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Chronic Toxicity of Binary Mixtures of Six Metals (Ag, Cd, Cu, Ni, Pb, and Zn) to the Great Pond Snail *Lymnaea stagnalis*

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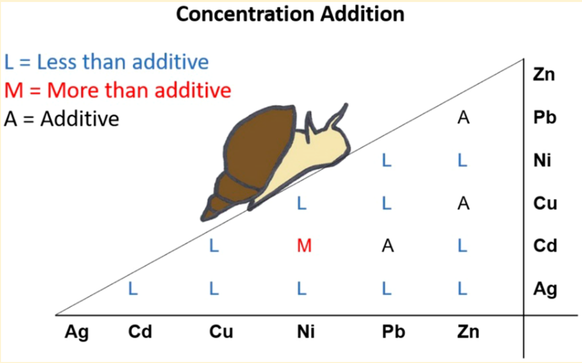
Supporting Information

ABSTRACT: Although metal-mixture toxicity has recently received increasing attention, there is still insufficient knowledge on joint effects occurring in chronic exposures to relatively low metal concentrations. We characterized the chronic toxicity of binary mixtures of six metals (Ag, Cd, Cu, Ni, Pb, and Zn) in 14 day growth tests with juveniles of the metal-sensitive freshwater snail *Lymnaea stagnalis*. Observations were compared with predictions from individual metals and from the two most frequently used mixture models: concentration addition (CA) and independent action (IA). Predictions based on measured total dissolved concentrations and on calculated free-ion activities did not differ greatly because multimetal geochemical interactions in the tests were limited. In around half of the tests, mixture toxicity was higher than the greatest effect caused by the individual metals, arguing in favor of considering joint effects. When the additive models were used, the great majority of interactions were either additive or less than additive (i.e., antagonism). In general, the IA model was the most accurate, while the CA model was the most conservative. Along with other studies, these findings suggest that, at least for binary combinations, the simple CA model may provide satisfactory protection from the chronic metal toxicity of metal mixtures to aquatic organisms.



INTRODUCTION

While metals are almost invariably found in mixtures in the environment, they are generally regulated on a metal-by-metal basis. Incorporating metal mixtures into regulatory frameworks has been an important focus over the past decade.[1−3] A pair of conceptual models are most commonly used to evaluate mixture toxicity on the basis of single-substance toxicity data: the concentration addition (CA) model and the independent action (IA) model. The CA model is applied to chemicals with similar modes of action (MoA), so that substances only differ in their potency and can be considered as dilutions of one another. Conversely, the IA model (also called the response addition model) is applied to chemicals with dissimilar MoA so that the probabilities of response to the chemicals are independent. These two "additive" models rely on the common assumption that chemicals in the mixture do not physically, chemically, and biologically interact. However, this condition is not always fulfilled because many substances have been shown to interact in either a synergistic ("more than additive") or an antagonistic ("less than additive") manner by, respectively, enhancing or lessening each other's toxicity. In the case of metal mixtures, no clear pattern has emerged so far because all potential outcomes have been observed, with interactions seemingly being metal-, organism-, and concentration-specific.[4−6] The inherent variability associated with toxicity testing may partially explain this unclear picture because patterns have also been shown to vary across studies and even within the same study.[7−9] Nevertheless, the two meta-analyses from Norwood et al.[5] and Vijver et al.[6] showed that, for around 70% of the cases, the additivity assumption produces a conservative prediction of toxicity (i.e., toxicity is either correctly predicted or over-predicted).[6,10] However, most of the examined studies address short-term acute toxicity at relatively high metal concentrations. In contrast, long-term chronic studies, performed at lower and more environmentally realistic concentrations, remain insufficient to draw conclusions on the validity of additive models.[3,11,12]

The present study aims at addressing the data gap on metal mixture chronic toxicity to aquatic organisms. Chronic toxicity of binary mixtures of six trace metals (Ag, Cd, Cu, Ni, Pb, and Zn) were assessed with the great pond snail, *Lymnaea stagnalis*. Recently, this freshwater pulmonate snail has been shown to be either the most-sensitive or the second-most-sensitive freshwater organism in chronic exposures to Co, Cu, Ni, and Pb, making it particularly relevant for the development of future water quality criteria.[13−17] These studies showed that juvenile-snail growth inhibition was a particularly sensitive end-point, potentially occurring via the disruption of Ca homeostasis.[14] Indeed, *L.*

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stagnalis has a high Ca requirement to sustain the rapid shell formation and very large growth rates observed during its early life stages (around 20% of body mass per day). Yet, it remains elusive whether this reported effect constitutes the primary MoA or if it is rather a secondary effect of chronic metal exposures.[16,19] In the face of these poorly understood mechanisms of metal chronic toxicity to *L. stagnalis*, the choice of applying CA or IA for mixture toxicity prediction based on a presumed MoA is therefore challenging.

The main objective of this study was to evaluate the performance of both additive models (CA and IA) at predicting 14 day growth inhibition in juvenile *L. stagnalis* exposed to Ag, Cd, Cu, Ni, Pb, and Zn in binary mixtures (15 combinations). So far, chronic studies with metals and *L. stagnalis* typically employed exposure times of ≥21 days, with growth and reproduction as end points.[13−16,18−20] However, recent chronic studies[19,21] have demonstrated that 14 day growth reliably predicts full life-cycle toxicity effects in snails for at least some metals. In the present work, deviations from additive models were quantified and interpreted as multimetal interactions with the MixTox statistical framework developed by Jonker et al.[22] According to now widely accepted bioavailability concepts such as the biotic ligand model (BLM), metal toxicity is better predicted by the free-ion activity rather than by the total metal dissolved concentration.[23] Hence, toxicity predictions were performed based on both measured total dissolved concentrations and estimated free-ion activities.

■ MATERIALS AND METHODS

Experimental Animals and Water Composition. Snails were obtained from an in-house culture at McMaster University (Hamilton, ON). They were kept at the University of British Columbia at 25 ± 1 °C, under a 16 h light/8 h dark photoperiod in Vancouver dechlorinated tap water amended with salts. These salt additions were intended to increase the water hardness of the very soft Vancouver water (from 3.3 to 120 $mg \cdot L^{-1}$ as $CaCO_3$) to promote healthier snail culture conditions. Additionally, for comparative purposes, we aimed at mimicking the water ionic composition of the University of Miami, where many of the metal chronic toxicity studies with *L. stagnalis* have been conducted.[14,16,17,19] The final water composition for this study was (as mean ± SD, $n = 252$): pH = 7.81 ± 0.20, [Ca] = 0.94 ± 0.01 mM, [Mg] = 0.22 ± 0.02 mM, [Na] = 1.7 ± 0.2 mM, [K] = 0.054 ± 0.015 mM, [Cl] = 1.0 mM (nominal), [SO_4] = 0.79 mM (nominal), [dissolved organic carbon, DOC] = 0.76 ± 0.08 $mg \cdot L^{-1}$, and alkalinity = 0.80 ± 0.05 $mEq \cdot L^{-1}$ (measured as [dissolved inorganic carbon, DIC]). The snail culture was maintained under static renewal conditions and was fed a mix of thoroughly washed peeled sweet potato (*Ipomoea batatas*) and romaine lettuce (*Lactuca sativa*).

Experimental Design. For each binary mixture, toxicity test concentrations were selected according to a fixed-ratio ray design, known to efficiently cover response surfaces.[24] An example of this experimental design is given for the Ag/Cd mixture in Figure SI.1. Briefly, data were collected along five binary fixed-ratio rays composed of the metals alone (1:0 and 0:1) and in mixtures (1:1, 3:1, and 1:3). Along each ray, a range of concentrations was tested for each metal, with a maximum concentration fixed at 2.5 times (metal alone) or 1.7 times (metal in mixtures) its individual 50% effect concentration (EC50, assessed in preliminary single metal toxicity tests with 13 treatments). These maximum concentrations were set to prevent obtaining many treatments with 100% effects. Overall with this design, 7 treatments per individual metal and 15 binary mixture treatments were simultaneously tested for each binary toxicity test. For example, for the Ag/Cd mixture, we tested 7 treatments of Ag alone, 7 treatments of Cd alone, and 15 Ag/Cd treatments (Figure SI.1). The simultaneous testing of single metals and their mixtures eliminates between-test variability as a factor that can lead to erroneous interpretations of mixture interactions.[7] Finally, because the toxicity data analysis was regression-based, treatments had only one replicate to maximize their numbers. This allows better coverage of the response curves and surfaces and, hence, increased reliability and power in the analysis.[22] Controls, however, were replicated six to eight times to assess variation within the toxicity tests.[25]

Exposure solutions were prepared by spiking stock solutions of the different metal salts ($AgNO_3$, $CdCl_2 \cdot 5H_2O$, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, $Pb(NO_3)_2$, $Zn(NO_3)_2 \cdot 6H_2O$, ACS grade, Fisher Scientific) in 2 L of the same water used for snail culture. Water was kept in polypropylene containers and was constantly aerated via a tubing system. Tests were performed at 25 ± 1 °C under a 16 h light/8 h dark photoperiod. Metal addition was performed 24 h prior to the beginning of tests to allow sufficient time for thermodynamic equilibration. At the beginning of each test, 5 snails of approximately 3 weeks age were added in bulk to each exposure container. The average snail wet weight was 7.5 mg ±0.2 mg (min = 5 mg and max = 10 mg) in each treatment. Every day, exposure water was renewed, snails were fed ad libitum with fresh and thoroughly washed romaine lettuce, and mortality was recorded. Note that these tests conditions (e.g., the snail density in the test solutions) were partly selected so that the water chemistry was kept as constant as possible over the duration of the tests to minimize the influence of abiotic factors (e.g., pH and DOC and Ca concentrations) on metal toxicity.[23] Water samples were filtered through a 0.45 μm poly(ether sulfone) (PES) membrane (Membrane Solution, Dallas, TX) on days 1, 7 and 14, prior to and after the water change, to measure pH and trace metals, Ca, Na, Mg, K, DOC, and DIC concentrations. However, while concentration analyses of metals were performed on all samples, the other physicochemical analyses were carried out on only 3 random samples at each collection time (resulting in $n = 252$ in total, for these latter analyses). Tests were terminated after 14 days, and snail wet weights were measured to the nearest 0.1 mg.

Water Analyses and Speciation Modeling. Inorganic elements were analyzed by atomic absorption spectrometry (AA240 FS, Varian) with a graphite furnace for Ag, Cd, Cu, Pb, and Ni and with a flame for Zn, Ca, Mg, K, and Na. The DOC and DIC concentrations were measured with a total organic carbon analyzer (V-series TOC analyzer, Shimadzu). Instrument calibrations were verified with certified reference waters TMDA-54.5 and TM-25.4 from Natural Resources Canada.

Measured water physico-chemistry was entered into the Windermere Humic Aqueous Model (WHAM, version VII) to calculate metal speciation in the different exposure solutions. The software's default thermodynamic database was unchanged, except for the metal carbonate constants, which were replaced by the NIST recommended values (Table SI.1). Metal binding to dissolved organic matter (DOM) was modeled with the assumptions that DOM is composed of 50% carbon (i.e. DOC) by weight[26] and corresponds to 65% chemically "active" fulvic acid.[27] These assumptions have been shown to provide good agreements between observed and WHAM-predicted metal speciation.[28,29]

Data Analyses. As previously stated, metal toxicity is usually assumed to be directly proportional to free-ion activity for a given pH and ionic composition.[23] Hence, data analyses were performed based on both measured total dissolved and calculated (as described in the above section) free-ion activities. This allowed multimetal geochemical interactions in the exposure water to be taken into account (e.g., changes in free metal concentration by multimetal competition for binding to DOM).

Juvenile *L. stagnalis* grow exponentially with time so that, for each treatment, the mean specific growth rate of the snails (SGR, in day^{-1}) was calculated as:

$$\text{SGR} = \frac{\ln(w_{\text{final}}) - \ln(w_{\text{initial}})}{t} \qquad (1)$$

where w_{initial} and w_{final} are, respectively, the initial and final average snail wet weight and t is the exposure period (i.e., 14 days).

The SGR was then normalized by the SGR of the snails exposed in the controls ($\text{SGR}_{\text{control}}$) to obtain relative growth rate (RGR, in percent):

$$\text{RGR} = \frac{\text{SGR}}{\text{SGR}_{\text{control}}} \times 100 \qquad (2)$$

For each metal M, single concentration−response curves of RGR against metal concentration were fitted with a log−logistic equation using SigmaPlot:

$$y = \frac{\max}{1 + \left(\frac{x_{\text{M}}}{\text{EC50}_{\text{M}}}\right)^{\beta_{\text{M}}}} \qquad (3)$$

where y is the RGR, x_{M} is the measured dissolved concentration or calculated free-ion activity of metal M, max is the upper limit (fixed at the RGR of snails in the control, i.e., at 100%), EC50_{M} is the median effective concentration (i.e., the concentration leading to a 50% decrease in RGR), and β_{M} is the slope parameter and describes the steepness of the curve.

For each mixture treatment, the relevance of taking into account mixture toxicity was assessed by comparing the observed mixture effect to the predicted greatest individual effect (GIE) in the mixture. This GIE prediction corresponded to the highest predicted effect from the two metals composing the mixture. The latter effect was calculated with eq 3 and the individual metal parameters estimated from the single metal tests.

The mixture effect between two metals M1 and M2 was then modeled with the CA model (eq 4) and the IA model (eq 5) using the parameter estimates obtained from the single metal data:

$$\frac{x_{\text{M1}}}{\text{EC50}_{\text{M1}} \times \left(\frac{\max - y}{y}\right)^{1/\beta_{\text{M1}}}} + \frac{x_{\text{M2}}}{\text{EC50}_{\text{M2}} \times \left(\frac{\max - y}{y}\right)^{1/\beta_{\text{M2}}}} = 1 \qquad (4)$$

$$y = \max \times \frac{1}{1 + \left(\frac{x_{\text{M1}}}{\text{EC50}_{\text{M1}}}\right)^{\beta_{\text{M1}}}} \times \frac{1}{1 + \left(\frac{x_{\text{M2}}}{\text{EC50}_{\text{M2}}}\right)^{\beta_{\text{M2}}}} \qquad (5)$$

Finally, the binary mixture effect was analyzed using the MixTox model developed in Excel by Jonker et al.[22] First, the above reference models were fitted to both the single metals and the mixture data (whole data set). For this regression, the best-fit parameters previously obtained from the single metal tests were used as initial parameters. Additionally, we set their upper and lower fitting constraints at their previously determined 95% upper and lower confidence limits. Second, statistical deviations from CA and IA models were assessed by fitting the whole data set again, with the synergy and antagonism deviation models noted as CA_S/A and IA_S/A, respectively. These CA_S/A and IA_S/A models, respectively, are composed of the CA and IA models implemented with an extra "deviation parameter" *a*, as described by Jonker et al.[22] A Chi-squared test was then performed to determine whether this additional parameter provided a significantly better fit than the original CA and IA models. When no significant improvement was observed ($p > 0.05$), no interaction or strict additivity was concluded. When a significant improvement was observed ($p < 0.05$), antagonism and less-than-additive toxicity ($a < 0$) or synergism and more-than-additive toxicity ($a > 0$) was concluded. Finally, the goodness of fit of each model was evaluated using the standard error of the regression (SE being the square root of the mean square error (MSE), with MSE being the residual sum of squares divided by the number of degrees of freedom), representing the average difference between the predicted and the observed RGR (i.e., the variations in RGR that are not explained by the model).

RESULTS AND DISCUSSION

Metal Speciation in Toxicity Tests. The variation of the physicochemical parameters (temperature, pH, metal concentrations, DOC concentration, etc.) was <20% across the range of the chronic toxicity tests. The average values are given in the Experimental Animals and Water Composition section.

According to WHAM VII predictions performed in the single metal toxicity tests, the free-ion activity at 50% of toxic effect represented 31%, 55%, 1.3%, 59%, 4.6%, and 53% of the total dissolved metal concentration for Ag, Cd, Cu, Ni, Pb, and Zn, respectively (see Table SI.2 for detailed chemical speciation of each metal). However, the percent of Cu^{2+} and Pb^{2+} increased by 7-fold and 1.4-fold over the range of total Cu and Pb dissolved concentration tested. These increases were the result a progressive saturation of the limited number of DOM binding sites by Cu and Pb, as their dissolved concentrations were increased across the treatments of their toxicity tests.

In the mixture toxicity tests, WHAM VII predicted that Cu^{2+} and, to a lesser extent, Pb^{2+} activities were noticeably increased by the presence of Ni and Zn in solution (results not shown). Indeed, Ni and Zn, present at relatively high concentrations in the mixture toxicity tests, significantly competed with Cu and Pb for binding on the limited number of complexation sites on the DOM. This effect was relatively small for Pb, with an increase of Pb^{2+} activity of only up to 1.4-fold with Zn (and no significant effect from Ni). The geochemical effect was larger for Cu, with Ni and Zn increasing the percent of Cu^{2+} in solution by up to 1.6-fold and 2.4-fold, respectively.

Single-Metal Toxicities. Note that the entire toxicity data set is provided in section 5 of the Supporting Information. For each metal, Figure 1 shows the RGR of *L. stagnalis* as a function of the measured dissolved metal concentrations. These concentration−response curves were obtained by compiling all the single metal toxicity tests performed in this study, at different times over a 2 year period. The number of single metal tests is not the same for each metal because multiple mixtures were sometimes analyzed at a same time. In all of the individual toxicity tests performed, the chronic effect concentration that represented 50% reduced growth rate relative to the controls (EC50) varied by 30% for Ag, by 10% for Cd, by 26% for Cu, by 8.8% for Ni, by 43% for Pb, and by 9.8% for Zn (relative standard

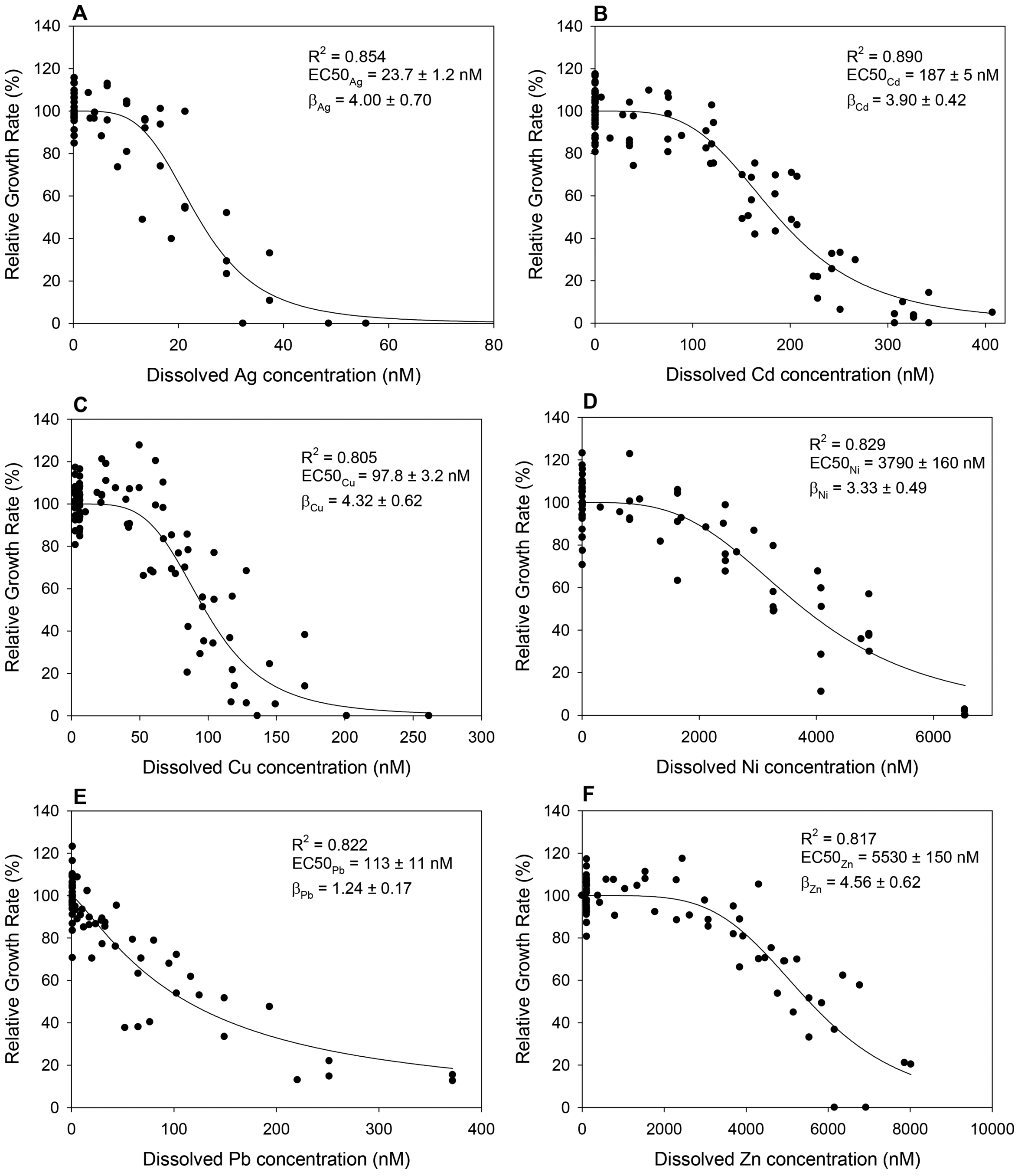

Figure 1. Relative growth rate of juvenile *L. stagnalis* as a function of measured dissolved metal concentration, for (A) Ag, (B) Cd, (C) Cu, (D) Ni, (E) Pb, and (F) Zn. Regression lines with eq 3 are given along with the fitted parameters in each panel. These concentration−response curves were obtained by compiling all of the different single-metal toxicity tests performed in this study (as detailed in the Results and Discussion section). Each point represents the mean response of five snails. Relative growth rates as a function of free ionic activity are given in Figure SI.2.

deviation). For the compiled data, the results of the regressions with eq 3 are given in Figure 1, and the corresponding chronic effect concentrations that represented 14 day EC10, EC20, and EC50 are given in Table 1. The chronic effect concentration of regulatory interest is generally the EC10 for the European Union and the EC20 for the U.S. Environmental Protection Agency. Due to the much lower slope of the Pb concentration−response curves (cf. β values given in Figure 1), the snail sensitivity to Pb compared with the other five metals varied slightly with the effect concentration considered. In other words, with a rather different slope, the degree of parallelism of the Pb concentration−response curve with the other single metal curves is lower, affecting the relative order of the EC values. Indeed, snail sensitivity in the tested water decreased by approximately 230-fold as Ag ≈ Pb > Cu > Cd > Ni > Zn based on EC10 values, as Ag > Pb > Cu > Cd > Ni > Zn based on EC20 values, and as Ag > Pb ≈ Cu > Cd > Ni > Zn based on EC50 values (comparison performed with a *t* test with a significance level of 0.05 and a false discovery rate correction). These observed sensitivities were similar to the sensitivities reported for juvenile *L. stagnalis* in other chronic studies for dissolved Ag (28 day EC50 (biomass) = 5.8 μg L^{-1}, comparable conditions),[30] Cu (30 day EC20 (biomass) = 1.8 μg L^{-1}, similar conditions[16]), Pb (30 day EC20 (biomass) = 3 μg L^{-1}, similar conditions[19]), Cd (31 day

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Table 1. Chronic Effect Concentrations (In Total Dissolved Concentration and in Free-Ion Activity) On the Relative Growth Rate of Juvenile *L. stagnalis* Exposed for 14 Days to Waterborne Ag, Cd, Cu, Ni, Pb, and Zn[a]

		total dissolved concentration			free-ion activity		
		EC10	EC20	EC50	EC10	EC20	EC50
Ag	nM	13.7 [11.1–16.5]	16.8 [14.5–19.0]	23.7 [21.3–26.1]	4.22 [3.42–5.02]	5.17 [4.44–5.89]	7.31 [6.54–8.07]
	$\mu g \cdot L^{-1}$	1.48 [1.20–1.78]	1.81 [1.56–2.05]	2.56 [2.30–2.82]	0.455 [0.367–0.542]	0.558 [0.480–0.636]	0.789 [0.706–0.877]
Cd	nM	107 [92.6–120]	131 [119–142]	187 [177–198]	58.4 [51.0–66.3]	72.2 [65.1–78.4]	103 [97.6–109]
	$\mu g \cdot L^{-1}$	12.0 [10.4–13.4]	14.7 [13.4–16.0]	21.0 [19.9–22.2]	6.57 [5.70–7.43]	8.11 [7.36–8.86}	11.6 [11.0–12.3]
Cu	nM	58.5 [50.5–67.0]	70.7 [63.6–77.8]	97.8 [91.4–104]	0.472 [0.344–0.600]	0.673 [0.545–0.801]	1.24 [1.08–1.39]
	$\mu g \cdot L^{-1}$	3.71 [3.21–4.25]	4.49 [4.04–4.94]	6.21 [5.81–6.61]	0.0300 [0.0219–0.0381]	0.0428 [0.0346–0.0509]	0.0787 [0.0686–0.0883]
Ni	nM	1960 [1590–2330]	2500 [2170–2830]	3790 [3470–4110]	1150 [943–1380]	1470 [1280–1660]	2240 [2050–2430]
	$\mu g \cdot L^{-1}$	115 [93.2–137]	147 [127–166]	222 [204–241]	67.6 [54.7–80.5]	86.5 [75.3–97.6]	131 [120–143]
Pb	nM	19.3 [11.7–26.8]	37.0 [26.9–47.2]	113 [90.6–135]	0.758 [0.421–1.10]	1.54 [1.08–2.00]	5.21 [4.09–6.33]
	$\mu g \cdot L^{-1}$	4.00 [2.43–5.56]	7.67 [5.57–9.77]	23.4 [18.8–28.0]	0.157 [0.0872–0.227]	0.320 [0.225–0.415]	1.08 [0.847–1.31]
Zn	nM	3410 [3010–3840]	4080 [3730–4400]	5530 [5240–5830]	1800 [1580–2020]	2160 [1980–2340]	2950 [2780–3110]
	$\mu g \cdot L^{-1}$	223 [196–250]	266 [244–287]	362 [342–381]	118 [103–132]	141 [129–153]	193 [182–203]

[a] Mean and 95% confidence intervals. These EC values were obtained from the toxicity data presented in Figure 1 and are compilations of multiple single-metal toxicity tests.

EC50 (shell length) ≈ 30 $\mu g\ L^{-1}$, comparable conditions),[31] and Zn (28 day EC10 (growth rate) = 200–1630 $\mu g\ L^{-1}$, various conditions).[18] Hence, the present study confirms that, in chronic exposures, *L. stagnalis* is highly sensitive to Ag, Cu, and Pb and relatively insensitive to Cd and Zn, in comparison with other aquatic organisms. However, the snails in our study did not show a high sensitivity to Ni, in contradiction with other chronic studies, which reported EC values 1 to 2 orders of magnitude lower (21 day EC20 (biomass) < 1.3 $\mu g\ L^{-1}$, similar conditions,[17] 30 day EC20 (biomass) = 1.6–27 $\mu g\ L^{-1}$ various conditions,[15] 28 day EC20 (SGR) = 10–32 $\mu g\ L^{-1}$, various conditions).[20] Note, however, that only the EC values based on SGR are directly comparable with our EC values. Indeed, for exponential growth, biomass gives lower numerical EC estimates than SGR, and these estimates decrease as toxicity test duration increases.[32] A priori, water chemistry does not appear to be the reason for the lower Ni toxicity to *L. stagnalis* observed in our study. Indeed, the lowest Ni EC20 value observed by Brix et al.[17] was obtained in Miami tap water, which was mimicked in the present study (cf. the Experimental Animals and Water Composition section). Further investigations are underway to elucidate the discrepancy with previous studies.

The single metal toxicity data are also presented as a function of the free-ion activity (in Table 1 and Figure SI.2). As a result of the increased proportion of free Cu^{2+} (7-fold) and Pb^{2+} (1.4-fold) with Cu and Pb concentration described in the previous section (cf. Table SI.2), the slope parameters (β) of the concentration–response curves were ~2-fold and ~1.1-fold lower for Cu and Pb, respectively, when expressed as a function of the free ion activities (Figure SI.2) rather than of total dissolved concentrations (Figure 1). This led to snail sensitivity decreasing as Cu ≈ Pb > Ag > Cd > Ni > Zn based on free-ion activity EC10 values and Cu > Pb > Ag > Cd > Ni > Zn based on free-ion activity EC20 and EC50 values.

Binary-Mixture Toxicities. The most important findings of this study are that (i) predictions based on total dissolved concentration and on free-ion activity did not greatly differ, with most differences occurring in mixtures containing Cu; (ii) GIE gave the lowest prediction of toxicity, followed by IA then CA; (iii) the MixTox analyses on CA and IA concluded very few more-than-additive toxicity mixtures (i.e., synergism, under-prediction of toxicity) and mostly strictly additive toxicity (i.e., no interaction, accurate toxicity prediction) or less-than-additive toxicity mixtures (i.e., antagonism, overprediction of toxicity); and (iv) IA was the most-accurate, while CA was the most-conservative and -protective additive model.

For all of the mixture treatments tested, the RGR predictions from the individual metal-by-metal (GIE) and additive (CA and IA) approaches are depicted as a function of RGR observations in Figure 2 based on the total dissolved concentrations and in Figure SI.3 based on the free-ion activities. The model under-predicts the mixture toxicity (i.e., over-predicts RGR) for data points above the 1:1 line, and conversely, the model over-predicts the mixture toxicity (i.e., under-predicts RGR) for data points below the 1:1 line. Data points on the 1:1 line correspond to a perfect prediction by the model. The dashed lines in these figures, representing an absolute error on RGR of 20%, are provided to better assess the extent of the deviation from the model prediction (i.e., the distance of the data points from the 1:1 line) and do not correspond to any kind of acceptability criteria.

The predictions in Figure 2 (based on total dissolved concentrations) and Figure SI.3 (based on ion activities) were made from the individual metal toxicity tests parameters given in Table SI.3 (based on total dissolved concentrations) and Table SI.4 (based on free-ion activities). For each individual metal mixture, Table 2 provides the standard error of the regression (SE, indicating the average distance that the observed RGR fall from the regression line) for the GIE, CA, and IA models and the type of interaction (no interaction = additive, antagonism = less than additive or synergism = more than additive) concluded for the CA and IA models by the MixTox analyses, based on total dissolved concentration and on free-ion activity (given in brackets when different). Details of the MixTox statistical analyses are given in Tables SI.5, SI.6, SI.7, and SI.8.

The following sections describe and discuss these different observations in more detail, including some additional discussion given on their potential biological meaning and implications for the environmental regulation of chronic metal mixture toxicity.

Comparison of Predictions Based on Total Dissolved Concentration and Free-Ion Activity. In our study, geochemical effects were limited and focused on mixtures with Cu and Pb, as detailed previously. The shallower slopes of Cu^{2+} and (to a much lesser extent) Pb^{2+} activity–response curves led to slightly closer CA and IA toxicity predictions than when based on total dissolved concentration (Table 2 versus Table SI.3). Indeed, the

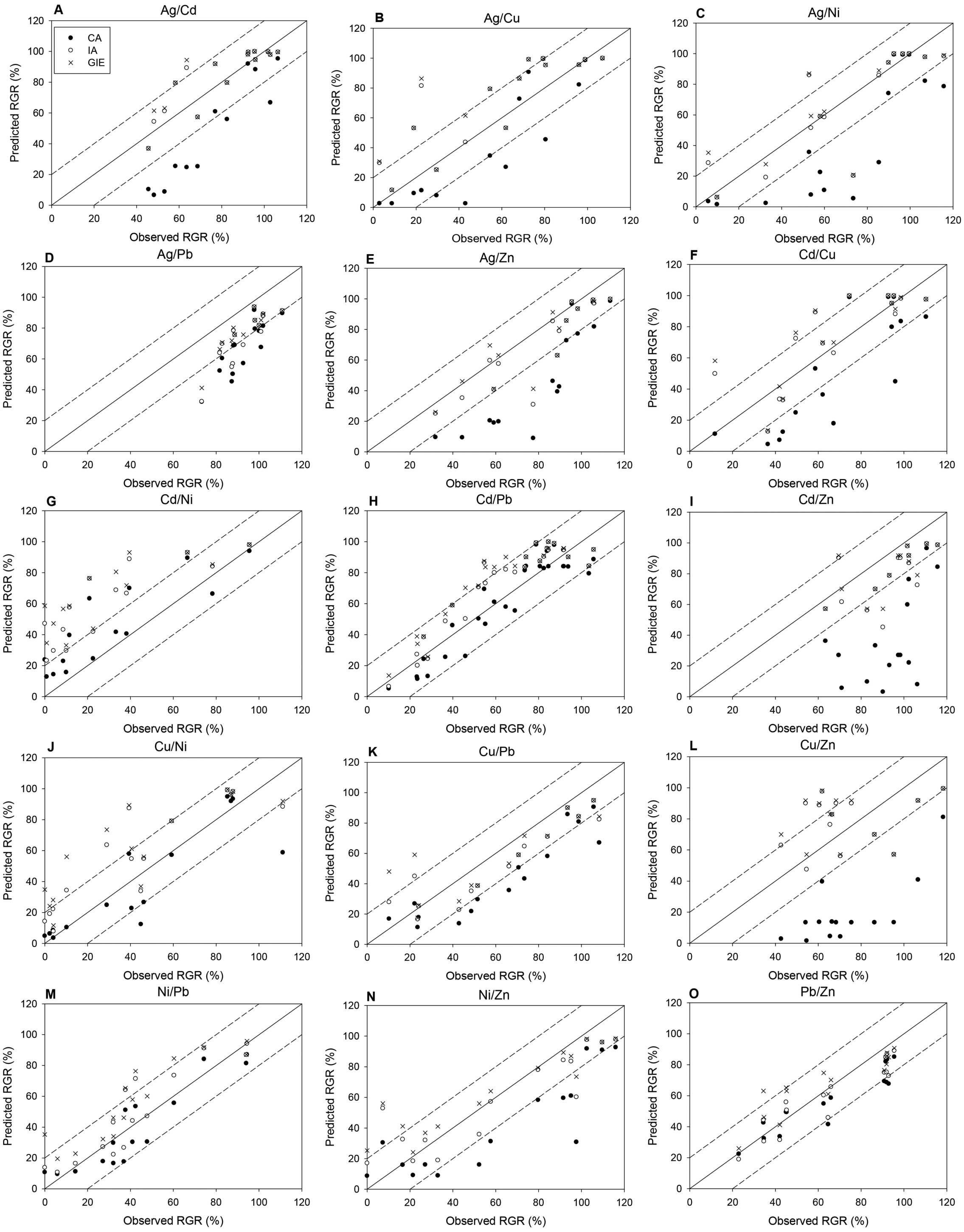

Figure 2. Predicted vs measured relative growth rates of juvenile *L. stagnalis* for the 15 binary combination of Ag, Cd, Cu, Ni, Pb, and Zn: (A) Ag/Cd, (B) Ag/Cu, (C) Ag/Ni, (D) Ag/Pb, (E) Ag/Zn, (F) Cd/Cu, (G) Cd/Ni, (H) Cd/Pb, (I) Cd/Zn, (J) Cu/Ni, (K) Cu/Pb, (L) Cu/Zn, (M) Ni/Pb, (N) Ni/Zn, and (O) Pb/Zn. Predictions are from the greatest individual effect (eq 3, shown by x), concentration addition (eq 4, shown by ●), and independent action (eq 5, shown by ○) approaches, based on measured total dissolved concentration and the individual metal toxicity parameters given in Table SI.3. The full line corresponds to the perfect fit (i.e., strict additivity) and the points above this line represent more-than-additive toxicity, while the points below this line represent less-than-additive toxicity. The dashed lines represent an absolute difference of 20% between the observed and the predicted RGR and are drawn for comparison only. The predictions based on free ionic activity are given in Figure SI.3.

difference between CA and IA predictions is greatly dependent on the steepness of the single toxicant concentration−response curves.[33] In the CA concept in which toxicants can theoretically replace one another, steep concentration−response curves imply that small concentration increments will yield large response increments. Hence, in the CA model, combined effects can be expected even at individual concentrations at which no effects are observed but not in the IA model. The activities of Cu^{2+} and Pb^{2+} were also affected (to a much lesser extent) by Ni and Zn. Overall, these geochemical effects had some influence on the

Table 2. Standard Error of the Regression from the GIE (eq 3), CA (eq 4), and IA (eq 5) Calculations and the Type of Interaction Concluded from the MixTox Analysis for the CA and IA Models (Details Given in Tables SI.5−8)[a]

	standard error of the regression (%)			MixTox conclusion on mixture toxicity[b]	
mixture	GIE	CA	IA	CA	IA
Ag/Cd	12	28	9.1	L	A
Ag/Cu	24	20 (15)	23	L (A)	A
Ag/Ni	19	34	18	L	A
Ag/Pb	16	27	21	L	L
Ag/Zn	14	36	16	L	L
Cd/Cu	19	27 (18)	18 (16)	L	A
Cd/Ni	46	30	34	M	M
Cd/Pb	16	11	14	A	M (A)
Cd/Zn	17	62	20	L	L
Cu/Ni	27 (22)	18	21 (18)	L (A)	M (A)
Cu/Pb	18 (23)	22 (26)	15 (23)	L	L
Cu/Zn	25 (23)	57 (60)	20 (28)	L	A (L)
Ni/Pb	18	11	13	A	A
Ni/Zn	18	28	19	L	A
Pb/Zn	13	13 (15)	11	A (L)	A

[a] Results are given based on measured total dissolved concentrations and, in brackets, based on free-ion activities calculated with WHAM VII when there is a difference ($\Delta SE > 1\%$ or different interaction type). [b] A = additive, L = less than additive, M = more than additive.

conclusions of the joint effects analyses (Table 2 and Tables SI.5−8). When based on free ion activities rather than total dissolved concentrations, the CA analysis of Pb/Zn changed from no interaction to a slightly significant antagonism (i.e., the model predicted greater toxicity than observed), and the IA analysis of Cd/Pb changed from a slightly significant synergism to no interaction. For Cu, these effects led to concluding the same type of interaction for both models for Ag/Cu (no interaction), Cu/Ni (no interaction), and Cu/Zn (antagonism) when analyses were based on free ion activities. More precisely, free-ion activity based analyses resulted in decreased CA-predicted toxicity of Ag/Cu and Cu/Ni, decreased IA-predicted toxicity of Cu/Zn, and increased IA-predicted Cu/Ni toxicity. Despite these changes in additivity, these geochemical effects on Cu and Pb had overall very little impacts on the accuracy (SE) of the different models.

Comparison of Model Performance. The mean SE for the 15 binary metal mixtures tested was 28% for CA, 18% for IA, and 20% for GIE (based on both total dissolved concentrations and free ion activities). These SE values suggest that the IA and GIE approaches had a similar accuracy, which was higher than the CA approach. These relative performances from the CA, IA, and GIE models did not change whether the analysis was based on total dissolved concentrations or free-ion activities. In this section, each model performance is primarily described based on total dissolved concentration, with a secondary description based on free-ion activity given in brackets when different by more than 1%.

The GIE approach logically provided the smallest estimates of mixture toxicity. In our study, the observed effects in 58% (55%, free-ion activity based) of all the mixture treatments tested were greater than estimated by the GIE. In 19% of them, the toxicity under-estimation (i.e., over-estimation on RGR) was >20%. However, the toxicity over-estimation (i.e., under-estimation on RGR) was >20% for 10% of the mixtures. Hence, the prediction error (both over- and under-estimation) was within ±20% for 71% of the mixtures.

The IA toxicity predictions were consistently higher than or equal to GIE predictions. Equal toxicity was observed in mixtures with a metal concentration ratio at which only one metal was present at a high enough concentration to elicit an individual effect. Indeed, according to the IA model, any mixture component present below its individual no-effect concentration will not contribute to the joint effect of the mixture. With this model, the percent of mixture treatments with an underestimated toxicity dropped to 48% (44%) of all the mixture treatments, with 17% (13%) of them with a RGR error >20%. However, the toxicity was overestimated in 52% (56%) of all the mixture treatments, with only 8% (14%) of them with an RGR error of >20%. Hence, the toxicity was estimated within ±20% error for 75% (73%) of all treatments. The MixTox analysis with the IA model concluded strictly additive toxicity (i.e., no interaction) for 8 mixtures (9), less-than-additive toxicity for 4 mixtures (5), and more-than-additive toxicity) for 3 mixtures (1).

The CA model consistently estimated a higher toxicity than the IA (and the GIE) model. This was expected from the relatively steep concentration−response curves of metals.[33] With the CA model, the toxicity was underestimated in 23% (26%) of all the mixture treatments, with only 4% of them having an RGR error of >20%. However, the toxicity was over-estimated in 77% (74%) of all the mixture treatments, with 43% of them with an RGR error of >20%. Hence, the toxicity was estimated within ±20% error for 53% of all treatments. The MixTox analysis with the CA model concluded strict additivity for 3 mixtures (4), less-than-additive toxicity for 11 mixtures (10), and more-than-additive toxicity for 1 mixture.

Biological Relevance of the CA and IA Performances. While the IA model generally predicted metal mixture toxicities quite well in this study, it is rather unlikely that all the different metals in the mixtures for which strict IA additivity was concluded have completely independent MoA. As discussed in the Introduction, the MoA of growth inhibition by metals in juvenile *L. stagnalis* remains to be elucidated. However, for all metals evaluated to date with this organism (Co, Cu, Pb, and Ni), a common significant reduction in Ca uptake rates has been linked to growth effects.[13,16,17,19] This association between Ca uptake and growth is not surprising because the remarkable growth rate of these snails (~20% day^{-1} in their first month post-hatch) is made possible by a high net Ca uptake rate (~8000 nmol g^{-1} h^{-1}; ~100-fold higher than in fish)[14] to support shell growth. An association between Ca uptake inhibition and Pb exposure is not surprising, as this metal is a known Ca antagonist.[34] More surprising are the high *L. stagnalis* apparent sensitivities to Ag and Cu, which are known Na antagonists,[35,36] as well as the apparent insensitivity to Cd and Zn, known Ca antagonists.[37−39] Yet according to Brix et al.,[19] the observed Ca uptake inhibition in Pb-exposed *L. stagnalis* is not caused by simple direct competition at Ca channels and transporters, and the exact mechanisms remain to be elucidated. Nevertheless, with Ca uptake inhibition appearing as an excellent diagnostic of metal-induced growth effects in snails, it seems unlikely that metals will exhibit completely independent MoAs, and thus, the IA model may not be appropriate. However, assuming a common MoA for metals and, thus, the validity of the CA concept would be overly simplistic because many different mechanisms can be responsible for Ca uptake inhibition.[40] To conclude, it is likely that neither of the two simplistic additive models is conceptually valid and that

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the reality falls somewhere between them (not entirely similar or dissimilar MoA).

Interactions, mostly of an antagonistic nature, were observed for both CA and IA models. Metals are known to be able to interact by competition for abiotic ligands in the water and for biotic ligands involved in uptake, toxicity, and detoxification. Bioavailability models, such as the BLM,[23] can account for these different types of interactions. In the present study, the former type of interaction, i.e., the geochemical interactions in the water, was accounted for by using the calculated free ion activities for the data analyses. Previous metal mixture studies have shown that more-than-additive toxicity could be explained by multimetal competition for a limited number of abiotic binding sites (typically on DOM), leading to increased free metal ions and, hence, to a more-than-additive mixture toxicity.[9,41] In the present study, however, when predictions were based on the free ion rather than the total metal, most resulting changes in mixture toxicity predictions were not attributed to multimetal interactions but rather to single-metal interactions at DOM and subsequent changes in slopes of concentration−response curves. When free-ion activities were used in the additive models, the remaining observed interactions should indicate interactions at biological sites. Less-than-additive toxicity can theoretically be explained by multimetal competition at biological target sites, such as metal surface transporters. For example, if a metal, present at a subtoxic concentration, prevents a second metal, present at a toxic concentration, from binding to its biological receptor, then the mixture toxicity will be less than additive. In the present study, the typical classification of metals between Ca antagonists (Cd, Zn, and Pb) and Na antagonists (Cu and Ag) did not predict accurately these types of interaction, as might be expected given these MoAs are most consistently observed in acute rather than chronic exposures. Indeed, this hypothesis worked for some strong antagonisms (e.g., Cd/Zn) but not for others (e.g., Ag/Pb). To provide insights into the current multimetal interactions, in a subsequent study (Crémazy et al. in preparation), we have investigated how the six metals interact for uptake in *L. stagnalis*, in binary mixtures, taking into account both known strong antagonisms and the issue of essentiality versus nonessentiality. In general, the results suggest that multimetal interactions exist at biological levels other than metal uptake. Nevertheless, it is encouraging for bioavailability-based models that only one more-than-additive mixture (for Cd/Ni) was observed for both CA and IA when toxicity was expressed on the basis of free-ion activity. Indeed, more-than-additive toxicity cannot be explained by competition at biological target sites. The reasons why Cd and Ni appear to enhance each others' toxicities are unclear and require further studies. Overall, our study indicates that a bioavailability-based approach for metal mixtures[42−45] has the mathematical potential to explain most of the joint effects observed in this study. However, knowledge on the toxicity mechanisms of the different metals and on the nature of their interactions is critically needed for the construction of such model.

Implications for Risk Assessment of Metal Mixtures. Currently, ecological-risk assessment mostly considers the effects of single substances in isolation. This approach is justified if there is evidence showing that the joint action of chemicals is not larger than the greatest individual effect (GIE) in the mixture. As detailed above, this was not the case for many treatments tested. A better protection was offered by the two additive reference models because they logically both predicted higher mixture toxicity. As discussed above, a definitive choice of reference model based on toxicological knowledge about toxicant MoA was not possible here. It has been proposed that both models could be used in risk assessment to determine a so-called prediction window,[46] between which mixture toxicity of substances with not entirely similar and dissimilar MoA may fall.[47] However, this was not the case for approximately a third of the binary mixtures in this study, with mixture toxicity being sometimes over-estimated (less-than-additive toxicity) or under-estimated (more-than-additive toxicity) by the models. Within the regulatory context, more-than-additive mixtures arguably constitute the greatest concern. Fortunately, more-than-additive toxicity was only observed for a very limited number of mixtures (only the Cd/Ni mixture when analysis was based on the free-ion activities). Hence, for most of the metal mixtures tested, both additive models gave either conservative or accurate predictions of toxicity, with the CA being the most conservative and the IA being the most accurate. Recent studies on the chronic toxicity of metal mixtures have made the same observation about CA protectiveness and IA accuracy (see the systematic evaluation from Nys et al.).[12] Generally, the combination of protectiveness and mathematical simplicity of CA makes this model a popular conservative choice for metal mixtures risk assessment.[1] In the absence of a more-mechanistic model, the present study supports the use of CA as a first line of decision-making. Improved risk evaluations, through the development of a bioavailability model, will require a better understanding of individual metals MoA and the nature of their interactions. Finally, it would be of great relevance to test higher order mixtures (e.g., ternary and quaternary combinations) to evaluate if the present conclusions on binary mixtures still hold in more-complex scenarios.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b06554.

> Figures showing toxicity test experimental design, individual metal-toxicity tests analyses, and predicted vs observed RGR based on free-ion activities. Tables showing metal-speciation calculations, individual metal-toxicity tests analyses, detailed MixTox statistical analyses, and the experimental data of all the single metal and mixture toxicity tests carried out in the present study. (PDF)

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Notes

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