

TOXICITY OF CADMIUM, COPPER, AND ZINC AND THEIR BINARY MIXTURES TO
DAPHNIA MAGNA IN LABORATORY AND FIELD-COLLECTED WATERS

by

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ABSTRACT

Many surface waters are impacted by elevated concentrations of multiple metals resulting from acid mine drainage and industrial inputs. Because these elevated concentrations of metal mixtures sometimes can be toxic to aquatic life, there is a need to better understand and predict the toxicity of metal mixtures in the environment. Laboratory toxicity tests are important for providing data to develop multi-metal toxicity models. However, many laboratory toxicity tests do not closely mimic the aqueous geochemistry at contaminated sites. Therefore, it is difficult to accurately predict the toxicity of metal mixtures in the field. I investigated differences in the toxicity of Cd, Cu, and Zn to neonates of a freshwater invertebrate (*Daphnia magna*) in laboratory waters and in waters from the Clear Creek Superfund Site, a Colorado stream impacted by acid mine drainage. I exposed *Daphnia magna* neonates to individual metals and binary combinations of metals. I also used the Biotic Ligand Model to evaluate how accurately the model can predict the toxicity of Cu and Zn in field water compared to standard laboratory waters. The Cu-Zn binary combinations tested provided evidence of response-additive to slightly more-than-additive toxicity in field water, and the Cd-Zn binary combinations tested provided evidence of less-than-additive toxicity in field water. The toxicity of Cu and Zn in field water was not accurately predicted by the Biotic Ligand Model. This study highlights the importance of water chemistry when extrapolating results of metal-mixture toxicity tests from the laboratory to the field, and it emphasizes the need for statistical models to quantify the differences in toxicity of metal mixtures between varying water types.

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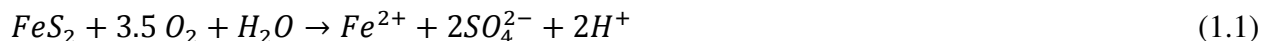
CHAPTER 1 INTRODUCTION

Metals are ubiquitous in the environment (Smith et al. 2015). At low concentrations, metals can be beneficial (i.e., essential elements such as Cu, Ni, and Zn) or at least cause no adverse effects (non-essential elements such as Cd; Janssen and Muysen 2001). However, inputs from mining, industry, municipalities, and agriculture can result in elevated environmental concentrations of metals that can be toxic to aquatic organisms (Meyer et al. 2007). In addition to these anthropogenic sources, natural mineral weathering can also introduce metals to the environment (Schmidt et al. 2012).

1.1 Metals in the Environment and Acid Mine Drainage Formation

Sulfide-rich mineralized areas, especially those in which hard-rock mines are present, can generate acid mine drainage (AMD; Baker and Banfield 2003). In addition to creating acidity, AMD releases metals to the environment. Because metals usually co-occur in ore bodies (Schemel et al. 1999), AMD usually releases metal mixtures instead of individual metals. Although the identity and proportions of the metals depend on the composition of the mineralized rock, metals including Cd, Cu, Ni, and Zn are of particular concern at many hard-rock mining sites. Acid mine drainage from legacy mines is a widespread and persistent pollution problem, particularly in Colorado and other western states that contain a large number of historic and abandoned mines (Schemel et al. 1999).

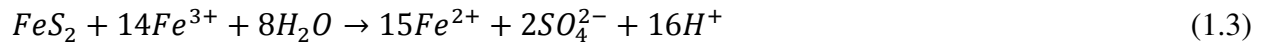
In the first step of AMD formation, sulfide-containing minerals in waste-rock and tailings piles are exposed to molecular oxygen (O_2) and water (Equation 1.1).



The ferrous iron (Fe^{2+}) liberated from the oxidation of pyrite (FeS_2) can then be oxidized to ferric iron (Fe^{3+} ; Equation 1.2).



The initial oxidation of ferrous iron is slow and considered rate-limiting in the formation of AMD (Baker and Banfield 2003). However, when enough ferric iron has accumulated, the generation of AMD proceeds rapidly because sulfide minerals are more readily oxidized by ferric iron than by O_2 . The oxidation of pyrite by ferric iron (Equation 1.3) can be considered an auto-catalytic step in the formation of AMD, and the catalysts in this reaction are microbes (Baker and Banfield 2003).



Two important microorganisms involved in the formation of AMD are *Acidithiobacillus ferrooxidans* and *Ferrobacillus ferrooxidans* (Colmer et al. 1950). Both species are autotrophs capable of oxidizing ferrous iron and reduced sulfur to obtain energy. These microorganisms are part of an important feedback loop in the formation of AMD and contribute to the generation of highly-acidic conditions.

The acidity generated in AMD is an important control on metal speciation. Because AMD creates an acidic environment, many metals are soluble in AMD. Dissolved metals can be present in water as free metal ions (i.e., aquo ions; Smith et al. 2015) and as complexes with OH, CO_3^{2-} , and other inorganic and organic ligands, depending on the pH of system (Figure 1.1). The free metal ions in solution are important because they are considered the most bioavailable form of most cationic metals (Meyer et al. 2007, Smith et al. 2015).

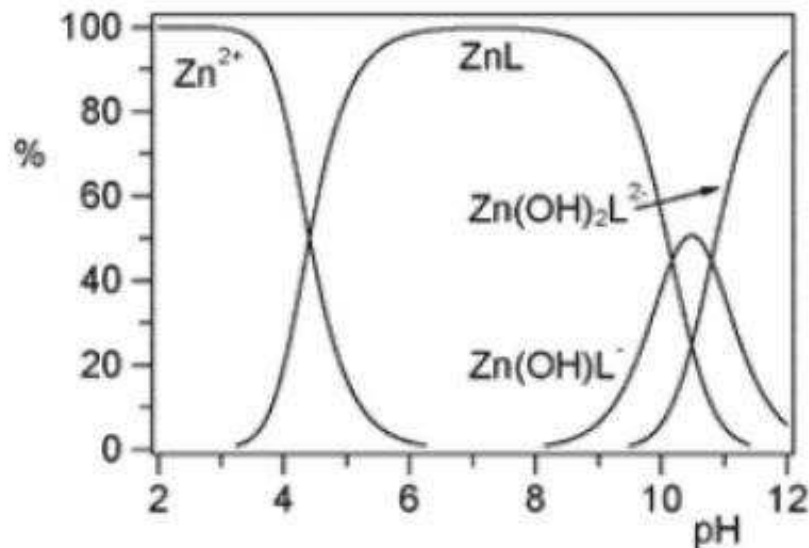


Figure 1.1. Zinc speciation across a range of pH values (Bianchini et al. 2003) . The vertical axis is the percent contribution of a given Zn species to the total concentration of dissolved Zn in the water, and L represents a ligand that may complex with metal, such as carbonate ions or dissolved organic matter. Metal chalcophiles (metals that bond stronger with sulfur than with nitrogen- or carboxylate-containing moieties) have similar speciation to Zn and will be present as M^{n+} at low to intermediate pH values, where n+ is the charge on the free metal ion. Other metal chalcophiles commonly found in AMD include Ag, Cd, Cu, and Pb (White 2013).

The concept of bioavailability includes several chemical, biological, and physical interactions in soil and water that determine the exposure of an organism to a toxicant. Bioavailable metal is

operationally defined as the amount of metal taken up by an organism (Ehlers and Luthy 2003). In order to predict the bioavailability and toxicity of metals, it is crucial to understand the geochemistry (i.e., the chemical components and their speciation) of the exposure water.

1.2 Metal Speciation and the Biotic Ligand Model

Because elevated concentrations of metals can be toxic to aquatic life, there is a need to better understand and predict the toxicity of metals in the aquatic environment. Toxicity is not simply related to total dissolved metal in solution but instead is related to metal-ligand complexation and metal interactions with other cations at sites of toxic action (Di Toro et al. 2001). Aquatic chemistry parameters such as pH, water hardness, alkalinity, and the concentration and composition of dissolved organic carbon (DOC) exert important controls on metal speciation and aquatic toxicity. The Biotic Ligand Model (BLM; Di Toro et al. 2001, Paquin et al. 2002) combines metal speciation and toxicity (Meyer et al. 2007), although other metal-speciation-based bioavailability models are also available (e.g., WHAM-F_{TOX}; Tipping and Lofts 2015).

Conceptually, the BLM treats an aquatic organism (or part of the organism, such as the gill) as a ligand in solution with which metals can complex. Other ligands, such as dissolved organic matter (DOM; for which DOC is a surrogate index of concentration), compete to bind the metal; and cations in solution, such as Ca²⁺, compete with the metal to bind at the biotic ligand. Therefore, the amount of metal that accumulates on the biotic ligand depends on the total concentration of dissolved metal, the amount of complexation with ligands, and the amount of binding competition from other cations (Playle 1998, Di Toro et al. 2001). In an example for Cu, Figure 1.2 illustrates some of the water chemistry parameters that the BLM uses to predict toxicity.

Although the BLM incorporates a variety of water chemistry parameters, pH and DOC are especially important to consider. These two parameters strongly influence the speciation and toxicity of metals. To a great extent, pH controls the amount of metal in the dissolved phase and the formation of metal complexes with inorganic ions (DeForest and Van Genderen 2012). Under acidic conditions, sulfate ions (SO₄²⁻) may be present and can complex with the metal.

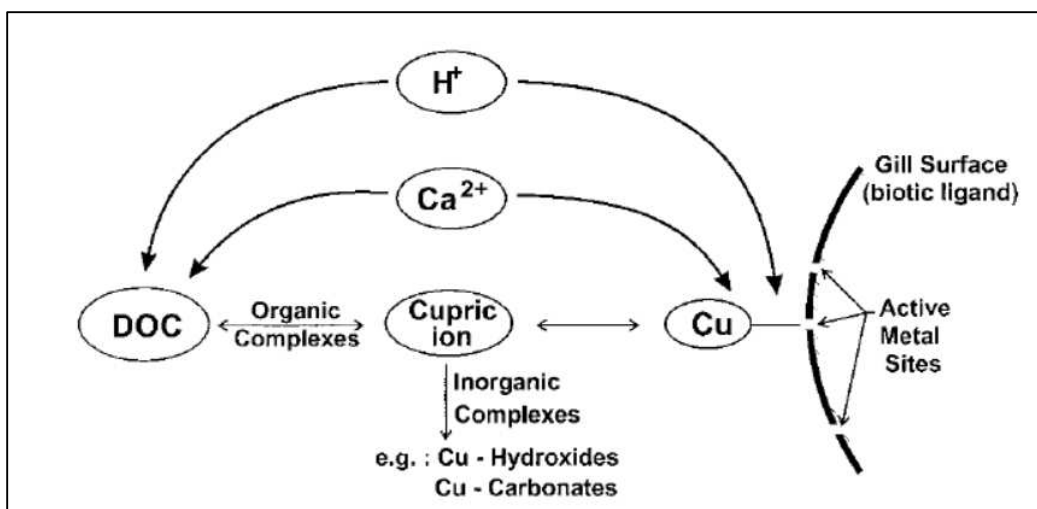


Figure 1.2. Conceptual diagram of the Biotic Ligand Model, which shows Cu speciation and complexation (Paquin et al. 2000). In this depiction, the cupric ion (Cu^{2+}) complexes with dissolved organic carbon (DOC), hydroxides, and carbonates; and it competes with Ca^{2+} and H^+ to bind at the biotic ligand site(s). However, other inorganic ligands (e.g., HCO_3^- , SO_4^{2-} , Cl^-) can also form complexes with Cu, and other cations (e.g., Na^+ , Mg^{2+}) can also compete with Cu^{2+} . Only the fraction of dissolved Cu that can bind to the biotic ligand site is toxic to the organism.

As the pH increases, hydroxide (OH^-), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) ions increase in concentration and become increasingly important for metal complexation (Smith et al. 2015). Those anions form complexes with free metal ions and thus help to control the concentrations of the free metal ions. Therefore, knowledge of inorganic ligands in an aquatic system is important to predict the bioavailability of a metal. However, organic ligands are also important. Dissolved organic matter can act as a sink for dissolved metals due to metal-binding functional groups such as carboxylates, phenols, amines, and thiols (McKnight et al. 1992). Predicting metal-DOM complexation in the natural environment can be difficult because natural DOM is composed of a heterogeneous, complex mixture of organic carbon constituents, and the binding affinities for metals to natural DOM can span several orders of magnitude (Craven et al. 2012).

The composition and concentration of DOM in the environment are important for predicting metal-DOM complexation and toxicity. In the natural environment, DOM can be subdivided into two classes: simple compounds and humic substances. Simple DOM compounds include biomolecules excreted by microorganisms. In contrast, humic substances are complex heterogeneous mixtures of many different organic compounds.

Humic substances are important because they comprise up to 95% of the DOM in aquatic systems and are often present at higher concentrations than inorganic ions (Stevenson 1994).

Humic substances can be subdivided into fulvic and humic acids based on solubility. Fulvic acids are soluble at all pH values, whereas humic acids are insoluble at pH values less than 2 (White 2013). Although the subdivision of humic substances into fulvic and humic acids is operational, humic and fulvic acids also differ in composition. Humic acid tends to have a greater abundance of aromatic structures than fulvic acid, and fulvic acid typically has a higher content of carboxylic acid groups (White 2013). Figures 1.3 and 1.4 show possible structures for humic and fulvic acids. Understanding the composition of DOM can be useful for interpreting BLM toxicity predictions because not all DOM has the same affinity or binding capacity for metals (Craven et al. 2012).

Computationally, the BLM combines these concepts of metal speciation with empirical biological data to predict metal accumulation on the biotic ligand and its consequent toxicity to aquatic organisms. Ten water chemistry parameters are required as inputs to the BLM: temperature, pH, DOC, Ca^{2+} , Mg^{2+} , Na^+ , K^+ , SO_4^{2-} , Cl^- , and alkalinity (USEPA 2007). The BLM uses the Windermere Humic Aqueous Model (WHAM) to predict metal-DOM complexation (Di Toro et al. 2001, Paquin et al. 2002). The WHAM model treats humic acid and fulvic acid differently, and there is a parameter in the BLM input for the percent of humic acid in the DOC. However, in studies in which the percent of humic acid was varied from 1% to 100%, there was negligible influence on the predicted LC50 values (Smith et al. 2015).

To predict metal complexation to the biotic ligand, equilibrium constants are needed for binding of the metal and competing cations to the biotic ligand (Santore et al. 2001). For some organisms, such as rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*), these binding constants were derived from measurements of metal adsorbed to fish gills over a range of metal concentrations. For other aquatic organisms, the binding constants were derived from toxicity studies (de Schampelaere and Janssen 2002).

Aquatic organisms can have numerous sites at which toxicants can bind, and not all of the sites respond to the same toxicant in the same way. For example, one biotic ligand site may be highly sensitive to a certain metal but another site may not be adversely affected (Niyogi and Wood 2004). When a specified amount of metal, called the critical accumulation, binds to the organism, a specified percentage response occurs (Santore et al. 2001). The accumulation of metal on the biotic ligand that causes 50% mortality is called the Lethal Accumulation 50 (LA50; i.e., the median lethal accumulation). Because the LA50 is assumed to be constant

regardless of the chemistry of the exposure water (Meyer et al. 2007), the median lethal concentration (the LC50) of dissolved metal in the exposure water can be calculated using the BLM.

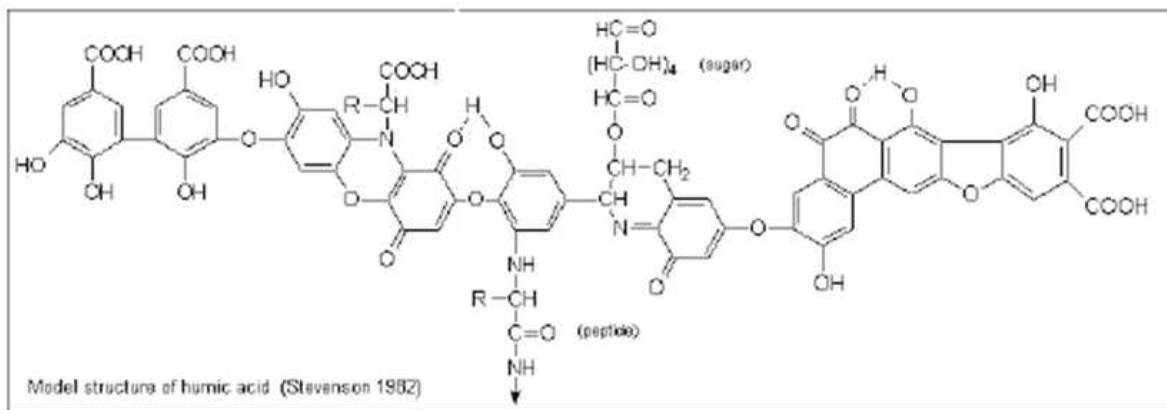


Figure 1.3 Representative structure of humic acid (Stevenson 1994).

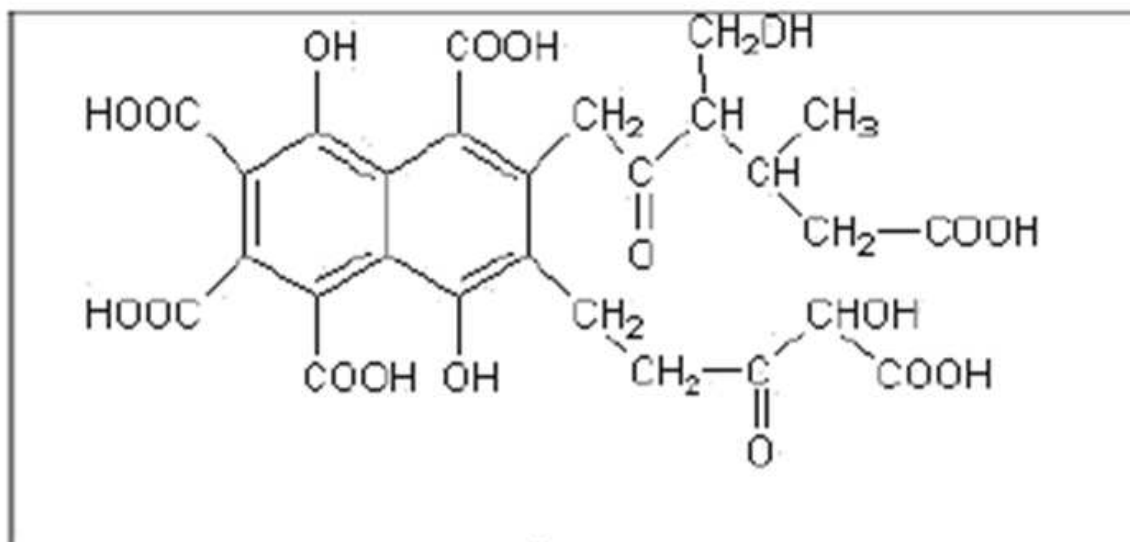


Figure 1.4. Representative structure of fulvic acid (Stevenson 1994). In most natural waters, the approximately circumneutral pH will cause many of the sites on this molecule to be deprotonated, thus allowing for the complexation of metals.

The most current BLM, version 2.2.3, includes metal speciation and acute toxicity models for Cd, Cu, Pb, and Zn for several aquatic organisms, including rainbow trout, fathead minnow, and water fleas (*Daphnia magna*, *D. pulex*, and *Ceriodaphnia dubia*). Versions of the BLM to predict

the toxicity of other metals (e.g., Ag, Ni) and metal mixtures in freshwater environments are under development (Santore and Ryan 2015). Additionally, versions of the BLM to predict Cu and Zn toxicity in marine environments have been developed, and versions to predict Cu and Ni toxicity in soils are being researched (Arnold et al. 2005; Di Toro et al. 2005).

1.3 Regulation of Individual Metals

In the United States, most metals are regulated on an individual-metal basis using hardness-based water quality criteria (e.g., USEPA 2002a). As water hardness increases, toxicity is typically ameliorated; thus, hardness-based criteria concentrations increase as hardness increases (Meyer et al. 2007, Smith et al. 2015). However, as discussed previously, a number of other water chemistry parameters including pH, DOC, and alkalinity are important for predicting toxicity. Because the science of metal bioavailability has advanced since the early 1980s when the hardness-based criteria for metals were introduced (Meyer et al. 2007) and the Cu BLM is a robust tool for predicting toxicity, the USEPA updated its recommended national water quality criteria for Cu in 2007 to include the BLM (USEPA 2007). However, the BLM has not yet been approved for deriving criteria concentrations for other metals. States and tribes can now choose to use the BLM in their water quality standards for Cu, and 18 states have adopted the BLM in some context for deriving Cu criteria concentrations. Most of these states only use the BLM for deriving site-specific criteria rather than replacing the state-wide hardness-based criteria with BLM-based criteria (Carleton 2015).

Replacing state-wide criteria with the BLM is challenging because water chemistry varies temporally and spatially (Carleton 2015). Although water hardness also varies spatially and temporally, implementation of BLM-based criteria is perceived as more challenging than implementation of hardness-based criteria. Because the BLM requires 10 water chemistry parameters, large datasets that span all four seasons are desirable to implement the BLM (although the same concern in concept should also apply to water hardness). Many states and tribes do not have sufficient databases of water chemistry to use the BLM, and adopting rigorous water sampling plans can be expensive. One way to deal with the absence of large databases of water chemistry is to develop conservative default values for various model inputs, especially pH, DOC, alkalinity, Ca^{2+} , and Na^+ (Carleton 2015). Such defaults could be developed on a regional or local basis using regional water quality data.

Although there are challenges to implementing the BLM, BLM-based water quality criteria are an important step in updating hardness-based water quality criteria for metals. Biotic Ligand Model predictions for metal toxicity are more accurate than hardness-based predictions and usually result in more appropriate regulations (USEPA 2007).

1.4 Regulation of Metal Mixtures

Although progress is being made in updating water quality criteria for metals, the criteria currently are applied to individual metals and not metal mixtures. This regulatory framework is problematic because metals are almost always present in the environment as metal mixtures instead of as individual metals (Meyer et al. 2015a). Hardness-based and BLM-based water quality criteria for individual metals often do not accurately predict the toxicity of metal-mixtures (DeForest and Van Genderen 2012). In order to better assess metal mixtures in aquatic environments, several metal industry organizations including the Copper Alliance, International Zinc Association, Nickel Producers Environmental Research Association, and Rio Tinto commissioned a Metal Mixtures Modeling Evaluation (MMME) project in 2011 (Van Genderen et al. 2015). My research was partially funded by those organizations. The goals of the MMME project include comparing and developing predictive mixture models and developing a better understanding of metal-mixture interactions.

Regulating and modeling metal mixtures is challenging for a variety of reasons. Metal mixtures are regulated on an individual-metal basis partly due to the complexity of metal-mixture toxicity (Meyer et al. 2015a). In the regulatory environment, metal-mixture toxicity is assumed to be additive; that is, the two metals in combination are predicted to produce a total effect that is the sum of the individual effects (Meyer et al. 2015a). However, numerous studies have shown that assuming response-additive toxicity is often not correct, and that toxicity can also be less-than-additive or more-than-additive (Norwood et al. 2003, Vijver et al. 2011, Meyer et al. 2015b, Traudt et al. 2016; see Figure 1.5 for examples). A meta-analysis of metal-mixture toxicity showed response-additive or less-than-additive toxicity in approximately 70% of the data (Vijver et al. 2011). Therefore, assuming response-additive toxicity in a regulatory framework results in protective or overly-conservative criteria 70% of the time. Although assuming response-additive toxicity will result in protective regulations most of the time, assuming response-additive toxicity will also result in cases in which toxicity is under-predicted (Meyer et al. 2015a). Assuming response-additive toxicity in the regulatory environment is also

problematic because it does not provide insight into which combinations of metals cause less-than-additive, additive, or more-than-additive toxicity (Meyer et al. 2015a). In order to develop appropriate regulations for metal mixtures that are not overly conservative or under-protective, toxicity data and accurate predictive models are needed. This study provides data and insights to help parametrize metal-mixture toxicity models.

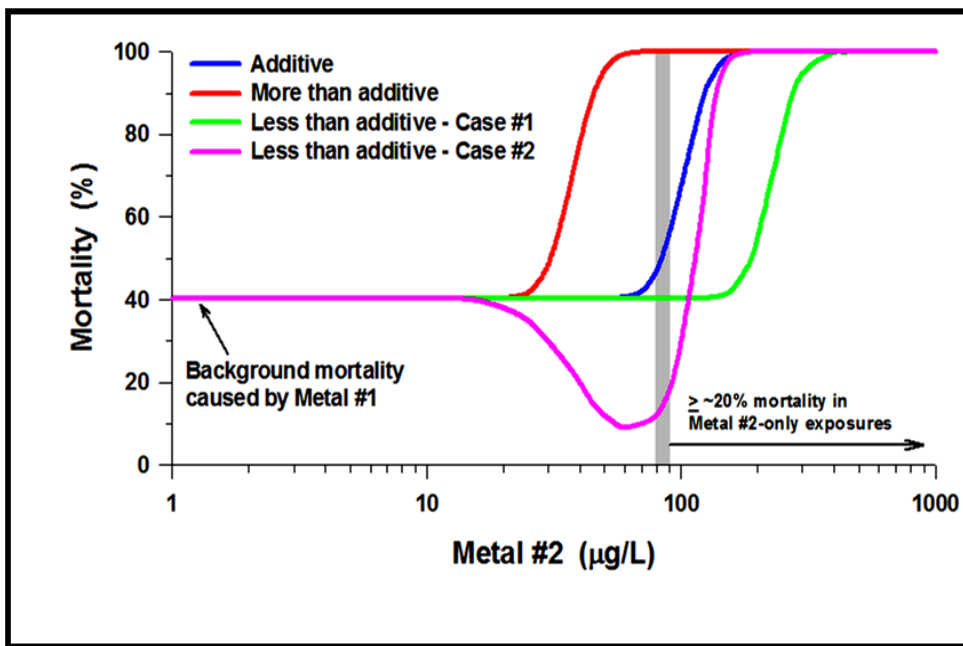


Figure 1.5. Conceptual examples of additive, more-than-additive, and less-than-additive toxicity in a binary-metal mixture. The vertical axis is the mortality caused by the binary mixture, and the horizontal axis is the concentration of Metal #2 being “titrated” into exposure water that contains a constant background concentration of Metal #1. The blue curve indicates additive toxicity. The red curve indicates more-than-additive toxicity, wherein a lower concentration of Metal #2 is needed to cause the same response as a higher concentration of Metal #2 in the additive-toxicity scenario (i.e., the concentration of Metal #2 at the inflection point of the more-than-additive curve is less than the concentration of Metal #2 at the inflection point of the response-additive curve). The green and pink curves indicate less-than-additive toxicity, wherein the concentration of Metal #2 at the inflection point is greater than in the response-additive case (Meyer et al. 2015a).

1.5 Thesis Objectives and Outline

The primary goal of this research is to compare individual-metal and metal-mixture toxicity in lab waters to toxicity in field-collected waters from the North Fork of Clear Creek in Blackhawk, Colorado, to assist in developing an accurate multi-metal BLM. The toxicity of these mixtures was determined in 48-h acute toxicity tests using the freshwater invertebrate *Daphnia*

magna. In addition to collecting the toxicity data, I used the BLM to predict the toxicity of Cu and Zn in field-collected waters. The objective of modeling toxicity with the BLM was to evaluate how accurately the model can predict the toxicity of Cu and Zn in field water compared to standard laboratory waters. These topics are addressed in the remaining three chapters of this thesis. Chapter 2 focuses on the methods, results, and interpretation of Cd, Cu, and Zn individual-metal toxicity tests; binary-metal toxicity test methods, results, and interpretations are presented in Chapter 3; and Chapter 4 summarizes the results of Chapters 2 and 3 and presents conclusions.

Although the major focus of this research is the toxicity of Cd, Cu, and Zn to *Daphnia magna* in laboratory and field-collected waters, I also conducted a small study to determine if two different sources of *D. magna* neonates have different sensitivity to Ni. For the majority of the experiments presented in this thesis, the source of *D. magna* was laboratory-cultured organisms purchased as neonates from Aquatic Biosystems, Inc. (Fort Collins, CO). This approach was facilitated by the close proximity of the Aquatic Biosystems facility, which allowed shipping times of less than one half day. The other source was *D. magna* ehippia (resting eggs) that could be stored for long periods of time in a dormant state and hatched when needed. This comparison is important because not all researchers, especially those at remote field sites, are close to toxicity-test organism suppliers and may need alternate sources of test organisms. This side study of Ni toxicity to two different sources of *D. magna* neonates was funded by a Sussman Fellowship from the Colorado School of Mines and United States Geological Survey and is described in Appendix A.

CHAPTER 2 INDIVIDUAL-METAL TOXICITY OF CD, CU, AND ZN IN FIELD AND LABORATORY WATERS

Laboratory toxicity tests are important for providing data to develop accurate toxicity models and establish regulatory limits for metals. However, the water used in many laboratory toxicity tests does not closely mimic the aqueous geochemistry at contaminated sites. Therefore, it is also important to conduct toxicity tests in field water.

2.1 Introduction

Aquatic chemistry characteristics such as water hardness, alkalinity, and the concentration and composition of dissolved organic matter exert important controls on aquatic toxicity, but often laboratory-water recipes do not match the characteristics of surface waters into which metals are discharged (Van Genderen et al. 2007). A standard matrix for performing laboratory toxicity tests is the U.S. Environmental Protection Agency (USEPA) moderately hard reconstituted (MHR) water (USEPA 2002b). In order to evaluate how well laboratory waters mimic field water, I performed individual-metal toxicity tests in a variety of laboratory reconstituted waters and in field-collected water from a stream contaminated with acid mine drainage (AMD). These various waters were used for testing Cd, Cu, and Zn toxicity, and the results from these tests were used to evaluate whether the biotic ligand model (BLM) can accurately predict metal speciation and toxicity in laboratory waters and the field water.

2.2 Materials and Methods

Many of the materials and methods in this study were modeled after the materials and methods used in standard USEPA freshwater toxicity tests. All materials and methods are described in detail in the following sections.

2.2.1 Field Site

Field water was collected from a relatively pristine site approximately 4 km upstream of the City of Blackhawk on the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado for use as a dilution water in some of the toxicity tests. A reach of NFCC is part of the Clear Creek/Central City Superfund Site and is impacted by AMD from abandoned mines (Figure 2.1; USEPA 2004). Downstream of the water-collection site, two major point sources discharge AMD into NFCC: Gregory Incline and National Tunnel. They enter the stream in Blackhawk and contribute to elevated concentrations of Al, Cu, Fe, Mn, and Zn in NFCC (USEPA 2004).

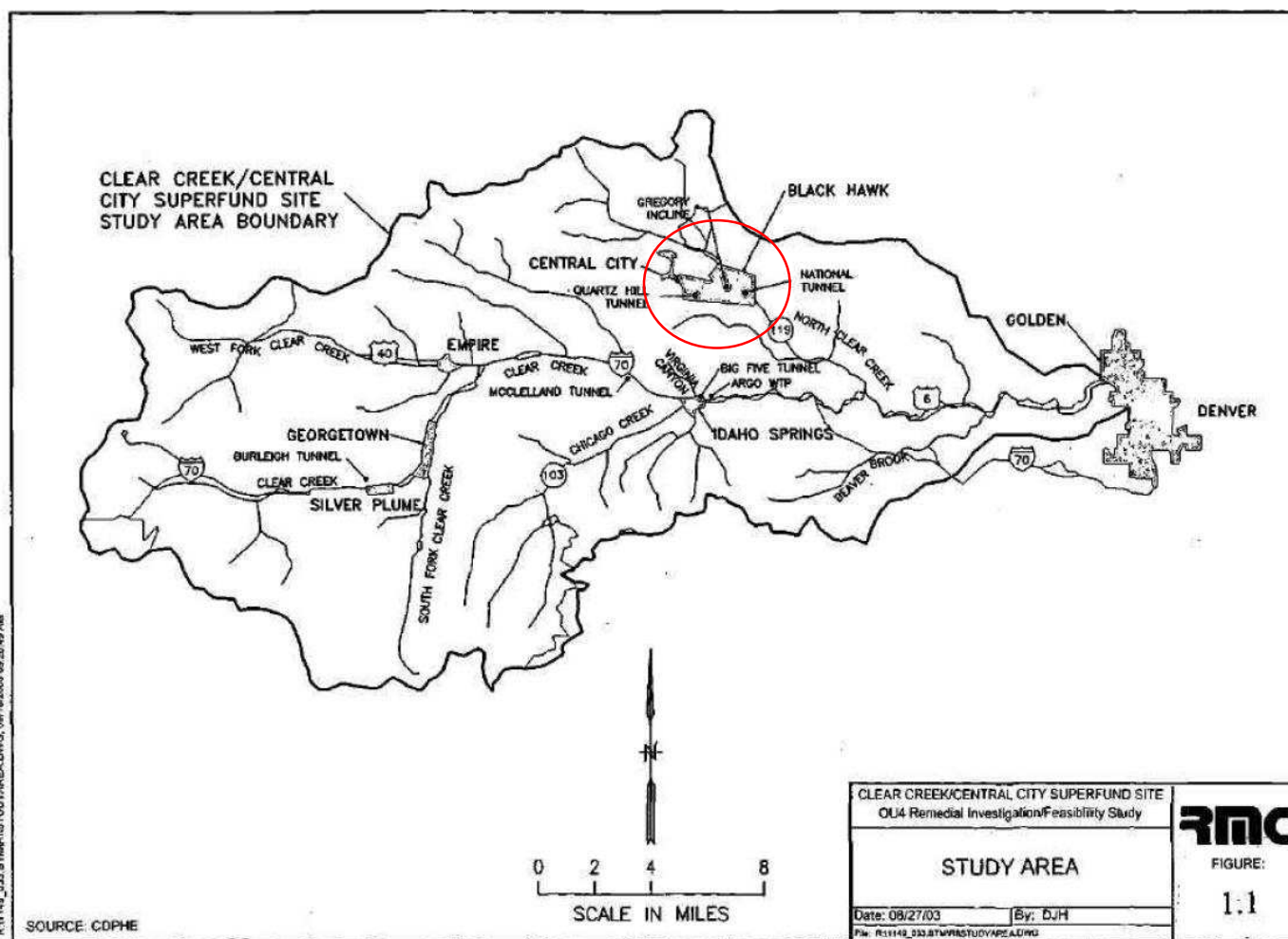


Figure 2.1. Clear Creek/Central City Superfund site in Gilpin County, central Colorado, where acid mine drainage has contaminated numerous stream reaches. The North Fork of Clear Creek (NFCC) is circled in red. Gregory Incline and National Tunnel are the major point sources of metals entering NFCC. Figure from USEPA (2004).

2.2.2 Test Organisms

Daphnia magna neonates were used as the test organisms in all the toxicity tests in this study. The natural habitat of *D. magna* includes large and small lakes as well as seasonally flooded depressions. *Daphnia magna* are relatively large (approximately 5 mm long) and therefore are at risk of predation from visual predators such as fish (Ebert 2005). For these reasons, *D. magna* are not usually found in Rocky Mountain streams. However, *D. magna* are still considered good model organisms for the toxicity tests in this study for a variety of reasons. They are relatively easy to culture in the laboratory, and laboratory raised *D. magna* typically produce an asexual (parthenogenetic) clutch of up to 100 eggs every 3 to 4 days (Ebert 2005). Because *D. magna* reproduce parthenogenetically, have high fecundity, and are economical to culture, USEPA (2002b) approves the use of *D. magna* for use in freshwater toxicity tests. In this study, parthenogenetically-produced (i.e., asexually-produced) *D. magna* neonates were ordered from Aquatic Biostystems, Inc. in Fort Collins, Colorado and shipped same day to the Colorado School of Mines in MHR water with algae (*Pseudokirchneriella subcapitata*) as food.

2.2.3 Toxicity-Test Water

The dilution waters in the acute toxicity tests included MHR water, dilutions of MHR water, and water collected from NFCC in the winter of 2015. Field water was filter-sterilized with a 0.45 μm Geotech filter (white acrylic copolymer coating over a non-woven substrate) from Geotech Environmental Equipment, Inc. (Denver, CO). Ambient dissolved-metal concentrations of Al, Cu, and Zn in NFCC field water were typically less than the detection limits in this study (see section 2.2.5). Ambient Fe concentrations were typically less than 10 $\mu\text{g/L}$, and ambient Mn concentrations were less than 1 $\mu\text{g/L}$. The MHR water was prepared according to standard recipes (USEPA 2002b; Table 2.1). Soft water and very soft water are simply dilutions of MHR water and are standard USEPA reconstituted waters. Intermediate softness water is also a dilution of MHR water that I prepared and is not a standard USEPA reconstituted water.

The differences in aqueous geochemistry of the laboratory waters and field-collected waters are important for interpreting the results of toxicity tests in varying water types. The NFCC field water has approximately half of the hardness and alkalinity as USEPA MHR water (Figure 2.2). Soft water, and water with intermediate softness were chosen as test waters because the hardness and alkalinity of these waters more closely mimicked the hardness and alkalinity in NFCC field water. Very soft water was chosen as a test water to show the differences in toxicity over a wide

range of water hardness. The amount of hardness and alkalinity in NFCC field water is most similar to USEPA soft water.

Table 2.1. Recipes and approximate water chemistry parameters for USEPA reconstituted laboratory waters (USEPA 2002b) and North Fork of Clear Creek field water. The intermediate softness water is not an official USEPA reconstituted-water recipe. The pH values for the USEPA waters are the pH values the USEPA recipe (2002b) gives as guidelines. The pH values for intermediate softness water and North Fork Clear Creek water were measured.

	NaHCO ₃	CaSO ₄	MgSO ₄	KCl	pH	Hardness	Alkalinity
	(mg/L)	(mg/L)	(mg/L)	(mg/L)		(mg/L as CaCO ₃)	(mg/L as CaCO ₃)
Very Soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Intermediate softness	24.0	15.0	15.0	1.0	7.4-7.8	20-25	12-15
North Fork Clear Creek	-	-	-	-	7.4-8.1	30-40	15-25
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64

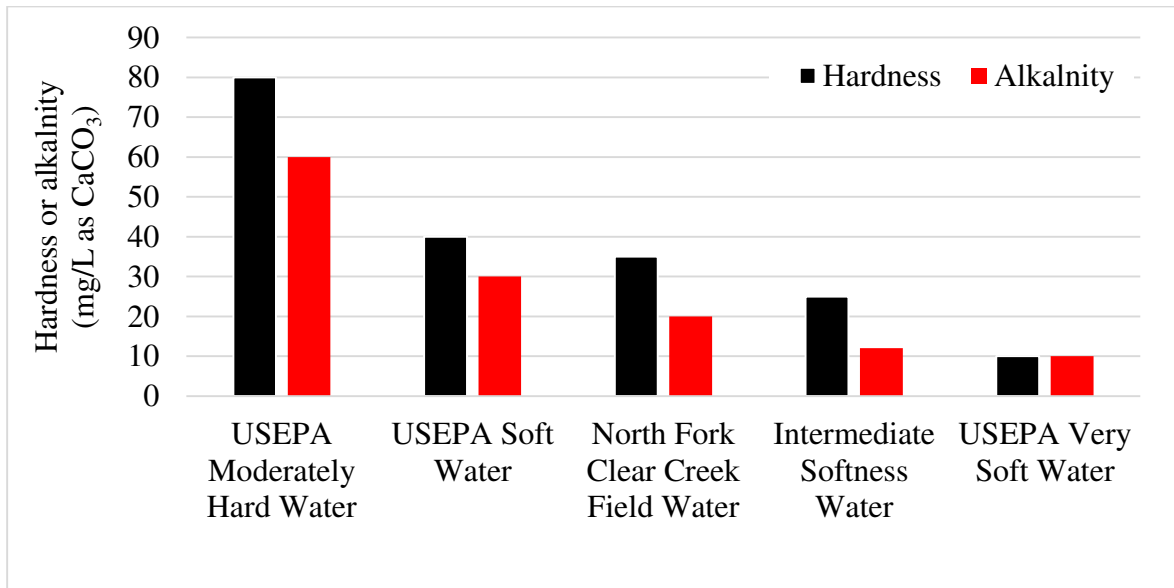


Figure 2.2. Hardness and alkalinity of laboratory reconstituted and field-collected waters used in the toxicity tests.

2.2.4 Toxicity Tests

I used 48-h lethality tests to evaluate the toxicity of individual metals to the freshwater invertebrate *D. magna*. The toxicity tests were performed in a variety of waters, including MHR water, dilutions of MHR water, and field-collected water. To all of the laboratory waters, I added 3 mg/L DOC as Suwanee River fulvic acid (SRFA) obtained from the International Humic Substances Society (IHSS). If the field water did not contain 3 mg/L of natural DOC, the water was amended with SRFA to increase the DOC concentration to 3 mg/L. The toxicity tests comprised a series of either 6 or 12 metal concentrations in a gradient designed to produce mortalities ranging from 0 to 100% (Figure 2.3). The following metal salts were spiked into the exposure waters: $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, and $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (Baker Chemical Company). Four replicate 40-mL beakers that contained 25 mL of dilution water were tested at each concentration in the gradient, and five organisms were placed in each beaker. Survival was monitored at 24 and 48 h, and immobilization was used as a proxy for mortality. All tests were conducted in VWR incubators at a temperature of $20 \pm 2^\circ\text{C}$, with a 16 h-8 h light-dark cycle.

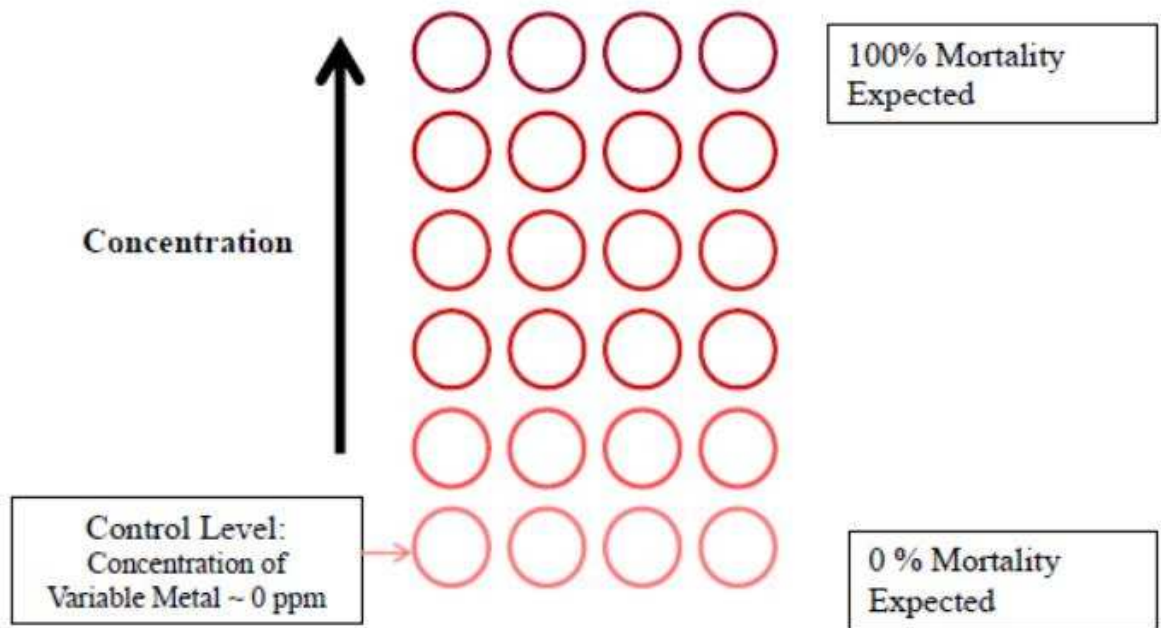


Figure 2.3. Schematic of a toxicity test with a series 6 different metal concentrations in a gradient designed to produce mortalities ranging from 0 to 100%. The first row were the control beakers in which no metals were added to solution, and the sixth row shows the highest concentration of metal. Before starting the test, the positions of the beakers were randomized to avoid potential positional bias.

2.2.5 Chemical Analyses

At the beginning and end of each toxicity test, the pH was recorded using a Thermo Orion 5-Star pH meter, and dissolved oxygen concentration and temperature were recorded using a YSI 55 probe. Alkalinity was measured by titration using a Hach Alkalinity Test Kit and 0.16 M sulfuric acid with bromocresol green-methyl red as an indicator.

Water samples from each suite of toxicity tests were analyzed for metals, inorganic cations, sulfur, and DOC. At each concentration gradient in the toxicity test, the water was analyzed for inorganic cations and sulfur (assumed to be present as sulfate) using inductively coupled plasma-optical emission spectroscopy (ICP-OES Perkin Elmer Optima 5300) with scandium as an internal standard. Two NIST certified standard reference materials 1640a and 1643e were analyzed at the beginning and at the end of each batch of samples, and continuing calibration verification (CCV) standards were run after every 10 samples and at the end of each batch of samples. The detection limits for selected metals on the ICP-OES are shown in Table 2.3. The concentrations for chloride and sulfate were calculated from the analyzed concentrations of K and S, respectively. This is because the chloride and sulfate anions were not measured directly with the ICP-OES, and it was assumed that all of the chloride came from the KCl used to prepare the USEPA MHR water and all of the sulfur was present as sulfate. For each exposure water, other samples were acidified with phosphoric acid before DOC concentration was measured using persulfate oxidation with a GE Sievers 900 TOC Analyzer.

Table 2.2. ICP-OES limits of detection for selected elements during this study.

Element	Detection limit ($\mu\text{g/L}$)
Al	4.5
Ca	4.7
Cd	0.3
Cl	36.6
Cu	0.4
Fe	0.3
K	40.4
Mg	1.3
Na	6.5
Ni	0.4
SO ₄	17.4
Zn	0.4

2.2.6. Data Analyses

I calculated the Effects Concentration 50 (EC50; i.e., the median effects concentration, at which 50% immobilization is predicted to occur) as an index of acute toxicity instead of the Lethal Concentration 50 (LC50; i.e., the median lethal concentration, at which 50% mortality is predicted to occur), because death of *D. magna* cannot be positively identified without checking the heartbeat of the organism. Immobilization, which I monitored by tapping the beaker and visual observation, is commonly used as a proxy for death and thus is a traditional mortality endpoint in *D. magna* toxicity tests (USEPA 2002b).

Concentration-response equations were determined using least-squares regression fits of logit-transformed mortality data in OriginPro 9.1 software (Northampton, MA):

$$y = A_1 + \frac{(A_2 - A_1)}{1 + 10^{(LogX_0 - X) * p}} \quad (2.1)$$

where A1 and A2 are the lower and upper mortality limits (%), respectively; X is the metal concentration; X₀ is the metal concentration at the center of the distribution (i.e., the EC50); p is the slope of the logit-regression curve; and y is the mortality (%). Confidence bands at the 84% confidence level were calculated for the concentration-response curves, because non-overlap of two independent 84% confidence intervals indicates a statistically significant difference at the 95% level (Payton et al. 2003).

2.2.7. Biotic Ligand Modeling

The BLM version 2.2.3 (Hydroqual, Inc., 2007) was used to predict Cu and Zn EC50 concentrations for *D. magna* in the various dilution-water chemistries. The water chemistry data were entered in the BLM input file, and the model was run in toxicity-prediction mode. In this mode, the user does not need to enter metal concentrations, and the BLM predicts the EC50 for a chosen organism based on a default median lethal accumulation (LA50) value. The LA50 is the accumulation on the biotic ligand that results in 50% mortality. The LA50 is expressed in units of nmol/g wet weight of the biotic ligand (Santore et al. 2001). In addition to toxicity-prediction mode, the BLM can be run in speciation mode. In speciation mode, the user must enter the metal concentrations and water chemistry data, and the BLM predicts inorganic and organic speciation in the water (Hydroqual, Inc., 2007).

Because the BLM uses data from many different strains of *D. magna*, the BLM-predicted EC50 values might not accurately predict the toxicity of metals to the strain of *D. magna* used in this study. Therefore, I performed a strain-specific calibration of the BLM to better predict the

toxicity of the metals to the strain of *D. magna* neonates used in this study. To perform this calibration, the default BLM was run in speciation mode, and metal concentrations and water chemistry data from toxicity tests in MHR water were entered into the BLM. The output files from running the BLM in speciation mode report the BLM-predicted accumulation of metal on the biotic ligand in nmol/ g wet weight of the organism in that water chemistry. This accumulation of metal on the biotic ligand at each exposure concentration was then plotted versus observed mortality, and an accumulation-response equation was determined using least-squares regression fits of logit-transformed data in OriginPro 9.1 software (Figure 2.4). The LA50 value was then found from this accumulation-response curve by calculating the metal accumulation on the biotic ligand at 50% mortality using logit regression in the OriginPro 9.1 software.

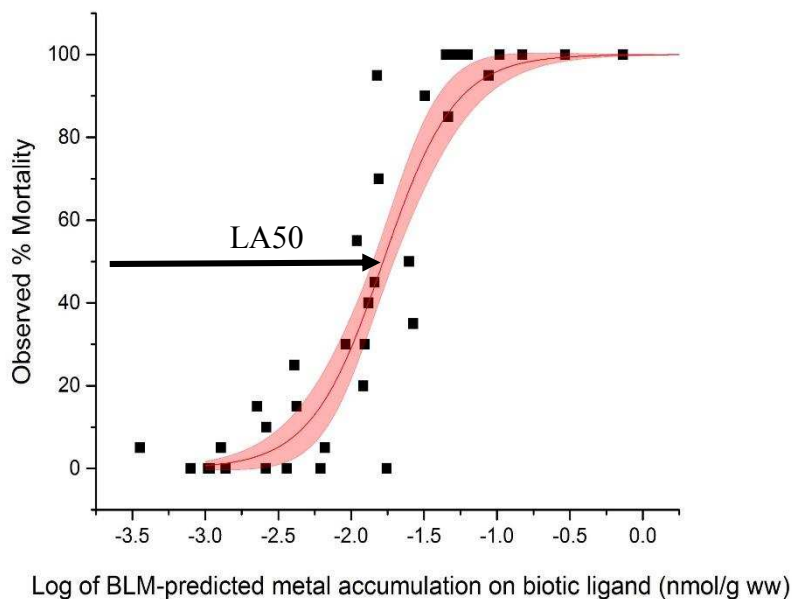


Figure 2.4. Example accumulation-response curve for a metal. This graph is a concentration-response curve in biotic-ligand (instead of dissolved-metal) space. The median lethal accumulation (LA50; i.e., the concentration of metal on the biotic ligand that causes 50% mortality) is indicated by the arrow. The salmon-colored shading is an 84% confidence band around the logit-regression curve.

After the LA50 was determined for the strain of *D. magna* that I used, the BLM was run in user-defined toxicity mode. User-defined toxicity mode allows the user to input an LA50

different than the default LA50. The BLM then predicts the strain-specific EC50 based on that user-defined LA50 instead of the default LA50 (Hydroqual, Inc., 2007; Figure 2.5). By definition, after calibrating a strain-specific BLM in MHR water, the predicted EC50 in MHR water will equal the observed EC50 in MHR water.

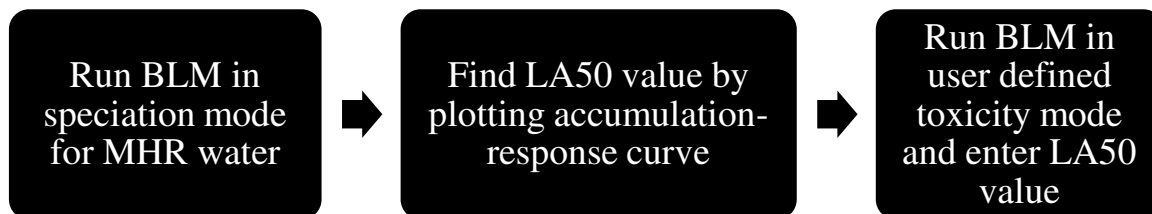


Figure 2.5. Flow chart for calibrating a strain-specific Biotic Ligand Model (BLM; LA50 = median lethal concentration of metal accumulated on the biotic ligand; MHR water = USEPA moderately hard reconstituted water).

2.3 Results and Discussion

Figures 2.6 to 2.11 show the concentration-response curves for Cu-only and Zn-only toxicity tests in MHR water, dilutions of MHR water, and NFCC water. The EC50 concentrations and 84% confidence intervals for Cu and Zn in laboratory and field-collected waters are reported in Table 2.3.

2.3.1 Individual-Metal Toxicity of Cu and Zn

Copper-only and Zn-only EC50 concentrations in NFCC field water were significantly lower than the corresponding EC50 concentrations in MHR water (Table 2.3) This shift in EC50 concentration was expected because MHR water has approximately twice the hardness of NFCC water, and Ca^{2+} and Mg^{2+} protect the biotic ligand from metals (de Schampelaere and Janssen 2002). The EC50 concentrations for Zn and Cu in NFCC field water were closer to the EC50 concentrations in USEPA soft water but were still significantly lower (Table 2.3). Therefore, although NFCC field water and USEPA soft water have approximately the same hardness and alkalinity, the USEPA soft water is more protective (i.e., has higher EC50 concentrations). This protective effect may be explained by the “effective” DOC concentration. Although both the NFCC field water and MHR water contained approximately 3 mg/L of DOC, the NFCC water contained both natural DOM and added SRFA. In contrast, all 3 mg/L DOC in the USEPA MHR water and soft water was SRFA. The natural DOC in the field water may not bind metals as

effectively as SRFA, resulting in an increase in Cu and Zn bioavailability and toxicity in NFCC water compared to USEPA soft water.

Finally, the EC50 concentrations for Cu and Zn in USEPA very soft water did not differ significantly from the EC50 concentrations in NFCC water at the 95% confidence level (Table 2.3). Figures 2.12 and 2.13 illustrate the trend in EC50 concentrations when MHR water is diluted to soft and very soft water.

Table 2.3. EC50 concentrations and 84% confidence intervals for Cu and Zn in laboratory and field-collected waters.

	USEPA MHR water	USEPA soft water	NFCC field water	USEPA very soft water
Zn EC50 (mg/L) (84% C.I.)	0.87 (0.78-0.97)	0.33 (0.28-0.39)	0.18 (0.16-0.21)	0.19 (0.17-0.23)
Cu EC50 (mg/L) (84% C.I.)	0.100 (0.097-0.103)	0.056 (0.054-0.058)	0.0267 (0.025-0.0279)	0.030 (0.027-0.034)

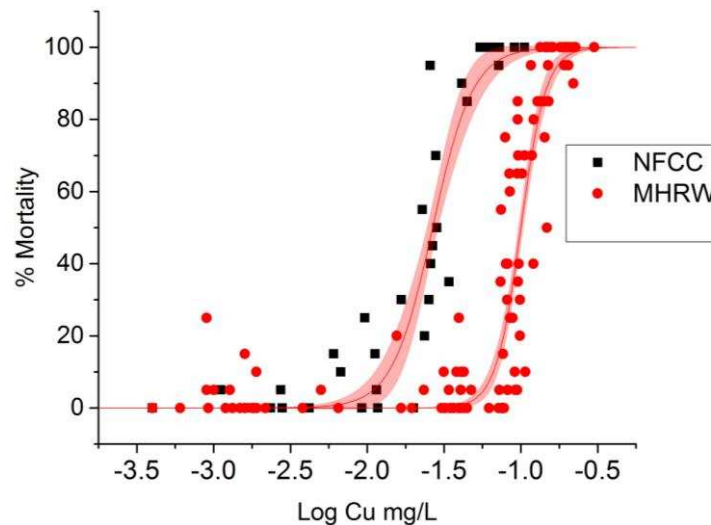


Figure 2.6. Logit concentration-response curves for *Daphnia magna* neonates exposed to Cu in 48-h lethality tests in USEPA (2002b) moderately hard reconstituted water (MHRW) and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Cu toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

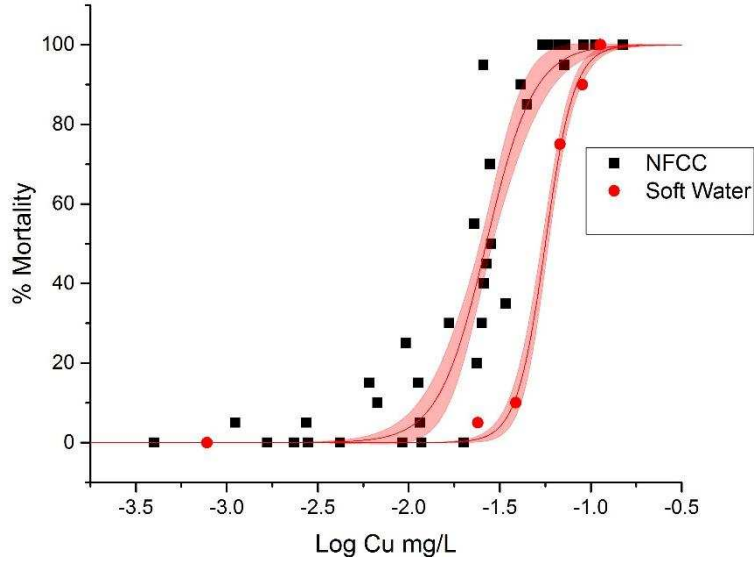


Figure 2.7. Logit concentration-response curves for *Daphnia magna* neonates exposed to Cu in 48-h lethality tests in USEPA (2002b) soft water and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Cu toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

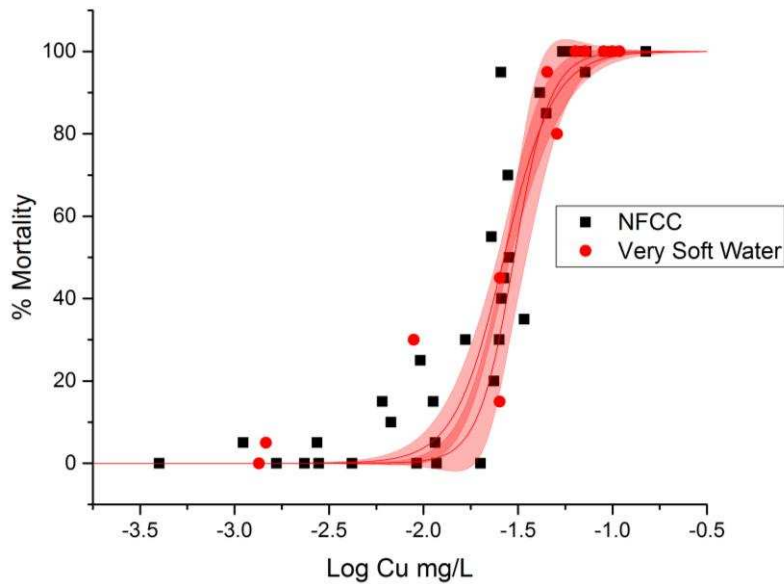


Figure 2.8. Logit concentration-response curves for *Daphnia magna* neonates exposed to Cu in 48-h lethality tests in USEPA (2002b) very soft water and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Cu toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

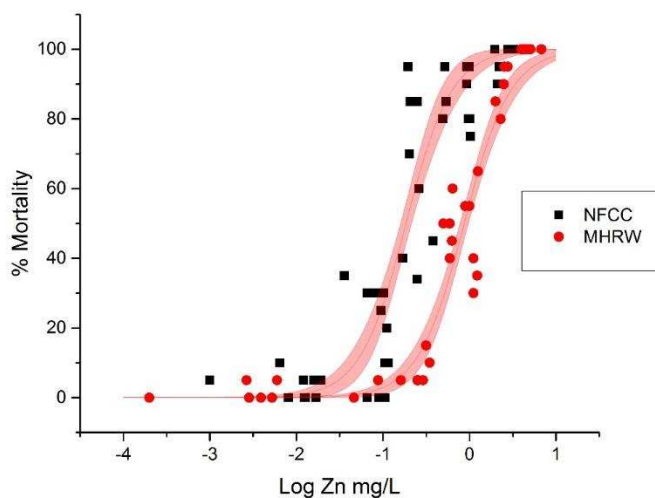


Figure 2.9. Logit concentration-response curves for *Daphnia magna* neonates exposed to Zn in 48-h lethality tests in USEPA (2002b) moderately hard reconstituted water (MHRW) and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Zn toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

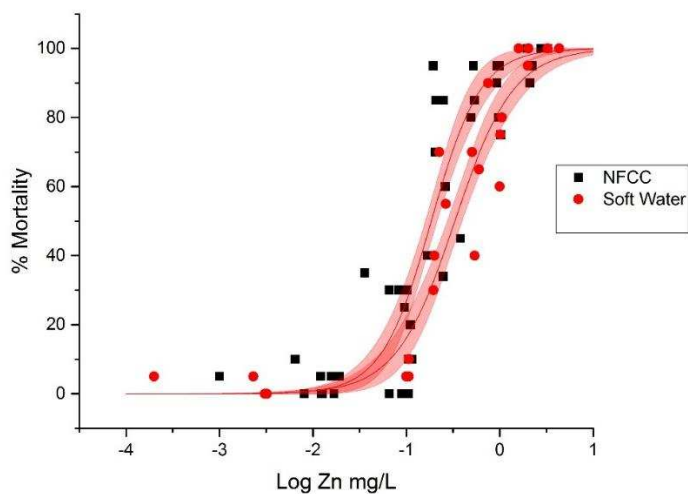


Figure 2.10. Logit concentration-response curves for *Daphnia magna* neonates exposed to Zn in 48-h lethality tests in USEPA (2002b) soft water and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Zn toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

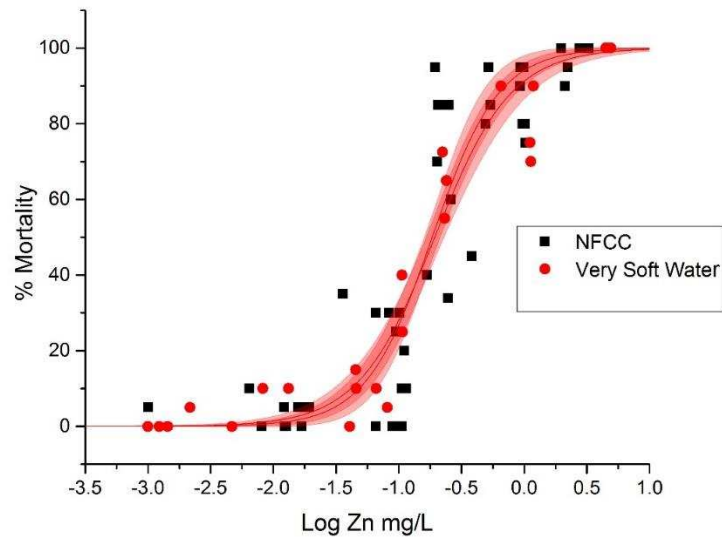


Figure 2.11. Logit concentration-response curves for *Daphnia magna* neonates exposed to Zn in 48-h lethality tests in USEPA (2002b) very soft water and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Zn toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

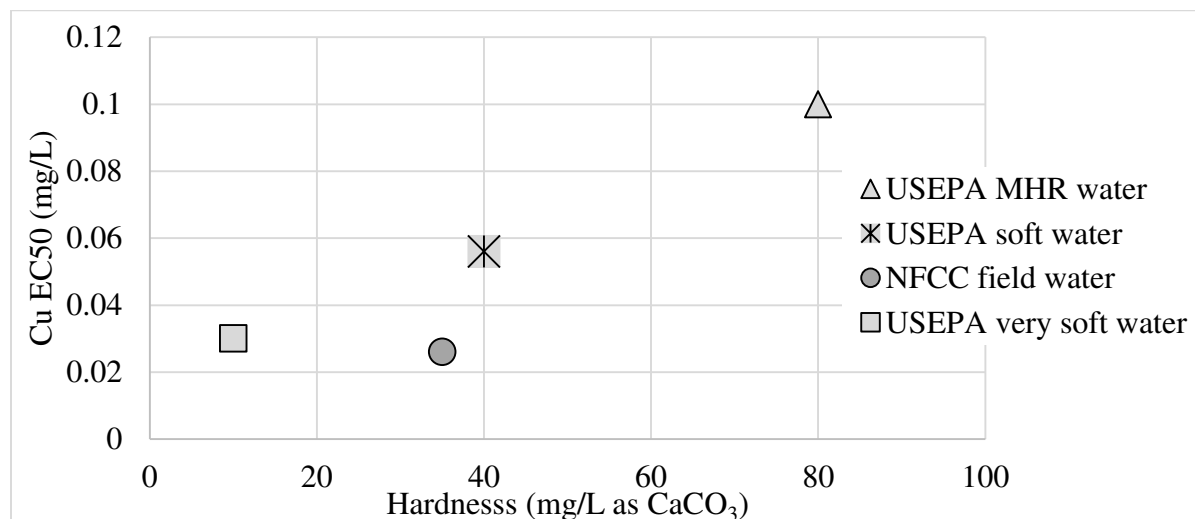


Figure 2.12. Relationship between median effect concentrations (EC50 values) of Cu and water hardness in the dilution waters used in this study (MHR = moderately hard water; NFCC = North Fork Clear Creek). As the hardness and alkalinity of the USEPA lab waters decreased, the Cu EC50 concentration decreased (i.e., Cu was more toxic at lower water hardness). Although the EC50 concentration for NFCC water is lower than might be expected based solely on hardness, a variety of geochemical characteristics in the field water could explain this, including the “effective” DOC concentration (see text for explanation).

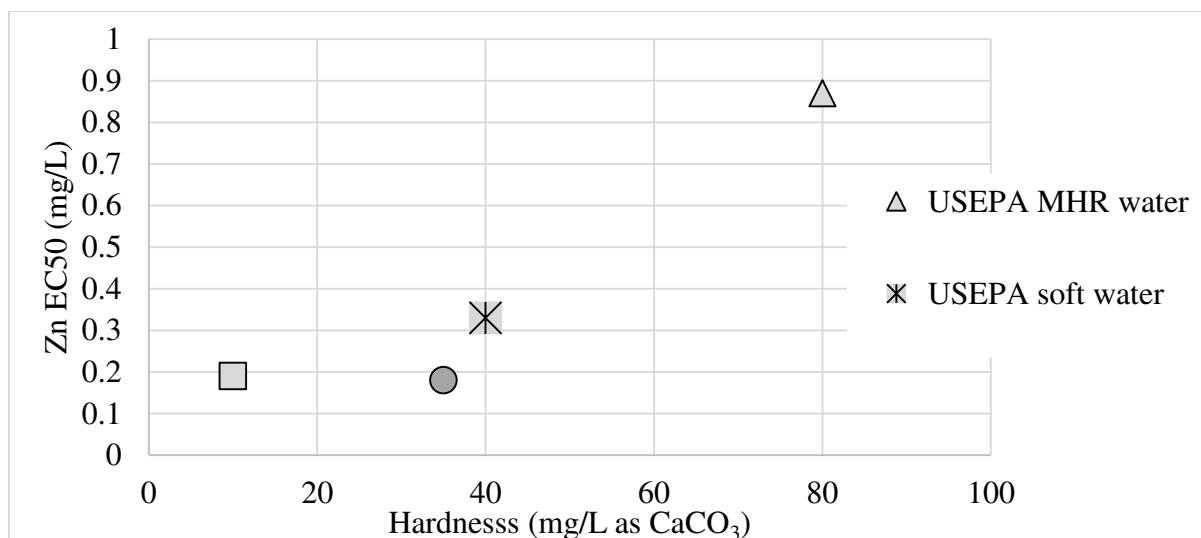


Figure 2.13. Relationship between median effect concentrations (EC50 values) of Zn and water hardness in the dilution waters used in this study (MHR=moderately hard water; NFCC=North Fork Clear Creek). As the hardness and alkalinity of the USEPA lab waters decreased, the Zn EC50 concentration decreased (i.e., Zn was more toxic at lower water hardness). Although the EC50 concentration for NFCC water is lower than might be expected based solely on hardness, a variety of geochemical characteristics in the field water could explain this, including the “effective” DOC concentration (see text for explanation).

2.3.2 Predictions of Cu-only and Zn-only Toxicity

Biotic ligand model predictions can be useful for interpreting the Cu-only and Zn-only toxicity results in the previous section. In order to better understand and model metal-mixture toxicity, there is a need to compare observed individual-metal toxicity to the BLM-predicted toxicity in MHR water and field-collected water. The BLM has been approved for deriving site-specific water quality criteria for Cu in the U.S., and several advances in predicting Zn toxicity may lead to the adoption of BLM-derived Zn criteria (DeForest and Van Genderen 2012). However, there continues to be a need to validate the BLM’s accuracy in predicting toxicity in a variety of water chemistries so the model can be used at a variety of sites with varying aqueous geochemistry.

The acute-toxicity Cu BLM has been tested in USEPA lab waters and field waters that have high hardness (> 250 mg/L as CaCO₃), and previous studies demonstrated that the Cu BLM can predict EC50 concentrations within a factor of two in field waters with high hardness (Van Genderen et al. 2007). The Zn BLM has also been tested in a variety of water chemistries. When several Zn binding constants were manipulated from the default BLM parameterization, acute

EC50 concentrations were predicted within a factor of two in lab waters (DeForest and Van Genderen 2012).

I used the default BLM version 2.2.3 to predict EC50 concentrations for Cu and Zn in MHR water, dilutions of MHR water, and NFCC field water with low hardness (< 40 mg/L as CaCO₃). The default BLM predicted the Cu and Zn EC50 concentrations in MHR water and USEPA soft water within a factor of 2; however, the BLM over-predicted the EC50 concentrations for Zn and Cu in NFCC water and USEPA very soft water by more than a factor 2 (Figure 2.14). In aquatic toxicology, a factor of 2 has become a generally-accepted index of good agreement between predicted and observed concentrations (Santore et al. 2001).

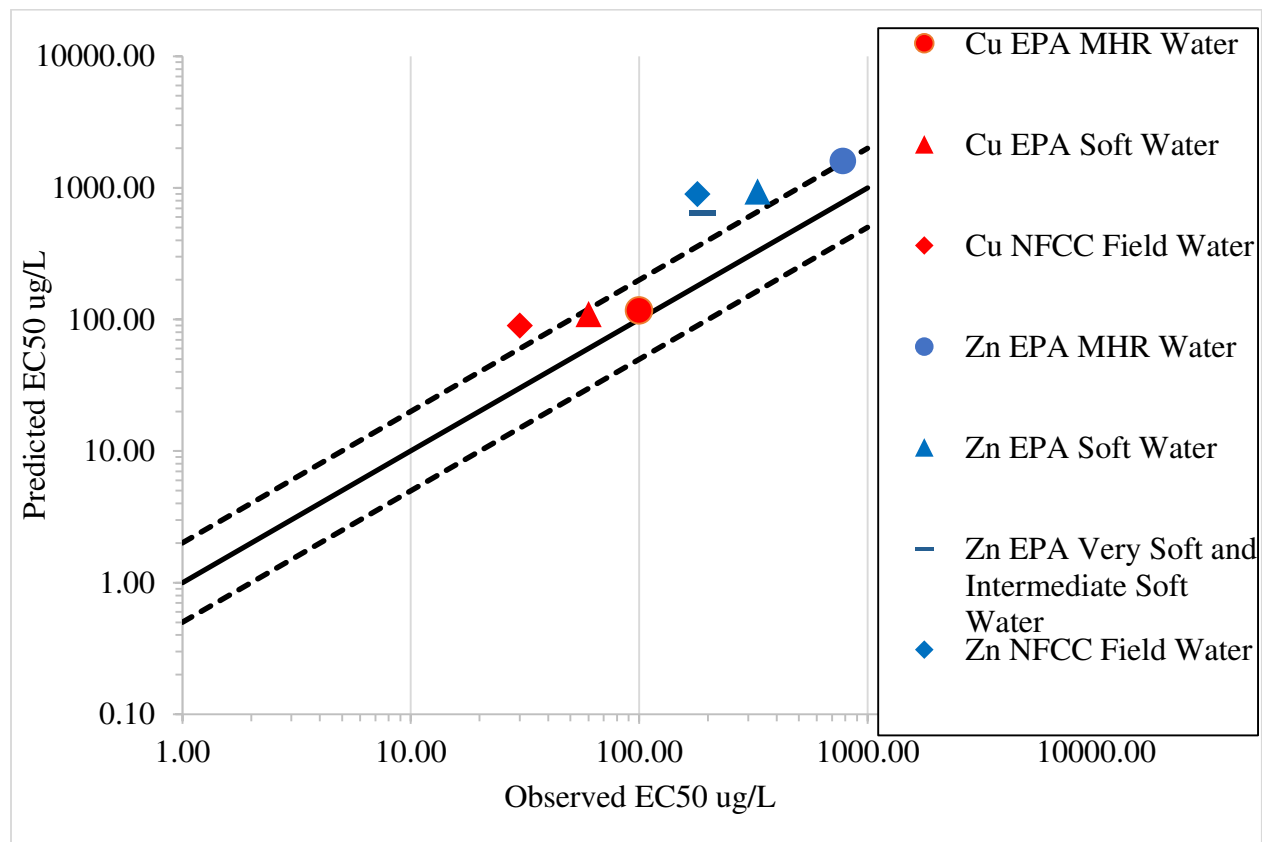


Figure 2.14. Default biotic ligand model-predicted median effect concentrations (EC50 values) for *Daphnia magna* neonates exposed to Cu and Zn in USEPA moderately hard reconstituted (MHR) water, soft water, and very soft water, and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. The solid diagonal line represents the 1:1 fit of perfect agreement between observed and predicted EC50 values. Dashed lines are \pm a factor of 2 from the 1:1 line.

This over-prediction of the Cu and Zn EC50 concentrations (i.e., under-prediction of Cu and Zn toxicity) in the softer waters can be explained by at least two causes. First, the strain of *D.*

magna used in this study may have a slightly different sensitivity to Cu and Zn than the composite data set of *D. magna* toxicity with which the BLM is parameterized. Second, the BLM might not account for an important aspect of the water chemistry in the USEPA very soft and NFCC waters.

To determine if a strain-specific sensitivity was responsible for the difference in BLM-predicted EC50 concentrations and observed concentrations, I calibrated the BLM to the toxicity of Cu and Zn to my strain of *D. magna* in USEPA MHR water. Consequently, by definition, the strain-specific predicted EC50 concentration equals the observed EC50 concentration in USEPA MHR water (Figure 2.15). This strain-specific BLM calibration also shifted the predicted EC50 concentrations for USEPA soft and very soft waters within a factor of 2 of the observed EC50 concentrations. However, the predicted Cu and Zn EC50 concentrations in NFCC field water were still greater than a factor of 2 from the observed EC50 concentrations.

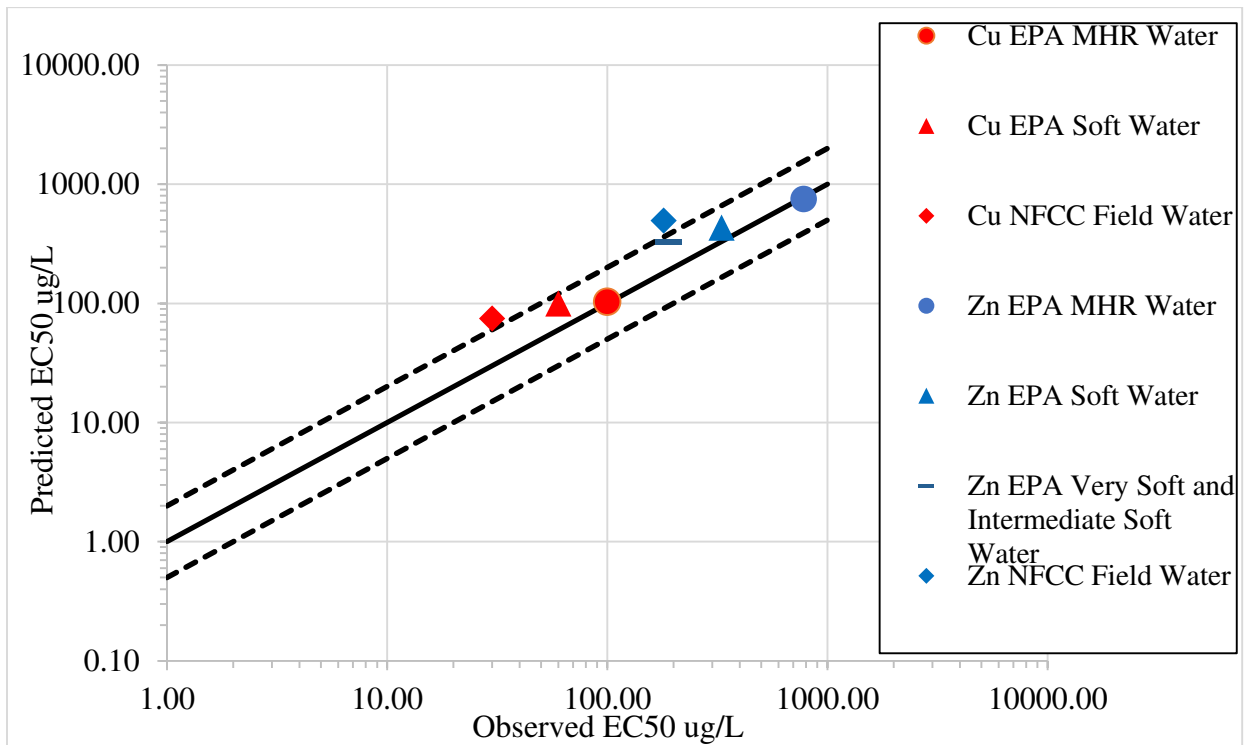
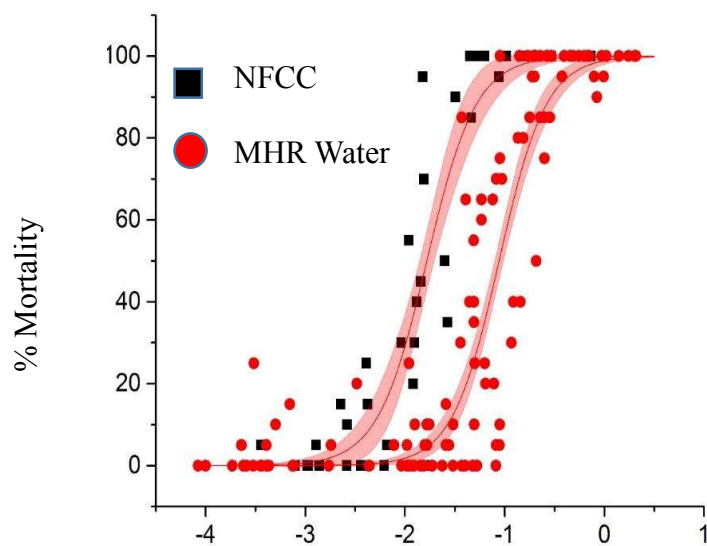


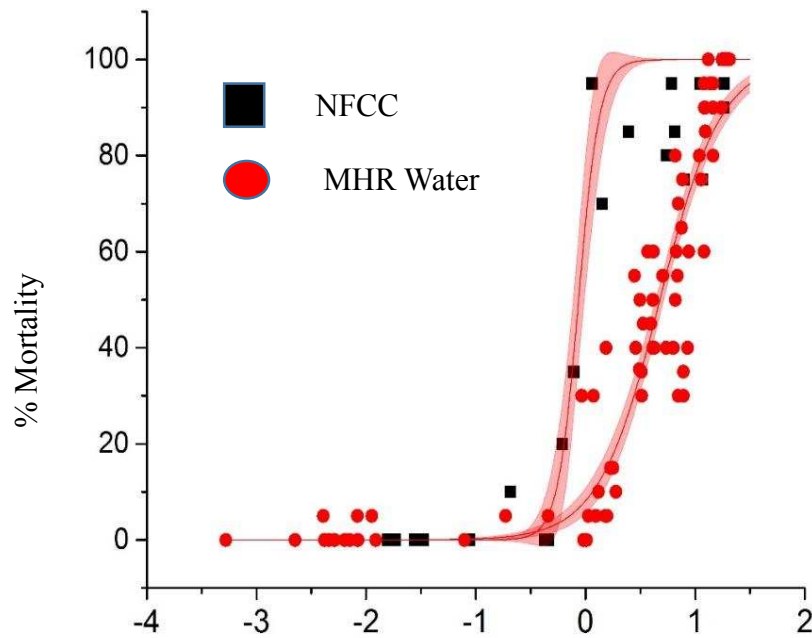
Figure 2.15. Strain-specific biotic ligand model-predicted median effect concentrations (EC50 values) for *Daphnia magna* neonates exposed to Cu and Zn in USEPA moderately hard reconstituted (MHR) water, soft water, and very soft water, and in water from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. After calibrating the BLM to the strain of *Daphnia magna* neonates used in this study, the BLM still over-predicted the Cu and Zn EC50 concentrations in NFCC water. The solid diagonal line represents the 1:1 fit of perfect agreement between observed and predicted EC50 values. Dashed lines are \pm a factor of 2 from the 1:1 line.

The BLM was also run in speciation mode to calculate the model-inferred accumulation of metal in nmol/g wet weight on the organism at the various concentrations of total dissolved metal that were in the exposure waters used in these Cu and Zn toxicity tests. Then I plotted the accumulation of metal versus mortality. These graphs are concentration-response curves in “biotic ligand space”. Figures 2.16 and 2.17 show the BLM-predicted Cu and Zn accumulation on the biotic ligand versus observed mortality. Mortality versus BLM-predicted Cu and Zn accumulation curves should overlap for MHR water and NFCC water, because one of the major assumptions of the BLM is that a given metal accumulation on the biotic ligand causes a specific amount of toxic response regardless of the water quality in which the organism is exposed to the metal if other toxicants are not present (Santore et al. 2001). Therefore, the same BLM-predicted metal accumulation on the biotic ligand in different waters should cause the same amount of mortality; and a given strain of *D. magna* should have the same LA50 for a given metal, regardless of the exposure-water chemistry. However, the LA50 values for *D. magna* were significantly different in NFCC field water and MHR water (Table 2.4).



Log of BLM-predicted Cu accumulation on biotic ligand (nmol/g ww)

Figure 2.16. Biotic ligand model (BLM)-predicted Cu accumulation on the biotic ligand versus mortality in USEPA moderately hard reconstituted (MHR) water and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. For these calculations, the BLM-input dissolved organic carbon concentration was ~3 mg/L for both waters.



Log of BLM-predicted Zn accumulation on biotic ligand (nmol/g ww)

Figure 2.17. Biotic ligand model (BLM)-predicted Zn accumulation on the biotic ligand versus mortality in USEPA moderately hard reconstituted (MHR) water and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. For these calculations, the BLM-input dissolved organic carbon concentration was ~3 mg/L for both waters.

One explanation for why the biotic-ligand-space curves do not overlap in MHR water and NFCC field water could be the quality of the DOC in the field water. The MHR water contained 3 mg/L DOC as SRFA. The NFCC field also had 3 mg/L DOC, but it contained approximately 2 mg/L as natural DOC and 1 mg/L as SRFA. It is possible that the natural DOC in the NFCC field water did not bind metals as effectively as the SRFA. Therefore, this difference in “effective DOC” could be responsible for the differences in the BLM predictions for metal accumulation on the biotic ligand in USEPA MHR water and NFCC water, because the default parameterization of the BLM assumes all DOM has the same binding affinity and capacity for a given metal. However, the binding affinities for metals to natural DOM can span several orders of magnitude, and modeling metal-DOM complexation in the environment can be challenging (Kuhn et al. 2015).

To test the effective-DOC hypothesis, I decreased the DOC concentration in the NFCC field water in the BLM input file from the measured concentration of approximately 3 mg/L to 1

mg/L, and then I ran the BLM in speciation mode. Figures 2.18 and 2.19 show the model-inferred metal accumulation on the biotic ligand in NFCC field water with 1 mg/L effective DOC and in USEPA MHR water with 3 mg/L DOC as SRFA. Adjusting for “effective” DOC concentration improved the predictions, but adjustment was not consistent between Cu and Zn. Reducing the DOC in the NFCC field water from 3 to 1 mg/L resulted in Cu LA50 values in USEPA MHR water and NFCC field water that did not differ significantly (Table 2.4). However, the Zn LA50 values in the two waters still differed significantly when the BLM-input DOC in the NFCC field water was reduced from 3 to 1 mg/L. The BLM-input DOC concentration in the NFCC field water would have to be <1 mg/L to have the same Zn LA50 value as in USEPA MHR water.

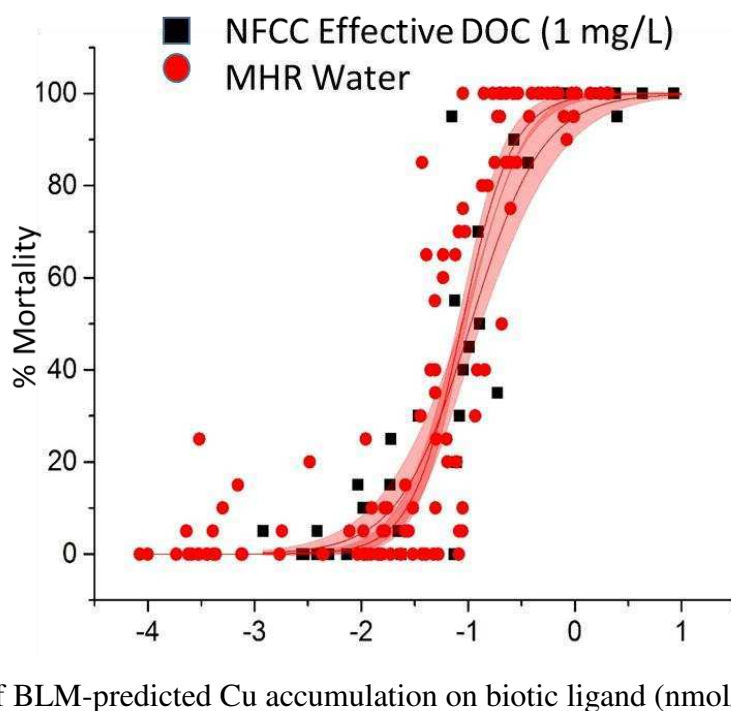


Figure 2.18. Biotic ligand model (BLM)-predicted Cu accumulation on the biotic ligand versus mortality in USEPA moderately hard reconstituted (MHR) water and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. For these calculations, the BLM-input dissolved organic carbon concentration was 1 mg/L for the NFCC water and 3 mg/L for the USEPA MHR water.

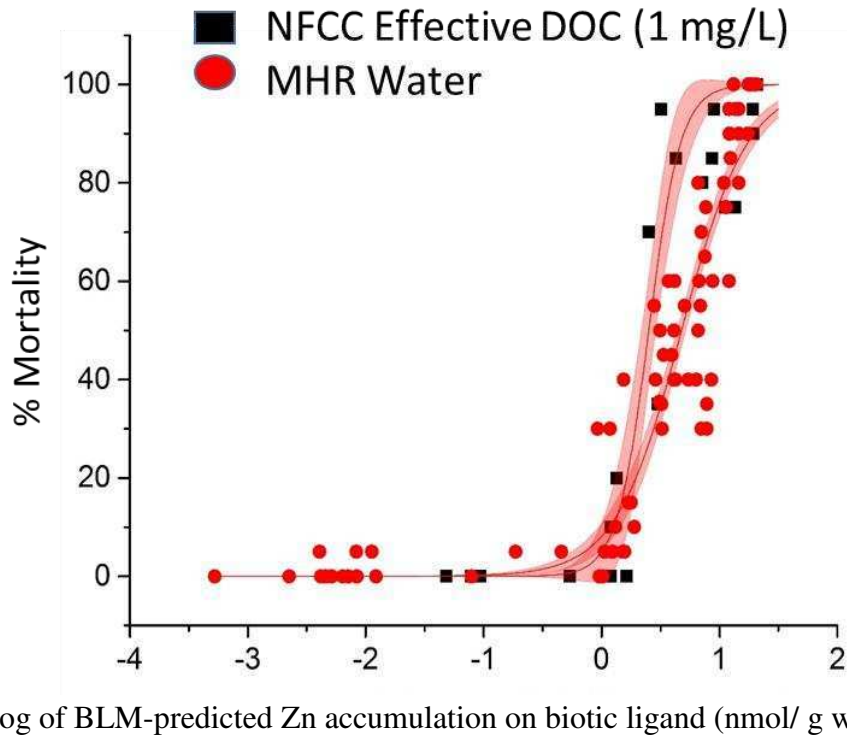


Figure 2.19. Biotic ligand model (BLM)-predicted Zn accumulation on the biotic ligand versus mortality in USEPA moderately hard reconstituted (MHR) water and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. For these calculations, the BLM-input dissolved organic carbon concentration was 1 mg/L for the NFCC water and 3 mg/L for the USEPA MHR water.

Table 2.4. BLM-predicted LA50s and 84% confidence intervals for Cu and Zn in USEPA MHR water and NFCC field water with 3 mg/L DOC and 1 mg/L “effective” DOC.

Metal	Water and DOC concentration	BLM-predicted LA50 (nmol/g ww) (84% C.I.)
Cu	USEPA MHR water 3 mg/L DOC	0.088 (0.080-0.097)
Cu	NFCC field water 3 mg/L DOC	0.017 (0.014-0.019)
Cu	NFCC field water 1 mg/L "effective" DOC	0.106 (0.087-0.129)
Zn	USEPA MHR water 3 mg/L DOC	4.77 (4.40-5.17)
Zn	NFCC field water 3 mg/L DOC	0.865 (0.778-0.962)
Zn	NFCC field water 1 mg/L "effective" DOC	2.52 (2.22-2.85)

Although the concept of “effective” DOC concentration improved the predictions for Cu and Zn LA50 values in the NFCC field water, the “effective” DOC concentrations are most likely not realistic. Using 1 mg/L “effective” DOC assumes that all of the natural DOC in NFCC field water (~2 mg/L) did not bind metals and that only the 1 mg/L of DOC added as SRFA was responsible for metal binding. This assumption is not valid because natural DOM in alpine streams impacted by AMD plays an important role in binding metals (McKnight et al. 1992). For example, in a study of Colorado alpine streams, natural DOM-Cu complexation played a crucial role in controlling the chemical activity of Cu^{2+} and thus the predicted toxicity of Cu to fish (Brooks et al. 2007a). Similarly, DOM in a montane wetland and in a high-elevation river in Wyoming modified the toxicity of Cu to fathead minnows (Brooks et al. 2007b). Natural DOM in alpine streams such as NFCC clearly plays a role in metal complexation, and it would not be mechanistically defensible to assume that none of the natural DOC in the NFCC field water interacted with Cu or Zn in the toxicity tests.

An alternate hypothesis that may explain the differences in the BLM-predicted Cu and Zn LA50 values in USEPA MHR water and NFCC field water may involve the ambient concentrations of Al and Fe in NFCC field water. Although the NFCC field site is a pristine reach of the stream, it contains low concentrations of Al and Fe. Ambient Fe concentrations were typically <10 $\mu\text{g/L}$ in NFCC field water, and Al concentrations were typically less than the detection limit of 4.5 $\mu\text{g/L}$. However, even those low concentrations of Al and Fe in the NFCC field water might compete with Cu and Zn for binding to DOM and thus leave higher Cu^{2+} and Zn^{2+} concentrations in the NFCC water than would be predicted by the BLM, which currently does not account for interactions with Al and Fe. As a consequence, the “effective” DOC concentration would be operationally decreased by the presence of Al and Fe, thus impacting the toxicity of Cu and Zn in the NFCC field water. A multi-metal biotic ligand model is in development (Santore and Ryan 2015), and that model could help test this hypothesis.

Overall, the default BLM parameterization can predict the observed Zn and Cu acute EC50 concentrations within a factor of 2 in USEPA MHR, soft, and very soft water but not in NFCC water. When a strain-specific calibration of the BLM was performed, the predicted Cu and Zn EC50 concentrations in NFCC water were still not within a factor of 2 of the observed EC50 concentrations. The concept of “effective” DOC may be useful for using the BLM in field waters if the BLM predictions are not within a factor of 2 of the observed EC50s. However, alternate

explanations of differences in BLM-predicted EC50s and observed EC50s (such as low background concentrations of metals like Al and Fe) are also plausible and potentially more likely.

2.3.3: Cd-only Toxicity in Laboratory and Field-Collected Waters

Cadmium toxicity tests were also performed in NFCC field collected water and USEPA laboratory waters. Unlike the results of the Cu and Zn toxicity tests in dilutions of USEPA MHR water, there was no clear relationship between the hardness of the water and the Cd EC50.

Although NFCC field water is much softer than USEPA MHR water and therefore should be less protective, Figure 2.20 and Table 2.5 show that the Cd EC50 was higher (instead of lower) in NFCC field water. However, the toxicity in USEPA MHR water varied considerably among the replicate tests. Some of this variation may be explained by the age of the organisms. Although all of the test organisms were neonates (less than 24 h old), a difference in a few hours of age impacts Cd toxicity to *D. magna* neonates. Contrary to traditional dogma, the Cd EC50 in toxicity tests started with 0- to 4-h-old neonates is greater than the Cd EC50 in tests started with 10- to 14-h-old neonates, which is greater than the Cd EC50 in tests started with 20- to 24-h-old neonates (Traudt et al. 201_). As *D. magna* neonates approach 24 h old, they begin to molt. This molting may make them more sensitive to cadmium toxicity (Traudt et al. 201_). Figure 2.21 compares Cd toxicity based on age at the start of the test in EPA MHR water to Cd toxicity in NFCC field water.

Interestingly, the Cd EC50 in NFCC water is captured by the confidence interval around the EC50 concentration for 0 to 4-h-old organisms in USEPA MHR water (Table 2.5). This result was unexpected because the organisms used in the NFCC field toxicity tests were not constrained to 0 to 4 h old, and the NFCC field water should have been far less protective based on hardness than the USEPA MHR water.

Cadmium toxicity in NFCC field water was also compared to toxicity in EPA soft water and a dilution of soft water I refer to as “intermediate” softness. The Cd EC50 was greater in NFCC field water than in USEPA soft water and equal to the EC50 in the intermediate softness water (Figures 2.22 and 2.23, and Table 2.5).

Table 2.5. Median Effect concentrations (EC50 values) and 84% confidence intervals for Cd in laboratory and field-collected waters.

Exposure water	Cd EC50 (mg/L) (84% C.I.)
USEPA MHR water	0.050 (0.042-0.058)
USEPA MHR water <i>D. magna</i> age 0-4 h (Traudt et al. 201_)	0.144 (0.116-0.179)
USEPA MHR water <i>D. magna</i> age 20-24 h (Traudt et al. 201_)	0.018 (0.014-0.022)
USEPA soft water	0.041 (0.032-0.052)
NFCC field water	0.160 (0.139-0.185)
Intermediate softness water	0.158 (0.134-0.187)

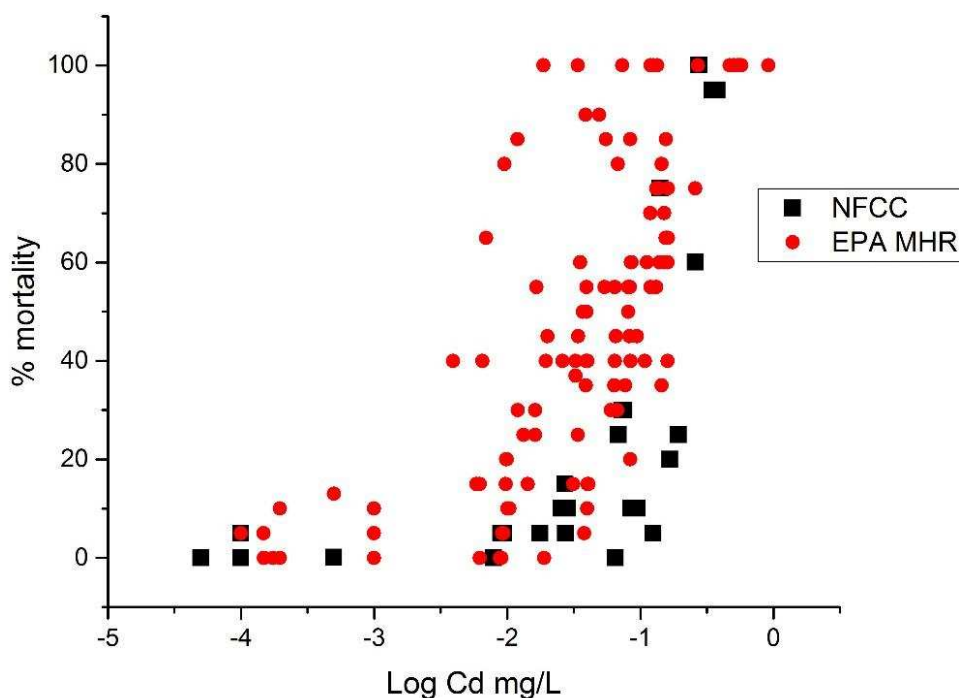


Figure 2.20: Mortality of *Daphnia magna* neonates exposed to Cd in 48-h lethality tests in USEPA moderately hard reconstituted water (MHR) and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Cd toxicity tests in each water were combined (22 tests in EPA MHR water and 6 tests in NFCC field water).

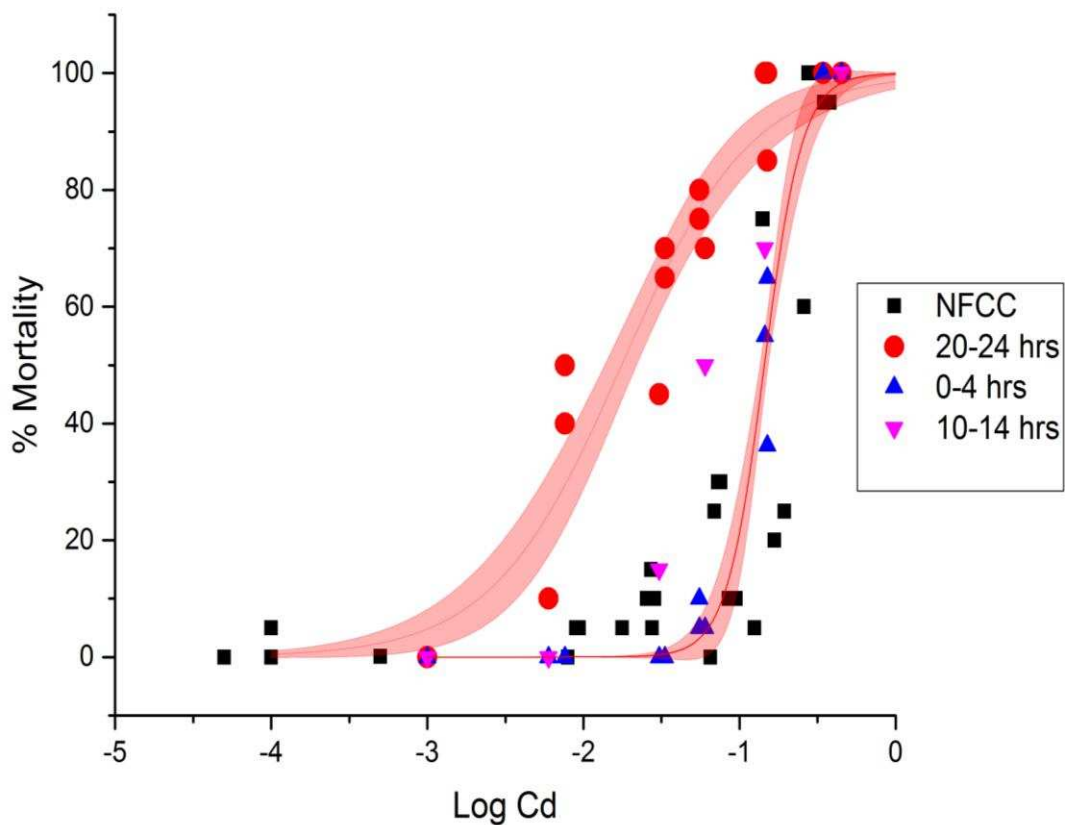


Figure 2.21: Logit concentration-response curves for mortality of *Daphnia magna* neonates exposed to Cd in 48-h lethality tests in USEPA moderately hard reconstituted (MHR) water and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. The age of the neonates at the start of the toxicity tests in MHR water was constrained to three intervals (0-4, 10-14, and 20-24 h), but the age of the neonates at the start of the toxicity tests in NFCC field water ranged from 4-24 h. Results of multiple Cd toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve. Age-related toxicity data from Traudt et al. (201_).

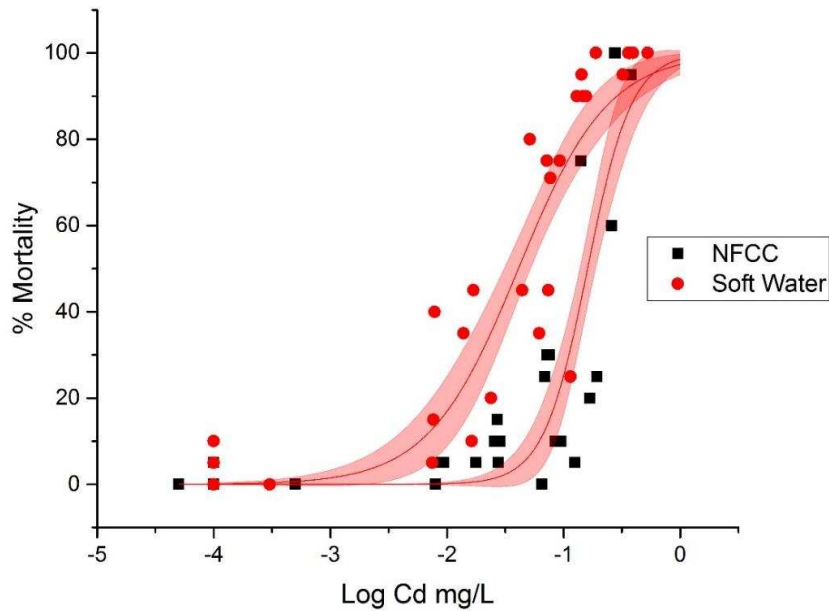


Figure 2.22: Logit concentration-response curves for *Daphnia magna* neonates exposed to Cd in 48-h lethality tests in USEPA soft water and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Cd toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

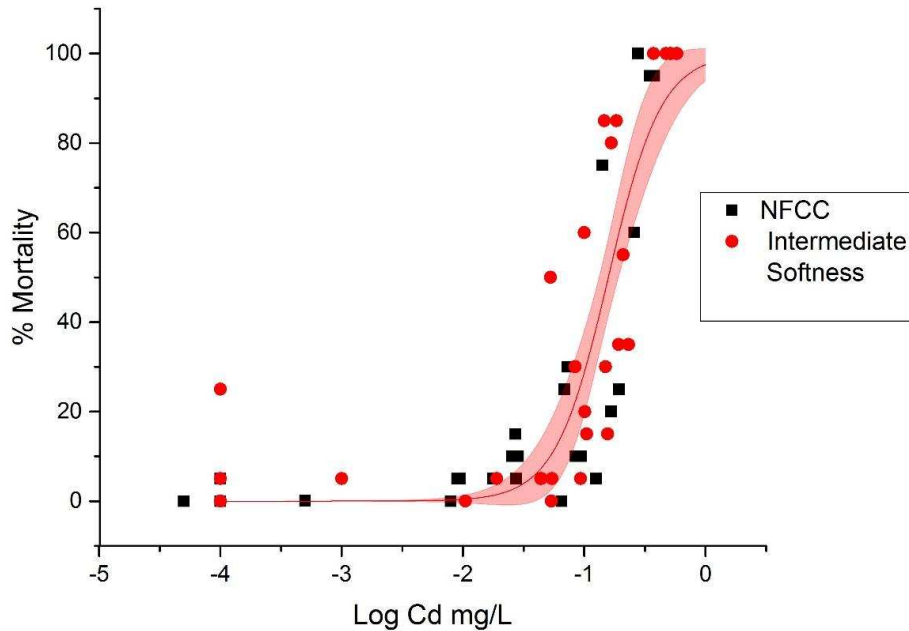


Figure 2.23: Logit concentration-response curves for *Daphnia magna* neonates exposed to Cd in 48-h lethality tests in water with intermediate softness and in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Results of multiple Cd toxicity tests in each water were combined to generate the logit-regression curves. The salmon-colored shading is an 84% confidence band around each curve.

There is no clear trend in the Cd EC50 concentrations in differing water chemistries, making it challenging to develop accurate predictive models for Cd toxicity (Figure 2.24). The neonates used in toxicity tests with NFCC water were not constrained to a narrow age window, but the Cd EC50 in NFCC water is not significantly different from the EC50 in EPA MHR water with neonates age 0 to 4 h old. Other Cd toxicity tests in the literature have shown high variability (Clubb et al. 1975), and there is no clear mechanistic explanation for this variability. One possibility may be related to metallothioneins, which are proteins involved in the transport and detoxification of Cd in organisms (Poynton et al. 2007). Age-related differences in concentrations of metallothioneins in *D. magna* could lead to variable sequestration of Cd and thus to variable toxicity of Cd. An emerging tool that could lead to insight into variable Cd toxicity is toxicogenomics. Toxicogenomics is the application of genomic studies to toxicology, and distinct gene-expression patterns have been reported in *D. magna* that were exposed to sub-lethal concentrations of Cd and other metals (Poynton et al. 2007). Further research in the field of toxicogenomics may lead to a mechanistic understanding of Cd toxicity to *D. magna*, which could help explain the age-related variability in Cd toxicity.

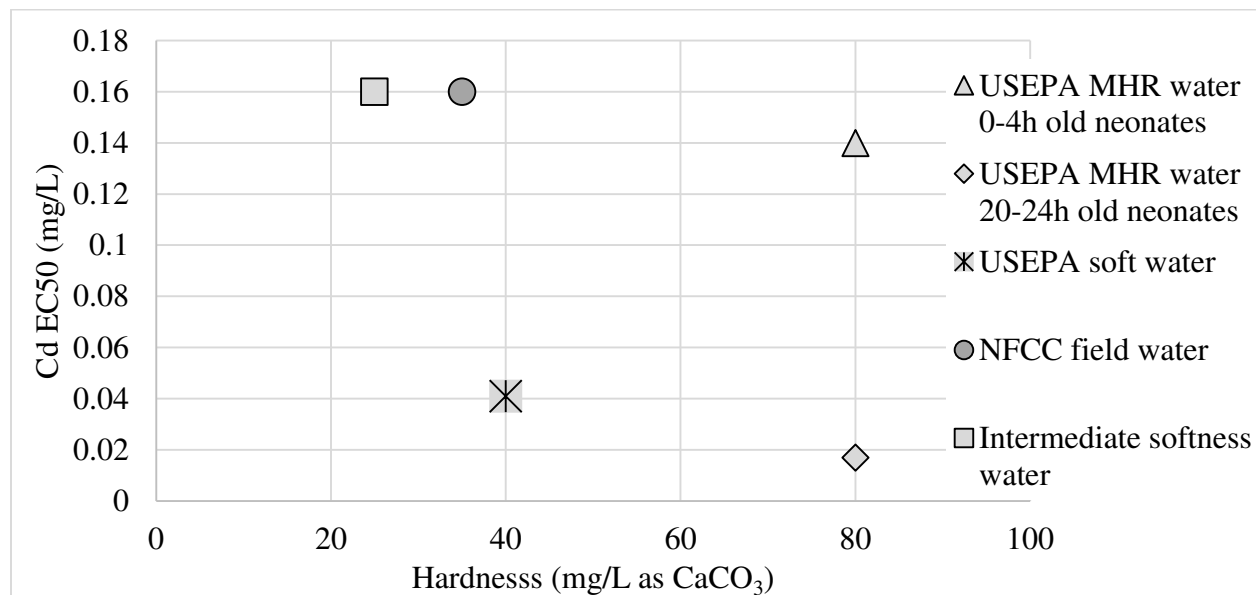


Figure 2.24 Relationship between median effect concentrations (EC50 values) of Cd and water hardness in the dilution waters used in this study (MHR=moderately hard water; NFCC=North Fork Clear Creek). The Cd EC50 was expected to decrease as the hardness of the dilution waters decreased (i.e., Cd was expected to be more toxic at lower water hardness). However, there is no clear hardness-dependent trend in Cd EC50 values. Age-related EC50 values in USEPA MHR water from Traudt et al. (201_).

CHAPTER 3

BINARY-METAL TOXICITY TESTS IN LAB AND FIELD WATERS

Although Chapter 2 focused on individual-metal toxicity, metals usually occur in the environment as mixtures (Meyer et al. 2015a). One of the goals of this thesis is to contribute to the understanding of metal-mixture toxicity and provide data that can be used to develop multi-metal toxicity models. To this end, I tested binary metal-mixtures of Cu-Zn and Cd-Zn in NFCC field water and in several laboratory-water chemistries.

3.1 Introduction

Cadmium, Cu, and Zn were chosen because they are present in the AMD that enters the North Fork of Clear Creek, and previous studies have examined the toxicity of these mixtures in USEPA MHR water (e.g., Meyer et al. 2015b). Acute toxicity to *D. magna* has been reported to be additive to slightly more-than-additive in Cu-Zn mixtures in USEPA MHR water and less-than-additive in Cd-Zn mixtures in USEPA MHR water (Meyer et al. 2015b), and herein I demonstrate similar responses in NFCC field water. This study highlights the importance of water chemistry when extrapolating results of metal-mixture toxicity tests from the laboratory to the field, and it emphasizes the need for statistical models to quantify the differences in toxicity of metal mixtures among various water chemistries.

3.2 Materials and Methods

The materials and methods for the metal-mixture toxicity tests are described in detail in this chapter, and new statistical methods are also introduced. The test organisms and chemical analyses in the binary-metal toxicity tests were the same as in the single-metal toxicity tests, as described in Chapter 2.

3.2.1 Toxicity Tests and Chemical Analyses

Acute 48-h Cu-Zn and Cd-Zn toxicity tests were performed with *D. magna* neonates in conjunction with the individual-metal toxicity tests discussed in Chapter 2. In these binary-metal toxicity tests, Metal #1 was held constant at a specified “background” concentration while Metal #2 was “titrated” into a series of exposure waters. The tests comprised a series of twelve combinations of metal concentrations in a gradient designed to produce mortalities ranging from 0 to 100%. This series of 12 metal-mixture combinations (11 varied-metal concentrations and one control) was used to identify toxicity decreases at low concentrations and toxicity increases

at higher concentrations of the varied metal. In the case of the Cu-Zn mixtures tested, Cu was Metal #1 (held constant) while Zn was varied in a concentration series. In Cd-Zn toxicity tests, Cd was Metal #1 and Zn was varied in a concentration series. The concentration of the background metal was varied from test to test to determine if more-than, less-than, or response-additive toxicity was dependent on the background concentration of Metal #1.

When a suite of binary-metal toxicity tests was conducted in the NFCC reference-site field water, the water all came from the same sampling event. For example, the Cu-Zn binary-metal toxicity tests were all performed in a matrix of NFCC field water that was collected in January 2015 and stored in a cold room. The water used in the Cd-Zn tests was also collected on the same date.

The toxicity of Cd-Zn mixtures was tested in USEPA MHR water, dilutions of MHR water, and NFCC field water. To further evaluate the toxicity of Cd-Zn in NFCC field water, a batch of synthetic NFCC water was prepared in the laboratory. This water mimicked the aqueous geochemistry in the stream water by matching the concentration of the major inorganic ions and water hardness, but the quality of DOC in the synthetic field water was different from the field-collected NFCC water. I added 3 mg/L DOC as SRFA to the synthetic NFCC water, and the field-collected NFCC water contained 3 mg/L DOC as a combination of SRFA (1 mg/L DOC) and natural DOM (approximately 2 mg/L DOC).

3.3 Statistical Analyses

Determining whether the toxicity of a metal-mixture is additive, more-than-additive, or less-than-additive using a quantitative, statistically robust method is challenging (Meyer et al. 2015a). Response addition assumes that the components of the metal mixture are functionally independent of each other, because the metals are assumed to have independent modes of toxicity. In contrast to the response-addition model, the concentration-addition model assumes that the components in the mixture have a similar mode of toxicity and affect the same site(s) of action (Parvez et al. 2009). Most aquatic toxicity tests do not explore enough (if any) physiological processes to determining underlying mechanisms of toxicity (Meyer et al. 2015a). In this study, the terms less-than-additive, additive, and more-than-additive toxicity are used because these terms do not require knowledge of specific toxic mechanisms. The response-addition toxicity model was chosen over the concentration-addition model because the specific interactions of Cd, Cu, and Zn at these sites of toxic action are not well characterized.

In this study, I quantify non-additive toxicity by evaluating changes in the concentration of the inflection points that occur in individual-metal and metal-mixture concentration-response curves (Traudt et al. 2016). In an individual-metal toxicity test, the concentration-response curve is generally a symmetrical sigmoidal curve, and the inflection point of the curve occurs at 50% effect level, defined as the median effects concentration (EC50). In a toxicity test of a binary mixture of metals in which Metal #1 is held constant and Metal #2 is “titrated” into the mixture in a series of treatments containing increasing concentrations of Metal #2, the initial mortality in the test will depend on the background concentration of Metal #1. In some cases, the initial mortality may be greater than 50%, making it impossible to determine an EC50. In that situation, the inflection point of the metal-mixture concentration response curve is not the EC50 but instead is the concentration at 50% of the residual survival. For example, if the initial mortality in a binary-metal toxicity test is 60%, the mortality at the inflection point will be 80%. I will call the concentration at the inflection point the EC_{infl} .

When the toxicity of the binary metal-mixture is strictly response-additive, the inflection point of the metal-mixture concentration-response curve occurs at a constant concentration of Metal #2, independent of the concentration of Metal #1 in the mixture (Figure 3.1; Traudt et al. 2016). If, on the other hand, the toxicity of a metal-mixture is not response-additive, the EC_{infl} shifts. When the toxicity of a metal mixture is more-than-additive, the inflection point of the metal-mixture concentration-response curve shifts to a lower concentration than the response-additive inflection point (i.e., less than the Metal #2-only inflection point). For less-than-additive toxicity, the inflection point of the metal-mixture concentration-response curve shifts to a higher Metal #2 concentration than the response-additive inflection point (i.e., greater than the Metal #2-only inflection point).

I calculated the predicted response-additive mortality (m) for a binary-metal mixture based on the individual-metal toxicity using Equation 3.1:

$$m = [1-(S_{M1}*S_{M2})]*100\% \quad (3.1)$$

In this equation, S_{M1} and S_{M2} are the survival proportions predicted for Metal #1 and Metal #2 at their concentrations in the mixture, but based on the survival that would occur in Metal #1-only and Metal #2-only toxicity tests. The survival proportions for Metal #1 and Metal #2 were predicted from the individual-metal concentration-response curves in toxicity tests conducted concurrent with the binary-metal toxicity tests.

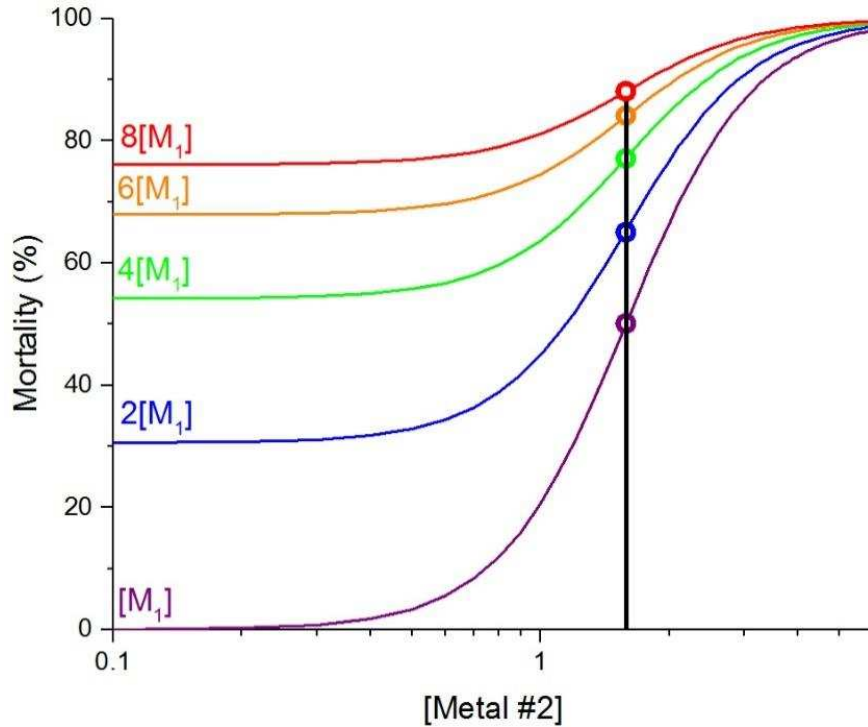


Figure 3.1. Examples of response-additive toxicity. The colored curves indicate different concentrations of Metal #1 in a series of binary metal-mixture toxicity tests, and the inflection points of these curves are circled. As the concentration of Metal #1 increases, the initial (background) mortality in the toxicity test increases. However, the inflection points of the metal-mixture concentration-response curves always occur at the same concentration of Metal #2, indicating response-additive toxicity. When the toxicity is more-than-additive, the inflection point of the concentration-response curve shifts to a lower concentration of Metal #2. Conversely, when the toxicity of the mixture is less-than-additive, the inflection point shifts to a higher concentration of Metal #2.

The concentration-response curves for predicted response-additive toxicity were determined from regressions of predicted response-additive mortality (calculated using Equation 3.1) versus Metal #2 concentration using OriginPro 9.1 software (OriginLab, Northampton, MA), based on the following transformed logit relationship:

$$y = A_1 + \frac{(A_2 - A_1)}{1 + 10^{((\text{Log}X_0 - X) \cdot p)}} \quad (3.2)$$

where A_1 and A_2 are the lower and upper mortality limits (%), respectively; X is the concentration of Metal #2, X_0 is the concentration of Metal #2 at the center of the distribution (i.e., the Metal #2 EC_{infl}); p is the slope of the logit-regression curve; and y is the mortality in the binary-metal mixture (%). Because the concentration-response curves calculated by OriginPro are symmetrical, the mortality at the inflection point occurs at 50% of the residual survival.

I used a one-sample t-test to determine if the toxicity of a metal mixture differed significantly from the predicted (Zn-only) toxicity. The null hypothesis was that the metal-mixture toxicity was response-additive (i.e., the Metal #2 EC_{infl} concentrations of the binary mixtures equal the Metal #2-only EC50). To perform the t-test, the ratio of the difference between the predicted and each observed inflection point to the predicted inflection point was calculated as:

$$EC_{infl} \text{ ratio} = \frac{\text{observed } EC_{infl} - \text{predicted } EC_{infl}}{\text{predicted } EC_{infl}} \quad (3.3)$$

Because the background concentration of Metal #1 was varied in a series of binary-metal toxicity tests, this ratio was averaged for all the various concentrations of Metal #1. The mean of the ratios from the series of binary metal-mixture toxicity tests was then compared to zero because, if the toxicity of the series of binary metal-mixtures was response-additive, the observed EC_{infl} concentrations should equal the predicted EC_{infl} concentrations. The t value was calculated as:

$$t \text{ calc} = \frac{EC_{infl} \text{ ratio} * \sqrt{N}}{s.d.} \quad (3.4)$$

The calculated t value was compared to a critical t value from a Student's t-table, as determined by the degrees of freedom (N-1) and the desired confidence level. The statistical significance of all comparisons in this study was determined at the 95% confidence level.

3.3 Results and Discussion

Concentrations-response curves, mortality data, and statistical analyses for the metal-mixture toxicity tests are presented in sections 3.3.1 and 3.3.2. The results and interpretations for the Cu-Zn toxicity tests and the Cd-Zn toxicity tests are presented in sections 3.3.1 and 3.3.2, respectively.

3.3.1 Cu-Zn Mixtures

The toxicity of five Cu-Zn mixtures with varying concentrations of background Cu was tested in NFCC field water. The background concentrations of Cu ranged from 10 to 25 µg/L. In six Cu-only toxicity tests in NFCC water, 10 µg Cu/L caused mortality ranging from 0 to 25%, and 25 µg Cu/L caused mortality ranging from 30 to 60%. Figure 3.2 shows the predicted response-additive toxicity curves for the Cu-Zn mixtures, and Table 3.1 shows the predicted response-additive EC_{infl} concentrations, initial mortality, and mortality at the inflection point for each Cu-Zn mixture that was tested.

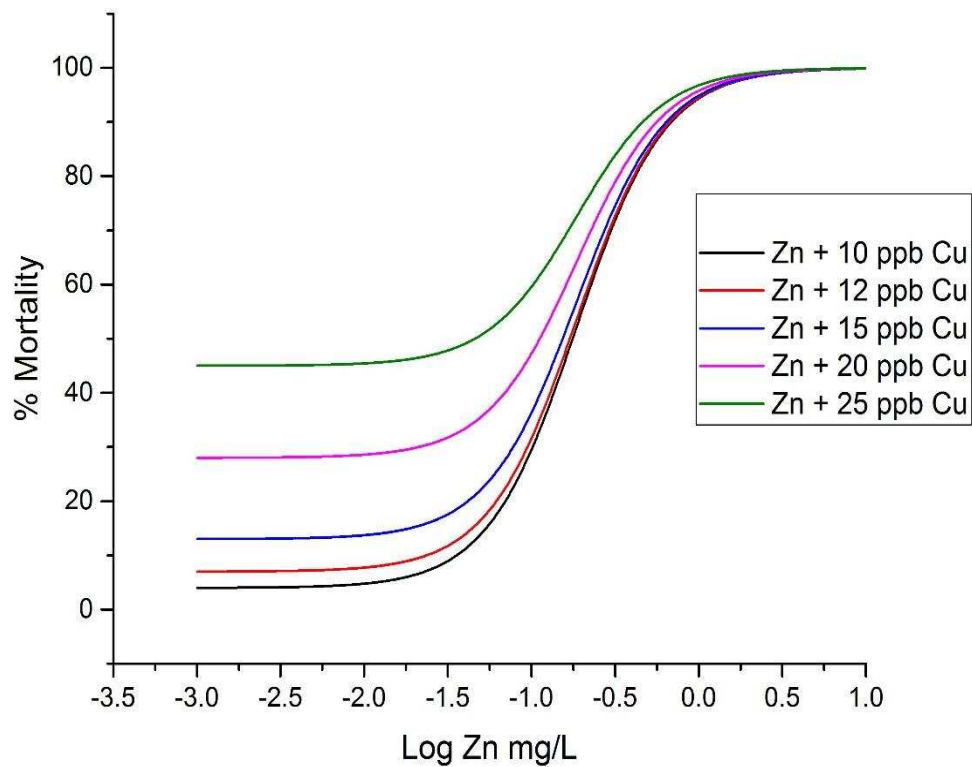


Figure 3.2. Predicted response-additive logit concentration-response curves for *Daphnia magna* neonates exposed to Cu-Zn mixtures in 48-h lethality tests in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Response-additive toxicity was predicted using the concentration-response curves for multiple Cu-only and Zn-only toxicity tests in NFCC field water.

Table 3.1. Observed and predicted EC_{infl} concentrations (with 84% confidence intervals in parentheses) for Cu-Zn mixture toxicity to *Daphnia magna* neonates in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. Note that the observed EC_{infl} is significantly less than the predicted EC_{infl} for each mixture except for Zn + 12 µg/L Cu. The mean of the ratios is - 0.169, and the standard deviation is 0.231.

Metal mixture	Predicted response-additive initial mortality (%)	Predicted response-additive mortality at inflection point (%)	Predicted response-additive Zn EC_{infl} (mg/L)	Observed initial mortality (%)	Observed mortality at inflection point (%)	Observed Zn EC_{infl} (mg/L) (84% C.I.)	Ratio [(obs-pred)/obs]
Zn only				0	50	0.185 (0.161-0.212)	
Zn + 10 mg/L Cu	4	52	0.185 (0.161-0.212)	20	60	0.133 (0.111-0.159)	-0.281
Zn + 12 mg/L Cu	7	53.5	0.185 (0.161-0.212)	10	55	0.158 (0.142-0.176)	-0.143
Zn + 15 mg/L Cu	13	56.5	0.185 (0.161-0.212)	10	55	0.234 (0.220-0.258)	0.267
Zn + 20 mg/L Cu	28	64	0.185 (0.161-0.212)	30	65	0.151 (0.098-0.160)	-0.324
Zn + 25 mg/L Cu	45	72.5	0.185 (0.161-0.212)	65	82	0.117 (0.098-0.141)	-0.365

The results of the toxicity tests are shown in Figures 3.3 to 3.7. In each figure, the Cu-Zn mixture is plotted along with the Zn-only toxicity results to show how the added background Cu concentration changed the toxicity when mortality is plotted versus Zn concentration.

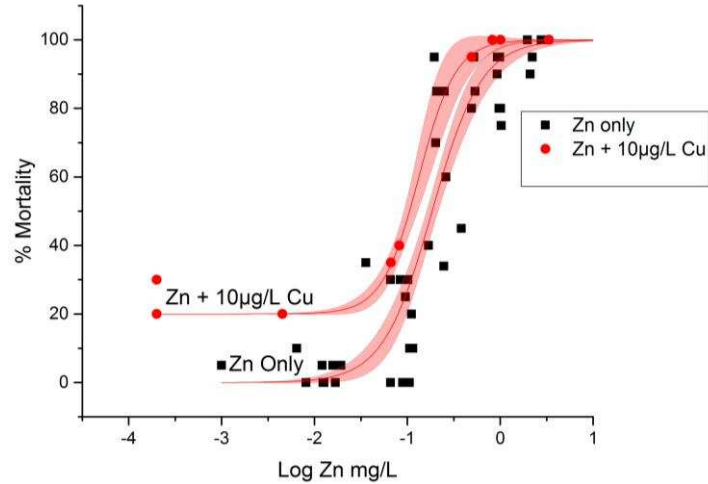


Figure 3.3. Logit concentration-response curves for *Daphnia magna* neonates exposed to Zn only and Zn + 10 µg/L Cu in 48-h lethality tests in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. That dilution water contained 3 mg/L dissolved organic carbon (DOC) composed of 2 mg/L natural DOC and 1 mg/L DOC added as Suwannee River fulvic acid.

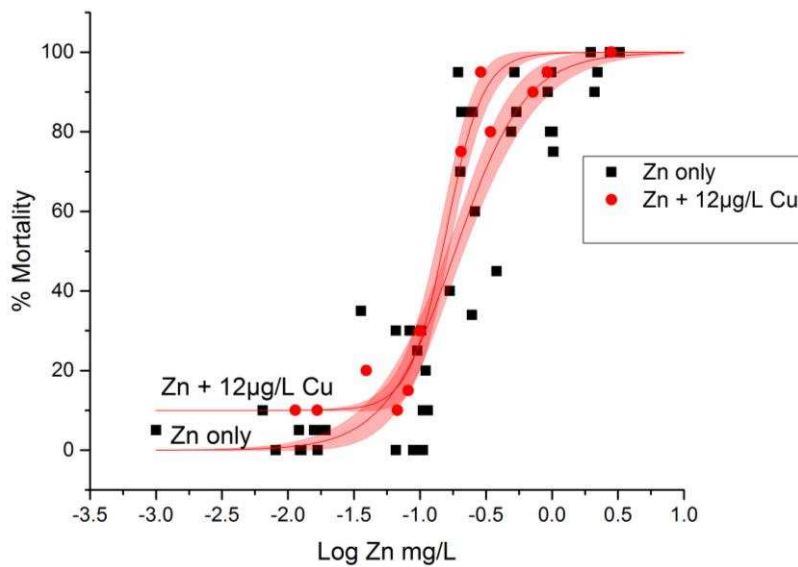


Figure 3.4. Logit concentration-response curves for *Daphnia magna* neonates exposed to Zn only and Zn + 12 µg/L Cu in 48-h lethality tests in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. That dilution water contained 3 mg/L dissolved organic carbon (DOC) composed of 2 mg/L natural DOC and 1 mg/L DOC added as Suwannee River fulvic acid.

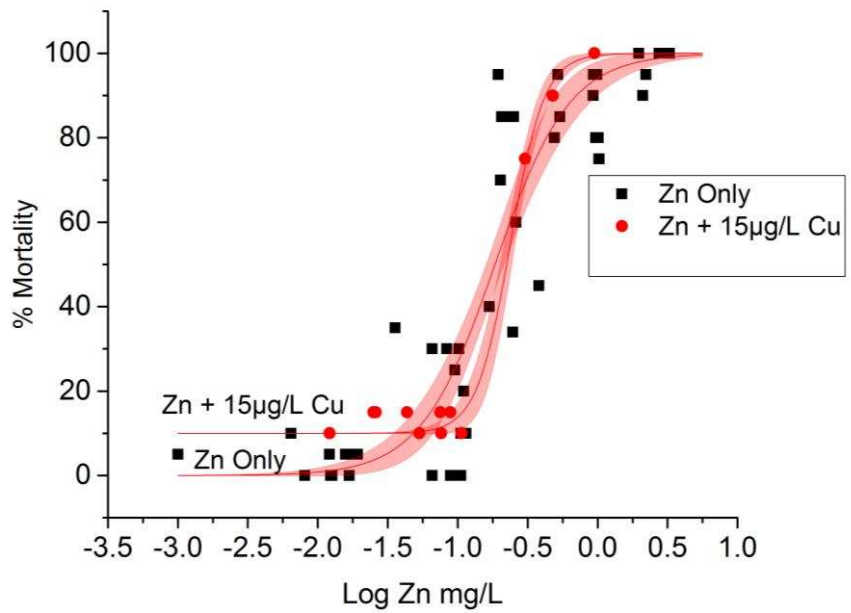


Figure 3.5. Logit concentration response curves for *Daphnia magna* neonates exposed to Zn only and Zn + 15 µg/L Cu in 48-h lethality tests in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. That dilution water contained 3 mg/L dissolved organic carbon (DOC) composed of 2 mg/L natural DOC and 1 mg/L DOC added as Suwannee River fulvic acid.

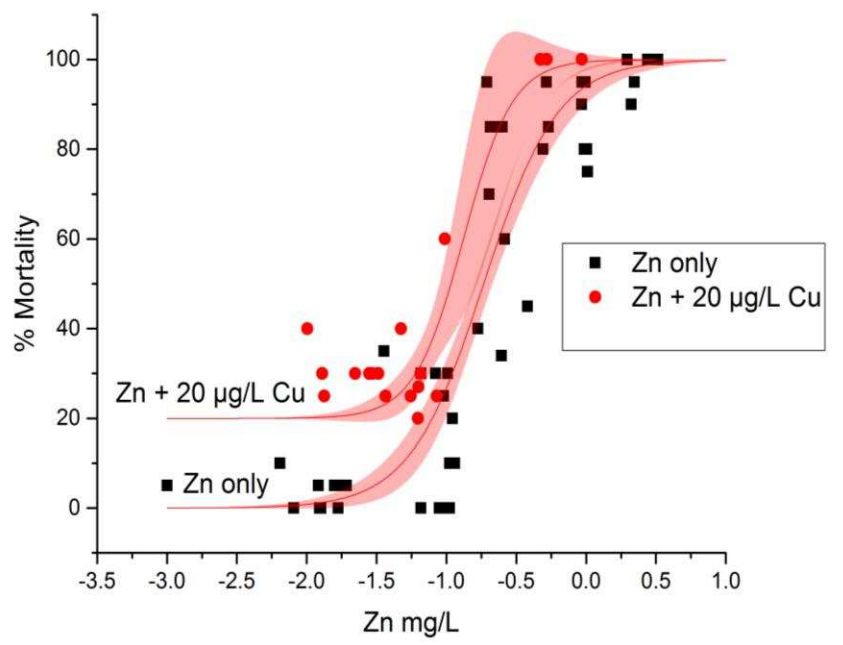


Figure 3.6. Logit concentration response curves for *Daphnia magna* neonates exposed to Zn only and Zn + 20 µg/L Cu in 48-h lethality tests in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. That dilution water contained 3 mg/L dissolved organic carbon (DOC) composed of 2 mg/L natural DOC and 1 mg/L DOC added as Suwannee River fulvic acid.

The initial mortality in the test with 10 $\mu\text{g/L}$ Cu (20%) was greater than the initial mortality with 12 $\mu\text{g/L}$ Cu (15%). This may be explained by the variability of mortality at this concentration of Cu seen in the individual-Cu toxicity tests. In 6 individual-metal Cu toxicity tests in NFCC field water, 10 $\mu\text{g/L}$ Cu caused mortality ranging from 0 to 25%. The initial mortality for Zn + 15 $\mu\text{g/L}$ Cu was 15% which is the same initial mortality as the Zn-Cu mixture with 12 $\mu\text{g/L}$ background Cu.

The initial mortality in the Zn + 20 $\mu\text{g/L}$ Cu test was 20% which is the same initial mortality as the Zn-Cu mixture with 12 $\mu\text{g/L}$ Cu. Two Cu-Zn mixture toxicity tests with 20 $\mu\text{g/L}$ Cu were performed on separate days. In one test, the initial mortality was 20% and in the second test, the initial mortality was 30%.

At low concentrations of Zn in the Zn + 25 $\mu\text{g/L}$ Cu tests, the initial mortality was clustered around 60%. This initial mortality was on the upper end of mortality caused by 25 $\mu\text{g/L}$ Cu in individual-Cu toxicity tests. In 6 individual-metal Cu toxicity tests, 25 $\mu\text{g/L}$ Cu caused mortality ranging from 30 to 60%.

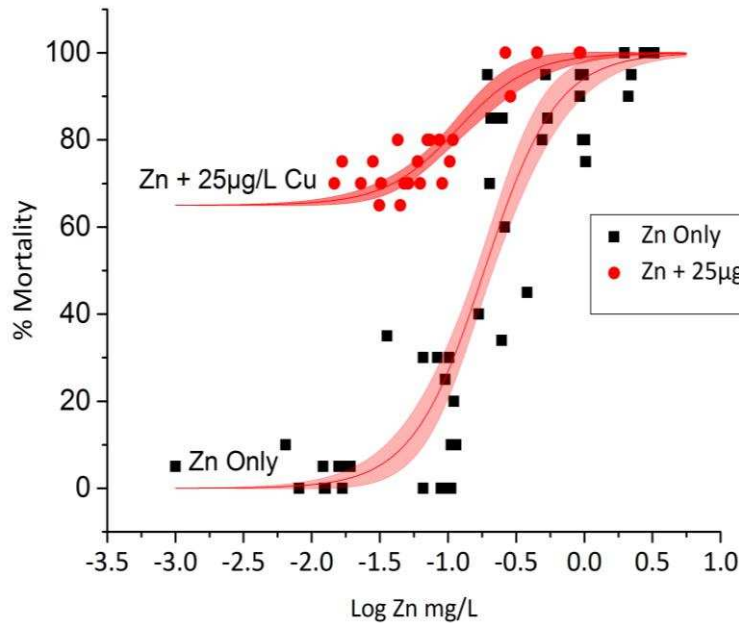


Figure 3.7. Logit concentration response curves for *Daphnia magna* neonates exposed to Zn only and Zn + 25 $\mu\text{g/L}$ Cu in 48-h lethality tests in water collected from the North Fork of Clear Creek (NFCC) in Gilpin County, Colorado. That dilution water contained 3 mg/L dissolved organic carbon (DOC) composed of 2 mg/L natural DOC and 1 mg/L DOC added as Suwannee River fulvic acid.

Visual examination of the Cu-Zn mixture concentration-response curves suggests that the toxicity of Cu-Zn mixtures was response-additive to slightly more-than-additive, and the calculated EC_{infl} concentrations were consistent with those observations (i.e., EC_{infl} concentrations were generally less than the Zn-only EC_{50} ; Table 3.1). The observed EC_{infl} for each Cu-Zn mixture, except Zn + 12 $\mu\text{g/L}$ Cu, was significantly different from the predicted EC_{infl} , as determined by non-overlap of the 84% confidence intervals. The EC_{infl} values for Zn + 10 $\mu\text{g/L}$ Cu, Zn + 20 $\mu\text{g/L}$ Cu, and Zn + 25 $\mu\text{g/L}$ Cu were significantly less than the predicted EC_{infl} , suggesting that the toxicity of these mixtures was more-than-additive. However, the EC_{infl} for the mixture of Zn + 15 $\mu\text{g/L}$ Cu was significantly greater than the predicted EC_{infl} , suggesting less-than-additive toxicity. Comparing each Cu-Zn mixture's EC_{infl} to the predicted EC_{infl} leads to the conclusion that the toxicity of Cu-Zn mixtures can be response-additive, less-than-additive, or more-than-additive, depending on the difference of a few $\mu\text{g/L}$ of added Cu. This point-by-point statistical analysis does not provide insight into the toxicity of Cu-Zn mixtures as a whole. To overcome this limitation, the toxicity of Cu-Zn mixtures was also analyzed globally. This analysis was done by comparing the average of the EC_{infl} ratios to zero using a t-test.

The t_{calc} value for the average EC_{infl} ratio was 0.216, which is less than the critical t value of 2.776. Therefore, the null hypothesis that Cu-Zn mixture toxicity is response-additive was not rejected. This conclusion is consistent with the conclusion by Meyer et al. (2015b) that Cu-Zn mixture toxicity in USEPA MHR water is response-additive to slightly more-than-additive.

In summary, when the Cu-Zn mixtures were analyzed individually, the toxicity of most mixtures was more-than-additive. However, when all of the mixtures were analyzed as a group, the null hypothesis that the toxicity of Cu-Zn mixtures is response-additive could not be rejected.

3.3.2 Cd-Zn Mixtures

The toxicity of Cd-Zn mixtures with varying concentrations of background Cd was tested in USEPA MHR water, dilutions of MHR water, and NFCC field water. The background concentrations of Cd ranged from 100 to 200 $\mu\text{g/L}$. In toxicity tests of Cd-Zn mixtures in USEPA MHR water, a clear protective effect was observed (i.e., the “dip” in the concentration-response curve in Figure 3.8, wherein mortality decreased as Zn concentration was increased at sublethal Zn concentrations until mortality began to increase at lethal Zn concentrations). This protective effect is most likely due to geochemical interactions. If Zn out-competes Cd for

binding at the biotic ligand, then the toxicity of the mixture will decrease at sublethal concentrations because Zn is less toxic than Cd.

Compared to the Cu-Zn toxicity tests, binary Cd-Zn test results are challenging to interpret. This is because the protective effect observed in Cd-Zn tests cannot be described with a symmetrical sigmoidal concentration-response curve. Therefore, it is not appropriate to use the EC_{infl} method to compare results of Cd-Zn toxicity tests to predicted response-additive toxicity.

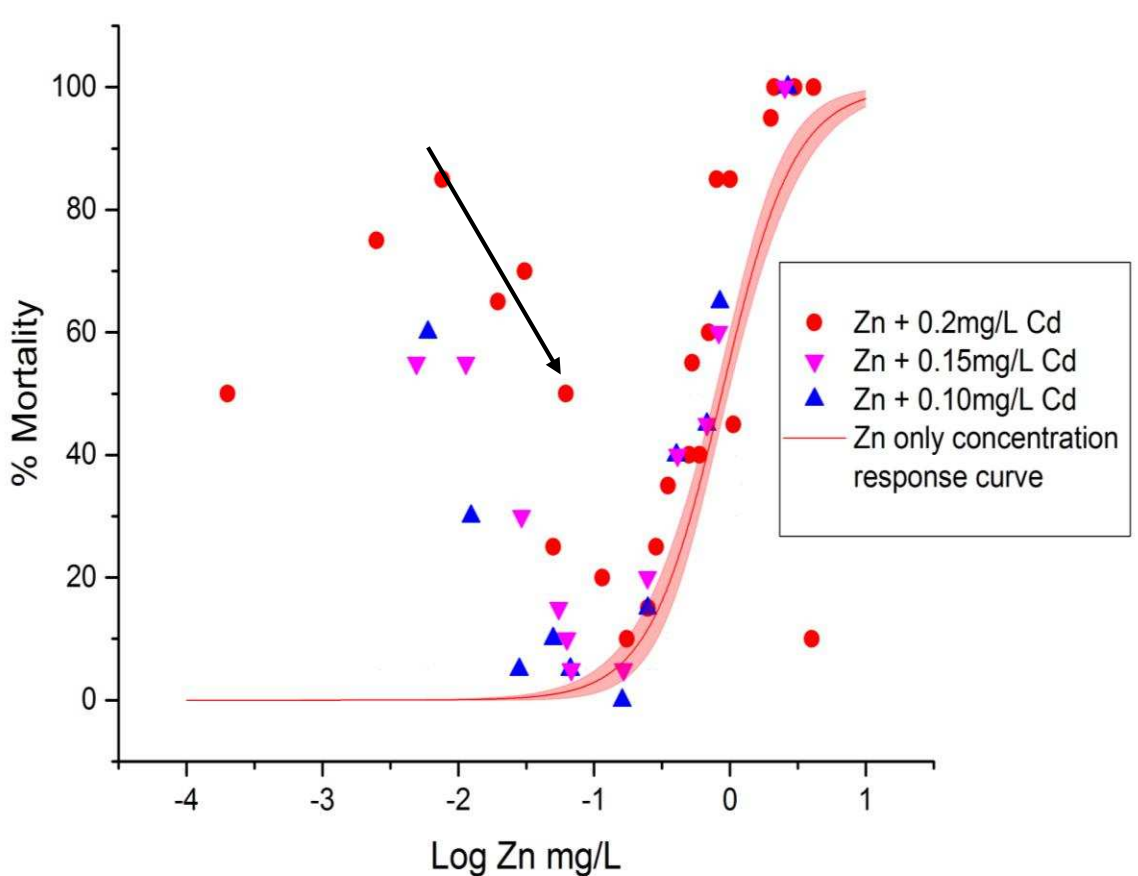


Figure 3.8. Mortality of *Daphnia magna* neonates exposed to Zn only, Zn + 0.10 mg/L Cd, Zn + 0.15 mg/L Cd, and Zn + 0.20 mg/L Cd in 48-h lethality tests in USEPA moderately hard reconstituted water. When background concentrations of Cd were added to the exposure waters, the initial mortality with no added Zn increased to between 50 and 90 %. As Zn concentration in the mixtures was increased to 0.1 mg/L, the mortality decreased to 0 to 15 %; but at Zn concentrations >0.1 mg/L, mortality increased as expected for Zn-only toxicity.

When the test water was changed from USEPA MHR water with 3 mg/L DOC as SRFA to NFCC field water with 3 mg/L DOC as a mixture of natural DOC and SRFA, the protective

effect in Cd-Zn mixtures was dampened. The initial mortality in NFCC field water due to Cd was less than the initial mortality observed in USEPA MHR water. This makes sense because individual-Cd tests in NFCC field water were less toxic than in USEPA MHR water, as shown in section 2.3. As Zn was titrated into the exposure waters, the mortality decreased to between 0 and 15%; but when the Zn concentration became lethal, the toxicity increased (Figure 3.9). Unlike the toxicity in USEPA MHR water, the toxicity of the Cd-Zn mixture did not increase almost exactly with the Zn-only concentration response curve. The toxicity of the Cd-Zn mixture in the synthetic field water differed from the toxicity in the field-collected NFCC water because the dip in mortality was not as pronounced in the synthetic NFCC water as in the actual field water (Figure 3.10).

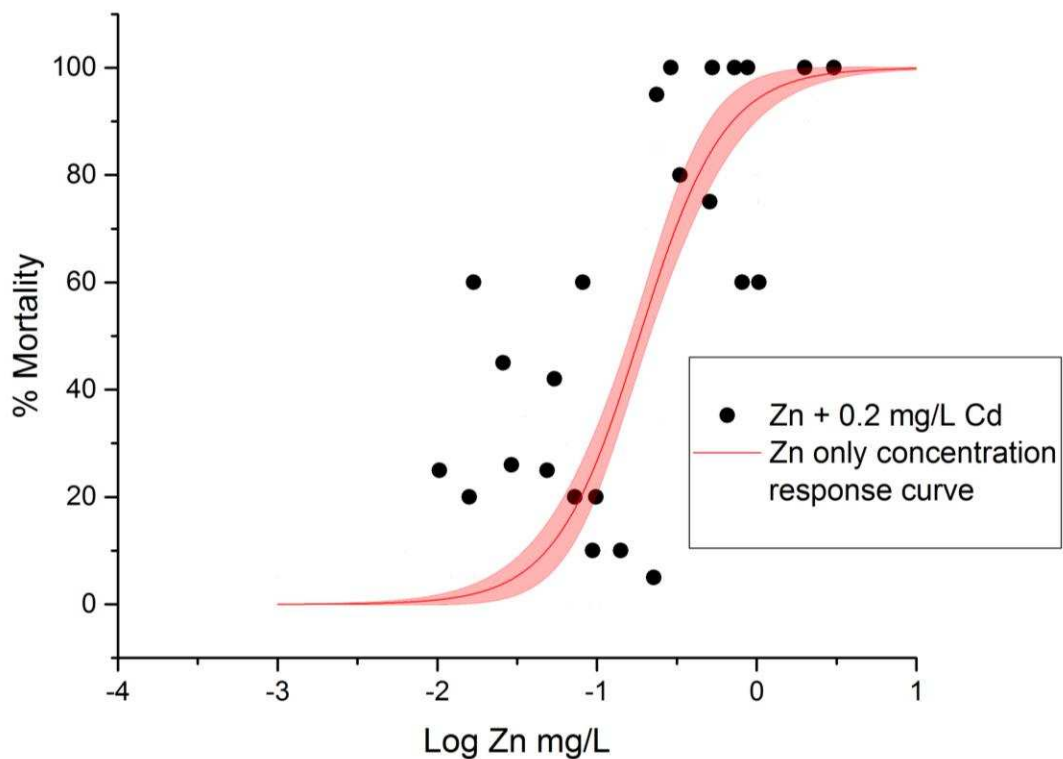


Figure 3.9. Mortality of *Daphnia magna* neonates exposed to Zn only and Zn + 0.20 mg/L Cd in 48-h lethality tests in water collected from the North Fork of Clear Creek (NFCC) in Gilpin, Colorado. When background concentrations of Cd were added to the exposure water, the initial mortality with no added Zn increased to between 20 and 60%. As Zn concentration in the mixtures was increased to 0.1 mg/L, the mortality decreased to 0%; but at Zn concentrations >0.2 mg/L, mortality increased.

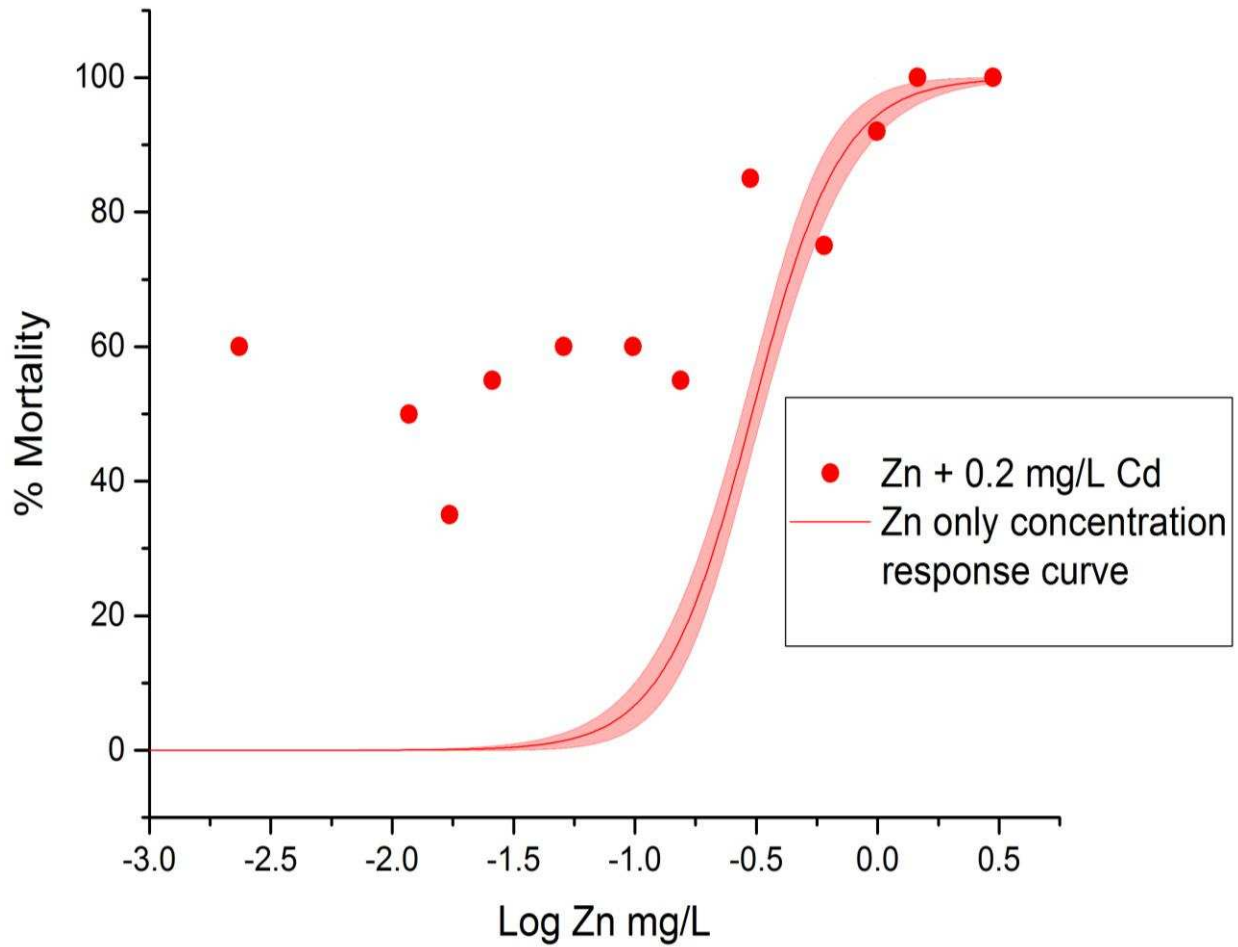


Figure 3.10. Mortality of *Daphnia magna* neonates exposed to Zn only and Zn + 0.20 mg/L Cd in 48-h lethality tests in synthetic laboratory NFCC field water. When background concentrations of Cd were added to the exposure water, the initial mortality with no added Zn increased to 60%. As Zn concentration in the mixtures was increased to 0.15 mg/L, the mortality decreased to 35%. The mortality hovered near 55 to 60% until the Zn concentration increased to 0.3 mg/L and mortality increased.

The toxicity of Cd-Zn mixtures in USEPA soft water containing 3 mg/L DOC is shown in Figure 3.11. When background concentrations of Cd were added to solution, the initial mortality increased to between 85 and 100%. This initial mortality due to Cd was much greater than the initial mortality observed in NFCC field water and was similar to the initial mortality in tests in USEPA MHR water. The “dip” in the mortality was not as pronounced in the tests in soft water. The soft water mimicked the NFCC field water most closely in terms of hardness and alkalinity, but Cd caused much greater initial mortality in the Cd-Zn tests in the soft water than in the NFCC field water.

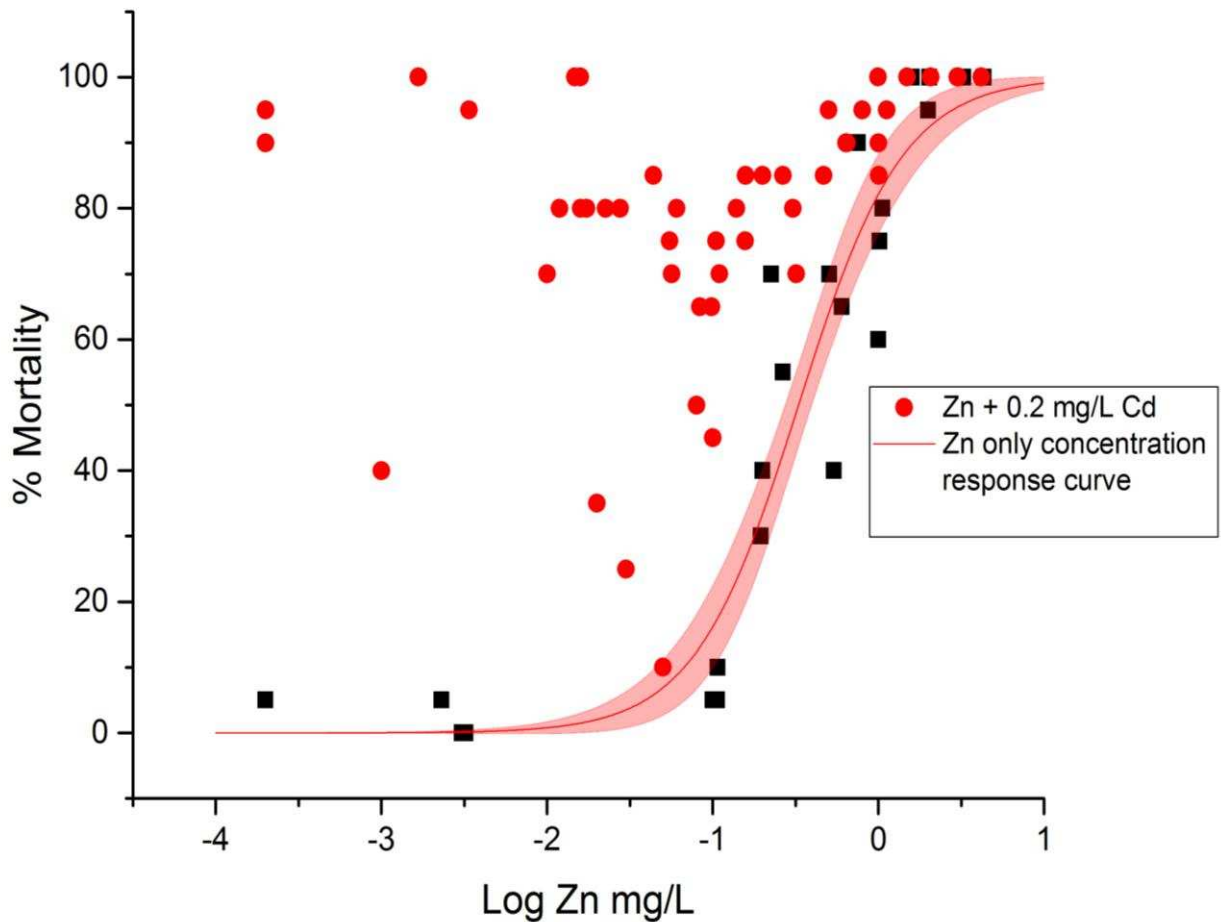


Figure 3.11. Mortality of *Daphnia magna* neonates exposed to Zn only and Zn + 0.20 mg/L Cd in 48-h lethality tests in USEPA soft water. When background concentrations of Cd were added to the exposure water, the initial mortality with no added Zn increased to 90%. As Zn concentration in the mixtures was increased to 0.1 mg/L, there was a slight decrease in mortality.

Finally, the “dip” in toxicity of Cd mixtures was dampened further when test water was changed to very soft water and water with intermediate softness (water with hardness of ~20-25 mg/L as CaCO₃) (Figures 3.12 and 3.13). The dip effect was dampened even more in the water with intermediate softness than in the soft water. In very soft water, a slight dip in mortality is observable, but the background concentration in this test was 0.12 mg/L in contrast to the 0.20 mg/L Cd in the other tests.

When USEPA MHR water was diluted to very soft water (hardness of ~10 mg/L as CaCO₃), there was still a small protective effect in the Cd-Zn mixture toxicity. In the Cd-Zn mixture tested, the mortality decreased from 30 to 0% as the Zn concentration was increased (Figure 3.13).

Overall, the toxicity of Cd-Zn mixtures is variable and cannot be predicted based on water hardness alone. Zinc protects against Cd toxicity in Cd-Zn mixtures, and this effect is most obvious in USEPA MHR water. The protective effect becomes more difficult to interpret in dilutions of MHR water. Although NFCC field water and USEPA soft water have very similar hardness and alkalinity, there was a larger “dip” in mortality in the NFCC field water than in the laboratory water. The toxicity of the Cd-Zn mixture in synthetic NFCC water more closely resembled the toxicity in USEPA soft water than in NFCC field water. As the laboratory water became softer and was changed from soft to intermediate softness to very soft, the protective effect was dampened. In conclusion, predicting Cd-Zn mixture toxicity is challenging. Zinc appears to protect against Cd toxicity in MHR water, but to a less degree in field-collected and soft waters.

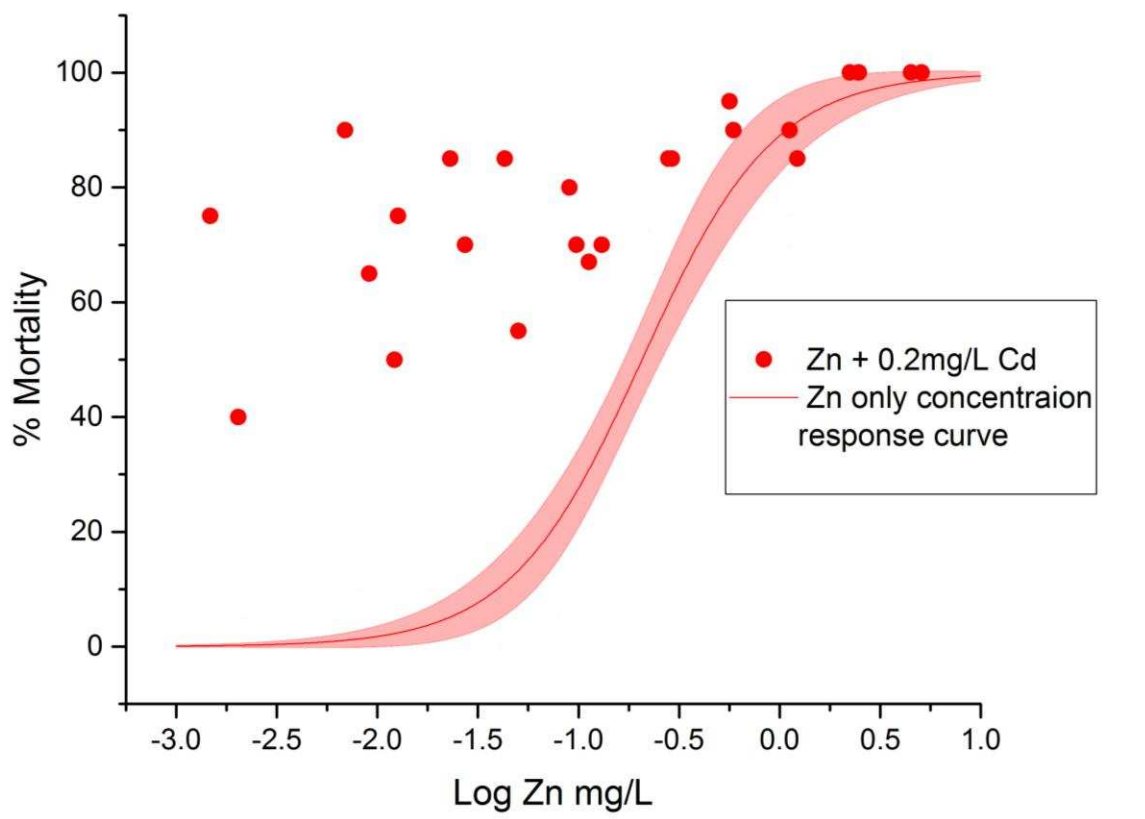


Figure 3.12. Mortality of *Daphnia magna* neonates exposed to Zn only and Zn + 0.20 mg/L Cd in 48-h lethality tests in water with intermediate softness. The protective effect in Zn-Cd mixtures that was evident in USEPA MHR water is almost completely absent in water with intermediate softness. When background concentrations of Cd were added to the exposure water, the initial mortality with no added Zn varied from 40 to 80 %. As Zn concentration in the mixtures was increased to 0.1 mg/L, there was a slight dip in mortality.

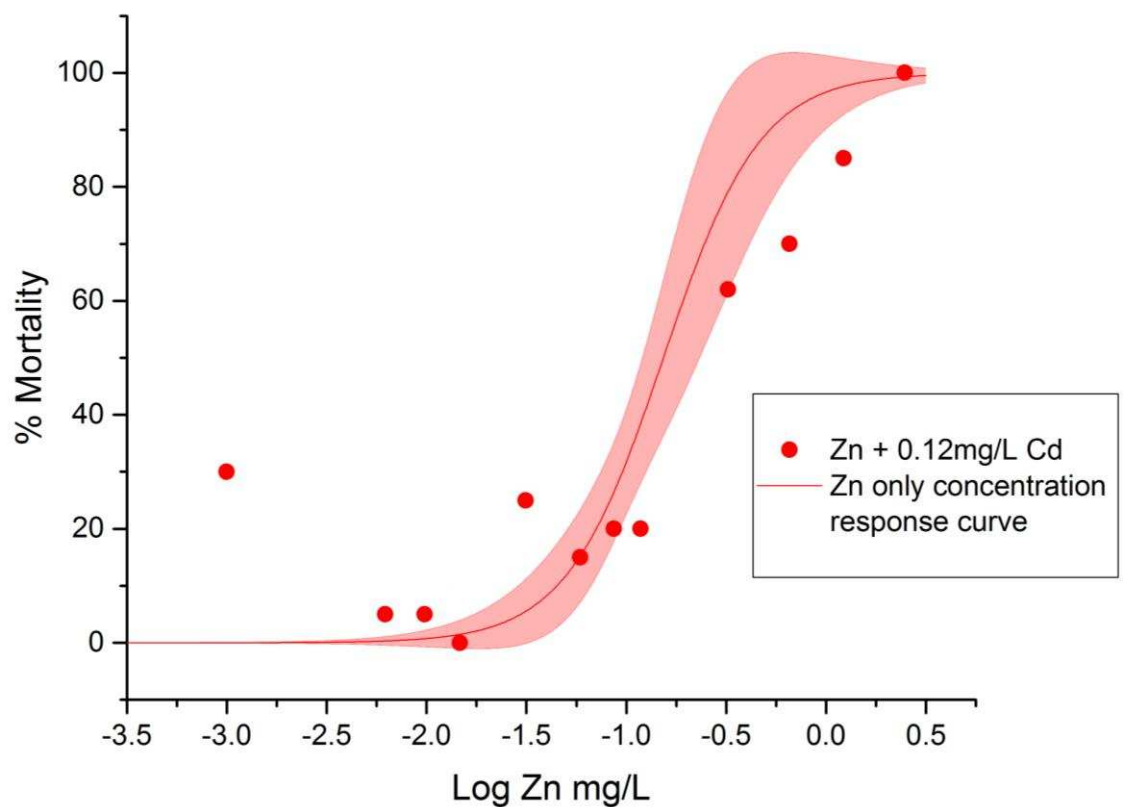


Figure 3.13. Mortality of *Daphnia magna* neonates exposed to Zn only and Zn + 0.12 mg/L Cd in 48-h lethality tests in very soft water. When background concentrations of Cd were added to the exposure water, the initial mortality with no added Zn increased to 30%. As Zn concentration in the mixtures was increased to 0.01 mg/L, the mortality decreased to 0 to 5%; but at Zn concentrations >0.1 mg/L, mortality increased as expected for Zn-only toxicity.

CHAPTER 4 SUMMARY AND CONCLUSIONS

Metals can enter aquatic ecosystems through a variety of pathways including agricultural and industrial processes, municipal effluents, natural mineral weathering, and acid mine drainage (AMD). Although these sources are quite different, they have one thing in common: they discharge metal mixtures instead of individual metals to the environment. Metals have the potential to be toxic to aquatic life depending on the combinations and concentrations of the metals and on the aqueous geochemistry of the environment. The results of tests conducted in this study demonstrate that the toxicity of individual metals and metal mixtures vary depending on water chemistry parameters such as hardness, alkalinity, and dissolved organic carbon concentration. Similar trends in the toxicity of Cd-Zn and Cu-Zn mixtures in USEPA moderately hard reconstituted (MHR) water were observed in field-collected waters, although some of these effects were dampened in the field water compared to the MHR water. Overall, this study highlights the importance of water chemistry when extrapolating results of individual-metal and metal-mixture toxicity tests from the laboratory to the field.

The range of EC50 concentrations for Cu-Zn and Cd-Zn metal-mixtures in exposure waters that had the same and very similar chemistry demonstrates the need for reliable modeling tools to develop appropriate water quality criteria for metal mixtures. The toxicity of metal mixtures in this study varied from response-additive to less-than-additive (i.e., to less toxic than would be predicted from a simple combination of the effects of the two individual metals). A better understanding of the mechanisms of metal-mixture toxicity may lead to more accurate predictive models. Ultimately, being able to accurately predict the toxicity of metal mixtures will facilitate the regulation of metals as mixtures instead of on an individual-metal basis.

More chemical and ecotoxicological research on individual metals and metal mixtures in varying water chemistries is needed to understand the sometimes puzzling trends in toxicity. In this study, the concentrations needed to produce acute-toxicity responses were, in many cases, greater than concentrations that would actually be found in the environment. For example, to adequately test for more-than-additive, additive, and less-than-additive toxicity in Cd-Zn and Cu-Zn mixtures, I sometimes increased the Zn concentration to 4 mg/L. However, in the AMD-impacted reach of the North Fork of Clear Creek, the Zn concentrations are usually less than 2 mg/L. Additionally, in surface waters affected by AMD and/or natural mineral weathering,

metals are present in predictable molar ratios. The molar ratios of metals in a mixture can have a significant impact on toxicity (Ralston et al. 2007).

Additional toxicity testing near the chronic criteria for metals and at representative metal ratios would be a good next step to developing accurate predictive models for multi-metal toxicity. These kinds of toxicity tests coupled with toxicogenomics could lead to insights into mechanisms of metal toxicity. Toxicogenomics enables the identification of genomic responses to toxicants even at very low concentrations; therefore, such studies may prove quite useful in developing water quality standards for metal-mixtures in the environment. Finally, in situ toxicity tests at field sites impacted by elevated concentrations of metals are important for fully understanding how the complex geochemistry at a site (including the presence of Al and Fe) can impact toxicity.

Because metals probably will always be present in surface waters from natural and/or anthropogenic sources, it is important to understand and be able to predict the toxicity of metal mixtures in aquatic environments. Results of the toxicity tests in this study with a variety of water chemistries provide a start for interpreting toxicity results from field studies. Interpreting toxicity results from a field test is challenging when many factors can vary among sites. Therefore, these controlled laboratory tests provide an important basis for understanding and predicting metal toxicity in the field.

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APPENDIX A

NICKEL TOXICITY TO EPHIPPIAL *DAPHNIA MAGNA* NEONATES

Culturing *Daphnia magna* in the laboratory is relatively economical and easy. However, the process requires personnel for daily monitoring of the organisms, which can be time-consuming. Some researchers choose not to culture their own organisms and instead order them from a company like Aquatic BioSystems, Inc. (ABS) in Fort Collins, Colorado, which supplied the organisms I used in the individual-metal and metal-mixture toxicity tests described in the main part of this thesis. However, not all researchers are located near a provider of toxicity test organisms. For example, toxicity tests are sometimes conducted at remote field sites such as mines in isolated mountain regions. In such situations, it may be desirable to order ephippia (resting eggs) that can be stored until hatching is induced, instead of trying to have parthenogenetically produced neonates shipped from a supplier within the 24-h time limit before they are too old to start a toxicity test per U.S. Environmental Protection Agency guidance (USEPA 2002b).

Under favorable conditions, *D. magna* females produce an asexual clutch of parthenogenetic offspring. However, if the organisms are stressed (e.g., have little or no food), they reproduce sexually and produce “resting eggs” that are encapsulated in a protective structure called an ephippium (Ebert 2005). The ephippia can be collected before they hatch, and hatching can be postponed by storing the ephippia in a dark, cool environment with an added preservative.

The purpose of this study was to test whether the acute toxicity of Ni to *D. magna* neonates differs between ephippial neonates and parthenogenetically-produced neonates. This was an extension of testing that was begun by Elizabeth Traudt in 2013 with ephippial *D. magna* neonates exposed to Ni and Cd, and binary Ni-Cd mixtures. Traudt conducted Ni-only toxicity tests in USEPA MHR water with ABS neonates and ephippial neonates in 2013; and during this study, I conducted more Ni-only toxicity tests with ephippial neonates in 2014. The 2014 ephippial neonates were from a different batch than the 2013 ephippial neonates. Nickel was chosen because there is generally low variability in the EC50 concentration from test to test (Traudt et al. 201_).

To test the sensitivity of ephippial neonates to metals, ephippia were obtained in DAPHTOXKITS™ from Environmental Bio-Detection Products, Inc. (Ontario, Canada), which

is a distributor for MicroBio Tests, Inc. (Belgium). After the ephippia were obtained from Environmental Bio-Detection Products, Inc., hatching was induced by rinsing the ephippia to remove the preservative and then exposing them to constant light for a minimum of 72 h. After they hatched, the ephippial neonates were treated the same as the neonates ordered from ABS (as described in section 2.2.2 in Chapter 2). Acute Ni toxicity tests with the neonates from both sources were conducted in 2013 according to standard procedures in USEPA moderately hard reconstituted (MHR) water, but with 3 mg/L of dissolved organic carbon added to the MHR water as Suwannee River fulvic acid (as described in sections 2.2.3 and 2.2.4 in Chapter 2). Nickel was spiked into the exposure water using $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (Mallinckrodt Chemical). In 2014, acute Ni toxicity tests with ephippial neonates were conducted according to the same procedures as in 2013.

The toxicity of Ni to ephippial neonates in MHR water amended with 3 mg DOC/L was similar to its toxicity to parthenogenetically-produced neonates in the same exposure water (Figure A.1 and Table A.1). The toxicity to the ephippial neonates was within the general variability of the toxicity to the ABS neonates.

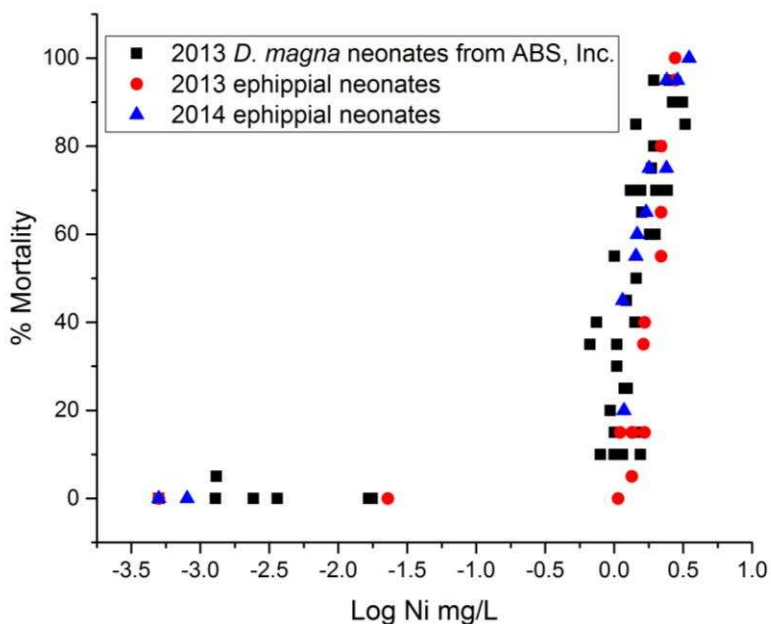


Figure A.1. Mortality of *Daphnia magna* neonates exposed to Ni in 48-h lethality tests in USEPA moderately hard reconstituted water. Sources of the neonates were parthenogenetically-produced neonates from Aquatic BioSystems, Inc. (ABS, Fort Collins, CO) and ephippial neonates from Environmental Bio-Detection Products, Inc. (Ontario, Canada). Results of multiple Ni toxicity tests with neonates from each source were combined. Data from 2013 are from Elizabeth Traudt, Colorado School of Mines (unpublished).

Table A.1. Median effect concentrations (EC50 values) and 84% confidence intervals for Ni toxicity to different sources of *Daphnia magna* neonates in USEPA moderately hard reconstituted water.

Source of <i>Daphnia magna</i> neonates	EC50 (mg/L) (84% C.I.)
2013 ABS, Inc. parthenogenetically-produced neonates	1.43 (1.34-1.54)
2013 ehippial neonates	1.91 (1.85-1.97)
2014 ehippial neonates	1.38 (1.31-1.46)

Based on these results, ehippia appear to be a reliable and comparable alternative to culturing or ordering parthenogenetically-produced neonates. The EC50 concentration from the 2014 test with ehippial neonates was not significantly different from the EC50 concentration from the 2013 tests with neonates from ABS, Inc. However, the EC50 concentration from the 2013 tests with ehippial neonates was significantly different from the EC50s from the other two tests. This difference suggests that there may be variability among batches of ehippia.

Although ehippia are used only on a very limited basis by the aquatic-toxicity testing community because of concerns about potential genotypic and phenotypic changes in the offspring as a result of sexual recombination of their gene complement, such changes could be tested for by conducting toxicity tests with ehippial neonates from a given batch that would be exposed to several standard reference toxicants (e.g., NaCl, CuSO₄). Demonstration of consistency among batches of ehippia might help to increase the acceptance and use of ehippial *D. magna* neonates within the aquatic-toxicity testing community.