Non-Additive Toxicity of Bi-Metal Mixtures to Fathead Minnows

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ABSTRACT

Much research has been conducted to assess the toxicity of metals to aquatic organisms. Most of the research has focused on the toxicity of individual metals. Recently, attention has been paid to metal mixture toxicity because metals are usually present as mixtures in contaminated environments. The literature review indicates that metal mixtures may be additive, synergistic, or antagonistic to freshwater species. However, the data is not consistent and is dependent on the metal and organisms. The goal of this research is to use a systematic experimental design to characterize the toxicity of Cu, Zn, Cd and Ni mixtures to *Pimephales promelas*. Standard 96h toxicity tests were conducted with larval *P. promelas* based on the US EPA methods to determine metal mixture effects. All experiments were conducted in synthetic moderately hard water. Results of this study indicate that the toxicity of Cu-Zn, Cu-Ni, and Zn-Ni bi-mixtures was synergistic. These results suggest a joint mechanism of toxicity of these metal bi-mixtures in larval *P. promelas*. However, a biphasic dose response was found for Cd and Zn mixtures and *P. promelas*. The effect was antagonistic over all Zn concentrations. Results of this study are important for developing a Biotic Ligand Model for metal mixtures and useful for setting mixture water quality guidelines for metals.

Keywords: metal mixture toxicity, additive effect, antagonistic effect, *Pimephales promelas*
CHAPTER ONE

INTRODUCTION

Metal pollution has been an environmental issue for many decades. Zinc (Zn), copper (Cu), and nickel (Ni), are the most concerning metals because of their popular application. Zinc and copper are considered essential metals, those which are necessary for biological function at trace levels. Nickel is essential to some species at trace levels, although a human deficiency has not been reported [1]. Cadmium (Cd) is a nonessential metal. All four metals enter the environment from natural and anthropogenic sources. Natural sources primarily come from volcanic eruptions and erosion of igneous rocks and are minor compared to anthropogenic sources. Copper and zinc emission from industrial activities (e.g., mining, metal processing and production), and runoff from agricultural application (Cu and Zn containing pesticides, herbicides, fungicides, etc.) are the main anthropogenic sources. Nickel is largely used for the production of stainless steel. Cadmium is commonly used in electroplating and battery production.

According to the US EPA, background concentrations of Cu, Zn, and Ni in freshwater are up to 30 µg/L, 120 µg/L, and 470 µg/L, respectively. Background cadmium concentrations in uncontaminated environments are generally less than 2 µg/L [2].

According to Cammarota [3], the world consumption of Zn has increased from $974 \times 10^3$ tons (1950) to $1195 \times 10^3$ tons (1977) in North America, $22 \times 10^3$ tons (1950) to $180 \times 10^3$ tons (1977) in South America, $933 \times 10^3$ tons (1950) to $2898 \times 10^3$ tons (1977) in North America, and $22 \times 10^3$ tons (1950) to $180 \times 10^3$ tons (1977) in South America.
(1977) in Europe, and 80 x 10^3 tons (1950) to 1221 x 10^3 tons (1977) in Asia. Cammarota also predicted that US demand for Zn in 2000 was between 1.5 and 3.2 million tons and 8.3 million tons for the world. Based on the National Academy of Science (1975), worldwide Cu emissions from industrial activities were approximately 12 x 10^6 kg/year in early 1970s. This number increased to 20.55 x 10^6 kg/year in late 1970s [4].

In the aquatic environment, metals exist as different species and may be present in different oxidation states. Speciation determines free ion activity, but interactions at the biotic ligand must also be evaluated when determining bioavailability [5]. Although Cu, Zn, Ni have been isolated in different oxidation states (I, II, III) including Cu^{+2}, Zn^{+2}, Ni^{+2}, CuCO_3, ZnCO_3, NiCO_3, CuHCO_3^+, NiHCO_3^+, CuSO_4, ZnSO_4, NiSO_4, Cu(OH)_2, Zn(OH)_2, NiOH^+; oxidation state II is the most dominant in aquatic environments.

Considerable interest has focused on the speciation of Cu and Zn in natural waters because of their biological importance. Ionic Cu (Cu^{2+}) and Zn (Zn^{2+}) have been assumed to be the most bioavailable species and produce toxicological effects to aquatic organisms at elevated levels [6, 7]. Zn and Cu speciation in the aquatic environment is dependent on physical and chemical water characteristics. Increased pH decreases the ionic form of metals, hence decreasing bioavailability. Increased alkalinity will result in the formation of Zn and Cu carbonate species (e.g., CuCO_3, ZnCO_3, CuHCO_3^+). This will decrease concentrations of ionic Zn and Cu. Cu and Zn also compete with other cations (e.g., Ca, Mg) for binding sites at biotic ligands (e.g., fish gills). Therefore, raised Ca and Mg concentrations in the environment will result in fewer interactions between Cu and Zn with the biotic ligands. This competition makes Cu and Zn less bioavailable to aquatic organisms, and therefore decreases toxicity. Cu and Zn also complex with natural organic
matter (NOM) and form non-bioavailable forms (e.g., Cu-NOM, Zn-NOM). Therefore, elevated concentrations of NOM will also decrease Cu and Zn bioavailability and toxicity. Water quality parameters such as pH, hardness, alkalinity, and dissolved organic carbon (DOC) may affect metal speciation and therefore bioavailability. Specifically, increasing any of these criteria will decrease the bioavailability of ionic metals and decrease toxicity.

Metals are ionoregulatory and osmoregulatory disruptors to *Pimephales Promelas* (fathead minnows). The wide range of toxic effects of metal mixtures may be due to different modes of action or speciation. Specifically, copper toxicity in fathead minnows targets sodium ion uptake channels in the gill membrane [8]. Copper inhibits the Na+/K+-ATP pump on the basolateral membrane of the gill chloride cells, reducing active transport of sodium. Copper also replaces calcium at tight paracellular junctions, causing an excessive loss of sodium. The yolk sac regulates ion flow in larval fathead minnows, although less is known about the mechanism of toxicity. Whole body sodium concentration has been used as a biomarker for acute metal exposure to larval fathead minnows [9].

Zinc toxicity is believed to inhibit calcium uptake, resulting in decreased plasma calcium concentrations, followed by hypocalcemia [10, 11]. To a lesser effect, Zn also offsets the acid/base balance in fish [12]. Nickel also exhibits toxicity by blocking several different calcium channels and disrupting calcium homeostasis [13]. The specific mechanism of Ni toxicity to fathead minnows has not yet been elucidated. Cadmium acts as a calcium mimic due to its similar chemical properties, including ionic radius [14].
In the natural environment, metals are usually present as mixtures. When metals are present as mixtures, there is competition for binding to the biotic ligand. This competition affects toxicity varyingly depending on the specific metal and ion channel through which it passes. An additive effect (the toxicity of two metals is equal to the sum of their individual toxicities) would be observed if metals compete for binding sites at the same ion channel. When competition between a nonessential toxic metal (e.g., Cd) and an essential metal (e.g., Zn) is present, the essential metal has a stronger binding affinity to the target site and an antagonistic effect (the toxicity of two metals is less than the sum of their individual toxicities) would be produced. Synergistic toxic effects (the toxicity of two metals is greater than the sum of their individual toxicities) may be found when metals enter organisms independently at different ion channels.

Although single contaminants may be found in aquatic environments at levels too low to cause adverse effects, multiple contaminants at low levels may trigger toxicity due to additive or synergistic effects [15]. Additionally, single metal toxicity data is often used to estimate the assumed additivity of mixtures, using the toxic unit approach [16].

The degree of the toxic effect of these mixtures likely derives from differing mechanisms of toxicity. Therefore, noninteractive toxicants with similar sites of toxic action should produce additive effects [17]. Several studies have been conducted to determine the effects of metal mixtures on aquatic organisms [15, 18, 19, 20, 21, 22]. However, the results were inconsistent and dependent on the test species and specific metal mixtures. Preston et al [18] and Franklin et al [19] found that the toxicity of Cu and Cd mixtures to microorganisms (Pseudomonas fluorescens) and freshwater alga (Chlorella sp.) was more than additive (synergistic). However, the effect of a Cu and Cd
mixture on aquatic plants (*Silene vulgaris*) was nonadditive (antagonistic) [20]. Franklin et al [19] also reported that the toxicity of their Cu and Zn mixture, and Cd and Zn mixture to *Chlorella sp.* was antagonistic. According to Spehar and Fiandt [23], the toxicity of six metal mixtures (As, Cd, Cr, Cu, Hg, Pb) was synergistic to fathead minnows (*Pimephales promelas*) but additive to daphnids (*Ceriodaphnia dubia*). Data on the toxicity of Cu and Zn mixtures to fathead minnows are lacking in the literature, and studies on the influence of water quality parameters on the toxicity of metal mixtures have not been conducted.

Although there is a significant body of data related to metal toxicity to aquatic and terrestrial organisms [9, 17, 18, 19, 21, 22, 24, 25, 26], there have been few studies conducted to test the toxicity of metal mixtures. This is problematic since most metal contamination is present as mixtures in the environment, making it difficult to estimate the contribution of the individual metals. It is also difficult to predict mixture toxicity based on individual metal data. Currently, most regulatory bodies treat mixtures as their separate components and assume additivity [15]. There are significant consequences if this assumption is not correct.

The goal of this research is to characterize the toxicity of binary metal mixtures of Cu, Zn, Ni, and Cd to fathead minnows, and to determine whether the toxicity of metal bi-mixtures of these four metals produce additive (1+1=2), more than additive (1+1>2), or less than additive (1+1<2) effects. Mixture effects are traditionally calculated via the toxic unit (TU) method, which assumes strict concentration additivity [16]. The toxic unit approach allows separate toxicants in a mixture to be expressed as fractions of that mixture [25]. Specifically, mixture components are expressed as a fraction of the LC50.
concentration. Fathead minnows were selected for these studies because they are an EPA recommended species for toxicity testing, and easy to culture.
CHAPTER TWO
MATERIALS AND METHODS

Experimental design

To evaluate the toxicity of metal bi-mixtures to larval fathead minnows, 96 hour renewal acute toxicity tests were conducted in synthetic moderately hard water. Experiments followed a specific design to establish a baseline for individual metal tests and to allow comparison for mixture tests (Table 1). The tests were conducted with individual metal and bi-metal mixtures. Treatment concentrations in the mixture tests were half of the treatment concentrations in the individual metal tests. This design allows for comparison for additive or non-additive effects of bi-metal mixtures (Table 1a). For example, the effect was more than additive if mortality of a mixture treatment with concentration A+B is higher than the total mortality of the individual metal treatments with the same concentration (A, B).

Results of single metal tests gave LC-50 values for larval fathead minnows and the active range of concentrations for each single metal. Although there is a significant body of data on single metal toxicity to larval fathead minnows, there may be some variation in toxicity between populations of the same age due to different water quality. Results of single metal tests determined the concentrations to be used for all metal mixture tests, to result in partial mortality with which we could analyze. Specific concentrations were chosen in order to produce partial mortality in mixture tests, so that a statistical analysis could be completed (Table 1). Single metal concentrations ranged
from 50-800 µg/L, 100-1600 µg/L, 500-8000 µg/L, for Cu, Zn, and Ni respectively. Metal concentrations in treatments with 100% mortality were not repeated in mixture tests so that any possible synergistic effects could be detected. Metal concentrations in the mixture tests ranged from 25-400 µg/L, 50-800 µg/L, and 250-2000 µg/L, for Cu, Zn, and Ni respectively. The endpoint in all single metal and mixture tests was mortality.

For Cd-Zn mixture tests, the experiment conducted followed a different experimental design in order to detect antagonistic toxicity (Table 1b). During this mixture test, Cd was held constant at a concentration known to produce partial mortality. Zn concentration was varied over seven treatments. The Cd concentration of 30 µg/L was chosen based on a preliminary test conducted with larval fathead minnows and Cd alone (unpublished data).

**Toxicity Testing**

All tests were performed using the EPA standard method [26]. Test water was prepared using 16MΩ (MilliQ) water (Barnstead E-pure) and an addition of sodium bicarbonate, calcium sulfate, magnesium sulfate, and potassium chloride based on the U.S. EPA standard methods for toxicity testing [26]. All test chambers were washed with nitric acid, and then rinsed with 16MΩ water to avoid any interference from contamination. Each test had at least five metal concentrations. All tests contained a control group for comparison. Replicates contained 10 or 20 fish each, depending upon the availability of healthy larval fish. However, the number of fish per replicate was the same within each test. Statistical analyses were strengthened with a greater number of fish, as variability between replicates was reduced. Tests were conducted at 25 ± 2°C with a photoperiod of 16h light and 8h dark.
Table 1. Experimental Design for Single and Bi-metal Mixture Tests

Copper, nickel, and zinc bi-mixture
(A= 50 µg/L Cu, B= 100 µg/L Zn, C= 500 µg/L Ni)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Individual</th>
<th>Mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test 1</td>
<td>Test 2</td>
</tr>
<tr>
<td>Cu</td>
<td>Zn</td>
<td>Ni</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>2</td>
<td>2A</td>
<td>2B</td>
</tr>
<tr>
<td>4</td>
<td>8A</td>
<td>8B</td>
</tr>
<tr>
<td>5</td>
<td>16A</td>
<td>16B</td>
</tr>
</tbody>
</table>

Cadmium and zinc mixture

<table>
<thead>
<tr>
<th>Concentration (µg/L)</th>
<th>Control</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Zn</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>800</td>
</tr>
</tbody>
</table>

Treatments of test solutions were made from prepared synthetic moderately hard water and desired quantities of metal stock solutions. For the stock solutions, Cu was added as CuSO\(_4\)·5H\(_2\)O, Zn was added as ZnCl\(_2\), Ni was added as NiSO\(_4\)·6H\(_2\)O, and Cd was added as CdSO\(_4\). Stock solutions of Cu, Zn, Ni, and Cd were made to desired concentrations, and then verified by ICPMS. Test solutions were prepared at least two hours before organisms were added to allow ample time for equilibration. Water quality such as dissolved oxygen (DO), pH, temperature, and conductivity was measured one hour after preparing test solutions. DO and temperature were measured using a YSI 550A dissolved oxygen meter (Fisher Scientific, Hanover Park, Illinois, USA). pH was measured using an Accumet AP 110 pH meter (Fisher Scientific, Hanover Park, Illinois, USA).
Conductivity was measured using a YSI 30 conductivity meter (Fisher Scientific, Hanover Park, Illinois, USA). Water quality parameters such as hardness and alkalinity were measured at test initiation and termination. Water hardness was determined by titration with 0.01M ethylenediaminetetraacetic acid (EDTA). Alkalinity was determined by titration with 0.02N H\textsubscript{2}SO\textsubscript{4}. Average DO, pH, and temperature values were 8.3 ± 0.4 mg/L, 7.65 ± 0.11, and 23 ± 0.5°C respectively. Average hardness and alkalinity was 103 ± 10 mg/L as CaCO\textsubscript{3}, and 67 ± 4 mg/L as CaCO\textsubscript{3} respectively.

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

Fish were then impartially distributed into test chambers one or two at a time, to ensure randomization. Only fish which appeared healthy were used for testing. Testing of individual metals was carried out side by side with the corresponding mixtures to confirm that previous individual metal sensitivity had not significantly changed. All tests were static exposures, and the containers were not aerated. Each day, test containers were moved to eliminate position effects.

DO, pH, and temperature were measured at exactly 24, 48, 72, and 96 hours after test initiation. After recording daily measurements, mortality was recorded for every replicate of each treatment. Any dead fish present were removed daily. After 96 hours of
testing, any surviving individuals were euthanized with methane tricaine sulfonate (MS-222) and discarded accordantly with standard procedure.

Water samples were collected for total and dissolved metal anion and cation analyses. Dissolved metal samples were filtered using a 0.45µm Whatman™ filter (GE Healthcare Life Sciences). All samples were acidified with HNO₃ to about pH 2 and stored at 4°C in a refrigerator prior to analysis. Analysis of metals and cations was performed with a NeXion 300S Inductively Coupled Plasma Mass Spectrometer (Perkin Elmer, Oak Brook, IL, USA). Samples for anion analysis were also filtered using a 0.45µm Whatman™ filter (GE Healthcare Life Sciences) and analyzed with an Ion Chromatograph (Metro Ω, Northbrook, IL, USA).

**Data Analysis**

Lethal effects concentrations (96h LC-50s) were used for effects analysis. LC50 values were calculated from mortality data using Toxcalc, a statistical package designed for environmental toxicity testing [27]. Mortality data generated a dose response curve, and data was evaluated by Dunnett’s multiple comparison test (p<.05).

Mortality data were used for effect analysis. A side by side treatment comparison for mortality of single metal tests and the corresponding mixture tests was conducted to test whether there was a significant difference between the two tests using the T-test method. For example, if mortality of a mixture treatment was not significantly different from the sum of mortality from corresponding individual metal treatments, then we could reasonably assume that toxicity was additive. If mixture mortality was significantly more or less than the sum of the corresponding individual mortality, we can reasonably say that toxicity was synergistic or antagonistic, respectively. An effect with a \( p \leq 0.05 \) is
considered significant. Data were also tested to make sure that the assumption of homogeneous variance and normal distribution was met prior to using it for treatment comparisons.

A side by side comparison of single metal tests and the corresponding mixture tests was conducted to test whether there was a significant difference between the two tests. Replicates from the same treatment were tested for equal variance to ensure consistency in all treatments. If there was no significant difference between replicates, a T-test was performed to generate a two tailed p-value. Mixture mortality was compared with the sum of the mortality from the two single metal tests of identical concentrations to test for a significant difference (p< 0.05).

In addition to cross treatment comparison, the TU approach was also used to determine additive and non-additive effects [16]. 96-h LC50 values for individual metals were used to calculate TU. For metal bi-mixture tests, exposure concentrations were normalized to TU for calculating lethal effect in TU. The total toxic unit (TTU) of two metals in each exposure treatment can be calculated using the below formula [28]:

\[
TTU = TU_a + TU_b = \frac{C_a}{LC50_a} + \frac{C_b}{LC50_b}
\]  

(1)

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 lethal effect concentrations of individual metals A and B, respectively. \(TU_a\) and \(TU_b\) are toxic units of metals A and B, respectively. Mortality data and TU were used to calculate lethal effect in TTU (96h-LTTU) for metal mixtures. 96h-LC50 and 96h-LTTU were calculated by Toxcalc software using the Probit method.
Based on formula (1), one TU of each individual metal is equivalent to 50% mortality. Therefore, a TTU of 2 for a bi-metal mixture would be equivalent to 100% mortality, and a TTU of 1 for that bi-metal mixture would be equivalent to 50% mortality. Using this concept, we can determine the mixture effect by comparing the 96-h LTTU with a TTU of 1. If LTTU is equal to 1, we can say the effect is additive. If LTTU is less than 1, we can say the effect is more than additive (synergistic). If LTTU is greater than 1, we can say the effect is less than additive (antagonistic) [28].
CHAPTER THREE

RESULTS AND DISCUSSION

Individual and Bi-mixture Toxicity of Cu, Zn, and Ni

Results of individual and bi-mixture tests with Cu, Zn, and Ni in *P. promelas* are shown in Table 2. For mixture tests, specific metal bi-mixtures produced a wide range of toxic responses to fathead minnows (Table 2). In general, mortality ranged from 0% to 100%. To avoid under evaluation of the effect, only treatments that produced partial mortality (< 100%) were chosen for effect analysis. Results of these treatments are presented in Figure 1. For all Cu-Zn, Cu-Ni, and Zn-Ni mixture tests, the effects were more than additive.

Since the range of concentrations of the second metal had been tested alone previously, we summed the individual mortality data of the corresponding concentrations for comparison. The summed data was then compared to the corresponding mixture data and was tested for equal variance followed by a p-test. If mixture mortality was not significantly different from the sum of mortalities from individual metal tests across all treatments, then we could reasonably assume that toxicity was additive. If mixture mortality was significantly more or less than the sum of mortalities from individual metal tests across all treatments, we can reasonably say that toxicity was synergistic or antagonistic, respectively.

Specific metal bi-mixtures produced a wide range of toxic responses to fathead minnows. Table 2 displays mortality data of the mixture tests. However, not all of these
treatments produced partial mortality. Therefore, treatments which could not be evaluated statistically have not been included in this cross treatment comparison. Table 3 shows all of the treatments where partial mortality was observed. For all Cu-Zn, Cu-Ni, and Zn-Ni mixtures the effects were more than additive. For Cu and Zn (Fig. 1a), no mortality was observed in individual metal tests at 50 µg/L Cu or 100 µg/L Zn. In the mixture test utilizing the same concentrations, 27% mortality was observed. At 100 µg/L Cu and 200 µg/L Zn, the sum of mortality from individual tests was 23%. This was significantly lower than the mixture mortality (60%). At 200 µg/L Cu and 400 µg/L Zn, the sum of individual mortalities was 40%, but mixture mortality was 97% (Table 3). There was no mortality in the control group for this test.

A similar pattern was observed for Cu-Ni mixtures (Figure 1b, Table 3). No significant mortality was observed in an individual test utilizing Ni concentrations of 1000 µg/L or less, and Cu concentrations of 50 µg/L or less. However, in a mixture of 50 µg/L Cu and 500 µg/L Ni, 30% mortality occurred. In a mixture containing 100 µg/L Cu and 1000 µg/L Ni, 97% mortality was observed. However, the sum of individual metal mortalities was only 21%. For 1200 µg/L Ni and 100 µg/L Cu, the sum of individual mortalities was 31% (likely due to Cu only), but mixture mortality was 97%. In a mixture containing 130 µg/L Cu and 1500 µg/L Ni, 97% mortality was observed while the sum of the individual mortalities was 44%. There was also no mortality in the control group.

For Zn-Ni mixtures (Figure 1c, Table 3), toxicity was also more than additive. At or below concentrations of 100 µg/L Zn and 500 µg/L Ni, no mortality was observed. At 200 µg/L Zn and 1000 µg/L Ni, the sum of individual mortalities was 3% which was not significantly different from the mixture mortality (7%). At 400 µg/L Zn and 1500 µg/L
Ni, the sum of individual mortalities was 13%. Mixture mortality at these same concentrations was 67%. At a concentration of 800 µg/L Zn and 2000 µg/L Ni the sum of the individual mortalities was 57%, but mixture mortality was 100%. No mortality was observed in the control groups of these tests. Results of these tests indicate that the effect of Zn and Ni was more than additive over most concentrations.

Results of 96h-LC50 for individual metal tests and 96h-LTTU for bi-metal mixture tests are shown in Table 4 and Figure 4. Using dissolved concentrations measured via ICPMS, 96h-LC50 values for individual Cu, Zn, and Ni were 124, 817, and 3309 µg/L, respectively. The 95% confidence intervals for these 96h-LC50s were 80-150, 428-2206 and 2283-4864 µg/L, respectively. These results indicate that the toxicity decreased in the order of Cu, Zn, and Ni. 96h-LTTU values for Cu-Zn, Cu-Ni, and Ni-Zn mixtures were 0.700, 0.458, and 0.704, respectively (Figure 4). The 95% confidence intervals for these 96h-LTTUs were 0.571-0.780, 0.406-0.515, and 0.629-0.779, respectively. All of the 96h-LTTUs were less than one indicating that the effect of the metal bi-mixtures was more than additive. With the lowest 96h-LTTU, the effect of Cu-Ni mixtures appeared to be the most profound among the three metal mixtures. Using the Probit model method for individual Cu, Ni, Zn, the slopes of the dose-response curves were 5.88, 4.18, and 3.75 (Figure 2). For Cu-Zn, Cu-Ni, and Zn-Ni mixtures, the slopes were 4.67, 8.13, and 9.59, respectively (Figure 3).

Since no antagonistic effect was observed for Cu, Ni, and Zn bi-mixtures tests, dose response curves were plotted for both individual and mixture tests for comparison of the relative toxicity within the individual metals and bi-metal mixtures (Figures 2, 3). For individual metals, the active concentration range for Cu was lowest and followed by Zn
and Ni (Figure 2). These results indicate that the relative toxicity of these metals increased in the order of Cu, Zn, and Ni. If these metals produced independent effects, the relative toxicity of bi-mixtures would have increased in the order of Cu-Zn, Cu-Ni, and Zn-Ni. However, the dose response curve for Cu-Ni mixture shifted to the low end of the TTU range and was followed by Zn-Ni and Cu-Zn mixtures (Figure 4).

The present study found that the effects of Cu, Zn, and Ni bi-mixtures were more than additive to fathead minnows. At the time of the literature review for this paper, no data existed characterizing the acute toxicity of Cu-Zn, Cu-Ni, or Zn-Ni bi-metal mixtures to larval fathead minnows. According to Sprague et al. [25], Cu-Zn mixtures were synergistic to juvenile salmon. However, according to Parrott et al. [29], Cu-Zn mixtures were antagonistic to DNA, RNA, and the protein content of larval fathead minnows. A difference in mechanism for aquatic metal ions to larval fathead minnows versus isolated groups of cells may account for this difference in toxicity. Kangarot [30] characterized Zn-Ni mixtures as more than additive to the common guppy when Ni was present in higher concentrations than Zn. Kangarot [30] also characterized Cu-Ni mixtures as synergistic to the common guppy, which is in agreement with our data.
Table 2. Toxicity of Individual Metal Exposures and Bi-metal Mixture Exposures to Larval Fathead Minnows

(A= 50 µg/L Cu, B= 100 µg/L Zn, C= 500 µg/L Ni)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Test 1 Cu (µg/L)</th>
<th>Mortality (%)</th>
<th>Test 2 Zn (µg/L)</th>
<th>Mortality (%)</th>
<th>Test 3 Ni (µg/L)</th>
<th>Mortality (%)</th>
<th>Test 4 Cu (µg/L)</th>
<th>Zn (µg/L)</th>
<th>Mortality (%)</th>
<th>Test 5 Cu (µg/L)</th>
<th>Ni (µg/L)</th>
<th>Mortality (%)</th>
<th>Test 6 Zn (µg/L)</th>
<th>Ni (µg/L)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>A 0 ± 0</td>
<td>0</td>
<td>B 0 ± 0</td>
<td>0</td>
<td>C 1 ± 2.5</td>
<td>0</td>
<td>0.5</td>
<td>A 0 ± 0</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>B 0 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2A 20 ± 0</td>
<td>2B 3 ± 6</td>
<td>2C 1 ± 2.5</td>
<td>A B 27 ± 6</td>
<td>A C 30 ± 20</td>
<td>B C 0 ± 0</td>
<td>0.5</td>
<td>A 0 ± 0</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>B 0 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4A 37 ± 0.2</td>
<td>4B 3 ± 0.6</td>
<td>2C 10 ± 10</td>
<td>2A 2B 60 ± 10</td>
<td>2A 2C 97 ± 6</td>
<td>2B 2C 7 ± 0.6</td>
<td>0.5</td>
<td>A 0 ± 0</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>B 0 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>8A 87 ± 15</td>
<td>8B 40 ± 17</td>
<td>4C 17 ± 10</td>
<td>4A 4B 97 ± 0.02</td>
<td>4A 3C 100 ± 0</td>
<td>4B 3C 67 ± 6</td>
<td>0.5</td>
<td>A 0 ± 0</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>B 0 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>16 A 100 ± 0</td>
<td>16 B 90 ± 10</td>
<td>6C 47 ± 17</td>
<td>8A 8B 100 ± 0</td>
<td>8A 4C 100 ± 0</td>
<td>8B 4C 100 ± 0</td>
<td>0.5</td>
<td>A 0 ± 0</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>B 0 ± 0</td>
<td>0</td>
</tr>
</tbody>
</table>


Table 3. Treatment Comparison for Additive and More Than Additive Effects of Metal Mixtures

<table>
<thead>
<tr>
<th>Concentration (µg/L)</th>
<th>Mixture mortality (%)</th>
<th>Sum of individual metal mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>Zn</td>
<td>Cu</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>8B</td>
<td>97 ± 6</td>
</tr>
<tr>
<td>2A</td>
<td>2B</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>4A</td>
<td>4B</td>
<td>97 ± 0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (µg/L)</th>
<th>Mixture mortality (%)</th>
<th>Sum of individual metal mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>Ni</td>
<td>Cu</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>30 ± 20</td>
</tr>
<tr>
<td>2A</td>
<td>2C</td>
<td>97 ± 6</td>
</tr>
<tr>
<td>2A</td>
<td>2.4C</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>2.6A</td>
<td>3C</td>
<td>97 ± 7.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (µg/L)</th>
<th>Mixture mortality (%)</th>
<th>Sum of individual metal mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>Ni</td>
<td>Zn</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3 ± 6</td>
</tr>
<tr>
<td>2B</td>
<td>2C</td>
<td>7 ± 0.6</td>
</tr>
<tr>
<td>4B</td>
<td>3C</td>
<td>67 ± 6</td>
</tr>
</tbody>
</table>
Figure 1. Total mortality of fathead minnows due to individual metals versus mortality due to bi-metal mixtures (Cu and Zn, Cu and Ni, Ni and Zn)
Figure 2. Mortality of fathead minnows due to individual metal exposures

Figure 3. Mortality of fathead minnows due to metal mixture exposures
Table 4. Lethal Effect Concentrations for Individual Metals and Lethal Effect in Toxic Unit for Metal Bi-mixtures

<table>
<thead>
<tr>
<th>Metals</th>
<th>96h-LC50 (95% CI)a (µg/L)</th>
<th>96h- LTTU (95% CI)</th>
<th>Slopes of dose response curvesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>124 (80 – 150)</td>
<td>NA</td>
<td>5.88</td>
</tr>
<tr>
<td>Zn</td>
<td>817 (428 – 2206)</td>
<td>NA</td>
<td>4.18</td>
</tr>
<tr>
<td>Ni</td>
<td>3309 (2283 – 4864)</td>
<td>NA</td>
<td>3.75</td>
</tr>
<tr>
<td>Cu + Zn</td>
<td>NA</td>
<td>0.700 (0.571 – 0.780)</td>
<td>4.67</td>
</tr>
<tr>
<td>Cu + Ni</td>
<td>NA</td>
<td>0.458 (0.406 – 0.515)</td>
<td>8.13</td>
</tr>
<tr>
<td>Zn + Ni</td>
<td>NA</td>
<td>0.704 (0.629 – 0.779)</td>
<td>9.59</td>
</tr>
</tbody>
</table>

aData in parentheses are 95% confidence intervals
bSlopes were determined by the Probit method
NA: not applicable

Figure 4. Total toxic units (TTUs) for Cu, Zn, and Ni bi-metal mixtures
Although the mechanism of bi-metal mixture toxicity cannot be elucidated from this study, the more than additive effects of Cu, Zn and Ni bi-mixtures found for fathead minnows, the common guppy, and salmon suggest a joint toxicity of metals in the mixtures. The mechanism of acute toxicity of individual Cu, Zn and Ni in fish is attributed to the inhibition of osmotic ions (e.g., Na, Ca) by metals at the chloride cell membrane of the fish gills. This inhibition would result in a decrease in the uptake of osmotic ions, causing imbalance of osmotic pressure in the plasma and exerting toxic effects. Therefore, for metals that inhibit the same osmotic ion, the toxic effect is expected to be additive. Metals that cause inhibition at different ion channels inhibit multiple ions and produce more than additive toxic effects. Cu primarily targets sodium channels, while Zn targets calcium channels [11]. Nickel is believed to target both Ca and Na channels [11, 31]. Therefore, when exposed to individual metals, the organisms are likely losing individual osmotic ions corresponding to the metal target channel. In metal mixture exposures, the organisms would lose multiple osmotic ions simultaneously. This likely would enhance the imbalance in osmotic pressure in the plasma and produce greater toxic effects to the organisms. This mechanism would explain the more than additive toxicity of Cu-Zn and Cu-Ni bi-mixtures found in the present study. The Zn-Ni bi-mixture also produced more than additive toxicity, despite their similar mechanism of toxicity via calcium channel inhibition. The observed synergism may be due to the secondary inhibition effect of Ni on Na uptake. This may also explain the most profound synergistic effect of Cu and Ni mixtures, because fish would lose more Na due to the secondary inhibition effect of Ni.
It is worthwhile to note the relative toxicity of metals used in this study. The slopes and order of the response curves are indicative of the metals’ relative toxicity to fathead minnows (Figures 2, 3). The steepness of the curve indicates high potency even at low concentrations seen in this study. Cu was the most toxic having the steepest slope (5.88) and lowest LC50 value (124 µg/L). Ni was the least toxic with flattest slope (3.75) and highest LC50 (3309 µg/L). For mixture tests, if the effect produced by each individual metal is independent from the others, the slope of the dose response curve would have increased in the order of Zn-Ni, Cu-Ni, and Cu-Zn mixtures. However, a reversed order of the slopes was found in this study. Cu-Zn mixtures had the flattest slope (4.67) followed by Cu-Ni (8.13) and Zn-Ni (9.59) mixtures (Figure 3). This indicates that the joint effect of Cu and Ni in the mixture was the most profound. This may be due to the secondary inhibition of Ni on Na uptake.

Fewer data points for Cu-Ni and Zn-Ni mixtures were produced from this study, but a trend was clearly observed for both mixtures. It is worth noting that this study was conducted using a specific set of water quality criteria, and that the toxic effects produced are specific to those criteria. If any of the values of pH, hardness, alkalinity, or DOC were changed, the same effects would not necessarily be observed.

**Individual and Bi-mixture Toxicity of Cd and Zn**

Results of Cd-Zn mixture toxicity are shown in Table 5. Dose repose curves for individual Zn and Cd-Zn mixtures were plotted in Figure 5. Antagonistic toxicity was observed over all treatment concentrations for Cd-Zn mixtures. In a treatment containing 30 µg/L Cd and no Zn, mortality reached 90%. Upon the addition of 25 µg/L Zn, mortality decreased to 63%; nearly a 30% reduction. With the addition of 50 µg/L Zn,
mortality continued to decrease to 43%. At 100 µg/L Zn, the maximum protective effect was observed. At this point, mortality was reduced to just 33%. Above 100 µg/L Zn, mortality began to increase. At 200 µg/L Zn, mortality rose to 70%. At 400 µg/L Zn, mortality was 67%. However, there was no significant difference in mortality of these two treatments. At 800 µg/L Zn, mortality was 100%.

In Cd-Zn mixtures, Zn has historically offered some protection against the deleterious effects of Cd [14, 32, 33, 34, 35]. The protective effect of Zn from Cd toxicity is attributed to competitive binding between Cd and Zn to the biotic ligand. Zinc finger proteins (ZFs), a large family of metalloproteins that utilize zinc ions for structural integrity, have been known as target proteins for Cd [33, 35, 36, 37, 38]. It has been reported that the affiliation between Zn and ZFs is stronger than the affiliation between Cd and ZFs [33, 36, 37, 38, 39, 40]. This competition, therefore, would prevent Cd from binding to the biotic ligand and reduce the toxic effect.

In this study, however, a biphasic dose-response occurred at a wide range of Zn concentrations. At Zn concentrations ≤ 100 µg/L, Zn protected against Cd toxicity (protective phase). This result is in agreement with the results of other studies conducted with a variety of species. Odendaal et al. [41] found that Cd-Zn mixtures were antagonistic to the terrestrial isopod *P. laevis*. Cd-Zn mixtures were also antagonistic to *D. magna* [42], *S. vulgaris* [42], the earthworm *A. caliginosa* [43] and the clam *A. cygnea* [44].

When the Zn concentration was greater than 100 µg/L, Zn likely contributed to toxicity (joint toxicity phase). At its most protective concentration, Zn decreased toxicity by 57%. However, after this point, mortality increased (eventually to 100%) with
increasing Zn concentration (Figure 5). Therefore, in the case of larval fathead minnows, more Zn does not necessarily mean more protection against Cd toxicity. The mechanism of competitive binding to the target biotic ligands can explain the effect in the protective phase but likely does not explain the effect in the later phase. At Zn concentrations of 200 or 400 µg/L, there was no significant mortality compared with control in the individual Zn exposure test (Table 5, Figure 5). However, about 70% mortality occurred in the Cd-Zn bi-mixture test (Figure 5). When Zn concentration increased to 800 µg/L, 100% mortality was observed in the Cd-Zn bi-mixture test but only 40% mortality was observed in the individual Zn experiment. These results indicate that even at the most protective concentration of Zn, Cd still plays a role in producing a toxic effect. It is unclear how much of the mortality is caused by Cd versus Zn. There must be an interaction between Zn and Cd which causes the observed increase in mortality.

Table 5. Toxicity of Cd-Zn Individual Metal and Bi-metal Mixture Exposure to Larval Fathead Minnows

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Individual Zn</th>
<th>Cd-Zn mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn (µg/L)</td>
<td>Mortality (%)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>3 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>6</td>
<td>400</td>
<td>40 ± 17</td>
</tr>
<tr>
<td>7</td>
<td>800</td>
<td>90 ± 10</td>
</tr>
</tbody>
</table>
Figure 5. Dose response curves for fathead minnows exposed to individual Zn and bi-mixtures of Cd and Zn

![Dose response curves](image)

**Speciation**

Water samples were collected at test initiation and termination for analysis of metals, cations (Ca, Mg, K, Na), and anions (Cl, CO$_3^-$, HCO$_3^-$, SO$_4^{2-}$, NO$_3^-$, NO$_4^-$). Metal and cation concentrations in these samples were analyzed using Inductively Coupled Plasma Mass Spectrometry (ICPMS) for all tests. Anions were analyzed by Ion Chromatography (IC).

The Minteq model is an equilibrium model used to calculate chemical speciation in natural waters. Metal, anion, and cation concentrations were used in this model to generate unique speciation for selected treatments Day zero and day four sample data were averaged and input to the Minteq model [45]. Input parameters for this model include: Cu, Zn, Ni, Cl, SO$_4^{2-}$, CO$_3^-$, Mg, Ca, Na, K, and pH. Ionic strength was calculated based on input parameters, and temperature was fixed at 25°C. Based on this data,
concentrations of the metal species presumed present were generated (Figures 6-14). These species were also expressed as percentages (Figures 15-23).

As previously stated, the ionic forms of Cu, Zn, and Ni are the species which are most bioavailable to produce toxicity (although not all bioavailable species will produce toxic effects). To a lesser extent, hydroxide species also contribute to toxicity. Therefore, metal species other than the toxic $^{2+}$ and OH$^-$ forms should be unavailable and virtually nontoxic to fathead minnows. Since synthetic moderately hard water constituted the base water for all tests, speciation was expected to be similar in individual and mixture tests.

Only data generated from one treatment producing partial mortality in Cu, Zn, and Ni individual and mixture tests were run through the model. In order to compare speciation and bioavailability between individual and mixture tests, the treatment which was closest to the LC50 value for each metal was input to the Minteq model, thus ensuring partial mortality which could be analyzed. Percentages of all metal species can be found in Figures 15-23. As expected, speciation was conserved when comparing individual metal tests and mixture tests (Figures 15-23), due to constant pH throughout all tests. Ni and Zn were the two most bioavailable metals, with about 80% of the total metal present in the ionic form for all tests. Cu was predominantly present as CuCO$_3$, with under 10% present in the ionic form for all tests. CuOH$^+$ constituted about another 10% of all species for all tests. Cu was less than 20% bioavailable in all tests, but produced the most mortality at the lowest concentrations. This supports the conclusion that Cu was the most toxic of the three metals.

From the data generated by the Minteq model, Table 6 was assembled to compare the bioavailable constituents of each metal from individual and mixture experiments.
Since the ionic and hydroxide forms may produce toxicity, these species’ concentrations were summed to generate total bioavailability. Bioavailability was lower across the mixture tests than in the individual tests. Despite lower bioavailability, these mixture treatments produced significantly more mortality than the sum of corresponding individual treatments. For example, in the Cu-Zn mixture, the concentration of bioavailable metals was 48% lower than the sum of the corresponding bioavailable individual metal concentrations (Table 6, Figures 6-9). However, this mixture treatment produced 97% mortality, while the sum of individual metal mortality was only 40%. Similar results can be observed from Cu-Ni mixtures (Table 6, Figures 6, 10-12), and Zn-Ni mixtures (Table 6, Figures 7, 10, 13, 14). Therefore, bioavailability is not necessarily directly proportional to toxicity. This may be related to the different mechanisms of multiple metals in mixtures contributing to the greater imbalance of osmotic ions.

A Biotic Ligand Model (BLM) was recently developed to evaluate the toxicity of metals based on water quality characteristics (46, 47, 48, 49). The BLM was developed using the data on the influence of water quality characteristics on the toxicity of individual metals and is currently used by the U.S. EPA for evaluating water quality criteria for Cu in freshwater ecosystems [50]. Given the frequency of metal mixture contamination in the natural environment, the current BLM is not always relevant.

The present data regarding the toxicity of metal mixtures are both species and specific mixture dependent. No research has been published regarding the toxicity of Cu, Zn, and Ni mixtures to Pimephales promelas (fathead minnows) while varying water quality parameters. Results of this study will also support development of a BLM for Cu, Zn, Ni and Cd mixtures that is most useful for evaluating water quality criteria for Cu and
Zn in freshwater ecosystems. By advancing the BLM, more accurate toxicity data will become available which can also assist in choosing which metal contaminated sites are the most appropriate choices for cleanup. This is especially applicable to poorer nations which may not be able to allocate a significant amount of money toward costly cleanup.

Table 6. Speciation of Cu, Zn, and Ni in Individual and Mixture Tests Expressed as Concentrations and Percentages Generated by the Minteq Model

<table>
<thead>
<tr>
<th>Tests</th>
<th>Metal Species</th>
<th>Percent of total metal</th>
<th>Bioavailability (mmol/L)</th>
<th>Total bioavailability (mmol/L)</th>
<th>Sum of Individual bioavailability</th>
<th>Sum of Mixture bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu alone</td>
<td>Cu^{2+}</td>
<td>6</td>
<td>0.00013</td>
<td>0.00034</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CuOH^-</td>
<td>10</td>
<td>0.00020801</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn alone</td>
<td>Zn^{2+}</td>
<td>78</td>
<td>0.0087929</td>
<td>0.00920</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ZnOH^-</td>
<td>4</td>
<td>0.00040738</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni alone</td>
<td>Ni^{2+}</td>
<td>83</td>
<td>0.03775</td>
<td>0.03789</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NiOH^-</td>
<td>0</td>
<td>0.00014878</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu-Zn</td>
<td>Cu^{2+}</td>
<td>9</td>
<td>0.00020</td>
<td>0.00041</td>
<td>0.00954</td>
<td>0.00457</td>
</tr>
<tr>
<td></td>
<td>CuOH^-</td>
<td>10</td>
<td>0.00021612</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zn^{2+}</td>
<td>82</td>
<td>0.00402</td>
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</tr>
<tr>
<td></td>
<td>ZnOH^-</td>
<td>3</td>
<td>0.00013963</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cu-Ni</td>
<td>Cu^{2+}</td>
<td>9</td>
<td>0.00007</td>
<td>0.01300</td>
<td>0.03824</td>
<td>0.02599</td>
</tr>
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<td></td>
<td>CuOH^-</td>
<td>10</td>
<td>0.00008295</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ni^{2+}</td>
<td>82</td>
<td>0.01293</td>
<td>0.01299</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NiOH^-</td>
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<td>0.000058703</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn-Ni</td>
<td>Ni^{2+}</td>
<td>83</td>
<td>0.02033</td>
<td>0.02040</td>
<td>0.04710</td>
<td>0.02420</td>
</tr>
<tr>
<td></td>
<td>NiOH^-</td>
<td>0</td>
<td>0.000072406</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zn^{2+}</td>
<td>84</td>
<td>0.00370</td>
<td>0.00380</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ZnOH^-</td>
<td>2</td>
<td>0.00010466</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6. Cu speciation in individual test

Figure 7. Zn speciation in individual test

Figure 8. Cu speciation in Cu-Zn mixture

Figure 9. Zn speciation in Cu-Zn mixture
Figure 6. Cu speciation in individual test

Figure 10. Ni speciation in individual test

Figure 11. Cu speciation in Cu-Ni mixture

Figure 12. Ni speciation in Cu-Ni mixture
Figure 7. Zn speciation in individual test

Figure 10. Ni speciation in individual test

Figure 13. Zn speciation in Zn-Ni mixture

Figure 14. Ni speciation in Zn-Ni mixture
Figure 15. Cu speciation as percentages in individual metal test

Figure 16. Zn speciation as percentages in individual metal test

Figure 17. Cu speciation as percentages in Cu-Zn mixture

Figure 18. Zn speciation as percentages in Cu-Zn mixture
Figure 15. Cu speciation as percentages in individual metal test

Figure 19. Ni speciation as percentages in individual metal test

Figure 20. Cu speciation as percentages in Cu-Ni mixture

Figure 21. Ni speciation as percentages in Cu-Ni mixture
Figure 16. Zn speciation as percentages in individual metal test

Figure 19. Ni speciation as percentages in individual metal test

Figure 22. Zn speciation as percentages in Zn-Ni mixture

Figure 23. Ni speciation as percentages in Zn-Ni mixture
CHAPTER FOUR

CONCLUSIONS AND SUGGESTIONS

Metal mixture toxicity is a complex process which is poorly understood. From this research, we have categorized bimetal mixture toxicity of four metals of popular application. Results of this study indicate that the toxicity of Cu, Zn, and Ni bi-mixtures were more than additive to larval fathead minnows, suggesting a joint and enhanced toxicity of metals in the mixtures. Mixture toxicity was greatest for Cu-Ni mixtures, followed by Cu-Zn, and Zn-Ni. For Cd and Zn bi-mixtures, the effect was antagonistic. A biphasic dose-response occurred. Zn protected fathead minnows from Cd toxicity at concentrations ≤ 100 µg/L but contributed to toxicity at concentrations above 100 µg/L.

Additional studies should be conducted to characterize the toxicity mechanism of metal mixtures. Results of this study are useful for development of a Biotic Ligand Model for metal mixtures and have implications for setting water quality guidelines for metal mixtures. As previously stated, the toxicity of binary metal mixtures varies with test species, and the specific metal mixture. Future testing will be necessary to evaluate the totality of metal mixture toxicity on species diversity and ecosystem health.
APPENDIX A

MINTEQ MODEL INPUTS
<table>
<thead>
<tr>
<th>Test</th>
<th>pH</th>
<th>Cl</th>
<th>SO₄</th>
<th>CO₃²</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Na⁺</th>
<th>Mg²⁺</th>
<th>Zn²⁺</th>
<th>Cu²⁺</th>
<th>Ni²⁺</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>mg/L</td>
<td>mg/L</td>
<td>mg/L</td>
<td>mg/L</td>
<td>mg/L</td>
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<td>mg/L</td>
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<tr>
<td>Cu alone</td>
<td>7.7825</td>
<td>1.733</td>
<td>49.119</td>
<td>68.55</td>
<td>2.323</td>
<td>16.4</td>
<td>29.05</td>
<td>14.07</td>
<td>0.0424</td>
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<tr>
<td>Zn alone</td>
<td>7.7525</td>
<td>1.888</td>
<td>42.253</td>
<td>68.55</td>
<td>2.403</td>
<td>16.32</td>
<td>29.04</td>
<td>13.98</td>
<td>0.3903</td>
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<tr>
<td>Ni alone</td>
<td>7.5775</td>
<td>1.771</td>
<td>51.381</td>
<td>68.57</td>
<td>1.430</td>
<td>10.569</td>
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<td>8.679</td>
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<td>Cu-Zn</td>
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<td>2.3735</td>
<td>47.8415</td>
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<td>2.083</td>
<td>14.87</td>
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<td>Cu-Ni</td>
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<td>66.65</td>
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<td>Zn-Ni</td>
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<td>2.216</td>
<td>50.306</td>
<td>66.65</td>
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<td>14.223</td>
<td>26.425</td>
<td>11.563</td>
<td>0.289</td>
<td>0.003</td>
<td>1.434</td>
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</table>
REFERENCE LIST


47. Paquin PR, Di Toro DM, Santore RC, Trivedi D, Wu KB. 1999. A biotic ligand model of the acute toxicity of metals. III. Application to fish and *Daphnia* exposure to silver, Section 3 in Integrated Approach to Assessing the Bioavailability and Toxicity of Metals in Surface Waters and Sediments, a submission to the EPA Science


50. U.S. EPA.
VITA

Lynch’s interest in chemistry and aquatic toxicology began during her undergraduate career at Loyola University Chicago. Upon completing her B.S. in environmental science, she began pursuing a master’s in chemistry. Although her degree will be in chemistry, her research focus has primarily been in aquatic ecotoxicology. In addition to conducting toxicity tests, she is also in charge of maintaining cultures of fathead minnows, Florida apple snails, and water fleas in Dr. Tham Hoang’s Lab at Loyola University Chicago.

Lynch received an award for best student presentation during the 2013 Society of Environmental Toxicology and Chemistry national meeting regarding her research on bimetal mixture toxicology. She plans to pursue a position in waste management upon finishing her degree.