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Characterization of the effects of binary metal mixtures on short-term uptake of Ag, Cu, and Ni by rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Single metal Biotic Ligand Models (BLMs) have been developed for a number of metals and model organisms. While these BLMs improve our ability to regulate metals in the aquatic environment, in reality, organisms are often simultaneously exposed to metal mixtures. Recently, several attempts have been made to develop mixture BLMs (mBLMs). Some of these models assume competitive interactions between all metals, while others assume only metals with a similar mode of action (e.g., Na^+ or Ca^{2+} antagonists) will competitively interact. To begin testing these assumptions in the mBLM framework, standard 3-h gill metal binding assays with Ag, Cu, and Ni (primary metals), were performed *in vivo* on freshwater rainbow trout. Fish were exposed across a range of concentrations encompassing the 96-h LC50 for that metal to characterize uptake kinetics for each of these three primary metals (radiolabelled) in the presence and absence of a secondary metal (Ag, Cd, Cu, Ni, Pb, or Zn; not radiolabelled). We observed a complex series of interactions in binary mixtures that frequently contradicted theoretical expectations. Metals with similar modes of action did competitively interact in some instances, but not others, and when they did compete the competition was not necessarily reciprocal (e.g., Cu inhibited Ag uptake but Ag did not inhibit Cu uptake). We also observed examples of interactions between metals with dissimilar modes of action and several examples of metals stimulating the uptake of other metals. The underlying mechanisms for these unexpected interactions are unclear, but suggest that many of the current assumptions in mBLMs regarding the number and types of metal uptake sites and corresponding metal interactions are not correct. Careful characterization of metal mixture interactions is clearly needed before a reliable mBLM can be developed.

1. Introduction

Historically, metals have been regulated on an individual basis in aquatic environments based on dissolved waterborne concentrations and frequently as a function of water hardness. More recently, the Biotic Ligand Model (BLM) has been developed as a tool for predicting 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). The BLM approach integrates physiology, toxicology, and geochemistry into a modeling framework that relates local water chemistry to metal accumulation on the external surfaces (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 nutrient ion transport. The basic premise of the BLM is that metal binding to these proteins can be represented by a complexation reaction and an associated conditional binding constant (log K). Competing reactions can decrease metal accumulation and the resultant toxic response. These competing reactions include competition at the biotic ligand by other cations (e.g., Na^+, H^+, Ca^{2+}) and competing complexation reactions for the metal by ligands in solution. These ligands include inorganic anions (e.g., chloride, hydroxide, sulfide) as well as organic ligands such as dissolved organic carbon (DOC).

The foundation for the BLM was first laid out by Playle and coworkers who demonstrated that the effects of various water quality parameters (pH, major ions, and dissolved organic carbon) on short-term (3 h) metal (Playle and Dixon, 1993; Playle et al., 1993, 1992) accumulation at the fish gill could be used to develop

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a conditional binding constant (log K). These log K's could then be incorporated into geochemical equilibrium models, in which 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, 1992). Short-term metal accumulation at the gill was also demonstrated to be a useful predictor of whole animal metal toxicity, with a strong correlation between the 96-h LC50 and measured metal concentrations at the gill after 3 or 24 h of exposure (termed the lethal accumulation or LA50) (MacRae et al., 1999; Meyer et al., 1999; Morgan and Wood, 2004).

There is a sound physiological and chemical basis for understanding the mechanisms underlying observed acute toxicity in fish. 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 and Wood, 2002; Morgan et al., 2004). Similar interactions have been described for Zn^{2+} interfering with Ca^{2+} uptake (Hogstrand et al., 1994). Pb^{2+} similarly appears to disrupt primarily Ca^{2+} homeostasis, but also interferes with Na^{+} homeostasis at high concentrations (Rogers et al., 2005; Rogers and Wood, 2004). Ni^{2+} appears to be different, at least in fish, acting primarily as a respiratory toxicant (Pane et al., 2004a).

Based on this knowledge we can hypothesize how metal mixtures might interact. A large, multi-investigator study (the Metal Mixture Modeling Evaluation; MMME) recently used these basic hypotheses to develop initial formulations of 3 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). In general, these models were able to predict with reasonable accuracy the toxicity of metal mixtures, although there were specific data sets where toxicity was less than additive that were problematic (Farley et al., 2015). Interestingly, although the models were all fundamentally based on consideration of metal speciation, complexation, and competition, there were significant differences in model assumptions. For example, some models assumed a single binding site for all metals and used a concentration addition approach to estimate toxicity (Iwasaki et al., 2015; Tipping and Lofts, 2015). Other models assumed two binding sites or separate binding sites for each metal with respect to toxicity (i.e., independent action), although other metals could compete for accumulation at each site (Balistrieri and Mebane, 2014; Santore and Ryan, 2015). Further, all of the models were calibrated to initial data sets by adjusting both log K's and LA50s in different ways. Consequently, although two models might perform similarly in terms of predicting the toxicity of a given mixture, the underlying mechanisms behind that prediction (i.e., high/low affinity binding, high/low metal potency, independent action/concentration addition) were different for the various models.

These results lead to several open questions regarding how metal mixtures interact at the biotic ligand, including the following: Which metals compete for the same uptake sites? Are these interactions consistent as a function of metal concentration? Are the same metal interactions occurring at sites of toxic action, which may be different from uptake sites? Is toxicity concentration additive for metals with the same physiological mechanism of action? How do metals with different mechanisms of action interact?

Conceptually, there are two distinct processes that must be characterized to develop a mBLM; (i) characterization of interactions between metals for uptake by the organism; and (ii) characterization of interactions at the proximate site(s) of toxic action. For example, the acute toxicity of Cd and Zn mixtures have been observed to be less than additive to *Daphnia magna* (Meyer et al., 2015). This observation could be the result of several scenarios including the following: (i) Cd and Zn compete for the same uptake site and target the same site of toxic action (which is different from the site of uptake) in an additive manner. (ii) Cd and Zn compete for some of the same uptake sites, but also have additional uptake sites (i.e., other transporters) where they do no compete for uptake and Cd and Zn target different sites of toxic action. (iii) Cd and Zn do not compete for uptake sites, but do compete at the same site of toxic action in an antagonistic manner, etc. Toxicity data by themselves do not allow one to distinguish between these scenarios and so it is unclear whether adjustment of the log K, LA50, or both values is appropriate.

The objective of this study, and a key recommendation of the MMME study (Van Genderen et al., 2015), was to provide data on one component of the model, the effects of binary metal mixtures on metal uptake. Specifically, this study provides data on the short-term (3 h gill-binding) concentration-dependent uptake relationships for three different metals (Ag, Cu, and Ni – primary metals) in juvenile rainbow trout over concentration ranges relevant for acute toxicity (i.e. 96-h LC50 ranges). 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 (Cd, Pb, Zn, Ag, Cu, or Ni – secondary metals) in binary combinations.

2. Materials and methods

2.1. Experimental design overview

The uptake of a single (primary) metal (radiolabelled) was first characterized over a range of concentrations that encompassed the 96-h LC50 for that metal. The experiment was then repeated in the presence of a potentially interacting (secondary) metal (not radiolabeled) 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; these are key parameters in the BLM (Di Toro et al., 2001). This facilitated characterization of how the secondary metal does or does not change uptake of the primary metal at the fish gill at toxicologically relevant concentrations. For a single-transporter uptake, a change in K_m can be associated with a metal competitive interactions while a change in the B_{max} can be associated with non-competitive interactions (e.g. a change in the number of transporters).

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 minimize the potential for these changes. 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. Fish were acclimated to these conditions for at least one week prior to use in testing. During holding, fish were fed ~1% of their weight daily with commercial trout pellets. Fish were fasted for 72 h prior to use in testing.

2.3. Metal uptake experiments

To characterize metal uptake at the gill of rainbow trout, the general experimental protocol developed by Playle and Dixon (1993)

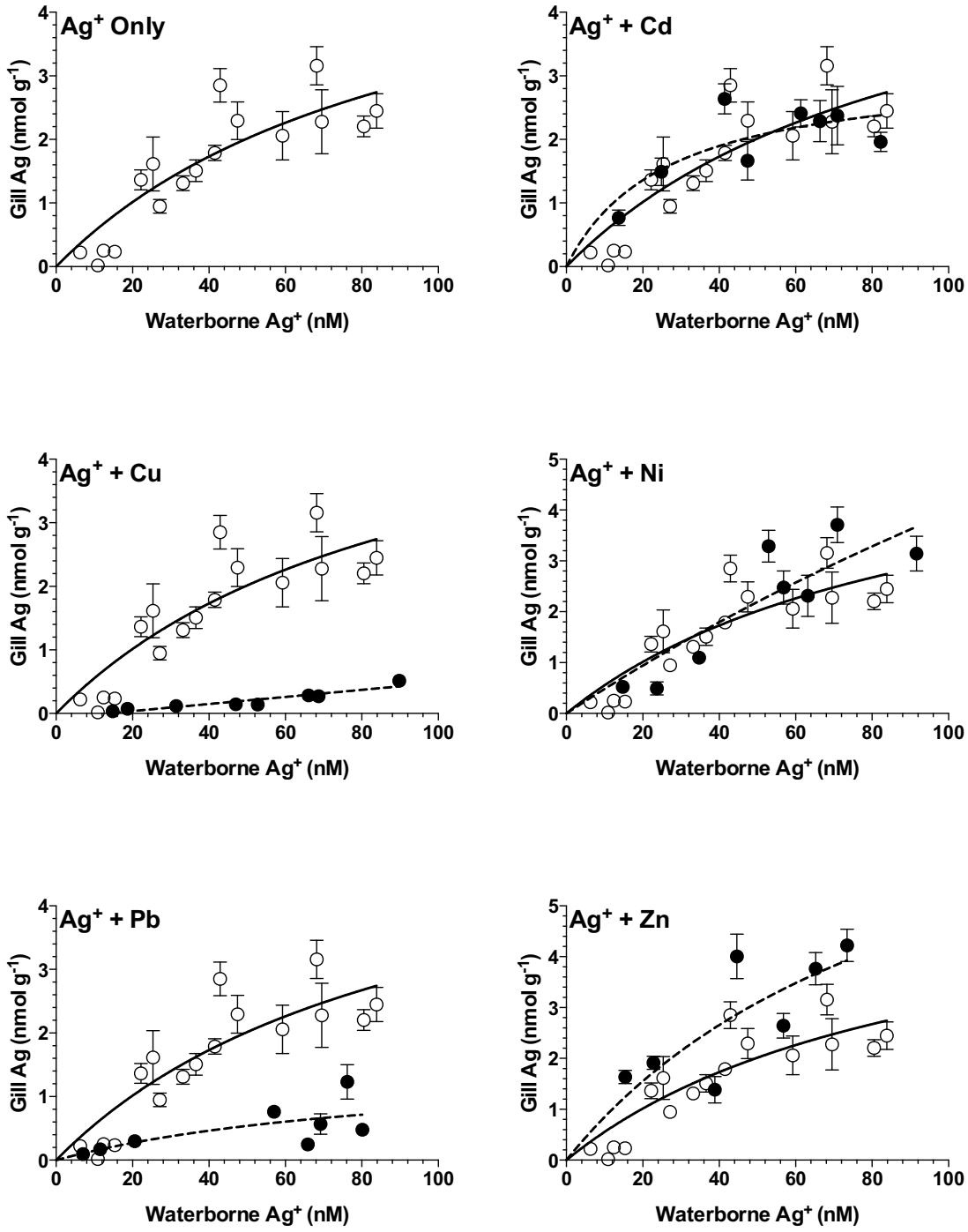

Fig. 1. Effect of Cd (0.18 μM), Cu (1.1 μM), Ni (260 μM), Pb (4.8 μM), and Zn (17 μM) (as secondary metals) on the uptake of Ag^+ (as primary metal) at the rainbow trout gill. The open symbols represent Ag^+ only exposures and closed symbols represent Ag^+ + secondary metal exposures. A Michaelis-Menten model was fitted to the data except for the Ag^+ + Cu experiment where a simple linear model provided a better fit. Model parameters and statistical test results are presented in Table 1.

was used, although we modified it by radiolabelling the primary metal. Fish were exposed to a range of 8–12 metal concentrations (n = 6 per treatment) to characterize the concentration-dependent accumulation of metal by the fish gill. For each treatment, fish were held in a single 800 ml polypropylene container with 650 ml of test solution (i.e., fish were treated as the unit of replication) that was provided with gentle aeration. Test solutions were prepared and allowed to equilibrate for 0.5 h prior to fish exposure and consisted of reagent grade metal salts of the appropriate secondary metal ($AgNO_3$, $Cd(NO_3)_2{\cdot}4H_2O$, $CuSO_4{\cdot}5H_20$, $NiSO_4{\cdot}6H_2O$, $Pb(NO_3)_2$, or $ZnSO_4$) plus various concentrations of the appropriate primary metal ($AgNO_3$, $CuSO_4{\cdot}5H_2O$, $NiSO_4{\cdot}6H_2O$) labeled with 3–4 $\mu Ci\,l^{-1}$ of the appropriate metal radioisotope (^{110m}Ag, ^{64}Cu, ^{63}Ni). The radioisotope was added at high specific activity in trace amounts so as not to appreciably alter the total concentration of the primary metal. Radioisotopes were obtained from commercial suppliers (^{63}Ni from Perkin-Elmer) (Boston, MA, U.S.A; ^{110m}Ag from Eckert and Ziegler Valencia, CA, USA) or made in the McMaster University Nuclear Reactor (^{64}Cu).

The range of concentrations for the primary metal being evaluated in each experiment was intended to bracket the 96-h LC50 based on previous studies conducted in our lab using the same

Table 1
Summary of model parameters and statistical tests for Ag^+ metal mixture experiments. Percentages represent AICc probability 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 uptake of Ag^+). Values in parentheses are p-values for F-tests comparing models for Ag^+ only versus Ag^+ + a secondary metal.

Mixture	B_{max} (nmol g^{-1})	K_m (nM)	r^2	Model	B_{max}	K_m
Ag Only	5.9	96	0.78	–	–	–
Ag + Cd	3.1	26	0.64	13% (0.40)	–	–
Ag + Cu[a]	–	–	–	–	–	–
Ag + Ni	19.3	389	0.77	16% (0.32)	–	–
Ag + Pb	1.6	95	0.45	>99% (<0.01)	18% (0.82)	17% (>0.99)
Ag + Zn	9.2	98	0.58	97% (<0.01)	19% (0.60)	17% (0.99)

[a] Ag^+ uptake in the presence of Cu was best described by a simple linear model: y = 0.00557- 0.073, r^2 = 0.88.

dilution water and conditions. 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., 1996), 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).

After fish were introduced to the exposure container, a water sample was collected at the beginning and end of the flux period for measuring radioactivity and dissolved (<0.45 μm) metal concentrations. At the end of the 3-h exposure, fish were euthanized by an overdose of MS-222 (tricaine methanesulfonate) and the gills excised. Gills were then rinsed in a 1 mM solution of "cold" (i.e. non-radioactive) metal to displace any loosely bound radioactive metal. For Ag and Cu, gills were subsequently placed in plastic vials for direct counting using a gamma counter. For Ni, samples were digested in 1 ml of 1N HNO_3 at 40 °C overnight before being assayed on a scintillation counter.

2.4. Analytical chemistry

Dissolved metal 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. 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 ^{110}Ag and ^{64}Cu was analyzed using an automated gamma counter (Perkin Elmer Wizard 1480 3" Auto Gamma Counter). Beta radioactivity for ^{63}Ni was measured in 1 ml of gill digest added to 5 ml of Ultima-Gold AB scintillation cocktail (Perkin Elmer, Toronto, Canada) by liquid scintillation counting (Tri-Carb 2900 TR; Perkin Elmer, Boston, MA, USA). Quench correction was performed using a quench curve constructed from various amounts of gill digests and the external standard method to correct gill cpm to the same efficiency as water cpm. Metal accumulation at the gill (nmol g^{-1}) was calculated based on the accumulation of radioactivity in the gill and the specific activity of the radioisotope in the water:

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

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 are presented and analyses were performed using measured test concentrations. Data are presented as mean ± SEM. Metal accumulation at the gill for the 3-h exposure period as a function of waterborne metal concentration was evaluated using the measured dissolved metal concentrations and subsequently estimated free metal ion concentrations. Free metal ion concentrations were estimated using the speciation software Visual Minteq (Version 3.0; courtesy of J.P. Gustaffson, Royal Institute of Technology, Sweden) using the embedded NICA-Donnan model for organic complexation.

We initially assumed that metal accumulation at the fish gill would approximate a Michaelis-Menten type model:

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

where, B_{max} is the maximum estimated concentration on the fish gill and K_m is the half saturation constant, and X is the free metal ion concentration. This assumption proved to be correct for Ag and Cu, but for Ni, there was no indication of binding site saturation up to the highest concentrations tested. Therefore a simple linear model (Y = mX + b, where b = 0) was used to describe Ni accumulation at the gill. Note, if one assumes that Ni uptake still conforms to Michaelis-Menten kinetics, then a linear model can be considered as a simplification of Eq. (1) when $X \ll K_m$, with $m = B_{max}/K_m$, where m is the slope of the line.

To test for differences in primary metal uptake between treatments (i.e., with and without the secondary metal present), we used Aikake Information Criterion (AIC) (Akaike, 1974) corrected for sample size (AICc) in GraphPad Prism (v. 6.0). This analysis evaluated whether data characterizing 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 whether a single model or two separate models is more appropriate. These probabilities are not directly analogous to a *p*-value 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). For comparison, we also evaluated models (and model parameters) in the absence and presence of a secondary metal using an F-test (Snedecor and Cochran, 1989).

3. Results

3.1. Silver

Silver speciation was predicted to be constant as a function of waterborne Ag concentration with 34% of dissolved Ag predicted to be Ag^+ (Table S1). None of the secondary metals had any influence on this speciation. The uptake of Ag^+ in the absence of a secondary metal exhibited saturable kinetics with a K_m of 96 nM Ag^+ and 3-

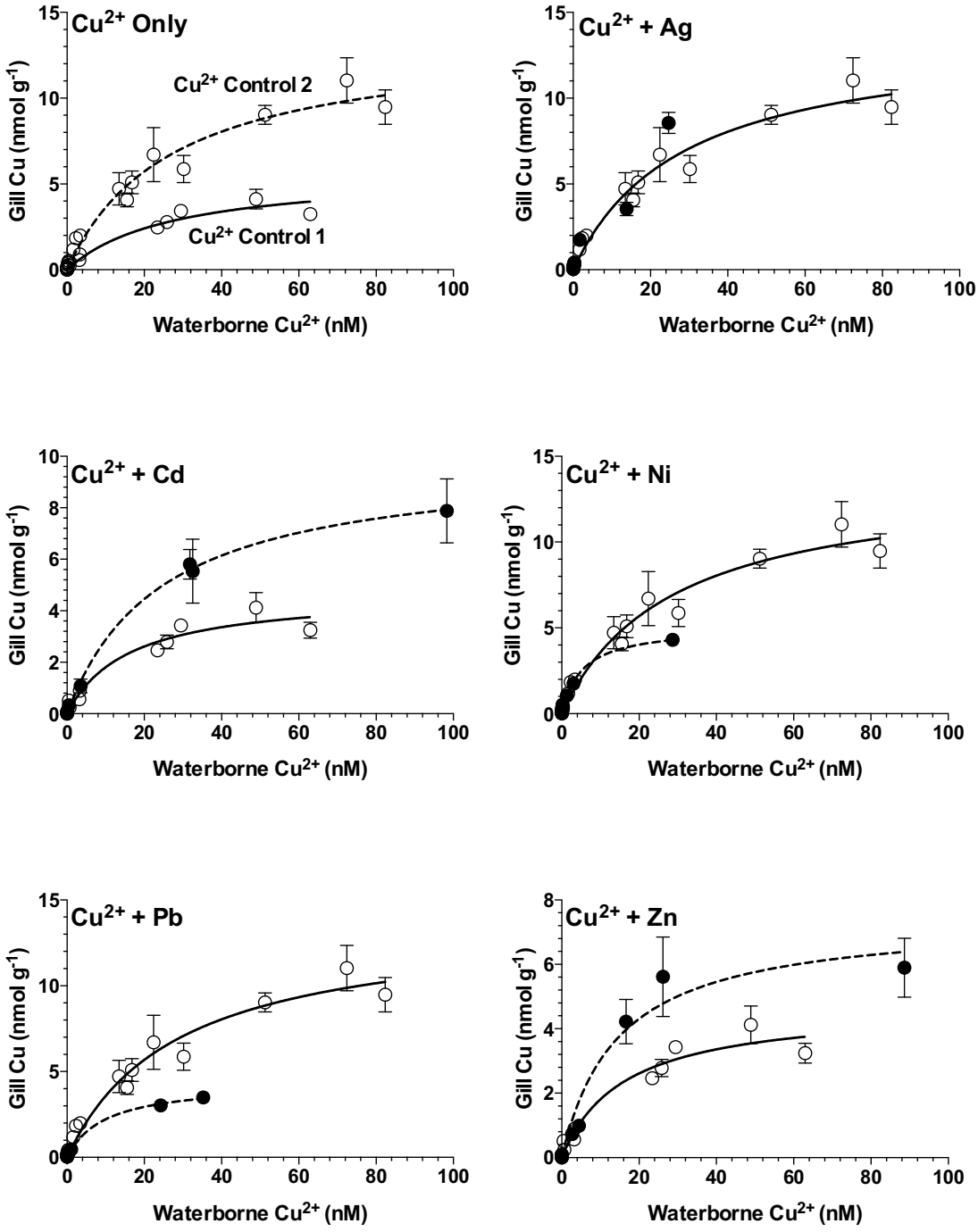

Fig. 2. Effect of Ag (0.11 μM), Cd (0.18 μM), Ni (260 μM), Pb (4.8 μM) and Zn (17 μM) (as secondary metals) on the uptake of Cu^{2+} (as primary metal) at the rainbow trout gill. The open symbols represent Cu^{2+} only exposures and closed symbols represent Cu^{2+} + secondary metal exposures. A Michaelis-Menten model was fitted to the data except for the Cu^{2+} + Ag experiment where data were insufficient to model. Model parameters and statistical test results are presented in Table 2.

h B_{max} of 5.9 nmol g^{-1} (Fig. 1, Table 1). There were no significant differences in the Ag^+ only uptake kinetics at the beginning and end of the experimental period, so data from these two experiments (i.e., Ag^+ only present) were pooled for analysis of the effects of secondary metals on Ag^+ uptake.

Addition of either Cd or Ni at their respective LC50 had no appreciable effect on Ag uptake kinetics with low AICc probabilities of differences between models (AICc = 13–16%; F-test, $p = 0.32$–0.40; Table 1). Therefore the apparent changes in calculated K_ms in the presence of both Cd and Ni, and in B_{max} in the presence of Ni, were not statistically meaningful (Table 1). Copper had the strongest inhibitory effect (AICc = 99%; F-test, $p < 0.01$) on Ag^+ uptake with, for example, a 90% inhibition of Ag^+ uptake at 50 nmol l^{-1} waterborne Ag^+. However, the inhibition resulted in Ag^+ uptake becoming linear as a function of waterborne Ag^+ in the presence of Cu precluding estimates of V_{max} and K_m (Fig. 1, Table 1). Pb also strongly inhibited Ag^+ uptake (Fig. 1, Table 1; AICc = > 99%; F-test, $p < 0.01$) but statistical differences in K_m and B_{max} could not be identified (Table 1), making it unclear whether this inhibition was competitive, non-competitive, or mixed. We note that while we were able to fit a Michaelis-Menten model to the Ag^+ + Pb uptake data, the fit was relatively poor ($r^2 = 0.45$) and, as observed for Cu, could also be

Table 2
Summary of model parameters and statistical tests for Cu^{2+} metal mixture experiments. Percentages represent AICc probability 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 uptake of Cu^{2+}). Values in parentheses are p-values for F-tests comparing models for Cu^{2+} only versus Cu^{2+} + a secondary metal.

Mixture	B_{max} (nmol g^{-1})	K_m (nM)	r^2	Model	B_{max}	K_m
Cu Only 1	4.7	16	0.97	–	–	–
Cu Only 2[a]	13.8	28	0.98	>99% (<0.01)	98% (<0.01)	32% (0.32)
Cu2 + Ag[b]	–	–	–	28% (0.31)	–	–
Cu1 + Cd	9.9	24	0.99	>99% (<0.01)	99% (<0.01)	32% (0.23)
Cu2 + Ni	5.0	5	0.98	>99% (<0.01)	93% (<0.01)	82% (0.02)
Cu2 + Pb	4.4	10	0.99	>99% (<0.01)	27% (0.36)	21% (0.61)
Cu1 + Zn	7.4	15	0.96	>99% (<0.01)	73% (0.03)	16% (0.84)

[a] Comparison of Cu Only 1 versus Cu Only 2.
[b] Data were insufficient to fit Michaelis-Menten, data compared using linear regression model on constrained data set; see Results for details.

modeled using simple linear regression ($r^2 = 0.45$). Unlike Cu and Pb, Zn stimulated Ag^+ uptake (Fig. 1, Table 1; AICc = 97%; F-test, $p < 0.01$) though again significant differences in model parameters were not identified (Table 1).

3.2. Copper

Copper speciation varied significantly as a function of waterborne Cu concentration with the predicted fraction as Cu^{2+} ranging from 0.00071% in the lowest Cu only treatment (19 nM) up to 2.3% Cu^{2+} at the highest Cu only treatment (3623 nM). In most cases, the presence of a secondary metal did not affect speciation. However, Cu^{2+} concentrations were ~15–30% higher (increasing with concentration) in the presence of Ni at comparable waterborne Cu concentrations, while Cu^{2+} concentrations were ~39–60% higher in the presence of Pb as a secondary metal. For both Ni and Pb, the increase in Cu^{2+} was caused by displacement of Cu^{2+} from DOC by the secondary metal causing an increase in all of the inorganic Cu species including the free ion.

Cu^{2+} uptake in the absence of a second metal exhibited Michaelis-Menten saturation kinetics but there were large differences (AICc = > 99%; F-test, $p < 0.01$) between the initial control experiment and final control experiment performed 1 week later (data not shown). This result would make evaluation of the effect of a second metal on Cu^{2+} uptake difficult. To address this issue, we repeated the Cu^{2+} study, this time with a Cu^{2+} only experiment performed concurrently with each experiment evaluating the effects of a second metal on Cu^{2+} uptake. Analysis of the Cu^{2+} only experiments associated with each metal indicated that data could be pooled into two groups where data within these two groups were statistically similar, but the two pooled data sets were different (AICc = > 99%; F-test, $p < 0.01$) (Fig. 2, Table 2). Specifically, the B_{max} was different between the two groups (AICc = 98%; F-test, $p < 0.01$) while K_m was similar (AICc = 32%; F-test, $p = 0.32$). Pooling of control data allowed for a more robust statistical analysis when evaluating the effects of a secondary metal on Cu^{2+} uptake.

In the experiment evaluating the effects of Ag on Cu^{2+} uptake, measured Cu concentrations were lower than anticipated due to an error in test solution spiking and did not allow for development of a Michaelis-Menten regression model for this experiment. However, visual inspection of the available data suggests that Ag had no effect on Cu^{2+} uptake, and this was supported by comparing linear regression models fitted to the Cu^{2+} alone data versus the Cu^{2+} + Ag data in the region of overlap (AICc = 28%; F-test, $p = 0.31$) (Fig. 2). Both Cd (AICc = 99%; F-test, $p \leq 0.01$) and Zn (AICc = 73%, F-test, $p = 0.03$) stimulated Cu^{2+} uptake with increases in B_{max} but no change in K_m (Table 2). Conversely, both Ni and Pb inhibited Cu^{2+} uptake (>99% AICc probabilities of different models; F-test, $p < 0.01$). For Ni, there was a reduction in both B_{max} (AICc = 93%; F-test, $p < 0.01$) and K_m (AICc = 82%; F-test, p = 0.02), while for Pb it is not clear whether this inhibition was competitive, non-competitive, or mixed (Table 2).

Table 3
Summary of model parameters and statistical tests for Ni^{2+} metal mixture experiments. Percentages represent AICc probability 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 uptake of Ni^{2+}). Values in parentheses are p-values for F-tests comparing models for Ni^{2+} only versus Ni^{2+} + a secondary metal. A simple linear model (y = mX + b), where m = the slope of the line and b = 0, was used.

Mixture	m	r^2	Differences in Slope?
Ni Only	0.39	0.90	–
Ni + Ag	1.78	0.87	>99% (< 0.01)
Ni + Cd	0.27	0.79	98% (<0.01)
Ni + Cu	0.82	0.86	>99% (< 0.01)
Ni + Pb	0.52	0.63	96% (<0.01)
Ni + Zn	0.39	0.83	23% (0.85)

3.3. Nickel

Nickel speciation varied slightly as a function of waterborne Ni concentration, with the predicted fraction present as Ni^{2+} increasing from 72% at the lowest Ni only treatment (0.22 μM) up to 81% in the highest Ni only treatment (375 μM). The presence of a secondary metal did not affect Ni speciation. Unlike Ag^+ and Cu^{2+}, Ni^{2+} uptake did not exhibit Michaelis-Menten saturation kinetics, with a simple linear model (y = mx, where m is the slope of the line) best describing Ni^{2+} uptake across the range of concentrations tested (Fig. 3). In the absence of a secondary metal, Ni^{2+} uptake was similar between the two experiments performed at the beginning and end of the study period, so control data were pooled for analysis. Because uptake was linear, statistical comparisons could only be made by testing for differences in the slope of Ni^{2+} uptake in the presence and absence of a secondary metal.

Zn was the only metal that had no effect (AICc = 24%; F-test, $p = 0.85$; Table 3) on Ni^{2+} uptake (Fig. 3). Cadmium (AICc = 98%; F-test, $p < 0.01$) and Pb (AICc = 96%, $p < 0.01$) elicited inhibition and stimulation, respectively, of Ni^{2+} uptake although the changes in slope were relatively modest (Fig. 3, Table 3). Both Ag and Cu elicited much larger stimulations of Ni^{2+} uptake with 4.4 and 2.1-fold increases in Ni^{2+} uptake when exposed to 150 μM waterborne Ni, respectively (AICc = > 99%; F-test, p < 0.01).

4. Discussion

4.1. The experimental approach

This study is part of an ongoing effort to understand the mechanisms of metal interaction at biotic ligands and to provide data on the effects of metal mixtures on metal uptake by aquatic organisms for the development of a mechanistically-based mBLM. It directly follows up a recent study from our laboratory (Niyogi et al., 2015) where an experimental approach exactly reciprocal to the present one was used. In binary combinations, Niyogi et al. (2015) examined the effect of one or more concentrations of a

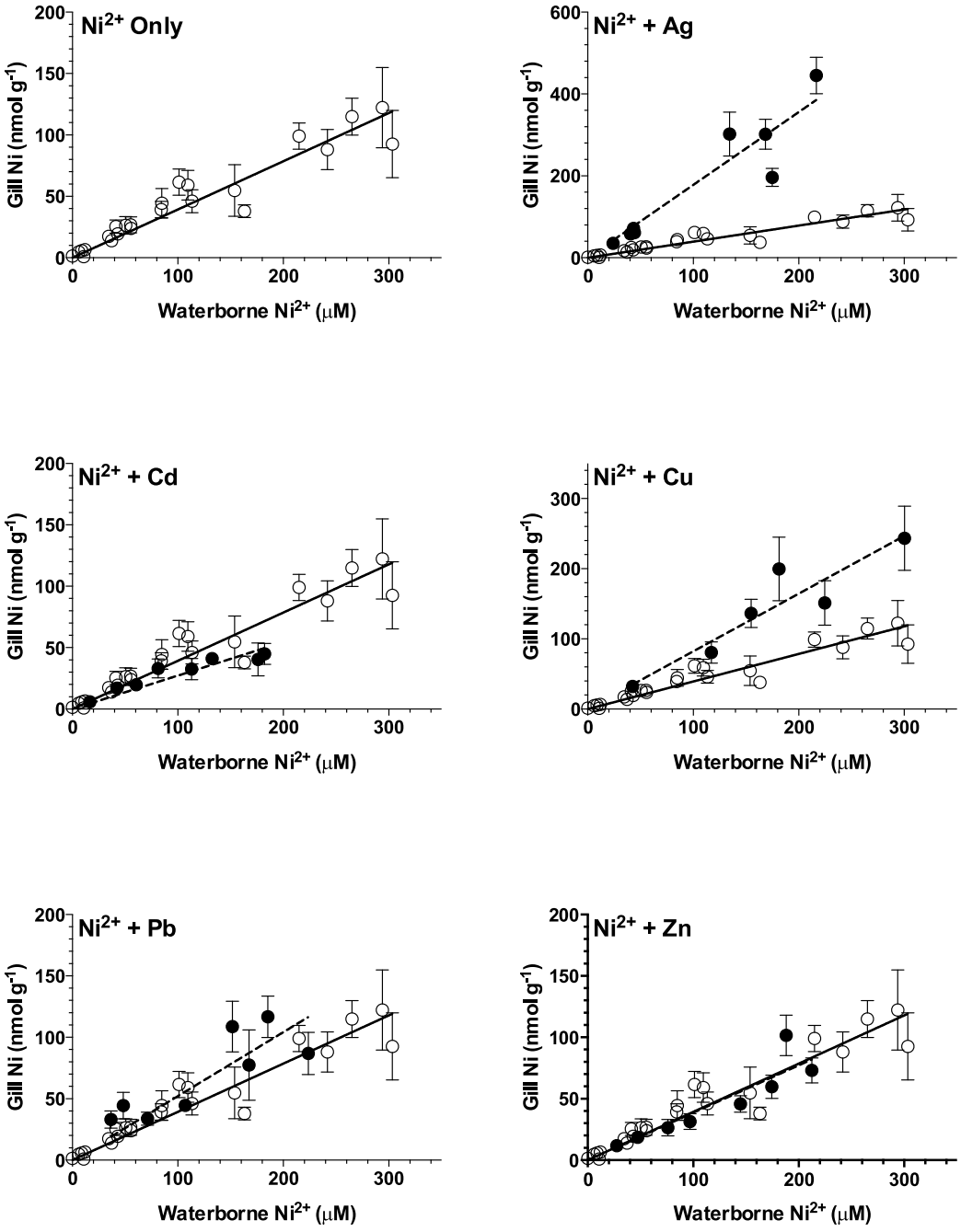

Fig. 3. Effect of Ag (0.11 μM), Cd (0.18 μM), Cu (1.1 μM), Pb (4.8 μM) and Zn (17 μM) (as secondary metals) on the uptake of Ni^{2+} (as primary metal) at the rainbow trout gill. The open symbols represent Ni^{2+} only exposures and closed symbols represent Ni^{2+} + secondary metal exposures. A linear model was fitted to the data. Model parameters and statistical test results are presented in Table 3.

secondary metal (equaling or bracketing the LC50) on the 3-h gill uptake of a primary metal presented at a single concentration (the LC50). While this approach detected both inhibitions and stimulations of metal uptake, it provided no information as to whether the effect was competitive or non-competitive. Furthermore, the number of binary combinations tested by Niyogi et al. (2015) was far less extensive. In the current study, we examined the effect of a single concentration (the 96-h LC50) of the "cold" secondary metal on the 3-h gill uptake of the primary metal (radiolabeled) over a range of concentrations that bracketed the 96-h LC50. The current approach therefore characterizes the concentration-dependent uptake kinetics of the primary metal (e.g., linear or Michaelis-Menten), potentially providing K_m and B_{max} values and how they change in the presence of the secondary metal (e.g., competitive or non-competitive interactions). This information will be of direct use in constructing an mBLM.

Considering that Ag and Cu are recognized as Na^+ antagonists, while Cd, Pb, and Zn are known Ca^{2+} antagonists, and Ni is a respiratory toxicant (see Introduction), we hypothesized that Ag and Cu would competitively inhibit uptake of each other while Cd, Pb, Ni, and Zn would not interact with Ag, Cu, or Ni uptake in any of the binary metal combinations tested.

4.2. Silver

In the absence of other metals, we estimated an affinity constant (K_m) of 96 nM Ag^+. This equates to a log K of 7.0 ($K = 1/K_m$), which is 1000-fold lower than the log K (10.0) originally estimated for Ag^+ (Janes and Playle, 1995), and ~3.5-fold lower than the log K (7.6) derived by McGeer et al. (2000) for the rainbow trout BLM. The log K estimated by Janes and Playle was based on a slightly different experimental protocol. Importantly, they used cold Ag to measure uptake and gills were rinsed with deionized water at the end of the exposure. This may have resulted in a significant over-estimation of Ag uptake as Ag loosely bound to the gill and mucus would have been measured using this method. The log K value derived by McGeer et al. is based on a toxicity rather than accumulation, so is not directly comparable.

Consistent with the premise that both Ag^+ and Cu^{2+} and taken up via Na^+ uptake pathways, Ag^+ uptake was strongly inhibited by Cu (Fig. 1). These results are inconsistent with our previous study (Niyogi et al., 2015) that found Cu had no significant effect on Ag^+ uptake in rainbow trout. The reason for this discrepancy is unclear, but results from the current study have more support than the single result from Niyogi et al. (2015), given that our observations were consistent across all 8 treatments evaluated (Fig. 1). Both metals can be taken up by an apical Na^+ channel in rainbow trout (Bury and Wood, 1999; Grosell and Wood, 2002) and so a competitive interaction might be expected. However, it is difficult to discern the nature of this inhibition, as Ag^+ uptake across the range of waterborne Ag^+ concentrations became linear in the presence of Cu. There is clearly a large non-competitive component to this inhibition as evidenced by the substantial reduction in B_{max} (Fig. 1), but it is not possible to rule out a competitive component as well. The potential mechanism underlying this inhibition is discussed later (Section 4.3) in the context of contradictory results for the effects of Ag on Cu^{2+} uptake.

Also consistent with our hypothesis was the lack of effect of Cd (Fig. 1) and Ni (Fig. 1) on Ag^+ uptake, given their different routes of uptake and different mechanisms of action. However, unexpectedly the two other Ca^{2+} antagonists, Pb and Zn, both affected Ag^+ uptake with Pb strongly inhibiting (Fig. 1) and Zn moderately stimulating uptake (Fig. 1).

While the AICc analysis indicates Pb had a significant effect on Ag^+ uptake, it did not provide support for competitive or non-competive inhibition (Table 1). However, visual inspection of the Pb experiment (Fig. 1) suggests at least a non-competitive component to inhibition of Ag^+ uptake. That the inhibition is non-competitive is consistent with Pb^{2+} being taken up via Ca^{2+} channels (Rogers and Wood, 2004) while Ag^+ is taken up via Na^+ channels. While Pb is typically considered a Ca^{2+} antagonist, at acutely toxic concentrations (4.8 μM) Pb also non-competitively inhibits Na^+ uptake in rainbow trout (Rogers et al., 2005, 2003). At these concentrations, Pb also inhibits carbonic anhydrase (CA) (Rogers et al., 2005), which generates H^+ by facilitating CO_2 hydration. These H^+ can then be used by the H^+-ATPase to generate an electrochemical gradient for Na^+ uptake via the Na^+ channel. Ag^+ uptake is sensitive to bafilomycin (H^+-ATPase inhibitor), suggesting that like Na^+, Ag^+ requires the electrochemical gradient generated by H^+-ATPase to pass through the apical Na^+ channel (Bury and Wood, 1999). Given that Ag^+ also potently inhibits CA activity (Morgan et al., 2004), the combined effects of Ag^+ and Pb^{2+} on CA activity in this experiment may explain the non-competitive inhibition of Ag^+ uptake.

The modest stimulation of Ag^+ uptake by Zn was also unexpected. One possible explanation for this observation is again related to the apical Na^+ channel. Recently, it was postulated that this Na^+ channel may be an acid sensing ion channel (ASIC) (Dymowska et al., 2014). In particular, ASIC4 has been demonstrated to be involved in Na^+ uptake in rainbow trout while ASIC4.2 is similarly involved in zebrafish (*Danio rerio*) (Dymowska et al., 2015, 2014). Most ASICs are H^+-gated ion channels, although ASIC4 is not H^+-gated in mammals (Baron et al., 2001). In zebrafish, ASIC4.1 is H^+-gated, while ASIC4.2 is gated by a currently unknown mechanism (Chen et al., 2007). In mammalian systems, Zn^{2+} potentiates activation of the H^+-gated ASIC2a, but inhibits activation of ASIC1a and ASIC3 (Baron et al., 2001; Lingueglia and Lazdunski, 2013). The effects of Zn^{2+} on ASIC4 have not been studied in any system, but Zn^{2+} potentiation of this channel could explain enhanced Ag^+ uptake in the presence of Zn.

4.3. Copper

In the absence of other metals we estimated a K_m of 16–28 nM Cu^{2+}, which equates to a log K of 7.6-7.8. These values are in reasonable agreement with the original log K of 7.4 developed for the BLM (Di Toro et al., 2001) for fish based on studies on fathead minnows (Playle et al., 1993). In contrast to Ag and Ni, there was considerable temporal variability in Cu B_{max} in the absence of other metals. To ensure that comparisons between Cu only and Cu + secondary metal experiments were valid, we performed side-by-side Cu only experiments with every secondary metal experiment for Cu. However, this does not obviate the fact that Cu B_{max} is quite variable, perhaps reflecting dynamically changing requirements of this essential metal in rapidly growing juvenile rainbow trout. How best to take this variability into account when parameterizing a mBLM or even single metal Cu BLM will require further evaluation.

Given the potential common uptake pathway for Cu^{2+} and Ag^+, we expected Ag would competitively inhibit Cu^{2+} uptake. While an error in test solution spiking prevented us from fully characterizing the effect of Ag on Cu^{2+} uptake, the available data indicate there is no interaction over the linear range of the uptake curve, strongly suggesting there is no interaction (Fig. 2). This is interesting given how strongly Cu inhibited Ag^+ uptake (Fig. 1) and differs from previous observations by Niyogi et al. (2015) that found Ag actually stimulated Cu^{2+} uptake in rainbow trout at the same concentration used in this study. A more detailed consideration of Ag^+ and Cu^{2+} uptake pathways may explain this discrepancy.

Copper uptake in rainbow trout occurs via Na^+-sensitive and Na^+-insensitive pathways (Grosell and Wood, 2002). The Na^+-sensitive pathway is the same Na^+ channel involved in Ag^+ uptake described above. The Na IC50 for the Na^+-sensitive pathway is 103 μM, so that at Na^+ concentrations present in the current experiments (600 μM), Cu^{2+} uptake is almost exclusively via the Na^+-insensitive pathway. This is in contrast to Ag^+, which is much less Na^+ sensitive, with only ~50% reduction in both affinity and uptake rate at 2.3 mM Na^+ and does not appear to move through some other pathway under high Na^+ conditions (Bury and Wood, 1999). The identity of the Na^+-insensitive pathway for Cu is not known, but is likely either DMT1 or Ctr1 (Grosell and Wood, 2002). DMT1 transports numerous metals but not Ag^+, while Cu^+-mediated uptake by Ctr1 is strongly competitively inhibited by Ag^+ in mammalian systems (Lee et al., 2002a, 2002b). The current results where Ag does not inhibit Cu^{2+} uptake suggests Cu^{2+} transport is via DMT1 rather than Ctr1 under these experimental conditions. This may not be the case in very low Na^+ waters which both metals may be taken up via a Na^+ channel, which will be a complicating factor for mBLM development.

So why does Cu inhibit Ag^+ uptake (Fig. 1) if these metals are using different transport proteins under our experimental conditions? We suggest this apparent non-competitive inhibition is again the result of compounding inhibition of CA by Ag^+ and Cu^{2+} (Zimmer et al., 2012) resulting in the reduction of intracellular H^+,

effectively inhibiting the H^+-ATPase that drives the electrochemical gradient for the Na^+-channel.

Both Ni and Pb (Fig. 2) inhibited Cu^{2+} uptake, results consistent with previous observations in zebrafish (Komjarova and Blust, 2009). In our study, this inhibition was apparently non-competitive although only Ni had a high AICc probability of a lower B_{max}. Inhibition of Cu^{2+} uptake by Pb (Fig. 2) is consistent with Pb inhibition for Ag^+ uptake (Fig. 1), suggesting a common mechanism of action. However, given that Cu^{2+} and Ag^+ are likely being transported by different proteins under our experimental conditions, this seems unlikely. Like Cu^{2+}, Pb^{2+} can be taken up by DMT1 (Cooper et al., 2007), but we would have expected a competitive rather than non-competitive inhibition if this was the mechanism of action. The inhibition by Ni is inconsistent with observations for Ag^+. While the observed result had a high AICc probability, given the truncated nature of the data characterizing Cu^{2+} uptake in the presence of Ni (Fig. 2), we suggest these results be treated with caution and recommend additional testing be performed before any conclusions are drawn regarding this interaction.

Stimulation of Cu^{2+} uptake was observed in the presence of Cd and Zn (Table 2, Fig. 2). Results for Cd are inconsistent with previous studies in rainbow trout and zebrafish that found Cd inhibited Cu^{2+} uptake in trout (Niyogi et al., 2015) and zebrafish (Komjarova and Blust, 2009), or had no effect in zebrafish (Komjarova and Bury, 2014). Indeed, we would have expected a competitive inhibition or no interaction if Cu was taken up by DMT1 or Ctr1, respectively. Stimulation of Cu^{2+} uptake by Zn (Fig. 2) is consistent with Zn-stimulated Ag^+ uptake (Fig. 1). This again suggests a common mechanism of action, but as previously discussed, Ag^+ and Cu^{2+} are likely being taken up by different pathways. Zn^{2+} is also taken up by DMT1 (Cooper et al., 2007), which should have resulted in a competitive inhibition, rather than stimulation of Cu^{2+} uptake. Collectively, these data suggest that in addition to DMT1 and/or Ctr1, Cu may be taken up by another mechanism in rainbow trout.

4.4. Nickel

Unlike Ag^+ and Cu^{2+}, Ni^{2+} uptake did not exhibit Michaelis-Menten saturation kinetics with uptake being linear across the range of concentrations tested (0.2–303 μM Ni^{2+}) (Fig. 3). To the best of our knowledge, the concentration-dependent kinetics of Ni uptake have not been previously characterized in fish. In invertebrates, Ni uptake in the pond snail *Lymnaea stagnalis*, was saturable at ~0.2 μM dissolved Ni, with a linear uptake component at higher concentrations (Niyogi et al., 2014), although Leonard and Wood (2013) demonstrated this linear component may also saturate at much higher concentrations (>10 μM). Saturable uptake kinetics have also been demonstrated in *Daphnia pulex*, *Lumbriculus variegatus*, and *Chironomus riparius* at very high dissolved Ni concentrations with saturation reached at ~50–5000 μM dissolved Ni (Leonard and Wood, 2013). Based on these data, it may be that the linear uptake observed in this study is just the linear component of a low affinity high capacity uptake system that does not saturate until concentrations above those that cause acute toxicity to rainbow trout.

Regardless, the observed results are difficult to quantify in terms of Ni uptake kinetics within the framework of a BLM. All of the metals except Zn had effects on Ni uptake, with Cd being inhibitory and Ag, Cu, and Pb being stimulatory (Fig. 3). These observations are somewhat similar to those in zebrafish where Cd was inhibitory, Cu had no effect, and Pb was stimulatory (Komjarova and Blust, 2009; Komjarova and Bury, 2014). Given the presumed similar uptake pathways for Cd, Pb, and Zn, it is interesting that these three metals all had different effects on Ni^{2+} uptake (Fig. 3). Perhaps most interesting is the robust stimulation of Ni^{2+} uptake by both Ag and Cu (Fig. 3).

The mechanism(s) of Ni^{2+} uptake in aquatic organisms is currently unknown. There have been no studies on fish investigating the mechanisms of Ni^{2+} uptake. Using pharmacological inhibitors, Niyogi et al. (2014) concluded that Ni^{2+} is not taken up via voltage-dependent Ca^{2+} channels, voltage-independent Ca^{2+} channels, or Ca^{2+}/H^+ exchangers in *L. stagnalis*. The linear nature of Ni uptake in fish has also led to the hypothesis that Ni^{2+} might be taken up via paracellular (i.e., through tight junctions) rather than transcellular pathways (T. Blewett, personal communication). This might provide an explanation for the substantial increase in Ni^{2+} uptake when fish were exposed to Cu, as Cu has been shown to increase paracellular permeability in rainbow trout gills (Jonsson et al., 2006) and other epithelia such as the mammalian intestine (Ferruzza et al., 2002). Ultimately though, until our understanding of the mechanisms by which Ni^{2+} is taken up in fish is improved, it is difficult to formulate a working hypothesis that might explain our observations regarding the effects of other metals on Ni^{2+} uptake.

4.5. 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 Ag, Cu, and Ni at the gill of rainbow trout. Considering our current understanding of the mechanisms underlying metal uptake (see Introduction), we hypothesized that Ag and Cu would competitively inhibit uptake of the other metal, while Cd, Pb, Ni, and Zn would exert no interactions on Ag, Cu, or Ni uptake in any of the binary metal combinations tested. This hypothesis most generally supports the framework of the mBLM developed by Balistrieri and Mebane (2014).

Our basic hypotheses were rejected in numerous ways. While Cu did inhibit Ag^+ uptake, the mechanism was non-competitive and there was not a reciprocal inhibition of Ag on Cu^{2+} uptake. There was both inhibition (Pb) and stimulation (Zn) of Ag^+ uptake by metals considered Ca^{2+} antagonists. Similarly, there were both stimulatory (Cd, Zn) and inhibitory interactions (Ni, Pb) with Cu^{2+} uptake that were not predicted. While our lack of knowledge of Ni uptake pathways precluded any hypothesis about interactions with other metals, it was surprising that Ag and Cu so significantly stimulated Ni uptake.

Considering all of these interactions in total, we suggest that the mBLM developed by Santore and Ryan (2015) in which each metal has its own site of uptake, may be the most appropriate modeling framework with respect to metal uptake in rainbow trout at concentrations that are acutely toxic. We emphasize that our results are strictly focused on metal uptake and should not be confused with actual toxicity. With respect to toxicity, there may be fewer target proteins leading to a model in which, despite there being independent uptake pathways for some metals, there are common mechanisms of action (e.g., inhibition of CA). For example, Niyogi et al. (2015) demonstrated that, despite likely having independent pathways for uptake, Ag^+ and Cu^{2+} acted in a simple additive manner with respect to inhibiting Na^+ uptake in rainbow trout. Additional mechanistic studies like those of Niyogi et al. (2015) assessing binary metal effects on Na^+ and Ca^{2+} uptake would be useful to understand the linkage between metal uptake and toxicity.

Finally, it is worth noting the multiple incongruent observations between this study and earlier studies on the effects of binary metal mixtures on metal uptake in fish. It could be argued that there are sufficient physiological differences between rainbow trout and zebrafish as well as several methodological differences to explain observed differences between our study and those of Komjarova and colleagues (Komjarova and Blust, 2009; Komjarova

and Bury, 2014). There were even some differences in binary metal interactions from those reported in the Niyogi et al. (2015) study performed in the same laboratory, with the same water supply and source of fish as the current study. However, as pointed out earlier, methodology differed between the two studies, and the current approach is more powerful. Additional experiments to understand the source(s) of this variability are clearly needed before data can be used in the development of a mBLM. It will also be important to extend these approaches to binary metal effects on the uptake of Zn^{2+}, Pb^{2+}, and Cd^{2+} (all thought to move through the Ca^{2+} uptake pathway) as primary metals.

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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.2016.10.008.

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