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ACUTE TOXICITY OF BINARY-METAL MIXTURES OF COPPER, ZINC, AND NICKEL TO *PIMEPHALES PROMELAS*: EVIDENCE OF MORE-THAN-ADDITIVE EFFECT

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Abstract: Metal mixture toxicity has been studied for decades. However, the results are not consistent, and thus ecological risk assessment and regulation of mixtures has been difficult. The objective of the present study was to use a systematic experimental design to characterize the toxicity of binary-metal mixture of Cu, Zn, and Ni to *Pimephales promelas*, typically to determine whether the effect of these binary-metal mixtures on *P. promelas* is more-than-additive. Standard 96-h toxicity tests were conducted with larval *P. promelas* based on US Environmental and Protection Agency methods to determine metal mixture effects. All experiments were conducted in synthetic moderately hard water with no addition of dissolved organic matter. Three different effect analysis approaches, the MixTox model, the Finney model, and the toxic unit method, were used for comparison. The results indicate that the toxicity of Cu+Zn, Cu+Ni, and Zn+Ni mixtures to *P. promelas* was more-than-additive. Among the 3 mixtures, the effect of the Cu+Ni mixture was the most profound. The results of the present study are useful for applications to models such as the metal mixture biotic ligand model. More research should be conducted to determine the mechanisms of acute and chronic toxicity of metal mixtures. *Environ Toxicol Chem* 2016;35:446–457. © 2015 SETAC

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INTRODUCTION

Metals are usually present in the natural environment as a mixture of multiple elements. Organisms living in such contaminated environments are exposed to metal mixtures. The toxicity of metal mixtures has been studied for decades [1–10]. In general, 3 different types of effects have been reported. An additive effect is indicated when the individual metals in the mixture interact with organisms and produce an effect but do not enhance or diminish each other's actions, resulting in the effect of mixture and the total effect of individual metals being equal [11]. If individual metals enhance each other's actions, the mixture would be considered to have a more-than-additive effect (also called synergism). A less-than-additive effect (also called antagonism) is observed when mixture components diminish each other's actions.

Although numerous metal mixture studies have been conducted, the results are not consistent, thus making ecological risk assessment and regulation difficult. Two major reviews of the acute toxicity of binary- or higher-metal mixtures (i.e., copper [Cu], zinc [Zn], cadmium [Cd]) by Norwood et al. [12] and Vijver et al. [13], and recently summarized by Meyer et al. [14], demonstrated that the mixture toxicity was less-than-additive in 40% to 51% of the tests, additive in 20% to 24% of the tests, and more-than-additive in 29% to 36% of the tests. The variety of results among studies might reflect real differences in additivity/nonadditivity of toxicity of metal mixtures, or the differences might have been the result of experimental artifacts such as variability of organism responses when metal mixtures are not tested concurrently with the paired individual-metal tests [15]. A coordinated experimental design and a clear mechanism of toxicity that allows one to choose an appropriate effect analysis method are also important [12,14,16,17].

Two general methods have been used for analysis of mixture effects. Concentration addition is used when individual toxicants in the mixture have a similar mode of action [18,19]. This assumes that a toxicant can be replaced totally or primarily by another toxicant at the biological target site of action without diminishing the overall combined effect [18]. Independent action (also called response addition) is recommended for mixtures with different (dissimilar) modes of action and assumes that the joint effect of a mixture can be calculated from the response of individual mixture components [18,19]. Based on the independent action method, individual mixture components that are present at concentrations below the effect thresholds do not contribute to the joint effect of the mixture [11]. However, the concepts of similar or dissimilar modes of action are sometime not accurately justified and may be dependent on organisms. For example, Cu is believed to inhibit sodium (Na) uptake at sodium channels in the fish gill membrane [20] but also replaces calcium at tight paracellular junctions, causing an excessive loss of sodium [21]. Our study with the Florida apple snail (*Pomacea paludosa*) indicated that, in addition to inhibiting Na uptake, Cu also significantly reduced Ca uptake by the snail (T. Hoang, unpublished data). Nickel is known to cause acute toxicity by disrupting respiratory capacity [22,23]. However, it also has been demonstrated that Ni exhibits toxicity by blocking several different calcium channels and disrupting calcium homeostasis [24]. Zinc toxicity is believed to inhibit calcium uptake, resulting in decreased plasma calcium concentration, followed by hypocalcemia [25,26]. To a lesser extent, Zn also offsets the acid/base balance in fish [27]. In a metal mixture biotic ligand model

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(mBLM), metals have been assumed to have multiple binding sites. For example, in addition to binding to its own site, Cu can also bind to the Zn site and vice versa [28]. The different possible target sites of metals, such as Cu, Zn, and Ni, make justification for similar or dissimilar modes of action less defensible. Consequently, choosing an appropriate effect analysis method, such as concentration addition versus independent action becomes problematic. Hoel [29] further detailed these concepts by considering simple similar (no interaction) and complex similar (interaction) and independent dissimilar and dependent dissimilar interactions. When developing a method for analysis of mixture effect, in addition to independent joint action (independent action method applied), and similar joint action (concentration addition method applied) Bliss [30] considered another type of action: synergistic action. The Finney model is usually used for synergistic effect [31,32].

Despite interaction at the biological site, metals also chemically interact with other inorganic ligands in the environment, and this would result in different bioavailability and toxic effects [28]. Meyer et al. [9] found slightly more-than-additive effects of Cu+Zn mixtures to *Daphnia magna* [9]. When using the mBLM for predicting toxicity, however, the model predicted a less-than-additive effect. Kortenkamp and Altenburger [11] analyzed the mixture effect of several datasets with different sites of action and found that the concentration addition method fit the experimental data better than the independent action method. From their study, Kortenkamp and Altenburger [11] suggested using both methods for comparison with observed effects when there is no absolute evidence for similar or dissimilar modes of action or when the evidence is unclear. Another reviewed study by Cedergreen et al. [33] demonstrated that both concentration addition and independent action methods can be used for mixtures with dissimilar modes of actions. Furthermore, so as to demonstrate the model-dependent nature of additivity assessment, Lee et al. [34] applied 8 models (or variants of models) to a given dataset, with 5 of these indicating additivity and the remaining 3 showing more-than-additive toxicity. Jonker et al. [7] developed an alternative approach (MixTox model) that used both concentration addition and independent action methods as reference models and included additional parameters to account for deviations from the reference models, such as synergism or antagonism parameters.

In addition, the Finney model has been used widely for analysis of the mixture effect of toxicants, especially for synergistic effects. The Finney model is a general approach that does not distinguish similar or dissimilar modes of action and is based on the concentration addition method. This is likely the more applicable approach because contaminant mixtures in the natural environment can contain more than 2 components with both similar and dissimilar modes of action. Use of the model can be found in many fields including botany, oncology, and entomology [32,35,36].

The objective of the present study was to characterize the toxicity of binary-metal mixtures of Cu, Zn, and Ni to fathead minnows (*Pimephales promelas*), to determine whether the effect of binary-metal mixtures of these metals is additive or nonadditive. We used a *ray design* and different data analysis approaches for comparison. We also report measured water chemistry of the test waters together with toxicity results for model application, such as with the mBLM, ecological risk assessment, and regulatory purposes. The SAS program code used for the Finney model is reported in the Supplemental Data.

MATERIALS AND METHODS

Experimental design

To evaluate the toxicity of binary-metal mixtures to larval fathead minnows, 96-h renewal acute toxicity tests were conducted in synthetic moderately hard water. The endpoint of the tests was 96-h cumulative mortality. The tests were conducted with individual metals and binary-metal mixtures. Experiments followed a ray (or fixed ratio) design that allowed us to determine additive or nonadditive effects of metal mixtures (Table 1). The ray design is used for determining the mixture effects of contaminants [16,37–39]. Tests included 5 treatment concentrations (except for the Ni-alone test) and a control. Our previous study with Ni [40] and preliminary tests indicated a wide range of response concentrations. Therefore, 2 additional Ni treatments were used for the Ni-alone test. For Cu, Ni, and Zn binary-metal mixture tests, treatment concentrations in the mixture tests were one-half of the treatment concentrations in the individual metal tests.

Although there is a significant body of literature data on individual metal toxicity to larval fathead minnows (US Environmental Protection Agency [USEPA] ECOTOX database [41] variations in toxicity are seen between studies of the same species because of differences in water quality [40,42,43]. To determine suitable metal concentrations for the present study, preliminary range-finding concentration tests were conducted with individual metals (Cu, Zn, Ni) and fathead minnows (unpublished data). Based on results of the preliminary tests, treatment concentrations in the individual metal tests were chosen to have partial mortality (between 0% and 100%) and to be within the ranges of 50 μg/L to 800 μg/L Cu, 100 μg/L to 1600 μg/L Zn, and 500 μg/L to 8000 μg/L Ni (Table 1). Metal concentrations that produced 100% mortality in individual metal tests were not repeated in mixture tests, so that any possible more-than-additive effects could be detected. Metal concentrations in the mixture tests ranged from 25 μg/L to 400 μg/L, from 50 μg/L to 800 μg/L, and from 250 μg/L to 2000 μg/L, for Cu, Zn, and Ni, respectively. Tests with individual metals and binary-metal mixtures were not conducted simultaneously, but an additional treatment with an individual metal was conducted with the mixture tests to confirm the consistent response of fathead minnow throughout the study period.

Toxicity testing

All tests were performed using the USEPA standard methods [44]. Test water was prepared using 18 mΩ Milli-Q water (Barnstead E-pure) and an addition of sodium bicarbonate, calcium sulfate, magnesium sulfate, and potassium chloride based on the USEPA standard methods for toxicity testing [44]. All test chambers were washed and rinsed with nitric acid and then with 18 mΩ water before using. Each test had at least 5 metal concentrations and a control. Three replicates were used for each metal concentration and the control. Replicates contained 10 larval fish. Tests were conducted at a temperature between 22 °C and 25 °C and a 16:8-h light:dark photoperiod at the Institute of Environmental Sustainability of Loyola University Chicago (Chicago, IL, USA).

Treatments of test solutions were made from prepared synthetic moderately hard water and desired quantities of metal stock solutions. The stock solutions were made from $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, and $NiSO_4 \cdot 6H_2O$. Stock solutions of Cu, Zn, and Ni were diluted to desired concentrations and then verified by inductively coupled plasma–mass spectrometry (ICP-MS;

Table 1. Exposure concentrations and mortality for individual metal and binary-metal mixture tests with larval *Pimephales promelas*[a]

Individual metals						Binary-metal mixtures					
Cu (μg/L)	Mortality (%)	Zn (μg/L)	Mortality (%)	Ni (μg/L)	Mortality (%)	Cu+Zn [TTU] (μg/L)	Mortality (%)	Cu+Ni [TTU] (μg/L)	Mortality (%)	Zn+Ni [TTU] (μg/L)	Mortality (%)
0(ND)	0	0(10)	0	0(0.9)	0	0(ND) + 0(20)	0	0(1) + 0(ND)	0	0(5) + 0(2)	3 ± 6
A(42)	0	B(88)	0	C(531)	1 ± 2.5	0.5A(18) + 0.5B(55) [0.21]	0	0.5A(16) + 0.5C(233) [0.19]	7 ± 6	0.5B(46) + 0.5C(243) [0.12]	0
2A(81)	20 ± 0	2B(190)	3 ± 6	2C(729)	1 ± 2.5	A(41) + B(97) [0.45]	27 ± 6	A(31) + C(473) [0.37]	30 ± 20	B(78) + C(484) [0.22]	0
4A(133)	37 ± 0.2	4B(390)	3 ± 0.6	3C(1465)	10 ± 10	2A(79) + 2B(159) [0.83]	60 ± 10	2A(53) + 2C(923) [0.66]	97 ± 6	2B(151) + 2C(294) [0.43]	7 ± 6
8A(153)	87 ± 15	8B(736)	40 ± 17	6C(2682)	17 ± 10	4A(138) + 4B(321) [1.49]	97 ± 0.02	4A(132) + 3C(1397) [1.41]	100 ± 0	4B(289) + 3C(1434) [0.72]	67 ± 6
16A(392)	100 ± 0	16B(1501)	90 ± 10	8C(2901)	47 ± 17	8A(265) + 8B(706) [2.98]	100 ± 0	8A(238) + 4C(1910) [2.39]	100 ± 0	8B(604) + 4C(1903) [1.22]	100 ± 0
				12C(6341)	86 ± 11	2A (75)	31 ± 13	3C (1.311)	0	8B (709)	50 ± 17
				16C(6900)	99 ± 2.5						

[a]A, B, and C represent nominal concentrations: A = 50 μg/L Cu; B = 100 μg/L Zn; C = 500 μg/L Ni. Data in parentheses are measured concentrations (μg/L). Data in brackets are total toxic units (TTUs). Mortality data are mean ± standard deviation.
ND = not detected.

NeXion 300S, PerkinElmer) prior to use. Test solutions were prepared at least 5 h before organisms were added. Water quality parameters such as dissolved oxygen, pH, and temperature were measured at test initiation and termination and daily during the test. Dissolved oxygen and temperature were measured using a YSI 550A dissolved oxygen meter, pH was measured using an Accumet AP 110 pH meter (Fisher Scientific), and conductivity was measured using a YSI 30 conductivity meter. Water quality parameters such as hardness and alkalinity were measured at test initiation and termination. Water hardness was determined by titration with 0.01 M ethylenediaminetetraacetic acid. Alkalinity was determined by titration with 0.02N H_2SO_4. The average measured dissolved oxygen, pH, and temperature were 8.3 mg/L, 7.62, and 22.4 °C, respectively. The average hardness and alkalinity were 104 mg/L and 68 mg/L as $CaCO_3$, respectively. Detailed water chemistry values of the test waters are shown in Table 2.

All test organisms were larval fathead minnows (≤4 d old). These fish were purchased from Aquatic Biosystems and were 1 d old or younger at the time they arrived. The fish were then acclimated to laboratory conditions for at least 24 h but no longer than 3 d prior to test initiation. During acclimation, fish were fed daily with freshly hatched brine shrimp (Brine Shrimp Direct). The fish used in all tests were fed at least 2 h prior to test initiation and at 2 h prior to the renewal of test water on day 2.

Fish were impartially distributed into test chambers 1 or 2 at a time to ensure randomization. Only fish that appeared healthy were used for testing. All tests were static exposures, and the test chambers were not aerated. Each day, test chambers were moved randomly to eliminate position effects. Mortality was recorded daily for every replicate of each treatment. Any dead fish were collected and removed daily. After 96 h of testing, any surviving individuals were euthanized with methane tricaine sulfonate (MS-222) and discarded in accordance with the standard procedures of the Loyola Institutional Animal Care and Use Committee.

Water samples were collected in separate sample vials at the beginning and the end of each exposure treatment, including control for total metal, dissolved metal, and cation and anion analyses. Total metal samples were collected directly from treatment solutions. Dissolved metal and cation and anion samples were filtered using a 0.45-μm polypropylene housing and a nylon membrane Whatman filter. A new filter was used for each sample of each type of analysis. A small amount of water sample (~5 mL) was first passed through the filter to wash out chemical residues on the filter (if any) before using for collecting samples. Total and dissolved metal and cation samples were acidified with HNO_3 to pH 2 and stored at 4 °C in a refrigerator prior to analysis. Analysis of metals and cations was performed by using a NeXion 300S ICPMS. Samples for anion analysis were analyzed with an 881 Compact Ion Chromatograph (Metro Ω USA). Samples for analysis of background dissolved organic carbon were collected from control water of each test and filtered with a new filter of the same filter type as used for dissolved metal and cation and anion samples. Concentrations of dissolved organic carbon were analyzed with a Shimazu TOC-L analyzer. The results of water chemistry analyses are shown in Table 2.

Effect analysis

Three approaches were used to analyze the data: the MixTox model developed by Jonker et al. [7], the Finney model, and the traditional toxic unit (TU) method based on additivity. The MixTox model used concentration addition and independent

Table 2. Test water chemistry[a]

Test	Ca^{2+} (mg/L)	Mg^{2+} (mg/L)	Na^{+} (mg/L)	K^{+} (mg/L)	Cl^{-} (mg/L)	SO_4^{2-} (mg/L)	DOC (mg/L)	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Temperature (°C)
Cu	16.41 ± 0.22	14.03 ± 0.15	29.66 ± 0.89	2.22 ± 0.27	1.73 ± 0.27	49.12 ± 13.33	0.74	7.66 ± 0.25	75 ± 5	111 ± 6	22.8 ± 0.4
Zn	16.41 ± 0.16	14.01 ± 0.05	29.00 ± 0.34	2.40 ± 0.09	1.93 ± 0.24	50.37 ± 10.41	0.61	7.71 ± 0.05	72 ± 2	110 ± 4	22.9 ± 0.2
Ni	13.14 ± 0.45	10.83 ± 0.18	24.53 ± 5.40	1.76 ± 0.26	1.95 ± 0.25	48.52 ± 4.05	0.81	7.64 ± 0.17	64 ± 4	106 ± 14	21.2 ± 1.52
Cu ± Zn	14.74 ± 0.21	12.57 ± 0.13	26.25 ± 0.43	2.11 ± 0.04	2.37 ± 0.019	47.84 ± 4.52	0.67	7.59 ± 0.17	65 ± 3	97 ± 6	22.9 ± 0.23
Cu ± Ni	13.74 ± 0.18	11.49 ± 0.09	27.42 ± 0.94	1.99 ± 0.19	1.95 ± 0.27	49.27 ± 4.04	0.79	7.58 ± 0.14	67 ± 3	102 ± 9	21.9 ± 0.07
Zn ± Ni	12.34 ± 3.77	10.33 ± 3.09	23.45 ± 6.87	1.78 ± 0.56	2.216 ± 0.05	50.31 ± 5.25	0.78	7.56 ± 0.08	65 ± 4	100 ± 12	22.6 ± 0.20
Average	14.80	12.43	27.28	2.05	2.02	49.24	0.73	7.62	68	104	22.4

[a]Data are mean ± standard deviation. No standard deviation for dissolved organic carbon (DOC) because only control sample was analyzed for each test.

action as the reference models and included deviation terms to detect synergism (more-than-additive) or antagonism (less-than-additive), dose level–dependent deviation, and dose ratio–dependent deviation effects. Two key parameters to be estimated in the MixTox model are parameter *a* and the sum of squared residuals between observed and predicted effect (SS). When parameter *a* is positive, the effect is considered less-than-additive. The effect is considered more-than-additive when *a* is negative. The SS also decreased when parameter *a* significantly differed from 0. The test statistic for parameter *a* significantly differing from 0 was determined using the χ^2 method with the degree of freedom equal to 1. More details can be found in Jonker et al. [7]. All analysis procedures were performed using the spreadsheet available from the Centre for Ecology & Hydrology [45]. Measured dissolved metal concentrations were used for the analysis.

With the Finney model, we analyzed the pairwise interaction between metals using the binary-logistic variant of the Finney model as introduced in Finney [31], and as further developed and extended in O'Brien [46]. To illustrate the Finney model for metals A and B, the model combines the data for these metals both singly and in mixed combination (simultaneously) using the so-called effective concentration *z* given by Equation 1

$$z = x_1 + \theta_1 x_2 + \theta_2 \sqrt{\theta_1 x_1 x_2} \quad (1)$$

In this equation, x_1 is the concentration of metal A; x_2 is the concentration of metal B; θ_1 is the relative potency parameter or conversion factor, which represents the relative toxicity between the 2 metals, such as median lethal effect concentration (LC50) of metal A $= \theta_1 \times$ LC50 of metal B; θ_2 is the coefficient of synergy; and $x_1 x_2$ is the interaction term of metals A and B and preserves additivity of the 2 metals. The square root included in $x_1 x_2$ expression is to maintain the same unit of concentration (i.e., $\sqrt{(\mu g/L)^2} = \mu g/L$). In this model, the effective concentration *z* is then related to the probability the tested organisms are alive (π) using the 2-parameter log–logistic dose–response function 2.

$$\pi = \frac{1}{1 + (z/\theta_3)^{\theta_4}} \quad (2)$$

In Equation 2, θ_3 is simply the LC50 for metal A alone, and θ_4 is the slope parameter or rate at which the curve descends in the mixture response surface. Furthermore, the LC50 for metal B alone can be determined by the ratio of θ_3 to θ_1 (θ_3/θ_1). We emphasize that the Finney model includes both of the above equations, and the statistical software (see SAS Program in the Supplemental Data) fits the 2 equations simultaneously.

The key parameter to be estimated in this model is the coefficient of synergy θ_2. Additive effect is indicated when θ_2 does not significantly differ from 0, less-than-additive effect is indicated for $\theta_2 < 0$, and more-than-additive effect is indicated for $\theta_2 > 0$. These 3 outcomes are demonstrated in Figure 1 for 2 arbitrary metals. The graphed isoboles or contours of toxic units (or equivalent concentration) are obtained by setting *z* equal to a constant—in the present study, this is such that $\pi = ½$ (i.e., $z = \theta_3$).

In Figure 1, for demonstration purposes, the coefficient of synergy is chosen to be $\theta_2 = 1.2$ for the more-than-additive effect isobole, $\theta_2 = 0$ for the additive effect isobole (line), and $\theta_2 = –0.5$ for the less-than-additive effect isobole. The more-than-additive effect isobole bows inward toward the origin, indicating that when the 2 metals are used in combination, a

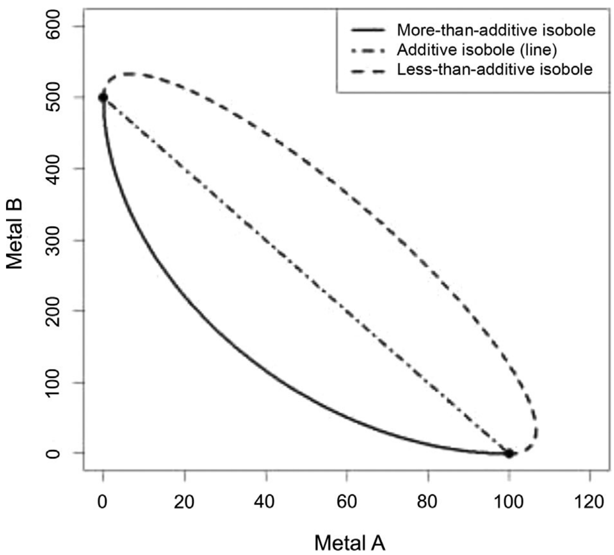

Figure 1. Isobole for 2 metals using the Finney model.

lesser amount is needed of the 2 metals to obtain the same overall effect. In contrast, the less-than-additive effect isobole bows outward because more of the metals are needed in combination to achieve the same effect, as the metals are interacting in a negative manner.

The mixture effects in the present study were analyzed using the Finney model and by SAS software package (Ver 9.3.1) with the nonlinear mixed model (NLMIXED) procedure. Measured dissolved metal concentrations (Table 1) were used for the effect analysis. The 96-h LC50 values for individual metal tests were calculated by the logistic method. Parameter estimates were obtained using maximum likelihood estimation by maximizing the log-likelihood, *LL*. Testing for additive effect (i.e., H_0:$\theta_2 = 0$ vs H_A:$\theta_2 \neq 0$) was achieved using the likelihood test statistic: $\chi^2 = 2(LL_{Full} - LL_{Reduced})$ (see Agresti [47] for more details). Here, LL_{Full} is the value of the log-likelihood for the unrestricted or full model (with no restriction on θ_2 as in H_A), $LL_{Reduced}$ is the value of the log-likelihood for the reduced model (i.e., imposing $H_0 : \theta_2 = 0$), and the chi-square test statistic has 1 degree of freedom.

With the TU approach, the total toxic unit (TTU) of 2 metals in each exposure treatment can be calculated using Equation 3 [16,19,37,38,48]. This procedure was also used in the MixTox model approach.

$$TTU = TU_a + TU_b = \frac{C_a}{LC50_a} + \frac{C_b}{LC50_b} \quad (3)$$

where C_a and C_b are exposure concentrations of metals A and B in each treatment, respectively; $LC50_a$ and $LC50_b$ are the median lethal effect concentrations for the metal A-alone exposure and B-alone exposure, respectively; TU_a and TU_b are toxic units of metals A and B in the mixture, respectively. After converting mixture concentrations to TTUs, the 96-h median lethal effect concentration in TU (96-h TTU50)—or the 10%, 20%, or 90% 96-h lethal effect concentration (96-h TTU10, 96-h TTU20, and 96-h TTU90, respectively; for risk assessment and regulation purposes)—can be calculated. The effect of a mixture can then be determined by comparing the 96-h TTU50 with 1. If the 95% confidence interval (CI) of the 96-h TTU50 contains 1, we consider the effect to be additive. The effect is considered more-than-additive when the 95% CI is less than 1 or less- than-additive when the 95% CI is greater than 1 [37,38]. The Toxcalc Program was used to determine 96-h TTU10s, 96-h TTU20s, 96-h TTU50s, 96-h TTU90s, and their 95% CIs using the logistic method. Comparison of the slopes of the concentration–response curves was conducted using the method published by Cohen et al. [49]. The method is also available online [50]. Measured dissolved metal concentrations were used for the analysis.

Comparisons of statistically significant differences between the mortalities in the individual metal treatments and in the concurrent binary-metal mixture test series were performed using *t* tests. Data met the assumptions of equal variance and normal distribution of residuals.

RESULTS

Responses of *P. promelas* in individual and binary-metal mixture tests with Cu, Zn, and Ni, are shown in Table 1. In general, mortality increased when metal concentrations were increased. Visibly, at a similar concentration, the total mortality attributable to individual metal exposures was less than the mortality attributable to mixture exposure. For example, at a 2A concentration of Cu and a 2B concentration of Zn, the total mortality was 23% (20% attributable to Cu, 3% attributable to Zn). This total mortality was less than the mortality (60%) attributable to Cu+Zn mixture exposure at 2A and 2B concentrations of Cu+Zn, respectively (Table 1). Similarly, for Cu and Ni, the total mortality attributable to a 2A concentration of Cu and a 2C concentration of Ni was 21%, which was less than the mortality (97%) attributable to Cu+Ni mixture exposure at similar concentrations. For a 4B concentration of Zn and a 3C concentration of Ni, the mortality attributable to mixture exposure was 67%. This is greater than the total mortality (13%) attributable to individual Zn and Ni exposure. These results reveal a more-than-additive effect of the binary-metal mixtures on *P. promelas*.

Results of individual metals tested concurrently with mixture tests showed 31%, 50%, and 0% mortality for Cu, Zn, and Ni, respectively (Table 1). When we compared these mortalities with the mortalities of the corresponding treatment in the individual tests (i.e., 31% compared with 20% for Cu [$p = 0.259$], 50% compared with 40% for Zn [$p = 0.08$]), there was no significant difference between them. These results indicate that fathead minnows responded consistently throughout the present study period.

Results of the effect analysis by the MixTox model are shown in Table 3 and Figures 2 and 3. With the independent action method, when a deviation term was added to describe a more- or less-than-additive effect, the SS decreased considerably. For example, with a Cu+Ni mixture, the SS decreased from 12 821 to 816. This indicates that the model predictions improved when either a more- or a less-than-additive effect term was included. This can also be seen in Figure 2, where the relationship between predicted and measured mortality was better when a more- or less-than-additive effect term was added. The value of parameter *a* was significantly less than 0 (Table 3), indicating a more-than-additive effect of the binary-metal mixtures. Using the concentration addition method, parameter *a* was only significantly negative for the Cu+Ni mixture. The SS also decreased considerably when a more- or less-than-additive term was added to the model for this mixture (Table 3). With the Cu+Zn mixture and the Zn+Ni mixture, parameter *a* was not significantly different from 0 (Table 3). Therefore, no deviation from the concentration addition model was indicated.

Table 3. Summary of MixTox model analysis of effect of binary-metal mixture on fathead minnow

		Independent action		Concentration addition	
Mixture	Parameter	Reference[a]	S/A[b]	Reference	S/A
Cu + Zn	SS	3065	767	1049	1477
	a	NA	–8.99	NA	–0.29
	$p(X^2)$	NA	2.5×10^{-6}	NA	ND[c]
Cu + Ni	SS	12 821	816	6230	2772
	a	NA	–25.47	NA	–3.23
	$p(X^2)$	NA	3.18×10^{-11}	NA	0.0001
Zn + Ni	SS	4395	473	3146	3092
	a	NA	–17.89	NA	–0.05
	$p(X^2)$	NA	2.36×10^{-10}	NA	0.58

[a]Reference: assumes additivity of toxicity.
[b]S/A = synergism/antagonism or more-than-additive/less-than-additive. The effect is considered more-than-additive when parameter *a* is significantly less than 0 ($p(X^2) < 0.05$).
[c]Data could not be determined (ND) because when S/A is included the SS is higher than the SS of the reference model. This does not allow calculation of $p(X^2)$.
SS = sum of squares of residuals between the observed and predicted mortality; NA = not applicable.

Using the Finney model to assess the mixture effect of Cu+Zn, the 96-h LC50 values (θ_3) for Cu alone and Zn alone were 125 μg/L and 821 μg/L, respectively. The 95% CIs of these LC50s for Cu and Zn were 109 μg/L to 141 μg/L and 685 μg/L to 957 μg/L, respectively (Table 4). The relative potency parameter θ_1 and the coefficient of synergy were found to be 0.152 and 1.155, respectively (Table 4). Substituting these values into Equation 4, the effective concentration z (isobole) for the Cu+Zn mixture became

$$z_{Cu/Zn} = [Cu] + 0.152[Zn] + 1.155\sqrt{0.152[Cu][Zn]} \qquad (4)$$

This effective concentration *z* illustrates the isobole curve (solid curve in Figure 4) and indicates the combination of the 2 metals that yields a probability that 50% of the fish are alive ($\pi = ½$). The curve bends in toward the origin because the coefficient of synergy is significantly non-0 ($H_0{:}\theta_2 = 0, H_A{:}\theta_2 \neq 0$; $\chi^2 = 66.8–47.5 = 19.3, p < 0.0001$). Because $\theta_2 > 0$, a more-than-additive effect on *P. promelas* is indicated for the Zn+Cu mixture. In Figure 4, retaining $H_0{:}\theta_2 = 0$ would have indicated that the dashed additive effect line would have been appropriate.

Similarly, for the Cu+Ni mixture, the model gave a 96-h LC50 of 3920 μg/L with a 95% CI of 3604 μg/L to 4236 μg/L for Ni alone (Table 4). The θ_1 and θ_2 coefficients were 0.033 and 2.969, respectively (Table 3). The effective concentration *z* for the Cu+Ni mixture was $z_{Cu/Ni} = [Cu] + 0.033[Ni] + 2.969\sqrt{0.033[Cu][Ni]}$ and is illustrated in Figure 5. Because θ_2 was significantly greater than 0 (Table 4), a more-than-additive effect was observed for the Cu+Ni mixture. Finally, the θ_1 and θ_2 coefficients for the Zn+Ni mixture were 0.233 and 1.264, respectively (Table 4). The effective concentration *z* for the Zn+Ni mixture was $z_{Zn/Ni} = [Zn] + 0.233[Ni] + 1.264\sqrt{0.233[Zn][Ni]}$ (Figure 6). A more-than-additive effect was also found for the Zn+Ni mixture because θ_2 was significantly greater than 0 (Table 4).

Using the TU approach, the TTU for each treatment of the Cu+Zn, Cu+Ni, and Zn+Ni mixture tests ranged from 0.21 to 2.98, from 0.19 to 2.39, and from 0.12 to 1.22, respectively (Table 1). Results of the 96-h TTU10, 96-h TTU20, 96-h TTU50, and 96-h TTU90 analyses are presented in Table 4. The 96-h TTU50 values for Cu+Zn, Cu+Ni, and Zn+Ni mixtures were 0.666, 0.419, and 0,641, respectively. The 95% CIs for these 96-h TTU50s were 0.616 to 0.719, 0.394 to 0.446, and 0.608 to 0.673, respectively. These 95% CIs of the 96-h TTU50 values were less than 1, indicating a more-than-additive effect of the binary-metal mixtures of Cu, Zn, and Ni on *P. promelas*.

The concentration–response curves smoothed by the logistic model for individual metals and binary-metal mixtures are shown in Figure 7. The slopes of the individual metal curves (and standard errors) were 10.31 (1.706), 7.85 (0.693), and 6.86 (0.171) for Cu, Zn, and Ni, respectively (Table 4). There was no significant difference between the slopes of individual metal curves (Table 4). The slopes (and standard errors) for Cu+Zn, Cu+Ni, and Zn+Ni mixture curves were 8.085 (0.654), 12.089 (1.183), and 16.103 (1.654), respectively (Table 4). Significant differences in the slopes of Cu+Zn versus Cu+Ni mixtures ($p = 0.025$) and Cu+Zn versus Zn+Ni ($p = 0.004$) mixtures were observed (Table 4). However, the slopes of the Cu+Ni mixture and Zn+Ni mixture were not significantly different ($p = 0.096$; Table 4).

DISCUSSION

Comparability of analysis approaches

The 3 analysis approaches used in the present study gave similar results. More-than-additive effects were observed for *P. promelas* exposed to the binary-metal mixtures of Cu, Zn, and Ni. The Finney model used exposure concentrations directly, whereas the MixTox model and the TTU approach converted the exposure concentrations to toxic units. The potency parameter in the Finney model is the conversion factor that allows the model to quantitatively express the total activity or exposure concentration of the mixtures via concentration of a toxicant in the mixture. For example, with the Cu+Zn mixture in the present study, the potency parameter was used to express the bioavailability or toxic concentration of Zn via Cu (e.g., Zn LC50 = Cu LC50/θ_1). An interaction term for toxicants in the mixture was included and represents the potential increase (more-than-additive effect, $\theta_2 > 0$) or decrease (less-than-additive effect, $\theta_2 < 0$) in the total (sum) activity or bioavailability of individual toxicants in the mixture. With the MixTox model and TU approaches, the concentration of individual toxicants was normalized to TUs, and the TTU of the mixture is the sum of TUs of all individual toxicants in the mixture. Compared with the Finney model, this is another way to convert concentrations of individual toxicants to a normalized parameter to express total exposure concentration of the mixture. The MixTox model allowed us to use both

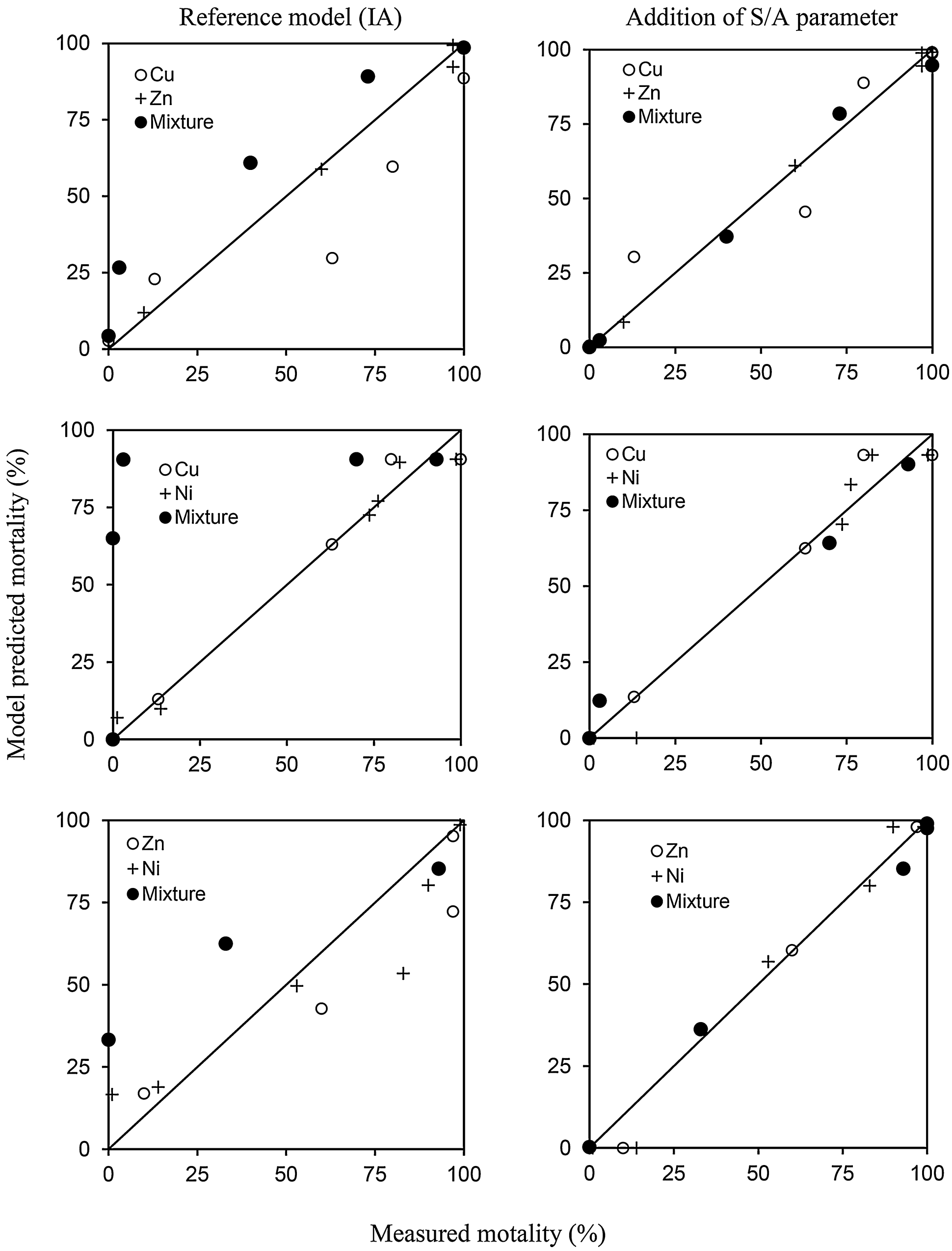

Figure 2. MixTox model (independent action [IA]) prediction versus measured toxicity. Data are not shown in the figure if the predicted mortality is more than 100% or below 0. For example, for the Zn and Ni mixture test, the reference model prediction for the 2 lowest concentrations were 1% and 6% below 0. S/A = synergism/antagonism or more-than-additive/less-than-additive.

concentration addition and independent action methods. In general, the results of the independent action method showed a better fit of predicted mortality to measured mortality (Figure 2). This favors a justification for dissimilar modes of action of Cu, Zn, and Ni. However, the concentration addition method also worked well with the Cu+Ni mixture, as the SS decreased significantly when a more or less additive term was added. This indicates that the requirement of similar mode of action for concentration addition is not critical for the Cu+Ni mixture.

The advantage of the Finney model and the MixTox model is that the methods provide a *p* value for hypothesis testing for coefficient of synergy that supports drawing conclusions on more- or less-than-additive effects of the mixtures. The Finney model has been used widely for determining the mixture effects of contaminants in the environment and drugs and toxicants in pharmacology and toxicology [19,32,39,46,51]. The toxic unit approach, however, provides 95% CIs of lethal TTUs (e.g., 96-h TTU50), which allows comparison with a TTU of 1. This approach has been used for analyzing mixture effects [10,37,38].

Binary-metal mixtures of Cu, Zn, and Ni

The present study found that the acute toxicity effects of Cu, Zn, and Ni binary mixtures were more-than-additive to *P. promelas*. Although more-than-additive toxicity is a less common outcome from metal mixture toxicity testing than additive or less-than-additive toxicity [12,13], there have been previous reports of more-than-additive toxicity from metal mixtures. For example, Khangarot et al. [3] reported more-than-additive toxicity with Cu–Ni mixtures and the common guppy. Khangarot et al. [3] also reported that when Ni was present at higher concentrations than Zn, the effect of Zn+Ni binary

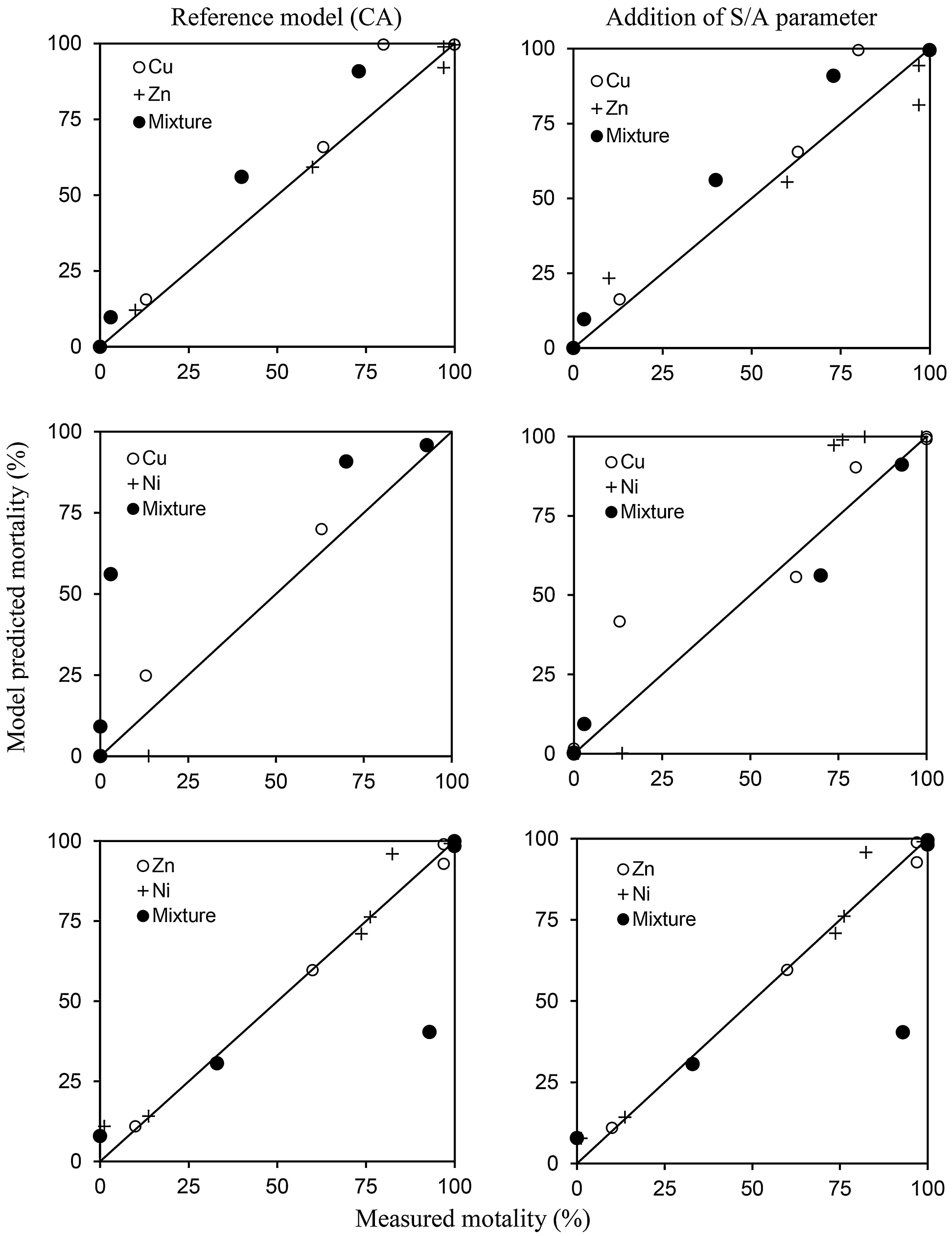

Figure 3. MixTox model (concentration addition [CA]) prediction versus measured toxicity. Data are not shown in the figure if the predicted mortality is more than 100% or below 0. For example, for individual Cu and Ni tests, most of the prediction by the reference model for low concentrations was approximately 3% below 0. S/A = synergism/antagonism or more-than-additive/less-than-additive.

mixtures on the common guppy was more-than-additive. Sprague and Ramsay [52] reported that the Cu+Zn binary mixture produced more-than-additive toxicity to juvenile salmon. According to Spehar and Fiandt [4], the toxicity of 6 metal mixtures (As, Cd, Cr, Cu, Hg, Pb) was more-than-additive to *P. promelas*. A recent study by Meyer et al. [9] found slightly more-than-additive acute toxicity of the Cu+Zn mixture to *D. magna*. Naddy et al. [10] found a more-than-additive effect of a Cu, Cd, and Zn mixture to *Ceriodaphnia dubia* in hard water. The mBLM also predicted a more-than-additive effect of Cu, Zn, Cd, and Pb to *Oncorhynchus mykiss*, *D. magna*, and *Hyalella azteca* based on dissolved concentrations [28].

Although the mechanism of acute toxicity of binary-metal mixtures cannot be elucidated from the present study, the more-than-additive effects of Cu, Zn, and Ni binary mixtures found for *P. promelas* suggest an enhancement of a metal to the bioavailability and/or toxicity of the other that results in an increase in the total effect of the mixtures. The negative parameter as determined by the MixTox model (Table 3) and the positive coefficients of synergy determined by the Finney model (Table 4) indicate an increase in total bioavailability and toxicity of the mixtures. Among the 3 binary-metal mixtures, the more-than-additive effect appears to be the most profound for the Cu+Ni mixture, with the most negative parameter *a* for the Cu+Ni mixture and the highest coefficient of synergy, regardless of the use of independent action or concentration addition methods. Visually, the distance between A and B in Figures, 5, and 6 4 was longest for the Cu+Ni mixture. The SS also decreased the most for this mixture when a more- or less-than-additive effect term was added. The more-than-additive toxicity of the binary-metal mixtures might be the result of external chemical interaction in the water and/or internal interaction at the biological target sites of action of the mixture. The mechanism of acute toxicity of metal mixtures in fish is

Table 4. Lethal effect concentrations for individual metals and lethal toxic unit for binary-metal mixtures[a]

Metals	Concentration response using the logistic model (standard error of slope/significant indicator for slope comparison)[b]	96-h LC50(95% CI) (μg/L)	96-h TTU10(95% CI)	96-h TTU20(95% CI)	96-h TTU50(95% CI)	96-h TTU90(95% CI)	θ^2	χ^2 (*p* value)
Cu	$y = 10.31x - 21.64$ (1.706/A,B,C)	125 (109–141)	NA	NA	NA	NA	NA	NA
Zn	$y = 7.85x - 22.91$ (0.693/A,B,C)	821 (685–957)	NA	NA	NA	NA	NA	NA
Ni	$y = 6.86x - 24.24$ (0.717/A,B,C)	3920 (3604–4236)	NA	NA	NA	NA	NA	NA
Cu+Zn	$y = 8.08X + 1.43$ (0.654/D)	NA	0.356 (0.307–0.400)	0.449 (0.400–0.492)	0.666 (0.616–0.719)	1.245 (1.118–1.431)	1.155	19.3 (<0.0001)
Cu+Ni	$y = 12.09X + 4.57$ (1.654/E,F)	NA	0.276 (0.245–0.301)	0.322 (0.294–0.345)	0.419 (0.394–0.446)	0.673 (0.583–0.719)	2.969	90.4 (<0.0001)
Zn+Ni	$y = 16.10X + 3.11$ (1.183/E,F)	NA	0.468 (0.422–0.504)	0.523 (0.485–0.558)	0.641 (0.608–0.673)	0.877 (0.822–0.958)	1.264	34.7 (<0.0001)

[a]Coefficient of synergy (θ_2) and Chi-squared statistics of the null hypothesis testing for the coefficient of synergy (H_0: $\theta_2 = 0$) were calculated by SAS using the Finney model. Others parameters were calculated by the Toxcalc program.

[b]*y* represents the Logit score; *x* represents $\log_{10}$ of exposure concentration; *X* represents total toxic unit. Slope comparison was conducted only within individual metals and binary-metal mixtures (no slope comparison between individual and binary-metal mixtures was conducted); slopes with the same indicative letters are not significantly different (A, B, C, D, E, and F denote Cu, Zn, Ni, Cu+Zn, Cu+Ni, and Zn+Ni, respectively).

LC50 = median lethal effect concentration; CI = confidence interval; TTU10 = total toxic units at 10% mortality; TTU20 = total toxic units at 20% mortality; TTU50 = total toxic units at 50% mortality; TTU90 = total toxic units at 90% mortality; NA = not applicable.

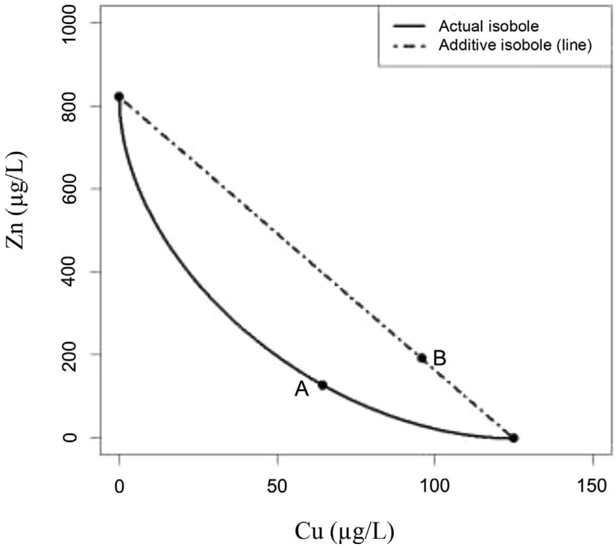

Figure 4. Fifty percent mortality isobole for Cu and Zn using the Finney model. Point A represents the actual median lethal concentration of the tested mixture (more than concentration additive). Point B represents the median lethal concentration if the effect was concentration additive in the tested mixture.

not clear. For individual metals (e.g., Cu, Zn), however, the mechanism of acute toxicity in fish is attributed to a block of transportation of osmotic ions, such as Na^+ by Cu or Ca^{2+} by Zn at the chloride cell membrane of the fish gills, which exerts the toxic effects [21,53,54]. Nickel is known to cause acute toxicity by disrupting respiratory capacity [22,23]. It is still unclear whether multiple stresses caused by multiple metals in the mixture, such as losing Na^+ and Ca^{2+} simultaneously because of Cu+Zn in the mixture, would result in more toxic effects.

Relative acute toxicity of individual metals and their binary-metal mixtures

It is not surprising that at similar concentrations the acute toxicity of individual metal exposure to *P. promelas* decreased

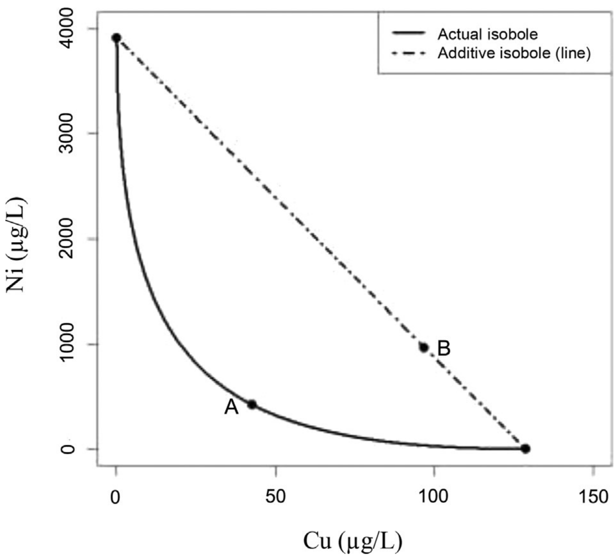

Figure 5. Fifty percent mortality isobole for Cu and Ni using the Finney model. Point A represents the actual median lethal concentration of the tested mixture (more than concentration additive). Point B represents the median lethal concentration if the effect was concentration additive in the tested mixture.

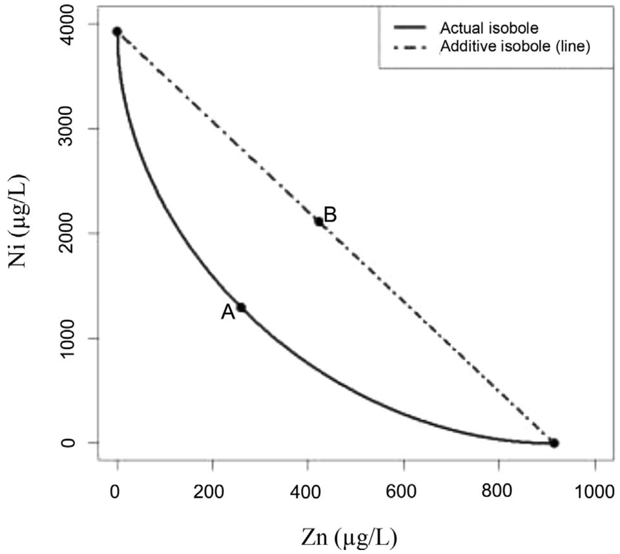

Figure 6. Fifty percent mortality isobole for Zn and Ni using the Finney model. Point A represents the actual median lethal concentration of the tested mixture (more than concentration additive). Point B represents the median lethal concentration if the effect was concentration additive in the tested mixture.

in the order of Cu > Zn > Ni. These results are in agreement with the literature results for *P. promelas* and other aquatic species (e.g., *D magna*, *H azteca*, *Lumbriculus variegatus)* [55,56]. The concentration–response curve of Cu is shifted to the left toward the lower range of concentrations. In contrast, the curve of Ni is shifted to the right toward the higher range of concentrations (Figure 7A). Given the ray design used in the present study, if metals act independently (no interaction between metals to cause more- or less-than-additive toxicity) in their binary-metal mixtures in the same way as the metals alone, the relative toxicity of their binary-metal mixtures would have decreased in the order of Cu+Zn, Cu+Ni, and Zn+Ni. However, our results indicate that the Cu+Ni mixture was the most toxic, with the lowest 96-h TTU10, 96-h TTU20, and 96-h TTU50 values (Table 4); the longest distance between points A and B in Figure 5; the most negative parameter *a*; and the greatest decrease in the SS (Table 3). The concentration–response curve of the Cu+Ni mixture also is shifted farthest to the left (Figure 7B). However, caution should be used when comparing the relative toxicities of the Cu+Zn and the Zn+Ni mixtures. These 2 mixtures had similar 96-h TTU50s; however, below these TTU points, the Cu+Zn mixture appeared to be more toxic than the Zn+Ni mixture. For example, the 96-h TTU10 for the Cu+Zn mixture (0.356) was lower than that for the Zn+Ni mixture (0.468; Table 4 and Figure 7B). An opposite relative effect can be seen for these 2 mixtures above the 96-h TTU50 points. The 96-h TTU90 was higher for the Cu+Zn mixture (1.245) than for the Zn+Ni mixture (0.877; Table 4 and Figure 7B). The difference in relative effects of these 2 mixtures can be seen by the difference in the slopes and intersection at the middle of their concentration–response curves. The Cu+Zn mixture had a lower slope than did the Zn+Ni mixture (Table 4). Although the slope of a concentration–response curve is not a reliable indicator for mechanism of toxicity, it is usually used as indicator for uptake and elimination rates of the toxicant (Rand et al. [57]). The steeper slope for the Zn+Ni mixture compared with the Cu+Zn mixture may indicate rapid absorption and rapid onset of effect after exposure to Zn+Ni simultaneously. Conversely, the shallower slope for the Cu+Zn mixture may be indicative of slow absorption, rapid excretion, or detoxification by fish when exposed to the Cu+Zn mixture.

In terms of individual metal exposures, the slopes of Cu, Zn, and Ni were not significantly different (Table 4). However, 2 of the 3 binary-mixtures slopes were higher than the slopes of their component individual-metal concentration–response curves. The mechanism for metals interacting in water and at the biological target sites of action that results in increasing slopes and toxicity in the binary-metal mixture is not clear.

Also, if the effect were simply additive, the concentration–response curves of the binary-metal mixtures would have crossed the dashed vertical line (TTU of 1) at their 96-h TTU50s. The shift of the 96-h TTU50s to the left of the dashed vertical line indicates more-than-additive effects (Figure 7B).

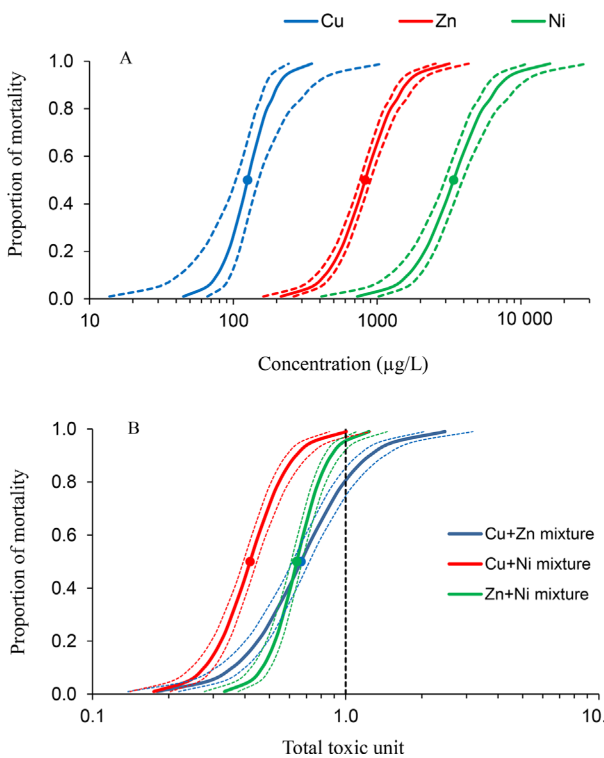

Figure 7. Concentration–response curves for individual metals (**A**) and binary-metal mixtures (**B**) fitted to the logistic model. Two boundary dashed curves of each response curve represent its 95% CI. The vertical dashed line crossing a total toxic unit (TTU) of 1 represents additive effect. The solid circles at the middle of each response curve represent the actual 96-h median lethal concentration (LC50) for individual metals and the 96-h median lethal effect concentration in toxic units (TTU50) of the binary-metal mixture.

CONCLUSIONS AND SUGGESTIONS

The results of the present study indicate that the acute toxicity of Cu, Zn, and Ni binary-metal mixtures to *P. promelas* was more-than-additive. These results suggest a joint and enhanced toxicity of metals in the mixtures. The present study has added to the literature of metal mixture toxicity, and the data can be applied to the BLM for metal mixtures in support of ecological risk assessment and regulatory purposes. Additional studies should be conducted to characterize the acute toxicity mechanism and chronic toxicity of metal mixtures.

Supplemental Data—Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3204.

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Data availability—Toxicity and water chemistry data are presented in the article. The SAS Program can be accessed via the Supplemental Data.

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