, in the absence of a secondary metal, was predicted to vary slightly as a function of dissolved Cd concentrations ranging from 71% to 75% Cd^{2+} over the range of concentrations tested and in the absence of a secondary metal (Table S1). Most of the secondary metals had a minimal effect on Cd speciation, with the exception of Ni which increased Cd^{2+} concentrations by ~5% across the range of concentrations.

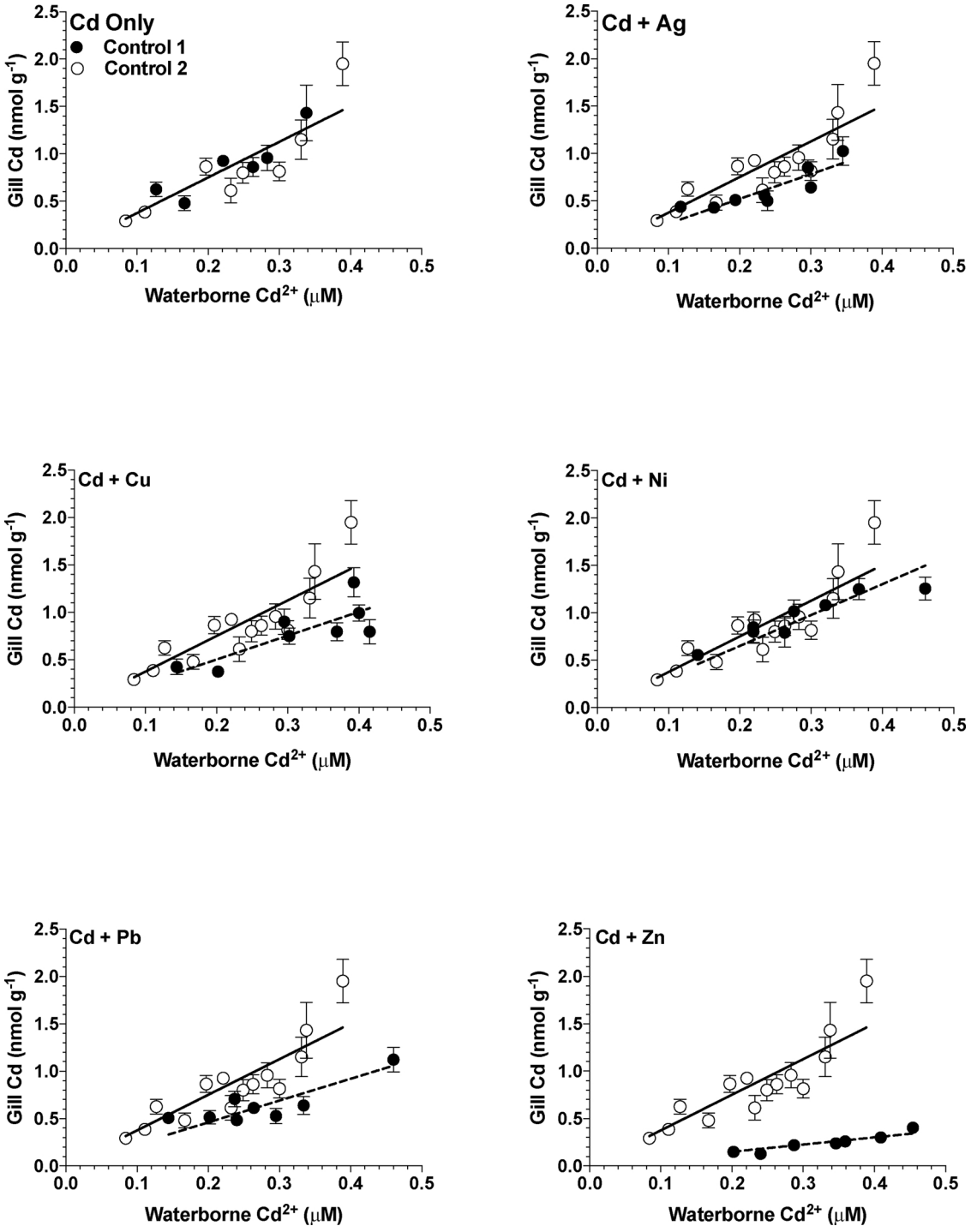

Fig. 1. Effect of Ag, Cu, Pb, Ni, and Zn (as secondary metals) on the uptake of Cd (as primary metal) at the rainbow trout gill. Control 1 and Control 2 are Cd only experiments conducted at the beginning and end of the experimental series. In all other panels, the open symbols represent Cd only exposures and closed symbols represent Cd + secondary metal exposures. A linear model (y = mx + b, where b = 0) was fitted to the data. Model parameters and statistical test results are presented in Table 1.

The uptake of Cd in the absence of a secondary metal appeared consistent with the presence of two transporters, a relatively high affinity saturable transporter and a lower affinity transporter that did not saturate over the range of concentrations tested. However, the range and number of Cd^{2+} concentrations tested were insufficient to discriminate between and characterize the two transporters in a statistically robust manner. Consequently, we opted to apply a linear regression model to the entire data set for analysis, but also present an alternative analysis in Supplemental Information that considers the two putative transporters.

There were no significant differences in the Cd only uptake kinetics at the beginning and end of the experimental period (AICc = 16%; F-test, $p = 0.94$), so data from these two experiments (i.e., Cd only present) were pooled for analysis of the effects of secondary metals on Cd uptake. Evaluation of the estimated slope of the pooled Cd only data set was 3.76 (Fig. 1, Table 1).

Cadmium uptake was significantly reduced in the presence of Ag, Cu, Pb, and Zn (Fig. 1, Table 1). Only Ni had no significant effect on Cd uptake. Zn clearly had the greatest effect on Cd uptake with, for example, an ~85% reduction in gill Cd observed at a waterborne Cd

Table 1
Summary of model parameters and statistical tests for Cd metal mixture experiments. Mean ± SE of slope (m). Percentages represent AICc probabilities that metal uptake kinetics in the presence and absence of a second metal are best described by two separate models (i.e., effect of the secondary metal on Cd uptake). All data were fit to a simple linear model (y = mx + b, where b = 0). Values in parentheses are *p*-values for F-tests comparing models for Cd only versus Cd + a secondary metal.

Mixture	m	r^2	Differences in Slope?
Cd Only	3.76 ± 0.22	0.77	–
Cd + Ag	2.61 ± 0.15	0.76	99% (< 0.01)
Cd + Cu	2.52 ± 0.20	0.63	> 99% (< 0.01)
Cd + Ni	3.25 ± 0.15	0.71	54% (0.10)
Cd + Pb	2.30 ± 0.15	0.64	> 99% (< 0.01)
Cd + Zn	0.75 ± 0.04	0.85	> 99% (< 0.01)

concentration associated with the LC50 in Cd only exposures (Fig. 1, Table S1). The use of a linear model precludes estimating B_{max} and K_m and so no inferences can be made regarding whether observed inhibitions are competitive or non-competitive in nature.

3.2. Lead

Lead concentrations were stable during the 3 h exposure period with initial and final concentrations generally within 10–20% of each other. Secondary metal concentrations were similarly stable except for Ag, where the final Ag concentration was only ~10% of the initial concentration. Additionally, the mean measured Ag concentrations in this experiment was only 0.026 μM compared to the target concentration of 0.11 μM. Lead speciation, in the absence of secondary metals, varied significantly as a function of waterborne Pb concentration with the predicted fraction as Pb^{2+} ranging from 0.09% in the lowest Pb only treatment (40 nM dissolved Pb) up to 1.07% Pb^{2+} at the highest Pb only treatment (3282 nM dissolved Pb). The presence of Ag, Cd, and Zn as secondary metals did not affect Pb speciation. However, Pb^{2+} concentrations were ~30% higher in the presence of Cu and ~10% higher in the presence of Ni due to competitive metal binding with organic matter (Table S2).

Lead uptake in the absence of a secondary metal exhibited Michaelis-Menten saturation kinetics with no significant differences between the initial control experiment and final control experiment performed 1 week later, allowing us to pool data from both experiments (Fig. 2). The pooled dataset had an estimated K_m of 4.3 nM Pb^{2+} and B_{max} of 51.2 nmol g^{-1} ww (Table 2).

In experiments with secondary metals, Ag, Cd, and Cu all significantly inhibited Pb uptake (AICc = > 99%; F-test, $p < 0.01$) while no significant effects on Pb uptake were observed in the presence of Ni or Zn (Table 2, Fig. 2). In experiments with Ag, Cu, and Cd, B_{max} was significantly reduced by 50–59% (AICc = 76-98%; F-test, p < 0.01-0.03; Table 2). Additionally, K_m estimates were lower (i.e., higher affinity) in the presence of all three metals, but none of these differences were statistically significant, even for Ag, where the K_m was estimated to be 4-fold lower compared to the Pb only experiment (Table 2).

3.3. Zinc

Zinc concentrations were stable during the 3 h exposure period with initial and final concentrations generally within 10–20% of each other. Secondary metal concentrations were similarly stable except for Ag, where the final Ag concentration was only ~30% of the initial concentration. In the experiments with Ag and Pb as the secondary metals, the mean dissolved Ag and Pb concentrations were 0.030 and 1.4 μM, which are significantly less than the intended concentrations equivalent to the LC50s of 0.11 and 4.8 μM, respectively. Zinc speciation was predicted to vary little as a function of dissolved Zn concentration or due to introduction of a secondary metal. Free Zn^{2+} concentrations ranged from 77 to 79% of dissolved Zn concentrations across all treatments in this study (Table S3).

Zinc uptake across the range of concentrations tested exhibited Michaelis-Menten saturation kinetics (Fig. 3). Similar to Cd, visual inspection of the data indicated the potential presence of high affinity (waterborne $Zn^{2+} < 2$ μM) and low affinity transporters, both of which appear to exhibit Michaelis-Menten saturation kinetics. As with Cd, these two apparent transporters could not be distinguished statistically. Further, the data distribution limited the number of reliable statistical comparisons that could be made between Zn only experiments and experiments with a secondary metal. Consequently, we opted to present our analysis based on a single transporter for Zn, but an alternative analysis assuming two transporters can be found in Supplementary Information (Tables S5 and S6, Fig. S2 and S3).

Assuming a single Zn transporter, and in the absence of a secondary metal, significant differences were observed in Zn uptake between the experiments at the beginning and end of the study period (AICc = 95%; F-test, $p < 0.01$) (Table 3). The trend in this difference between models is being driven by an increase in B_{max}, but the difference was not statistically significant (AICc = 55%; F-test, $p = 0.06$; Table 3). Evaluation of the effect of a secondary metal was made by comparing uptake with the Zn^{2+} only experiment conducted closest in time with the secondary metal experiment.

Neither Ag or Pb had a significant effect on Zn uptake (Fig. 3, Table 3). Both Cd and Cu significantly inhibited Zn uptake while Ni significantly stimulated Zn uptake (Fig. 3; AICc = > 99%; F-test, $p < 0.01$; Table 3). The inhibition of Zn uptake by Cd was also associated with a significant reduction in B_{max} (AICc = 99%, F-test, $p < 0.01$) but no change in K_m (AICc = 41%; F-test, $p = 0.11$). There were no significant changes in B_{max} or K_m associated with Cu inhibition of Zn^{2+} uptake (Table 3). The stimulation of Zn uptake in the presence of Ni was associated with a significant reduction in K_m as indicated by the F-test, although the AICc score was less convincing (AICc = 64%, F-test, $p = 0.04$; Table 3).

4. Discussion

This study forms part of an ongoing effort to develop mechanistically-based mBLMs by understanding the nature of interactions between components of metal mixtures at biotic ligands. We previously conducted a set of experiments using the same experimental design in which Ag, Cu, and Ni were the primary metals (Brix et al., 2016). There have also been several previous studies in which the effects of binary metal mixtures on metal uptake and toxicity to rainbow trout have been similarly studied using radio-isotopic analyses of short-term gill metal binding (Birceanu et al., 2008; Niyogi et al., 2015).

Considering that Cd, Pb, and Zn are known Ca^{2+} antagonists, while Ag and Cu are Na^+ antagonists, and Ni is a respiratory toxicant (see Introduction), we hypothesized that Cd, Pb, and Zn would reciprocally inhibit uptake of each other in a competitive manner, while Ag, Cu, and Ni would not interact with these metals in any of the binary metal combinations tested. The current results, together with those of Brix et al. (2016), indicate that these hypotheses have some validity but are far too simplistic.

With respect to the following discussion for Cd and Zn, it is important to note that our analysis was unable to statistically discriminate the presence of multiple transporters for each metal. While there is considerable evidence using a variety of techniques that multiple transporters are involved in both Cd and Zn uptake, in the absence of statistical support for these transporters in the current data set, we have analyzed the data assuming a single transport system for each metal. However, the following discussion does consider these different transporters in our interpretation of results with the intent of highlighting what appears to be a relatively complex set of metal–metal interactions involving multiple transport proteins. An alternative, more speculative discussion based on two transporter systems (but more limited statistical support) is presented in Supplemental Information.

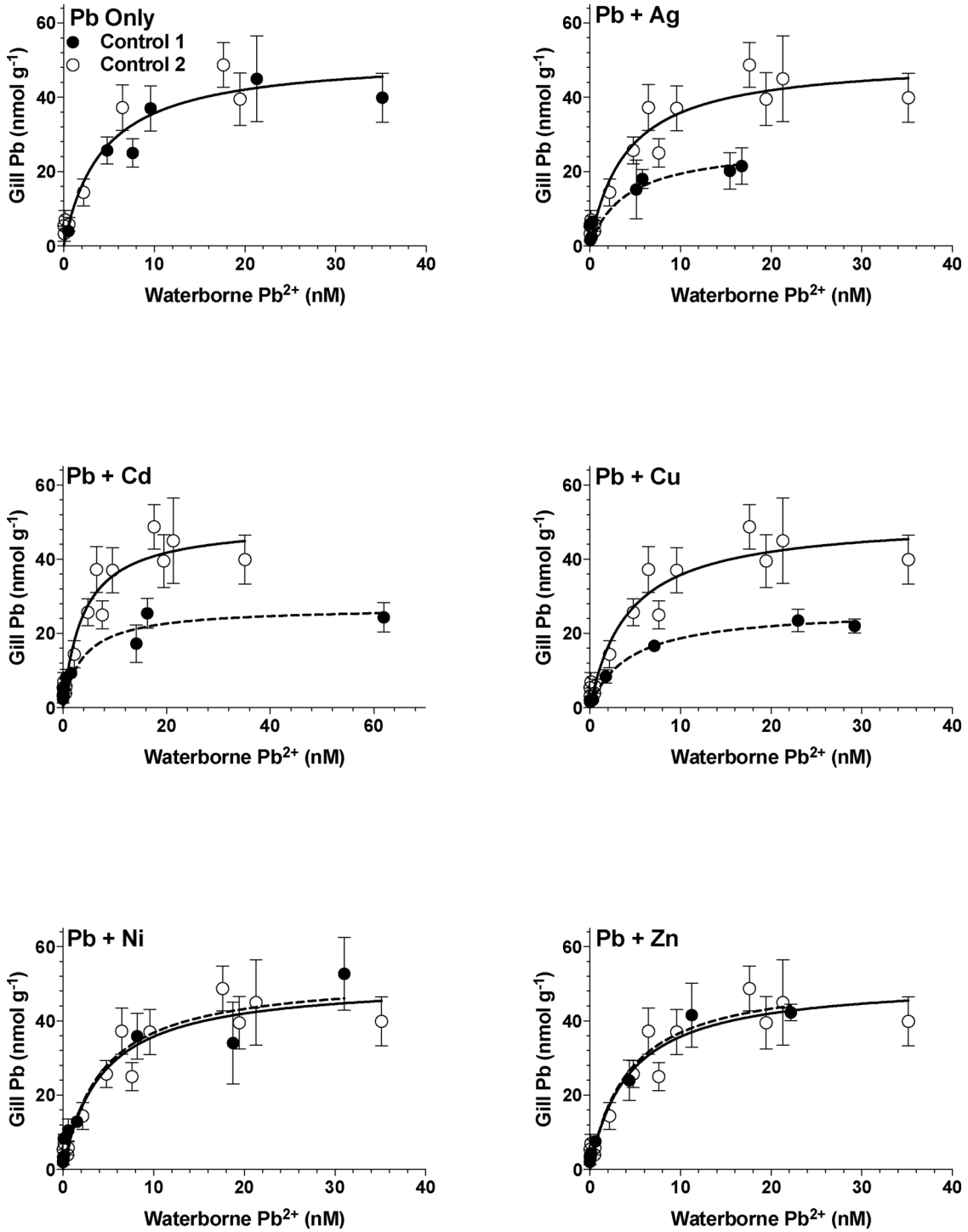

Fig. 2. Effect of Ag, Cd, Cu, Ni, and Zn (as secondary metals) on the uptake of Pb (as primary metal) at the rainbow trout gill. Control 1 and Control 2 are Pb only experiments conducted at the beginning and end of the experimental series. In all others panels, the open symbols represent Pb only exposures and closed symbols represent Pb + secondary metal exposures. A Michaelis-Menten model was fitted to the data. Model parameters and statistical test results are presented in Table 2.

4.1. Cadmium

Over the range of concentrations tested (84–460 nM Cd^{2+}), Cd uptake appeared to be non-saturable assuming a single-transporter system (Fig. 1). Applying a linear model to the data precludes estimation of an affinity constant (K_m) or maximum gill binding capacity (B_{max}). Previous studies have identified two saturable transporter involved in Cd uptake in rainbow trout. The high affinity transporter has an estimated K_m of 25–50 nM Cd^{2+} and saturates at a waterborne Cd concentration of ~300 nM Cd^{2+} in waters with comparable ionic composition and DOC (Birceanu et al., 2008; Niyogi et al., 2008; Niyogi and Wood, 2004). The low affinity transporter has a K_m of 1448 nM Cd^{2+} (B_{max} of 13.7 nmol g^{-1} ww) and saturates at a waterborne Cd concentration of ~6000 nM Cd^{2+} (Birceanu et al., 2008). Importantly, Cd uptake by this low affinity transporter approximates a linear function over the range of Cd concentrations tested in the current study.

Our experiments with Cd provided clear evidence for Ag, Cu, Pb, and Zn inhibiting Cd uptake and Ni having no effect on Cd uptake

Table 2
Summary of model parameters and statistical tests for Pb metal mixture experiments. Mean ± SE Michaelis-Menten parameters. Percentages represent AICc probabilities that metal uptake kinetics in the presence and absence of a second metal are best described by two separate models (i.e., effect of the secondary metal on Pb uptake). Values in parentheses are *p*-values for F-tests comparing models for Pb only versus Pb + a secondary metal.

Mixture	B_{max} (nmol g^{-1})	K_m (nM Pb^{2+})	r^2	Model	B_{max}	K_m
Pb Only	51.2 ± 4.8	4.3 ± 1.4	0.93	–	–	–
Pb + Ag	21.3 ± 2.1	1.1 ± 0.6	0.92	> 99% (< 0.01)	76% (0.03)	26% (0.30)
Pb + Cd	24.1 ± 2.4	1.7 ± 0.8	0.78	> 99% (< 0.01)	98% (< 0.01)	29% (0.26)
Pb + Cu	25.7 ± 1.4	3.6 ± 0.8	0.99	> 99% (< 0.01)	77% (0.03)	16% (0.81)
Pb + Ni	51.5 ± 7.1	3.9 ± 2.1	0.92	4% (0.93)	–	–
Pb + Zn	51.8 ± 4.9	4.1 ± 1.3	0.98	4% (0.92)	–	–

(Fig. 1, Table 1). The data did not allow us to distinguish between competitive and non-competitive inhibition as Cd uptake appeared to be linear over the range tested. The effects of Ag, Cu, and Pb were quite modest compared to Zn which strongly (~85%) inhibited Cd uptake, although we note that measured Ag, Cu, Pb concentrations were only 15%, 31%, and 47% of the target concentration (i.e., the LC50) and so likely underestimate the degree of inhibition compared to Zn. The results for Pb, Ni, and Zn are consistent with the hypothesis that only metals with the same mode of action will interact, while inhibition by Cu is inconsistent with this hypothesis.

Observations in this study are largely consistent with previous studies. For example, Birceanu et al. (2008) observed no inhibition of Cd uptake when fish were exposed to a range of Cd concentrations in the presence of 100 nM Pb. This is not surprising given the modest inhibition of Cd uptake we observed in the presence of 2270 nM Pb. Niyogi et al. (2015) used an experimental design reciprocal to ours (single concentration of primary metal and range of concentrations of secondary metal) and observed that both Cu and Zn significantly inhibited Cd uptake while Ni did not. Hence, while the inhibition of Cd uptake in the presence of Cu is inconsistent with our mechanism of action hypothesis, it has been previously observed.

There is strong evidence that an important Cd^{2+} transporter is the epithelial calcium channel (ECaC; TRP6). Several studies have demonstrated reciprocal competitive inhibition of Cd and Ca^{2+} (Hollis et al., 1997; Niyogi and Wood, 2004; Verbost et al., 1987; Verbost et al., 1989). Further, exposure of rainbow trout to elevated dietary Ca results in a down-regulation of Ca uptake, Cd uptake, and ECaC gene expression in the gill (Baldisserotto et al., 2004; Galvez et al., 2007). Similarly, the robust inhibition of Cd uptake by Zn is expected given that there is also reciprocal competitive inhibition between Zn and Ca^{2+} (Alsop and Wood, 1999; Hogstrand et al., 1994). Thus, all three ions appear to be competing for uptake via ECaC. The mechanism of Cd transport across the basolateral membrane is not clear, but Cd is known to be a potent inhibitor of the Ca^{2+}-ATPase (PMCA) (Verbost et al., 1988).

The more modest inhibition of Cd uptake in the presence of Ag, Cu, and Pb is more difficult to explain. As discussed below, there is strong evidence that Pb is also taken up by ECaC, so it is unclear why Zn but not Pb would strongly inhibit Cd uptake, but this may be a function of the relatively affinities of each metal for ECaC versus the exposure concentrations used in this study.

It is also possible that the inhibition by Cu and Pb is actually associated with the divalent metal transporter (DMT1, SLC11a2). DMT1 is primarily an Fe^{2+}/H^+ symporter but has been shown to also transport Cd and to a much lesser extent Ni and Pb in mammals (Garrick et al., 2003; Mackenzie et al., 2007). Additionally, Cd, Cu, Pb, Ni, and Zn have been demonstrated to inhibit Fe^{2+} uptake in mammalian and/or rainbow trout isoforms of DMT1 (Cooper et al., 2006; Cooper et al., 2007; Garrick et al., 2003; Mackenzie et al., 2007).

4.2. Lead

In the absence of other metals, we estimated a K_m of 4.3 nM Pb^{2+} (log K = 8.4). This value is lower than the K_m of 100 nM Pb^{2+} estimated by Birceanu et al. (2008) using a similar method, or the K_m of 1000 nM Pb^{2+} estimated by Macdonald et al. (2002) developed by measuring Pb uptake at relatively high concentrations in the presence and absence of several ligands (DOC, ethylenediamine, citrate) and competing cations (Ca, Na, Mg). The difference in K_m between the Birceanu et al. (2008) study and our study appears to be largely related to differences in speciation model selection. Birceanu et al. (2008) used the older Gaussian DOM model while we used the NICA-Donnan DOM model, with the latter predicting ~10-fold lower Pb^{2+} concentrations under the conditions used in both studies. Macdonald et al. (2002) had the same issue with Pb^{2+} speciation predictions which explains some of the discrepancy with the remaining difference likely associated with the different methodology used to estimate the K_m.

In the mixture experiments, Pb uptake was significantly inhibited in a non-competitive manner by Ag, Cd, and Cu, while Ni and Zn had no effect on Pb uptake (Table 2, Fig. 2). Again, we note that the measured Ag concentration was only 24% of the LC50 and so likely underestimates the impact of Ag on Pb uptake compared to the other metals evaluated. While inhibition by Cd might be expected given both Pb and Cd are Ca antagonists, the non-competitive nature of the inhibition suggests inhibition of the basolateral Ca^{2+}-ATPase rather than competitive interactions at the apical Ca^{2+} channel as the mechanism of action. The potent non-competitive inhibition by Ag and Cu is contrary to the hypothesis that only metals with the same mode of action would interact. Equally unexpected was the lack of inhibition by Zn, also a strong Ca antagonist. Rogers and Wood (2004) previously observed 38% and 47% inhibition of Pb uptake by 10 and 100 μM Zn, respectively (only the latter was significantly different than the control). It is unclear why exposure to 17 μM Zn in our study elicited no effect on Pb uptake. Consistent with our observations, previous studies on rainbow trout have also observed an inhibition of Pb uptake in the presence of Cd (Birceanu et al., 2008; Rogers and Wood, 2004).

Like Cd and Zn, reciprocal inhibition between Pb and Ca^{2+} uptake has been observed in rainbow trout suggesting Pb is being taken up by Ca^{2+} channels and experiments with pharmacological inhibitors indicate uptake is via a voltage independent Ca^{2+} channel, most likely ECaC (Rogers and Wood, 2004). As discussed above, Pb is also known to be transported by DMT1 (Cooper et al., 2007; Mackenzie et al., 2007). At the basolateral membrane, Pb is known to be transported by the Ca^{2+}-ATPase in humans red blood cells (Simons, 1988) and also to inhibit this protein in rainbow trout (Rogers and Wood, 2004). Finally, Pb is known to non-competitively inhibition Na^+ uptake pathways by binding to carbonic anhydrase and Na^+-K^+-ATPase, but Pb does not appear to be transported by apical Na^+ uptake pathways like the Na^+ channel (Rogers et al., 2005). It is difficult to entirely reconcile this information with our observations of non-competitive inhibition of Pb uptake by Ag, Cd, and Cu and no inhibition by Zn. The most obvious non-competitive mechanism would be inhibition of the Ca^{2+}-ATPase. While Cd is a potent Ca^{2+}-ATPase inhibitor (Verbost et al., 1988), there is no evidence Ag and Cu inhibit Ca^{2+}-ATPase activity. Further, Zn is also a potent Ca^{2+}-ATPase inhibitor, but Zn had no effect on Pb uptake (Hogstrand et al., 1996b).

In addition to the mechanism of non-competitive inhibition

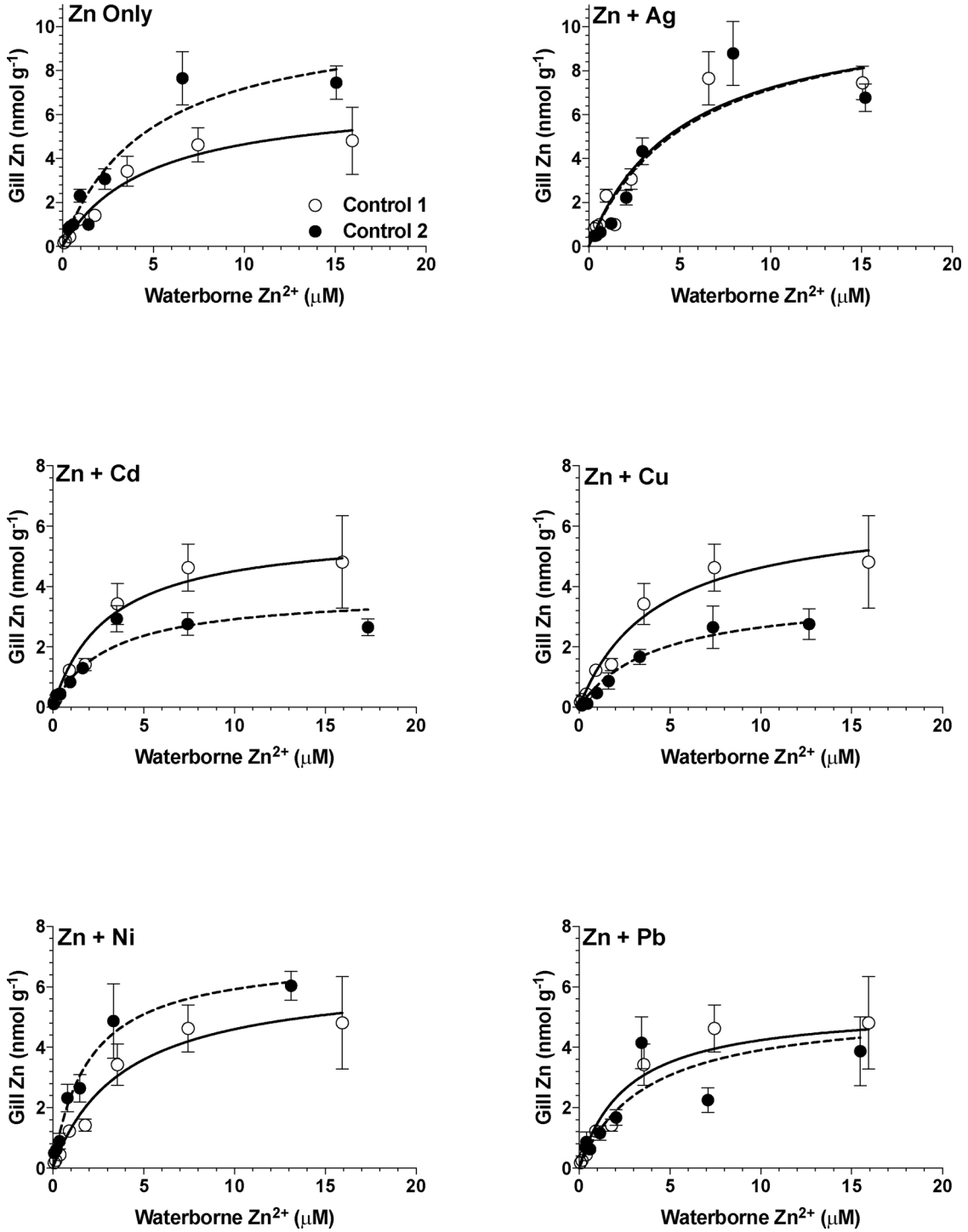

Fig. 3. Effect of Ag, Cd, Cu, Pb, and Ni (as secondary metals) on the uptake of Zn (as primary metal) at the rainbow trout gill. Control 1 and Control 2 are Zn only experiments conducted at the beginning and end of the experimental series. In all others panels, the open symbols represent Zn only exposures and closed symbols represent Zn + secondary metal exposures. A Michaelis-Menten model was fitted to the data. Model parameters and statistical test results are presented in Table 3.

described above, the lack of competitive inhibition is also informative with respect to apical uptake pathways for Pb. The lack of competitive inhibition by Cu and Cd suggests Pb uptake is not primarily occurring via DMT1 as Cd is known to be taken up by trout DMT1 (Cooper et al., 2007) and both Cd and Cu are known to inhibit DTM1 in mammalian systems (Garrick et al., 2003). The lack of competitive inhibition by Zn on Pb uptake is inconsistent with the premise that Pb is taken up by ECaC or DMT1, as Zn is known to be taken up by ECaC and inhibit DMT1 (Cooper et al., 2007; Hogstrand et al., 1994; Mackenzie et al., 2007). Additional study is clearly needed to further elucidate these mechanisms and interactions before an mBLM for Pb can be fully developed.

4.3. Zinc

Michaelis-Menten saturation kinetics were also observed for Zn uptake with a significant increase in B_{max} in the Zn only experiments conducted at the beginning and end of the study period (Fig. 3,

Table 3
Summary of model parameters and statistical tests for Zn metal mixture experiments. Mean ± SE Michaelis-Menten parameters. Percentages represent AICc probabilities that metal uptake kinetics in the presence and absence of a second metal are best described by two separate models (i.e., effect of the secondary metal on Zn uptake of Zn). Values in parentheses are *p*-values for F-tests comparing models for Zn only versus Zn + a secondary metal.

Mixture	B_{max} (nmol g^{-1})	K_m (μM Zn^{2+})	r^2	Model	B_{max}	K_m
Zn Only 1	6.4 ± 0.7	3.9 ± 1.1	0.97	–	–	–
Zn Only 2[a]	10.9 ± 2.1	5.1 ± 2.2	0.91	95% (< 0.01)	55% (0.06)	12% (0.65)
Zn2 + Ag	11.1 ± 2.9	5.5 ± 3.2	0.87	2% (0.99)	–	–
Zn1 + Cd	3.3 ± 0.4	1.8 ± 0.8	0.91	> 99% (< 0.01)	99% (< 0.01)	41% (0.11)
Zn1 + Cu	4.3 ± 0.5	5.8 ± 1.5	0.98	> 99% (< 0.01)	38% (0.12)	16% (0.38)
Zn1 + Ni	7.1 ± 0.5	2.0 ± 0.4	0.98	> 99% (< 0.01)	13% (0.40)	64% (0.04)
Zn1 + Pb	4.2 ± 1.0	2.0 ± 1.3	0.73	16% (0.17)	–	–

[a] Comparison of Zn Only 1 versus Zn Only 2.

Table 3). We previously observed this same pattern for Cu (Brix et al., 2016), and hypothesized it may be related to increasing essential element demands in rapidly growing juvenile rainbow trout. The similar observation for Zn, but not Cd or Pb, further supports this hypothesis and reinforces the problem of how to account for rapid changes in B_{max} within a BLM framework.

In the absence of a secondary metal we observed a mean K_m of 4500 nM Zn^{2+} (log K = 5.3). Previously, Alsop and Wood (2000) estimated a K_m of 593 nM Zn^{2+} for juvenile rainbow trout in the same water. This substantially lower K_m is based on only 4 data points, but suggests that a high affinity transporter may be present that was not easily discerned across the wide range of treatments used in our study to encompass the Zn 96-h LC50. We explore this possibility further in a more speculative data analysis described in Supplemental Information.

In mixture experiments (assuming a single Zn transporter), Zn uptake was significantly inhibited by Cd and Cu (Fig. 3, Table 3). Inhibition by Cd was non-competitive, while the mechanism for Cu could not be determined. Niyogi et al. (2015) also previously observed Cd inhibited Zn uptake. Previously, and in this study, we observed that Zn inhibited both Cd and Cu uptake, providing the best examples of reciprocal inhibition of metal uptake (Brix et al., 2016). The lack of inhibition by Pb was somewhat suprising, but may have been the result of measured Pb concentrations being only 29% of the LC50.

Stimulation of Zn uptake by Ni included a significant reduction in the K_m (i.e., increased affinity) (AICc = 64%; F-test, $p = 0.04$). Previously, we had observed no effect of Zn on Ni uptake (Brix et al., 2016). Given the disparate mechanisms of action for Zn and Ni, it is unclear why Ni stimulated Zn uptake.

Although we are unable to distinguish them in a statistically robust manner in these experiments, a number of transporters appear to be involved in Zn uptake at the fish gill. Reciprocal inhibition of uptake between Zn and Ca^{2+} suggest that ECaC is likely one mechanism of Zn uptake (Hogstrand et al., 1994). Additionally, similar to Cd, rainbow trout fed a high-Ca diet reduce both Ca^{2+} and Zn uptake at the gill (Niyogi and Wood, 2006). As described above, although Zn inhibits DMT1, available data suggests Zn transport by DMT1 is negligible (Cooper et al., 2007; Mackenzie et al., 2007).

There is also an array of Zn transporters belonging to the ZIP (SLC39) and ZnT (SLC30) transport families. In zebrafish (*Danio rerio*), ZnT1-ZnT5, ZnT7-ZnT9, ZIP1, ZIP3-ZIP10, and ZIP13 are all expressed in the gill (Feeney et al., 2005). Of these, ZnT5, ZIP1, ZIP3, and ZIP10 are known to be located on the apical membrane and thought to be involved in Zn uptake at the gill (Feeney et al., 2005; Zheng et al., 2008). Both ZIP1 and ZIP3 have been isolated and characterized in cell expression systems. ZIP1 (SLC39A1) is a high affinity (K_m < 385 nM Zn^{2+}) transporter (Qiu et al., 2007; Qiu et al., 2005) while ZIP3 (SLC39A3) is a relatively low affinity transporter (K_m = 10.5 μM Zn^{2+}) (Qiu and Hogstrand, 2005). Although only a high (30 μM Cu) concentration was tested, Cu was shown to inhibit Zn uptake by ZIP3, providing a potential mechanism for Cu-Zn interactions observed is this study and others.

We are aware of no mechanism by which Zn uptake by ZIP1 is stimulated by Ni. Indeed, similar to Cu, Ni has been shown to inhibit Zn uptake by human ZIP1 (Gaither and Eide, 2001).

While our experiments provide some insights into the interactions between Zn and other metals for uptake at the gill, it is clear that additional studies are needed to more fully characterize the transport kinetics of various Zn transporters. Additionally, experiments in which individual transporters are evaluated in an isolated system (e.g., expression and testing in *Xenopus* oocytes) are needed to better understand the relatively complex set of interactions between other metals and the various Zn transporters.

4.4. Implications for mixture BLM (mBLM) development

The objective of this study was to characterize the effects of Ag, Cd, Cu, Ni, Pb, and Zn on accumulation of Cd, Pb, and Zn in binary mixtures at the rainbow trout gill. We hypothesized that Ag, Cu, and Ni would not interact with uptake of the three primary metals while all binary combinations of the Cd, Pb, and Zn would competitively interact for uptake at the gill. This hypothesis is generally consistent with the mBLM framework developed by Balistrieri and Mebane (2014) in which separate binding sites for Na and Ca antagonists are utilized.

As in our earlier study on Ag, Cu, and Ni (Brix et al., 2016), the current series of experiments did not consistently support these basic hypotheses. Binary mixtures of Cd-Zn and Cd-Pb, which exhibited reciprocal inhibition, were generally consistent with a Ca antagonist specific binding domain, but confusingly, no interactions were observed in Pb-Zn mixtures. Even for the Cd-Zn and Cd-Pb mixtures, all of the interactions were attributed to non-competitive inhibition rather than competitive inhibition as might be expected for two Ca antagonists competing for the same Ca uptake pathway. Contrary to the Ca/Na antagonist hypothesis, Ag and Cu strongly inhibited Cd and Pb uptake, but only Cu inhibited Zn uptake. Finally, Ni, which would not be expected to interact with any of the three primary metals, surprisingly stimulated Zn uptake with a significant increase in affinity.

Overall, the interactions characterized in this study and our previous study on Ag, Cu, and Ni, support the modeling framework developed by Santore and Ryan (2015) in which each metal has its own site of uptake, although our data and other metal transporter studies suggest multiple binding sites may be needed for many metals, each with its own unique set of interactions with secondary metals. However, looking across the six metals we have now evaluated in this study and in Brix et al. (2016), we note that there were no statistically robust examples of competitive inhibition. In contrast, we have identified at least 4 examples of non-competitive inhibition (Pb-Ag, Pb-Cd, Pb-Cu, Zn-Cd; primary metal-secondary metal). This suggests that, at least with respect to metal uptake at the biotic ligand, there must be a fundamental change in the mBLM framework in which less than additive metal mixtures are often due to a change B_{max}, which in turn elicits changes in metal accumulation and toxicity.

Finally, it is important to recognize that this study only considers

metal interactions with respect to binding and uptake and does not consider additional potential interactions that may occur at the site of toxic action, if it differs from the site of uptake. Interactions at sites of toxicity are likely to involve at least a few additional proteins (e.g., Na^+/K^+-ATPase, Ca^{2+}-ATPase, carbonic anhydrase) that are not directly involved in metal uptake, although they may influence uptake via secondary active transport processes. Initial studies such of those of Niyogi et al. (2015) suggest that acute toxicity of binary mixtures (as measured by inhibition of Na^+ and Ca^{2+} uptake) may follow simple additivity. Additional studies that consider both metal mixture uptake, toxicity, and more importantly how to integrate these two physiological processes are needed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.10.015.

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