EVALUATION OF TOXICITY AND BIOAVAILABILITY OF METAL MIXTURES TO TWO FRESHWATER INVERTEBRATES

By

Elizabeth Marie Traudt

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Golden, Colorado

Date _____

Signed: _____
Elizabeth M. Traudt

Signed: _____
Dr. James F. Ranville
Thesis Advisor

Golden, Colorado

Date _____

Signed: _____
Dr. David T. Wu
Professor and Head
Department of Chemistry

ABSTRACT

Multiple metals are often present in natural water systems, leading to mixture toxicity that currently is difficult to predict. To help develop predictive toxicity models, the toxicity of Cd, Cu, Ni and Zn was measured in individual-metal, binary, and ternary mixtures in acute toxicity tests following USEPA protocol using *Daphnia magna* neonates (< 24 hour old). Toxicity tests in which *D. magna* were exposed to binary mixtures of Ni combined with Cd, Cu, or Zn demonstrated a protective effect (Cd-Ni), a greater-than-additive effect (Cu-Ni), and a slightly less-than-additive toxic effect (Ni-Zn). Following these results, I tested ternary mixtures of Cd-Ni with either Cu or Zn in an attempt to observe multiple interactions occurring concurrently (i.e. – mixing less-than-additive interaction with a more-than-additive interaction). In Cd-Cu-Ni mixtures, the toxicity was less-than-additive, additive, or greater-than-additive, depending on the concentration of the varied metal. In Cd-Ni-Zn mixtures, the toxicity was always less-than-additive or approximately additive. These results demonstrate that complex interactions of potentially competing toxic mechanisms can occur in metal mixtures but should be predictable by mechanistic models of metal-mixture toxicity.

In these studies, variability was high among replicate acute Cd lethality tests (e.g., >10-fold range of median effects concentrations [EC50s]). I hypothesized that age-related differences in sensitivity to metals might occur even within that relatively narrow age range. *Daphnia magna* neonates collected during three 4-h age windows were used to start acute toxicity tests. In repeated sets of tests, the Cd EC50 of the youngest neonates was approximately 10-fold greater than the EC50 of the oldest neonates. These results demonstrate that decreasing the age range of *D. magna* used in toxicity tests could help to improve the accuracy and precision of toxicity models, particularly for metal mixtures.

To continue my work with metal-mixture toxicity, I traced the flux of Cu in isotopically-labeled freshwater snails (*Lymnaea stagnalis*) that were fed flocculent material rich in Fe and other metals (e.g., Cu, Zn) collected from sediment in the North Fork of Clear Creek (NFCC). The uptake and depuration of Cu were measured over a 48-h exposure in which the organisms consumed control or contaminated food. The assimilation efficiency, which describes the extent to which the particle-associated Cu is taken up by an organism, can be used to quantify the bioavailability of Cu in the particulate-metal mixture. This study evaluated the biodynamic parameters of three types of flocculent contamination found at NFCC sites. The assimilation efficiency remained elevated around 40-50% in all three flocculent types. These results will have direct implications to understanding and predicting potential in-stream toxicity of contaminated sediments before, during, and after remediation of AMD.

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LIST OF ABBREVIATIONS USED

Ag.....Silver

Al.....Aluminum

AMD.....Acid mine drainage

ANOVA.....Analysis of variance

As.....Arsenic

BL.....Biotic Ligand

BLM.....Biotic Ligand Model

°C.....Degrees Celsius

Ca.....Calcium

CA.....Concentration addition

CCV.....Continuing calibration verification

Cd.....Cadmium

$Cd(NO_3)_2$.....Cadmium nitrate

CI.....Confidence interval

Cl^-.....Chloride

cm.....centimeter

CPCC.....Canadian Phycological Culture Centre

Cu.....Copper

$Cu(NO_3)_2$.....Copper nitrate

CV.....Coefficient of variation

d.....Day

DOC.....Dissolved organic carbon

DOM ..... Dissolved organic matter

DO..... Dissolved oxygen

EC50 ..... Concentration causing 50% toxicological effect

EC_x ..... Concentration causing x% toxicological effect

$EC_{x\ infl}$..... Concentration at the inflection point of the concentration-response curve

Fe^{2+} ..... Ferrous iron

Fe^{3+} ..... Ferric iron

$Fe(OH)_3$ ..... Ferric hydroxide

FeS_2 ..... Iron sulfide, pyrite

FIAM..... Free ion activity model

g..... gram

GSIM..... Gill Surface Interaction Model

h..... Hour

HFO..... Hydrous ferric oxide

Hg..... Mercury

HNO_3..... Nitric acid

H_3PO_4 ..... Phosphoric acid

H_2SO_4 ..... Sulfuric acid

IA ..... Independent action

IDL ..... Instrument detection limit

ICP-MS ..... Inductively coupled plasma-mass spectrometer

ICP-OES ..... Inductively coupled plasma optical emission spectroscopy

K..... Potassium

LOEC ..... Lowest observed effect concentration

L ..... Liter

MEAM ..... Metal effects addition model

mg ..... Milligram

Mg ..... Magnesium

MHRW ..... Moderately hard reconstituted water

ml ..... Milliliter

mm ..... Millimeter

MMMS ..... Mixed-metal, multi-site

MT ..... Metallothionein

M_x ..... Metal (generic)

M^{z+} ..... Free metal ion (generic)

n ..... Number of toxicity tests conducted

NFCC ..... North Fork of Clear Creek

NFP ..... North Fork particles

Ni ..... Nickel

$Ni(NO_3)_2$ ..... Nickel nitrate

NIST ..... National Institute of Science and Technology

NIEHS ..... National Institute of Environmental Health Sciences

NOEC ..... No observed effect concentration

NRCC ..... Nation Research Council of Canada

O_2 ..... Oxygen (molecular)

OECD ..... Organization for Economic Co-operation and Development

Pb .....Lead

ppm ..... Part per million

ppb.....Part per billion

pdf .....Probability Density Function

QA/QC ..... Quality assurance/quality control

RA .....Response addition

RSD.....Relative standard deviation

SD ..... Standard deviation

S .....Sulfur

SO_4 ..... Sulfate

TOC..... Total organic carbon

µg .....Microgram

µL ..... Microliter

µm .....Micrometer

µM..... Micromolar

USEPA..... United States Environmental Protection Agency

UV..... Ultraviolet

WHAM .....Windermere Humic Aqueous Model

WHAM-F_{Tox}.....Windermere Humic Aqueous Model-Toxicity Function

XRD ..... X-ray diffraction

Zn ..... Zinc

$Zn(NO_3)_2$ .....Zinc nitrate

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CHAPTER 1

INTRODUCTION

The goal of this thesis is to provide insight into bioaccumulation, bioavailability and toxicity to aquatic organisms affected by metal mixtures. Due to the large number of pathways by which metals can enter the environment, metals in surface waters tend to exist as mixtures rather than as a single contaminant. Currently there is a need for research on how metal mixtures may interact in surface waters, to help to develop models that are capable of predicting mixture toxicity by accounting for geological, chemical, and biological interactions. Ultimately, the goal is to advance regulatory and management approaches to control metal pollution.

1.1 Implications of metal contamination in the environment

Surface waters can be contaminated by metals through a variety of pathways including natural mineral weathering, mine drainage, and industrial, municipal, and agricultural discharges. Surface-water regulations are intended to maintain healthy aquatic ecosystems while also allowing for some industrial, private, and recreational uses of the waters. Worldwide, regulatory decisions for various ecosystems are generally based on results of toxicity tests that were conducted with individual metals [1]. But because metals usually do not occur alone in surface waters, efforts are being undertaken to determine how to effectively regulate metal mixtures [2]. Metal mixtures resulting in greater-than-predicted toxicity can pose an unforeseen risk to ecological and human health, because these effects are not accounted for in current metal-by-metal regulations and are not accounted for with standard assumptions of additive toxicity. On the other hand, metal mixtures resulting in less-than-predicted toxicity may be of concern to industries and municipalities, because the standard assumption of additive toxicity would lead to overly restrictive regulation that is not cost effective.

Based on the results of recent toxicity studies (including some of the results presented in this thesis), it is evident that assessment approaches for determining water quality standards may need to be re-evaluated using mechanistic models that integrate principles of metal bioavailability and computerized-software modeling. This may help to provide adequate, but not overly-restrictive environmental protection, by specifically examining how the combined bioavailabilities of the metals in a mixture will influence the toxicity of the mixture. Although numerous metal-mixture toxicity tests have been reported in the literature (e.g., see reviews by Norwood et al. 2003, Vijver et al. 2011, Meyer et al. 2015), few have reported adequate chemistry and/or toxicity results to support development of mechanistically-based metal-mixture toxicity models based on bioavailability concepts. Herein, bioavailability is defined as the ability of a metal to be internalized by an organism; thereby, it must be capable of passing across a biological membrane. One of the goals of my research has been to contribute to an understanding of metal bioavailability in metal mixtures and to provide data that can be used to improve bioavailability-based metal-mixture toxicity models.

1.2 History and current state of predictive toxicity models

In early metal-toxicity studies, the toxicity potential was evaluated with emphasis on the target organism and associated biological variables rather than on analysis of the exposure medium and its parameters [3]. However, since the 1970's, numerous studies have demonstrated a correlation between water chemistry parameters (pH, alkalinity, hardness, dissolved organic carbon [DOC] concentration) and metal toxicity to aquatic organisms[4]. Metal speciation is an important component in determining the toxicity potential because it is hypothesized that only a fraction of the total metal in a system will cause toxicity to an organism [5].

The importance of chemical complexation in influencing metal bioavailability was first conceptualized in the Free Ion Activity Model (FIAM) and the Gill Surface Interaction Model (GSIM). One assumption of the FIAM is that variations in the concentration of metal accumulated on a biotic ligand (BL; e.g., fish gills or ionoregulatory surfaces in aquatic invertebrates) site follow variations in the concentration of the free metal ions, thus making uptake rates into organisms dependent on the concentrations of free-metal ions in the aquatic environment [5]. This implies that complexation of metal by dissolved inorganic and organic ligands and/or sorption of a metal onto particles in the exposure water control the bioavailability and thus the toxicity of the metal [6]. However, it was later demonstrated that the free ion concentration by itself is not a good predictor of toxicity, because it does not account for water chemistry parameters such as hardness that also affect the toxic potential [7]. Proposed in 1983, the GSIM took into account the importance of some water chemistry parameters by demonstrating a correlation between water hardness and metal toxicity through competition at 'physiologically active' fish gill sites [8,9]. As a step beyond the FIAM and GSIM, the biotic ligand model (BLM; [10]) explicitly takes into account competition between major inorganic cations (e.g., Ca^{2+}, Mg^{2+}, Na^{+}, H^{+}) and metals for binding to BL sites, in addition to metal complexation/sorption by inorganic and organic ligands, when predicting toxicity.

The BLM assumes that a free metal ion must compete with other constituents in the water in order to be taken up by an organism and thereby result in toxicity. Specifically, a BL is postulated to be a type of binding site on an organism that can bind toxicants at a site of toxic action [10]. On any given organism, there are theorized to be a large number of metal-binding sites, some of which may contribute to an adverse effect to the organism. Naturally-abundant inorganic cations (i.e., H^{+}, Ca^{2+}, Mg^{2+}, Na^{+}) can compete directly with the metal ions for binding

to the BL, thereby providing protection against metal toxicity. However, dissolved ligands (e.g., DOM, major inorganic anions [e.g., CO_3^{2-}, HCO_3^-]) compete with the BL for the cations. As the complexation of major inorganic cations to dissolved ligands increases, those ligands become less efficient at complexing metals, thereby potentially increasing the toxicity of that solution by increasing its free-metal-ion concentration(s). On the other hand, increased complexation of major cations to the BL decreases the amount of metal that is able to interact at a target site, thereby decreasing the metals toxicity [11]. Thus, the BLM provides an elegant geochemical explanation of the differences of a metal's toxicity in different water chemistries, and it lends itself to quantitative predictions of metal toxicity. However, limitations of the BLM are that (1) it only considers interactions of "dissolved" metals with other "dissolved" components in the water (currently assumed to be forms that pass through a 0.45-µm filter, even though some of the metal in a 0.45-µm filtrate might be bound to colloidal material and thus might not be truly dissolved), (2) it does not predict sorption/binding of metals to particles (forms that do not pass through a 0.45µm filter), thus requiring input of dissolved-metal concentrations instead of total-metal concentrations into the model, and (3) current publicly-available versions of the BLM do not predict toxicity of metal mixtures.

Consequently, because metals usually occur as mixtures in natural waters, it is important to develop a "multi-metal, multi-site" biotic ligand model (MMMS-BLM) capable of predicting the expected toxicity in a system that is complicated by the presence of multiple metals. A MMMS-BLM model is currently in development by Windward Environmental. Similar to the individual-metal BLM that was developed by Windward Environmental, this metal-mixtures model will take into account various water chemistry parameters and the strength of binding of the metals to the BL(s). Similarly, the WHAM-F_{TOX} model (Lancaster Environment Centre,

Lancaster University) uses the same geochemical speciation principles of the BLM but uses humic acid as a generalized surrogate for organismal binding of metals. In this model, exposure is expressed by the accumulation of cations at such a site [12,13]. Finally, a more direct approach, known as the Metal Effects Addition Model (MEAM, Environment Canada, University of Waterloo), is an empirical approach that takes into account the amount of metal accumulated by organisms in laboratory exposures to metal mixtures [14].

1.3 Challenges in statistical determination of additivity

The complexities of mineral compositions and their weathering, and the variety of pathways for a variety of anthropogenic releases of metals to surface waters and groundwaters, make it unlikely that a metal will occur on its own in a surface water. Instead, a given dissolved metal will be part of a mixture of several metals, each present in multiple chemical species (e.g., free-metal ion and metal complexed with a variety of dissolved inorganic and organic ligands). Despite this, risk assessment approaches for metal contamination are still largely based on results of individual-metal toxicity tests and sometimes on the assumption that when more than one metal is present in a waterway, the metals will interact in an additive manner.

Currently, there are two general models for defining additivity: independent action and concentration addition [1,15]. The independent-action model (also known as the response-addition or effects-addition model) is defined by the assumption that the toxicants are 'functionally independent' of one another [16] and as a result, multiple substances used in combination produce a total effect that is a simple combination of the individual effects. In this model, one substance in a mixture does not change the determined outcome of a second or third substance in the system [17]. Conversely, the concentration-addition model is based on the toxic unit approach in which the combined effect of the mixture is assumed to be proportional to the

sum of the potency-normalized concentrations of the individual metals in the mixture [16,18]. Concentration addition is generally assumed to be appropriate when the chemicals have the same mechanism of toxic action (e.g., Cd and Zn both impair Ca homeostasis [19]). In contrast, independent action is generally assumed to be appropriate when the chemicals have different mechanisms of action (e.g., impairment of Na homeostasis by Ag and Cu versus impairment of Ca homeostasis by Cd and Zn [19]).

Unfortunately, recent studies have demonstrated that additive predictions of toxicological effects may not be representative of all metal-mixture systems [20-23]. In fact, two major literature reviews in which several decades of metal-mixture data were analyzed [24,25] indicate that approximately 70% of metal-mixture tests resulted in additive or less-than-additive toxicity (40-50% had less-than-additive toxicity, 20-25% had additive toxicity), whereas approximately 30% of mixtures exhibited more-than-additive toxicity. As a result, an assumption of additivity of metal-mixture toxicity might overall be considered conservative from a regulatory perspective, because predicting additive toxicity when the toxicity actually is less-than-additive is also protective of aquatic life. Despite that positive interpretation, those results mean that the toxicity of approximately 3 of every 10 metal mixtures might be underestimated by the additive-toxicity assumption (i.e., the regulatory approach would be under-protective); and in general, toxicity-prediction models based on additivity will be incorrect by being either under-protective or over-protective a large percentage of the time (i.e., approximately 75-80% of the time). The inability to accurately predict mixture toxicity in part stems from the use of dissolved-metal concentrations as predictors of toxicity, because factors like aqueous metal speciation, metal-metal competition for binding to dissolved ligands and BLs, and metal interactions within an organism can complicate this system to an extent that metal-mixture toxicity cannot be accurately explained using

dissolved-metal concentrations with either a concentration-addition or a response-addition assumption [1].

Challenges arise in the investigation of additivity of mixture toxicity because there is currently no unifying statistical method or model to determine significant differences when defining additivity vs. non-additivity, though several models have been proposed. Additionally, most researchers ignore the statistical uncertainty in their predictions of additive toxicity (i.e. the null hypothesis result to which their observed result is compared).

Hypothesis testing is a useful tool for comparing a dataset against a null hypothesis (for example, determining whether the toxicity exhibited by a given mixture is statistically different from the toxicity that would be exhibited from a single metal). In a basic manner of statistical differentiation, analysis of variance (ANOVA) tests are able to determine whether the effect at any given concentration and the control differ significantly. This technique is most commonly applied through a Dunnett's test. Dunnett's is a multiple-comparison test in which each treatment level is compared with a control. This method can define the lowest effect concentration (LOEC) and no-observed effect concentration (NOEC). LOEC and NOEC values are commonly used in regulatory platforms to determine acceptable threshold concentrations [26]. However, the reliance on hypothesis-based toxicity data, such as LOEC and NOEC values, has been widely debated in the literature [27,28] because the variability of NOEC and LOEC results are influenced by test design and because of the overall ambiguity of the protection level afforded by using LOEC and NOEC values to establish regulatory limits [28]. A Dunnett's test considers each treatment with a control separately, so it does not make use of the fact that underlying toxicant effects are ordered by increasing concentration, with no regard for the presumed concentration-response ordering of effects. In 1971, Williams suggested a new statistical design intended to detect an increasing

concentration-response [29,30], therefore making it more powerful than a Dunnett's Test. William's test is also a hypothesis-based statistical analysis, but it determines the NOEC iteratively by starting with the highest concentration in a mixture, to determine which concentration is statistically more toxic than the control. The ability for a statistical test to account for an increasing response throughout treatment levels is conceptually a key element for toxicity studies, and as a result, a few researchers have suggested modifications and manipulations performed on the William's Test [31-34] in an attempt to increase statistical power. Other statistical methods have been suggested that involve indices of additivity [35] to define synergistic, antagonistic, and additive toxicities.

Alternatively, in many cases researchers and regulators are more concerned with the extent of non-additive interactions and are interested in being able to predict the mortality for mixtures that behave non-additively. To gain insight into the magnitude of a mixture interaction, a point estimation approach is useful. Contrary to hypothesis testing, the point estimation approach uses the observed effects on the exposure concentrations to predict a specified effects concentration, such as the concentration that causes 50% mortality (EC50) [26], by transforming the concentration-response relationship. The probit and logit methods of transformation are two of the most common approaches [26,36,37]used to generate a point estimate; however other tests [38,39]have been proposed.

One of the greatest challenges in the statistical analysis of toxicological data is that many mixtures result in responses that do not follow an expected concentration-response relationship. Jonker [15] proposed a model to determine non-additive effects in mixture concentration-response results, even when the data do not follow a normal distribution, which is common in toxicological data. One limitation of statistically testing for non-additivity in metal mixturesis, in many cases,

toxicologists do not account for the uncertainty in the individual-metal toxicity data that are used to predict additive metal interactions. To address this oversight, Meyer et al. [1] recommended a randomization procedure that uses the parameters of individual-metal concentration-response curves (slope, center of curve, and standard error) to generate uncertainty bounds around a predicted mixture toxicity that can be compared to the observed mixture mortality, to determine if a mixture demonstrates non-additive toxicity.

Though there are many potential statistical models that could be followed, differences in experimental design, modes of toxicity, and the chosen additivity model (independent action vs concentration addition) make it difficult to choose one statistical method as being superior to another. Differences between response-additive predicted metal-mixture toxicity and the observed toxic effects of metal mixtures demonstrate a need for more sophisticated models that account for non-additive interactions, and development of water quality standards that protect against the toxic effects of metal mixtures.

1.4 Thesis objectives

Part of my goal with this thesis project is to create an effective and functional method to analyze toxicological data, in order to distinguish between response-additive, more-than-additive, and less-than-additive mixture toxicities. Before the research summarized in Chapters 2-4, I conducted binary-metal mixture toxicity tests with Cd-Ni, Cu-Ni and Ni-Zn. The results of these toxicity tests, along with a statistical analysis of the mixtures using a paired t-test, were presented in my 2014 Master's thesis [40] at Colorado School of Mines. After completion of the thesis, the statistical approach to defining non-additivity in the mixtures was reworked and presented in a publication in *Environmental Toxicology &Chemistry* in 2015 [21]. Although the analysis of the

data has been significantly altered from the work presented in my Master's thesis, I have included this work as an appendix instead of as a chapter in this thesis (Appendix A).

The toxicity tests for the study presented in Appendix A were designed to elicit a wide range of mortality by holding the concentration of one metal constant while the concentration of the second metal is varied along a concentration gradient. For many binary mixtures, this approach resulted in a concentration-response type curve that ranged from 0 to 100% mortality across the gradient of concentrations tested. The new approach I took was novel in that I chose to compare the inflection point (ECx_{infl}) of the concentration-response curve for a binary-metal mixture to the inflection point of the concentration-response curve for the varied-concentration metal alone (i.e. the EC50 of the individual-metal) [21]. The comparison of ECx_{infl} values to the individual-metal EC50 is valid because, assuming response-additive toxicity, 50% residual immobilization will always occur at the individual-metal EC50 (see Figure A.1 in Appendix A).

The research presented in Chapter 2 is a continuation of my metal-mixture toxicity work, this time evaluating ternary-metal mixtures of Cd-Cu-Ni and Cd-Ni-Zn. Analogous to the experimental design used in the binary metal mixtures study, the concentration of one metal (Metal 1) was varied in a gradient designed to result in mortalities ranging from 0 to 100%, while two other metals (Metal 2 and Metal 3) were held at constant "background" concentrations throughout the entire series. Then the "background" concentration of Metal 2 was changed in different tests, but the concentration of Metal 3 remained constant in all of the tests. Many challenges arose in the analysis of ternary-metal mixture results. Unlike the analysis of the binary-mixtures, in which I was able to plot concentration-response curves, the third metal present in these ternary mixtures complicated the concentration-response relationship to the extent that such a display provides little useful information regarding the interactions of the multiple metals. For

example, in ternary mixtures, the initial data point in a concentration-response curve (indicating no added concentration of Metal 1) was already the result of a complex interaction between the two constant "background" metal concentrations (Metal 2 and Metal 3). From here, as the concentration of the varied metal increased, the resulting effect is a complicated combination of interactions between Metals 1, 2, and 3 and the multiple potential mixture effects are no longer necessarily discernible from simple comparison of the inflection points of a concentration-response curve. To address this challenge, the individual-metal toxicity data that were collected in tandem with the mixture toxicity data were used to generate predictions of additive mixture toxicity (a null hypothesis to which observed toxicity results could be compared) and to evaluate the repeatability of results of individual-metal toxicity tests that were performed at different times. This approach was coupled with a Monte-Carlo-type randomization to test the statistical significance of each metal combination on a point-by-point basis. It was necessary to use the individual-metal toxicity data that were collected the same day as the mixture-toxicity data because I had routinely observed high day-to-day variability in individual-metal exposures that had yet to be explained.

In Chapter 3, I present an explanation for the variability I had previously observed in results of individual-metal toxicity tests, in which the among-test variability in Cd toxicity tests was very high, followed by Zn, Ni and Cu in decreasing order of variability of toxicity. Because the toxicity of individual-metals must be well understood before mixture effects can be interpreted and predicted, it is important to understand and incorporate all factors that may influence variability in the toxicity of individual metals. The simple explanation of the high among-test variability is that there are large age-related differences in toxicity even within the

first 24 h post-birth in *D. magna*; however, in routine practice, controlling for that age-related variability is not easy to put into practice.

The overarching goal of my projects is to assist in the development of a multi-site, multi-metal biotic ligand model by providing detailed and reliable data related to the toxicity of metal mixtures. The toxicity of the individual-metal and metal-mixture toxicity tests was determined with 48-h acute toxicity tests using a freshwater invertebrate, *Daphnia magna*. Data that was provided to the modelers included the metal-mixture toxicity data and water chemistry and results from individual-metal toxicity tests.

Finally, in Chapter 4 I extend my research dealing with toxic effects of metal mixtures into a natural setting contaminated by several metals. In these experiments, I used a relatively novel technique for reversing the isotopic signature of Cu in freshwater snails (*Lymnaea stagnalis*) and then tracking changes in isotopic composition of the Cu in the snails after they consumed sediment particles from an acid mine drainage-contaminated site in Blackhawk, Colorado. Particles presented to the snails contained the natural isotopic abundance of Cu, allowing me to trace the reverse-labeled organisms' uptake and elimination of Cu. This approach differs from the previous toxicity tests that were discussed in Chapters 2 and 3 in that this experiment utilizes the dietborne pathway of a metal contaminant, rather than the dissolved aqueous exposure. In most cases, only the results of aqueous exposures are considered in regulatory decisions even though the majority of the metal accumulation in some organisms sometimes comes from dietary uptake [41]. Furthermore, Wilding et. al. [42] concluded that when Zn was simultaneously exposed to the crustacean *Gammarus pulex* through dietary and aqueous pathways, the toxicity could not be predicted from the response to the aqueous exposure alone. Ultimately, although no direct conclusions can be drawn between the aqueous exposures of *D.*

magna to the dietary exposures of *L. stagnalis*, the experiments provide valuable insight into the interactions and effects of metal mixture toxicity as it relates to AMD-contaminated sites.

Finally, Chapter 5 presents an overall summary of the research performed, relates the results and implications of my studies to a natural AMD site, and suggests direction for future work.

1.5 References cited

1. Meyer JS, Farley KJ, Garman ER. 2015. Metal Mixtures Modeling Evaluation Project: 1. Background. *Environmental Toxicology and Chemistry* 34:726-740.

2. Van Genderen E, Adams W, Dwyer R, Garman E, Gorsuch J. 2015. Modeling and interpreting biological effects of mixtures in the environment: Introduction to the Metal Mixture Modeling Evaluation Project. *Environmental Toxicology and Chemistry* 34:721-725.

3. Tessier A, Turner DR. 1995. *Metal speciation and bioavailability in aquatic systems*. J. Wiley.
4. Meyer JS, Clearwater SJ, Doser TA, Rogaczewski MJ, Hansen JA. 2007. *Effects of Water Chemistry on Bioavailability and Toxicity of Waterborne Cadmium, Copper, Nickel, Lead, and Zinc to Freshwater Organisms*. Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.

5. Campbell P. 1995. Interactions between trace metals and aquatic organisms: A critique of the free-ion activity model. *Metal speciation and bioavailability in aquatic systems*.

6. Parent L, Twiss MR, Campbell PG. 1996. Influences of natural dissolved organic matter on the interaction of aluminum with the microalga *Chlorella*: A test of the free-ion model of trace metal toxicity. *Environmental Science and Technology* 30:1713-1720.

7. Meyer JS, Santore RC, Bobbitt JP, DeBrey LD, Boese CJ, Paquin PR, Allen HE, Bergman HL, DiToro DM. 1999. Binding of nickel and copper to fish gills predicts toxicity when water hardness varies, but free-ion activity does not. *Environmental Science and Technology* 33:913-916.

8. Pagenkopf GK. 1983. Gill surface interaction model for trace-metal toxicity to fishes: Role of complexation, pH, and water hardness. *Environmental Science and Technology* 17:342-347.

9. Janssen C, Heijerick D, De Schamphelaere K, Allen H. 2003. Environmental risk assessment of metals: Tools for incorporating bioavailability. *Environment International* 28:793-800.

10. Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PG, Delos CG, Di Toro DM, Dwyer RL, Galvez F. 2002. The biotic ligand model: A historical overview.

Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 133:3-35.

11. Di Toro DM, Allen HE, Bergman HL, Meyer JS, Paquin PR, Santore RC. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environmental Toxicology and Chemistry* 20:2383-2396.

12. Tipping E, Lofts S. 2013. Metal mixture toxicity to aquatic biota in laboratory experiments: application of the WHAM-F_{TOX} model. *Aquatic Toxicology* 142-143:114-22.

13. Tipping E, Lofts S. 2015. Testing WHAM-F_{TOX} with laboratory toxicity data for mixtures of metals (Cu, Zn, Cd, Ag, Pb). *Environmental Toxicology and Chemistry* 34:788-798.

14. Norwood WP, Borgmann U, Dixon DG. 2013. An effects addition model based on bioaccumulation of metals from exposure to mixtures of metals can predict chronic mortality in the aquatic invertebrate *Hyalella azteca*. *Environmental Toxicology and Chemistry* 32:1672-1681.

15. Jonker MJ, Svendsen C, Bedaux JJ, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environmental Toxicology and Chemistry* 24:2701-2713.

16. Könemann W, Pieters M. 1996. Confusion of concepts in mixture toxicology. *Food and Chemical Toxicology* 34:1025-1031.

17. Unkelbach H-D, Pöch G. 1988. Comparison of independence and additivity in drug combinations. *Arzneimittel-Forschung* 38:1-6.

18. Drescher K, Boedeker W. 1995. Assessment of the combined effects of substances: The relationship between concentration addition and independent action. *Biometrics* 51: 716-730.

19. Niyogi S, Wood CM. 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environmental Science and Technology* 38:6177-6192.

20. Meyer JS, Ranville JF, Pontash M, Gorsuch JW, Adams WJ. 2015. Acute toxicity of binary and ternary mixtures of Cd, Cu, and Zn to *Daphnia magna*. *Environmental Toxicology and Chemistry* 34:799-808.

21. Traudt EM, Ranville JF, Smith SA, Meyer JS. 2015. A test of the additivity of acute toxicity of binary-metal mixtures of Ni with Cd, Cu, and Zn to *Daphnia magna*, using the inflection point of the concentration-response curves. *Environmental Toxicology and Chemistry*: Accepted 16 Dec 2015.

22. Nys C, Asselman J, Hochmuth JD, Janssen CR, Blust R, Smolders E, De Schamphelaere KA. 2015. Mixture toxicity of nickel and zinc to *Daphnia magna* is noninteractive at low effect sizes but becomes synergistic at high effect sizes. *Environmental Toxicology and Chemistry* 34:1091-1102.

23. Newton K. 2012. The influence of Dissolved Organic Carbon and Surface Chemistry on the Toxicity of Silver Nanoparticles in *Daphnia magna*. Ph.D. thesis, Clemson University, Clemson, South Carolina, USA.

24. Norwood W, Borgmann U, Dixon D, Wallace A. 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. *Human and Ecological Risk Assessment* 9:795-811.

25. Vijver MG, Elliott EG, Peijnenburg WJ, de Snoo GR. 2011. Response predictions for organisms water-exposed to metal mixtures: A meta-analysis. *Environmental Toxicology and Chemistry / SETAC* 30:1482-7.

26. Rand GM. 1995. *Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment*. CRC Press, Pensacola, Florida, USA.

27. Newman MC. 2008. "What exactly are you inferring?" A closer look at hypothesis testing. *Environmental Toxicology and Chemistry* 27:1013-1019.

28. Warne MSJ, van Dam R. 2008. NOEC and LOEC data should no longer be generated or used. *Australasian Journal of Ecotoxicology* 14:1.

29. Williams D. 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics*:103-117.

30. Williams D. 1972. The comparison of several dose levels with a zero dose control. *Biometrics*:519-531.

31. Chase GR. 1974. On testing for ordered alternatives with increased sample size for a control. *Biometrika* 61:569-578.

32. Marcus R, Peritz E, Gabriel KR. 1976. On closed testing procedures with special reference to ordered analysis of variance. *Biometrika* 63:655-660.

33. Bretz F. 1999. Powerful modification of Williams' test on trend.

34. Bretz F, Hothorn LA. 2000. A powerful alternative to Williams' test with application to toxicological dose-response relationships of normally distributed data. *Environmental and Ecological Statistics* 7:135-154.

35. Marking L. 1977. Method for assessing additive toxicity of chemical mixtures. *in Aquatic toxicology and hazard evaluation*. ASTM International.

36. Stephan CE. 1977. Methods for calculating an LC 50. *in Aquatic toxicology and hazard evaluation*. ASTM International.

37. Lee G, Ellersieck MR, Mayer FL, Krause GF. 1995. Predicting chronic lethality of chemicals to fishes from acute toxicity test data: Multifactor probit analysis. *Environmental Toxicology and Chemistry* 14:345-349.

38. USEPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Pages 37-46 *in*. U.S. Environmental Protection Agency, Washington, DC, USA.

39. Hamilton MA, Russo RC, Thurston RV. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environmental Science and Technology* 11:714-719.

40. Traudt EM. 2014. Toxicity of binary mixtures of nickel, copper, cadmium and zinc to *Daphnia magna*. M.S. thesis, Colorado School of Mines, Golden, Colorado, USA.

41. Meyer JS. 2005. *Toxicity of dietborne metals to aquatic organisms*. Allen Press/ACG Publishing.

42. Wilding J, Maltby L. 2006. Relative toxicological importance of aqueous and dietary metal exposure to a freshwater crustacean: Implications for risk assessment. *Environmental Toxicology and Chemistry* 25:1795-1801.

CHAPTER 2

ACUTE TOXICITY OF TERNARY CD-CU-NI AND CD-NI-ZN MIXTURES TO *DAPHNIA MAGNA*

A paper to be submitted to *Environmental Science and Technology*

Elizabeth M. Traudt[1,2], James F. Ranville[3], Joseph S. Meyer[4]

2.1 Abstract

Multiple metals are often present in the natural water systems, leading to mixture toxicity that currently is difficult to predict. Previous toxicity tests in which *Daphnia magna* were exposed to binary mixtures of Ni combined with Cd, Cu, or Zn demonstrated that Ni protects against the toxicity of Cd, greater-than-additive toxicity occurred in Cu-Ni mixtures, and slightly less-than-additive toxicity occurred in Ni-Zn mixtures. Those results provided evidence for the importance of competition among metals for binding to biological and/or dissolved chemical ligands, depending on the metals in the binary mixture; however, the relative importance of each mechanism is unknown in mixtures containing more than two metals. To advance beyond binary mixtures of metals, we exposed *D. magna* neonates to Cd, Cu, Ni, or Zn alone and in ternary Cd-Cu-Ni and Cd-Ni-Zn combinations in standard 48-h lethality tests conducted in USEPA moderately hard reconstituted water with 3 mg DOC/L added as Suwannee River fulvic acid. In these ternary mixtures, two metals were held constant at specified concentrations while the third metal was varied through a series that ranged from nonlethal to lethal concentrations. In Cd-Cu-Ni mixtures, the toxicity was less-than-additive, additive, or greater-than-additive, depending on the concentration of the varied metal. However, in Cd-Ni-Zn mixtures, the toxicity was always

1. Graduate student, Department of Chemistry and Geochemistry, Colorado School of Mines
2. Primary researcher and author.
3. Associate Professor, Department of Chemistry and Geochemistry, Colorado School of Mines
4. Applied Limnology Professionals LLC; and Affiliated Faculty Member, Colorado School of Mines

less-than-additive or approximately additive, again depending on the concentration of the varied metal. These findings demonstrate that complex interactions of potentially competing toxicity-controlling mechanisms can occur in metal mixtures but should be predictable by mechanistic bioavailability-based models of metal-mixture toxicity.

2.2 Introduction

Metals are ubiquitous in the environment and are considered a major component of freshwater pollution from industrial, municipal, and agricultural discharges [1]. In addition, metal contamination is a major concern in regions that receive acid rock drainage, because metals are solubilized at low pH [2] and, as a result, become more bioavailable and potentially toxic to aquatic organisms [3]. Furthermore, metals almost never occur alone in surface waters and instead are in mixtures of several metals that occur as various chemical species. Despite this, most regulatory approaches and risk assessment procedures for metals are largely based on individual-metal toxicity exposures and the assumption that when more than one metal is present in a waterway, the metals will behave in a way than can be predicted by the simple interaction of each contaminate, referred to as an additive interaction [4].

Currently, there are two general models for predicting mixture effects: independent action (IA, also referred to as the response-addition (RA) model) and concentration addition (CA) [4,5]. The CA model is generally assumed to be appropriate when the chemicals have the same mechanism of toxic action (e.g., Cd and Zn both impair Ca homeostasis [6]), while the IA model is generally assumed to be appropriate when the chemicals have different mechanisms of action (e.g., impairment of Na homeostasis versus impairment of Ca) [7].

However, additive toxicity does not appear to occur in most metal-mixture systems [8-10]. For example, Norwood et al.[11] and Vijver et al.[12] conducted integrative literature reviews in

which decades of metal-mixture data were analyzed and determined that only approximately 20 to 25% of metal-mixture tests resulted in additive, whereas approximately 30% of the metal mixtures resulted in more-than-additive toxicity, and approximately 40 to 50% resulted in less-than-additive toxicity [4].

The inability to accurately predict mixture toxicity based on an assumption of additivity stems from the use of dissolved-metal concentrations as predictors of toxicity, in which factors like aqueous metal speciation, metal-metal competition for binding to dissolved ligands, and metal-metal interactions within an organism can complicate this system to an extent that metal-mixture toxicity cannot be accurately explained using either a concentration-addition or a response-addition assumption [4]. It is not currently well-understood how the interactions of multiple metals, water chemistry parameters, and aquatic organisms affect the toxicity of metal mixtures [4]. Compounding this conundrum, an even greater challenge is to predict the outcome when multiple interactions may be contributing to toxicity in a mixture. For example, if the resulting toxicity of a mixture containing Metal 1 and Metal 2 was less than expected, but the resulting toxicity of a mixture with Metal 2 and Metal 3 was generally greater than expected, a ternary mixture containing Metals 1, 2, and 3 could exhibit a somewhat complicated combined effect of those interactions.

In this study, my goal was to test for and understand competing interactions among metals in ternary mixtures. To do this, I used Cd and Ni (two metals that had been previously determined to result in a less-than-additive toxicity [13]) in all analyses, while the third metal in the ternary mixture was either Cu or Zn. Previous work I conducted with Cd-Ni and Cu-Ni mixtures demonstrated that these binary combinations can produce less-than-additive and greater-than-additive acute toxicity to *D. magna*, respectively [13]. However, Cd-Cu mixtures caused less-

than-additive acute toxicity to *D. magna* when Cu was titrated into a constant background concentration of Cd, but greater-than-additive toxicity when the roles of the two metals were reversed [8]. Because the effects of the binary mixtures differed greatly depending on the metals in the mixture, Cd-Cu-Ni mixtures were chosen to test which effect would prevail in a ternary mixture in which both types of interactions (i.e., less-than-additive and more-than-additive toxicity) might occur.

Previous work with Cd-Ni and Cd-Zn mixtures demonstrated pronounced less-than-additive toxicity that was qualitatively similar for the two binary-metal mixtures [8,13]. Less-than-additive toxicity also occurred in Ni-Zn mixtures, although to a much lesser extent [13]. Therefore, in Cd-Ni-Zn mixtures, I expected less-than-additive toxicity at the lowest concentrations of the varied-concentration metal (Ni or Zn), but I was uncertain whether multiple less-than-additive effects would result in an overall less-than-additive toxicity. The hypothesis I generated for the ternary-metal mixtures can be visualized in Figure 2.1.

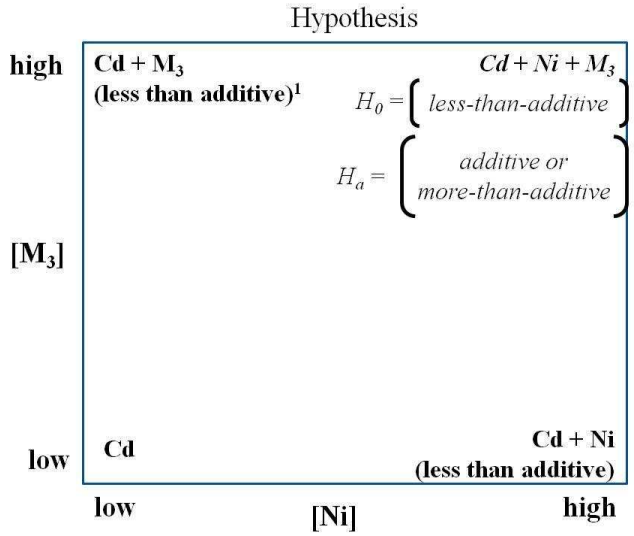

Figure 2.1: Matrix diagram illustrating potential outcomes of ternary-metal mixture toxicity tests. In this schematic, as well as in the experimental design, the Cd concentration is held constant at 0.9 μM for all of the toxicity tests. Protective effects were observed in binary combinations of Cd + Ni [13], Cd + Cu [8], and Cd + Zn [8], therefore, the null hypothesis related to this experimental design is that a ternary combination of Cd + Ni + M3will result in a less-than-additive toxicity.

2.3 Experimental

2.3.1 Test organisms

Daphnia magna neonates were used in all toxicity tests and were obtained from Aquatic Biosystems, Inc, in Fort Collins, Colorado in moderately hard water reconstituted water (MHRW) [14] with the green alga *Pseudokirchneriella subcapitata* as food. The neonates were sent via same-day shipping to ensure that all organisms were less than 24 h old at the start of the toxicity tests. Because the tests were started as soon as a shipment arrived and they only lasted 48 h, the *D. magna* were not fed again.

2.3.2 Exposure water

The exposure water in the toxicity tests was MHRW to which dissolved organic carbon (DOC; at 3 mg/L) was added as Suwannee River fulvic acid obtained from the International Humic Substances Society (http://www.humicsubstances.org/). Fulvic acid was added to the exposure waters to provide a concentration of organic carbon that is more representative of surface waters than the low background concentration of ≤0.5 mg DOC/L in Milli-Q water [8,15]. Metal salts [$Ni(NO_3)_2$, $Cd(NO_3)_2$, $Cu(NO_3)_2$, $Zn(NO_3)_2$ (Regaent Grade, Mallinckrodt Chemical (Ni) and Baker Chemical Company (Cd, Cu, Zn))] were added to that exposure matrix. Exposure solutions were prepared 24 to 36 h before the start of a toxicity test, to allow equilibration of the metals with the DOC [16].

2.3.3 Acute toxicity tests

The toxicity of individual metals and ternary mixtures was determined in 48-h static, non-renewal lethality tests, following procedures recommended by the U.S. Environmental Protection Agency [14]. The ternary-metal tests comprised a series of either 6 or 12 metal combinations in a gradient designed to produce mortalities ranging between 0 and 100%. Series of 6 metal

concentrations were used in all individual-metal tests and in ternary mixtures that contain high metal concentrations, where nearly 100% mortality was expected to occur at all concentrations. In the ternary mixtures, the concentrations of Cd and Ni were held constant throughout an entire series while the concentration of the third metal (M_x; either Cu or Zn) was increased incrementally in the series; and then the roles of Ni and M_x were reversed in separate tests (i.e., the former M_x became a constant-concentration metals along with Cd, and Ni became the varied-concentration metal). The concentration of Cd was held constant at 0.9 μM (0.1 mg/L) throughout all of the ternary-metal toxicity tests. Individual-metal toxicity tests were conducted concurrently with each ternary mixture and comprised a dilution series of 6 concentrations (including a control that contained no added metals) for each metal, and these controls also served as the no-added-metal controls for the concurrent metal- mixture tests. For example, concurrent with the ternary Cd-Cu-Ni tests, individual-metal toxicity tests were conducted with Cd, Cu, and Ni.

In all tests, each metal concentration or mixture was tested in 4 replicate chambers, each containing 25 mL of exposure water and 5 organisms. Therefore, a total of 20 organisms were exposed to each concentration in the individual- or ternary-metal gradient. The number of dead organisms was recorded at 24 and 48 h, with immobilization as the indicator of mortality [14].

The ternary-metal tests were conducted during an 8-month period from January to August 2014. All tests were conducted in incubators (VWR International) at a temperature of 20±2°C, with a 16h-8h light-dark cycle. To test for variability in responses, each set of a metal-mixture series and its associated individual-metal toxicity tests was repeated on a different week during the study (i.e., duplication of each set of tests).

2.3.4 Chemical analyses

Water samples from all controls and exposure concentrations were analyzed at the beginning of the toxicity tests for total concentrations of metals (including Cd, Cu, Ni, and Zn), major inorganic cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), and sulfur using an Optima 5300 ICP-OES inductively coupled plasma optical emission spectrophotometer (PerkinElmer). The exposure waters were not filtered before analysis because particles were unlikely in these synthetic laboratory waters and preliminary tests demonstrated that commercial filters either sorb metals from, or leach metals into, initial volumes of water that are passed through the membranes [8]. Consequently, the small volumes (< 100 ml) of exposure waters used in these *D. magna* toxicity tests were not sufficient to adequately rinse the filters only or exceed their sorption capacity. Additional preliminary tests, in which sufficient sample was used to saturate the filter membrane, demonstrated that the metals added to MHRW were >90% dissolved [8]. Therefore, the total-metal concentrations were assumed to closely approximate the dissolved-metal concentrations.

Temperature and dissolved oxygen were measured using a YSI 55 probe (YSI Incorporated), and pH was measured using an Orion ROSS electrode and Orion 2 STAR meter (Thermo Fisher Scientific) calibrated with pH 4, 7, and 10 buffers. Alkalinity was analyzed in the MHRW that was used to prepare all the exposure waters, by titration with H_2SO_4 to the bromo-cresol green endpoint [17]. Total organic carbon (TOC) concentrations were analyzed by UV-catalyzed persulfate oxidation using a Sievers Model 900 TOC Analyzer (GE Analytical Instruments).

Sulfate concentrations in all controls and metal exposures were calculated by assuming all the sulfur measured by ICP-OES was present as SO_4^{2-}. Chloride concentrations were calculated

by assuming the molar Cl^- concentration equaled the measured molar K^+ concentration (because the only Cl^- in the MHRW recipe was added as KCl [14]).

In all ICP-OES analytical runs, a Sc internal-calibration standard was continuously introduced into the plasma along with each sample, and samples were analyzed in triplicate. Quality assurance/quality control (QA/QC) samples included deionized water blanks (Barnstead Nanopure system, Thermo Fisher Scientific) that contained trace-metal-grade HNO_3 (Thermo Fisher Scientific), and certified continuing calibration verification (CCV) standards. The QA/QC samples were analyzed immediately after instrument calibration, after every 10 samples, and at the end of each set of samples. Additionally, NIST certified standard reference materials 1640a and 1643e [18] were analyzed for trace elements before and at the end of each set of samples. All samples were reanalyzed in any analytical run in which acceptable QA/QC results were not obtained. Those unacceptable results could include: deviations of the internal Sc standard greater than 20% from the known concentration, deviations of the CCV samples greater than 10% from the known concentrations, or relative standard deviations (RSDs) of triplicate analyses of a sample greater than 10%. The ranges of instrument detection limits for the metals, major cations, and sulfur during the 8-month study were (in μg/L): 4.7-7.0 Ca, 0.1-0.3 Cd, 0.3-0.4 Cu, 3-40 K (equivalent to 2.7-36.3 Cl), 0.1-0.4 Mg, 6-8 Na, 5.8-6.5 S (equivalent to 17.4-19.5 SO_4), 0.1-0.4 Ni, and 0.5-0.7 Zn.

Unfiltered water samples for analysis of TOC concentration were collected from the control and the highest metal concentration at the beginning of a test and preserved by addition of H_3PO_4 to pH <2. The alkalinity of each batch of MHRW was determined before testing using a Hach field titration kit. At the beginning of each test, unfiltered water from each treatment in the

metal-concentration series was acidified to pH $\leq$2 with 2% Optima HNO_3 and then submitted for elemental analysis.

2.3.5 Data analyses

Although it is traditional to compare median effect concentrations (EC50 values) determined from 2 or more concentration-response series to quantitatively characterize and compare toxicity results, this approach was not ideal in the present study because such an analysis does not have the resolution to evaluate multiple metal-metal interactions that may occur throughout a concentration-response curve. Instead, we calculated the ratio of the observed mixture mortality to the mortality predicted by assuming IA. The IA model was chosen to evaluate the toxicity of the ternary metal mixtures because the exact mechanism of toxic action of Ni, which was included in each ternary-metal mixture, is not known. In addition, toxicants usually have the same concentration-response slopes that target the same molecular sites [19,20]. However, my analysis of individual-metal toxicity tests that were conducted in-tandem with the mixtures revealed that the slopes of the dose-response curves where highly dependent on the metal in the exposure (Table 2.1), leading to the conclusion that the metals do in fact target different channels.

An observed-to-predicted ratio less than 1 indicates less-than-additive toxicity, a ratio of 1 indicates additive toxicity, and a ratio greater than 1 indicates more-than-additive toxicity.

The software @RISK (Palisade Corporation) was used to compute Monte-Carlo-type randomizations in which the uncertainty associated with the observed mortality and the uncertainty in predicted response-additive mortality in each combination of metal concentrations was used to test for non-additivity on a point-by-point basis. This randomization test is a modification of the test for additivity used in Meyer et al. [4].In each randomization run, 100,000

iterations were performed. In each iteration, random concentration-response curves were generated for each of the three metals by randomly drawing EC50 and log-logistic slopes from normal distributions defined by the average and standard error associated with each variable. Each concentration-response curve was a log-logistic relationship in the form:

$$M = \frac{1}{1+\left(\frac{EC50}{C}\right)^{slope}} \quad (2.1)$$

where M is the mortality proportion (0 to 1) and C is the metal concentration (in the same units as the EC50). To avoid randomly selecting unrealistic zero or negative values for the EC50 and slope the lower of each of those normal distributions was truncated at 0.1. Then, the mortality in a ternary mixture for a null hypothesis of IA was predicted as:

$$\text{Mixture mortality proportion} = 1 - (S_{Cd} * S_{Ni} * S_{M_x}) \quad (2.2)$$

where S_{Cd}, S_{Ni}, and S_{M_x} are the survival proportions (= 1 - M) that are predicted from the individual-metal concentration-response relationships for Cd, Ni, and M_x at the concentrations of those metals in the Cd-Ni-M_x mixture. Concurrently, a random value of the observed mortality was drawn from a beta distribution defined by the average and standard error of the observed mortality. The limits of the beta distribution were 0.0 (0% mortality) and 1.0 (100% mortality) Then, the ratio of observed mortality to predicted mortality was calculated for each of the 100,000 iterations, and the 2.5 and 97.5 percentiles of the distribution of 100,000 ratios were calculated as the 95% confidence interval. If the 95% confidence interval on that ratio did not include a value of 1 (which is the value of the mortality ratio assuming the null hypothesis of independent-action toxicity), the mixture toxicity was inferred to be non-additive at the 95% confidence level.

Due to high age-dependent variability of EC50 values for *D. magna* neonates exposed to some metals in individual-metal toxicity tests [21], it was important to calculate the predicted toxicity based on the individual-metal tests that had been conducted on the same day as a given

mixture test (instead of consolidating all of the individual-metal data to obtain a central-tendency concentration-response curve for each metal).Therefore, the uncertainty in predicted IA mortality was computed from the standard errors of the EC50 and slope of each individual-metal concentration-response curve that was tested concurrently with a given mixture.

In this study, the EC50 values and the slopes of the associated concentration-response curves were calculated using the logit-regression method in OriginPro 9.1 (OriginLab Corporation). Microsoft Excel was used to calculate the 95% confidence intervals on the averages of several EC50 values or slopes and to perform a 1-way analysis of variance (ANOVA) with Tukey HSD post-hoc comparisons to test for significant differences in the EC50 values and slopes for the individual metals.

2.4 Results and Discussion

Water chemistry parameters measured in all individual-metal and metal-mixture toxicity tests and the calculated log-logit regression slopes and intercepts (EC50s) are tabulated in the Supplementary Information file. Average measured concentrations of the constant-concentration metals in the metal-mixture tests are reported in the captions and legends of Figures 2.1 through 2.4, but the measured concentrations for each trial are tabulated in the Supplementary Information file. Measured concentrations of the varied-concentration metals are plotted on the horizontal axes in Figures 2.1 to 2.4 and are also tabulated in the Supplementary Information file. Survival in all controls during the 8-month study was greater than or equal to 90%.

2.4.1 Individual Metals

Based on the molar EC50 values in the individual-metal toxicity tests, Ni was the least toxic (i.e., it had the highest EC50) of the 4 metals tested in this DOC-supplemented MHRW, followed by Zn, Cu, and Cd in sequence of increasing toxicity (Table 2.1). These results are

consistent with results that Traudt et al [13] and Meyer et al [8] reported for the same exposure-water recipe.

2.4.2 Cu-Cd-Ni Mixtures

When the concentration of Cd was held constant at 0.9 μM, the concentration of Ni was held constant at 0.25, 3.8 or 37 μM, and Cu was increased from sublethal to lethal concentrations, less-than-additive, approximately additive, and more-than-additive toxicity occurred in that sequence (Figure 2.2). At low Cu concentrations in the mixture, the predicted mortality was greater than the observed mortality (i.e., the observed:predicted mortality ratio was less than 1, indicating less-than-additive toxicity). This result is consistent with the less-than-additive toxicity in Cd-Ni binary mixtures [13], because the Cu concentrations were negligible to minor in the low-Cu ternary mixtures (less than approximately 0.02 μM Cu). However, as the concentration of Cu was increased to approximately 0.3 to 0.4 μM, the observed:predicted mortality ratio increased to approximately 1, indicating approximately additive toxicity; and the ratio was greater than 1 at Cu concentrations greater than 0.6 μM, indicating greater-than-additive toxicity. The highest concentration of Ni tested (37 μM) exhibited the least predominant less-than-additive toxicity at concentrations less than 0.2 μM Cu, as well as the generally least predominant more-than-additive toxicity (or even merely additive toxicity) at concentrations greater than 0.2 μM Cu. This is likely due to the high "background" concentration of Ni in this series, for which high "background" mortality would be predicted based on results of the Ni-only tests. Because the predicted and observed mortality cannot exceed 100%, those mortality percentages converged and the ratio of observed:predicted mortality did not differ greatly from 1. Similarly, at the highest Cu concentrations tested, the observed:predicted mortality ratio approached 1 because both the observed and predicted mortality percentages approached the maxima of 100% (see Supplemental

Information spreadsheet). Therefore, the ability to resolve non-additive toxicity disappears at very high metal concentrations, as it also disappears at very low metal concentrations that cause little to no toxicity.

Table 2.1: Individual-metal toxicity data for Cd, Cu, Ni, and Zn. The average 48-h median effect concentration (EC50), slope of log-logit concentration-response curve, and number of toxicity tests conducted (n) is reported for immobilization of *Daphnia magna* neonates exposed to Cd, Cu, Ni, or Zn in repeated individual-metal static, non-renewal lethality tests conducted from January through August 2014. Numbers in parenthesis are the 95% confidence interval (CI) on the EC50 or slope. Different capital letters within a column indicate statistically significant ($p \leq 0.05$) differences among the metals.

Metal	Average EC50 (uM) (95% CI)	Slope (95% CI)	n
Cd	0.836 A (0.580-1.091)	1.679 A (1.210-2.148)	8
Cu	1.412 B (1.210-1.696)	6.294 B (3.440-9.149)	5
Ni	31.440 C (23.350-39.531)	2.851 C (2.301-3.402)	8
Zn	12.124 D (6.896-17.353)	1.705 A (1.254-2.157)	5

When the roles of Cu and Ni were reversed (i.e., the Cu and Cd concentrations were held constant at 0.9 μM Cd and 0.5, 0.6, 0.9, 1.4, or 3.6 μM Cu while Ni was increased from sublethal to lethal concentrations), the responses were more varied (Figure 2.3). At the lowest Cu concentration (0.5 uM), the mixture toxicity was less-than-additive, additive, and more-than-additive, depending on the Ni concentration in the mixture. Mixtures that contained 0.6 μM Cu were additive or significantly less-than-additive at Ni concentrations less than 10 μM, but they were more-than-additive at approximately 20 μM Ni. Results at 0.9 and 1.4 μM Cu were qualitatively consistent with the first series of Cd-Cu-Ni mixture tests shown in Figure 2.2 (i.e., additive or more-than-additive toxicity at all Cu concentrations greater than 0.6 μM). As expected from Cd-Cu binary mixture tests with no added Ni [8], the ratio of observed:predicted mortality

that occurred in the 0.9 μM Cd-3.6 μM Cu-0.03 μM Ni ternary mixture was less than 1; however, this ratio was not statistically different than 1. At all higher Ni concentrations mixed with those Cd and Cu concentrations, the observed:predicted mortality ratio was equal to or approximately 1 because both the observed and predicted mortality percentages approached the maxima of 100% (see Supplemental Information spreadsheet).

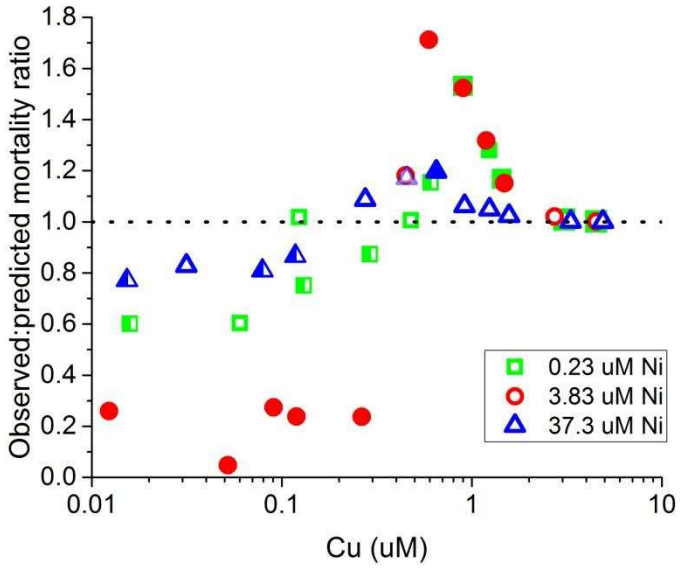

Figure 2.2: Additivity of toxicity of Cd-Cu-Ni mixtures that contained constant concentrations of Cd (0.9 μM in all exposure waters) and Ni (concentrations in the 3 separate sets of tests shown in the legend) while Cu was varied along a concentration gradient within each of the 3 sets of tests. Additivity of toxicity data was evaluated by the ratio of observed mortality to predicted independent-action mortality, with each data point showing the average of duplicate runs of each combination of metal concentrations. Closed symbols = significantly non-additive toxicity ($p \leq 0.05$) in both replicate trials; open symbols = not significantly non-additive toxicity in both replicate trials; half-filled symbols = significant non-additive toxicity in 1 of the 2 replicate trials. Ratios less than 1 indicate less-than-additive toxicity; ratios greater than 1 indicate more-than-additive toxicity.

This more-than-additive toxicity at high Cu or Ni concentrations in Cd-Cu-Ni mixtures is similar to the more-than-additive toxicity in Cu-Ni binary mixtures [13], and it almost entirely overshadows any less-than-additive toxicity that would be expected from either Cd-Ni [13] or Cd-

Cu [8] binary interactions. Although the results of these ternary Cd-Cu-Ni tests support the existence of the less-than-additive toxicity that has been demonstrated in Cd-Cu and Cd-Ni binary mixtures, the results also suggest that the more-than-additive Cu-Ni interaction can overshadow these protective effects at high Cu or Ni concentrations.

Although using this method of analysis made differentiating a more-than-additive effect from a less-than-additive effect easier to quantify, the resolving power of this method is limited when the metal concentrations considerably exceed the EC50 for one of the varied metals (i.e., greater than 1.4 μM Cu and 31 μM Ni in these tests) because both the observed and predicted mortalities approach 100%, therefore forcing the ratio to approach a value of 1. Nonetheless, this analysis of observed:predicted toxicity ratios is instructive when the observed and predicted mortality in a mixture are greater than 0% and less than 100%.

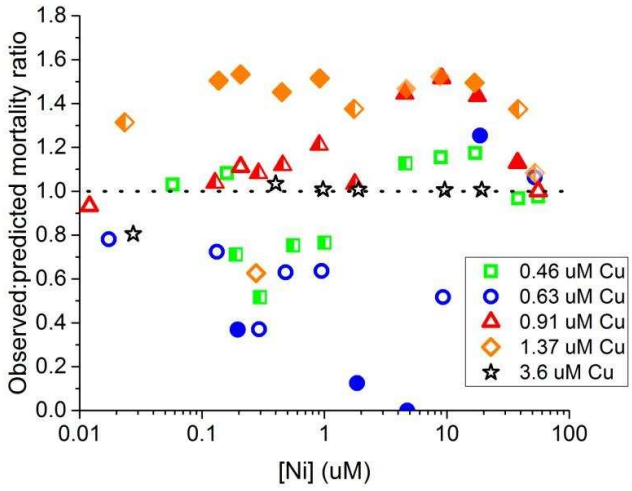

Figure 2.3: Additivity of toxicity of Cd-Cu-Ni mixtures that contained constant concentrations of Cd (0.9 μM in all exposure waters) and Cu (concentrations in the 5 separate sets of tests shown in the legend) while Ni was varied along a concentration gradient within each of the 5 sets of tests. Additivity of toxicity data was evaluated by the ratio of observed mortality to predicted independent-action mortality, with each data point showing the average of duplicate runs of each combination of metal concentrations. Closed symbols = significantly non-additive toxicity ($p \leq 0.05$) in both replicate trials; open symbols = not significantly non-additive toxicity in both replicate trials; half-filled symbols = significant non-additive toxicity in 1 of the 2 replicate trials. Ratios less than 1 indicate less-than-additive toxicity; ratios greater than 1 indicate more-than-additive toxicity.

2.4.3 Cd-Ni-Zn Mixtures

When Ni and Cd concentrations were held constant while Zn was increased through an exposure-concentration series (Figure 2.4) and when Cd and Zn concentrations were held constant while Ni was increased through an exposure-concentration series (Figure 2.5), the observed mortality was almost always less than the predicted response-additive mortality (i.e., the observed:predicted ratio was less than 1 for nearly all mixture concentrations). In Figure 2.4 at low concentrations of Zn, only a single data point had an observed:predicted mortality ratio greater than 1, and it did not differ significantly from 1 and it occurred at the lowest tested concentrations of both Ni and Zn in the ternary mixture. Therefore, this data is effectively representative of a Cd-only toxicity test and is particularly interesting because, when the same Cd concentration was tested with higher concentrations of Ni, Zn, or both Ni and Zn, in all instances these metals protected against the Cd toxicity (the observed:predicted mortality ratio was less than 1). At high Zn concentrations greater than 20 μM, the curves collapse to or approach a ratio of 1 because of nearly 100% observed and predicted mortalities.

In Figure 2.5, where Zn and Cd concentrations were held constant and Ni was varied across a gradient, the lowest constant-concentration of Zn (3.4 μM Zn) initially displayed the most considerable less-than-additive effect at very low Ni concentrations. This result is in agreement with prior evidence of a less-than-additive interaction between Zn and Cd [13] that is evident even at fairly low Zn concentrations. At intermediate and high constant concentrations of Zn (16.8 and 33.8 μM, respectively), the toxicity was dominantly additive. This may be partially due to a 'limit of detection' because the concentrations of Zn that were tested exceeded the EC50 of Zn (approximately 12 μM (Table 2.1)). As a result, I observed that multiple competing less-than-additive mechanisms did not combine to form a more-than-additive interaction.

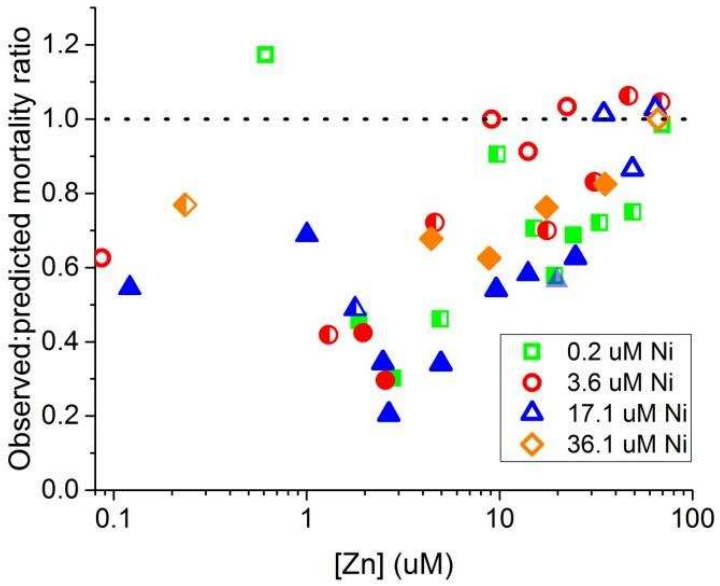

Figure 2.4: Additivity of toxicity of Cd-Ni-Zn mixtures that contained constant concentrations of Cd (0.9 μM in all exposure waters) and Ni (concentrations in the 4 separate sets of tests shown in the legend) while Zn was varied along a concentration gradient within each of the 4 sets of tests. Additivity of toxicity data was evaluated by the ratio of observed mortality to predicted independent action mortality, with each data point showing the average of duplicate runs of each combination of metal concentrations. Closed symbols = significantly non-additive toxicity ($p \leq 0.05$) in both replicate trials; open symbols = not significantly non-additive toxicity in both replicate trials; half-filled symbols = significant non-additive toxicity in 1 of the 2 replicate trials. Ratios less than 1 indicate less-than-additive toxicity; ratios greater than 1 indicate more-than-additive toxicity.

2.4.4 Synthesis

In my earlier work on binary mixtures when using the same metal (Ni) in binary mixtures with a variety of other metals (Cd, Cu, and Zn), I noted that the range of additive and non-additive toxicity demonstrates why a predictive model that can account for the various chemical interactions of metals with each other in a mixture, with various components of the exposure water (e.g., pH, alkalinity, major cations, and DOC), and with biotic ligands would help to improve water quality criteria/guidelines to incorporate metal mixtures instead of regulating on a metal-by-metal basis [13]. The same conclusions can be drawn from the results of my ternary mixture work, in that the additivity/non-additivity of the mixture toxicity differed considerably when two of the metals (Ni, Cd) were held constant but the concentration and identity of the third

metal were varied. Additional coordinated datasets with complete water chemistry and acute (for USA regulations) and/or chronic (for European regulations) toxicity data for these and other metal mixtures will be needed to improve the mechanistic basis for predictive models.

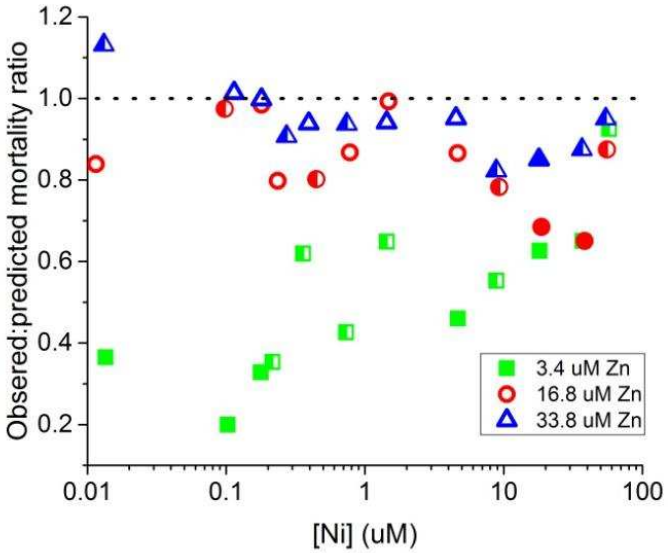

Figure 2.5: Additivity of toxicity of Cd-Ni-Zn mixtures that contained constant concentrations of Cd (0.9 μM in all exposure waters) and Zn (concentrations in the 3 separate sets of tests shown in the legend) while Ni was varied along a concentration gradient within each of the 3 sets of tests. Additivity of toxicity data was evaluated by the ratio of observed mortality to predicted response-additive mortality, with each data point showing the average of duplicate runs of each combination of metal concentrations. Closed symbols = significantly non-additive toxicity ($p \leq 0.05$) in both replicate trials; open symbols = not significantly non-additive toxicity in both replicate trials; half-filled symbols = significant non-additive toxicity in 1 of the 2 replicate trials. Ratios less than 1 indicate less-than-additive toxicity; ratios greater than 1 indicate more-than-additive toxicity.

Similar results for ternary metal mixtures have been reported for mixtures including Cd, Cu, Ni, and Zn in which a range of less-than-additive, additive, or more-than-additive toxicity was observed (based on dissolved-metal concentrations), depending on the combinations of metals and their concentrations. In general, less-than-additive effects are some of the most commonly observed interactions in ternary metal mixtures involving Cd [8,12,22]. Xu et al [23] conducted toxicity tests on binary, ternary, and quaternary metal mixtures and also observed a less-than-additive toxicity in binary mixtures of Cd and Zn, but a non-interactive toxicity in all

ternary and quarternary mixtures. However, most of the mixture toxicity studies to date either did not have a robust set of binary mixture toxicity data with which to compare ternary results to, or the experiments were not organized in a way in which they could directly infer consistency or discrepancies between the binary and ternary mixture results.

Several factors could result in non-additivity of metal mixtures. The dissolved organic matter (DOM) used in these toxicity tests was in the form of fulvic acid. Fulvic acids consist of a variety of molecules containing functional groups including (but not limited to) aromatic rings with hydroxyl groups and carboxylic acids. At mid-range pH values (including those generally found in natural environments), the majority of the acidic functional groups will be deprotonated [24]. As a consequence of this, DOM is capable of acting as a 'sink' for positively charged ions and thus greatly influences the ions that remain as hydrated cations (M^{z+}) in solution. Therefore, DOM greatly influences the resulting toxicity of metals.

Furthermore, DOM is not the only ligand in natural waters that may influence the toxicity of a metal mixture. Anions such as carbonates, chlorides, sulfates and hydroxides tend to form complexes with metals in a solution [2]. After being complexed, the reactivity of that metal ion and the bioavailability of the metal tend to be diminished if not entirely eliminated [25]. Cations that are naturally abundant (e.g., H^+, Ca^{2+}, Mg^{2+}, Na^+) also can compete directly with the metal ions for binding to the biotic ligands (BLs; e.g. fish gills). Not only will natural ligands (DOM, anions) act as a sink for all these cations, but the major cations may also compete with metals for binding to the sites of action directly on the BL. As the complexation of major cations to natural ligands increases, those ligands become less efficient at complexing metals, thereby potentially increasing the toxicity of that solution. On the other hand, increased complexation of major cations to the BL decreases the amount of metal that is able to interact at a target site, which will

decrease the toxicity. Some or all of these interactions occur for any given mixture site, making it difficult to predict toxicity of metal mixtures unless all geological, chemical, and biological parameters are adequately accounted for.

Although testing binary, ternary, and quarternary mixtures is a start, metal combinations in natural systems often contain more than just a few metals and thus have almost unlimited possible combinations of metals and their concentrations. Additionally, the types and extents of interactions among metals might vary as water chemistry varies. Because testing a large number of combinations of metals and their concentrations in a wide variety of water chemistries would be expensive and time consuming, many combinations of metals and water chemistry likely will never be evaluated. To this end, incorporating mechanistic models that include both geochemical interactions among metals and differences between the bioavailability of metals in mixtures could offer a solution.

2.5 Conclusions

Because the understanding of mechanisms underlying metal-mixture toxicity and the ability to predict those effects are still being developed, it is important to test interactions in mixtures that contain a relatively small number of metals before progressing to mixtures containing many metals. Based on the results of this study of ternary-metal mixtures, and similar to studies with binary-metal mixtures, I conclude that more-than-additive, less-than-additive, and additive toxicities can all be observed in metal mixtures containing more than two metals, depending on the metals in the mixture and their concentrations. Because none of the results were inconsistent with the interactions expected from results of binary-metal mixtures, predictive models that can accurately describe a binary-metal system may be sufficient for predicting the toxicity of mixtures that contain more than 2 metals. And because the results were consistent with

metal-metal competition for binding to dissolved ligands and/or biotic ligands, bioavailability-based models that incorporate these types of biogeoechemical interactions might be useful for predicting multiple-metal toxicity.

2.6 Acknowledgements

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2.7 References cited

1. Allen HE, Luther GW, Garrison W. 1997. *Metals in Surface Waters*. Ann Arbor Press, Chelsea, MI, USA.

2. Morel FMM, Hering JG. 1993. *Principles of Aquatic Chemistry*. John Wiley & Sons, Inc., New York.

3. Campbell P. 1995. Interactions between trace metals and aquatic organisms: A critique of the free-ion activity model. *Metal Speciation and Bioavailability in Aquatic Systems* 3.

4. Meyer JS, Farley KJ, Garman ER. 2015. Metal Mixtures Modeling Evaluation Project: 1. Background. *Environmental Toxicology and Chemistry* 34:726-740.

5. Jonker MJ, Svendsen C, Bedaux JJ, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environmental Toxicology and Chemistry* 24:2701-2713.

6. Niyogi S, Wood CM. 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environmental Science and Technology* 38:6177-6192.

7. Könemann W, Pieters M. 1996. Confusion of concepts in mixture toxicology. *Food and Chemical Toxicology* 34:1025-1031.

8. Meyer JS, Ranville JF, Pontash M, Gorsuch JW, Adams WJ. 2015. Acute toxicity of binary and ternary mixtures of Cd, Cu, and Zn to *Daphnia magna*. *Environmental Toxicology and Chemistry* 34:799-808.

9. Lynch NR, Hoang TC, O'Brien TE. 2015. Acute toxicity of binary-metal mixtures of copper, zinc, and nickel to *Pimephales promelas*: Evidence of more-than-additive effect. *Environmental Toxicology and Chemistry* 35:446-457.

10. Martin HL, Svendsen C, Lister LJ, Gomez-Eyles JL, Spurgeon DJ. 2009. Measurement and modeling of the toxicity of binary mixtures in the nematode *Caenorhabditis elegans*—a test of independent action. *Environmental Toxicology and Chemistry* 28:97-104.

11. Norwood W, Borgmann U, Dixon D, Wallace A. 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. *Human and Ecological Risk Assessment* 9:795-811.

12. Vijver MG, Elliott EG, Peijnenburg WJ, de Snoo GR. 2011. Response predictions for organisms water-exposed to metal mixtures: A meta-analysis. *Environmental Toxicology and Chemistry* 30:1482-1487.

13. Traudt EM, Ranville JF, Smith SA, Meyer JS. 2015. A test of the additivity of acute toxicity of binary-metal mixtures of Ni with Cd, Cu, and Zn to *Daphnia magna*, using the inflection point of the concentration-response curves. *Environmental Toxicology and Chemistry*: Accepted 16 Dec 2015.

14. USEPA. 2002. *Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms*, 5th edition, EPA-821-R-02-012. U.S. Environmental Protection Agency, Washington, DC.

15. Thurman EM. 1985. *Organic Geochemistry of Natural Waters*, volume 2. Springer.

16. Ma H, Kim SD, Cha DK, Allen HE. 1999. Effect of kinetics of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* 18:828-837.

17. American Public Health Association, American Water Works Association, Water Environment Federation. 2012. *Standard Methods for the Examination of Water and Wastewater,* 22nd ed. American Water Works Association, Denver, CO, USA.

18. Zeisler R, Murphy KE, Becker DA, Davis WC, Kelly WR, Long SE, Sieber JR. 2006. Standard Reference Materials® (SRMs) for measurement of inorganic environmental contaminants. *Analytical and Bioanalytical Chemistry* 386:1137-1151.

19. Greco WR, Bravo G, Parsons JC. 1995. The search for synergy: A critical review from a response surface perspective. *Pharmacological Reviews* 47:331-385.

20. Gennings C, Carter W, Carchman R, Teuschler L, Simmons J, Carney E. 2005. A unifying concept for assessing toxicological interactions: Changes in slope. *Toxicological Sciences* 88:287-297.

21. Traudt EM, Ranville JF, Meyer JS. 2016. Effect of age on acute toxicity of Cd, Cu, Ni, and Zn in individual-metal exposures to *Daphnia magna* neonates. *Environmental Toxicology and Chemistry*. Accepted May 23, 2016.

22. Nys C, Van Regenmortel T, Janssen CR, Blust R, Smolders E, De Schamphelaere KAC. Submitted 2016. Comparison of chronic mixture toxicity of Ni-Zn-Cu and Ni-Zn-Cu-Cd mixtures between *Ceriodaphnia dubia* and *Pseudokirchneriella subcapitata*. *Environmental Toxicology and Chemistry*.

23. Xu X, Li Y, Wang Y, Wang Y. 2011. Assessment of toxic interactions of heavy metals in multi-component mixtures using sea urchin embryo-larval bioassay. *Toxicology in Vitro* 25:294-300.

24. Leenheer JA, Croué J-P. 2003. Characterizing aquatic dissolved organic matter: Understanding the unknown structures is key to better treatment of drinking water. *Environmental Science and Technology* 37:18A-26A.

25. Allen HE, Hall RH, Brisbin TD. 1980. Metal speciation. Effects on aquatic toxicity. *Environmental Science and Technology* 14:441-443.

CHAPTER 3

EFFECT OF AGE ON ACUTE TOXICITY OF CD, CU, NI, AND ZN IN INDIVIDUAL-METAL EXPOSURES TO *DAPHNIA MAGNA* NEONATES

Modified slightly from a paper accepted in *Environmental Toxicology and Chemistry*[1]

Elizabeth M. Traudt[2,3], James F. Ranville[4], Joseph S. Meyer[5]

3.1 Abstract

In previous studies in our laboratory, variability was high among replicate acute Cd *Daphnia magna* lethality tests (e.g., >10-fold range of median effects concentrations [EC50s]), less among Zn tests, and relatively low for Cu and Ni tests. Although the U.S. Environmental Protection Agency's (USEPA's) protocol includes starting toxicity tests with neonates less than 24 h old, I hypothesized that age-related differences in sensitivity to metals might occur even within that relatively narrow age range. *Daphnia magna* neonates were collected during three 4-h age windows (0-4, 10-14, and 20-24 h old) and immediately exposed to each of the four metals for 48 h using the standard USEPA protocol. In repeated sets of tests during different weeks, the Cd EC50 of the youngest neonates was approximately 10-fold greater than the EC50 of the oldest neonates (i.e., Cd was less toxic to the youngest neonates), and the EC50 of neonates aged 10-14 h was intermediate. Age-related differences were negligible in Cu, Ni and Zn tests. Therefore, variability in toxicity of Cd may partly be caused by temporal variability in neonate age at the start of toxicity tests. Decreasing the age range of *D. magna* used in toxicity tests could help to improve the accuracy and precision of toxicity models, particularly for metal mixtures.

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2. Graduate student, Department of Chemistry and Geochemistry, Colorado School of Mines
3. Primary researcher and author.
4. Associate Professor, Department of Chemistry and Geochemistry, Colorado School of Mines
5. Applied Limnology Professionals LLC; and Affiliated Faculty Member, Colorado School of Mines

3.2 Introduction

The cladoceran *Daphnia magna* is one of the most commonly-used invertebrate species for evaluating the toxicity of chemicals to freshwater organisms [1,2]. Although toxicity tests have been conducted with *D. magna* for over half a century [3], methodology for evaluating acute toxicity with these organisms was not standardized until the mid-1980s [4,5]. To control for age-related differences in sensitivity to toxicants, the United Sates Environmental Protection Agency (USEPA) and the Organization for Economic Co-operation and Development (OECD) stipulate the age of *D. magna* should be less than or equal to 24 h at the start of acute toxicity tests [6,7]. However, based on reported variability in the toxicity of some metals to *D. magna* neonates [8,9], I hypothesized even the relatively narrow 24-h age window might introduce considerable variability in acute lethality tests with some toxicants.

The typical life span of a *D. magna* is approximately 2 months, with the organisms reaching reproductive maturity in the first 5 to 10 d [10]. *D. magna* neonates grow 0.126 to 0.388 mm d^{-1} [11] and shed their first carapace [10] within 24 h after being released from their mother's brood chamber. As a result of this rapid life cycle, the variability in toxicity to contaminants may be more apparent if the organisms' sensitivity is highly age-dependent. Previous authors have reported that changes in age-dependent sensitivity of <1- to 7-d-old *D. magna* were often not statistically significant [12], and adult daphnids tend to be less sensitive than younger organisms [2]. However, those studies were limited in their temporal resolution, which prevented their ability to evaluate changes in neonate sensitivity during the first 24 h after release from the brood chamber.

Daphnia magna neonates were previously exposed to binary and ternary mixtures of Cd, Cu, Ni, and Zn in acute lethality tests in the authors' laboratory [8,9]. When individual metals

were tested concurrently with the mixtures at concentrations ranging from non-lethal to lethal, variability of mortality was very high in the Cd-only tests (e.g., >10-fold range of median effects concentrations [EC50 values]), less in Zn-only tests, and relatively low in Cu-only and Ni-only tests. This same pattern of variability was evident in the binary and ternary mixtures, which makes predicting mixture toxicity from variable individual-metal results even more challenging than if one only had to account for physical-chemical metal-metal interactions [13]. In the current study, I demonstrate that the among-test variability was caused at least in part by large age-related differences in sensitivity of *D. magna* neonates to some metals during their first 24 h post-birth. Therefore, in addition to the influence of test design [14], early post-natal age of daphnids at the start of single-metal and metal-mixture toxicity tests can influence their results and interpretation.

3.3 Experimental

3.3.1 Test organisms

Daphnia magna were purchased from Aquatic BioSystems, Inc. in Fort Collins, Colorado. Gravid *D. magna* females were shipped to the Colorado School of Mines in Golden, Colorado, in USEPA moderately hard reconstituted water (MHRW) [6] with the green alga *Pseudokirchneriella subcapitata* as food (approximately 3×10^7 cells/L or 3.8×10^5 cells/*D. magna* adult). Upon arrival, the organisms were transferred to a skimmer tank, which allowed for the removal of neonates that had emerged during shipping. The gravid *D. magna* were maintained in skimmer tanks containing freshly-prepared MHRW and *P. subcapitata*. All neonates that emerged during the next 4 h were collected and either (1) immediately placed into exposure water to begin a toxicity test or (2) held in a separate tank of MHRW for either 10 or 20 h before being placed into exposure water. Neonates that were held in the separate tank for an additional amount

of time were fed *P. subcapitata* (approximately 3.6×10^7 cells/L or 4.5×10^5 cells/neonate) 4 h before being placed in the exposure waters, and were not fed during the exposure.

3.3.2 Exposure water

The exposure water in the toxicity tests was MHRW to which 3 mg DOC/L was added as Suwannee River fulvic acid (International Humic Substances Society; http://www.humicsubstances.org/). Metal salts [$Ni(NO_3)_2$, $Cd(NO_3)_2$, $Cu(NO_3)_2$, $Zn(NO_3)_2$ (Regaent Grade, Mallinckrodt Chemical (Ni) and Baker Chemical Company (Cd, Cu, Zn))] were added to that exposure matrix. Exposure solutions were prepared 24 to 36 h before the start of a toxicity test, to allow equilibration of the metals with the dissolved organic carbon (DOC) as recommended by Ma et al. [15].

3.3.3 Toxicity tests

The toxicity of individual metals was determined in 48-h static, non-renewal lethality tests, following USEPA-recommended procedures [6]. The toxicity tests comprised a series of 6 metal concentrations in a gradient designed to produce mortalities ranging from 0 to 100%. In all tests, each metal concentration was tested in 4 replicate chambers (50-mL polycarbonate cups), each containing 25 ml of exposure water and 5 organisms (i.e., a total of 20 organisms were exposed to each concentration in the metal gradient). The number of immobilized organisms was recorded at 24 and 48 h, with immobilization as the indicator of mortality [6].

Individual-metal toxicity tests that had not been controlled for age were conducted from September 2012 through November 2013 in a previous study [8]. Age-controlled individual-metal tests were conducted from September through December 2014. All tests were conducted in incubators (VWR International) at 20±2°C, with a 16h/8h light-dark cycle.

3.3.4 Chemical analyses

At 0 and 48 h, temperature and dissolved oxygen were measured using a YSI 55 probe (YSI Incorporated), and pH was measured using an Orion ROSS electrode and Orion 2 STAR meter (Thermo Fisher Scientific) calibrated with pH 4, 7, and 10 buffers. Alkalinity was analyzed in the MHRW that was used to prepare all the exposure waters by titration with H_2SO_4 to the bromo-cresol green/methyl red endpoint [16]. Unfiltered water samples for analysis of total organic carbon (TOC) concentration were collected from the control and the highest metal concentration at the beginning of each test and preserved by addition of H_3PO_4 (reagent grade, EM Science) to pH <2. Total organic carbon concentrations were analyzed by UV-catalyzed persulfate oxidation using a Sievers 900 TOC Analyzer (GE Analytical Instruments).

At the beginning of each test, unfiltered water from each treatment in the metal-concentration series was acidified to pH <2 with concentrated Optima HNO_3 (Mallinckrodt Chemical) and then submitted for elemental analysis. The exposure waters were not filtered before analysis because preliminary tests demonstrated that commercial filters can either sorb metals from, or leach metals into, initial volumes of water that are passed through the membranes [9]. Consequently, the small volumes (<100 ml) of exposure waters used in these *D. magna* toxicity tests were not sufficient to adequately rinse the filters or exceed their sorption capacity. Because additional tests demonstrated that the metals added to MHRW were >90% dissolved [9], the total-metal concentrations were assumed to closely approximate the dissolved-metal concentrations.

All controls and exposure concentrations were analyzed for total concentrations of metals (including Cd, Cu, Ni, and Zn), major inorganic cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), and sulfur using an Optima 5300 ICP-OES inductively coupled plasma optical emission spectrometer

(PerkinElmer). Sulfate concentrations in all controls and metal exposures were calculated by assuming all the sulfur measured by ICP-OES was present as SO_4^{2-}. Chloride concentrations were calculated by assuming the molar Cl^- concentration equaled the measured molar K concentration (because the only Cl^- in the MHRW recipe was added as KCl; [6]).

Quality assurance/quality control (QA/QC) procedures for the toxicity tests that were controlled for age (September-December 2014) were the same as in the individual-metal toxicity tests that had not been controlled for age (September 2012 - November 2013) [8]. The pH probe was calibrated daily using certified buffers at pH 4, 7, and 10. In all ICP-OES analytical runs, a Sc internal-calibration standard was continuously introduced into the plasma along with each sample, and samples were analyzed in triplicate. Quality assurance/quality control samples included deionized water blanks (Barnstead Nanopure system, Thermo Fisher Scientific) that contained trace-metal-grade HNO_3 (Thermo Fisher Scientific), and certified continuing concentration verification (CCV) standards. The QA/QC samples were analyzed immediately after instrument calibration, after every 10 samples, and at the end of each set of samples. Additionally, NIST certified standard reference materials 1640a and 1643e [17] were analyzed before and at the end of each set of samples. All samples were reanalyzed in any analytical run in which acceptable QA/QC results were not obtained. Unacceptable QA/QC results could include: deviations of the internal Sc standard greater than 20% from the known concentration, deviations of the CCV samples greater than 10% from the known concentrations, or relative standard deviations (RSDs) of triplicate analyses of a sample greater than 10%. The ranges of instrument detection limits for the metals, major cations, and sulfur during the age-controlled toxicity tests were: 4.2-7.0 μg Ca/L, 0.1-0.3 μg Cd/L, 0.3-0.4 μg Cu/L, 18-40 μg K/L (equivalent to 16-36 μg

Cl/L calculated), 0.1-0.4 μg Mg/L, 6.0-7.0 μg Na/L, 1.8-6.5 μg S/L (equivalent to 5.4-19.5 μg SO_4/L calculated), 0.1-0.4 μg Ni/L, and 0.3-0.6 μg Zn/L.

3.3.5 Data analyses

Non-overlap of 84% confidence intervals was used to infer significant differences between two means at the 95% confidence level, as recommended by statisticians [18,19]. In this study, the EC50 values and the slopes of the associated concentration-response curves were calculated using the logit-regression method in OriginPro 9.1 (OriginLab Corporation). The 84% confidence intervals on the averages of several EC50 values or slopes were calculated at n-1 degrees of freedom using Microsoft Excel.

3.4 Results and Discussion

3.4.1 General variability

In the individual-metal toxicity tests started with 0- to 24-h-old neonates (i.e., using the standard USEPA protocol for neonate age [6]), Cu EC50 values were the least variable (i.e., lowest CV) of the 4 metals tested, followed by Zn, Ni, and Cd in sequence of increasing variability (Table 3.1). Similar results have been reported elsewhere for Cd, Cu, and Zn toxicity [9], supporting this observation that the acute toxicity of Cd to 0- to 24-h-old *D. magna* neonates is highly variable. The intermediate variability of Zn toxicity to *D. magna* was similar to the ranking of toxicity variability among Cd, Cu, and Zn reported by Meyer et al. [9] in the same exposure-water recipe.

The logit-regression slopes differed significantly among all 4 metals (Table 3.1). In this analysis, Cd had the shallowest slope and Cu had the steepest slope, with the slopes of Ni and Zn falling in the intermediate range (Table 3.1). The steepness of the concentration-response curves

was inversely related to the CV of the EC50s of the metals (average slope = $0.3024 \cdot CV^{-1.686}$; R^2 = 0.815); however, this might have been a statistical artifact whereby more-disperse data generally have a shallower least-squares regression slope than less-disperse data.

Table 3.1: Individual-metal toxicity data for Cd, Cu, Ni, and Zn in *Daphnia magna* neonates 0-24h old at initial exposure. Data includes the average 48-h median effect concentration (EC50) and relevant parameters for immobilization of neonates exposed to Cd, Cu, Ni, or Zn in repeated individual-metal static, non-renewal lethality tests conducted with 0- to 24-h-old neonates from September 2012 through November 2013. Modified from Traudt et al. [8].

Metal	Average EC50 (mg/L)[a] (84% CI)[b]	Range (mg/L)	SD[c] (mg/L)	CV[d]	Slope[a,e] (84% CI)[b]	n[f]
Cd	0.054 A (0.039-0.069)	0.002-0.125	0.036	0.661	0.683 A (0.642-0.724)	13
Cu	0.100 B (0.093-0.108)	0.074-0.128	0.019	0.192	6.654 B (6.372-6.936)	15
Ni	1.633 C (1.510-1.756)	0.606-2.45	0.475	0.291	2.933 C (2.846-3.020)	31
Zn	0.928 D (0.773-1.083)	0.604-1.28	0.256	0.276	1.428 D (1.336-1.521)	7

[a] Different capital letters within a column indicate statistically significant differences among the metals.
[b] CI = confidence interval.
[c] SD = standard deviation.
[d] CV = coefficient of variation = SD/average.
[e] Slope of logit(mortality) versus log(concentration) curve.
[f] n = number of individual-metal toxicity tests conducted.

3.4.2 Age-related differences in sensitivity of neonates

Within the first 24 h after a daphnid was released from the brood chamber, its sensitivity to some metals was highly age-dependent. Similar to the results observed for the general variability of Cd, in which the EC50 values spanned over an order of magnitude for neonates that started the tests at 0 to 24 h old, the average EC50 values for neonates that started the age-controlled Cd toxicity tests at 0 to 4, 10 to 14, and 20 to 24 h old ranged from 0.020 to 0.141 mg Cd/L (Figure 3.1 and Table 3.2). The youngest neonates were the most tolerant of Cd (average EC50 [84% CI]: 0.141 mg Cd/L [0.114-0.168], n = 3 tests), while the oldest neonates experienced statistically significant greater toxicity from the same exposures (average EC50 [84% CI]: 0.020 mg Cd/L [0.004-0.035], n = 3 tests). Consistent with these results, the EC50 in the single toxicity

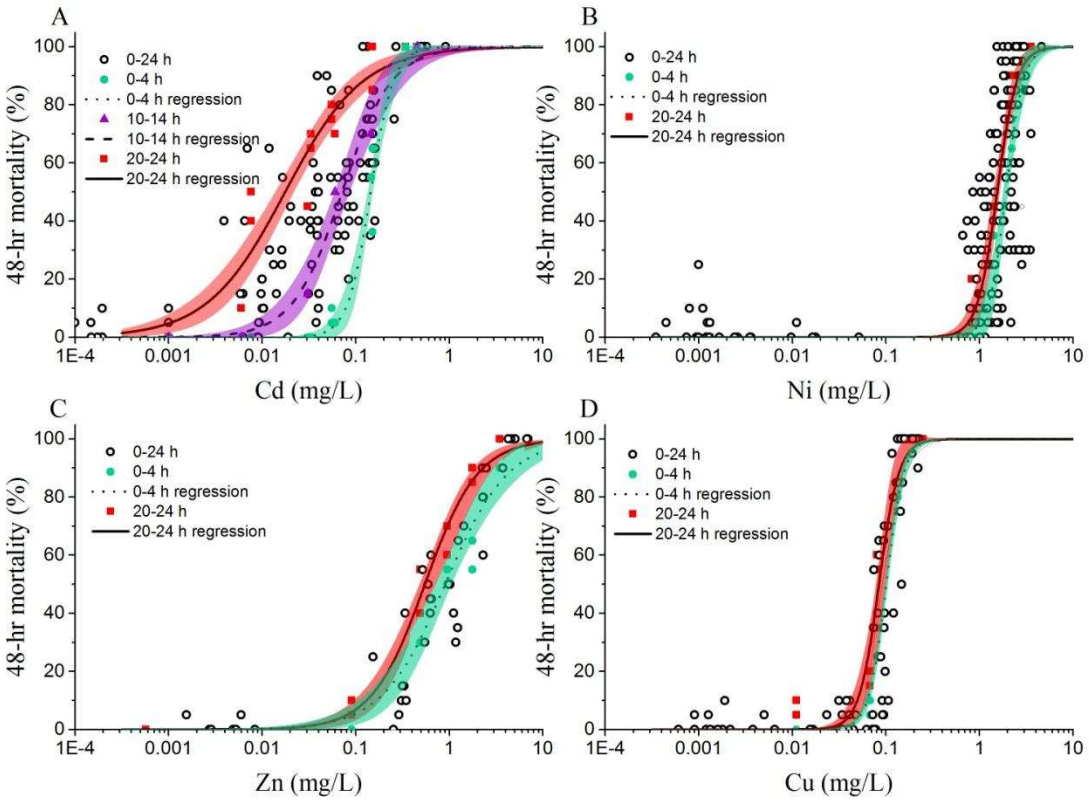

Figure 3.1: Concentration-response curves for *Daphnia magna* neonates exposed to (A) Cd, (B) Ni, (C) Zn, or (D) Cu as a function of age at the start of 48-h static, non-renewal lethality tests. "0-24 h" refers to individual-metal toxicity tests conducted from September 2012 through November 2013, in which the age range of the neonates at the start of the test was 0-24 h. In age-controlled trials with all 4 metals, two 4-h age windows of neonates (0-4 h and 20-24 h post-birth) were used to start the toxicity tests. A third 4-h age window (10-14 h post-birth) was used in Cd toxicity tests. The regression curves were fitted to the composite data set of each age-controlled group, and the shaded region represents the 84% confidence interval [18].

Table 3.2: Individual-metal toxicity data for Cd, Cu, Ni, and Zn at controlled age ranges of *Daphnia magna* neonates. Data includes the average 48-h median effect concentration (EC50) and other relevant parameters for immobilization of neonates exposed to Cd, Cu, Ni, or Zn in repeated individual-metal static, non-renewal lethality tests designed to evaluate the sensitivity of the organisms within several different 4-h age windows during the first 24 h post-birth. The tests were conducted from September through December 2014.

Metal	Organism age at start of toxicity test (h)	Average EC50 (mg/L)[a] (84% CI)[b]	Range (mg/L)	SD[c] (mg/L)	CV[d]	Slope[e] (84% CI)[b]	n[f]
Cd	0-4	0.141 (0.114-0.168)*	0.122-0.164	0.021	0.150	4.613 (1.94-7.29)*	3
	10-14	0.071 (N/A)	N/A	N/A	N/A	1.640 (N/A)	1
	20-24	0.020 (0.004-0.035)*	0.010-0.034	0.012	0.619	1.222 (0.65-1.80)*	3
Cu	0-4	0.101 (0.101-0.102)*	0.101-0.101	0.00017	0.0020	4.715 (3.49-5.94)	2
	20-24	0.082 (0.071-0.093)*	0.079-0.085	0.0040	0.049	6.820 (0-20.6)	2
Ni	0-4	2.065 (2.043-2.087)*	2.059-2.071	0.0080	0.0039	6.520 (0-14.5)	2
	20-24	1.560 (1.251-1.868)*	1.481-1.639	0.112	0.072	4.470 (3.07-5.87)	2
Zn	0-4	0.904 (0.767-1.040)	0.869-0.939	0.050	0.055	1.244 (0.48-2.01)	2
	20-24	0.553 (0.132-0.975)	0.445-0.662	0.153	0.277	1.637 (1.48-1.79)	2

[a] Asterisks (*) indicate statistically different EC50 values or slopes (as determined by non-overlapping confidence intervals) between 0-4 h and 20-24 h tests for each metal.
[b] CI = confidence interval.
[c] SD = standard deviation.
[d] CV = coefficient of variation = SD/average.
[e] Slope of logit(mortality) versus log(concentration) curve.
[f] n = number of toxicity tests conducted.

test conducted with the intermediate-age group (0.071 mg Cd/L) was intermediate between the other EC50 values.

Along with the gradient in Cd EC50 values, the steepness of and variability around the Cd concentration-response curves were also age-dependent (Table 3.2). The youngest neonates, which had the highest average Cd EC50, also had the steepest toxicity curves and lowest variability. In contrast, the oldest neonates had a 3.8-fold shallower concentration-response curve (as indicated by the slope) and 4-fold greater variability around the average Cd EC50 (as indicated by the CV) within a 4-h age window. These results may suggest that the daphnids were released from the parent daphnid with a physiological defense mechanism [20]; however, such a mechanism might have waned as they aged and the neonates became susceptible to a variety of external factors that may have led to increased variability in the susceptibility to some metals. Alternatively, Cd (and to some extent Zn, see below) might have interfered with important age-dependent developmental/physiological processes that occur between 48 and 72 h post-birth (i.e., after a test started with 0- to 4-h-old daphnids had ended, when the daphnids were 48-52 h old; but before a test started with 20- to 24-h-old daphnids had ended, when the daphnids were 68-72 h old). However, as discussed above for the general differences among the 4 metals, the shallower slope for the 20- to 24-h-old organisms could have been a statistical artifact resulting from increased variability of toxicity responses in that age class.

In tests started with 0- to 24-h-old neonates, the Zn EC50 values ranged from 0.3 to 2.0 mg Zn/L. The among-age variability with Zn was considerably smaller than with Cd (i.e., the average Zn EC50 for 0- to 4-h-old neonates was only 1.6 times the average Zn EC50 for 20- to 24-h-old neonates), and the average Zn EC50 values did not differ significantly between the youngest and oldest neonates (Figure 3.1 and Table 3.2). Once again, the older neonates exhibited

greater variability around the EC50 than the younger organisms; however, in contrast to Cd, the concentration-response curves for the older neonates were slightly steeper (but not significantly different) than for the younger neonates. This difference between Cd and Zn could indicate different modes of toxicity of the two metals at that early life stage in *D. magna*.

Sensitivity to Cu and Ni also differed significantly as a function of age (Figure 3.1 and Table 3.2). However, the average EC50 of the 0- to 4-h-old neonates was only 1.2 to 1.3 times the average EC50 of the 20- to 24-h-old neonates for Cu and Ni, compared to 7-fold and 1.6-fold differences for Cd and Zn, respectively. Therefore, the age-related differences in EC50 values were small for Cu and Ni. Additionally, the average slopes of the concentration-response curves for either age group were similar for Cu and Ni and did not differ significantly between the two age groups for either of those 2 metals.

3.4.3 Age-related differences in 24-h mortality

Following the trend in total mortality at 48-h exposure, the older organisms were more likely than the younger organisms to become immobilized during the initial 24 h of exposure to some of the metals. Again, Cd exhibited the greatest difference in sensitivity among age groups, with only approximately 20% mortality after 24 h in the highest exposure concentration (~400 µg Cd/L) in tests started with 0- to 4-h-old neonates, while tests started with 20- to 24-h-old neonates had approximately the same mortality percentage in the lowest exposure concentration (~8 µg Cd/L) (Figure 3.2).

After Cd, Zn exhibited the next greatest difference in immobilization between 0- to 4-h-old and 20- to 24-h-old neonates at 24 h of exposure (Figure 3.3). In the tests started with 20- to 24-h-old neonates, at least 70% of the total number of deaths in each exposure concentration

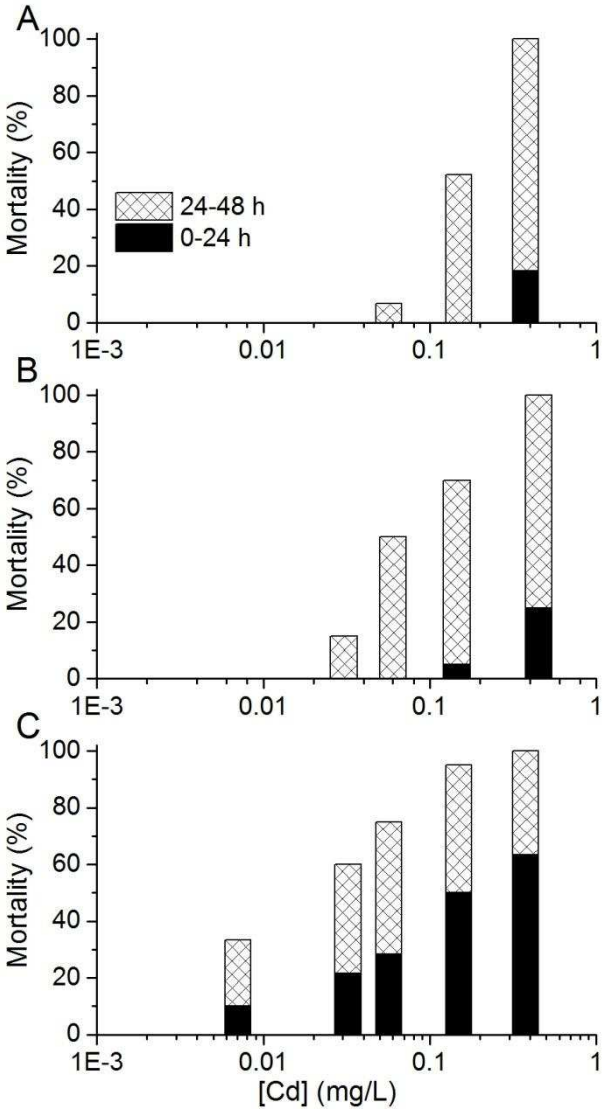

Figure 3.2: Cd mortality percentages during 0-24 h and 24-48 h intervals. In 48-h static, non-renewal lethality tests with *Daphnia magna* neonates exposed to Cd. Three 4-h age windows of neonates were used to start the toxicity tests: (A) 0-4 h, (B) 10-14 h, and (C) 20-24 h post-birth. For age groups A and C, the averages of 3 tests are shown; only 1 test was conducted for age group B.

occurred in the first 24 h of exposure, while much lower percentages of the total mortality occurred in the first 24 h of exposure in the tests started with 0- to 4-h old neonates.

With Ni, the tests started with 0- to 4-h-old neonates had only slightly less mortality in the first 24 h of exposure than the tests started with 20- to 24-h-old neonates (Figure 3.4). Even smaller differences between tests started with the 2 different age groups occurred with Cu (Figure 3.5).

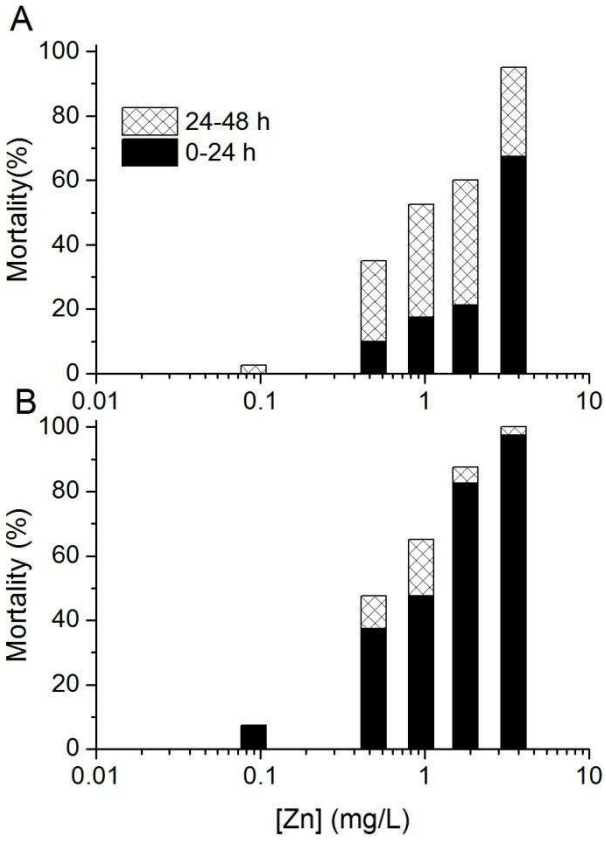

Figure 3.3: Zn mortality percentages during 0-24 h and 24-48 h intervals. In 48-h static, non-renewal lethality tests with *Daphnia magna* neonates exposed to Zn. Two 4-h age windows of neonates were used to start the toxicity tests: (A) 0-4 h and (B) 20-24 h post-birth. The averages of 2 tests are shown for each age group.

3.4.4 Synthesis

These results are consistent with Nebeker et al. [12], who exposed various age groups of *D. magna* to Cd and Cu in sediments to determine if the sensitivities changed as a function of the

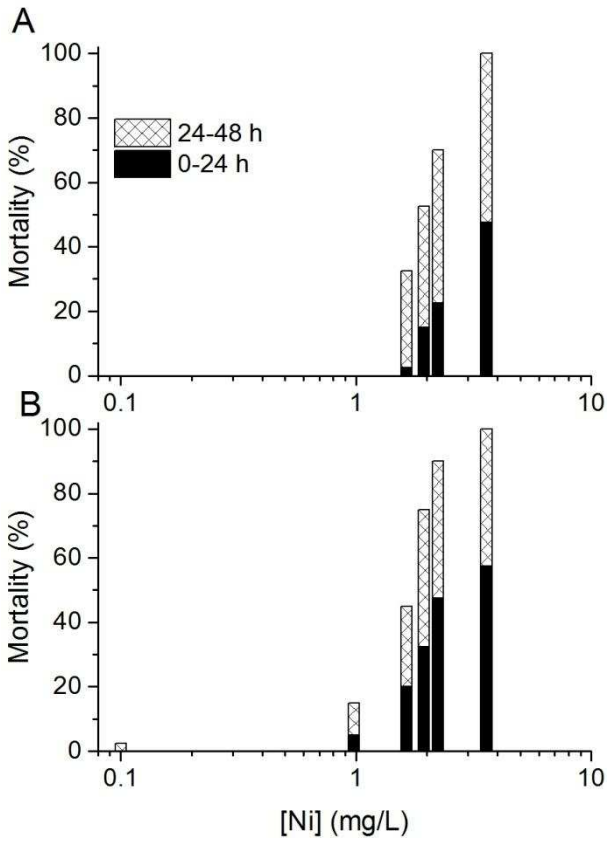

Figure 3.4: Ni mortality percentages during 0-24 h and 24-48 h intervals. In 48-h static, non-renewal lethality tests with *Daphnia magna* neonates exposed to Ni. Two 4-h age windows of neonates were used to start the toxicity tests: (A) 0-4 h and (B) 20-24 h post-birth. The averages of 2 tests are shown for each age group.

organism's age. Those age groups ranged from less than 4 h old up to 6 d old, with intervals relevant to the current study at <4-h, <24-h, 1-d and 2-d. Although their younger daphnids qualitatively appeared to be less sensitive to Cd than the older daphnids, Nebeker et al. [12] concluded that, at the 95% confidence level, the EC50 values for Cd and Cu did not often differ significantly among the age groups. However, those authors did not report the standard deviations or 95% confidence intervals on the average EC50 values, thus precluding the more appropriate comparison of 84% confidence intervals to infer statistically significant differences at the 95% confidence level [18]. In another study, 1-d-old *D. magna* were significantly more sensitive to dichloroaniline than were 7-d-old *D. magna*, but the two age groups had the same sensitivity to

trichloroethane, dieldrin and pentachlorophenol regardless of the organism's age [2]. That author did not test for age-related differences within the 0- to 24-h-old age group.

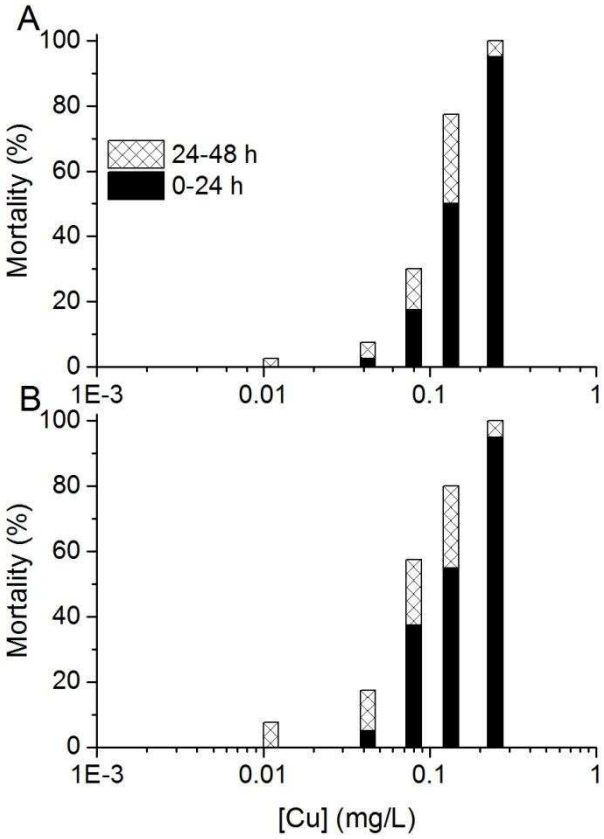

Figure 3.5: Cu mortality percentages during 0-24 h and 24-48 h intervals. In 48-h static, non-renewal lethality tests with *Daphnia magna* neonates exposed to Cu. Two 4-h age windows of neonates were used to start the toxicity tests: (A) 0-4 h and (B) 20-24 h post-birth. The averages of 2 tests are shown for each age group.

De Laender et. al. [14] concluded that non-simultaneous testing of individual chemicals and chemical mixtures can greatly reduce the confidence of accurately classifying a mixture as additive or non-additive. The age-dependent sensitivity to some metals within the first 24 h post-birth that we have demonstrated in *D. magna* contributes to the variability introduced by non-simultaneous testing and thus should be considered when interpreting results of metal-mixture toxicity tests.

Several mechanisms might explain age-related differences in sensitivity to some metals during the first 24 h post-birth. For example, 0- to 4-h-old neonates might retain a yolk that provides nutrition [20] that is mostly consumed or no longer available in 20- to 24-h-old neonates, thus increasing the susceptibility of the 20- to 24-h-old neonates to Cd and Zn. This difference would not be related to general nutrition, because the 20- to 24-h-old neonates in the current study were fed immediately before their toxicity tests were begun and were treated identically to the 20- to 24-h-old neonates in the Cu and Ni tests, in which minimal age-related toxicity differences occurred. Instead, this explanation depends on a yet-to-be-identified attribute of the egg yolk that would preferentially protect against specific metals like Cd. Another possible explanation is that Cd might interfere with important age-dependent developmental or physiological processes, such as the molting of the carapace, that occur between 24 and 72 h post-birth (i.e., after the first 24 h of exposure ends in toxicity tests begun with 0- to 4-h-old daphnids). In these 48-h toxicity tests, the oldest organisms (i.e., those started at age 20- to 24-h-old) usually molted before the end of the tests, but the youngest organisms (i.e., those started at age 0- to 4-h-old) only rarely molted before the end of the tests.

3.5 Conclusions

The sensitivity of *D. magna* neonates to Cd (and to some extent Zn, Ni, and Cu) is highly variable even within their first 24 h post-birth. Although the physiological cause of the age-related differences in sensitivity to metals is not known, the consequences to the precision of toxicity tests involving these metals and the implications for predictive models of toxicity that use those results could be important. For some routine purposes, less-precise EC50 values might suffice; however, the imprecision inherent in testing neonates born during the 0- to 24-h age window recommended by the USEPA and OECD might introduce greater uncertainty to the determination and modeling of the toxicity of metal mixtures that contain Cd and Zn. Furthermore, in testing

mixtures for additivity, uncertainties in each individual-metal test may result in very large windows of uncertainty around the computed additive toxicity. Thus small, but real non-additive effects may be missed. Additionally, some other metals that have not yet been tested for age-related differences in sensitivity might also be of concern.

3.6 Acknowledgements

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3.7 References cited

1. Koivisto S. 1995. Is *Daphnia magna* an ecologically representative zooplankton species in toxicity tests? *Environmental Pollution* 90:263-267.

2. Adema D. 1978. *Daphnia magna* as a test animal in acute and chronic toxicity tests. *Hydrobiologia* 59:125-134.

3. Naumann E. 1934. Über die Anwendung von *Daphnia magna* Straus als Versuchstier zur experimentellen Klarlegung der Lebensverhältnisse im Wasser. *Internationale Revue der gesamten Hydrobiologie und Hydrographie* 31:421-431.

4. Peltier WH, Weber CI. 1985. *Methods for measuring the acute toxicity of effluents to freshwater and marine organisms*, 3rd edition, EPA/600/4-85/013. U.S. Environmental Protection Agency, Cincinnati, Ohio.

5. OECD. 1984. *Test No. 202: Daphnia sp. Acute Immobilisation Test*. OECD Publishing.

6. USEPA. 2002. *Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms*, 5th edition, EPA-821-R-02-012. U.S. Environmental Protection Agency, Washington, DC.

7 OECD. 2004. *Test No. 202: Daphnia sp. Acute Immobilisation Test*. OECD Publishing.

8. Traudt EM, Ranville JF, Smith SA, Meyer JS. 2015. A test of the additivity of acute toxicity of binary-metal mixtures of Ni with Cd, Cu, and Zn to *Daphnia magna*, using the inflection

point of the concentration-response curves. *Environmental Toxicology and Chemistry*: Accepted 16 Dec 2015.

9. Meyer JS, Ranville JF, Pontash M, Gorsuch JW, Adams WJ. 2015. Acute toxicity of binary and ternary mixtures of Cd, Cu, and Zn to *Daphnia magna*. *Environmental Toxicology and Chemistry* 34:799-808.

10. Ebert D. 2005. Introduction to the Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia*. National Center for Biotechnology Information, Bethesda, MD, USA.
11. Mc Kee D, Ebert D. 1996. The effect of temperature on maturation threshold body-length in *Daphnia magna*. *Oecologia* 108:627-630.

12. Nebeker AV, Cairns MA, Onjukka ST, Titus RH. 1986. Effect of age on sensitivity of *Daphnia magna* to cadmium, copper and cyanazine. *Environmental Toxicology and Chemistry* 5:527-530.

13. Meyer JS, Farley KJ, Garman ER. 2015. Metal Mixtures Modeling Evaluation Project: 1. Background. *Environmental Toxicology and Chemistry* 34:726-740.

14. De Laender F, Janssen CR, De Schamphelaere KAC. 2009. Non-simultaneous ecotoxicity testing of single chemicals and their mixture results in erroneous conclusions about the joint action of the mixture. *Chemosphere* 76:428-432.

15. Ma H, Kim SD, Cha DK, Allen HE. 1999. Effect of kinetics of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* 18:828-837.

16. American Public Health Association, American Water Works Association, Water Environment Federation. 2012. *Standard Methods for the Examination of Water and Wastewater,* 22nd ed. American Water Works Association, Denver, CO, USA.

17. Zeisler R, Murphy KE, Becker DA, Davis WC, Kelly WR, Long SE, Sieber JR. 2006. Standard Reference Materials® (SRMs) for measurement of inorganic environmental contaminants. *Analytical and Bioanalytical Chemistry* 386:1137-1151.

18. Julious SA. 2004. Using confidence intervals around individual means to assess statistical significance between two means. *Pharmaceutical Statistics* 3:217-222.

19. Payton ME, Greenstone MH, Schenker N. 2003. Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical significance? *Journal of Insect Science* 3: article 34.

20. Mittmann B, Ungerer P, Klann M, Stollewerk A, Wolff C. 2014. Development and staging of the water flea *Daphnia magna* (Straus, 1820; Cladocera, Daphniidae) based on morphological landmarks. *Evo Devo* 5: article 12.

CHAPTER 4

USING REVERSE-ISOTOPIC LABELING OF *LYMNAEA STAGNALIS* TO UNDERSTAND CU BIOAVAILABILITY VIA DIETBORNE PATHWAYS AT AN ACID MINE DRAINAGE CONTAMINATED SITE

4.1 Abstract

The flux of Cu was traced in isotopically-labeled freshwater snails (*Lymnaea stagnalis*) that were fed flocculent material rich in Fe and other metals (e.g., Cu, Zn) collected from sediment in the North Fork of Clear Creek (NFCC) in Black Hawk, Colorado. This study is part of a project to evaluate contamination and potential remediation of acid mine drainage (AMD) in NFCC. In these dietborne-metal experiments, snails are used as an easily-cultured surrogate for other invertebrates. The isotopic signature of the snails is reversed by culturing them in moderately hard reconstituted water containing a sub-lethal concentration of isotopically-enriched ^{65}Cu for five weeks before the dietary exposure begins. Variable amounts of the metal-contaminated flocculent material is mixed with a diatom (*Nitzschia palea*) to produce a series of dietborne-metal concentrations, and each mixture is collected on a filter to create a mat the snails eat. Using mass balance and isotopic-ratio conversions, the uptake and depuration of Cu are traced over a 48-h exposure in which the organisms consumed control or contaminated food. The assimilation efficiency, which describes how well the particles are taken up by an organism, can be used to quantify the bioavailability of metals. This study evaluated the biodynamic parameters of three types of metal-contaminated particles collected from NFCC sites: (1) loosely-coated flocculent that was easily washed off coarse-rocky sediment, (2) an armored coating that was isolated after the sediment was agitated in a rock tumbler; and (3) a mixture of loosely-coated flocculent and armored coating material collected by rinsing and scraping the sediment with a plastic spatula. The uptake rate of Cu was the greatest in the loosely-coated flocculent material,

followed in decreasing order by the mixture of loosely-coated flocculent material and armored coating, and the armored coating alone. As the concentration of Cu in the dietborne exposure was increased, the food-ingestion rate decreased drastically for the loosely-coated flocculent, but remained relatively constant for the mixture and only slightly decreased for the armored coating alone. Finally, the Cu assimilation efficiency remained elevated around 40-50% and did not differ significantly among all three flocculent types. These results will be compared to previous studies where the same parameters were analyzed for Cu at a different AMD site, the Animas River, in Durango, Colorado.

4.2 Introduction

Acid mine drainage (AMD) can be a major source of metals in waterways near mining regions. Water can become acidified when metallic sulfide ores oxidize to form sulfuric acid [1]; and AMD occurs when the acidic waters flow through waste-rock or tailings piles. The complete oxidation of a particularly common ore, iron disulfide (pyrite, FeS_2) is:

$$FeS_{2(s)} + \frac{15}{4} O_2 + \frac{7}{2} H_2O \rightarrow 4H^+ + 2SO_4^{2-} + Fe(OH)_{3(s)} \quad (4.1)$$

The acidic runoff can enter surface waters through storm-water and snowmelt runoff and interact along the way with metal-rich mineral ores, thereby releasing metals by acidic dissolution. Acidic interactions with metal sulfides other than pyrite are of particular concern, because those sulfides can also be oxidized and exacerbate acidification of the ecosystem and the release of metals. In this reaction, hydrous ferric oxides (HFOs) precipitate, but the resulting flocculent material will remain highly porous. As such, HFOs have a large relative surface area and have been hypothesized to have binding sites available to adsorb a variety of metals [2].

Under acidic conditions, dissolved metals tend to exist predominantly as free metal ions [1]. With respect to the total metal concentration in a system, the concentration of free metal ions

has been most directly linked to toxicological effects in organisms [3]. It is hypothesized that free metal ion is the most bioavailable to an organism. Bioavailability is loosely defined as the degree at which a substance is made available at a site of toxic action [4].

The impact AMD will have on an ecosystem is often difficult to predict due to vast seasonality changes in the composition and flow of the adits and receiving waters [5], as well as substantial variations in native mineral composition between sites. Nonetheless, it is important to be able to accurately estimate the impact in order to select appropriate remediation. Furthermore, dietborne exposures are not often considered in many regulatory and/or management decisions, which has led to some concern that ecological impact may be drastically underestimated when organisms may be taking up metals via both waterborne and dietborne pathways [6].

Metals frequently found in relation to sulfide ore mine sites include: Al, As, Cd, Cu, Ni, Pb and Zn. Some of these metals (Cu, Zn) are considered micronutrients to various aquatic species, while others (Cd, Pb, As) are non-essential elements [7]. If an organism does not have sufficient levels, deficiency of micronutrients could result in stunted growth, infertility and even toxicity.

Because the element of interest in this study, Cu, is a micronutrient to *L. stagnalis*, it is present at background concentrations in healthy organisms that do not necessarily reflect the amount of Cu in the surrounding environment. In fact, even when the external exposure concentration of Cu changes, organisms often continue to regulate Cu concentrations effectively [8], which makes it extremely difficult to detect small changes in elemental flux in tissues. However, artificially loading an organism with a stable isotope that has a low natural abundance makes it feasible to subsequently track the uptake and depuration of a more-abundant isotope, rather than attempting to quantify relatively small changes in the background concentration of the

metal [9]. Specifically, Cu has two stable isotopes: ^{63}Cu and ^{65}Cu, with 69% and 31% relative natural abundance, respectively. In this study, the isotopic signature of the snails was reversed via waterborne exposure to ^{65}Cu before the snails were exposed to metal-contaminated particles containing the natural isotopic ratio of $^{65/63}$Cu.

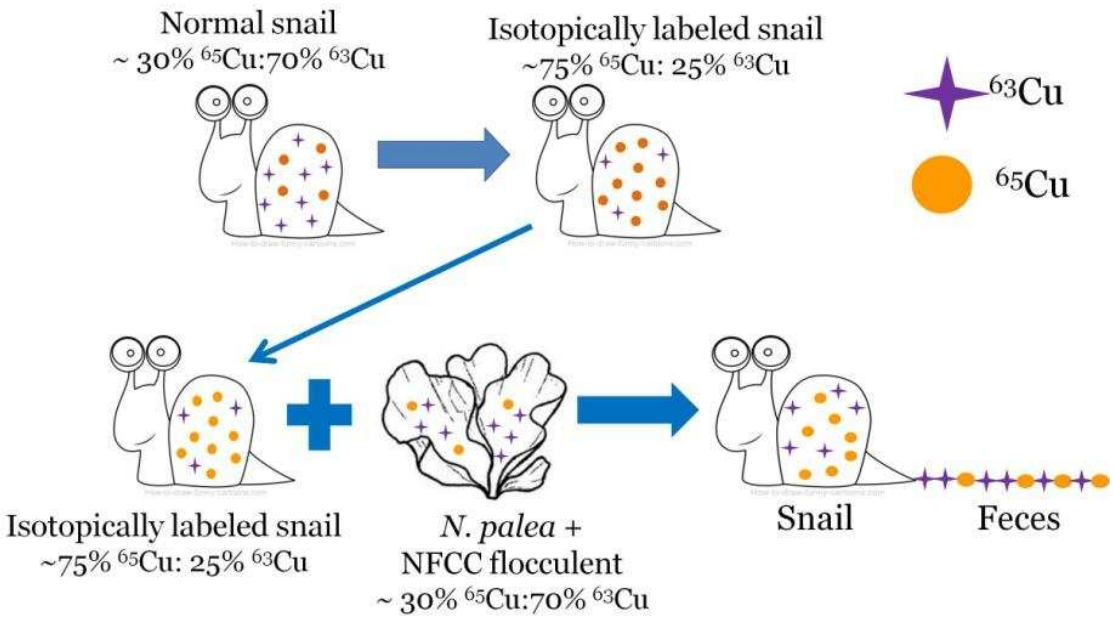

Figure 4.1: Schematic of the reverse-isotope tracing process. The isotopic signature of the organism is reversed through waterborne exposure to ^{65}Cu (upper) before the organism is fed particles at the natural isotopic abundance from an AMD-contaminated site. At the end of the exposure, the snail and feces are collected and analyzed to quantify the amount of ^{63}Cu that was accumulated by the organism during the dietborne exposure (lower).

The goal of this research is to quantify the proportion of Cu that is bioavailable via a dietborne pathway in the North Fork of Clear Creek in Gilpin County in central Colorado, and AMD-contaminated stream that contains a mixture of metals. To do this, the flux of ^{63}Cu was traced in a freshwater snail during dietborne exposure to NFCC particles, followed by a depuration period in which the organisms were purged of the NFCC particles. Using a series of isotopic ratio conversions and mass balances to account for the amount of Cu taken up and eliminated from the snail, the assimilation efficiency was determined. Assimilation efficiency is a

parameter that describes the extent to which a chemical is taken up by an organism [10,11] and therefore is an index of the bioavailability of the chemical.

The US Environmental Protection Agency has approved the construction of a treatment plant to ameliorate the AMD contamination that enters NFCC in the city of Blackhawk, Colorado so this stretch of the creek can be restored. Because this treatment plant is scheduled to be operational by spring 2017, preremediation data are needed so the effectiveness of the treatment plant can be evaluated. Currently, the streambed is coated with metal-containing HFOs that are present as flocculent material loosely covering the sediment and as an armored coating on the rocks. However, recent simulations conducted by Jacob Williamson at the Colorado School of Mines indicate that when the current adits are no longer allowing AMD to enter NFCC, the loose flocculent material will be washed away relatively quickly; but the tougher, armored coating may remain for a longer period of time. As such, the geochemical changes during the remediation process may have direct implications to the bioavailability of metals in the stream, depending on the type of sediment coating that the organisms interact with.

4.3 Experimental

The following procedure is based on method developed by Croteau et al. [12].

4.3.1 Organisms

Lymnaea stagnalis were used in all Cu bioavailability tests. They are aquatic pulmonate gastropod mollusks (freshwater snails) [13] that have been commonly used in bioavailabilty and biomonitoring studies [14,15] due to their high bioconcentration capacity, particularly for Cu [16]. The snails were cultured in 20L glass aquaria that contained approximately 10L of USEPA moderately hard reconstituted water (MHRW) [17] and fed romaine lettuce *ad libitum* on alternating days. Approximately 2-3 weeks post-birth, 10 μg/L of ^{65}Cu (Trace Sciences 99.4%,

$Cu(NO_3)_2$) [12] was added to the MHRW containing the organisms. The media was replaced with fresh MHR and ^{65}Cu on a weekly basis. To ensure complete reversal of the organisms' isotopic signature, the snails were exposed to ^{65}Cu for 5 weeks before the exposure to the NFCC particles.

4.3.2 North Fork of Clear Creek particles

Acid-mine-drainage-impacted sediment was collected in November 2015 (experiment 1) and April 2016 (experiments 2 and 3) from the streambed of the North Fork of Clear Creek in Blackhawk, Colorado, immediately downstream from the AMD input at the National Tunnel drainage pipe. The sediment consisted of gravel and cobble ranging in size from about 1 to 8 cm that was heavily coated with orange-yellow precipitate. For experiment 1, the sample was transported to the Colorado School of Mines in Golden, Colorado, where the sediment was rinsed in deionized water and scraped with a polypropylene spatula to remove and isolate both "loose" and "armored" flocculent coating. The resulting particulate material was transported on ice via next-day shipping to the U.S. Geological Survey in Menlo Park, California, and stored at 4˚C in the dark. For experiment 2, the sediment was rinsed with deionized water to isolate "loosely-coated" flocculent material. Experiment 3 tested the armored coating on the rocks. This portion of the coating was isolated by placing previously-rinsed sediment in a rock tumbler for 6 h to agitate the rocks and remove the armored material. Experiments 2 and 3 were conducted at the Colorado School of Mines.

4.3.3 Diatom culturing

The benthic diatom *Nitzschia palea* was mixed with the NFCC particles before the dietborne exposure. The diatom was added to provide a palatable food source for the snails, because they do not eat abiotic particles that are not mixed with natural food items [12]. *Nitzschia palea* (strain CPCC 160, University of Waterloo) was inoculated in S-diatom media [12] having a

natural-isotopic Cu abundance and grown for 2-3 weeks before being used in each dietborne-metal exposure.

4.3.4 Uptake experiments

A total of 5 exposure treatments containing 10 snails per treatment were tested in each experiment. To create the diatom + particle mats that were presented to the snails as food during the dietborne-metal exposure, a constant mass of *N. palea* was mixed with increasing concentrations of the NFCC particle at each treatment (Table 4.1). This mixture was filtered onto a 1.2μm polycarbonate Isopore membrane (Millipore) using vacuum filtration that was monitored so the pressure drop across the membrane did not exceed 10-15 mm Hg. A small portion of the membrane from each treatment level was retained for direct digestion and analysis. The remaining portion of the membrane was placed into a feeding chamber.

Feeding chambers consisted of 150-ml acid-washed polypropylene cups with holes cut on either side to allow water to flow through the feeding cup. The holes in the chamber were covered with mesh (~250 μm) to prevent the snails from escaping. During the exposure, the 5 feeding cups were partially submerged in approximately 2-L MHRW water in a 40-L aquarium.

Snails were removed from the culturing tank and segregated so that enough snails of approximately the same size (soft tissue dry weight <1 mg) were collected for the exposure. Fifty snails of the chosen size were randomly separated into the 5 treatment feeding chambers (10 snails per treatment level). In addition, 10 snails were immediately rinsed and placed in the freezer to serve as a reversely-labeled “control” treatment having no exposure to diatoms or NFCC particles. Snails in the feeding chambers were allowed to feed on the mats for 4 h. At the end of the feeding period, the snails were rinsed in MHRW to remove any loose flocculent

material from the surface of the organism, and each organism was placed in an individual depuration chamber.

The depuration chambers were 50-mL acid-washed polypropylene containers with holes cut in either side and secured with 250-μm mesh to retain the snail and any feces released during the depuration period. Additionally, the top of the depuration chamber was covered by mesh to allow for flow of O_2 into the chamber. The snails were supplied with organic romaine lettuce (~1 cm^2) in each chamber to eat during the 48-h depuration period. The depuration chambers were contained in a 40-L glass aquarium filled with MHRW, which was continuously filtered using a charcoal filter to keep the water fresh. Samples of the MHRW in the chambers were collected and analyzed for metal content to account for any dissolved Cu. After the 48-h period, the snails were removed from their chamber, rinsed and placed in a freezer to euthanize them.

4.3.5 Digestions

All feces were collected from the depuration chamber and transferred directly into teflon digestion vials using a 1-mL polypropylene transfer pipet. The soft tissue each frozen snail was separated from its shell and placed onto an acid-washed teflon square (~1 cm^2). The filter membrane containing the diatom + particle mat, the feces, the snail soft tissue, and a sample of the lettuce fed during the depuration period were allowed to dry for 24 h in an oven at 40˚C. The dried diatom + particle mat and the snail soft tissue were weighed separately before the samples were transferred into a teflon digestion vial. Two hundred μL of 16 N HNO_3 (Fisher Scientific) were added to each vial for the entire the digestion period (5-7 days), to which 80 μL of >30% H_2O_2 (Macron Fine Chemicals) was added for the final 24 h of digestion. Certified reference materials (DOLT-5 (NRCC), TORT-2 (NRCC), and Mussel (NIST)) were incorporated into the

procedure by measuring a mass of the standards that was similar to the mass of the products and following the same digestion protocol alongside the food mat, snail, and feces samples.

4.3.6 Sample analysis

Digested samples were brought to a final volume of 4 mL, and the naturally occurring Cu isotopes were analyzed using a Perkin Elmer Nexion 300 Inductively Coupled Plasma- Mass Spectrometer (ICP-MS, Instrumental Detection Limit (IDL): 0.011 μg ^{63}Cu/L). Analyte analysis consisted of 10 individual measurements of each sample that were averaged. Germanium was added to each sample as an internal standard (8 μL/mL, Spex CertiPrep). An acid blank and quality control sample of 100 μg Cu/L were run every ten samples.

The particulate materials isolated from the AMD impacted sediment were analyzed for metals and inorganic cations using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES Perkin Elmer Optima 5300, IDL: 0.4 μg Cu/L). Scandium was used as the internal standard; continuing calibration verification (CCV) and NIST certified reference materials 1640a and 1643e were run before and after the analysis.

4.3.7 Data analysis

The results calculated from these experiments included assimilation efficiency of Cu in NFCC particles, the ingestion rate of the exposure material, and accumulation and influx of ^{63}Cu. The input information for these calculations is listed in Table 4.1. The influx of ^{63}Cu refers only to the excess Cu that is accumulated in an organism over the course of the exposure period; this value is used to determine the uptake rate of Cu in North Fork particles (NFP) as a function of the concentration of Cu that the organism was exposed to. This quantity is calculated by subtracting the average ^{63}Cu concentration (ug g^{-1}) that was measured in the "control" organisms (those that

were isolated without being exposed to NFP) from the final ^{63}Cu concentration (ug g^{-1}) measured in each snail that had been exposed to natural particles (Equation 4.2).

$$\text{Influx } {}^{63}\text{Cu} = \frac{\left[{}^{63}\text{Cu}\right]_{\text{snail}} - \left(\frac{1}{n}\sum_{i=1}^{n}\left[{}^{63}\text{Cu}\right]_{\text{control}}\right)}{T_1} \quad (4.2)$$

The initial ^{63}Cu concentration in each snail ($\left[{}^{63}\text{Cu}\right]^0_{\text{snail}}$) was estimated using Equation 4.3 by subtracting the amount of ^{63}Cu taken up during the exposure from the final ^{63}Cu concentration measured in the organism ($\left[{}^{63}\text{Cu}\right]_{\text{snail}}$).

$$\left[{}^{63}\text{Cu}\right]^0_{\text{snail}} = \frac{{}^{63}\text{Cu}_{\text{snail}} - \left(\frac{k_{uNFP}\left[{}^{63}\text{Cu}\right]_{NFP}}{k_e}\left(1-\exp^{-k_e T_1}\right) + \frac{k_{ulett}\left[{}^{63}\text{Cu}\right]_{lett}}{k_e}\left(1-\exp^{-k_e T_2}\right)\right)}{\exp^{-k_e(T_1+T_2)}} \quad (4.3)$$

^{63}Cu taken up during the exposure was attributed to NFP in the food mat and from lettuce during the depuration phase. In Equation 4.3, k_u refers to the specific uptake rate for NFP or lettuce [18], respectively, determined from the slope of the influx of ^{63}Cu versus the Cu concentration in the food mat. k_e is the elimination rate for Cu from *L. stagnalis* reported by Croteau et al.[18]. Using the initial and final ^{63}Cu concentrations and the dry mass of each snail (wt_{snail}), I calculated the amount of ^{63}Cu accumulated in each organism (Equation 4.4).

$${}^{63}\text{Cu}_{\text{accumulated}} = \left(\left[{}^{63}\text{Cu}\right]_{\text{snail}} - \left[{}^{63}\text{Cu}\right]^0_{\text{snail}}\right) \times \text{wt}_{\text{snail}} \quad (4.4)$$

The particle ingestion rate is determined in Equation 4.5 by dividing the total ^{63}Cu that was taken up by the organism (Cu that was accumulated within the organism, as well as the Cu that was excreted during the depuration phase) by the ^{63}Cu in the food mat and accounting for the exposure time and weight of the snail. The loss of ^{63}Cu to the water was assumed to be negligible during the exposure. Finally, in Equation 4.6, the assimilation efficiency is the ^{63}Cu that was accumulated within the organism at the end of the depuration period divided by the sum of the accumulated Cu and Cu lost through elimination (i.e. in the feces) during the experiment.

$$\text{Ingestion Rate} = \frac{(^{63}\text{Cu}_{\text{accumulated}} + {}^{63}\text{Cu}_{\text{feces}})}{[^{63}\text{Cu}]_{\text{food}} \times \text{wt}_{\text{snail}} \times \text{T}_1} \quad (4.5)$$

$$\text{Assimilation Efficiency (\%)} = \frac{^{63}\text{Cu}_{\text{accumulated}}}{^{63}\text{Cu}_{\text{accumulated}} + {}^{63}\text{Cu}_{\text{feces}}} \times 100 \quad (4.6)$$

The resulting ^{65}Cu:^{63}Cu ratio, ^{63}Cu influx rate, food ingestion rate, and Cu assimilation efficiency values are the mean of 10 individual measurements for each treatment level. Copper concentrations in the diatom + particle mats were the mean of five individual measurements for each treatment level. The 84% confidence interval around the means was calculated to help infer significant differences between two means at the 95% confidence level, as recommended by statisticians [19,20].

Table 4.1: Input information for calculating relative isotopic abundance, uptake and ingestion rates, and assimilation efficiencies in *Lymnaea stagnalis*.

Variable	Unit	Definition
$[\,^{x}\text{Cu}]^{0}_{\text{snail}}$	μg g^{-1}	Background snail Cu concentration
$[\,^{x}\text{Cu}]_{\text{NFP/ lett}}$	μg g^{-1}	Dietary Cu concentration
$[\,^{x}\text{Cu}]_{\text{Snail/Control}}$	μg g^{-1}	Cu concentration in a given snail (or control snail)
p^{x}_{snail}		Relative abundance of ^{63}Cu or ^{65}Cu in the snails soft tissues
k_u	g g^{-1} day^{-1}	Rate constant of Cu uptake from food source
k_e	day^{-1}	Rate constant of elimination
T_1	day	Exposure duration
T_2	day	Depuration duration
n		Number of snails in ‘control’ subgroup
wt_{snail}	g	Mass of snail soft tissue
$^{x}\text{Cu}_{\text{accumulated}}$	g	Mass of Cu accumulated in soft tissues during exposure
$^{x}\text{Cu}_{\text{feces}}$	g	Mass of Cu in feces collected post-exposure

4.4 Results

General analysis of NFCC flocculent material was conducted in December 2015 at the U.S. Geological Survey in Menlo Park, California. In this analysis, samples of sediment were

collected from NFCC and flocculent material was isolated by rinsing the sediment with deionized water, as well as light scraping of the sediment with a polypropylene spatula. Therefore, in this section, I will refer to this sample in section 4.4.1 as "unfractionated" particles in reference the method of collection of the NFCC floc.

In section 4.4.2, analysis of loosely-coated NFCC flocculent material was conducted in May 2016 at Colorado School of Mines in Golden, Colorado. In this analysis, samples of sediment were collected from NFCC and flocculent material was isolated solely by rinsing the sediment with deionized water. Although a great deal of the orange ferric-hydroxide floc was rinsed off in this isolation method, and although the sediment was rinsed until the rinse ran clear, there was still a visible red-orange tint to the rocks, indicating a more armored coating existed on the sediments. In this section, I will refer to this sample as "loose-coated floc" to reference the method of collection of the NFCC floc.

Finally, section 4.4.3 details the analysis of the armored-coating on NFCC flocculent material that was conducted in June 2016 at Colorado School of Mines in Golden, Colorado. In this analysis, the samples of sediment were collected from NFCC and previously rinsed clear in the loose-coated flocculent material experiment were used to isolate the residual "armored" coating. Specifically, a rock tumbler was used to agitate the sediment, forcing collisions to remove the armored coating from the rocks. The red-orange color that initially tinted these rocks appeared to have been removed during this process. It should be noted that some underlying rock could have been abraided-off in addition to the armored coating during the collisions in the rock tumbler. In this section, I will refer to the isolated flocculent as "armored-coating particles". The final Cu concentrations for each treatment among the different particle types is shown in Table 4.2.

Table 4.2: Measured Cu concentrations in each dietary exposure treatment to *Lymnaea stagnalis*. The three experiments were conducted from December 2015-June 2016.

Treatment	Final [Cu] (ug/g)		
	Mixed	Loose	Armored
1	151.8	175.3	180.5
2	536.4	466.5	516.7
3	811.5	877.1	856.5
4	1011	1336	1260
5	1298	1880	1550

4.4.1 NFCC unfractionated sediment coatings

In this exposure of unfractionated particles collected from NFCC, the ratio of ^{65}Cu:^{63}Cu in the snails ranged from 2.9, which occurred in organisms that were collected as controls and not exposed to any food source with a natural abundance of isotopes, to approximately 1.5, which was the final isotopic ratio in organisms exposed to high concentrations of Cu having the natural isotopic abundance (Figure 4.2). The influx rate of ^{63}Cu (expressed as $\mu g\ ^{63}Cu\ g_{snail}^{-1}\ d^{-1}$) increased throughout the entire ^{63}Cu concentration gradient tested (Figure 4.3), with an average exposure-concentration-normalized influx rate (determined from the regression slope) of 0.195 $g_{food}\ g_{snail}^{-1}\ d^{-1}$ (i.e., 0.195 $\mu g\ ^{63}Cu\ g_{snail}^{-1}\ d^{-1}\ [\mu g\ ^{63}Cu\ g_{food}^{-1}]^{-1}$, n = 5). The food ingestion rate with the unfractionated particles collected from NFCC remained constant at 0.48 ± 0.06 $g_{ingested\ food}\ g_{snail}^{-1}\ day^{-1}$ in all Cu concentrations (average ± SD, n = 5; Figure 4.4). The assimilation efficiency averaged 45 ± 6% (± SD, n = 5; Figure 4.5). The standard deviation was relatively high primarily due to the assimilation efficiency determined for Cu ingested from the diatom-only food mat. This treatment contained only a low background ^{63}Cu concentration that was near detection limit. The variability in this treatment was much wider than in the trials

containing NFCC particles. If I only used the assimilation efficiencies in treatments containing NFCC particles, the average assimilation efficiency was 47 ± 3% (average ± SD, n = 4).

4.4.2 NFCC flocculent material from loose coatings

In this exposure involving only loosely-coated flocculent, the ^{65}Cu:^{63}Cu ratio in the snails ranged from 3.6, which occurred in organisms that were collected as controls and not exposed to any food source with a natural abundance of isotopes, to approximately 1.9, which was the final isotopic ratio in organisms exposed to high concentrations of Cu in the natural isotopic abundance (Figure 4.2). The influx of ^{63}Cu increased as the dietborne Cu exposure concentration increased in the four lowest ^{63}Cu concentrations tested (Figure 4.3), up to an influx of 296 ug Cu g_{snail}^{-1} day^{-1} at 616 ug ^{63}Cu g_{food}^{-1}. The final influx rate using the four lowest ^{63}Cu concentrations was 0.45 g_{food} g_{snail}^{-1} day^{-1} (i.e., 0.45 μg ^{63}Cu g_{snail}^{-1} d^{-1} [μg ^{63}Cu g_{food}^{-1}]$^{-1}$, n = 4). The food ingestion rate with the loosely-coated flocculent material collected from NFCC decreased drastically from approximately 1.58 to 0.42 $g_{\text{ingested food}}$ g_{snail}^{-1} day^{-1}as the dietborne Cu concentration increased (n = 5; Figure 4.4). In this experiment, the assimilation efficiency averaged 48 ± 5% (average ± SD, n = 5; Figure 4.5).

4.4.3 NFCC armored coatings

In the exposure with only armored coating, the ^{65}Cu:^{63}Cu ratio in the snails ranged from 2.5, which occurred in organisms that were collected as controls and not exposed to any food source with a natural abundance of isotopes, to approximately 1.6 (Figure 4.2). The influx of ^{63}Cu increased incrementally in the four lowest ^{63}Cu concentrations tested (Figure 4.3), up to an influx of 89 ug Cu g_{snail}^{-1} day^{-1} at 869 ug ^{63}Cu g_{food}^{-1}. The final influx rate was determined using the slope of the lowest ^{63}Cu concentrations to be 0.098 g_{food} g_{snail}^{-1} day^{-1}(i.e., 0.098 μg ^{63}Cu g_{snail}^{-1} d^{-1} [μg ^{63}Cu g_{food}^{-1}]$^{-1}$). The ingestion rate of the armored-coated particles collected from NFCC

remained constant at approximately 0.32 ± 0.07 $g_{\text{ingested food}}\, g_{\text{snail}}^{-1}\, \text{day}^{-1}$ in all Cu concentrations (average ± SD, n = 5; Figure 4.4). In this experiment, the assimilation efficiency averaged 38 ± 3% (average ± SD, n = 5; Figure 4.5).

4.5 Discussion

Using the same procedure as in my study, other researchers have reported Cu influx rates, food ingestion rates, and Cu assimilation efficiencies from several media including lettuce, diatoms, synthetic hydrous ferric oxides (HFO), and particles from another AMD source, the Animas River in Durango, Colorado (Table 4.3). The uptake rate of Cu (k_{uf}) was lowest from the diatom exposure [21] and highest from HFO exposure [22]. The uptake rates do not appear to correlate with the assimilation efficiencies because the diatom and HFO exposures had some of the highest assimilation efficiencies at 72% and 99%, respectively. Both of these experiments also resulted in significant suppression of the food ingestion rate as the dietborne Cu concentration increased [21,23].

Table 4.3: Summary of related studies that have been completed using the same method of reverse-isotopic tracing to analyze various media types related to dietborne uptake and AMD-contamination exposures in *Lymnaea stagnalis*.

Particle Type	Uptake Rate (k_u) (g food/g snail/d)	Assimilation Efficiency (%)	Reference
^{65}Cu in lettuce	0.16	86	Croteau (2008)
^{65}Cu in N. palea diatom	0.06	72	Croteau (2009)
^{65}Cu in HFO	0.69	>70	Cain (2013)
NFCC loosely-coated floc	0.45	48	Traudt Thesis (2016)
NFCC unfractionated particles	0.19	47	Traudt Thesis (2016)
Animas River (AMD) particles	0.14	44	Croteau (2013)
NFCC armored coating	0.10	36	Traudt Thesis (2016)

Particles from different AMD sites had largely similar biodynamic properties: both the Animas River particles and the North Fork particles (unfractionated particles) resulted in

assimilation efficiencies of approximately 45% [12] and uptake rates of 0.14 and 0.19 g g^{-1} day^{-1}, respectively. Further, in both AMD treatments, the ingestion rate of the particles remained approximately constant regardless of the dietary Cu concentration.

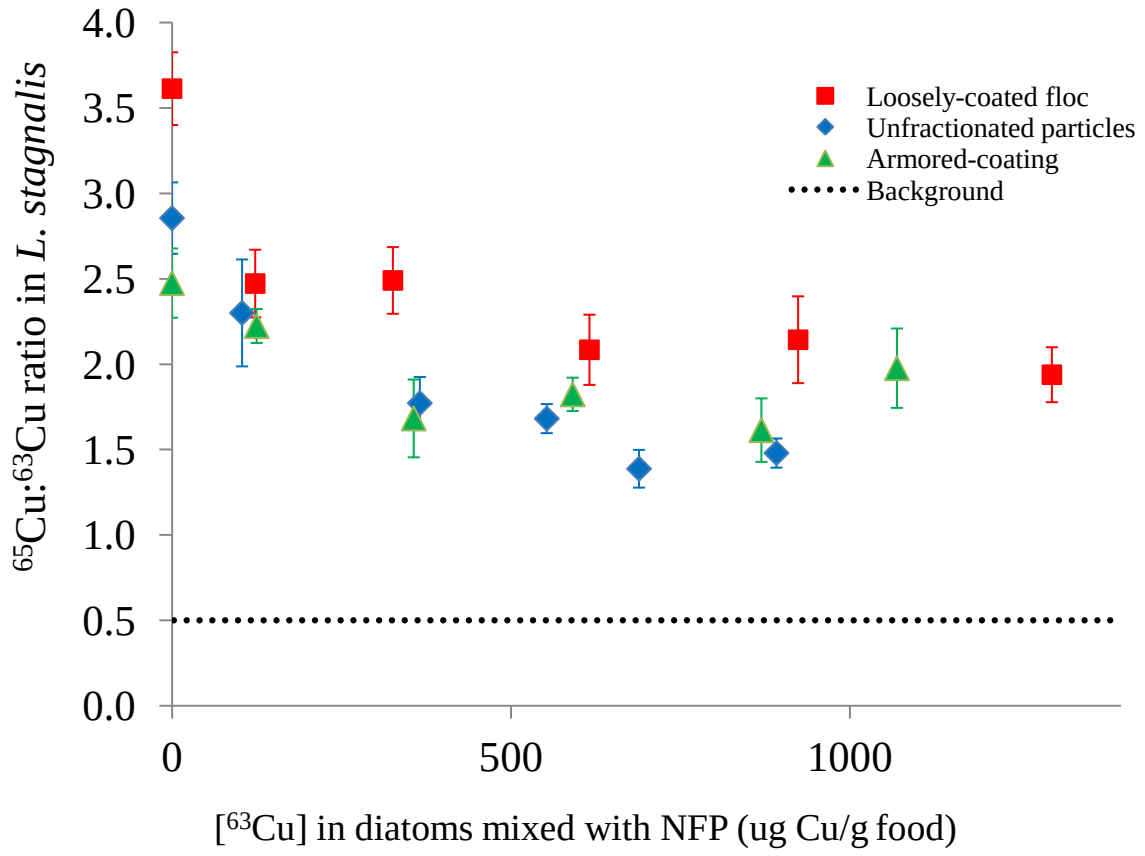

Figure 4.2: $^{65}Cu:^{63}Cu$ ratio in isotopically labeled snails (*Lymnaea stagnalis*) at different dietborne Cu concentrations collected from the North Fork of Clear Creek (NFCC) and tested in 3 batch sets consisting of (1) loosely-coated flocculent, in which the sediment was gently rinsed to remove and collect surface coatings, (2) unfractionated particles, in which the sediment was rinsed to remove the surface coating and also scraped gently with a plastic spatula to remove some of the armored coating, and (3) armored particles, in which the loosely-coated surface material was removed before to agitating the sediment in a rock tumbler to isolate and collect material that had formed an armored coating on the sediment. The background ratio indicates the natural isotopic $^{65}Cu:^{63}Cu$ ratio in unlabeled snails, 0.5:1. As the concentration of AMD-contaminated particles (and thus of particulate Cu) was increased in the food, the $^{65}Cu:^{63}Cu$ ratio in the snails decreased toward the background ratio. This indicates uptake of Cu from AMD-contaminated particles that have a natural $^{65}Cu:^{63}Cu$ ratio. Error bars represent 84% confidence intervals.

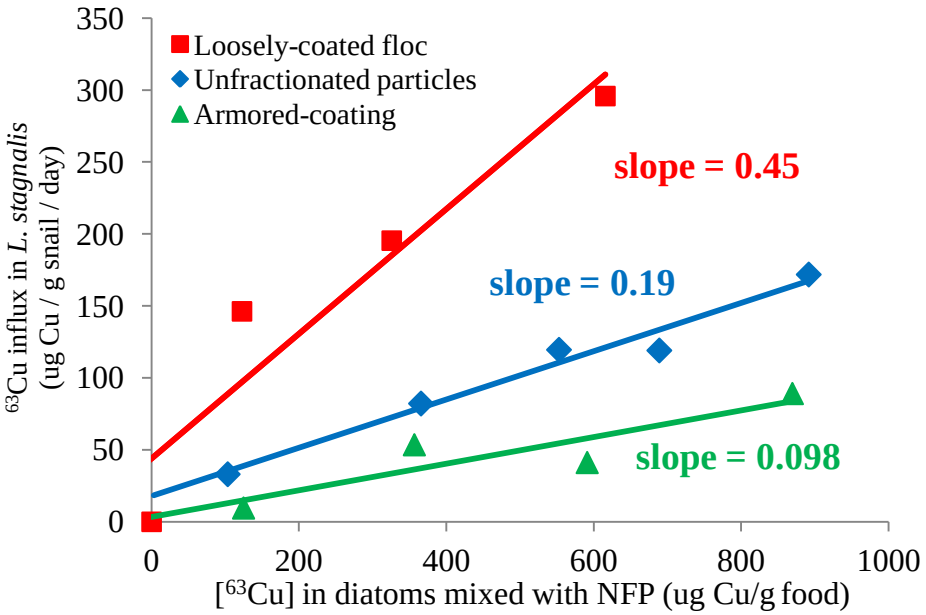

Figure 4.3: Influx of ^{63}Cu into snails (*Lymnaea stagnalis*) from three types of particulate material collected from an NFCC acid mine drainage-contaminate site. The influx determines the Cu uptake rate, or the amount of Cu (in ug) that is internalized per gram of snail (dry weight) over a time period (day). In this figure, the uptake rate of loose-coated flocculent material is considerably greater than the Cu uptake rates of unfractionated particles or armored-coating.

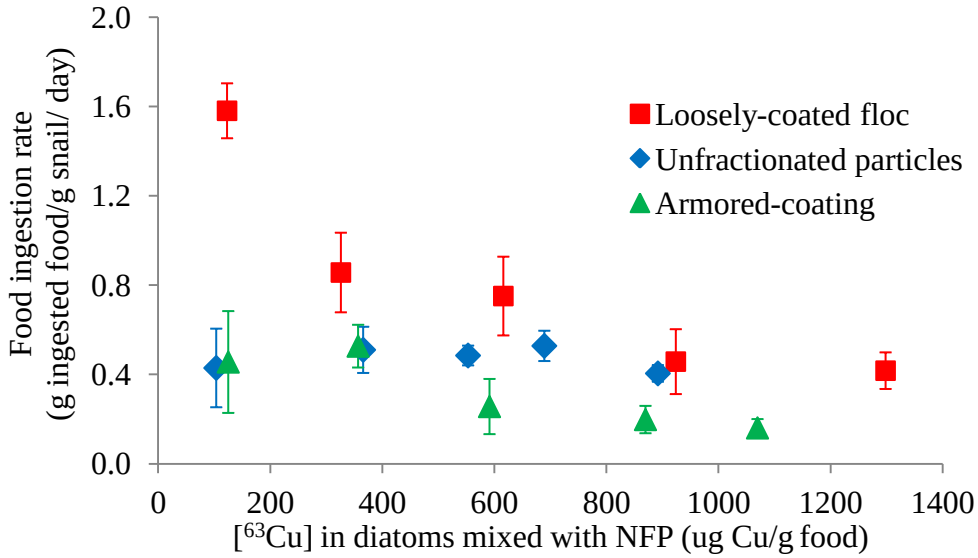

Figure 4.4: Ingestion rate of ^{63}Cu into snails (*Lymnaea stagnalis*) from three types of particulate material collected from an NFCC acid mine drainage-contaminate site. Ingestion rate quantifies the amount of food that was eaten by each organism over the course of the exposure, but does not necessarily correspond with the concentration of Cu that is internalized. Ingestion rate of NFCC particles either decreased (loosely-coated flocculent) or remained relatively constant (unfractionated particles and armored coating) while the Cu concentration was increased in different treatments. Error bars represent an 84% confidence interval.

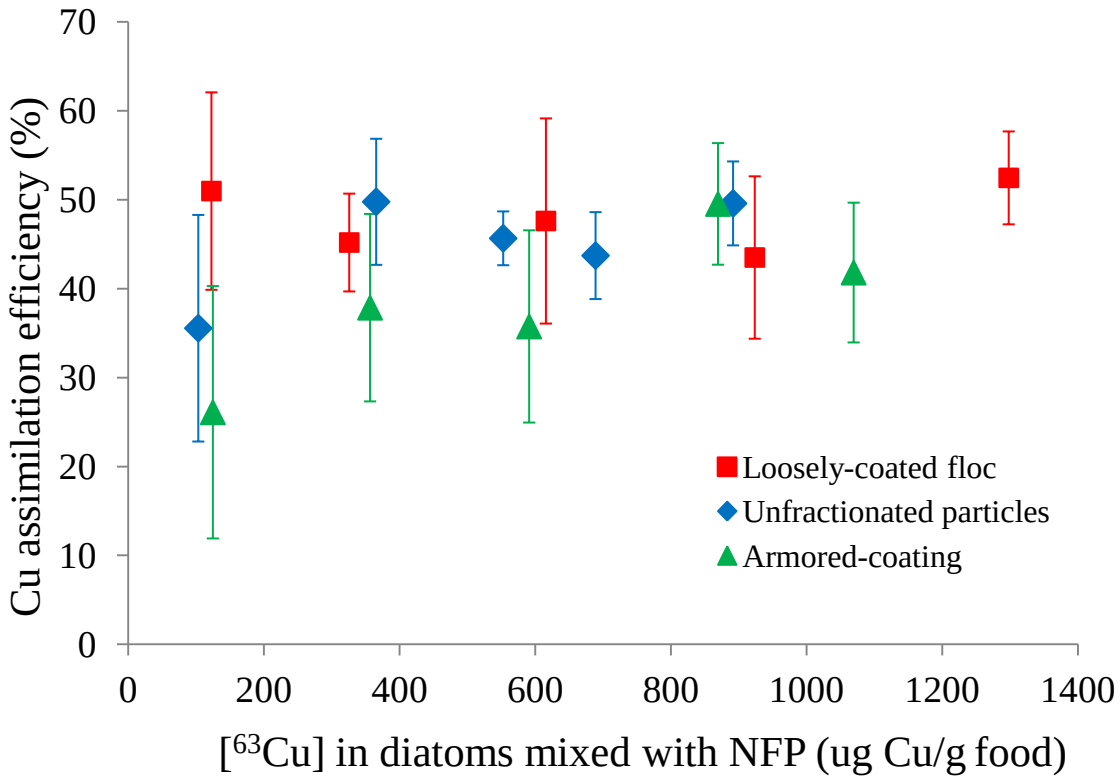

Figure 4.5: Assimilation efficiency of ^{63}Cu from three types of particulate material collected from NFCC. The assimilation efficiency, which may be used to describe the bioavailablility of a particle, was relatively high at approximately 40-50% efficiency for all types of particulate material that was collected from NFCC. The lowest Cu concentrations appeared to be more variable, but this is most likely due to those concentrations being very low and near the detection limit, rather than an actual effect.

These results generally do not agree with the more in-depth analysis of the loosely-coated flocculent and armored coating material from the North Fork that was analyzed in this study. My results indicated a considerably greater Cu uptake rate from the loose floc (0.45 g g^{-1} day^{-1}) than the unfractionated particles (0.19 g g^{-1} day^{-1}) and armored particles (0.09 g g^{-1} day^{-1}). According The loose floc had an influx rate only slightly lower than the one reported for HFO [22], but noticeably larger than any other particle type in my study. In the experiments examining the loosely coated flocculent material, when the ^{63}Cu concentration reached 924 and 1298 ug^{63}Cu g_{food}^{-1}, the influx decreased or leveled out to 170 and 310 ug Cu g_{snail}^{-1} day^{-1}, respectively. A similar trend was also observed for the highest Cu concentration of the armored coating

material, when the ^{63}Cu concentration reached 1069 ug ^{63}Cu g_{food}^{-1}, the influx had decreased to 68 ug Cu g_{snail}^{-1} day^{-1}. This is consistent with previous bioaccumulation studies, in which high concentrations of Cu inhibited or suppressed the Cu influx from increasing linearly [21].

The food ingestion rate also differed among the types of particles in my study. The loose floc exhibited a very high initial ingestion rate that decreased in each subsequent treatment at higher Cu concentrations. However, the unfractionated particles and armored coating caused much lower initial ingestion rates and only decreased slightly, if at all, across the treatment levels. Finally, the Cu assimilation efficiency from the armored particles (~38%) was much lower as compared to the unfractionated particles or loose floc (~47% and 48%, respectively). Although the former two types of particles generally had Cu assimilation efficiencies similar to another acid mine drainage site [12], the assimilation efficiency in the armored coating particles was the lowest that has yet been reported using this method.

4.6 Conclusions and future work

These results provide evidence that Cu associated with natural AMD-contaminated particles is not only bioavailable, but also suggests that the bioavailability may be similar among different AMD sites. However, when the particulate material was separated and analyzed based on loosely-coated material vs armored particles, many of the biodynamic parameters differed based on the type of flocculent material.

Much work still needs to be done to fully understand differences in the type of flocculent, which should ultimately help to understand factors affecting bioavailability. For example, an x-ray diffraction (XRD) analysis may be able to differentiate key mineralogic characteristics of the different types of particulate material; or a microbial analysis might determine whether biofilms could have played a role in determining bioavailability. For the analyses presented in this thesis,

all of the samples were collected and prepared for immediate exposure, without freezing. However, due to sometime limited proximity of sampling locations, expenses, and time constraints, preparing fresh samples for immediate exposure may not always be practical. To determine if it would be feasible to freeze samples prior to exposure, I would like to repeat experiments done with NFCC particles after the sediments have been frozen for various time periods. Finally, in an attempt to relate the dietborne exposures to my previous observations in waterborne metal-mixture exposures, it might be interesting to trace the uptake of individual metals as well as multiple metals in a mixture to determine if the flux of the metals could elucidate mechanisms resulting in non-additive toxicity. Thus far, using reverse-labeling to trace the flux of Cu has demonstrated promising potential for assessing bioavailability from particles.

4.7 Acknowledgements

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4.8 References cited

1. Morel FMM, Hering JG. 1993. *Principles of Aquatic Chemistry*. John Wiley & Sons, Inc., New York.

2. Benjamin MM, Leckie JO. 1981. Multiple-site adsorption of Cd, Cu, Zn, and Pb on amorphous iron oxyhydroxide. *Journal of Colloid and Interface Science* 79:209-221.

3. Campbell P. 1995. Interactions between trace metals and aquatic organisms: A critique of the free-ion activity model. *Metal Speciation and Bioavailability in Aquatic Systems* Volume 3.

4. Meyer JS, Clearwater SJ, Doser TA, Rogaczewski MJ, Hansen JA. 2007. *Effects of Water Chemistry on Bioavailability and Toxicity of Waterborne Cadmium, Copper, Nickel, Lead, and Zinc to Freshwater Organisms*. Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.

5. Gray N. 1998. Acid mine drainage composition and the implications for its impact on lotic systems. *Water Research* 32:2122-2134.

6. Wilding J, Maltby L. 2006. Relative toxicological importance of aqueous and dietary metal exposure to a freshwater crustacean: Implications for risk assessment. *Environmental Toxicology and Chemistry* 25:1795-1801.

7. Forstner U, Wittmann GT. 1979. *Metal Pollution in the Aquatic Environment*. Springer-Verlag.

8. Adams WJ, Blust R, Borgmann U, Brix KV, DeForest DK, Green AS, Meyer JS, McGeer JC, Paquin PR, Rainbow PS. 2011. Utility of tissue residues for predicting effects of metals on aquatic organisms. *Integrated Environmental Assessment and Management* 7:75-98.

9. Croteau M-N, Luoma SN, Topping BR, Lopez CB. 2004. Stable metal isotopes reveal copper accumulation and loss dynamics in the freshwater bivalve Corbicula. *Environmental Science and Technology* 38:5002-5009.

10. Wang W. 1987. Factors affecting metal toxicity to (and accumulation by) aquatic organisms—Overview. *Environment International* 13:437-457.

11. Lee B-G, Griscom SB, Lee J-S, Choi HJ, Koh C-H, Luoma SN, Fisher NS. 2000. Influences of dietary uptake and reactive sulfides on metal bioavailability from aquatic sediments. *Science* 287:282-284.

12. Croteau M-N, Cain DJ, Fuller CC. 2013. Novel and nontraditional use of stable isotope tracers to study metal bioavailability from natural particles. *Environmental Science and Technology* 47:3424-3431.

13. Lagadic L, Coutellec M-A, Caquet T. 2007. Endocrine disruption in aquatic pulmonate molluscs: Few evidences, many challenges. *Ecotoxicology* 16:45-59.

14. Ballance S, Phillips PJ, McCrohan CR, Powell JJ, Jugdaohsingh R, White KN. 2001. Influence of sediment biofilm on the behaviour of aluminum and its bioavailability to the snail *Lymnaea stagnalis* in neutral freshwater. *Canadian Journal of Fisheries and Aquatic Sciences* 58:1708-1715.

15. Coeurdassier M, De Vaufleury A, Badot P-M. 2003. Bioconcentration of cadmium and toxic effects on life-history traits of pond snails (*Lymnaea palustris* and *Lymnaea stagnalis*) in laboratory bioassays. *Archives of Environmental Contamination and Toxicology* 45:102-109.

16. V.-Balogh K, Fernandez DS, Salánki J. 1988. Heavy metal concentrations of *Lymnaea stagnalis* in the environs of Lake Balaton (Hungary). *Water Research* 22:1205-1210.

17. USEPA. 2002. *Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms*, 5th edition. EPA-821-R-02-012. U.S. Environmental Protection Agency, Washington, DC.

18. Croteau M-N, Luoma SN. 2008. A biodynamic understanding of dietborne metal uptake by a freshwater invertebrate. *Environmental Science and Technology* 42:1801-1806.

19. Julious SA. 2004. Using confidence intervals around individual means to assess statistical significance between two means. *Pharmaceutical Statistics* 3:217-222.

20. Payton ME, Greenstone MH, Schenker N. 2003. Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical significance? *Journal of Insect Science* 3:article 34.

21. Croteau M-N, Luoma SN. 2009. Predicting dietborne metal toxicity from metal influxes. *Environmental Science and Technology* 43:4915-4921.

22. Cain DJ, Croteau M-N, Fuller CC. 2013. Dietary bioavailability of Cu adsorbed to colloidal hydrous ferric oxide. *Environmental Science and Technology* 47:2869-2876.

23. Cain D, Croteau MN, Fuller CC, Ringwood AH. 2016. Dietary uptake of Cu sorbed to hydrous iron oxide is linked to cellular toxicity and feeding inhibition in a benthic grazer. *Environmental Science and Technology* 50:1557-1560.

CHAPTER 5

CONCLUSIONS

5.1 Summary of results and implications

Metals are ubiquitous in surface waters and usually are present as mixtures instead of as lone metals. Anthropogenic discharges from municipalities, industries, and agriculture further contribute to mixtures of metals at various concentrations. Metals have the potential to impair aquatic biota, depending on the concentrations of the metals relative to their toxic effects, but prediction of toxicity of metal mixtures to aquatic organisms remains challenging.

The range of toxic effects I have observed in ternary, binary and even single-metal exposures demonstrates why a reliable predictive model of metal-mixture toxicity would help advance water quality standards to incorporate metal mixtures instead of regulating on a metal-by-metal basis. However, in order to fully explain the mechanism(s) affecting the toxicological outcome of metal mixtures in freshwater, it is necessary to understand not only the interactions of metals with other components of the water chemistry, but also to understand the interactions of metals with the biotic ligand. In addition, it is necessary to develop a quantitative and robust statistical approach with which the additivity or non-additivity of metal-mixture toxicity can be inferred.

Challenges associated with statistical determination are rooted in the concept that a specific mixture of two or more metals will be distinctly defined as either additive or non-additive. In reality, mixture additivity may fluctuate depending on the chemical concentration. For instance, if the concentration of one metal is varied across a gradient that causes from 0 to 100% mortality, while a very low concentration of a second metal is held constant throughout the entire treatment, this mixture may yield a concentration-response curve that is not statistically

different from the concentration-response curve of the first metal alone. However, the same experiment using higher concentrations of the background metal might easily be determined not-statistically the same as the original concentration-response curve, and therefore non-additive. In this hypothetical instance, although most toxicologists would characterize the mixture of these two metals as non-additive, the mixture is only conclusively non-additive when the background metal is held at (or perhaps, above) a specific concentration. To avoid over generalizing, especially at non-ecologically relevant levels, I argue that implementing more specific defining factors, such as a numerical value, to non-additive toxicities would result in conclusions that may be more pertinent to governing bodies.

Specifically, defining mixtures by the concentrations at which the "greatest response-additive effect" or the "lowest non-additive effect" occur would both succinctly characterize a particular mixture and convey whether or not the non-additive effect would be applicable to a specific system. This scheme is not without flaws, as numerous studies have reported conflicting results regarding additivity of specific metal mixtures, depending on factors such as: the type of organism tested, the solution matrix, and chronic v. acute toxicity tests. As such, these parameters still need to be defined in the literature alongside the quantitative evaluation of the mixture. This method may also not be applicable in cases of multiple concurrent interactions (i.e. ternary mixtures in Chapter 2), because trends in non-additivity may be greater-than-additive or less-than-additive rather than have a single transition between only response-additivity and non-additivity.

In another publication, Meyer, Farley, and Garman 2015 suggested that it may be advantageous to advance beyond the additivity vs. non-additivity framework and instead focus on developing models that can accurately predict the toxicity of metal mixtures. In any case, contradicting results resulting from variability in experimental design among a plethora toxicity

tests that have been conducted make a strong case for the argument that classification toxicity of mixtures may need to be reevaluated to result in meaningful and consistent conclusions.

My work with *Lymnaea stagnalis* in quantifying the bioavailability of Cu stemmed from our need to more fully understand biogeochemical interactions in the AMD-contaminated North Fork of Clear Creek. The results of these studies indicated that the assimilation efficiency (or bioavailability) of Cu is approximately independent of the type of flocculent material that the organisms are exposed to. This implies that even during remediation in the NFCC, Cu will remain bioavailable and potentially toxic to organisms inhabiting the stream until both the loosely-coated flocculent material and the armored coating have been removed or diminished to undetectable concentrations. As of yet, there is no reliable timetable to determine when this goal can be met.

5.2 Recommendations for future research

Natural water compositions can vary considerably from location to location, even within the same region. As such, it is difficult to draw extensive generalizations about the effects of different metals and mixtures of metals without relying on predictive models. Considerable work remains in order to fully understand the mechanisms behind metal-mixture toxicity before reliable predictive models can be implemented into regulatory frameworks. The overlying goal of my research is to gain a deeper understanding into the mechanisms that influence bioavailability and toxicity, and to determine which factors must be accounted for in the development of predictive models. Such factors may be site-specific (water chemistry, geology, microbial ecology) or organism-specific (BL binding sites, type of organism, organism-specific sensitivity).

To continue this line of work, I plan to continue studying the bioaccumulation and bioavailability models by developing methods that expand the reverse-isotopic tracing methods detailed in Chapter 4 to make the procedure applicable to ecologically relevant species, such as

chironomids, that would better represent montane, AMD-contaminated lotic systems. In addition, I plan to perform additional chemical and toxicological analyses in NFCC to better characterize the Fe^{2+}/Fe^{3+} and determine factors that control the oxidation of iron; and to directly deploy organisms *in-vivo* to understand how variations in the iron composition at an AMD site may affect metal bioavailability and toxicity.

APPENDIX A

A TEST OF THE ADDITIVITY OF ACUTE TOXICITY OF BINARY-METAL MIXTURES OF NI WITH CD, CU, AND ZN TO *DAPHNIA MAGNA*, USING THE INFLECTION POINT OF THE CONCENTRATION-RESPONSE CURVES

Modified slightly from a paper published in *Environmental Toxicology and Chemistry*[1]

Elizabeth M. Traudt[2,3], James F. Ranville[4], Samantha A. Smith[5], Joseph S. Meyer[6]

A.1 Abstract

Mixtures of metals are often present in surface waters, leading to toxicity that is difficult to predict. To provide data for development of multi-metal toxicity models, *Daphnia magna* neonates were exposed to individual metals (Cd, Cu, Ni, Zn) and to binary combinations of those metals in standard 48-h lethality tests conducted in USEPA moderately hard reconstituted water with 3 mg DOC/L added as Suwannee River fulvic acid. Toxicity tests were performed with mixtures of Ni and (1) Cd, which is considerably more toxic than Ni, (2) Cu, which is less toxic than Cd but more toxic than Ni, and (3) Zn, which has a toxicity threshold similar to Ni. For each combination of metals in the binary mixtures, the concentration of one metal was held constant while the second metal was varied through a series that ranged from nonlethal to lethal concentrations; then the roles of the metals were reversed. Inflection points of the concentration-response curves were compared, to test for additivity of toxicity. Sub-lethal concentrations of Ni caused less-than-additive toxicity with Cd, slightly less-than-additive toxicity with Zn, and greater-than-additive toxicity with Cu. One explanation of these results might be competition

1. Reprinted with permission of ET&C, 2015
2. Ph.D. Candidate, Department of Chemistry and Geochemistry, Colorado School of Mines
3. Primary researcher and author
4. Associate Professor, Department of Chemistry and Geochemistry, Colorado School of Mines
5. Undergraduate student, Department of Chemistry, Colorado School of Mines
6. Applied Limnology Professionals LLC; and Affiliated Faculty Member, Colorado School of Mines

among the metals for binding to biological ligands and/or dissolved organic matter. Therefore, models might have to incorporate sometimes competing chemical interactions in order to accurately predict metal-mixture toxicity.

A.2 Introduction

Surface waters can be contaminated by metals through a variety of pathways including point sources such as discharges from industries and municipal wastewater treatment plants, and nonpoint sources such as storm-water runoff, agricultural runoff, and mineral weathering [1]. Because of the variety of anthropogenic sources and the natural occurrence of combinations of metals in ore bodies, metals are rarely present alone in natural systems. Instead, metals usually occur as mixtures in soils, ground waters, and surface waters. However, metals usually are regulated on an individual basis instead of as mixtures, in part because current understanding does not allow accurate prediction of the toxicity of metal mixtures to aquatic organisms [2]. Therefore, in order to help develop more comprehensive approaches to water quality regulations, it is important to have a better understanding of metal-mixture toxicity that can lead to improved predictive models.

Numerous studies have demonstrated a relationship between water chemistry (e.g., pH, alkalinity, hardness, dissolved organic carbon [DOC] concentration) and the toxicity of metals to aquatic organisms [3,4]. In general, those water chemistry parameters can protect against metal toxicity either by complexing with the metals to form less bioavailable forms (e.g., as with HCO_3^-, CO_3^{2-}, and DOC) or by competing with the metals for binding at sites of toxic action on or in organisms (e.g., as with H^+, Ca^{2+}, and Mg^{2+}) [5,6]. However, it is not currently well-understood how the interactions of multiple metals with those water chemistry parameters and with aquatic organisms affect the toxicity of metal mixtures [2]. For example, metal-metal competition for

binding to dissolved ligands such as DOC might increase the concentration of the free metal ion of 1 or more metals in the exposure water, thus potentially leading to more toxicity than would be expected. Metal-metal competition for binding at sites of toxic action might decrease the accumulation of 1 or more metals at those sites, thus potentially leading to less toxicity than would be expected [2]. Therefore, metal-mixture toxicity to a given organism might be less-than-additive (i.e., less than expected from a simple combination of the effects of the individual-metal exposures [2]), additive (i.e., as expected), or more-than-additive (i.e., more than expected), depending on the metals in the mixture. Meyer et al. [7] demonstrated examples of each of those possible situations with *Daphnia magna* exposed to binary mixtures of Cd, Cu, and Zn.

Bioavailability models such as the Biotic Ligand Model (BLM) [8] and the Windermere Humic Aqueous Model-Toxicity Function (WHAM-F_{TOX}) [9] are being developed to predict the effects of metal mixtures on aquatic organisms in a variety of water chemistry conditions, based on metal accumulation on a target tissue (i.e., the biotic ligand [BL] in the BLM) or a chemical surrogate (i.e., WHAM humic acid in WHAM-F_{TOX}) [10]. Both models assume that the aquo ("free" metal) ion is bioavailable and capable of causing toxic effects to an organism, but that it must compete with other cations (including the other metals in the mixture) in order to accumulate at sites of toxic action and potentially result in toxicity. However, only a small number of studies that have adequate water chemistry and toxicity data are currently available to parameterize such models [11-13].

Two general approaches are used to predict mixture toxicity from the toxicity of the individual components of the mixture: concentration additivity and response additivity [2]. Concentration addition is generally assumed to be appropriate when the chemicals have the same mechanism of toxic action (e.g., Cd and Zn impair Ca homeostasis [14]). Response addition is

generally assumed to be appropriate when the chemicals have different mechanisms of action (e.g., impairment of Na homeostasis by Ag or Cu [14] versus impairment of Ca homeostasis by Cd and Zn). In the present study, response addition was chosen to evaluate the toxicity of the metal mixtures, because the exact mechanism of toxic action of one of the metals (Ni) that was included in each tested binary mixture is not known [14].

In response addition (also referred to as independent action [2]), one substance in a mixture does not change the determined outcome of another substance in the system [15], because the toxicants in a chemical mixture are assumed to act independently at different sites of toxicity [16]. As a result, substances in a mixture produce an effect that is the joint but independent probability of their individual effects. However, recent studies have shown that the toxicity of metal mixtures sometimes deviates from response-additive predictions [7]. Aqueous metal speciation, metal-metal competition for binding to dissolved ligands, and metal interactions within an organism can complicate this system to an extent that metal-mixture toxicity cannot be accurately explained using either a concentration-addition or a response-addition assumption if dissolved-metal concentrations are used as the predictor of toxicity [2]. This is important because the toxicity of more-than-additive metal mixtures will be under-predicted, thus potentially causing unanticipated ecological effects; whereas less-than-additive metal mixtures may be of concern to industrial and municipal dischargers because over-predicted toxicity may lead to overly-restrictive and cost-ineffective regulation. Better understanding of metal-mixture toxicity should lead to improved, more accurate metal-mixture models that can then be used for more cost-effective regulation [2].

In the present study, results are presented from acute toxicity tests in which *D. magna* were exposed to binary mixtures of Ni combined with Cd, Cu, or Zn. These 4 metals were chosen

because their toxicity to aquatic organisms has been studied extensively as individual metals and because they also can be found in association in surface waters that receive discharges from industry, municipalities, or mine drainage [17]. The present study extends an earlier study of the acute toxicity of binary mixtures of Cd, Cu, and Zn to *D. magna* [7] and provides additional evidence of the less-than-additive, additive, and more-than-additive toxicity interactions reported by those authors.

A.3 Materials and methods

A.3.1 Test organisms

Daphnia magna were used in all the toxicity tests. Neonates were obtained from Aquatic Biosystems, Inc, in Fort Collins, Colorado in moderately hard water reconstituted water (MHRW) [18] with the green alga *Pseudokirchneriella subcapitata* as food. The neonates were sent via same-day shipping to ensure that all organisms were less than 24 h old at the start of the toxicity tests. Because the toxicity tests were started as soon as a shipment arrived at the lab, the *D. magna* were not fed again.

A.3.2 Exposure water

The exposure water in the toxicity tests was MHRW to which 3 mg DOC/L was added as Suwannee River fulvic acid obtained from the International Humic Substances Society (http://www.humicsubstances.org/). Fulvic acid was added to the exposure waters to provide a concentration of organic carbon that is more representative of surface waters than the low background concentration of ≤0.5 mg DOC/L in Milli-Q water [7,19]. Metals were added to that exposure matrix as acidified (2-5% nitric acid) standards that are used for atomic absorption and inductively coupled plasma spectroscopy analyses (EM Science, Merck). Exposure solutions were

prepared 24 to 36 h before the start of a toxicity test, to allow equilibration of the metals with the DOC [20].

A.3.3 Toxicity tests

The toxicity of individual metals and binary mixtures was determined in 48-h static, non-renewal lethality tests, following procedures prescribed by the U.S. Environmental Protection Agency [18]. The binary-metal tests comprised a series of either 6 or 12 combinations of metal concentrations in a gradient designed to produce mortalities ranging between 0 and 100%. In the binary mixtures, the concentration of Metal #1 was held constant throughout the entire series while the concentration of Metal #2 was increased incrementally in the series; and then the metals were reversed in separate tests (i.e., the former Metal #2 became the constant-concentration metal, and the former Metal #1 became the varied-concentration metal), as in the experimental design used by Meyer et al. [7]. Individual-metal toxicity tests were conducted concurrently with each binary mixture and comprised a dilution series of 6 concentrations (including a control) for each metal. For example, if the metals in the binary mixture were Ni and Zn, Ni-only and Zn-only toxicity tests were also conducted. In all tests, each metal concentration or mixture was tested in 4 replicate chambers, each containing 25 mL of exposure water and 5 organisms. Therefore, a total of 20 organisms were exposed to each concentration in the individual- or binary-metal gradient. The number of dead organisms was recorded at 24 and 48 h, with immobilization as the indicator of mortality [18].

Testing of the binary-metal mixtures extended over a 14-month period from September 2012 to November 2013. All tests were conducted in incubators (VWR International) at a temperature of 20±2°C, with a 16h-8h light-dark cycle.

A.3.4 Chemical analyses

At 0 and 48 h, the pH, dissolved oxygen concentration, and temperature were recorded. Unfiltered water samples for analysis of total organic carbon (TOC) concentration were collected from the control and the highest metal concentration at the beginning of a test and preserved by addition of H_3PO_4 to pH <2. The alkalinity of each batch of MHRW was determined before testing. At the beginning of a test, unfiltered water from each treatment in the metal-concentration series was acidified to pH $\leq$2 with 2% Optima HNO_3 and then submitted for elemental analysis.

Water samples from all controls and exposure concentrations were analyzed at the beginning of the toxicity tests for total concentrations of metals (including Cd, Cu, Ni, and Zn), major inorganic cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), and sulfur using an Optima 5300 ICP-OES inductively coupled plasma optical emission spectrophotometer (PerkinElmer). The exposure waters were not filtered before analysis because particles were unlikely in these synthetic waters and preliminary tests demonstrated that commercial filters either sorb metals from, or leach metals into, initial volumes of water that are passed through the membranes [7]. Consequently, the small volumes (< 100 ml) of exposure waters used in these *D. magna* toxicity tests were not sufficient to adequately rinse the filters or exceed their sorption capacity. Additional preliminary tests, in which sufficient sample was used to saturate the filter membrane, demonstrated that the metals added to MHRW were >90% dissolved [7]. Therefore, the total-metal concentrations were assumed to closely approximate the dissolved-metal concentrations.

Temperature and dissolved oxygen were measured using a YSI 55 probe (YSI Incorporated), and pH was measured using an Orion ROSS electrode and Orion 2 STAR meter (Thermo Fisher Scientific) calibrated with pH 4, 7, and 10 buffers. Alkalinity was analyzed in the MHRW that was used to prepare all the exposure waters by titration with H_2SO_4 to the bromo-

cresol green endpoint [21]. Changes in alkalinity at the various metal concentrations were modeled using WHAM VII software (http://www.ceh.ac.uk/products/software/wham/). Total organic carbon concentrations were analyzed by UV-catalyzed persulfate oxidation using a Sievers Model 900 TOC Analyzer (GE Analytical Instruments).

Sulfate concentrations in all controls and metal exposures were calculated by assuming all the sulfur measured by ICP-OES was present as SO_4^{2-}, and chloride concentrations were calculated by assuming the molar Cl^- concentration equaled the measured molar K^+ concentration (because the only Cl^- in the MHRW recipe was added as KCl; [18]).

In all ICP-OES analytical runs, a Sc internal-calibration standard was continuously introduced into the plasma along with each sample, and samples were analyzed in triplicate. Quality assurance/quality control (QA/QC) samples included deionized water blanks (Barnstead Nanopure system, Thermo Fisher Scientific) that contained trace-metal-grade HNO_3 (Thermo Fisher Scientific), and certified continuing calibration verification (CCV) standards. The QA samples were analyzed immediately after instrument calibration, after every 10 samples, and at the end of each set of samples. Additionally, NIST certified standard reference materials 1640a and 1643e [22] were analyzed for trace elements before and at the end of each set of samples. All samples were reanalyzed in any analytical run in which acceptable QA/QC results were not obtained. Those unacceptable results could include: deviations of the internal Sc standard greater than 20% from the known concentration, deviations of the CCV samples greater than 10% from the known concentrations, or relative standard deviations (RSDs) of triplicate analyses of a sample greater than 10%. The ranges of instrument detection limits for the metals, major cations, and sulfur during the study were: 3-14 μg/L Ca, 0.1-0.2 μg/L Cd, 0.3-0.4 μg/L Cu, 15-27 μg/L K

(equivalent to 13-25 μg/L Cl), 0.1-0.2 μg/L Mg, 6-8 μg/L Na, 1.5-12.1 μg/L S (equivalent to 4.5-37 μg/L SO_4), 0.1-0.4 μg/L Ni, and 0.2-0.4 μg/L Zn.

A.3.5 Data analyses

Toxicity data were analyzed using OriginPro 9.1 Software (OriginLab Corporation). Although it is traditional to compare median effect concentrations (EC50 values) determined from 2 or more concentration-response series to quantitatively characterize and compare toxicity results, this approach was not appropriate in the present study when high background mortality was caused by the constant-concentration metal (i.e., Metal #1) in the binary-metal series. In such cases, it was more appropriate to ask what concentration of Metal #2 was needed to immobilize 50% of the test organisms that would not have been immobilized by the background concentration of Metal #1 (i.e., what concentration of Metal #2 was needed to halve the residual survival). Therefore, for quantitative comparisons, it was necessary to determine an alternative to the traditional EC50 (i.e., the concentration at which 50% of all the organisms were immobilized). This alternative is denoted herein as the ECx_{infl} (i.e., the concentration at the inflection point of the binary-mixture concentration-response curve, at which a total of x% immobilization would occur) and was determined using normalized distributions of a probability density function (pdf) for the predicted toxicities [23]. The pdfs were established by taking the first derivative of the concentration-response curves that were fitted to the immobilization data using the least-squares log-logit regression (Equation A.1). In this equation, y is the observed immobilization proportion; A_1 is the lower mortality limit; C is the metal concentration (either the concentration in an individual-metal test or the concentration of the varied metal in a binary-metal test); C_0 is the concentration at the center of the distribution (i.e., the ECx_{infl}); and p is the slope of the log-logit regression curve at C_0.

$$y = A_1 + \frac{(100 - A_1)}{1 + 10^{[\log(C_0) - \log(C)]\,p}} \quad \text{(A.1)}$$

The first derivative of these concentration-response curves is the change in immobilization proportion per unit change in the logarithm of the metal concentration. The maximum first derivative (i.e., the slope at the inflection point) in each binary-metal test series was scaled to a maximum possible value of 1.0 (i.e., the maximum first derivative in each test series was normalized by dividing it by the first derivative at the EC50 determined in the corresponding individual-metal toxicity test), from which an x% mortality at the inflection point (x_{infl}) was determined (Equation A.2). In this equation, D is the maximum of the normalized derivative for each curve. This is akin to setting $x_{infl} = 100 \cdot [A_1 + (1 - A_1)/2]$ (i.e., akin to the mortality percentage at which the residual survival is halved).

$$x_{infl} = \left(1 - \frac{D}{2}\right) \text{ x } 100 \quad \text{(A.2)}$$

For a given binary-metal toxicity test, the concentration of Metal #2 at the inflection point of the concentration-response curve (i.e., the ECx_{infl}) was then plotted on the vertical axis of a graph in which the concentration of Metal #1 in the same toxicity test was plotted on the horizontal axis. As the concentration of Metal #1 is increased in a sequence of separate binary-metal tests, a significant decrease or increase of the ECx_{infl} from the EC50 determined in the individual-metal test for Metal #2 provides evidence for more-than-additive or less-than-additive toxicity, respectively, in mixtures containing those two metals. The statistical significance of those deviations was tested by determining whether the 84% confidence interval for a given ECx_{infl} was outside the 84% confidence interval determined for the EC50 values in all individual-metal toxicity tests that were conducted with Metal #2. Overlap of 84% confidence intervals provides a more correct test of a significant difference between two mean values at the 95%

confidence level than the more commonly used but overly-conservative overlap of 95% confidence intervals [24]. In the current study, the widths of 95% confidence intervals on EC50 values calculated using OriginPro 9.1 were divided by the square root of 2 to estimate the widths of the corresponding 84% confidence intervals, as recommended by Meyer et al. [2]. The comparison of ECx_{infl} values to the individual-metal EC50 is valid because, assuming response-additive toxicity, 50% of the residual organisms that would survive exposure to Metal #1 alone will be immobilized regardless of the percentage of the organisms that would be immobilized in the absence of Metal #2 (i.e., 50% residual immobilization will always occur at the individual-metal EC50, Figures A.1 and A.2).

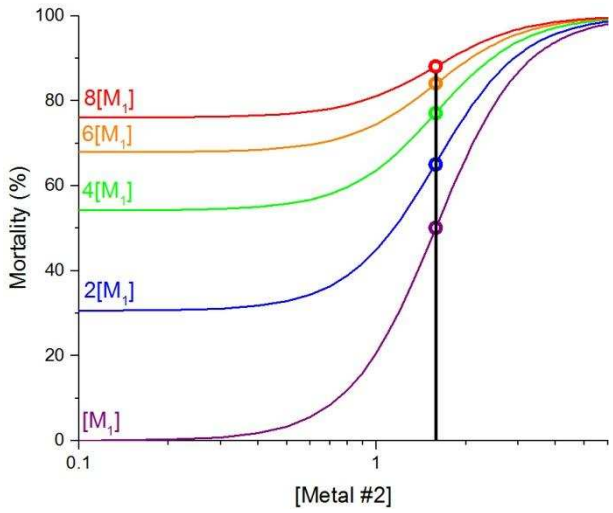


Figure A.1: Hypothetical example of response-additive toxicity. This example is representative of a system with 2 metals in 5 different binary mixtures. In this example, Metal #1 was held constant at 5 different background concentrations (represented by the 5 different colors; [Purple] < [Blue] < [Green] < [Orange] < [Red]) while Metal #2 was increased along a concentration gradient at each background metal concentration. Higher concentrations of Metal #1 cause higher initial mortality, but the inflection point of each curve will occur at the same concentration of Metal #2 (at a concentration of approximately 1.6 in this example) if the toxicity of the metal mixture is response additive.

A.4 Results and Discussion

A.4.1 Individual metals

Based on the EC50 values in the individual-metal toxicity tests, Ni was the least toxic (i.e., it had the highest EC50) of the 4 metals tested in this MHRW, followed by Zn, Cu and Cd in sequence of increasing toxicity (Table A.1). In the Ni-only, Cu-only, and Zn-only tests, the EC50 values among all tests conducted during the 14-month study had minimal variability around a steep concentration-response curve (see coefficients of variation in Table A1). In contrast, the concentration-response curves for Cd were shallower and more variable among the separate tests (see coefficient of variation in Table A1). Meyer et al. [7] reported similar results for Cd toxicity in a variety of organisms, indicating that Cd has a high variability in toxicity that is, as of yet, unexplained. The toxicity of Ni and Zn were more variable than for Cu, but less than for Cd. This intermediate variability of Zn toxicity to *D. magna* is similar to the ranking of toxicity variability among Cd, Cu, and Zn reported by Meyer et al. [7] in the same exposure-water recipe.

A.4.2 Ni and Cd binary mixtures

In MHRW containing 3 mg DOC/L, Ni was approximately 2 orders of magnitude less toxic than Cd (Table A1); and concentrations of Ni in uncontaminated surface waters are generally more than 4 times greater than the concentrations of Cd [25]. For these reasons, the concentrations of Cd used in binary mixture toxicity tests were considerably lower than the Ni concentrations.

When the concentration of Cd was held constant as the Ni concentration was increased, increasing Ni concentrations depressed or, in some cases, wholly eliminated the initial Cd toxicity at Ni concentrations <1 mg/L (Figure A.3). For example, initial mortality in 0.1 mg Cd/L was nearly 100% (caused only by Cd toxicity, because the initial Ni concentration in the several repeat

Table A.1: Summary of toxicity in individual-metal exposures. This table displays the average 48-h median effect concentration (EC50), standard deviation (*SD*), coefficient of variation (*CV* = standard deviation/average), and the number of individual toxicity tests (*n*) performed for immobilization of *Daphnia magna* neonates exposed to Cd, Cu, Ni, or Zn in repeated individual-metal toxicity tests during the 14-month study.

Metal	Average EC50		SD (mg/L)	CV	n
	(mg/L)	(μM)			
Cd	0.054	0.48	0.036	0.661	13
Cu	0.100	1.57	0.019	0.192	15
Ni	1.633	27.8	0.475	0.291	31
Zn	0.928	14.2	0.256	0.276	7

tests always was approximately 100-1,000 times less than the Ni-only EC50). As the added Ni concentration increased, the mortality steadily decreased to a minimum of 5% at approximately 0.5 mg Ni/L, demonstrating a large protection against Cd toxicity by Ni. However, mortality increased when Ni concentrations were high enough to cause Ni-induced toxicity (i.e., when the concentration-response curve of the Cd-Ni mixture became approximately identical to the Ni-only concentration-response curve on the right half of Figure A.3). These results are similar to the large protection against Cd toxicity to *D. magna* by Zn, until the Zn concentration became high enough to cause Zn-induced toxicity (Figure 3B in [7]).

When the roles of Cd and Ni were reversed in a test in which the Ni concentration was held constant and Cd was increased through a concentration series, the concentration-response curves shifted to the right. These shifts also demonstrate a protective effect of Ni against Cd toxicity because, as Ni concentration was increased, higher Cd concentrations were needed to cause the same amount of Cd-induced immobilization. The results of these Cd-Ni toxicity tests are illustrated in Figure A.4E, where the concentration of Cd at the inflection point of the Cd-Ni concentration-response curve (i.e., the ECx_{infl}) is plotted versus the constant Ni concentration in

each test. These ECx_{infl} concentrations can be compared to the solid horizontal line that represents the Cd-only EC50 (i.e., the Cd concentration at the inflection point of the Cd-only curve). If the 2 metals had acted independently of each other (and thus the toxicity of the Cd-Ni mixtures had been response-additive), the inflection point of each Cd-Ni toxicity curve should have occurred at approximately the Cd-only EC50. Instead, the Cd ECx_{infl} values increased as the Ni concentration in the exposure waters increased in the separate sets of Cd-Ni mixture tests, demonstrating less-than-additive toxicity.

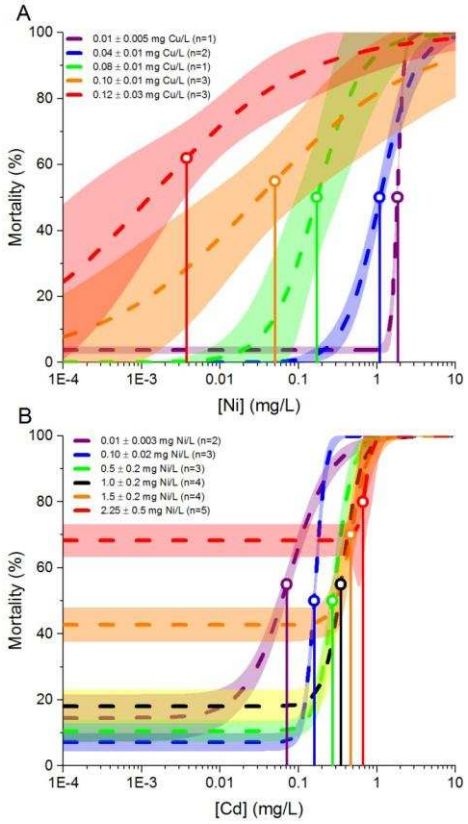


Figure A.2: Concentration-response curves of select binary-metal mixtures. (A) Concentration-response relationship when a constant concentration of Cu is present with increasing concentrations of Ni. The inflection points of the curves shift towards lower concentrations as the concentration of Cu is increased. Because less Ni is required to elicit mortality as the background Cu concentration is increased, the toxicity of this mixture is more-than-additive. (B) Mixtures of constant Ni concentrations with increasing concentrations of Cd. The inflection points of the curves shift to a higher concentration of Cd as the concentration of Ni is increased, thus illustrating less-than-additive toxicity.

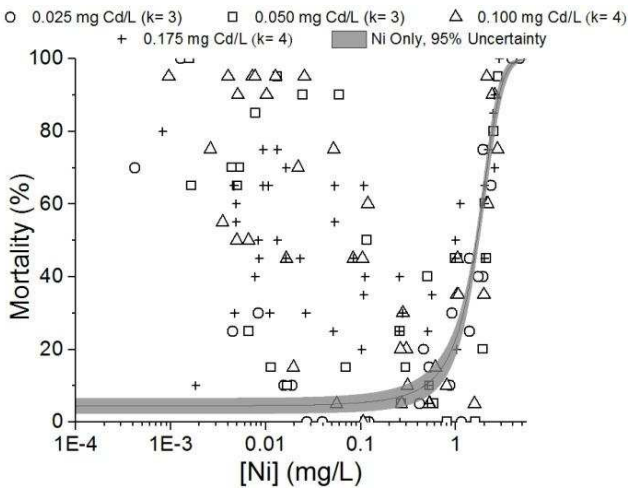


Figure A.3: Toxicity of constant Cd concentrations with varied concentrations of Ni. This graph illustrates the mortality (determined by immobilization of *Daphnia magna* neonates) in 48-h exposures to Cd-Ni mixtures, in which a constant background concentration of Cd was maintained while Ni was varied along a concentration gradient. Different background concentrations of added Cd were used in different sets of toxicity tests, but with replicate tests conducted at each Cd concentration (k equals the number of replicate tests, indicated in the legend). As the concentration of Ni was increased, the Cd-induced mortality decreased until Ni concentrations reached the range at which Ni-induced mortality began to increase, represented by the rising portion of the shaded Ni-only mortality band.

The literature contains few reports of Cd-Ni binary toxicity tests. However, our results differ from Martin et al. [26], who reported that reproduction of a terrestrial nematode (*Caenorhabditis elegans*) in a binary combination of Cd and Ni did not deviate from predictions of the independent-action model. This lack of agreement between studies may be a result of differences in (1) bioavailability of the metals in the exposure media (growth-medium agar for the nematode, and water for *D. magna*), (2) the physiology of the organisms, (3) acute and chronic toxicity of Cd-Ni mixtures, and/or (4) geochemical interactions of the metals in the different concentration ranges in which the Cd-Ni mixtures were tested.

The mechanism(s) underlying the protective effects of one metal against the toxicity of another metal are difficult to determine. The lessening of toxicity may be caused by chemical interactions between the metals while in the bulk exposure water, chemical interactions between the metals at the BL site (e.g., competitive metal binding that alters the impairment of the

organism's uptake of major ions like Na^+ and Ca^{2+}), physiological interactions between the metals after they enter the organism, or any combination of those processes. However, the results of the present study are consistent with the hypothesis that chemical interactions between the metals at the BL caused the less-than-additive toxicity in the Cd-Ni mixtures, wherein Ni at sublethal concentrations out-competed Cd at lethal concentrations for binding to the BLs and, as a result, decreased the Cd-induced toxicity. This is similar to the mechanism suggested by Meyer et al. [7] for *D. magna* exposed to Cd-Cu and Cd-Zn mixtures, and it is consistent with the suppression of Cd uptake by *D. magna* when the concentration of Ni in Cd-Ni mixtures is increased [27]. Bioavailability models like the BLM and WHAM-F_{TOX} account for chemical interactions of metals among each other and with major inorganic ions and dissolved organic matter (DOM), but they do not yet account for potential physiological interactions.

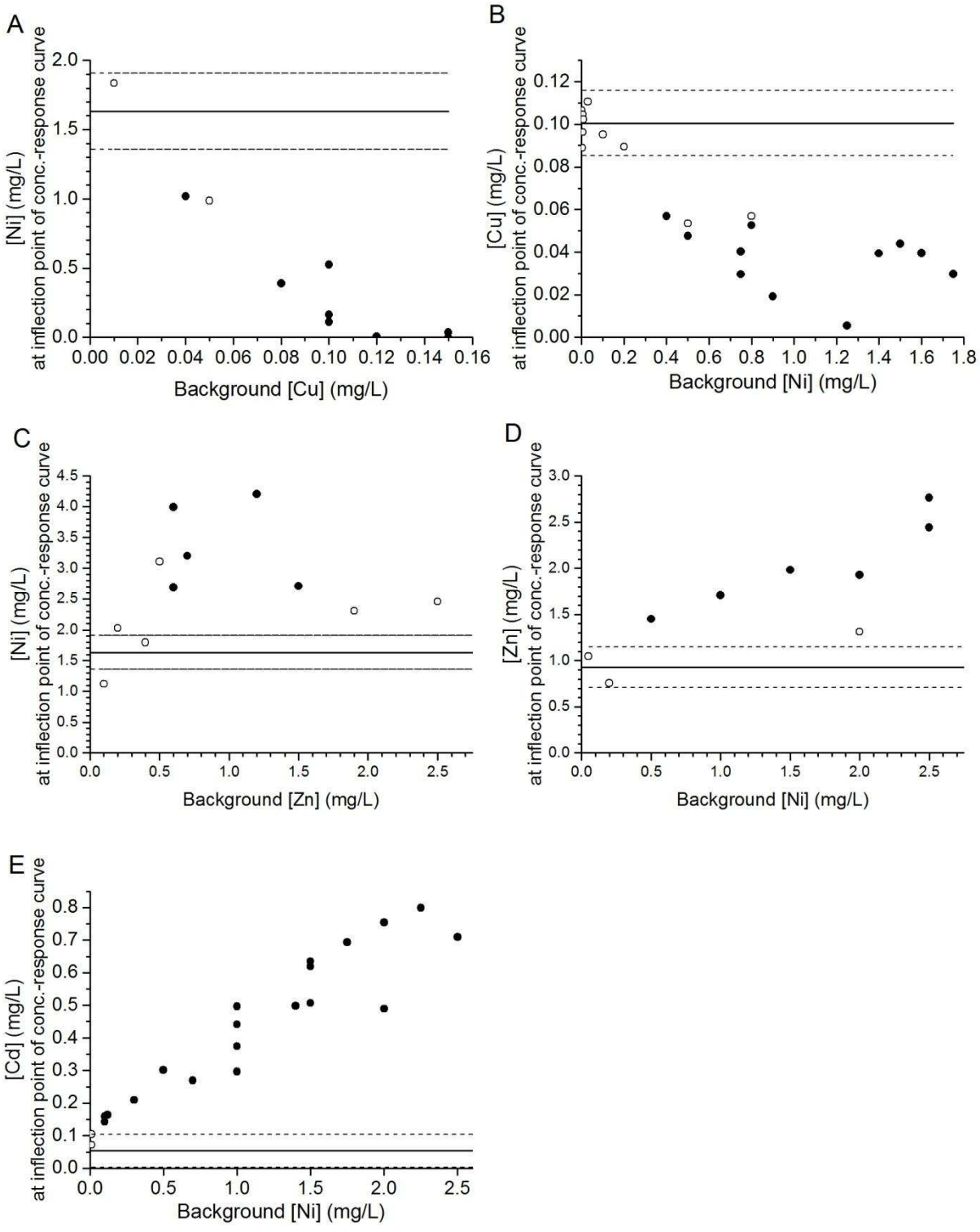
A.4.3 Ni and Cu binary mixtures

In MHRW containing 3 mg DOC/L, Ni is approximately 1 order of magnitude less toxic than Cu (Table 1). Although the ratios of the concentrations of Cu and Ni used in these binary-metal toxicity tests do not necessarily reflect their natural abundance ratios, the concentrations produced a full range of immobilization percentage with emphasis on determining the inflection points on the concentration-response curves for the mixtures.

Each binary combination of Cu and Ni produced evidence of more-than-additive toxicity, because the inflection points of the concentration-response curves for the mixtures (i.e., the ECx_{infl} values) shifted to progressively lower concentrations than the individual-metal EC50 of the varied metal, as the concentration of the constant "background" metal was increased (Figures A.4A and A.4B). In mixtures in which the Cu concentration was held constant and the Ni concentration was varied, the Ni ECx_{infl} values should have remained constant at approximately

1.63 mg Ni/L if the mixture toxicity had been additive; but instead, the ECx_{infl} concentrations began decreasing at background Cu concentrations as low as approximately 0.02 to 0.04 mg Cu/L and eventually decreased to less than 0.1 mg Ni/L in the presence of background Cu concentrations greater than or equal to approximately 0.1 mg Cu/L (i.e., at greater than or equal to approximately the Cu-only EC50; Figure A.4A). Similarly, in mixtures in which the Ni concentration was held constant and the Cu concentration was varied, the Cu ECx_{infl} values should have remained constant at approximately 0.10 mg Cu/L if the mixture toxicity had been additive; but instead, the ECx_{infl} concentrations decreased to less than or equal to 0.06 mg Cu/L in the presence of background Ni concentrations greater than or equal to approximately 0.4 mg Ni/L (i.e., at greater than or equal to approximately 1/4 the Ni-only EC50; Figure A.4B). This more-than-additive toxicity in mixtures of Cu and Ni supports results reported for *Lebistes reticulatus* [28] but is not consistent with the less-than-additive effect of Cu on Ni uptake in *D. magna* [27]. Results of the present study are consistent with the hypothesis that chemical interactions in the bulk exposure water caused the more-than-additive toxicity in the Cu-Ni mixtures. If hydrated "free" nickel ions (represented as Ni^{2+}) competed with hydrated "free" Cu ions (represented as Cu^{2+}) for binding to carboxylic or phenolic sites on the DOM, the concentrations of Cu^{2+} and/or Ni^{2+} (the purportedly most bioavailable forms of those metals) in the exposure water would have increased as the total dissolved concentration of either Cu or Ni was increased. Although Cu is generally assumed to have a higher affinity for DOM than Ni has [29,30], the outcome of competitive binding is determined by the mathematical product of the free-ion concentration and the binding affinity of the ion (i.e., the outcome is determined by $[M^{2+}]\cdot K_{M\text{-}DOM}$ instead of only by $K_{M\text{-}DOM}$; where $[M^{2+}]$ is the molar concentration of the "free" ion, and $K_{M\text{-}DOM}$ is the binding constant between the "free" ion and DOM) [2]. Therefore, the higher concentration of Ni than Cu

Figure A.4: Toxicity of Cd-Ni, Cu-Ni, and Zn-Ni mixtures to *Daphnia magna* neonates. Toxicity tests took place in 48-h exposures in which the concentration of the metal that remained constant throughout a test is plotted on the horizontal axis versus the concentration at the inflection point of the varied metal's concentration-response curve (the ECx_{infl}) plotted on the vertical axis. The solid horizontal line is the average median effect concentration (EC50) of the varied metal, as determined by single-metal toxicity tests run in conjunction with the mixture tests, and the dashed lines represent the 84% confidence limits. Open circles indicate the ECx_{infl} of the binary mixture was not statistically different ($p > 0.05$) from the EC50 of the single-metal toxicity, based on overlap of the 84% confidence intervals for the single-metal EC50 and the mixture ECx_{infl} values (see text). Closed circles indicate trials in which the ECx_{infl} differed significantly from the single-metal EC50 ($p \leq 0.05$). The tests represented in each graph are: (A) a constant background concentration of Cu while Ni was varied along a concentration gradient, (B) a constant background of concentration of Ni while Cu was varied along a concentration gradient, (C) a constant background concentration of Zn while Ni was varied along a concentration gradient, (D) a constant background concentration of Ni while Zn was varied along a concentration gradient, and (E) a constant background concentration of Ni while Cd was varied along a concentration gradient.


A
[Ni] (mg/L) at inflection point of conc.-response curve
Background [Cu] (mg/L)
B
[Cu] (mg/L) at inflection point of conc.-response curve
Background [Ni] (mg/L)
C
[Ni] (mg/L) at inflection point of conc.-response curve
Background [Zn] (mg/L)
D
[Zn] (mg/L) at inflection point of conc.-response curve
Background [Ni] (mg/L)
E
[Cd] (mg/L) at inflection point of conc.-response curve
Background [Ni] (mg/L)

could have allowed Ni to compete effectively with Cu. Because Cu is more toxic than Ni, increased Cu^{2+} concentrations in the exposure water (relative to the concentrations that would be expected in the absence of Ni) probably account for the more-than-additive toxicity in the Cu-Ni mixtures. This metal-metal competition for binding to DOM is similar to the mechanism suggested by Meyer et al. [7] for *D. magna* exposed to Cu-Zn mixtures.

A.4.4 Ni and Zn binary mixtures

In MHRW containing 3 mg DOC/L, Ni and Zn had similar toxicity (Table A.1). The EC50 values for Ni and Zn were 1.63 ± 0.48 and 0.93 ± 0.28 mg/L (average $\pm$ s.d.), respectively, which allowed the Ni-Zn mixture concentrations to be representative of concentrations possibly seen in heavily-contaminated acid mine drainage sites [31,32]. Furthermore, Ni and Zn are used extensively in current technologies, which make them likely co-occurring constituents of industrial discharges into surface waters [33].

In mixtures in which the Ni concentration was held constant as the Zn concentration was increased, the toxicity was additive to less-than-additive (i.e., as the "background" Ni concentration was increased, the Zn ECx_{infl} values generally increased or did not differ significantly from the Zn-only EC50; Figure A.4D). That pattern is consistent with a hypothesis that chemical interactions at the BL caused the less-than-additive toxicity in the Ni-Zn mixtures, wherein Ni at sublethal concentrations competed with Zn at lethal concentrations for binding to the BL(s). That pattern is also consistent with the slight inhibition of Zn uptake by *D. magna* in the presence of low Ni concentrations (0.1-0.25 uM) [27], although that decrease of Zn uptake rate was not statistically significant. Two of the 3 Ni-Zn tests in which the mixture toxicity was not significantly non-additive had low background Ni concentrations (i.e., less than or equal to 0.2 mg Ni/L; Figure A.4D), at which Ni-Zn competitive interactions might have been relatively

small and thus more difficult to detect. Only 1 of the 9 Ni-Zn tests in which Ni was the constant-concentration background metal had a Zn ECx_{infl} that did not differ significantly from the Zn-only EC50 (i.e., the Zn ECx_{infl} was only 1.3 times the Zn EC50). That test had at a high background Ni concentration (2.0 mg Ni/L; Figure A.4D), and the reason for that relatively low Zn ECx_{infl} is unknown (when all other Zn ECx_{infl} values in that range of background Ni concentrations from 1.5 to 2.5 mg Ni/L were at least twice as high as the Zn-only EC50). However, in general, metal-metal competition likely decreased the Ni- or Zn-induced toxicity, which may be analogous to the mechanism hypothesized above for the less-than additive toxicity in Cd-Ni mixtures.

Although also suggesting less-than-additive toxicity, the response was more complicated in mixtures in which the Zn concentration was held constant as the Ni concentration was increased. In those mixtures, the Ni ECx_{infl} doubled at low Zn concentrations of 0.7 mg Zn/L and then tended to decrease but still remained greater than the Ni-only EC50 through Zn concentrations as high as 1.4 mg Zn/L (Figure A.4C). The Ni ECx_{infl} values would be expected to gradually increase as occurred in the Cd-Ni mixtures (Figure A.2B), if the only mechanism controlling the mixture toxicity was competition between Ni and Zn for binding to the BL(s). Therefore, an alternative or additional physiological process might have occurred in the mixtures in which the Zn concentration was held constant while the Ni concentration was varied. Previous studies on the toxicity of Ni-Zn binary mixtures in *L. reticulatus* [28] and *D. magna* [13] indicated that the interaction is difficult to classify. Khangarot et al. [28] reported that in acute toxicity tests with *L. reticulatus*, the toxicity was less than additive at high Zn:Ni ratios but was greater than additive at low Zn:Ni ratios. In contrast, Nys et al. [13] reported that in chronic toxicity tests with *D. magna*, Ni and Zn did not have an interaction when each metal

concentration was below its EC20, but the toxicity became more than additive when both metals were present at a concentration greater than their EC20.

A possible physiological explanation for the results in Figure A.4C is the induction of metallothionein (MT) synthesis by Zn [34], which was also suggested by Meyer et al. [7] as a potential explanation for some unexpected results in Cd-Zn mixtures. If a near-maximal rate of MT production was induced by 0.5 mg Zn/L, and higher Zn concentrations thus would not have induced higher rates of MT production, the Ni $EC_{x_{infl}}$ values would remain approximately constant because the same concentration of Ni would be needed to exceed its sequestration by MT. Analyses of MT concentrations in *D. magna* exposed to various background Zn concentrations as Ni is increased through a concentration series would be needed to test this hypothesis.

A.4.5 Synthesis

Similar to the results reported by Meyer et al. [7] for binary mixtures of Cd, Cu, and Zn and in general similar to results of other metal-mixture studies [35,36], the toxicity of the Cd-Ni, Cu-Ni, and Ni-Zn mixtures in the present study was either less-than-additive, additive, or more-than-additive when based on dissolved-metal concentrations, depending on the combinations of metals and their concentrations. All of the tests were conducted in the same water chemistry, including the same DOC concentration and source, and replicate tests of the same mixture concentrations produced similar results, thus supporting that the results were not due to random variability.

The range of additive and non-additive toxicity when using the same metal (Ni) in binary mixtures with a variety of other metals (Cd, Cu, and Zn) demonstrates why a predictive model that can account for the various chemical interactions of metals with each other in a mixture, with

various components of the exposure water (e.g., pH, alkalinity, major cations, and DOC), and with BLs would help to improve water quality criteria/guidelines to incorporate metal mixtures instead of regulating on a metal-by-metal basis. However, physiological interactions between metals cannot be excluded as an alternative or complementary explanation for the non-additive toxicity among some metals that is apparent when conclusions are based on dissolved-metal concentrations. Additional coordinated datasets with complete water chemistry and acute and/or chronic toxicity data for these and other metal mixtures will be needed to improve the mechanistic basis for predictive models. Acute and chronic toxicity data are needed because different regulatory jurisdictions use acute (e.g., USA) or chronic (e.g., Europe) results as the basis for most of their water criteria or guidelines for metals.

Although testing binary mixtures is a start, metal combinations in natural systems often contain more than just 2 metals and thus have almost unlimited possible combinations of metals and their concentrations. Additionally, the types and extents of interactions among metals might vary as water chemistry varies. Because testing a large number of combinations of metals and their concentrations in a wide variety of water chemistries would be expensive and time consuming, many combinations of metals and water chemistry likely will never be evaluated. Nonetheless, the next logical steps to help parameterize metal-mixture toxicity models would be to systematically study a set of ternary-metal mixtures (e.g., comprising Cd, Cu, Ni, and Zn) and to determine the effects of varying water chemistry on the additivity or non-additivity of the toxicity of the binary- and ternary-metal mixtures. Additionally, testing at lower, more environmentally representative concentrations would be beneficial for modeling the potential chronic toxicity of metal mixtures.

A.5 Conclusions

Considerable work remains to fully understand the mechanisms underlying metal-mixture toxicity and thus to develop predictive, bioavailability-based models. Simple assumptions about additivity of toxicity in metal mixtures sometimes can be under-protective (e.g., more-than-additive toxicity demonstrated for Cu-Ni mixtures in the present study and for some Cu-Zn mixtures in Meyer et al. [7]) and sometimes can be over-protective (e.g., less-than-additive toxicity demonstrated for Cd-Cu, Cd-Ni, and Cd-Zn mixtures in the present study and in Meyer et al. [7]). Competitive chemical interactions of the metals with the components of the exposure water and with BLs undoubtedly will play major roles in bioavailability-based models (e.g., multiple-metal BLMs and WHAM-F_{TOX} [10]), but metal interactions with physiological processes might also have to be incorporated. Simple reliance on dissolved-metal concentrations as predictors of metal-mixture toxicity leaves a cloudy picture, but measured or model-calculated concentrations of "free" metal ions and organism-accumulated metals [37] might help to clarify the picture. To that end, techniques to improve measurement of free-ion activities in metal mixtures and to measure metal accumulation at sites of toxic action (or to measure, for example, genetic markers of the bioavailability of metals in mixtures) could provide independent information for parameterization of metal-mixture models.

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A.7 References

1. Allen HE, Luther GW, Garrison W. 1997. *Metals in Surface Waters*. Ann Arbor Press, Chelsea, MI, USA.

2. Meyer JS, Farley KJ, Garman ER. 2015. Metal Mixtures Modeling Evaluation Project: 1. Background. *Environmental Toxicology and Chemistry* 34:726-740.

3. Bury N, Shaw J, Glover C, Hogstrand C. 2002. Derivation of a toxicity-based model to predict how water chemistry influences silver toxicity to invertebrates. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 133:259-270.

4. Meyer JS, Clearwater SJ, Doser TA, Rogaczewski MJ, Hansen JA. 2007. *Effects of Water Chemistry on Bioavailability and Toxicity of Waterborne Cadmium, Copper, Nickel, Lead, and Zinc to Freshwater Organisms*. Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.

5. Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PG, Delos CG, Di Toro DM, Dwyer RL, Galvez F. 2002. The biotic ligand model: A historical overview. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 133:3-35.

6. Playle R. 1998. Modelling metal interactions at fish gills. *Science of the Total Environment* 219:147-163.

7. Meyer JS, Ranville JF, Pontash M, Gorsuch JW, Adams WJ. 2015. Acute toxicity of binary and ternary mixtures of Cd, Cu, and Zn to *Daphnia magna*. *Environmental Toxicology and Chemistry* 34:799-808.

8. Santore R, Ryan A. 2014. Development and application of a multimetal multibiotic ligand model for assessing aquatic toxicity of metal mixtures. *Environmental Toxicology and Chemistry* 34: 777-787.

9. Tipping E, Lofts S. 2015. Testing WHAM-F_{TOX} with laboratory toxicity data for mixtures of metals (Cu, Zn, Cd, Ag, Pb). *Environmental Toxicology and Chemistry* 34:788-798.

10. Farley KJ, Meyer JS, Balistrieri LS, De Schamphelaere KA, Iwasaki Y, Janssen CR, Kamo M, Lofts S, Mebane CA, Naito W. 2015. Metal Mixture Modeling Evaluation Project: 2. Comparison of four modeling approaches. *Environmental Toxicology and Chemistry* 34:741-753.

11. Van Genderen E, Adams W, Dwyer R, Garman E, Gorsuch J. 2015. Modeling and interpreting biological effects of mixtures in the environment: Introduction to the Metal Mixture Modeling Evaluation Project. *Environmental Toxicology and Chemistry* 34:721-725.

12. Lynch NR, Hoang TC, O'Brien TE. 2015. Acute toxicity of binary-metal mixtures of copper, zinc, and nickel to *Pimephales promelas*: Evidence of more-than-additive effect. *Environmental Toxicology and Chemistry* 35:446-457.

13. Nys C, Asselman J, Hochmuth JD, Janssen CR, Blust R, Smolders E, De Schamphelaere KC. 2015. Mixture toxicity of nickel and zinc to *Daphnia magna* is noninteractive at low effect sizes but becomes synergistic at high effect sizes. *Environmental Toxicology and Chemistry* 34:1091-1102.

14. Niyogi S, Wood CM. 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environmental Science and Technology* 38:6177-6192.

15. Unkelbach H-D, Pöch G. 1988. Comparison of independence and additivity in drug combinations. *Arzneimittel-Forschung* 38:1-6.

16. Drescher K, Boedeker W. 1995. Assessment of the combined effects of substances: The relationship between concentration addition and independent action. *Biometrics* 51:716-730.

17. Butler BA, Ranville JF, Ross PE. 2008. Direct versus indirect determination of suspended sediment associated metals in a mining-influenced watershed. *Applied Geochemistry* 23:1218-1231.

18. USEPA. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th Ed. USEPA, editor, Washington, DC.

19. Thurman EM. 1985. *Organic Geochemistry of Natural Waters*, volume 2. Springer.

20. Ma H, Kim SD, Cha DK, Allen HE. 1999. Effect of kinetics of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* 18:828-837.

21. American Public Health Association American Water Work Association, Water Environment Federation. 2012. *Standard Methods for the Examination of Water and Wastewater*, 22nd ed. American Water Works Association, Denver, CO, USA.

22. Zeisler R, Murphy KE, Becker DA, Davis WC, Kelly WR, Long SE, Sieber JR. 2006. Standard Reference Materials® (SRMs) for measurement of inorganic environmental contaminants. *Analytical and Bioanalytical Chemistry* 386:1137-1151.

23. Tanaka T, Ciffroy P, Stenberg K, Capri E. 2010. Regression approaches to derive generic and fish group-specific probability density functions of bioconcentration factors for metals. *Environmental Toxicology and Chemistry* 29:2417-2425.

24. Julious SA. 2004. Using confidence intervals around individual means to assess statistical significance between two means. *Pharmaceutical Statistics* 3:217-222.

25. Forstner U, Wittmann GT. 1979. *Metal pollution in the aquatic environment*. Springer-Verlag.

26. Martin HL, Svendsen C, Lister LJ, Gomez-Eyles JL, Spurgeon DJ. 2009. Measurement and modeling of the toxicity of binary mixtures in the nematode *Caenorhabditis elegans*—a test of independent action. *Environmental Toxicology and Chemistry* 28:97-104.

27. Komjarova I, Blust R. 2008. Multi-metal interactions between Cd, Cu, Ni, Pb and Zn in water flea *Daphnia magna*, a stable isotope experiment. *Aquatic Toxicology* 90:138-144.

28. Khangarot BS, Durve VS, Rajbanshi VK. 1981. Toxicity of interactions of zinc—nickel, copper—nickel and zinc—nickel—copper to a freshwater teleost, *Lebistes reticulatus* (Peters). *Acta Hydrochimica et Hydrobiologica* 9:495-503.

29. Di Toro DM, Allen HE, Bergman HL, Meyer JS, Paquin PR, Santore RC. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environmental Toxicology and Chemistry* 20:2383-2396.

30. Tipping E, Hurley M. 1992. A unifying model of cation binding by humic substances. *Geochimica et Cosmochimica Acta* 56:3627-3641.

31. Sheoran A, Sheoran V. 2006. Heavy metal removal mechanism of acid mine drainage in wetlands: A critical review. *Minerals Engineering* 19:105-116.

32. Matlock MM, Howerton BS, Atwood DA. 2002. Chemical precipitation of heavy metals from acid mine drainage. *Water Research* 36:4757-4764.

33. Hall RM. 1977. The evolution and implementation of EPA's regulatory program to control the discharge of toxic pollutants to the nation's waters. *Natural Resources Lawyer*:507-529.

34. Klaassen CD, Liu J, Diwan BA. 2009. Metallothionein protection of cadmium toxicity. *Toxicology and Applied Pharmacology* 238:215-220.

35. Norwood W, Borgmann U, Dixon D, Wallace A. 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. *Human and Ecological Risk Assessment* 9:795-811.

36. Vijver MG, Elliott EG, Peijnenburg WJ, de Snoo GR. 2011. Response predictions for organisms water-exposed to metal mixtures: A meta-analysis. *Environmental Toxicology and Chemistry* 30:1482-7.

37. Norwood WP, Borgmann U, Dixon DG. 2013. An effects addition model based on bioaccumulation of metals from exposure to mixtures of metals can predict chronic

mortality in the aquatic invertebrate *Hyalella azteca*. *Environmental Toxicology and Chemistry* 32:1672-1681.

APPENDIX B

SUPPLEMENTAL ELECTRONIC FILES

Supplemental spreadsheet in Appendix B contains data for all single-, binary-, and ternary-metal mixture toxicity tests performed over the course of this study. The mixture combinations (Ni-Cu, Ni-Cd, Ni-Zn, Cd-Ni-Cu, and Cd-Ni-Zn; referenced in Chapter 2 and Appendix A) are divided onto different tabs within the spreadsheet. The spreadsheet also contains a tab that displays the toxicity data for the age related variability (referenced in Chapter 3). Each table contains the date of analysis, the major constituent ions of the water chemistry, pH, temperature, alkalinity, and mortality data of the toxicity test. File is in Excel 2003 format.

Traudt_Thesis_Supplemental.xlsx	Compilation of raw data from acute toxicity tests performed.