2016

Chronic Toxicity of Binary Metal Mixtures of Cadmium-Zinc and Cadmium-Nickelon Daphnia magna

Edgar Ramiro Perez
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

CHRONIC TOXICITY OF BINARY METAL MIXTURES OF CADMIUM-ZINC AND CADMIUM-NICKEL ON DAPHNIA MAGNA

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN BIOLOGY

BY

EDGAR PEREZ

CHICAGO, ILLINOIS

AUGUST 2016

ACKNOWLEDGEMENTS

It is with immense gratitude that I acknowledge the generosity of Fr. Michael J. Garanzini, S.J., past President of Loyola University Chicago and the Loyola University Chicago Council of Regents; they first offered me an opportunity to attend Loyola University with a scholarship so as to complete my Bachelor's degree in environmental science. Without their generosity and trust this thesis would have only remained a dream.

I would also like to especially thank my thesis advisor Dr. Tham Hoang from the Institute of Environmental Sustainability who welcomed me into his Ecotoxicology and Risk Assessment lab, first as an undergraduate volunteer and then as a graduate student and for his guidance throughout all the phases of this project.

I am very much indebted to Dr. Nancy Tuchman, Director of the Institute of Environmental Sustainability and Dr. Theresa Grande, Director of the Biology Graduate School Program for finding me the financial means to complete my graduate studies and whose confidence and emotional support allowed me to reach my dream.

I would also like to thank the remarkable scientists and teachers who were involved in my scientific training here at Loyola University Chicago, especially within the Biology Department. I consider it an honor to have worked and learned from them these past few years. I would especially like to acknowledge my gratitude to Dr. Martin Berg, Dr. Rodney M. Dale, and Dr. Theresa Grande of that same department who

generously served on my committee of readers and whose input and wise advice made me think clearer and deeper and write more succinctly.

Others I would like to extend a thank you include fellow students Ellen Cole, Erik Reynolds, Nadia Ali, Anthony Signa, Tien Trinh, Sylvia Sadowicz, Tam Nguyen, and Ritesh Kashyap for their assistance in the experimental phases of this project. Furthermore, heartfelt thanks go out to the men and woman who work behind the scenes within the Biology Department and the Institute of Environmental Sustainability and whose contributions often go unrecognized but whose work was essential; folks like Audrey Berry and Christine Wolff.

I cannot find words to express my gratitude to Fr. Stephen Mitten, S.J., my best friend for his unfailing support and continuous encouragement. And I owe my deepest gratitude to my wife Deisy Avila for her unconditional love and support, and my son Zyden Pérez for being the center of my motivation and drive.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

This study characterizes binary-metal mixture effects of cadmium (Cd^{2+}) + zinc (Zn^{2+}) and Cd^{2+} + nickel (Ni^{2+}) on *Daphnia magna*. Although acute studies have shown protective Ni^{2+} (Traudt et al. 2016) and Zn^{2+} effects (Meyer et al. 2015) against Cd^{2+} toxicity, no study has fully characterized a protective effect on *D. magna* at several endpoints (survival, reproduction, growth, and accumulation). In this study, the titration design was selected to characterize the 21 day (21-d) chronic effects of the binary-metal mixtures on survival, growth, reproduction, and metal accumulation in *D. magna*. Using this design, increasing concentrations of Zn^{2+} (10, 20, 40, 80, 120, 160 and 200µg/L) and Ni^{2+} (20, 40, 80, 100, 120, 140, and 160µg/L) were titrated against a constant concentration of 1.5µg/L Cd^{2+}. A single-metal assay (Zn^{2+}/Ni^{2+} alone), a Cd^{2+} alone treatment, and a control treatment were concurrently conducted for comparison. The results in this thesis demonstrate that Cd^{2+} alone was highly toxic to *D. magna*. In a mixture with Cd^{2+} and Zn^{2+}, sublethal concentrations of 10 and 20µg/L Zn^{2+} were consistently insufficient to protect *D. magna* from chronic Cd^{2+} toxicity since Cd^{2+} toxic effects were observed on the survival, reproductive, and growth endpoints (Figs. 1, 2, 9), whereas mixtures containing 40, 80, and 120µg/L Zn^{2+} provided strong protective effects to *D. magna* at all the endpoints examined. Higher Zn^{2+} concentrations of 160 and 200µg/L exceeded the necessary concentrations needed to protect *D. magna*, and no protective effects were observed.

In contrast, sublethal and moderate Ni^{2+} concentrations of 20, 40, and 80µg/L were found to strongly protect *D. magna* from chronic Cd^{2+} toxicity, whereas higher Ni^{2+} concentrations (≥100µg/L) exceeded the necessary concentration needed to protect *D. magna*, and Ni^{2+} protective effects were absent. Interestingly, 1.5µg/L Cd^{2+} was found to be protective in the Cd^{2+}-Ni^{2+} mixture containing 100µg/L Ni^{2+}. Additionally, no concentration of Ni^{2+} or Zn^{2+} was found to provide complete protective effects to *D. magna* (i.e. 100% protective effects). Therefore, suggesting that no concentration of Zn^{2+} or Ni^{2+} used in this study, entirely outcompeted the binding of Cd^{2+} ions to the biotic ligand. Embryos analyzed for morphological alterations in both the Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures demonstrate severe developmental defects. The results of the present study are useful for development of environmental quality guidelines for metal mixtures.

CHAPTER ONE

INTRODUCTION

Overview

Through anthropogenic activities, metals are consistently encountered in surface waters at elevated concentrations (Komjarova and Bury, 2014). In this way, humans have degraded the earth through the excessive pollution of aquatic ecosystems with metals introduced from agricultural run-off, waste-water discharges from industries, manufacturing companies, metal-mining facilities, etc. Therefore, humans have negatively impacted aquatic life, and created unhealthy aquatic habitats, driving the decline of biological diversity. Currently, there is a greater need to understand how the toxic mixture of metals affect aquatic organisms in chronic exposures, as opposed to acute exposures. This knowledge is important because most studies have invested large efforts in understanding acute toxicity, rather than chronic toxicity. Although acute toxicity data provide a short-term understanding of how a metal may affect an organism, its usefulness is limited because metals are persistent pollutants in the environment and undergo many chemical transformations during migration between ecosystems (aquatic, terrestrial, atmospheric). Therefore, understanding the mechanisms of metal mixture toxicity and their interactions with biota, will enable scientists to derive more accurate risk assessments for metal mixtures, and may or will stimulate the development of more stringent water quality criteria for chronic metal exposures.

Environmental Concentrations and Sources

The introduction of metals into aquatic ecosystems is a growing global environmental concern. Metals such as cadmium (Cd^{2+}), nickel (Ni^{2+}), and zinc (Zn^{2+}) are of particular concern due to their extensive industrial use. These metals occur naturally in the environment and are continually introduced into aquatic ecosystems through natural processes such as volcanic emissions, and natural processes such as chemical weathering of bedrock, sediment, minerals, and soil. In fact, heavy metals are generally present at low concentrations in aquatic ecosystems, usually too low to cause serious human health effects; however, due to anthropogenic inputs, heavy metal concentrations have been detected at elevated levels in surface waters (lakes, lagoons, river and streams) (Ntakirutimana et al. 2013, Kadirvelu et al. 2011), making the anthropogenic route the principal mode of entry.

Zinc enters the aquatic environment primarily through factory wastewater discharge from the manufacturing of brass and other alloys, automobile equipment, medical tools, domestic appliances, pharmaceuticals, construction materials, and synthesis of animal feed and fertilizers (International Zinc Association (IZA), 1997). Similarly, Ni^{2+} is chiefly introduced due to its expansive use in the manufacturing of stainless steel and other nickel comprising alloys, in the fabrication of nickel-cadmium batteries, production of electronic equipment, smelting, refining, electroplating, and its principal use as a catalyst in the chemical industry (Adriano1986, Eisler 1998). Likewise, Cd^{2+} is principally introduced into the environment through the fabrication of nickel-cadmium batteries, metal-mining facilities, atmospheric deposition, agricultural

run-off, leachate from contaminated sites, and industrial wastewater discharges (Bodar et al. 1988).

Natural Zn^{2+} concentrations may vary substantially in the aquatic environment depending on local geology. For example, in mountain rivers that have very old and heavily weathered mineral formations, Zn^{2+} concentrations may be <10µg/L, whereas concentrations of Zn^{2+} in rivers that flow through zinc-rich mineral formations may reach as high as 200µg/L (IZA 1997). In urban stormwater runoff however, Zn^{2+} concentrations can range between 10 and 2400µg/L (Cole et al. 1984), and concentrations can be even higher near industrial sites because urban stormwater generally has less Zn^{2+} than industrial stormwater. On the other hand, natural Ni^{2+} concentrations in surface and ground water are known to range between 3 and 10µg/L (Agency for Toxic Substances and Disease Registry (ATSDR), 1997); but in urban stormwater runoff, Ni^{2+} concentrations can range between 1 and 182µg/L (Cole et al. 1984). In fact, near nickel ore mining sites, surface water Ni^{2+} concentrations have been detected to be as high as 75 to 200µg/L (Cempel and Nikel, 2006), and 2 to 20,000µg/L at hazardous national priority list (NPL) sites (ATSDR 2005). Groundwater Ni^{2+} concentrations have been documented to range between 4.2 to 11,400µg/L at NPL sites (ATSDR 2005). Natural concentrations of Cd^{2+} in surface and groundwater tend to be relatively low, usually <1µg/L (ATSDR 2012). However, Cd^{2+} concentrations ≥1,000µg/L have been documented near Cd-bearing mineral deposits (Thornton, 1992). Cole et al. (1984), reported Cd^{2+} concentrations ranging between 0.1 to 14µg/L in urban stormwater run-off, but polluted

aquatic ecosystems near Cd-emitting industries have been reported to contain higher concentrations, as high as 77µg/L (ATSDR 2012).

According to the EPA aquatic life criteria (EPA 2016 update), the chronic concentrations of Cd^{2+}, Ni^{2+}, and Zn^{2+} pose no health risks to the majority of aquatic organism if they are less than 0.72µg/L, 52µg/L, and 120µg/L respectively. The value in the criteria was expressed as a function of 100mg/L as $CaCO_3$ hardness and in terms of dissolved equivalents of the metals. It is important to note that the toxicity of these metals may be enhanced in soft water, or reduced in hard water, depending on the concentration of Ca^{2+} and Mg^{2+} ions (hardness).

Speciation and Bioavailability

In most aquatic ecosystems, the dominant species of Cd, Ni, and Zn is the hydrated divalent cation Cd^{2+}, Ni^{2+}, and Zn^{2+} (ATSDR 2005, 2012). In surface water, these metals can exist in both suspended and dissolved forms. However, higher importance is given to the dissolved ionic forms of metals because they are assumed to be the most bioavailable species to aquatic biota (Pagenkopf 1983, Paquin et al. 2002). Metal speciation and bioavailability is heavily dependent on water quality parameters such as pH, alkalinity, hardness, suspended particulate solids, and dissolved organic matter (DOC) (Amra, 2012). When DOC is present in the environment, free ionic metals (e.g., Ni^{2+}, Cd^{2+}, Zn^{2+}) can complex with DOC (i.e. humic and fulvic acids) and form metal-DOC complexes. This complexation will decrease concentrations of free ionic metals and therefore decrease metal bioavailability and toxicity.

Metals can also adsorb onto suspended particles and bind to inorganic ligands, such as anions resulting in decreased bioavailability. For example, in surface waters, Zn^{2+} binds to suspended solids or sediments through adsorption onto clay minerals, organic substances, or hydrous iron and manganese oxides (ATSDR 2005). Similarly, Ni^{2+} and Cd^{2+} adsorb onto hydrous mineral surfaces of aluminum, iron, and manganese oxides (Rai and Zachara 1984; Evans 1989; ATSDR 2012). For instance, in surface waters containing high concentrations of organic matter, more than half of the available Cd^{2+} will form complexes, or chelate with the organic material (McComish and Ong 1988). Indeed, Cd^{2+} forms strong bonds with organic matter (ATSDR 2012), and its affinities with complexing agents tend to follow this order: humic acids > CO_3^{2-} > OH^- > Cl^- > SO_4^{2-} (EPA 1979). Similarly, most of the Ni^{2+} in water is associated with organic matter (ATSDR 2005). Ultimately, complexation and adsorption reduces the concentration and bioavailability of metals in the water column, therefore, reducing toxicity.

The fate and mobility of heavy metals such as Cd^{2+}, Ni^{2+}, and Zn^{2+} depends primarily on partitioning between solid and soluble phases, which is driven predominantly by surrounding pH. This suggests that pH plays a crucial role in facilitating chemical transformations that lead to precipitation, complexation, and adsorption. For instance, Zn^{2+} remains as a hydrated free ion at low pH values, thereby enabling its transport in unpolluted water, because it easily adsorbs onto suspended solids (ATSDR 2005). However, in polluted waters containing high Zn^{2+} concentrations and pH values greater than 8, Zn^{2+} precipitates by complexing with OH^- ions to form solid zinc hydroxide ($Zn(OH)_2$) (ATSDR 2005). Similarly, in unpolluted waters with low pH

values, the hydrated Cd^{2+} ion is expected to predominate; but in polluted waters with pH ranges between 8.5 and 11, Cd^{2+} precipitates (Alabaster, 1982). Cadmium (Cd^{2+}) generally precipitates to form insoluble cadmium carbonate ($CdCO_3$), cadmium oxide (CdO), and cadmium sulfide (CdS: under reducing conditions) (ATSDR 2012). The same concept follows in the precipitation and availability of Ni^{2+}. In its entirety then, precipitation is an important process that drives the removal of metals from the water column to the sediment under high pH values, reducing the toxicity and bioavailability of a metal. In contrast, as pH values decrease, insoluble precipitates become soluble and partition into the water phase (EPA 1979). Thus, as pH decreases, the solubility of the precipitates increases ($Zn(OH)_2$, $CdCO_3$, etc.), subsequently increasing the toxicity and bioavailability of the metals in the water column. Moreover, metals that form complexes with organic and inorganic matter, or humic acids may also dissociate under different pH values. For example, Guy et al. (1976) found that Zn^{2+} and humic acid complexes are approximately 50% dissociated at pH 5.5, and may dissociate faster under lower pH values.

Another factor that affects speciation, mobility, and bioavailability of a metal in the water column is the alkalinity of water (i.e. buffering capacity). Increased alkalinity indicates higher concentrations of carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions. Higher alkalinity results in the formation of Cd^{2+}, Ni^{2+}, and Zn^{2+} carbonate species such as : $NiCO_3$, $ZnCO_3$, $CdCO_3$, $Ni(HCO_3)_2$, $Zn(HCO_3)_2$, and $Cd(HCO_3)_2$, reducing the bioavailability and the toxicity of a metal. Additionally, bioavailable metals also compete with essential ions such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), for binding sites on

biotic ligands (i.e. fish gills) (Lynch 2013). Therefore, increased concentrations of Ca^{2+} and Mg^{2+} (i.e. increased hardness), prevents Cd^{2+}, Ni^{2+}, and Zn^{2+} metals from adsorbing unto biotic ligands. Consequently, hardness reduces the toxicity of a metal, but not its bioavailability. Overall, increasing pH, alkalinity, hardness, suspended solids, organic matter, and DOC in a water body, subsequently decreases the toxicity and/or bioavailability of a metal due to processes such as precipitation, competition, complexation with both organic and inorganic matter, and adsorption unto DOC and suspended organic particles.

Cellular Uptake

In the aquatic environment, metal complexation with organic or inorganic ligands results in the production of different metal species with varying sizes, oxidation states, and charges. Therefore, the hydrated metal ion, and all its chemically reactive species, or complexes, determines the mechanistic route of cellular uptake (Dawson and Ballatori, 1995). For the most part, aquatic toxicologists agree that ionic metal species dominate Ca^{2+} channel uptake, simply because complexed metal species are essentially impermeable due to their large sizes (Simkiss, 1998). However, recent research has suggested that a significant portion of metals such as Cd^{2+} and Zn^{2+} can enter through ZIP8 and ZIP14 (Fujishiro et al. 2012, Jenkitkasemwong et al. 2012), and Cd^{2+}, Ni^{2+}, and Zn^{2+} through the divalent metal transporter-1 (DMT1) (Bannon et al. 2003, Mackenzie et al. 2006, Kwong 2011). Also note that complexed metal species can enter through other routes such as: endocytosis, or hydrophobic routes (direct penetration of the plasma membrane). Nevertheless, note that the permeating efficiency of metal ions across the

plasma membrane is driven by fluctuating environmental factors. Changes in water quality parameters such as hardness, alkalinity, and pH, may enhance, or retard cellular uptake, by affecting bioavailability or free metal ion activity, directly increasing or decreasing competition for interactions at the biotic ligand (i.e. interacting with sub-cellular structures such as channels that traverse the plasma membrane). Generally, the plasma membrane is the first site of action for heavy metals (Viarengo, 1994), and the degree of toxicity depends on the form of the metal, and its route of entry.

Ion Channels

Ion channels are large, multimeric, transmembrane proteins that form an aqueous passageway for ions with a narrow selectivity filter that allows for the transport of specific essential cations such as Ca^{2+}, Na^{+}, and K^{+} (Hille, 2008). Ion channels are of major importance in toxicology because they can transfer ions at high rates across the cell membrane (Hille, 1992), rapidly altering cytoplasmic ion concentrations and ionic homeostasis (Shafer, 2000). For this very reason, ion channels provide a major route of entry for metals, because they create an opportunity for metal cations to parasitize the channel and enter the cytoplasm of the cell. Nevertheless, ion channels not only serve as routes for ion transfer across the cell membrane, but they are also biologically essential in mechanistic signaling functions that are critically important for cellular communication (Shafer, 2000).

Voltage-gated Ca^{2+} channels have received considerable attention, for they are the most abundant cationic channels on the plasma membrane of muscle cells (skeletal, smooth, cardiac), neuronal cell bodies and dendrites (central nervous system and

peripheral nervous system), and endocrine cells providing a major route of entry for most metal cations (Simkiss, 1998). In particular, a diverse group of divalent and trivalent cations use those channels as their primary mode of entry (Lansman, 1990). Perhaps, the movement of divalent metal cations through Ca^{2+} channels is due to their similarity to the essential physiologic Ca^{2+} ion (Shafer, 2000), or due to their smaller ionic radii. For example, the Cd^{2+} ion has an ionic radius of 0.97 Å while the Ca^{2+} ion has an ionic radius of 0.99 Å (Barbalace K.), chemically making the Cd^{2+} ion and the Ca^{2+} ion very similar. On the other hand, Zn^{2+} and Ni^{2+} ions have an ionic radius of 0.74 Å and 0.69 Å, respectively (Barbalace K.), making those metal ions much smaller than the Ca^{2+} ion. Consequently, Cd^{2+}, Ni^{2+}, and Zn^{2+} metal ions inevitably interact with, and trespass Ca^{2+} channels.

The mechanistic movement of metal ions through Ca^{2+} channels has been long-established using a variety of cell types such as: heart cells (Lansman et al. 1986; Hess and Tsien, 1984; Hess et al. 1986), myotubes (Lansman, 1990), and neurons (Chow, 1991; Swandulla and Armstrong, 1989; Taylor, 1988). Therefore, any cell containing voltage-gated Ca^{2+} channels must be considered a potential route for metal entry (Shafer, 2000). Although many types of voltage-gated Ca^{2+} channels have been described - L-,N-,P-,Q-,R-, and T-types (Nowycky et al. 1985); the mechanistic movement of metal ion permeation seems to be similar for L-, N-, P-, Q-, and R-type Ca^{2+} channels, and researchers suspect that the same mechanistic processes are involved in the permeation of the T-type Ca^{2+} channel (Shafer, 2000). Nevertheless, differences are apparent because

Ca^{2+} channels are distinguished by their sensitivity to organic and inorganic channel blockers such as: nifedipine, nimodipine, Cd^{2+}, and Ni^{2+} metal ions (Shafer, 2000).

Other studies have shown that binding sites (glutamate residues) in voltage-gated Ca^{2+} channels, recruit Cd^{2+} ions with relatively high affinity, form strong bonds with the metal ion, and release the metal very slowly. This reveals that Cd^{2+} is a competitive inhibitor of Ca^{2+} transport, making the Cd^{2+} ion a potent blocker of Ca^{2+} currents (Lansman et al. 1986; Taylor, 1988). In fact, studies that induced point mutations on an L-type Ca^{2+} channel α_1-subunit, confirmed that the Cd^{2+} ion efficiently binded to the four glutamate residues in the aqueous pore loops of the Ca^{2+} channel, subsequently preventing Ca^{2+} entry into the cell (Ellinor et al. 1995; Mikala et al. 1993; Yang et al. 1993). Other studies using cerebellar granule cells (Usai et al. 1997), optic nerve cells (Fern et al. 1996), and PC12 cells (Hinkle and Osborne, 1994), have shown that in the presence of agonists or antagonists, Cd^{2+} entry via L-type voltage-gated Ca^{2+} channels either increased or decreased, respectively. Nimodipine, another potent L-type Ca^{2+} channel blocker, protected PC12 cells against Cd^{2+} cytotoxicity, whereas L-type Ca^{2+} channel agonists enhanced Cd^{2+} uptake, increasing cytotoxicity (Cd^{2+} LC_{50} went from 12 to 6μM) (Hinkle and Osborne, 1994). Likewise, studies examining Zn^{2+} (Wakamori et al. 1998; Winegar and Lansman, 1990) and Ni^{2+} (Wakamori et al. 1998; Winegar et al. 1991; Zamponi et al. 1996) permeation through Ca^{2+} channels, have reported similar blocking potencies, preventing Ca^{2+} transport and current induction. Therefore, Zn^{2+} and Ni^{2+} are also competitive inhibitors of Ca^{2+}. As with Cd^{2+}, Zn^{2+} can be blocked from entering L-type voltage gated Ca^{2+} channels in heart cells (Atar et al. 1995) and neurons (Sensi et al.

1997; Weiss et al. 1993) using channel specific antagonists. Similarly, Ni^{2+} permeation across the cell membrane is likely to be blocked by Ca^{2+} channel antagonists, and enhanced by Ca^{2+} channel agonists.

With respect to trout, Ca^{2+} is assumed to enter fish gills along a concentration gradient (Hogstrand et al. 1994). Normally, Ca^{2+} is transported from the water column, into the gill cells, via voltage gated Ca^{2+} channels on the apical epithelium, and exits via a basolateral Ca^{2+} ATPase pump (Hogstrand et al. 1994). Hogstrand and colleagues (1994) found that Zn^{2+} metal ions inhibit Ca^{2+} uptake, leading to decreased plasma Ca^{2+} (hypocalcaemia) in the gill cells. However, because Cd^{2+}, Ni^{2+}, and Zn^{2+} compete with Ca^{2+} for the same biotic ligand (voltage gated Ca^{2+} channels, ZIP8, ZIP14, DMT-1), this suggests that all these metals would lead to hypocalcaemia through competitive inhibition. Studies that manipulated Ca^{2+} concentrations in the testing media found that Cd^{2+} toxicity (Calamari et al. 1980), Cd^{2+} uptake (Part et al. 1985; Wicklund 1990), and Cd^{2+} accumulation (Carroll et al. 1979), were all reduced in fish gills when Ca^{2+}concentrations were increased. Therefore, competition for binding sites on the biotic ligand would decrease the toxicity of Cd^{2+}, Ni^{2+}, and Zn^{2+} if the Ca^{2+} ion concentration in the water column was very high (i.e. hard water).

Hydrophobic Route

Metal ions in the environment are generally in their ionic, hydrated form, so it is unlikely that ions penetrate the cell membrane through a hydrophobic route (Simkiss, 1998). However, metals do associate with organic substrates in the water column, and in some cases, yield organometallics. The physicochemical properties of organometallics

can generally be described as neutral, nonpolar species of high lipophilic character (Newman, 2015). This suggests that organometallics are expected to permeate the lipid layer of the cell membrane relatively easily as well as to transport the trace metal in that form (Newman, 2015). For example, compounds such as dithiocarbamates and xanthates form hydrophobic organometallic complexes with many heavy metals. Studies conducted with these organic ligands and Cd^{2+} have shown that in their presence, the free Cd^{2+} ion preferably forms organometallic complexes; hence, direct permeation across the plasma membrane becomes the primary route of entry in many aquatic organisms (Block, 1991; Block and Part, 1986; Gottofrey et al. 1988; Poldoski, 1979). Due to the latter results, and given that dithiocarbamates and xanthates are used extensively in the mining industry (Dobson, 1992), these organic pollutants are of high environmental concern. Xanthate concentrations between 4 and 400µg/L have been reported in receiving waters near metal mining facilities (Waltersson, 1984).

Other Routes of Uptake

The plasma membrane not only houses ion channels, but it also houses other transmembrane proteins such as carrier proteins, or transporters. The plasma membrane is generally coated with transporters that are specifically designed to recognize, and transport very specific ions (i.e. essential metals, major cations and anions), or other molecules that are vital to the cells survival. Because Cd^{2+} is a non-essential element that has no biological function, it is unlikely that cells will not house Cd^{2+} transporters. However, since Zn^{2+} and Ni^{2+} are essential elements, transporters for those metals do exist. Recent research studies have proposed that another major route of uptake for the

non-essential Cd^{2+} ion is through zinc transporters known as ZIP8 and ZIP14 (Fujishiro et al. 2012, Jenkitkasemwong et al. 2012), and through another transporter known as (DMT1) (Bannon et al. 2003, Mackenzie et al. 2006, Kwong, 2011). It is likely that Cd^{2+} ions may hitch a ride through those transporters, since movement of ions through these types of transporters requires no energy consumption (passive facilitated diffusion).

Another possible route of uptake is through endocytosis. Although the uptake of a metal species through this process may be quantitatively insignificant, it cannot be over looked. Through endocytosis, particulate solids are engulfed by the cell's plasma membrane, and transported into the cell via that form. For example, particulate iron oxides are engulfed by the gill cells of mussels. Similarly, the pharyngeal gill cells of tunicates are able to engulf particulate vanadium (Newman, 2015). Therefore, it is likely that endocytosis might be the mechanism of entry for metallonanoparticles, a recent class of environmental contaminants that have received considerable attention (Newman, 2015).

A dietary route of uptake has also recently been proposed to be of crucial importance in aquatic toxicology (Newman, 2015). As aforementioned, metal ions do form complexes with biotic ligands, and also adsorb unto the surfaces of organic tissues. Therefore, it is possible for metal ions to adsorb unto the surfaces of algal cells, or zooplankton, and then detach in the digestive tract when consumed by organisms higher in the trophic chain. Similarly, other organisms that sequester toxic metal ions in the form of insoluble granules, or as chelates with metallothioneins (cysteine rich proteins) transfer the metal in that form when consumed. Because the gastrointestinal tract contains

powerful digestive enzymes, metal ions are likely released from their sequestered form, and become bioavailable for uptake, and transported via the epithelial gut cells of the organism (Newman, 2015). Additionally, because gut cells are generally adapted to reabsorb essential organic molecules such as amino acids, it is likely that the free metal ions form complexes with those organic molecules, and are therefore co-transported with them (Newman, 2015).

Overall, it is crucial to recognize that the cellular uptake of metal ions such as Cd^{2+}, Ni^{2+}, and Zn^{2+} can occur via several mechanisms of cellular uptake, and, in fact, several mechanisms concurrently. Therefore, quantifying cellular uptake of a metal through all possible routes at any given time would seem appropriate. However, it practically impossible to quantify and distinguish the proportion of metals that enter through each mechanistic route at any given time (Hille, 1992), because not all mechanisms of uptake are equally important. Accordingly, given that most toxicologists agree that the ionic metal species dominates channel uptake (Simkiss, 1998), and that free metal ions use Ca^{2+} channels for transport across the plasma membrane, this route of uptake is assumed to be the most important in metal toxicology, although other routes of uptake via ZIP transporters and DMT-1 are gaining research interest. In addition, other factors to consider is that permeation of a heavy metal species across a cell membrane not only depends on the chemical form of the metal, but also on its physicochemical properties, the species of organism, its age, stage of development, its physiological health, the type of epithelium it enters, the location of the epithelium, and its physiological role within the organism (Newman, 2015).

Environmental Toxicity

A great deal of research has focused on toxicity of a single metal. However, in natural aquatic ecosystems, metals do not exist as single elements, but rather as mixtures of multiple elements. Therefore, aquatic organisms are generally exposed to a cocktail of metal contaminants. For that reason, cellular uptake of Cd^{2+}, Ni^{2+}, and Zn^{2+} metal ions will depend on their competitive binding affinities for the biotic ligand (i.e. voltage gated Ca^{2+} channels, and transporters). As previously mentioned, the major route of entry for transport across the plasma membrane for Cd^{2+}, Ni^{2+}, and Zn^{2+} is through Ca^{2+} channels. On exit, those metals may further manifest toxic responses depending on their competitive interactions with target proteins holding metal-specific binding sites (metallothioneins, and metalloenzymes). Thus, the degree of competitive interactions with cellular ligands (i.e. membrane and cytoplasmic proteins) will basically result in varying degrees of toxic effects that deviate from additivity. Hence, three possible toxic effects can be described: additive, less-than-additive, or more-than-additive.

Generally, additive toxicological effects would be observed when two metals competitively enter the same ion channels, or transporters, but also possess similar binding affinities for the same cellular ligands. In effect, the mixture toxicity of the two metals would be equal to the sum of the toxicity of the individual metals. On the contrary, a less-than-additive toxicological effect would be observed when two metals competitively enter through the same ion channels, or transporters, but possess dissimilar binding affinities for the same cellular ligands. In this case, the mixture toxicity of the two metals would be less than the sum of their individual toxicities (antagonistic). Lastly,

a more-than-additive toxicological effect would be observed when two metals utilize different routes of entry (i.e., use different transporters, or other ion channels), and also possess dissimilar binding affinities for different cellular ligands. The mixture toxicity produced by the two metals in this case would be more than the sum of their individual toxicities (synergistic).

In many aquatic ecosystems, metals may be present at, or below EPA national water quality criteria. Therefore, metal concentrations may be too low to induce adverse effects when considered as single elements. However, when considering those same metal concentrations as mixtures, interactive effects that are additive or synergistic in character may result (Montvydienė and Marčiulionienė 2004). For example, in evaluating chronic Dutch water quality criteria, Enserink et al. (1991) exposed *D. magna* and *S. gairdneri* to metal concentrations that were at the maximum allowable toxicant concentrations (MATC). As single elements, the metals were not toxic to either organisms, but as mixtures, they were highly toxic to both species. Likewise, Masnado and colleagues (1995) acutely exposed *Ceriodaphnia dubia* to a synthetic metal mixture effluent that represented permitted discharge concentrations of metals; and found those mixtures to be exceedingly toxic to the species. Therefore, allowable metal concentrations in U.S waters are of concern, given that the EPA chronic water quality criteria only represent toxicity for single metals.

Little is known about the cumulative effects of long-term metal mixture exposures, their potential interactions, and their mechanisms of toxicity. Given that metals are persistent pollutants in the environment, chronic metal mixture toxicity studies

are more environmentally relevant, because they allow insight into other mechanistic ecological and toxicological factors that acute studies cannot provide insight into. Why? Acute studies generally range between 48 and 96 hours and organisms are exposed to very high concentrations of metals. On the other hand, chronic studies are largely conducted using sub-lethal metal concentrations, and organisms are exposed to these concentrations for a longer portion of their life cycle. Furthermore, in acute toxicity testing, only the survival endpoint is usually assessed, and other endpoints of toxicity are ignored, whereas chronic toxicity testing seeks to evaluate survival, reproductive, and growth endpoints. Such studies broaden the scope of a pollutants toxic effect. Due to the varying degrees of toxic effects previously mentioned, acute toxicity tests are short-sighted, because the same effect may not be observed in all endpoints (i.e., if an antagonistic effect is observed in the survival endpoint that may not be the case in the reproductive endpoint). In other words, a mixture of metals may interact differently with the epithelial cells of the reproductive system, versus the epithelial cells of the digestive system, the nervous system, the respiratory system, the circulatory system, or the excretory system thereby resulting in different effects on different endpoints of interest.

While most research has focused on single metal toxicity, metal mixture toxicity has nevertheless been studied for decades (Meyer et al. 2015). However, a great portion of those studies have only invested large efforts in understanding acute toxicity. For example, in recent meta-analyses conducted by Norwood et al. (2003) and Vijver et al. (2011) only 35% and 9% of the studies, respectively, reported chronic toxicity. A recent metal mixture evaluation conducted by Meyer et al. (2015), indicated the need for

chronic mixture studies as the lack of chronic toxicity data in the literature makes it difficult to arrive at meaningful deductions concerning the interactions of metals in long-term chronic exposures. As a result, studies investigating chronic toxicity of metal mixtures at sub-lethal concentrations are needed and are essential for improving our scientific understanding of the interactions between metals and organisms and long-term metal mixture toxicity.

A literature review showed that the acute and chronic toxicity of metal mixtures on aquatic organisms is not consistent. Studies evaluating the interactive toxicity of metal ions have demonstrated that the same mixture of metals can produce different results, depending on the species being tested, and on the exposure period (Negilski et al. 1981; Kraak et al. 1994; Masnado et al. 1995). In addition, differences exist even within a species population, and the toxicity of a metal mixture may further be influenced by an organism's age, and its stage of development. For these reasons, binary-metal mixtures of Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} have shown both additive (non-interactive) and non-additive (interactive) effects on many species of aquatic organisms, ranging from less-than-additive to more-than-additive toxicity (Norwood et al. 2003).

Mixtures of Cd^{2+}-Zn^{2+} have demonstrated **less-than-additive toxicity** on the survival of the water fleas *Ceriodaphnia dubia*, *Daphnia ambigua*, *Daphnia pulex* (Shaw et al. 2006), survival of *D. magna* (Meyer et al. 2015; Cañizares-Villanueva et al. 2000; Attar and Maly, 1982), survival of the freshwater shrimp *Paratya tasmaniensis* (Thorpe and Lake, 1974), survival of the rainbow trout *Oncorhynchus mykiss* (Mebane et al. 2012), survival of the flag fish *Jordanella floridae* (Spehar et al. 1978); uptake in

duckweed *Lemna Polyrhiza* (Noraho and Gau, 1995), uptake in *D. magna* (Komjarova and Blust, 2008), uptake in the mud shrimp *Corophium volutator* (Bat et al. 1998), uptake in the polychaete *Capitella capitate* (Goto and Wallace, 2007), uptake in the gastro-intestinal system of the rainbow trout *Oncorhynchus mykiss* (Ojo and Wood, 2008), uptake in the gills of zebrafish *Danio rerio* (Glynn, 2001), feeding behavior of *D. magna* (Barata et al. 2002), development of larval purple sea urchins *Strongylocentrotus purpuratus* (Phillips et al. 2003), growth of the marine diatoms *Phaeodactylum tricornutum* and *Skeletonema costatum* (Braek et al. 1980), regeneration of the fiddler crab *Uca pugilator* (Weis, 1980), and adsorptive capacity of the freshwater algae *Chlamydomonas reinhardtii* (Lavoie et al. 2012a,b), *Chlorella vulgaris*, and the cyanobacteria *Anacystis nidulans* (Awasthi and Rai, 2004). However, **more-than-additive toxicity** have also been reported on the survival of freshwater copepods (Borgmann, 1980), survival of the marine shrimp *Callianassa australiensis* (Negilski et. al. 1981), reproduction of the water flea *D. magna* (Biesinger et al. 1986), growth of the marine diatoms *halassiosira pseudonana* and *Skeletonema costatum* (Braek et al. 1980). Yet, other studies have also documented **additive toxicity** on the survival of *D. magna* (Shaw et al. 2006), survival of juvenile minnows *Phoxinus phoxinus* (Wicklund Glynn et al. 1992), survival of the freshwater shrimp *Paratya tasmaniensis* (Thorpe and Lake, 1974), survival of the marine copepod *Tisbe holothuriae* (Verriopoulos and Dimas, 1988), survival of the amphipod *Allorchestes compressa* (Ahsanullah et. al. 1988), and on the adsorptive capacity of the freshwater algae *Scenedesmus quadricauda* (Awasthi and Rai, 2004).

Similarly, mixtures of Cd^{2+}-Ni^{2+} have shown **less-than-additive toxicity** on the survival of the water flea *D. magna* (Traudt et al. 2016), uptake in *D. magna* (Komjarova and Blust, 2008), uptake in the zebra fish *Danio rerio* (Komjarova and Blust, 2009), growth of the freshwater bacteria *Nitrosomonas europaea* at an ammonia concentration between 2.5 and 40mg/L as Nitrogen (Sato et al. 1986), and adsorption capacity of the freshwater algae *Chlorella vulgaris* (Awasthi and Rai, 2004) while **more-than-additive toxicity** has also been reported on the growth of the freshwater bacteria *Nitrosomonas europaea* at an ammonia concentration >40mg/L as Nitrogen (Sato et al. 1986), and adsorptive capacity of the freshwater alga *Scenedesmus quadricauda* (Awasthi and Rai, 2004). Still, other experiments have also demonstrated **additive toxicity** on the growth of the freshwater bacteria *Aeromonas hydrophila* (Babich et al. 1986), growth of the freshwater bacteria *Nitrosomonas europaea* at an ammonia concentration <2.5mg/L as Nitrogen (Sato et al. 1986), adsorptive capacity of the freshwater cyanobacteria *Anacystis nidulans* (Awasthi and Rai, 2004), and development of larval purple sea urchins *Strongylocentrotus purpuratus* (Phillips et al. 2003).

Norwood et al. (2003) proposed that the variability in organism responses might be concentration-dependent, and due to inter/intraspecific species differences in sensitivity. In addition, random variability in organism responses may have led to inaccurate interpretation of mixture toxicity assessments as demonstrated by De Laender et al. (2009). If both metal alone and metal mixture toxicity tests were not simultaneously conducted, truly nonadditive (less-than-additive, and/or more-than-additive) effects could have been misinterpreted as additive effects. As a result, in the present study, it was

crucial and essential that Zn^{2+} alone, Cd^{2+} alone, Ni^{2+} alone, Cd^{2+}-Zn^{2+}, and Cd^{2+}-Ni^{2+} mixture tests be conducted concurrently to avoid misleading toxicity interpretations.

The objective of the present study is to characterize the chronic effect of Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures using a titration experimental design as used by Meyer et al. (2015) to detect any potential interactive effects of the metals. The measured endpoints include: survival (percent mortality), reproduction (total neonates reproduced, total live and dead neonates, neonates reproduced/adult/day, time to first brood, undeveloped embryos), growth (average dry weight of surviving adults), and metal uptake (tissue metal concentration in surviving organisms) of *D. magna* when exposed to Zn^{2+} alone, or Ni^{2+} alone and mixtures of Cd^{2+}-Zn^{2+} or Cd^{2+}-Ni^{2+} at sub-lethal concentrations.

CHAPTER TWO

METHODOLOGY AND EXPERIMENTAL DESIGN

Test Organisms

Laboratory cultured *Daphnia magna* from the Ecotoxicology and Risk Assessment Laboratory in the Loyola Institute of Environmental Sustainability (IES) were used in this study. *Daphnia magna* cultures were housed in 1L glass beakers (30 per beaker). Beakers were filled with 900-1000 ml reconstituted moderately hard water made from 16-18mΩ MilliQ water and laboratory grade chemicals ($CaSO_4.2H_2O$, $MgSO_4$, $NaHCO_3$, and KCl) based on the USEPA method for chronic toxicity testing (EPA 2002). The water quality measures for the culture media were as follows: hardness = 80-84 mg/L as $CaCO_3$, alkalinity = 54.54-60.6 mg/L as $CaCO_3$, pH = 7.19-7.89, and dissolved oxygen (DO) = 6.87-8.32 mg/L. Cultures were maintained at a temperature ranging from 21.2 – 25.6°C and at a photoperiod of 16h light: 8h dark. *Daphnia magna* culture water was changed on Mondays, Wednesdays, and Fridays. Dissolved oxygen, pH, and temperature were recorded following subsequent water changes. Dissolved oxygen and temperature were measured with a DO instrument: model YSI 550A, and pH was measured with a Fisher Scientific accumet portable laboratory meter: model AP110. Cultured *D. magna* were fed 6ml of an algal suspension (*Selenastrum capricornutum*) with a concentration of $3x10^7$ cells/ml, and 3ml YCT (a food suspension of yeast, cereal leaves and trout chow) at

a concentration of 12-13 mg solids/L daily. The algae and YCT were cultured and prepared in the Loyola Ecotoxicology and Risk Assessment Laboratory.

Metal Alone and Binary-Metal Toxicity Tests

The titration method was used in this study to characterize the chronic toxicity of Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures on *D. magna*. The titration design was chosen because it affords for the detection of graded changes in organism responses, across graded increases in metal concentrations (Meyer et al. 2015). Using this design, Cd^{2+} concentration was kept constant at 1.5µg/L across all treatments and Zn^{2+} (10, 20, 40, 80, 120, 160 and 200 µg/L) and Ni^{2+} concentrations (20, 40, 80, 100, 120, 140, and 160µg/L) varied. Concentrations of Cd^{2+}, Zn^{2+}, and Ni^{2+} were chosen based on screening chronic toxicity tests conducted in our laboratory (unpublished data). To compare the chronic metal alone, and mixture effects to *D. magna*, a control was used. In addition, to assess the mixture effects, a chronic Zn^{2+} alone and Ni^{2+} alone test was conducted with the same concentrations used in the mixture test. A twenty-one day (21) static renewal toxicity test method was used in the present study (American Society for Testing and Materials (ASTM, 2012)). To avoid potential organism health effects, the Zn^{2+} alone, Ni^{2+} alone and mixture tests were conducted simultaneously using neonate *D. magna* coming from the same batch (5^{th} brood). Tests were conducted with 48 hour test solution renewals in an aquatic toxicology testing room located in the Loyola IES. The aquatic toxicology testing room was set at a temperature of 25°C and a photoperiod of 16h light: 8h dark. The test concentrations were prepared from stock solutions of laboratory grade metal salts ($CdSO_4$ and $ZnCl_2$). The Zn^{2+} alone and the Ni^{2+} alone toxicity tests consisted of 7

treatments and 4 replicates, whereas the binary-metal toxicity tests consisted of 8 treatments and 4 replicates, which included a Cd^{2+} alone treatment. Replicates were comprised of 600ml polypropylene cups containing 10 neonate *D. magna* (<24 hours old) in 200ml of test solution from day 0 to day 10, and 350ml test solution from day 11 to day 21 to compensate for *D. magna* growth.

Due to the long term nature of the study, organisms grew over time, therefore, varying amounts of a suspension of *S. capricornutum* with a concentration of $3x10^7$ cells/ml and YCT of a concentration of 12-13 mg solids/L were fed daily. Food rations were adjusted depending on the feeding rate of the organisms. Feeding rate was low when organisms were small but increased when the organisms grew (see appendix A: table 1).

Mortality was recorded daily. *Daphnia magna* were considered dead if no mobility was observed after gentle probing with a pipette, and if no organ movements were observed after close examination. Dead organisms were removed from the test chambers and discarded. Reproductive output was also observed daily when *D. magna* became sexually mature and began reproducing. All neonates, both dead and alive, were separately counted, recorded, removed from the test chambers, and discarded. The same procedure was used if any undeveloped embryos were observed in the test chambers. More undeveloped embryos were observed on days 15 and 17 in the Cd^{2+}-Zn^{2+} mixtures and on days 16 and 19 in the Cd^{2+}-Ni^{2+} mixtures. These embryos were collected for morphological examination. All the embryos from a specific treatment were placed in a 30ml polypropylene beaker for further analysis. Two to four embryos from each mixture

treatment were observed after collection on a Zeiss SteREO Discovery.v12 and images were processed using Zeiss ZEN imaging software.

Twenty-four hours prior to the termination of the experiment, digestion tubes were labelled, placed in a Fisher Drying Oven, and dried at 60°C. At the end of the experiment, all surviving adults in each test chamber were collected, transferred to labelled digestion tubes, and carefully rinsed three times with DI water. All organisms in each replicate were collectively weighed in digestion tubes with a Metro Toledo Excellence Balance Model XS64, and their wet-weight was recorded. Organisms were then dried in the same oven at 60°C for 48 hours and reweighed to determine dry weight. The average dry weight for each individual organism was obtained by dividing the total weight in each replicate by the number of surviving organisms in that replicate.

Body Metal Accumulation

To determine body metal accumulation, surviving daphnids were digested with HNO_3 based on the US EPA Method 3050B (EPA 1996). Digested solutions were used for analysis of metal content in surviving daphnids. Analysis of metals was performed using a NexION Inductively Coupled Plasma-Mass Spectrometer (ICP-MS), model 300X (Perkin Elmer Inc.)

Water Quality and Chemical Analyses

Two 190L batches of reconstituted moderately hard water were prepared in the Ecotoxicology and Risk Assessment Lab during the Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures study. The moderately hard water was prepared from 16-18mΩ MilliQ water and laboratory grade chemicals ($CaSO_4.2H_2O$, $MgSO_4$, $NaHCO_3$, and KCl) based on the

ASTM method for chronic toxicity testing. Water quality measures for all toxicity tests (Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures) and (Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures) were taken once a week on days 0, 8, 14, and 21. Dissolved oxygen and temperature were measured with a dissolved oxygen instrument: model YSI 550A, and pH was measured with a Fisher Scientific accumet portable laboratory meter: model AP110. Hardness and alkalinity were measured by titration methods. Hardness was titrated against 0.01 M Ethylenediaminetetraacetic acid (EDTA). Alkalinity was titrated against 0.02 N H_2SO_4. The average and standard deviation of hardness, alkalinity, pH, DO, and temperature for the Zn^{2+} alone test were: 88.7 ± 7.18 mg/L as $CaCO_3$, 61.5 ± 3.97 mg/L as $CaCO_3$, 7.6 ± 0.16, 7.3 ± 0.24 mg/L, and 24.6 ± 0.40°C, respectively. Those same water quality parameters for the Cd^{2+}-Zn^{2+} mixture test were: 90.6 ± 4.52 mg/L as $CaCO_3$, 60.4 ± 3.85 mg/L as $CaCO_3$, 7.7 ± 0.15, 7.7 ± 0.34 mg/L, and 24.3 ± 0.53°C respectively. On the other hand, the water quality parameters for the Ni^{2+} alone test were: 90.8 ± 5.81 mg/L as $CaCO_3$, 62.3 ± 3.93 mg/L as $CaCO_3$, 7.8 ± 0.24, 7.4 ± 0.50 mg/L, and 25.4 ± 0.30°C respectively. As for the Cd^{2+}-Ni^{2+} mixture test the water quality parameters were as follows: 93.7 ± 8.48 mg/L as $CaCO_3$, 63.1 ± 4.23 mg/L as $CaCO_3$, 7.9 ± 0.11, 7.6 ± 0.31 mg/L, and 25.3 ± 0.49°C, respectively.

Water samples for total and dissolved metal, cations and anions were also collected weekly at the same time as water quality measurements. Approximately14 ml of test water was collected into 15ml polypropylene sample vials. Total metal samples were taken directly from the test water, but dissolved metal and cation and anion samples were filtered through polyvinyldifluoride (PVDF) filters with a pore diameter of 0.45µm.

Water samples for total and dissolved metals were preserved with one drop of concentrated nitric acid, and refrigerated for later analysis. Approximately 25 ml of test water was filtered through a new filter of the same filter type used for dissolved metal and cations and anions and collected into a 30 mL amber glass bottle for analysis of total organic carbon (TOC). Total metal, dissolved metal, and cation concentrations were analyzed using a NexION ICP-MS (Perkin Elmer Inc.), while concentrations of TOC were analyzed with a TOC analyzer (Shimazu Inc.).

Data Analyses

For each test, data were analyzed for statistical significance between control and exposure treatments. For the mixture test and to compare control and exposure treatments, data were also analyzed for statistical significance between Cd^{2+} alone and Cd^{2+}-Zn^{2+}, or Cd^{2+}-Ni^{2+} mixture treatments. A one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) multiple comparisons test were used to detect treatment differences within each endpoint. Differences across respective treatments of Zn^{2+} alone or Ni^{2+} alone, and Cd^{2+}-Zn^{2+} or Cd^{2+}-Ni^{2+} mixtures (e.g., treatment 1 for Zn^{2+} alone and its respective Cd^{2+}-Zn^{2+} mixture) were also analyzed using a one-way ANOVA. To test for interaction effects of Cd^{2+} and Zn^{2+}, a two-way ANOVA was used, treating metal exposure type as a factor (Zn^{2+} alone or Ni^{2+} alone vs. its Cd^{2+}-Zn^{2+} or Cd^{2+}-Ni^{2+} mixture) and the concentration of Zn^{2+} or Ni^{2+} as another factor.

CHAPTER THREE

RESULTS

Zn^{2+} Alone and Cd^{2+}-Zn^{2+} Mixtures

On average, measured total and dissolved metal concentrations deviated approximately 0-12% from the calculated nominal concentrations in the Cd^{2+}-Zn^{2+} mixtures and Zn^{2+} alone treatments (see appendix A: table 2). Due to those minute deviations nominal concentrations were used to report findings for the results. In addition, no significant differences were detected between the total and dissolved metal concentrations in either toxicity test.

Surviving Effects

The average 21-day cumulative percent mortality in the Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+} were significantly lower than Cd^{2+} alone ($p<0.01$) (Fig. 1) while the Cd^{2+}-Zn^{2+} mixtures containing 160 and 200µg/L Zn^{2+} were significantly higher ($p<0.01$) (Fig. 1). There was no statistically significant difference in cumulative mortality between the Cd^{2+}-Zn^{2+} mixtures containing 10 and 20µg/L Zn^{2+} and Cd^{2+} alone. This indicates that when Zn^{2+} is present at sublethal concentrations, such as 10 and 20µg/L in a mixture with 1.5 µg/L Cd^{2+}, mortality remains high and the Cd^{2+} effect on survival is still pronounced. However, as Zn^{2+} concentrations increase in the mixtures (40, 80, and 120µg/L), mortality significantly drops, reaching a low at 80µg/L (Fig. 1).

The results reveal that at a sufficient concentration, Zn^{2+} protects (buffers) *D. magna* from chronic Cd^{2+} toxicity, an evidence of a less-than-additive effect. Additionally, given that the cumulative mortality was significantly different between Cd^{2+}-Zn^{2+} mixtures and Cd^{2+} alone at higher Zn^{2+} concentrations (i.e., 160, 200 µg/L), suggested that Zn^{2+} concentrations exceeded the necessary concentrations needed to protect *D. magna* from chronic Cd^{2+} toxicity, Zn^{2+} becomes toxic to the organisms, and the toxicity is likely due to a joint toxicity between both metals. co

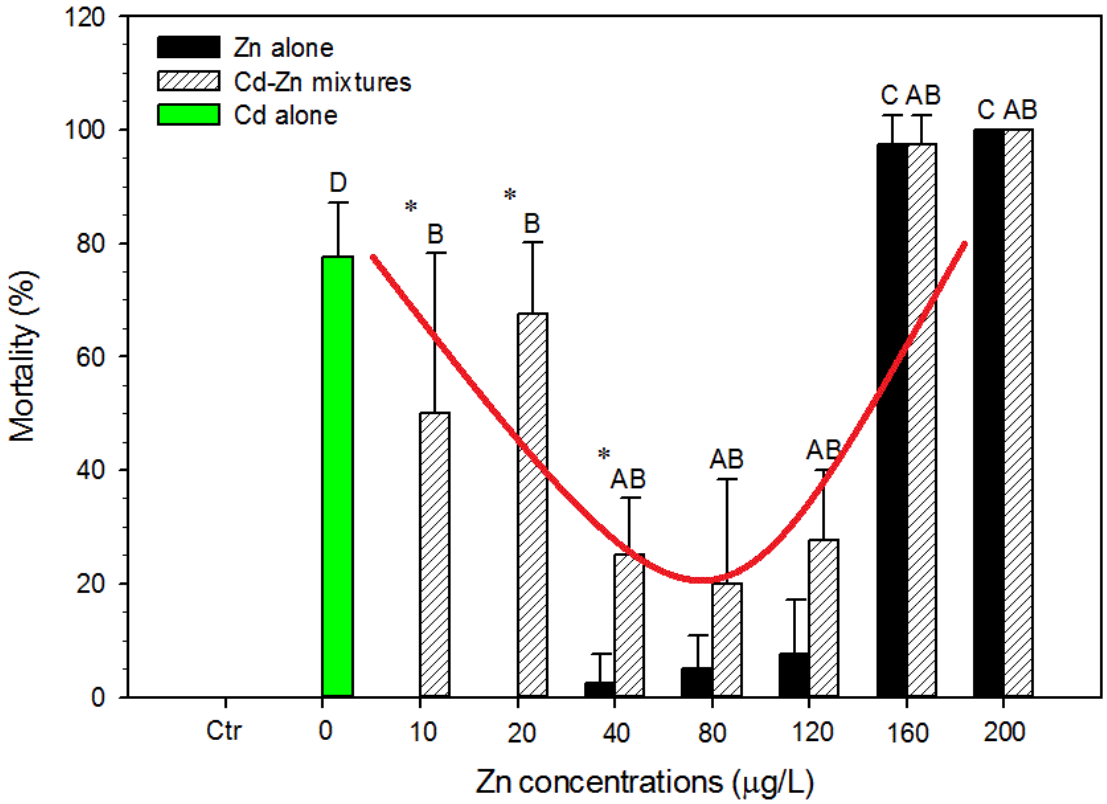

Figure 1. 21-day cumulative mortality of *D. magna* exposed to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.001$). C: significant differences between control and Zn^{2+} alone ($p<0.001$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation. Solid line represents a fitted polynomial regression.

In comparing the cumulative percent mortality of Zn^{2+} alone with its corresponding Cd^{2+}-Zn^{2+} mixture, the treatments containing 10, 20, or 40µg/L Zn^{2+} were found to be significantly different ($p<0.05$) (Fig. 1). At these concentrations of Zn^{2+} alone, there was no mortality (10 and 20µg/L Zn^{2+}) or no significant difference (40 µg/L Zn^{2+}) in mortality between the control and Zn^{2+} alone treatments (Fig. 1). These results suggest that the toxicity in those Cd^{2+}-Zn^{2+} mixtures would be due to Cd^{2+} exposure. There was no statistically significant difference found in mortality between Zn^{2+} alone and its corresponding Cd^{2+}-Zn^{2+} mixture at the higher Zn^{2+} concentrations (80 to 200 µg/L). However, given that Zn^{2+} alone treatments containing 80 and 120µg/L were not significantly different from the control; the toxicity observed in the corresponding Cd^{2+}-Zn^{2+} mixtures was also due to Cd^{2+} exposure. At higher Zn^{2+} concentrations (160 and 200µg/L), the toxicity observed in the Cd^{2+}-Zn^{2+} mixtures was likely due to Zn^{2+} exposure.

Reproductive Effects

Total Neonates, Live Neonates, and Reproductive Rates

In examining the reproductive effects of Cd^{2+} and Zn^{2+} on *D. magna*, several parameters were examined. One consideration was to count the total neonates reproduced, which included all living and dead neonates found in the test chamber. The second consideration was to count only the live neonates. The importance of assessing only the live neonates was to examine if protective Zn^{2+} effects were also apparent on the newly born neonates. Accounting for both total and live neonates allowed for the examination and comparison of two types of calculated reproductive rates; one rate, the

overall reproductive rate, looked at the total number of neonates reproduced per adult per day. The second, the live neonate reproductive rate counted only the surviving neonates per adult per day.

The effects of the Cd^{2+}-Zn^{2+} mixtures on the reproductive effort of *D. magna* indicated that total number of neonates ($p<0.05$) (Fig. 2), and the number of live neonates ($p<0.05$) (Fig. 3) reproduced by *D. magna* in the Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+} were significantly higher in comparison to Cd^{2+} alone.

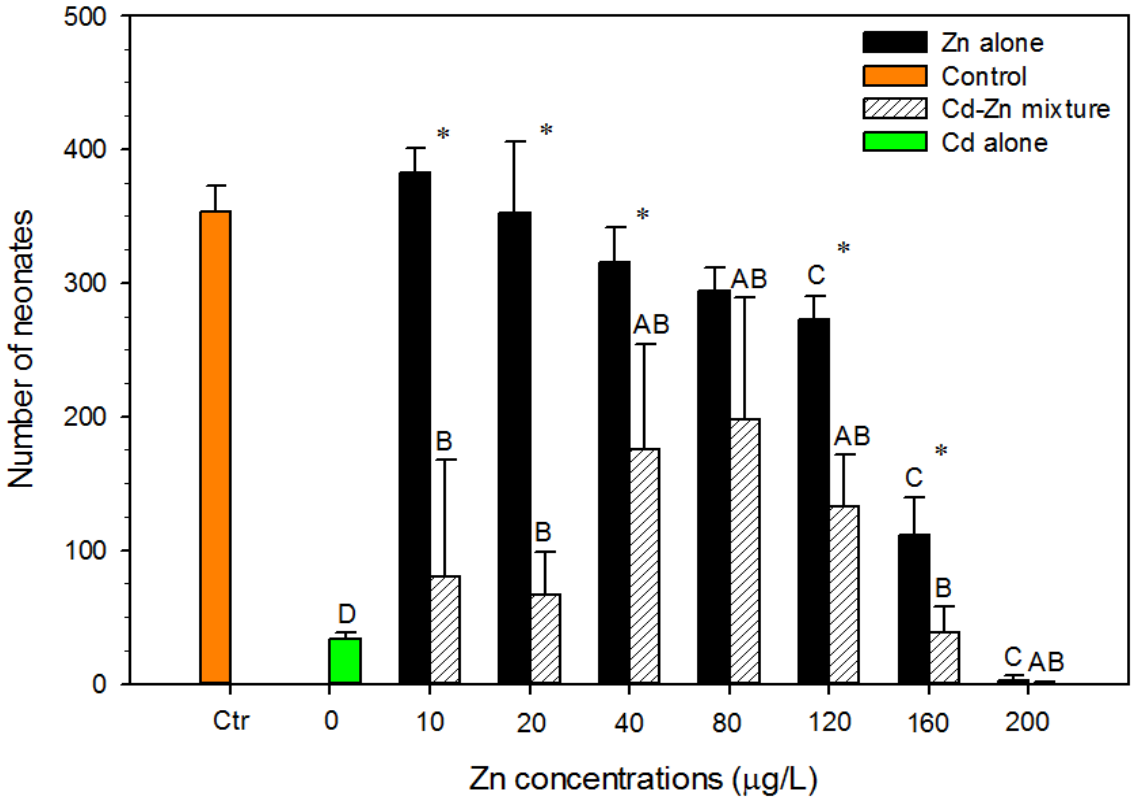

Figure 2. Total neonates reproduced over 21-days of exposure to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). C: significant differences between control and Zn^{2+} alone ($p<0.05$). D: significant difference between control and Cd^{2+} alone ($p<0.001$).*: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

Similarly, the overall reproductive rate ($p<0.05$) (Fig. 4), and the live neonate reproductive rate ($p<0.05$) (Fig. 5) were also significantly higher between the Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+}, and Cd^{2+} alone. No other Cd^{2+}-Zn^{2+} mixture was found to have a significantly higher number of neonates or reproductive rate than that of Cd^{2+} alone. These results suggest that Cd^{2+}-Zn^{2+} mixtures containing 10 and 20µg/L Zn^{2+} are not sufficient to protect *D. magna* from Cd^{2+} reproductive toxicity. *Daphnia magna* in those mixtures did not reproduce a higher number of total neonates or a higher number of living neonates (Figs. 2, 3), thereby reflecting the reduction in their respective reproductive rates (Figs. 4, 5). However, increasing Zn^{2+} concentrations in the mixtures (40, 80, and 120µg/L) inhibited Cd^{2+} reproductive toxicity (evidence of a less-than-additive effect), which translated into a significantly higher number of both total neonates (Fig. 2) and live neonates (Fig. 3). These are represented in the higher overall reproductive rate shown in Fig. 3 and the higher live neonate reproductive rate reflected in Fig. 5. On the other hand, in mixtures containing higher Zn^{2+} concentrations of 160 or 200µg/L, antagonistic Zn^{2+} effects were not observed. Interestingly, only the Cd^{2+}-Zn^{2+} mixture containing 200µg/L Zn^{2+} was found to have a significantly lower number of both total neonates ($p<0.05$) (Fig. 2), and living neonates ($p<0.05$) (Fig. 3) in comparison to Cd^{2+} alone. Again mixtures containing 160 and 200µg/L suggested that Zn^{2+} exceeded the necessary concentration needed to protect *D. magna* from chronic Cd^{2+} toxicity, and thereafter became toxic to the organisms. Note that the same trend was observed regardless of whether the data were analyzed with or without the total number of dead

neonates (Figs. 2, 3, 4, 5). Similar to the surviving results, reproductive results also show less-than-additive effect of Cd^{2+}-Zn^{2+} mixtures when Zn^{2+} concentration is sufficient.

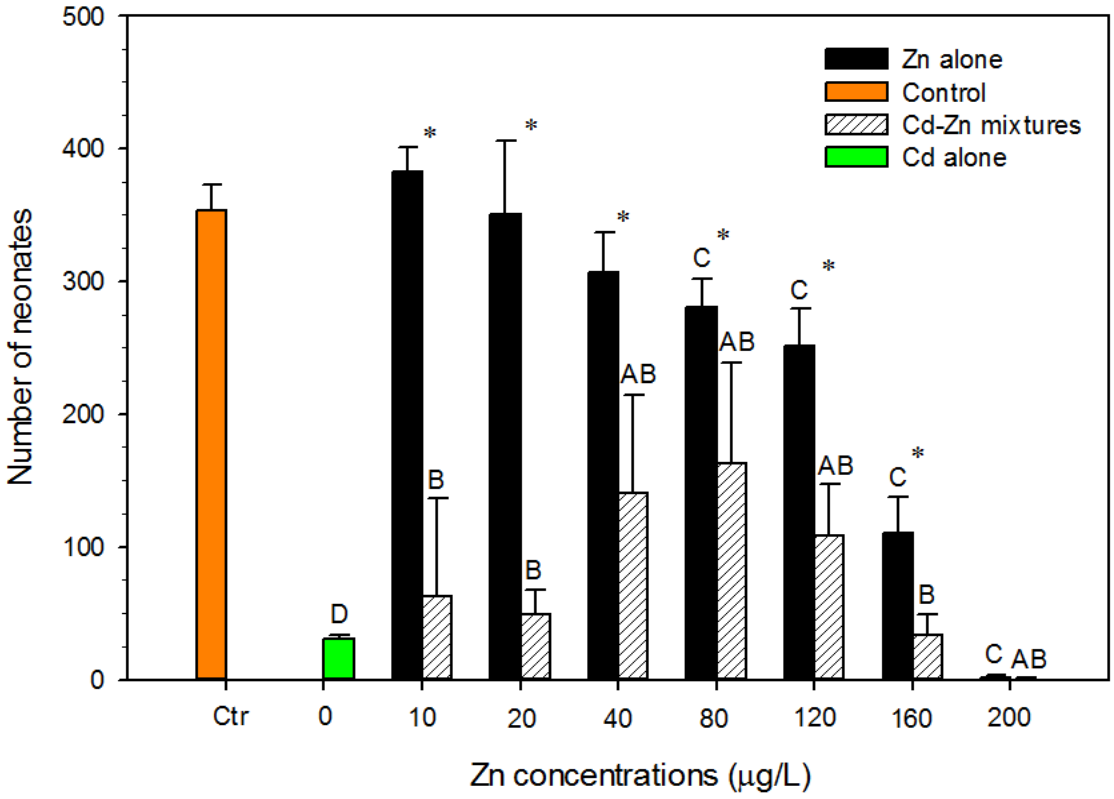

Figure 3. Total live neonates reproduced over 21-days of exposure to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). C: significant differences between control and Zn^{2+} alone ($p<0.05$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). n=4. Error bars represent standard deviation.

The total number of neonates and the overall reproductive rate in Zn^{2+} alone treatments containing 10, 20, 40, or 80µg/L showed no difference with control (Fig. 2 and Fig. 4). Similarly, Zn^{2+} alone concentrations of 10, 20, or 40µg/L were found to be non-toxic to *D. magna* reproduction of live neonates (Fig. 3), while Zn^{2+} alone concentrations of 10, 20, 40, or 80µg/L were found to be non-toxic on the live neonate

reproductive rate (Fig. 5). On the other hand, treatments containing higher Zn^{2+} alone concentrations of 120, 160, or 200µg/L were found to be toxic to *D. magna's* reproduction of total neonates (Fig. 2), and live neonates (Fig. 3), since those treatments contained a significantly fewer live neonates than the control. Additionally, with respect to the reproductive rates, the overall reproductive rate in Zn^{2+} alone did not reveal toxic manifestations in any treatment, except at the highest Zn^{2+} alone concentration (200µg/L) (Fig. 4). Likewise, in the live neonate reproductive rate, only the Zn^{2+} alone treatments containing 120 and 200µg/L were found to be significantly affected (Fig. 5).

In comparing the Zn^{2+} alone treatments with their corresponding Cd^{2+}-Zn^{2+} mixture, sublethal Zn^{2+} alone treatments containing 10, 20, or 40µg/L had a significantly higher number of total neonates ($p<0.05$) (Fig. 2), and a significantly higher overall reproductive rate ($p<0.05$) (Fig. 4) in comparison to their Cd^{2+}-Zn^{2+} mixture complement. This result suggested that the toxicity observed in those $Cd2^{+}$-Zn^{2+} mixtures was due to Cd^{2+} exposure. The Zn^{2+} alone treatment containing 80µg/L did not exhibit a significantly higher number of total neonates (Fig. 2), nor a higher overall reproductive rate (Fig. 4) in comparison to its corresponding Cd^{2+}-Zn^{2+} mixture. Thus the observed toxic effect in the mixture was still due to Cd^{2+} exposure and not due to Zn^{2+}, because the Zn^{2+} alone effects were not significantly different from the control. With the exception of the Zn^{2+} alone treatment containing 200µg/L (Figs. 2, 4), the higher Zn^{2+} alone treatments containing 120 and 160µg/L were also found to have a significantly higher number of total neonates (Fig. 2) and a higher overall reproductive rate (Fig. 4) than their Cd^{2+}-Zn^{2+} mixture correspondents. This result suggests that the observed toxicity in the Cd^{2+}-Zn^{2+}

mixtures containing 120 and 160µg/L in figure 2 were due to a joint toxicity between both Cd^{2+} and Zn^{2+}, since Zn^{2+} alone toxicity in those treatments were significantly lower than the control. Also, given that the overall reproductive rate in the Zn^{2+} alone treatments containing 120 and 160µg/L were not significantly different from the control (Fig. 4), indicates that the toxicity observed in the Cd^{2+}-Zn^{2+} mixtures was due to Cd^{2+} exposure.

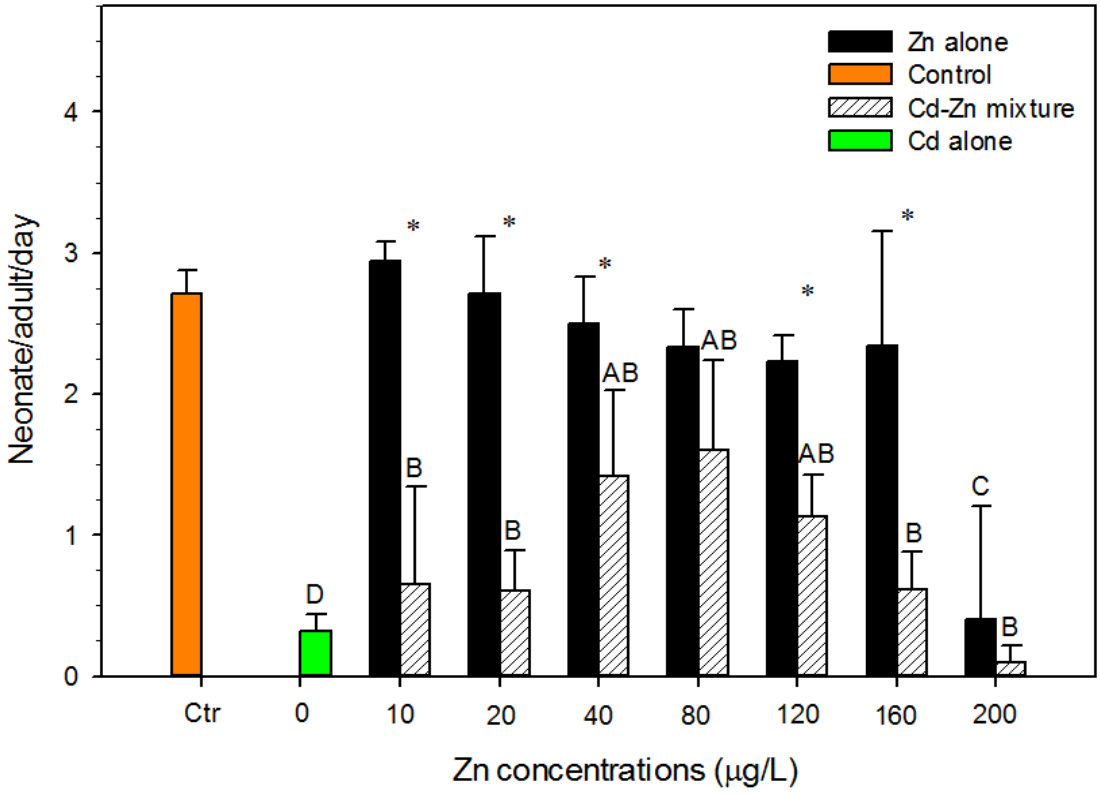

Figure 4. Overall reproductive rate (dead + live neonates) of *D. magna* when exposed to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). C: significant differences between control and Zn^{2+} alone ($p<0.05$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

The results in the analysis of total live neonates (Fig. 3) and the live neonate reproductive rate (Fig. 5) were similar to the results obtained in the analysis of total neonates (Fig. 2) and the overall reproductive rate (Fig. 4). The only difference was that the Zn^{2+} alone treatment containing 80µg/L was found to be significantly lower in comparison to the control when only live neonates were considered (Fig. 3). Moreover, because that same treatment was also found to contain a significantly higher number of live neonates than its corresponding Cd^{2+}-Zn^{2+} mixture (Fig. 3); the result indicated that the toxic effect observed in the mixture was due to both Cd^{2+} and Zn^{2+}, a result that was not detected when both dead and live neonates were considered collectively (Fig.2). Lastly, with respect to the live neonate reproductive rate (Fig. 5), the Zn^{2+} alone treatment containing 120µg/L was found to be significantly lower than the control, signifying that the toxicity observed in its corresponding Cd^{2+}-Zn^{2+} mixture was due to both Cd^{2+} and Zn^{2+}. Again, a result not detected when total neonates were considered (Fig. 4).

Total Dead Neonates and Undeveloped Embryos

Cd^{2+}-Zn^{2+} mixtures of 40, 80, or 120µg/L Zn^{2+} had a significantly higher number of dead neonates when compared to the Cd^{2+} alone treatment ($p<0.05$) (Fig. 6). The other concentrations of Cd^{2+}-Zn^{2+} mixtures were not significantly different with Cd^{2+} alone (Fig. 6). Similar to the results found in the surviving effects, the number of dead neonates in the Cd^{2+}-Zn^{2+} mixtures containing 10 and 20µg/L Zn^{2+} signify that those Zn^{2+} concentrations are not sufficient to protect *D. magna* from Cd^{2+} reproductive toxicity (Fig. 6). In other words, the toxic effects observed in those Cd^{2+}-Zn^{2+} mixtures would be

comparable to the effects observed in Cd^{2+} alone, since Cd^{2+} significantly alters the reproductive system. Conversely, Cd^{2+}-Zn^{2+} mixtures containing higher Zn^{2+} concentrations of 160 and 200µg/L would exceed the necessary concentration needed to protect *D. magna* from chronic Cd^{2+} reproductive toxicity, and Zn^{2+} would become toxic to the reproductive system of the organism.

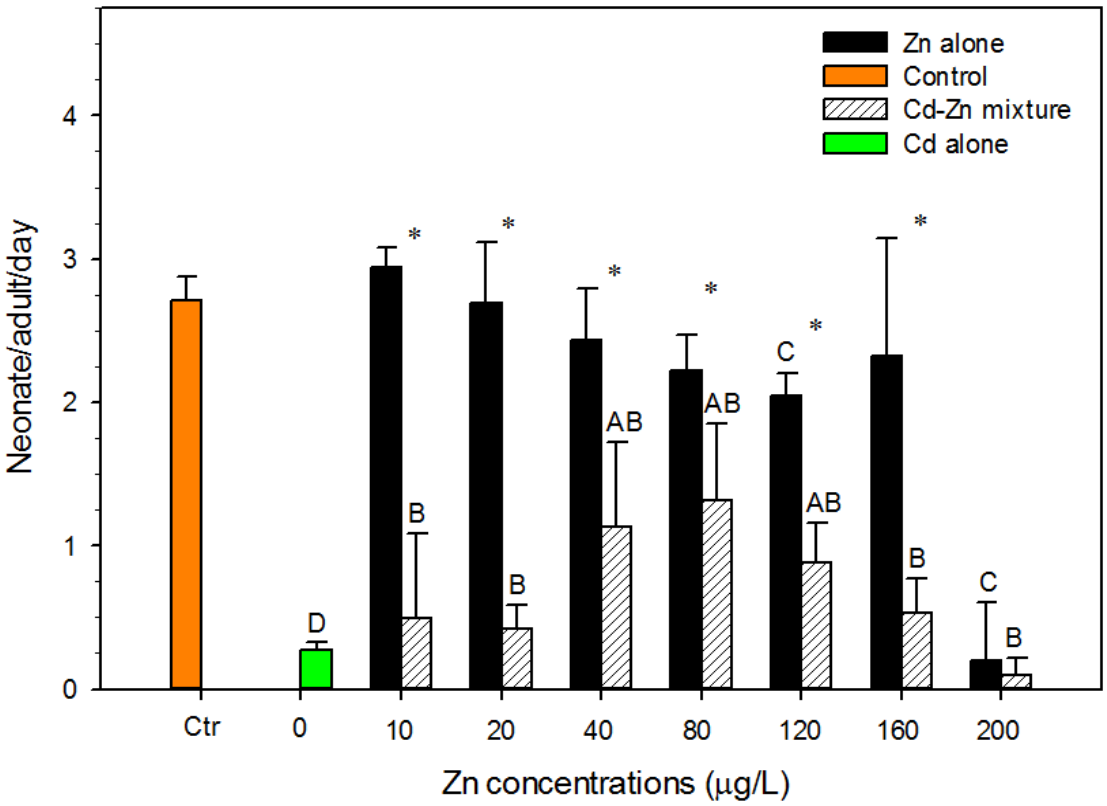

Figure 5. Reproductive rate (live neonates) of *D. magna* when exposed to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). C: significant differences between control and Zn^{2+} alone ($p<0.05$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

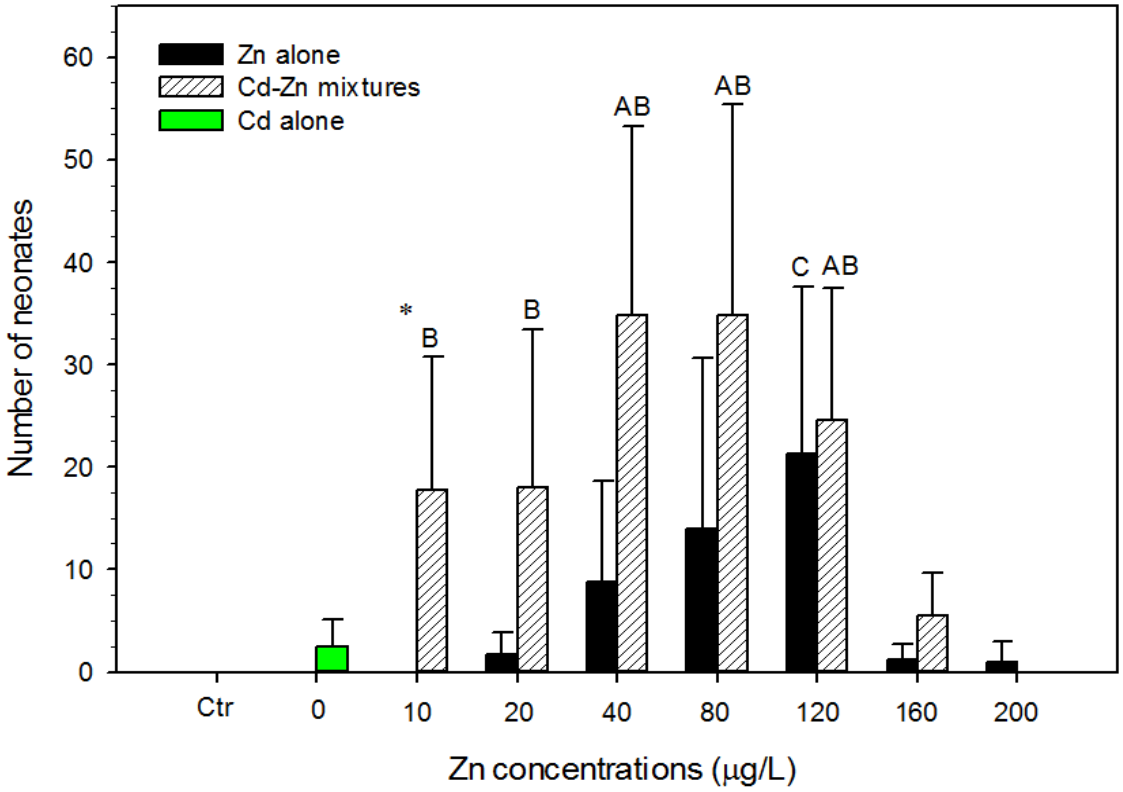

Figure 6. Total dead neonates reproduced over 21-days of exposure to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). C: significant differences between control and Zn^{2+} alone ($p<0.05$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

The number of dead neonates observed in all of the Cd^{2+}-Zn^{2+} mixtures were not statistically significant with their Zn^{2+} alone counterparts, except for the Cd^{2+}-Zn^{2+} mixture containing10µg/L Zn^{2+} ($p<0.05$) (Fig. 6). In addition, only the Zn^{2+} alone treatment containing 120µg/L was found to contain a significantly higher number of dead neonates in comparison to the control ($p<0.05$) (Fig. 6). The results of the experiment imply a number of things: 1) that the number of dead neonates observed in the Cd^{2+}-Zn^{2+} mixtures containing 10, 20, 40, and 80µg/L Zn^{2+} were most likely due to Cd^{2+} exposure,

because the number of dead neonates in their Zn^{2+} alone counterparts were not significantly different from the control (Fig. 6); 2) the number of dead neonates observed in the mixture containing 120µg/L Zn^{2+} was most likely due to both Cd^{2+} and Zn^{2+}, because the number of dead neonates observed in its corresponding Zn^{2+} alone treatment was significantly higher than the control ($p<0.05$) (Fig. 6). Furthermore, given that the number of undeveloped embryos observed in all Zn^{2+} alone treatments were not significantly different from the control (Fig. 7), and the number of undeveloped embryos in the Cd^{2+}-Zn^{2+} mixtures containing 10, 20, 40, 80, 120, and 160µg/L Zn^{2+} were all significantly higher than the control ($p<0.01$) (Fig. 7), and all of the Cd^{2+}-Zn^{2+} mixtures held a significantly higher number of undeveloped embryos in comparison to their Zn^{2+} alone counterparts ($p<0.01$) (Fig. 7), indicate to me that the embryotoxicity observed in those Cd^{2+}-Zn^{2+} mixtures was likely due to Cd^{2+} exposure.

The undeveloped embryos observed in the Cd^{2+}-Zn^{2+} mixtures were observed under a microscope for the presence of developmental obstructions, or morphological alterations which likely prevented the full development of a *D. magna* embryo. Compared to the control embryos, the embryos collected from the Cd^{2+}-Zn^{2+} mixtures revealed several morphological defects that either shutdown the process of mitotic cell division (cleavage), or disrupted cellular arrangement and organization that likely prevented the development of an embryo beyond stage 2 (see images in Appendix B).

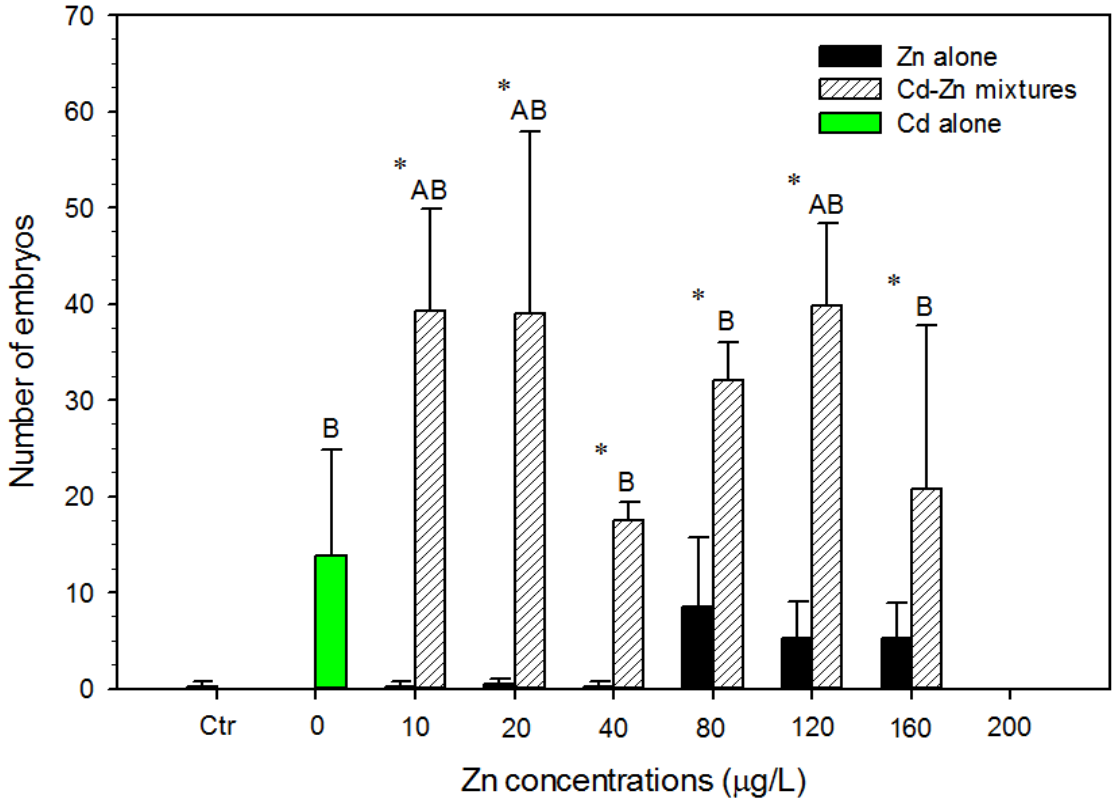

Figure 7. Total undeveloped embryos observed over 21-days of exposure to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). C: significant differences between control and Cd^{2+} alone ($p<0.05$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). n=4. Error bars represent standard deviation.

Time to First Brood

The time to first brood for Cd^{2+} alone was significantly earlier (9.25 days on average) in comparison to the control (11 days on average) ($p<0.01$) (Fig. 8). Furthermore, the time to first brood in the Cd^{2+}-Zn^{2+} mixture containing 20µg/L Zn^{2+} was not significantly higher than that of Cd^{2+} alone (Fig. 8). This result indicates that 20µg/L Zn^{2+} was not sufficient to protect *D. magna* from chronic Cd^{2+} toxicity, even though the Cd^{2+}-Zn^{2+} mixture containing 10µg/L Zn^{2+} did not show the manifestation of a toxic

effect. On the other hand, only the Cd^{2+}-Zn^{2+} mixture containing 200µg/L Zn^{2+} was shown to retard the time to first brood (Fig. 8).

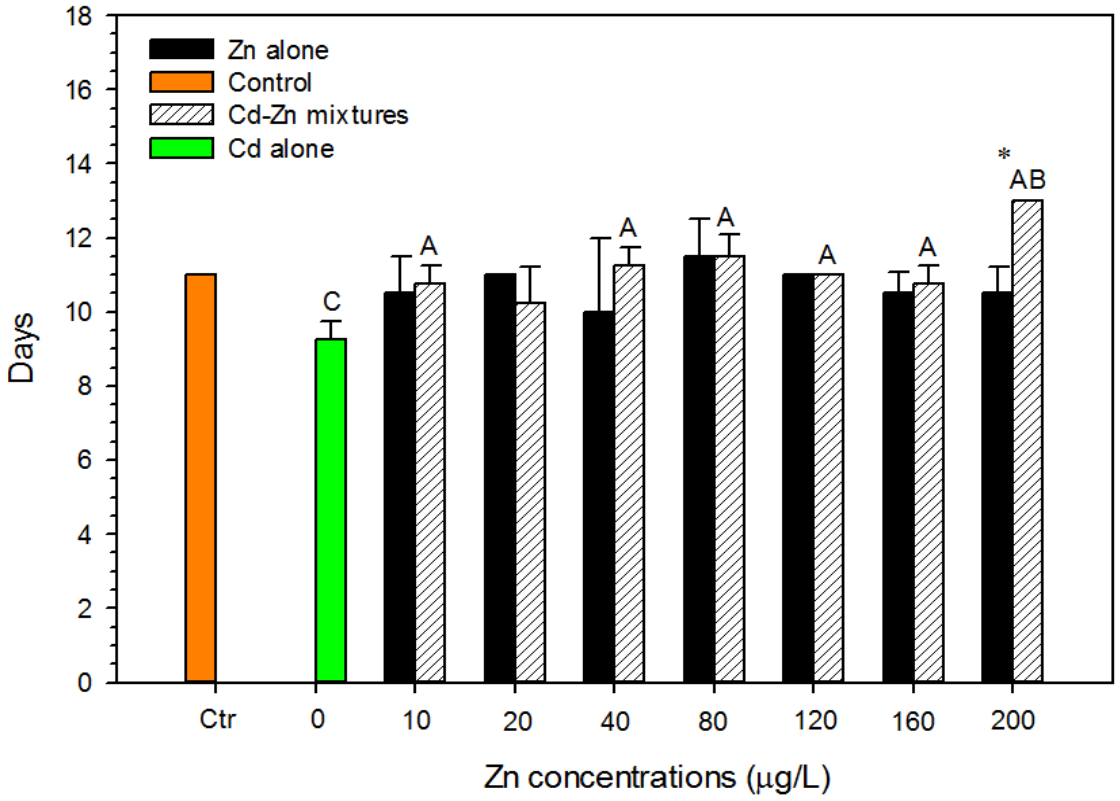

Figure 8. Time to first brood over 21-days of exposure to Cd^{2+} alone, Zn^{2+} alone, and Cd^{2+}-Zn^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Zn^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). C: significant differences between control and Cd^{2+} alone ($p<0.01$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). n=4. Error bars represent standard deviation.

Only the Cd^{2+}-Zn^{2+} mixture containing 200µg/L Zn^{2+} had a significantly higher time to first brood than its corresponding Zn^{2+} alone treatment. The result suggests that the toxicity is likely due to Cd^{2+} exposure, since *D. magna* in 200µg/L Zn^{2+} alone reproduced around the same time as control (Fig. 8).

Growth Effects

Dry Weight of Surviving Adults

The dry weights of the surviving *D. magna* exposed to Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+} were significantly higher than the dry weight of surviving organisms in Cd^{2+} alone ($p<0.05$) (Fig. 9). Furthermore, the dry weights of the surviving organisms in the other Cd^{2+}-Zn^{2+} mixtures were not significantly higher in comparison to Cd^{2+} alone (Fig. 9). Once more, this result implies that Cd^{2+}-Zn^{2+} mixtures containing 10 and 20µg/L Zn^{2+} were not sufficient to induce protective Zn^{2+} effects on *D. magna*. However, Cd^{2+}-Zn^{2+} mixtures containing higher Zn^{2+} concentrations of 40, 80, and 120µg/L strongly protected *D. magna* from Cd^{2+} growth toxicity, reaching a peak at 80µg/L (evidence of less-than-additive effects). Conversely, a protective Zn^{2+} effect was not observed in the Cd^{2+}-Zn^{2+} mixture containing 160µg/L Zn^{2+}. This result indicated that 160µg/L Zn^{2+} exceeded the necessary concentration needed to protect *D. magna* from chronic Cd^{2+} growth toxicity.

The dry weights of the daphnids in the Cd^{2+}-Zn^{2+} mixtures containing 10, 20, 40, 80, and 120µg/L Zn^{2+} were significantly less in comparison to their corresponding Zn^{2+} alone counterparts ($p<0.01$) (Fig.9). These same Zn^{2+} alone treatments were not significantly different from the control, except for the treatment containing 120µg/L Zn^{2+} ($p<0.05$) (Fig. 9). Therefore, the toxicity observed in the Cd^{2+}-Zn^{2+} mixtures containing 10, 20, 40, and 80µg/L Zn^{2+} were likely due to Cd^{2+} exposure. However, the toxicity observed in the Cd^{2+}-Zn^{2+} mixture containing 120µg/L Zn^{2+} was most likely due to both Cd^{2+} and Zn^{2+}, since that Zn^{2+} alone treatment was significantly different from the

control. At higher Zn^{2+} concentrations of 160µg/L, Zn^{2+} becomes toxic to the organisms, and the effect observed in that Cd^{2+}-Zn^{2+} mixture was most likely due to Zn^{2+} exposure.

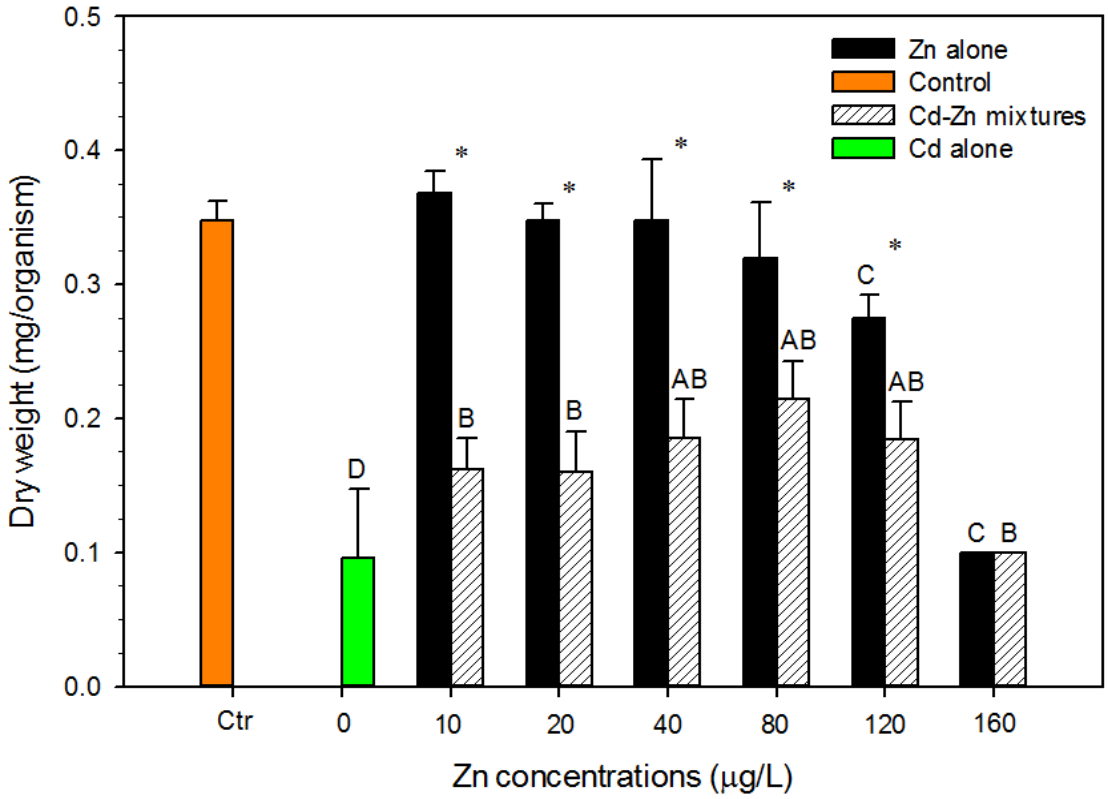

Figure 9. Dry weight of surviving adults at test termination in the Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). C: significant differences between control and Zn^{2+} alone ($p<0.05$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.01$). n=4. Error bars represent standard deviation.

Accumulation Effects

Body Metal Concentration

The average measured Cd^{2+} and Zn^{2+} concentrations in the tissues of surviving daphnids exposed to Cd^{2+}-Zn^{2+} mixtures are represented in figure 10. The results show that the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed to

Cd^{2+} alone, and to a mixture of Cd^{2+}-Zn^{2+} were all significantly higher than the measured Cd^{2+} concentration in control organisms. The results also show that as Zn^{2+} concentrations increased in the exposure media, Zn^{2+} concentrations gradually increased in the tissues of the surviving daphnids, fitting the trend of a power function (Fig. 10). Subsequently, a gradual decrease in Cd^{2+} tissue concentrations followed, also fitting the trend of a power function (Fig. 10).

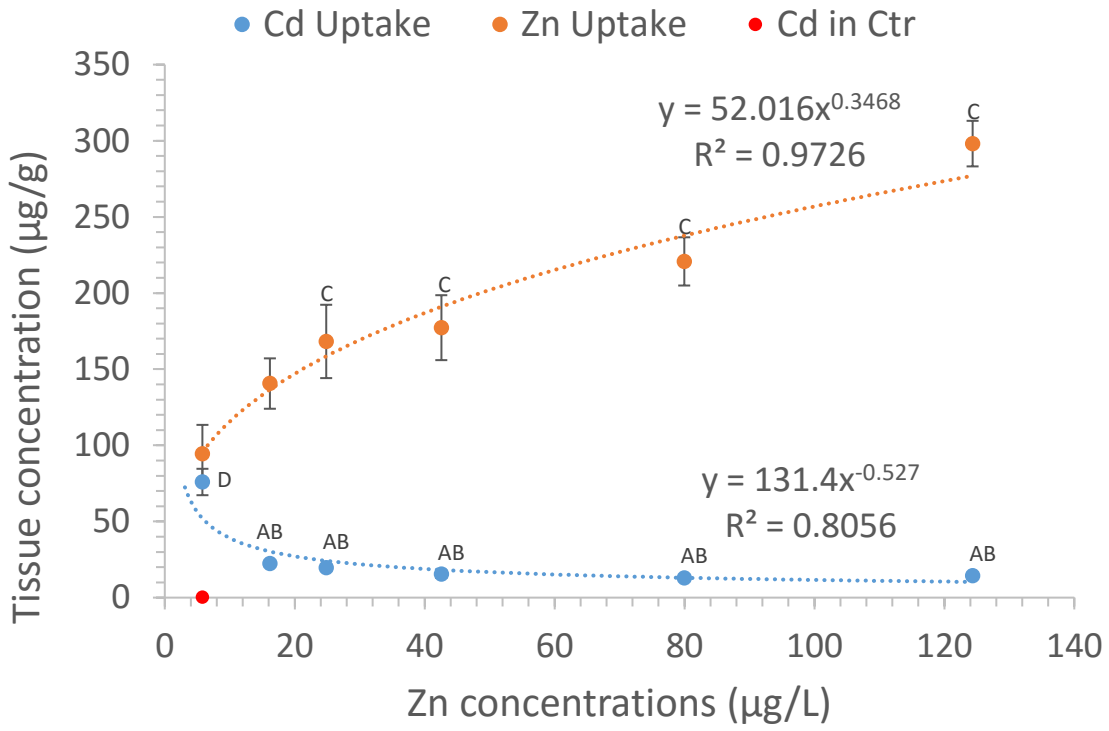

Figure 10. Metal concentrations in the tissues of surviving daphnids exposed to a mixture of Cd^{2+}-Zn^{2+} over 21 days. A: significant differences between Cd^{2+} alone and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Zn^{2+} mixtures ($p<0.05$). C: significant differences between control and Zn^{2+} alone ($p<0.05$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). Regressions are non-linear fitted power functions. n=4. Error bars represent standard deviation.

The results obtained from the tissue analysis of the surviving daphnids in the Cd^{2+}-Zn^{2+} mixtures is consistent with the hypothesis that metals compete for binding sites on the biotic ligand. The organisms exposed to Cd^{2+} alone accumulated significantly more Cd^{2+} than organisms exposed to a mixture of Cd^{2+}-Zn^{2+} ($p<0.01$) (Fig 10). In fact, the measured Cd^{2+} concentration in the tissues of the surviving daphnids in all Cd^{2+}-Zn^{2+} mixtures were found to be significantly lower than the measured Cd^{2+} in the tissues of the surviving daphnids exposed to Cd^{2+} alone. This result enforces strong evidence of a less-than-additive effect between Cd^{2+}-Zn^{2+} mixtures.

Sublethal Zn^{2+} concentrations of 10 and 20µg/L in a mixture with 1.5µg/L Cd^{2+} were consistently insufficient to significantly protect *D. magna* from chronic Cd^{2+} toxicity; this was because those low Zn^{2+} concentrations could not inhibit the accumulation of toxic levels of Cd^{2+} on the BL. On the other hand, moderate Zn^{2+} concentrations of 40, 80, and 120µg/L in a mixture with 1.5µg/L Cd^{2+} were consistently sufficient to induce protective Zn^{2+} effects on *D. magna*, consequently reducing the more toxic Cd^{2+} effect. This was because 40, 80, and 120µg/L Zn^{2+} prevented the accumulation of toxic levels of Cd^{2+} on the BL. However, when Zn^{2+} concentrations were too high (≥160µg/L), Zn^{2+} toxic effects were observed because the lower concentration of Cd^{2+} could not impede the accumulation of toxic levels of Zn^{2+} on the BL. Accordingly, the results suggest that Cd^{2+} and Zn^{2+} are interactive, and produce responses that are less-than-additive in character.

The results of the Cd^{2+}-Zn^{2+} experiment consistently demonstrated that *D. magna* exposed to a Cd^{2+}-Zn^{2+} mixture containing 40, 80, or 120µg/L Zn^{2+} were sufficient to

induce protective Zn^{2+} effects on all endpoint evaluated (Figs. 1-9). Consequently, tissue analysis (Fig. 10) also showed that the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed to Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+} were significantly lower than the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed Cd^{2+} alone (Fig. 10). In fact, the daphnids exposed to those Cd^{2+}-Zn^{2+} mixtures accumulated the lowest concentrations of Cd^{2+} (Fig. 10). For this reason, the Cd^{2+} toxic effects in those Cd^{2+}-Zn^{2+} mixtures were substantially decreased or almost completely inhibited, and translated into a decreasing trend in *D. magna* mortality (Fig. 1), an increasing trend in reproductive effort (Figs. 2, 3), and an increasing trend in growth (Fig. 9). *D. magna* were able to survive for a longer period of time in Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+}, which allowed the organisms to allocate more resources and energy into reproduction and growth, rather than investing more energy and resources into detoxifying mechanisms. However, *D. magna* exposed to Cd^{2+}-Zn^{2+} mixtures containing 10 and 20µg/L Zn^{2+} were consistently insufficient to induce protective Zn^{2+} effects. Even though the measured Cd^{2+} concentrations in the tissues of the surviving daphnids exposed to Cd^{2+}-Zn^{2+} mixtures containing 10 and 20µg/L Zn^{2+} was significantly lower than the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed Cd^{2+} alone (Fig. 10), the concentration of Cd^{2+} entering the tissues of *D. magna* was still sufficient to induce toxic effects. Therefore, the organisms exposed to Cd^{2+}-Zn^{2+} mixtures containing 10 and 20µg/L Zn^{2+} probably invested more energy and resources into detoxifying mechanisms, rather than into growth and reproduction, which explains why all the measured endpoints were substantially effected.

(Figs. 1, 2, 4, 9). Unfortunately, tissue analysis were not conducted on the Cd^{2+}-Zn^{2+} mixtures containing 160 and 200µg/L Zn^{2+} because *D. magna* in those treatments experienced 97.5% and 100% mortality respectively.

Ni^{2+} Alone and Cd^{2+}-Ni^{2+} Mixtures

On average, measured total and dissolved metal concentrations deviated approximately 0-10% from the calculated nominal concentrations in the Cd^{2+}-Ni^{2+} mixtures and Ni^{2+} alone treatments (see appendix A: table 3). Due to those minute deviations nominal concentrations were used to report findings for the results. In addition, no significant differences were detected between the total and dissolved metal concentrations in either toxicity test.

Surviving Effects

The survival effects of Cd^{2+}-Ni^{2+} mixtures had the following results. The average 21-day cumulative percent mortality in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, 80, or 100µg/L Ni^{2+} were significantly lower in comparison to Cd^{2+} alone ($p<0.01$) (Fig. 11). There was no statistically significant difference in cumulative mortality between other Cd^{2+}-Ni^{2+} mixture treatments and Cd^{2+} alone. When Ni^{2+} is present at concentrations such as 20, 40, 80, or 100µg/L in a mixture with 1.5 µg/L Cd^{2+}, mortality substantially drops, peaking at the Cd^{2+}-Ni^{2+} mixture containing 20µg/L Ni^{2+} (Fig. 11). The cumulative percent mortality of the Cd^{2+}-Ni^{2+} mixture containing 20µg/L Ni^{2+} was low (2.5%) and was not significantly different from the control (0%) (Fig. 11). The high survival rate indicates that at a sufficient concentration, such as 20, 40, or 80µg/L, Ni^{2+} protects *D. magna* from Cd^{2+} toxicity - evidence of a less-than-additive effect. However, at 100µg/L

Ni^{2+} in a mixture with 1.5µg/L Cd^{2+}, the protective agent is no longer Ni^{2+}, but Cd^{2+}. Therefore, Cd^{2+} is also protective when present at a sufficient concentration (Fig. 11). At higher Ni^{2+} concentrations (i.e. 120, 140, 160 µg/L) there was no statistically significant difference in cumulative mortality between the Cd^{2+}-Ni^{2+} mixtures and Cd^{2+} alone (Fig. 11). This indicates that while Ni^{2+} protects *D. magna* from chronic Cd^{2+} toxicity at lower concentrations, it does become toxic to the organism at higher concentrations.

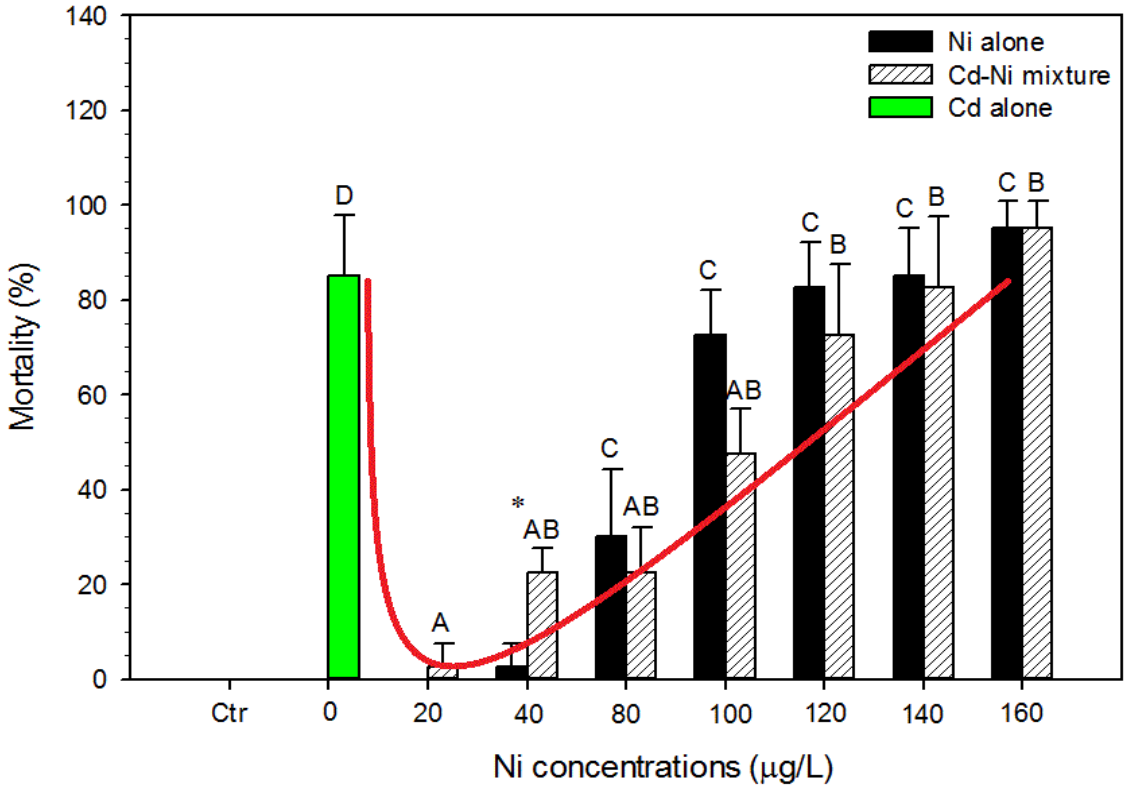

Figure 11. 21-days cumulative mortality of *D. magna* due to exposure to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.01$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.01$). C: significant differences between control and Ni^{2+} alone ($p<0.001$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation. Solid line represents a fitted polynomial regression.

In comparing the cumulative percent mortality between Ni^{2+} alone treatments and their corresponding Cd^{2+}-Ni^{2+} mixtures, only the treatment containing 40µg/L Ni^{2+} was found to be significantly different ($p<0.05$) (Fig.11). At a concentration of 20 and 40µg/L Ni^{2+} alone, there was a non-significant difference in mortality in comparison to control (Fig. 11). Therefore, the toxicity observed in those Cd^{2+}-Ni^{2+} mixtures was due to Cd^{2+} exposure. At higher Ni^{2+} alone concentrations (80 to 160 µg/L), percent mortality was significantly higher than control ($p<0.001$) (Fig.11). In addition, Ni^{2+} alone treatments containing ≥80µg/L also held non-significant differences in mortality with their corresponding Cd^{2+}-Ni^{2+} mixtures (Fig.1). These results suggest that the toxicity in those Cd^{2+}-Ni^{2+} mixtures was likely due to Ni^{2+} exposure.

Reproductive Effects

Total Neonates, Live Neonates, and Reproductive Rates

In examining the reproductive effects of Cd^{2+} and Ni^{2+} on *D. magna*, several parameters were examined. One consideration was to count the total neonates reproduced which included all living and dead neonates found in the test chamber. The second consideration was to count only the live neonates. The importance of assessing only the live neonates was to examine if protective Ni^{2+} effects were also apparent on the newly born neonates. Nonetheless, accounting for both total and live neonates allowed for the examination and comparison of two types of calculated reproductive rates. One rate, the overall reproductive rate, looked at the total number of neonates reproduced per adult per day. The second, the live neonate reproductive rate counted only the surviving neonates per adult per day.

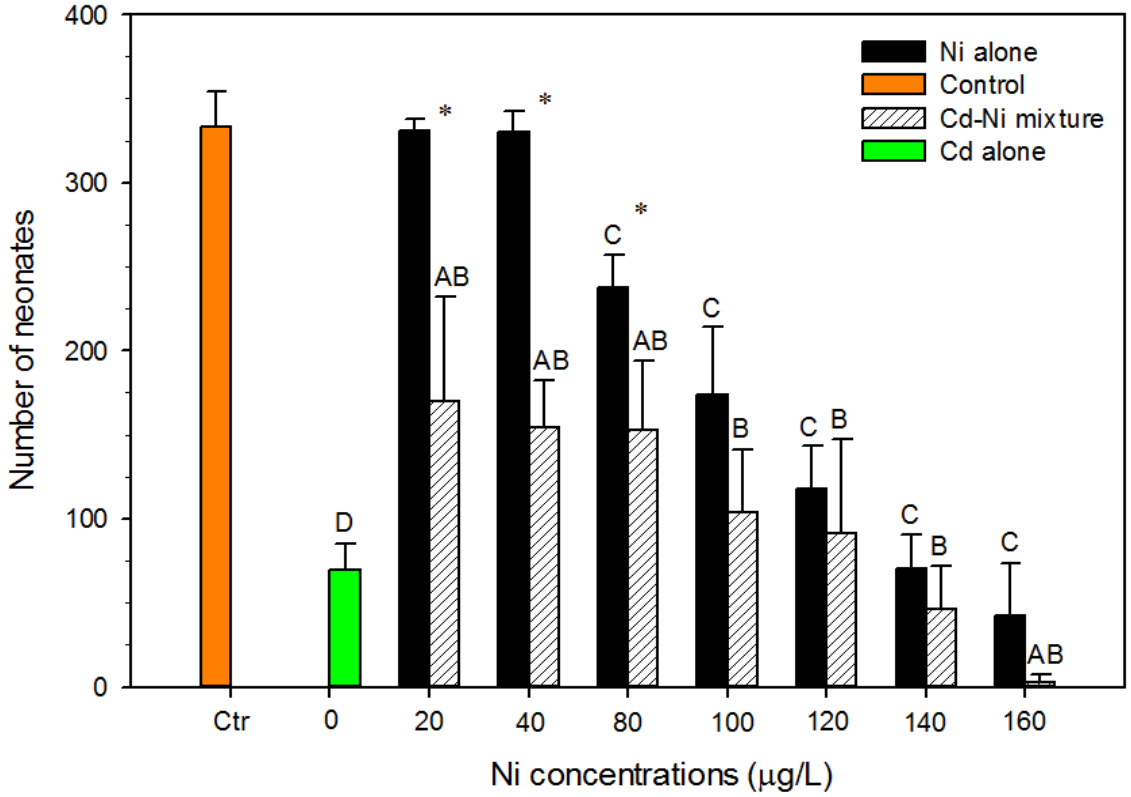

Figure 12. Total neonates reproduced over 21-days of exposure to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). C: significant differences between control and Ni^{2+} alone ($p<0.001$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

Mixtures containing 20, 40, or 80µg/L Ni^{2+} reproduced a significantly higher number of total neonates in comparison to the daphnids in Cd^{2+} alone ($p<0.05$) (Fig. 12). This is evidence of a less-than-additive effect. No other Cd^{2+}-Ni^{2+} mixtures contained a significantly higher number of total neonates in comparison to Cd^{2+} alone (Fig. 12) and only the Cd^{2+}-Ni^{2+} mixture containing 160µg/L Ni^{2+} had a significantly lower number of total neonates in comparison to that of Cd^{2+} alone ($p<0.05$) (Fig. 12). Since antagonistic Ni^{2+} effects were not observed in mixtures containing higher Ni^{2+} concentrations (100 to

160µg/L); Ni^{2+} exceeded the necessary concentration needed to protect *D. magna* from chronic Cd^{2+} reproductive toxicity and Ni^{2+} became toxic to the organisms.

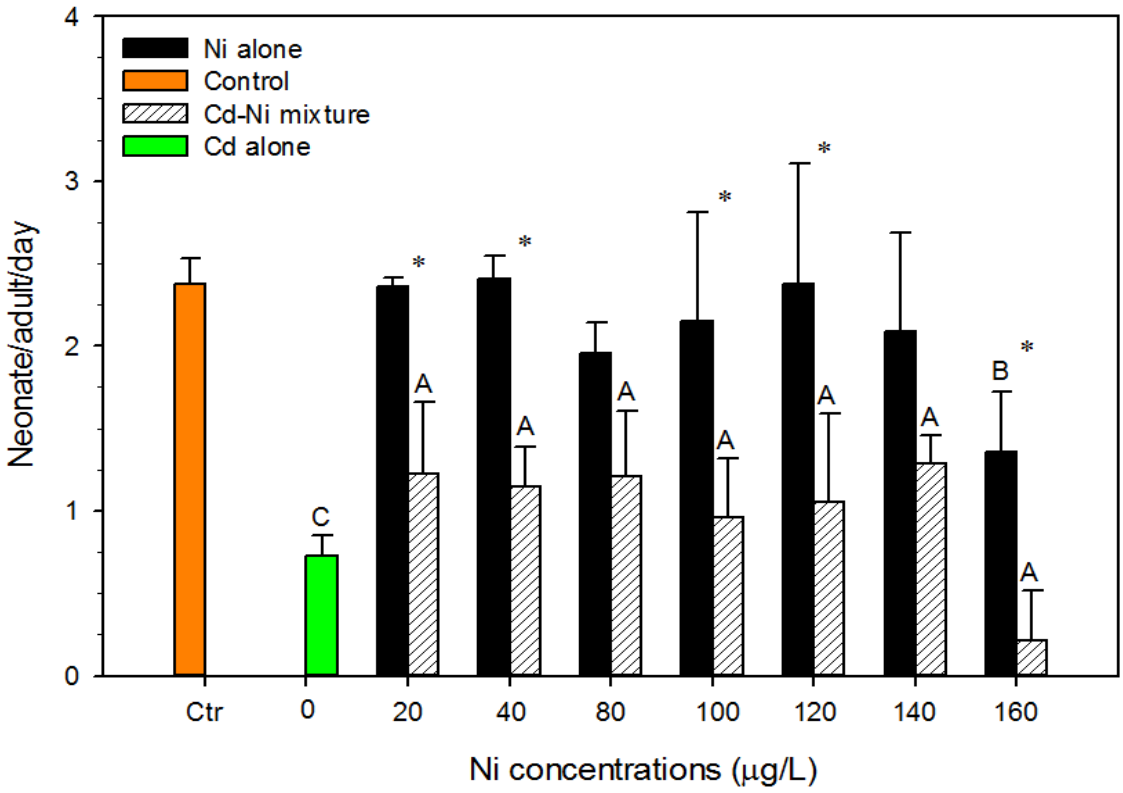

Figure 13. Overall reproductive rate (dead + live neonates) of *D. magna* when exposed to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.01$). B: significant differences between control and Ni^{2+} alone ($p<0.05$). C: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

Dissimilarly however, none of the Cd^{2+}-Ni^{2+} mixtures were found to contain a significantly higher number of live neonates when compared to the Cd^{2+} alone treatment (Fig. 14). This suggested that Ni^{2+} protective effects were not apparent, or possibly completely absent, when only live neonates were considered in analysis. Similarly, the results obtained for the overall reproductive rate (Fig. 13), and the live neonate reproductive rate (Fig. 15) established that none of the Cd^{2+}-Ni^{2+} mixtures had a

significantly higher or lower reproductive rate in comparison to Cd^{2+} alone (Fig. 13, 15). Hence, the results suggested that Ni^{2+} does protect adult *D. magna* from chronic Cd^{2+} reproductive toxicity, but not the neonates. Overall, like the surviving results, a less-than-additive toxicity is observed on adult *D. magna* when Ni^{2+} concentration is sufficient. Treatments containing sublethal Ni^{2+} alone concentrations of 20 and 40µg/L were not toxic to *D. magna* reproduction, since the total number of neonates (Fig. 12), and the total number of live neonates (Fig. 14) did not differ with control (Fig. 12, 14). However, since the responses in those same Ni^{2+} alone treatments were significantly different with their corresponding Cd^{2+}-Ni^{2+} mixtures in both the total number of neonates ($p<0.05$) (Fig. 12) and the total number of live neonates ($p<0.001$) (Fig. 14), meant that the reproductive toxicity observed in those Cd^{2+}-Ni^{2+} mixtures was likely due to Cd^{2+} exposure. On the contrary, Ni^{2+} alone treatments containing ≥80µg/L depicted a significant reduction in the number of total neonates ($p<0.001$) (Fig. 12), and live neonates ($p<0.001$) (Fig. 14) when compared to the control. With respect to figure 12, a higher Ni^{2+} alone concentration of 80µg/L was found to contain a significantly higher number of total neonates in comparison to its corresponding Cd^{2+}-Ni^{2+} mixture ($p<0.05$). This signifies that the toxicity observed in that Cd^{2+}-Ni^{2+} mixture was most likely due to both Ni^{2+} and Cd^{2+} exposure, since Ni^{2+} alone effects were significantly different from the control. However, since no other Ni^{2+} alone treatment was significantly different with its corresponding Cd^{2+}-Ni^{2+} mixture indicated that the reproductive toxicities observed in the Cd^{2+}-Ni^{2+} mixtures containing 100, 120, 140, and 160µg/L N^{2+} were most likely due to Ni^{2+} exposure (Fig. 12). Similarly, but not entirely, the Ni^{2+} alone treatments containing 80

and 100µg/L had a significantly higher number of total live neonates than their corresponding Cd^{2+}-Ni^{2+} mixtures ($p<0.001$) (Fig. 14).

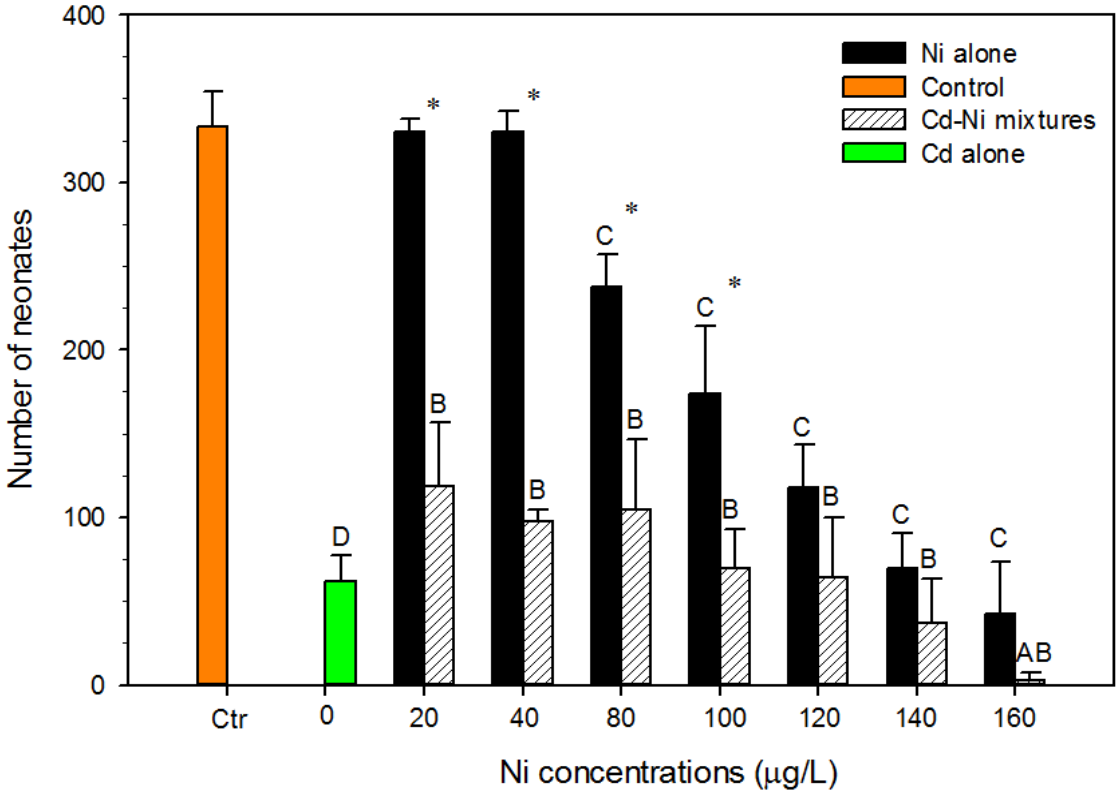

Figure 14. Total live neonates reproduced over 21-days of exposure to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between Cd^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). C: significant differences between control and Ni^{2+} alone ($p<0.001$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). n=4. Error bars represent standard deviation.

Given that those same Ni^{2+} alone treatments had a significantly lower number of total live neonates than control suggests that the toxicity observed in those Cd^{2+}-Ni^{2+} mixtures was most likely due to both Cd^{2+} and Ni^{2+} exposure (Fig. 14). Note that this result was absent when total neonates were collectively considered in analysis (Fig. 12). Furthermore, since Ni^{2+} alone treatments containing 120, 140, and 160µg/L did not reproduce a significantly

higher number of total live neonates in comparison to their corresponding Cd^{2+}-Ni^{2+} pair, meant that the toxicity observed in those Cd^{2+}-Ni^{2+} mixtures were most likely due to Ni^{2+} exposure (Fig. 14). In terms of the reproductive rate, only the Ni^{2+} alone treatment containing 160µg/L was found to hold a significantly lower overall reproductive rate ($p<0.05$) (Fig. 13), and a significantly lower live neonate reproductive rate ($p<0.05$) (Fig. 15) in comparison to the control. In addition, given that all Ni^{2+} alone treatments were significantly higher than their corresponding Cd^{2+}-Ni^{2+} mixtures in both the overall reproductive rate ($p<0.05$) (Fig. 13) and the live neonate reproductive rate ($p<0.05$) (Fig. 15) suggested that the toxicity observed in those Cd^{2+}-Ni^{2+} mixtures was most likely due to Cd^{2+} exposure.

Total Dead Neonates and Undeveloped Embryos

The average total number of dead neonates was significantly higher in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, 80, or 100µg/L Ni^{2+} in comparison to the Cd^{2+} alone treatment ($p<0.05$) (Fig. 16). The Cd^{2+}-Ni^{2+} mixture containing 160µg/L Ni^{2+} had on the other hand a significantly lower number of dead neonates compared to that of Cd^{2+} alone ($p<0.05$) (Fig. 16). No other Cd^{2+}-Ni^{2+} mixture was significantly different with Cd^{2+} alone (Fig. 16). This result revealed that *D. magna* in Cd^{2+}-Ni^{2+} mixtures containing 20, 40, 80, or 100µg/L Ni^{2+} are able to increase reproductive effort in comparison to Cd^{2+} alone (Fig. 16). However, Ni^{2+} does not seem to provide protective effects to the neonates in those Cd^{2+}-Ni^{2+} mixtures, as evident by the higher number of dead neonates (Fig. 16).

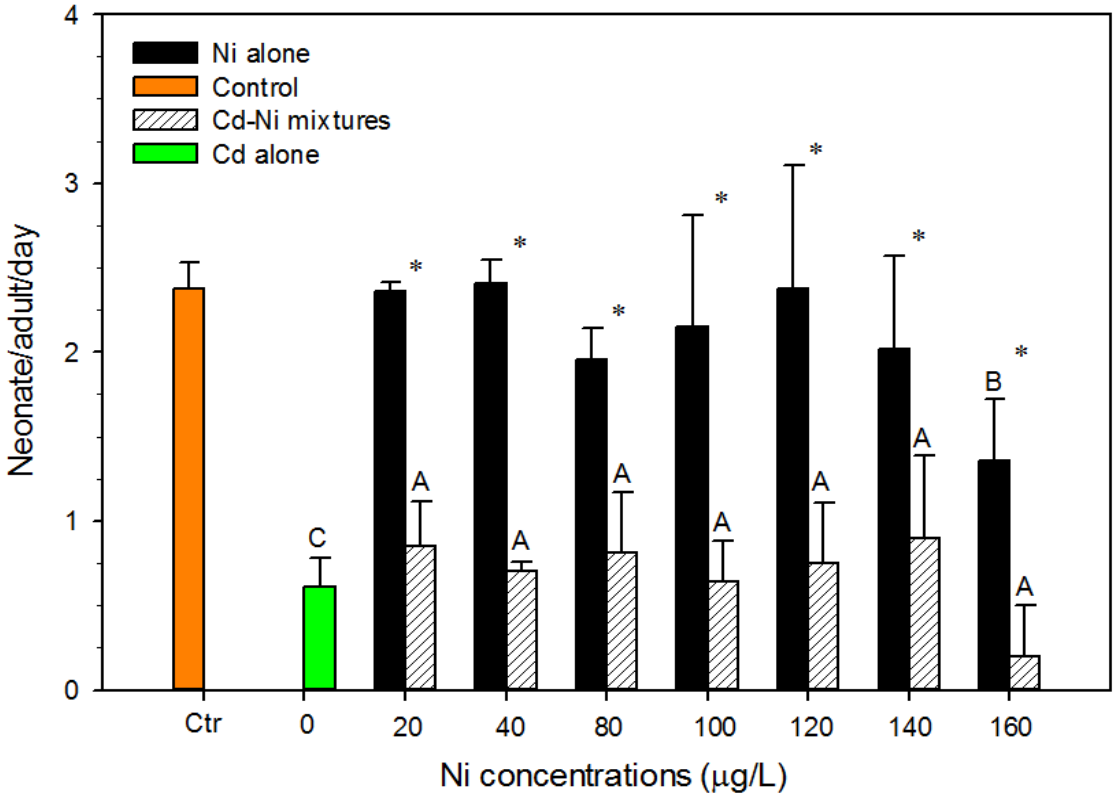

Figure 15. Reproductive rate (live neonates) of *D. magna* when exposed to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). B: significant differences between control and Ni^{2+} alone ($p<0.05$). C: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

Given the number of total dead neonates in the Cd^{2+}-Ni^{2+} mixtures containing ≥120µg/L Ni^{2+} were either not significantly higher, or were significantly lower (160µg/L) than Cd^{2+} alone (Fig. 16), implied that Ni^{2+} exceeded the necessary concentration needed to protect *D. magna* from chronic Cd^{2+} reproductive toxicity. Nickel became toxic to not only the newly born *D. magna* neonates, but to the reproductive system of adult *D. magna* as well.

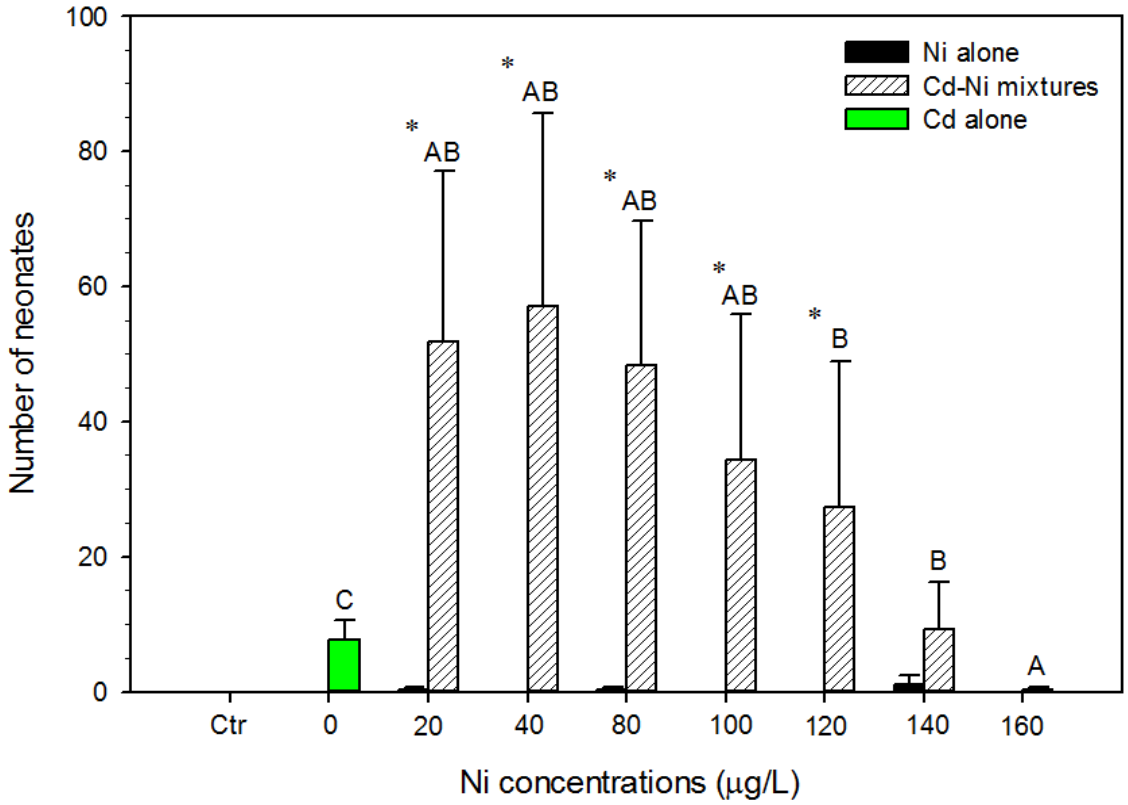

Figure 16. Total dead neonates reproduced over 21-days of exposure to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between Cd alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). C: significant difference between control and Cd^{2+} alone ($p<0.01$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). n=4. Error bars represent standard deviation.

The average number of undeveloped embryos decreased with increasing Ni^{2+} concentrations in the Cd^{2+}-Ni^{2+} mixtures (Fig. 17). Only the Cd^{2+}-Ni^{2+} mixtures containing 20 and 40µg/L Ni^{2+} had a significantly higher number of undeveloped embryos in comparison to Cd^{2+} alone ($p<0.05$) (Fig. 17). No significant differences in the number of undeveloped embryos were apparent between Cd^{2+} alone and Cd^{2+}-Ni^{2+} mixtures containing 80 to 120µg/L Ni^{2+}. Nonetheless, Cd^{2+}-Ni^{2+} mixtures containing 140 and 160µg/L Ni^{2+} held a significantly lower number of undeveloped embryos than Cd^{2+} alone ($p<0.05$) (Fig. 17). Since Cd^{2+}-Ni^{2+} mixtures containing 20 and 40µg/L had a

significantly higher number of undeveloped embryos than Cd^{2+} alone indicated no protective effect on *D. magna* embryos by Ni^{2+}. Furthermore, Cd^{2+}-Ni^{2+} mixtures containing higher Ni^{2+} concentrations ≥80µg/L were not protective either, since Ni^{2+} exceeded the necessary concentration needed to protect *D. magna* embryos from Cd^{2+} embryotoxicity. In mixtures containing ≥140µg/L Ni^{2+}, the reproductive system of *D. magna* was considerably disrupted (Figs. 12, 14).

None of the Ni^{2+} alone treatments had a significantly higher number of dead neonates in comparison to the control (Fig. 16). However, Cd^{2+}-Ni^{2+} mixtures containing 20, 40, 80, 100, or 120µg/L Ni^{2+} were all found to contain a significantly higher number of dead neonates in comparison to the control (Fig. 16). Since the number of dead neonates observed in the Ni^{2+} alone treatments containing 20, 40, 80,100, or 120µg/L were all significantly lower than their corresponding Cd^{2+}-Ni^{2+} mixtures ($p<0.05$) (Fig. 16), suggested that the toxicities observed in all those Cd^{2+}-Ni^{2+} mixtures were most likely due to Cd^{2+} exposure. However, the toxicities observed in the Cd^{2+}-Ni^{2+} mixtures containing ≥140µg/L Ni^{2+} were likely due to Ni^{2+} exposure.

The average number of undeveloped embryos observed in all Ni^{2+} alone treatments were not significantly different with the control (Fig. 17) while the number of undeveloped embryos observed in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, 80, or 100µg/L Ni^{2+} were all significantly higher in comparison to the control ($p<0.001$) (Fig. 17). These results indicated that the embryotoxicity observed in those Cd^{2+}-Ni^{2+} mixtures was likely due to Cd^{2+} exposure. On the other hand, the toxicity observed in the Cd^{2+}-Ni^{2+} mixtures containing ≥120µg/L Ni^{2+} was likely due to Ni^{2+} exposure.

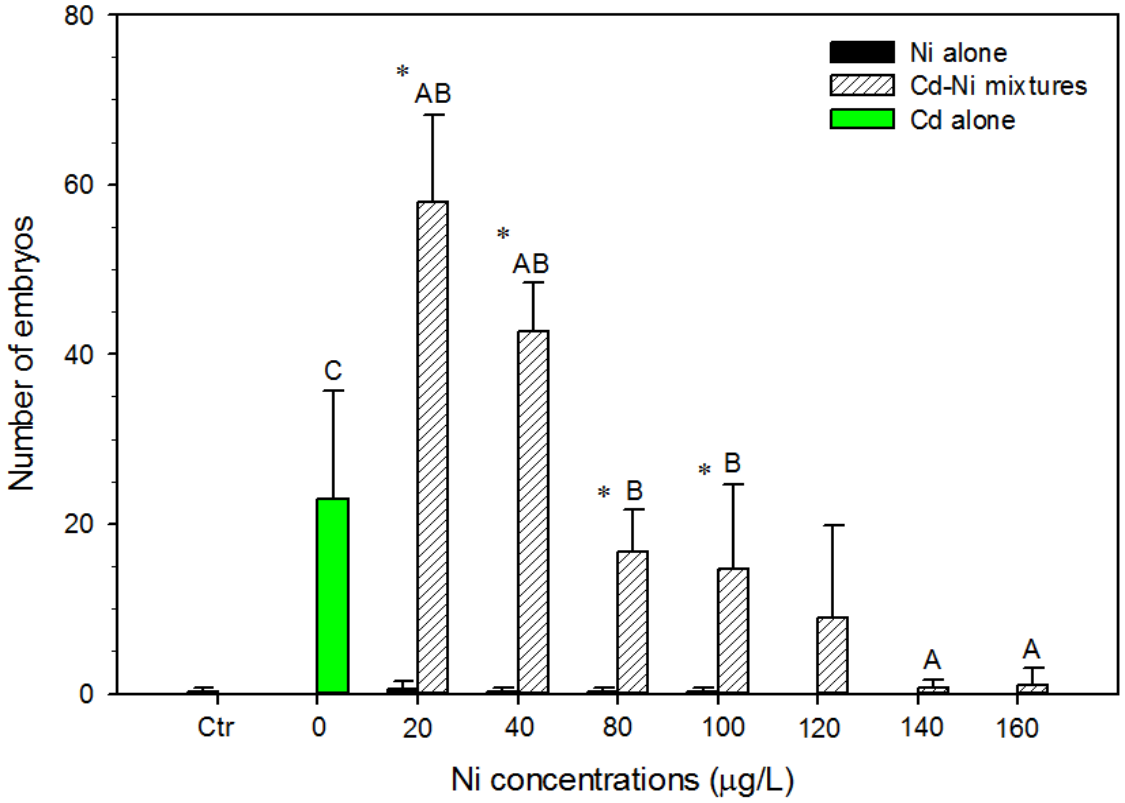

Figure 17. Total undeveloped embryos observed over 21-days of exposure to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between Cd alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). C: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.01$). n=4. Error bars represent standard deviation.

The undeveloped embryos observed in the Cd^{2+}-Ni^{2+} mixtures were scanned for the presence of developmental obstructions or morphological alterations which prevented the full development of a *D. magna* embryo. Compared to control embryos, the embryos from the Cd^{2+}-Ni^{2+} mixtures revealed several morphological defects that either shutdown the process of mitotic cell division (cleavage), or disrupted cellular arrangement and organization which prevented the development of a *D. magna* embryo beyond stage 5 (see Appendix B).

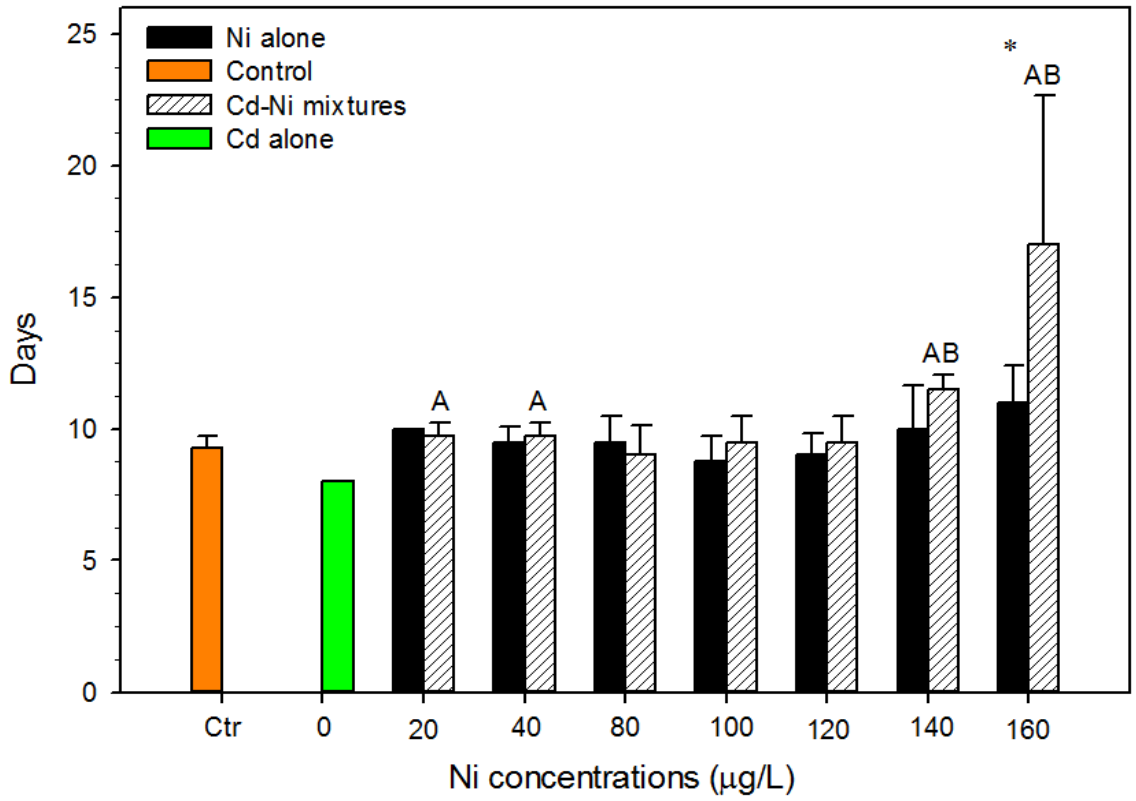

Figure 18. Time to first brood over 21-days of exposure to Cd^{2+} alone, Ni^{2+} alone, and Cd^{2+}-Ni^{2+} mixtures containing constant Cd^{2+} concentration of 1.5µg/L and varied Ni^{2+} concentrations. A: significant differences between Cd alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.01$). n=4. Error bars represent standard deviation.

Time to First Brood

The average time to first brood in Cd^{2+} alone seemed to be significantly earlier than the time to first brood in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, 140, and 160µg/L Ni^{2+} ($p<0.05$) (Fig. 18). These results indicate that 20 and 40µg/L Ni^{2+} were sufficient to protect *D. magna* from the toxicity Cd^{2+} exerts on the time to first brood, evidence of a less-than-additive effect. On the other hand, since the time to first brood was retarded in the Cd^{2+}-Ni^{2+} mixtures containing 140 and 160µg/L Ni^{2+}, revealed that Ni^{2+} exceeded the necessary concentration needed to protect *D. magna* from chronic Cd^{2+} toxicity (Fig. 18).

The time to first brood for Cd^{2+} alone was not significantly earlier (8 days on average) in comparison to the control (9.3 days on average) (Fig. 18). Only the Cd^{2+}-Ni^{2+} mixtures containing 140 and 160µg/L Ni^{2+} were found to hold a significantly higher time to first brood in comparison to the control ($p<0.05$) (Fig. 18). With respect to Ni^{2+} alone, none of those treatments were found to hold a significantly higher time to first brood with that of the control (Fig. 18). Moreover, only the Ni^{2+} alone treatment containing 160µg/L was found to hold a significantly lower time to first brood than its corresponding Cd^{2+}-Ni^{2+} pair. This suggested that the effects seen in that Cd^{2+}-Ni^{2+} mixture was likely due to Cd^{2+} exposure, because the time to first brood in that Ni^{2+} alone treatment was not significantly different with the control (Fig. 8).

Growth Effects

Dry Weight of Surviving Adults

The average dry weights of the surviving *D. magna* exposed to Cd^{2+}-Ni^{2+} mixtures containing 20, 40, 80, or 100µg/L Ni^{2+} were found to be significantly higher than the dry weights of the surviving *D. magna* in Cd^{2+} alone ($p<0.05$) (Fig. 19). This result implied a less-than-additive toxicity, since Cd^{2+}-Ni^{2+} mixtures containing 20, 40, or 80µg/L Ni^{2+} strongly protected *D. magna* from Cd^{2+} growth toxicity. Reciprocally, Cd^{2+} protected *D. magna* from Ni^{2+} toxicity in the Cd^{2+}-Ni^{2+} mixture containing 100µg/L Ni^{2+} (evidence of a less than additive effect by Cd^{2+}) (Fig. 19). In addition, since the dry weights of the surviving *D. magna* in other Cd^{2+}-Ni^{2+} mixtures did not differ significantly with that of Cd^{2+} alone (Fig. 19); indicated that the concentration of Ni^{2+} in those

mixtures exceeded the necessary concentration needed to protect *D. magna* from chronic Cd^{2+} growth toxicity.

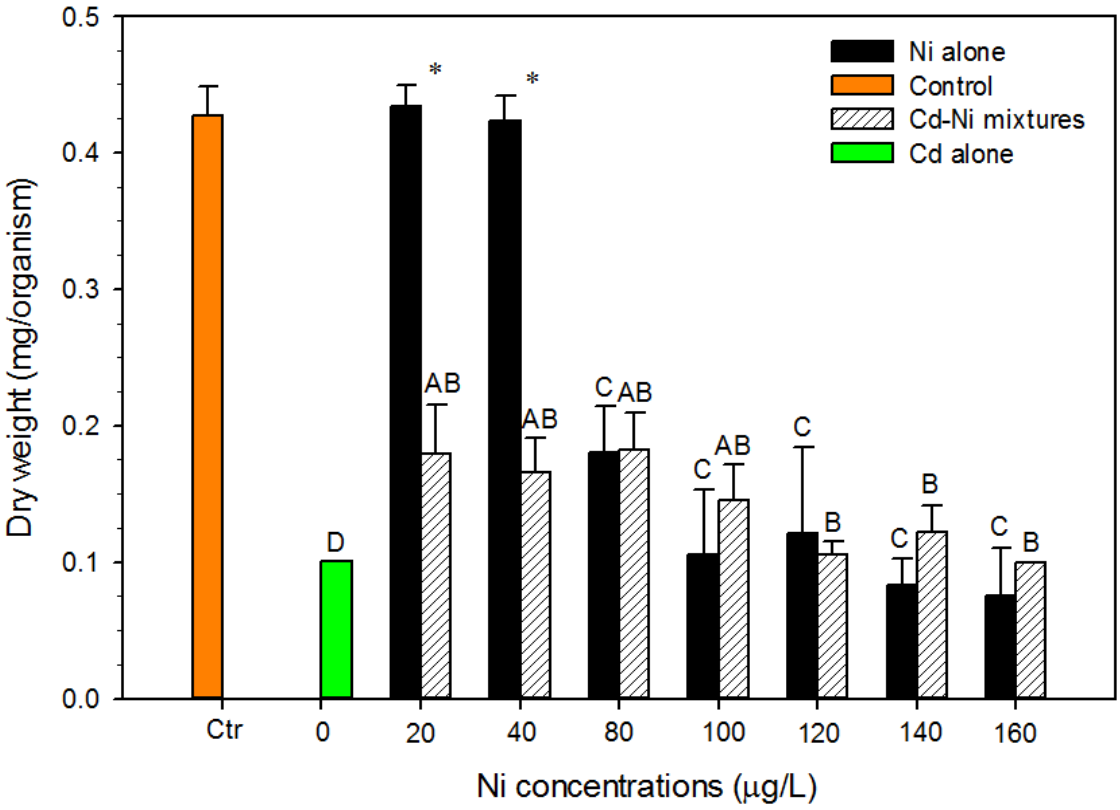

Figure 19. Dry weight of surviving adults at test termination in the Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures. A: significant differences between Cd^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.05$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). C: significant differences between control and Ni^{2+} alone ($p<0.001$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). *: significant differences between Ni^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). n=4. Error bars represent standard deviation.

The dry weights of the surviving *D. magna* in the Ni^{2+} alone treatments containing 20 and 40µg/L Ni^{2+} were significantly higher in comparison to their corresponding Cd^{2+}-Ni^{2+} mixtures ($p<0.001$) (Fig. 19). Plus, the dry weights of the surviving *D. magna* in those same Ni^{2+} alone treatments were not significantly different with that of the control (Fig. 19). As a result, the toxic effects observed in the Cd^{2+}-Ni^{2+} mixtures containing 20 and 40µg/L Ni^{2+} were most likely due to Cd^{2+} exposure.

However, the dry weights of the surviving *D. magna* in the Ni^{2+} alone treatments containing ≥80µg/L were not found to be significantly higher than their Cd^{2+}-Ni^{2+} pair (Fig. 19), but they were significantly lower than the control ($p<0.001$) (Fig. 19). This result suggested that the toxicity observed in Cd^{2+}-Ni^{2+} mixtures containing ≥80µg/L was likely due to Ni^{2+} exposure. Nickel at ≥80µg/L was toxic to *D. magna* growth at the end of 21 days of exposure.

Accumulation Effects

Body Metal Concentration

The average measured Ni^{2+} and Cd^{2+} concentrations in the tissues of the surviving daphnids exposed to Cd^{2+}-Ni^{2+} mixtures are represented in figure 20. The results show that the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed to Cd^{2+} alone, and to a mixture of Cd^{2+}-Ni^{2+} were all significantly higher than the measured Cd^{2+} concentration in control organisms. The results also show that as Ni^{2+} concentrations increased in the exposure media, Ni^{2+} concentrations gradually increased in the tissues of the surviving daphnids, fitting the trend of a power function (Fig. 20). Subsequently, a gradual decrease in Cd^{2+} tissue concentrations followed, also fitting the trend of a power function (Fig. 20). This result provides evidence of a less-than-additive effect between Cd^{2+}-Ni^{2+} mixtures.

The results obtained from tissue analysis of the surviving daphnids in the Cd^{2+}-Ni^{2+} mixtures is consistent with the hypothesis that metals compete for binding sites on the biotic ligand. The organisms exposed to Cd^{2+} alone accumulated significantly more Cd^{2+} than organisms exposed to a mixture of Cd^{2+}-Ni^{2+} ($p<0.01$) (Fig. 20). Interestingly,

the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed to Cd^{2+}-Ni^{2+} mixtures containing 20 and 40µg/L Ni^{2+} were not significantly lower than the measured Cd^{2+} in the tissues of the surviving daphnids exposed to Cd^{2+} alone. However, the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed to Cd^{2+}-Ni^{2+} mixtures containing ≥80µg/L Ni^{2+} were found to be significantly lower than the measured Cd^{2+} in the tissues of the surviving daphnids exposed to Cd^{2+} alone (Fig. 20).

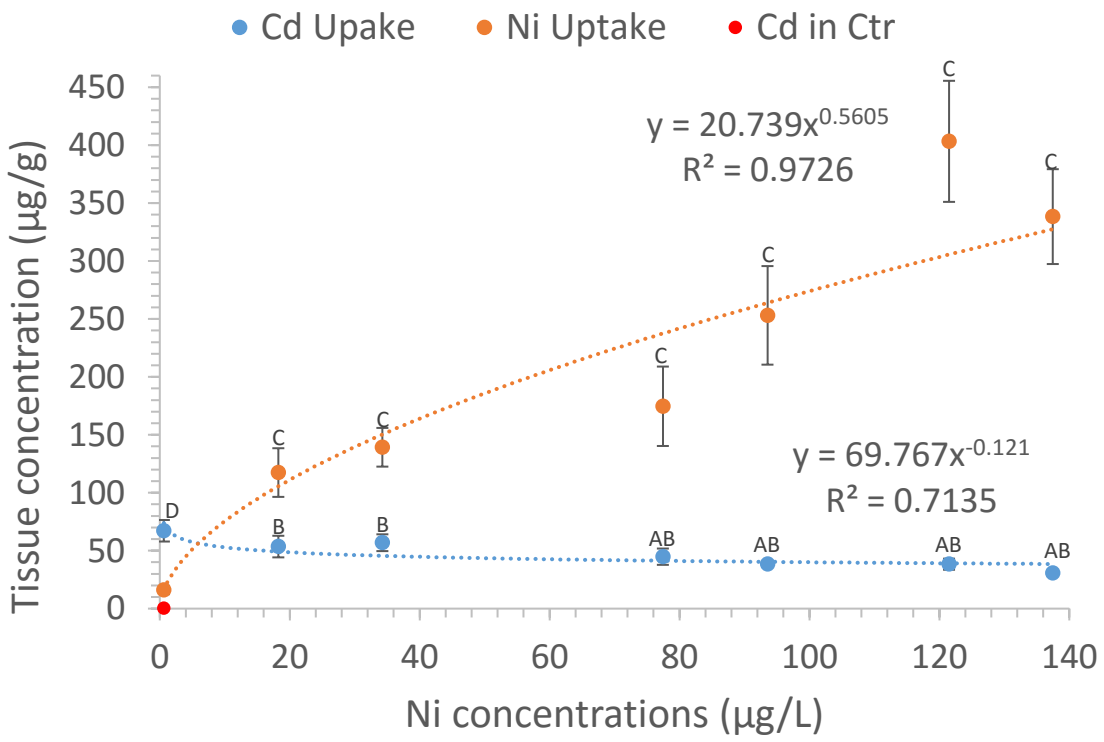

Figure 20. Metal concentrations in the tissues of surviving daphnids exposed to a mixture of Cd^{2+}-Ni^{2+} over 21 days. A: significant differences between Cd^{2+} alone and Cd^{2+}-Ni^{2+} mixtures ($p<0.01$). B: significant differences between control and Cd^{2+}-Ni^{2+} mixtures ($p<0.001$). C: significant differences between control and Ni^{2+} alone ($p<0.001$). D: significant difference between control and Cd^{2+} alone ($p<0.001$). n=4. Regressions are non-linear fitted power function. Error bars represent standard deviation.

Note that in the Cd^{2+}-Ni^{2+} mixtures, low and moderate Ni^{2+} concentrations of 20, 40, or 80µg/L in a mixture with 1.5µg/L Cd^{2+} were sufficient to significantly protect *D.*

magna from chronic Cd^{2+} toxicity, this was because those Ni^{2+} concentrations were (1) not in excess and (2) sufficient to impede the accumulation of toxic levels of Cd^{2+} on the BL's. Furthermore, since toxic Ni^{2+}effects were evident at 100µg/L Ni^{2+} alone in comparison to the control (Figs. 11, 19), but the toxicity slightly decreased in its corresponding Cd^{2+}-Ni^{2+} mixture suggested that the low concentration of Cd^{2+} prevented the accumulation of toxic levels of Ni^{2+} on the biotic ligand (Figs. 11, 19). However, when the Ni^{2+} concentrations were too high (≥120µg/L), Ni^{2+} became toxic to the organisms because the lower concentration of Cd^{2+} could not inhibit the accumulation of toxic levels of Ni^{2+} on the BL's. Similarly, the results suggest that Cd^{2+} and Ni^{2+} are interactive, and produce responses that are less-than-additive in character. Overall, it is essential to note that the interactive effects of the metals only occurred within a certain concentration range when one metal was not in excess, or when the binding sites on the BL's were not completely occupied.

The results in the Cd^{2+}-Ni^{2+} experiment demonstrate that Cd^{2+}-Ni^{2+} mixtures containing 20, 40, or 80µg/L Ni^{2+} were consistently sufficient to induce protective Ni^{2+} effects on *D. magna*. However, the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed to Cd^{2+}-Ni^{2+} mixtures containing 20 and 40µg/L Ni^{2+} were not significantly lower than the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed Cd^{2+} alone (Fig. 20). Therefore, this result suggests that although high concentrations of Cd^{2+} were penetrating the tissues of *D. magna* in those Cd^{2+}-Ni^{2+} mixtures, sublethal concentrations if Ni^{2+} (20 and 40µg/L) were still able to efficiently counteract the more toxic Cd^{2+} effect (Figs. 11, 12, 19). On the contrary, the

measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed to the Cd^{2+}-Ni^{2+} mixture containing 80µg/L Ni^{2+} was significantly lower than the measured Cd^{2+} concentration in the tissues of the surviving daphnids exposed Cd^{2+} alone (Fig. 20). However, since its corresponding Ni^{2+} alone pair (80µg/L) was consistently significantly different with control, implies that the effect observed in the Cd^{2+}-Ni^{2+} mixture was probably due to a joint toxicity between both Cd^{2+} and Ni^{2+} exposure. Lastly, the Cd^{2+}-Ni^{2+} mixtures containing higher Ni^{2+} concentrations (≥100µg/L) also accumulated significantly less Cd^{2+} than the organisms in Cd^{2+} alone. However, since the effects in those same Ni^{2+} alone treatments were always significantly lower than control, this suggests that the toxicity observed in the Cd^{2+}-Ni^{2+} mixtures was also due to both Cd^{2+} and Ni^{2+} exposure, or entirely due to Ni^{2+} exposure. Tissue analysis was not conducted on the Cd^{2+}-Ni^{2+} mixture containing 160µg/L Ni^{2+} because *D. magna* in that treatment experienced 95% mortality.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

Discussion

In the present study, Cd^{2+} alone was found to be very toxic to *D. magna*. Feeding observations showed that *D. magna* exposed to Cd^{2+} alone did not consume most of the algae fed to them routinely. Therefore, Cd^{2+} at a sublethal concentration of 1.5µg/L seemed to significantly disrupt resource consumption which resulted in reduced allocation of energy for reproduction and growth. This observation was apparent in both the Cd^{2+}-Zn^{2+} (Figs. 1-9) and the Cd^{2+}-Ni^{2+} (Figs. 11-19) experiments. For example, after 21 days of exposure, *D. magna* exposed to Cd^{2+} alone were found to have experienced a 71.4% decrease in average body weights in comparison to control organisms (Cd^{2+}-Zn^{2+} experiment) (Fig. 9). Similarly, in the Cd^{2+}-Ni^{2+} mixtures experiment, *D. magna* exposed to Cd^{2+} alone experienced a 76.7% decrease in average body weights in comparison to control organisms after 21 days of exposure (Fig. 19). Likewise, Bodar et al. (1988) exposed *D. magna* neonates to 1 and 5µg/L Cd^{2+} and found that just after 14 days of exposure, the body weights of *D. magna* in both Cd^{2+} treatments had dropped to approximately 40% in comparison to the control. Bodar and colleagues (1988) reported that Cd^{2+} directly disrupted feeding behavior and digestive mechanisms, which resulted in altered metabolism, subsequently reducing resource consumption. With respect to Cd^{2+}

reproductive toxicity, Jemec et al. (2007) found that Cd^{2+} concentrations ≥0.656µg/L significantly affected *D. magna* reproduction and survival. Likewise, Elnabaraway et al. (1986) demonstrated that a sublethal Cd^{2+} concentration of 2.5µg/L significantly reduced fecundity of reproducing *D. magna* in as early as 14 days of exposure.

The results of the present study support the hypothesis that Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures compete for binding sites on biotic ligands (BL's). As Zn^{2+} or Ni^{2+} concentrations gradually increased in the exposure media of the Cd^{2+}-Zn^{2+} or Cd^{2+}-Ni^{2+} mixtures, *D. magna* tissue concentrations of Zn^{2+} or Ni^{2+} subsequently increased, while the concentration of Cd^{2+} decreased (Figs. 10, 20). Thus, the principal process defining chronic toxicity between binary mixtures of Cd^{2+}-Zn^{2+} or Cd^{2+}-Ni^{2+} is competition for binding to similar biotic ligands. Work by Komjarova and Blust, (2008) found that the uptake rates of Cd^{2+}, Ni^{2+}, and Zn^{2+} were highly correlated with each other, indicating that the metals shared similar uptake and interaction pathways. In fact, the similar slopes of Ni^{2+}-Zn^{2+} and Cd^{2+}-Zn^{2+} (0.21and 0.23 respectively) suggested that the metals competitively interacted at similar biotic ligands, resulting in competitive inhibition (Komjarova and Blust 2008).

Recently, other scientists have proposed that a significant portion of metals such as Cd^{2+} and Zn^{2+} can enter through membrane transporters such as ZIP8 and ZIP14 (Fujishiro et al. 2012, Jenkitkasemwong et al. 2012), and Cd^{2+}, Ni^{2+}, and Zn^{2+} can enter through the divalent metal transporter-1 (DMT1) (Bannon et al. 2003, Mackenzie et al. 2006, Kwong 2011). However, because the exact mechanisms of transport are unknown, and the proportion of transported metals through these transmembrane proteins have not

been precisely measured, it is unclear whether the greatest proportion of metals permeate through Ca^{2+} channels, or through these transporters. More research on metal uptake routes need to be conducted so as to decipher which route of uptake is the most important.

Strong protective Zn^{2+} effects were observed in Cd^{2+}-Zn^{2+} mixtures containing 40, 80, and 120µg/L Zn^{2+}, allowing adult *D. magna* to reproduce a significantly higher number of total neonates (dead and alive) in comparison to Cd^{2+} alone ($p<0.05$) (Fig. 4). However, it is obvious that Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+} not only protected adult *D. magna*, but also the newly born neonate *D. magna* (Figs. 4, 5). From an evolutionary standpoint, this outcome is especially advantageous because this suggests that those newly born neonates were already adapted to the pollution in their surroundings, or they were rapidly adapting to the contaminants. A recent study conducted by Ward and Robinson (2005) showed that just after exposing a genetically diverse group of neonate *D. magna* for 48-hours to their Cd^{2+} EC50 (61µg/L), and then allowing those daphnids to recover and reproduce in clean hard water, significantly raised the EC50 of the first generation of neonates from 61 to 110µg/L Cd^{2+}. Given that the first generation of neonates had never been exposed to Cd^{2+} before, this signified that the neonates already possessed a resistant gene against Cd^{2+} toxicity that was passed on from the mother to the young, even though the mother was only exposed for 48-hours when she was <24 hours old. Therefore, it is likely that the live neonates encountered in the Cd^{2+}-Zn^{2+} mixtures containing 40, 80, or 120µg/L Zn^{2+} already possessed a resistant gene to counteract the effects of the more toxic Cd^{2+}. Further studies examining the

evolutionary resistance to chronic metal mixture toxicity is crucial to enhance scientific understanding of natural pollution phenomena, and biological adaptation. From an ecological perspective, this result is also attractive since the neonates that survived would be able to contribute and add variation to the gene pool of the population, and if survival extended into adulthood, they would be able to add to the growth of the population. Furthermore, although a protective Zn^{2+} effect was detected on the neonates, the protective Zn^{2+} effect was not 100% effective since those same Cd^{2+}-Zn^{2+} mixtures also contained a significantly higher number of dead neonates when compared to the Cd^{2+} alone treatment ($p<0.05$) (Fig. 6). Approximately 17.6 to 20% of the total neonates reproduced were observed dead on average.

Unfortunately, even though adult *D. magna* experienced a protective Ni^{2+} effect in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, or 80µg/L Ni^{2+}, a protective Ni^{2+} effect was not observed on neonate *D. magna* that were born in the exposure media. It is apparent that adult *D. magna* in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, or 80µg/L Ni^{2+} were able to increase reproductive effort in comparison to Cd^{2+} alone (Fig. 12). However, the average number of live neonates encountered in those same Ni^{2+}-Cd^{2+} mixtures were not significantly different with Cd^{2+} alone (Fig. 14). Approximately 30.5 to 36.8% of the neonates were found dead on average in those Cd^{2+}-Ni^{2+} mixtures. Given that there was a high percentage of neonate mortality, this explained why the number of total live neonates in those same Cd^{2+}-Ni^{2+} mixtures were not significantly different with Cd^{2+} alone (Fig. 14). The counter argument however, is that the number of live neonates in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, or 80µg/L Ni^{2+} were not significantly different

with Cd^{2+} alone because the generation of *D. magna* used was less sensitive to Cd^{2+} toxicity. In addition, given that the Cd^{2+}-Ni^{2+} mixtures containing 20 and 40µg/L Ni^{2+} did not hold a significantly higher number of live neonates in comparison to Cd^{2+} alone (Fig. 14), but held a significantly higher number of dead neonates (Fig. 16) and a higher number of undeveloped embryos (Fig. 17) than Cd^{2+} alone, signifies that 20 and 40µg/L Ni^{2+} in a mixture with 1.5µg/L Cd^{2+} are not sufficient to induce protective effects on neonate *D. magna*. Therefore, in the Cd^{2+}-Ni^{2+} mixture test, a possible transfer of resistant genes to the neonates to counteract Cd^{2+} toxicity is not observed, which means that the neonates probably died due to other reasons.

An explanation for observing dead neonates in the Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixture experiments is unclear. However, a recent study by Yu and Wang (2002) demonstrated that up to 44-67% from the aqueous phase, and 16-47% from the dietary phase of selenium uptake was lost from *D. magna* by allocating more energy into reproduction. Hence, the mother transferred a significant portion of accumulated metals to her offspring as a detoxification mechanism. Could *D. magna* in the Cd^{2+}-Zn^{2+} mixtures containing 40, 80, and 120µg/L Zn^{2+}, and *D. magna* in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, and 80µg/L Ni^{2+} be reducing Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} toxicity by increasing reproduction, and maternally transferring toxic levels of the metals to the embryos? This may be the case, as Yu and Wang (2002) found that the main route for Cd^{2+} and Zn^{2+} release from *D. magna* was through molting (50-70%) and (20-70%), respectively, within the first 4 days of aqueous exposure, but decreased to <20 and <30% after a dietary exposure, respectively. Hence, it is likely that the adult *D. magna* exposed

to Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures maternally transferred a great proportion of the metals to their young following a dietary contact, since the organisms in the present study were fed during the 21 days of exposure. In addition, since a non-protective Zn^{2+} effect was detected on the undeveloped embryos in the Cd^{2+}-Zn^{2+} mixtures containing 40, 80 and 120µg/L Zn^{2+} (Fig. 7), and in the Cd^{2+}-Ni^{2+} mixtures containing 20, 40, and 80µg/L Ni^{2+} (Fig. 17), indicated that *D. magna* embryos were accumulating toxic levels of the metals, preventing their full development. Nonetheless, given that many embryos also completed development, as observed by the total number of neonates in the Cd^{2+}-Zn^{2+} mixtures (Fig. 2) and in the Cd^{2+}-Ni^{2+} mixtures (Fig. 12), indicated to me that not all of the embryos would have accumulated equitoxic concentrations of the metals. Thus, it is likely that some embryos accumulated a sufficient Zn^{2+}, or Ni^{2+} concentration that antagonized the more toxic Cd^{2+} effect, and so underwent complete development. Studies that seek to measure the metal concentrations in the embryos of *D. magna* will shed some light on this hypothesis.

Equally however, *D. magna* neonates could have been exposed to the metals while in the brood pouch. Fisher et al. (2000) demonstrated that metals can directly adsorb onto developing embryos/neonates in the brood chamber. Therefore, the total accumulated metals in the tissues of the offspring might not be entirely due to maternal transfer. So, the accumulation of the metals in the tissues of the offspring could possibly be higher than anticipated. As a result, it is possible that after complete development some neonates were probably not mechanically functional and possessed deformities that rendered them unable to survive. A recent study investigating Cd^{2+} embryotoxicity

revealed that *D. magna* embryos experienced severe morphological defects after acute exposures to 60, 80, or 100µg/L Cd^{2+} (Djekoun et al. 2015). Neonates were observed to have many deformities which ranged from caudal spine malformations, to poorly developed carapaces, and no antennas or eyes (Djekoun et al. 2015). Although the concentrations of Cd^{2+} used in that study were between 1 and 2 orders of magnitude higher than the concentration of Cd^{2+} used in the present study, it is possible that under a chronic stress, 1.5µg/L Cd^{2+} is sufficient to induce embryonic deformities. Therefore, the dead neonates observed in the mixtures containing 40, 80, or 120µg/L Zn^{2+} probably possessed developmental malformations that disrupted their swimming, feeding, and metabolizing capacity. Other studies assessing that hypothesis should be conducted. Note though that it is also necessary to consider a dietary exposure. The young epithelial gut cells of a neonate could be more sensitive to Cd^{2+} toxicity. Neonates generally start feeding as soon as they are born, and if high concentrations of metals had adsorbed on the algal surfaces of *S. capricornutum*, it is likely that the young gut cells of some neonates were unable to tolerate it. Other studies seeking this information should also be conducted.

In examining embryonic morphological alterations, the study conducted by Mittmann et al. (2014) was strongly used as a reference (see all images in appendix B for clarity). Embryos collected from Cd^{2+} alone, and both the Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures showed that the developing offspring experienced morphological deformities, which translated into either the shutdown of mitotic cell division (early or late cleavage) (images 2, 3, 5, 6); disruption of cellular arrangement and organization (gastrulation)

(images 8, 9, 14, 15), or complete prevention from further development beyond stage 5 (images 11, 12) in comparison to control embryos that displayed normal cleavage, and a high degree of cellular arrangement, organization, and differentiation (images 1, 4, 7, 10, 13).

Noticeably, the results further demonstrate that embryos exposed to Cd^{2+} alone consistently only underwent early cleavage, giving rise to a morula (images 2, 3), but embryos exposed to a mixture containing 1.5µg/L Cd^{2+} or low to high concentrations of Zn^{2+} or Ni^{2+} underwent late cleavage, giving rise to a blastula (images 5, 6), gastrulation (8, 9), or development to stage 5 (images 11, 12). It should be noted that even though some embryos were able to form a blastula and initiate gastrulation, the blastomeres were disorganized or deformed, and conglomerated into a blob of cells (images 8, 9, 14). This signifies that at some point the blastomeres lose their ability to successfully communicate, and are unable to faithfully arrange and organize themselves. In totality, 45 and 36 embryos were processed from the Cd^{2+}-Ni^{2+} and Cd^{2+}-Zn^{2+} mixtures, respectively. All embryos exposed to a mixture did not develop beyond stage 5 (image 11, 12). Nonetheless, a trend is not clear because embryos within a specific treatment did not arrest at the same stage of development. Therefore, as previously mentioned, it seems that some embryos are accumulating toxic levels of a combination of the metals due to maternal transfer or adsorption, while others are not, and are able to push through further in development. An observation that begs further questioning. In general, it is likely that Cd^{2+} is disrupting biochemical signals that are essential to the successful development of *D. magna* embryos, or it could be acting as a genotoxic agent that leads to the premature

death of developing embryos. Recently, Metka and Hei (2004) established that Cd^{2+} has high mutagenic activity since it predominantly induces large deletion mutations. In addition, Cd^{2+} is able to interact with reactive oxygen species (ROS), disrupting cellular signaling and interfering with DNA repair (Metka and Hei, 2004). Studies exploring Cd^{2+} effects on mitotic signaling have not been conducted. Therefore, it would be interesting to examine how Cd^{2+} alters mitotic protein configurations.

Because Cd^{2+}, Ni^{2+}, and Zn^{2+} inhibit Ca^{2+} uptake, it is likely that these metals disrupt calcium homeostasis, and alter Ca^{2+}-dependent cellular signaling. Cytoplasmic Ca^{2+} signaling is known to regulate genes associated with differentiation, growth, and apoptosis (Kasprzak and Salnikow, 2007), and disruption of those signaling pathways may very well lead to deleterious effects, including DNA impairment, transcriptional abnormalities, and malignant cellular growths (Kasprzak and Salnikow, 2007). Once heavy metal ions make their way into the cytoplasm, they have direct access to many signaling proteins, and cellular signaling pathways which they can parasitize by altering protein configurations, binding sites, and activity (either activating or deactivating proteins). For example, Protein Kinase C, a family of enzymes involved in Ca^{2+}-dependent signaling, controls protein functions by phosphorylating serine and threonine residues on proteins. Studies have shown that Cd^{2+} is able to inappropriately activate Protein Kinase C (PKC) at nM concentrations, but also reversibly, inhibit its activity at μM concentrations (Long, 1997; Saijoh et al. 1988). In addition, metal ions may also replace the Ca^{2+} ion on Ca^{2+}-dependent signaling proteins, disrupting the normal functions of those proteins (Shafer, 2000). For example, calmodulin, another Ca^{2+}

dependent signaling protein, is able to strongly bind to, modify, and interact with many target proteins, including kinases and phosphatases (Chin and Means, 2000) inducing several cellular signaling pathways. Cadmium (Cd^{2+}) has been shown to replace Ca^{2+} on calmodulin's Ca^{2+} binding sites, therefore, inappropriately activating Ca^{2+} dependent transduction pathways (Flik et al. 1987). Thus, disruption of cellular Ca^{2+} homeostasis, and inappropriate activation, or deactivation of cellular signaling, is hypothesized to be the central mechanism driving metal toxicity.

Using a series of acute 48hr Cd^{2+}-Zn^{2+} exposures, Meyer et al. (2015) detected a 100% protective Zn^{2+} effect on *D. magna* survival at a sublethal Zn^{2+} concentration of 13µg/L. On the contrary, this study documents that on a chronic basis, 10 and 20µg/L Zn^{2+} in a mixture with 1.5µg/L Cd^{2+} are insufficient to prompt protective Zn^{2+} effects on *D. magna* survival, reproduction or growth (Figs. 1-9). In addition, based on the significantly different degrees of responses between the binary-metal and single-metal assays, these results emphasize the need for more comprehensive chronic metal mixture studies as they are more realistic in nature. Acute single metal toxicity assays are not representative of natural aquatic pollution, and so do not provide insight into detailed mechanistic biological, ecological, and toxicological dynamics which may thwart the true evaluation of a metal's toxicity in the environment, and lead to the production of inaccurate ecological risk assessments.

The results of this study are consistent with other literature studies indicating a protective Zn^{2+} effect from Cd^{2+} toxicity. For example, Meyer et al. (2015), found a less-than-additive toxicity between Cd^{2+}-Zn^{2+} mixtures. Cañizares-Villanueva et al. (2000),

reported reduced Cd^{2+} toxicity to *D. magna* when Zn^{2+} was present in a mixture after biological treatment with suspended cultures of *Chlorella vulgaris* in a 48 hour assay (less-than-additive). Attar and Maly (1982), also documented reduced Cd^{2+} toxicity to *D. magna* after acute exposures to Cd^{2+}-Zn^{2+} mixtures. Other studies using test organisms such as shrimp, flag fish, minnows, trout, and green algae have also reported less-than-additive toxicity between Cd^{2+} and Zn^{2+} mixtures (Thorp and Lake 1974; Spehar et al. 1978; Wicklund Glynn et al. 1992; Lavoie et al. 2012a, b; Mebane et al. 2012 respectively). Furthermore, other studies have also demonstrated a protective Ni^{2+} effect counteracting Cd^{2+} toxicity. Specifically, mixtures of Cd^{2+}-Ni^{2+} have shown less-than-additive toxicity on the survival of the water flea *D. magna* (Traudt et al. 2016), on the uptake in *D. magna* (Komjarova and Blust, 2008), on the uptake in the zebra fish *Danio rerio* (Komjarova and Blust, 2009), on the growth of the freshwater bacteria *Nitrosomonas europaea* at an ammonia concentration between 2.5 and 40mg/L as Nitrogen (Sato et al. 1986), and on the adsorption capacity of the freshwater algal *Chlorella vulgaris* (Awasthi and Rai, 2004).

Finally, it is important to point out that during the 21 days of exposure, *D. magna* were fed an algae suspension of *Selenastrum capricornutum* of $3*10^7$ cells/ml, and a food suspension of yeast, cereal leaves, and trout chow (YCT) of concentration 12-13 mg solids/L. Therefore, it is likely that organic carbon was introduced into the exposure media. Total organic carbon analysis (TOC) showed that the Cd^{2+}-Zn^{2+} mixtures contained an average of 4.44mg/L TOC, while the Cd^{2+}-Ni^{2+} mixtures contained an average of 6.07mg/L TOC (see appendix A: table 4). Unfortunately, this study did not

seek to assess the concentrations of metals that complexed with the organic carbon, nor did it seek to assess the concentrations of metals that adsorbed unto the surfaces of the algae. Recently, Awasthi and Rai, (2004) showed that bioavailable metal species such as Cd^{2+}, Ni^{2+}, and Zn^{2+} can adsorb strongly unto algal and bacterial surfaces, and compete for the binding sites that are available on those biotic ligands (BL's). In fact, the adsorptive capacity of one metal on the surface of algae or bacteria may create an interactive or non-interactive microenvironment, affecting the adsorptive capacity of a second metal. For instance, when Cd^{2+} and Ni^{2+} were simultaneously added to immobilized cells of the green algae *Scenedesmus quadricauda,* a synergistic adsorptive effect was observed, while a non-interactive adsorptive effect was observed when Cd^{2+} and Zn^{2+} were simultaneously added (Awasthi and Rai, 2004). On the other hand, when Cd^{2+} and Ni^{2+}, or Cd^{2+} and Zn^{2+} were simultaneously added to the immobilized cells of the green algae *Chlorella vulgaris*, an antagonistic adsorptive effect was observed in both instances (Awasthi and Rai, 2004). On the immobilized cells of the cyanobacteria *Anacystis nidulans* however, Cd^{2+} and Ni^{2+} were shown to adsorb in a non-interactive manner, while Cd^{2+} and Zn^{2+} were shown to adsorb in an antagonistic manner when both metals were added simultaneously (Awasthi and Rai, 2004). Therefore, it is highly likely that in the Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures, the metals interacted with the algal surfaces, and with the bacteria on their surfaces, creating a microenvironment with varying degrees of interactive and non-interactive adsorptive capacities. As a result, the organisms in this study were not only exposed to metals in the water column, but they were also exposed to metals through a dietary route.

The degree of the dietary effect is not explicitly known because the interactive adsorptive capacity of Cd^{2+} and Ni^{2+}, or Cd^{2+} and Zn^{2+} on the surfaces of *S. capricornutum* were not measured. It is likely though that Cd^{2+} and Zn^{2+} adsorbed in an antagonistic manner on the surfaces of *S. capricornutum* given that protective Zn^{2+} effects were observed in all endpoints measured (Figs. 1-10). In other words, Cd^{2+} and Zn^{2+} did not only interact antagonistically for permeation across the cell membrane through Ca^{2+} channels of different cell types, but it is likely that the metals also adsorbed unto the cells of *S. capricornutum* in the same manner. Therefore, when the algal cells were ingested, digestive enzymes detached the metals in the gut, and again, the metals antagonistically entered the epithelial gut cells of *D. magna*. Since protective Ni^{2+} effects were also observed on adult *D. magna* in the Cd^{2+}-Ni^{2+} mixtures, it is likely that the metals also adsorbed on the algae in the same manner. These claims are merely educated guesses, and not an answer to the interactive adsorptive capacity of the metals on the surfaces of *S. capricornutum*. Other studies investigating the competitive adsorptive capacity of Cd^{2+}, Ni^{2+}, and Zn^{2+} on the algae *S. capricornutum* are essential to further our understanding of the dietary effect.

Conclusion

On a chronic basis, Cd^{2+} alone at 1.5µg/L was found to be very toxic to *D. magna* at all endpoints evaluated (survival, reproduction, and growth). However, in a mixture with the more toxic Cd^{2+}, moderate Zn^{2+} concentrations of 40, 80, and 120µg/L were sufficient to strongly protect *D. magna* at all endpoints (evidence of less-than-additive effect) while sublethal Zn^{2+} concentrations of 10 and 20µg/L were not sufficient, and high

concentrations of 160 and 200µg/L were too excessive. The highest protective Zn^{2+} effect was consistently observed in the Cd^{2+}-Zn^{2+} mixture containing 80µg/L Zn^{2+}. Similarly, sublethal and moderate Ni^{2+} concentrations of 20, 40, and 80µg/L were sufficient to strongly protect *D. magna* at all endpoints (less-than-additive effect), while Ni^{2+} concentrations ≥100µg/L were too excessive. The highest protective Ni^{2+} effect was recorded in the Cd^{2+}-Ni^{2+} mixture containing 20µg/L Ni^{2+}. With respect to the neonates, Zn^{2+} concentrations of 40, 80, and 120µg/L were found to be protective to the newly born neonates as demonstrated in (Fig. 4), while 20, 40, and 80µg/L Ni^{2+} were not found to be protective (Fig. 14). Furthermore, no concentration of Ni^{2+} or Zn^{2+} was found to be protective to *D. magna* embryos. Therefore, indicating that some embryos are sacrificed by possibly accumulating higher concentration of metals. The embryos analyzed and processed in both the Cd^{2+}-Zn^{2+} and Cd^{2+}-Ni^{2+} mixtures showed severe morphological alterations which disrupted cleavage, gastrulation, and development beyond stage 5 (see appendix B). In fact, embryos within the same treatment did not arrest at the same stage of development. Lastly, tissue analysis supports the hypothesis that metals such as Cd^{2+}, Ni^{2+} and Zn^{2+} compete for similar biotic ligands since Cd^{2+} concentrations in the tissues of surviving *D. magna* decreased, as Zn^{2+} or Ni^{2+} concentrations increased in the exposure media.

APPENDIX A

SUPPLEMENTAL DATA

Table 1. Nourishment rations of *S. capricornutum* and YCT fed to *D. magna* during chronic exposure to Zn^{2+} alone and Cd^{2+}-Zn^{2+} mixtures over 21 days of exposure.

Day	Algae	YCT
0	2 drops	1 drop
1	4 drops	2 drops
2	4 drops	2 drops
3	0.3 ml	0.1 ml
4	0.5 ml	0.2 ml
5	0.5 ml	0.2 ml
6	1 ml	0.5 ml
7	1 ml	0.5 ml
8	1 ml	0.5 ml
9	1 ml	0.5 ml
10	2 ml	1 ml
11	2 ml	1 ml
12	2 ml	1 ml
13	2 ml	1 ml
14	3 ml	1.5 ml
15	3 ml	1.5 ml
16	3 ml	1.5 ml
17	3 ml	1.5 ml
18	3 ml	1.5 ml
19	3 ml	1.5 ml
20	3 ml	1.5 ml

Note: organisms were not fed on day 21 because the experiment ended on that day.

Table 2. Nominal and average measured total and dissolved metal concentrations (µg/L) in the test media of Zn^{2+} alone Cd^{2+}-Zn^{2+} mixtures.

			Cd^{2+}-Zn^{2+} mixture test		Zn^{2+} alone test
Sample Type	Nominal Zn^{2+}	Nominal Cd^{2+}	Avg. Zn^{2+}	Avg. Cd^{2+}	Avg. Zn^{2+}
Total	Control	0	4.22 ± 1.82	nd	4.22 ± 1.82
	0	1.5	5.88 ± 6.75	1.26 ± 0.18	na
	10	1.5	11.56 ± 2.90	1.44 ± 0.24	15.76 ± 5.63
	20	1.5	21.74 ± 9.34	1.41 ± 0.21	25.60 ± 5.95
	40	1.5	38.10 ± 6.80	1.42 ± 0.16	46.37 ± 8.05
	80	1.5	70.31 ± 14.11	1.41 ± 0.24	84.19 ± 11.88
	120	1.5	110.09 ± 20.22	1.41 ± 0.17	126.87 ± 18.48
	160	1.5	144.92 ± 16.23	1.41 ± 0.14	163.86 ± 24.81
	200	1.5	184.29 ± 3.99	1.51 ± 0.11	230.16 ± 21.08
Dissolved	Control	0	4.17 ± 1.80	nd	4.17 ± 1.80
	0	1.5	5.80 ± 1.24	1.13 ± 0.03	na
	10	1.5	16.18 ± 20.36	1.24 ± 0.18	11.10 ± 14.44
	20	1.5	24.84 ± 4.84	1.30 ± 0.08	20.94 ± 6.73
	40	1.5	42.56 ± 13.00	1.30 ± 0.11	45.11 ± 6.99
	80	1.5	79.95 ± 17.80	1.35 ± 0.07	89.13 ± 17.67
	120	1.5	124.35 ± 33.54	1.33 ± 0.12	131.37 ± 20.04
	160	1.5	151.43 ± 19.86	1.33 ± 0.12	160.29 ± 46.23
	200	1.5	188.74 ± 37.36	1.32 ± 0.12	222.89 ± 36.49

Note. Data is represented as average ± standard deviation; nd stands for not detected; na stands for not applicable.

Table 3. Nominal and average measured total and dissolved metal concentrations (µg/L) in the test media of Ni^{2+} alone Cd^{2+}-Ni^{2+} mixtures.

			Cd^{2+}-Ni^{2+} mixture test		Ni^{2+} alone test
Sample Type	Nominal Ni^{2+}	Nominal Cd^{2+}	Avg. Ni^{2+}	Avg. Cd^{2+}	Ave. Ni^{2+}
Total	Control	0	0.83 ± 0.15	nd	0.83 ± 0.15
	0	1.5	0.62 ± 0.12	1.31 ± 0.14	na
	20	1.5	19.01 ± 2.09	1.33 ± 0.23	20.86 ± 0.51
	40	1.5	38.56 ± 4.76	1.38 ± 0.19	37.74 ± 2.47
	80	1.5	76.93 ± 11.20	1.36 ± 0.15	80.38 ± 4.40
	100	1.5	95.10 ± 11.24	1.34 ± 0.13	96.23 ± 8.57
	120	1.5	113.86 ± 14.85	1.39 ± 0.12	118.30 ± 16.35
	140	1.5	133.84 ± 19.80	1.37 ± 0.16	141.73 ± 5.27
	160	1.5	148.02 ± 15.04	1.31 ± 0.13	161.46 ± 9.94
Dissolved	Control	0	0.71 ± 0.16	nd	0.71 ± 0.16
	0	1.5	0.63 ± 0.23	0.59 ± 0.19	na
	20	1.5	18.25 ± 4.92	0.63 ± 0.23	18.32 ± 7.09
	40	1.5	34.26 ± 6.22	0.66 ± 0.14	36.63 ± 13.74
	80	1.5	77.44 ± 10.81	0.75 ± 0.21	66.39 ± 8.84
	100	1.5	93.54 ± 12.63	0.71 ± 0.18	83.25 ± 11.07
	120	1.5	121.49 ± 22.58	0.83 ± 0.21	101.75 ± 11.72
	140	1.5	137.46 ± 22.96	0.80 ± 0.20	119.15 ± 8.79
	160	1.5	150.74 ± 21.48	0.74 ± 0.13	130.82 ± 6.57

Note. Data is represented as average ± standard deviation; nd stands for not detected; na stands for not applicable.

Table 4. Average measured total organic carbon (TOC) in the Zn^{2+} alone, Ni^{2+} alone, Cd^{2+}-Zn^{2+}, and Cd^{2+}-Ni^{2+} mixtures.

Zn^{2+} alone	Cd^{2+}-Zn^{2+} mixture	Ni^{2+} alone	Cd^{2+}-Ni^{2+} mixture
4.44 ± 1.46	4.21 ± 0.70	6.05 ± 0.77	6.07 ± 1.16

Note. Data is represented as average ± standard deviation.

APPENDIX B

IMAGES OF CONTROL EMBRYOS AND UNDEVELOPED EMBRYOS IN THE CADMIUM-ZINC AND CADMIUM-NICKEL MIXTURES

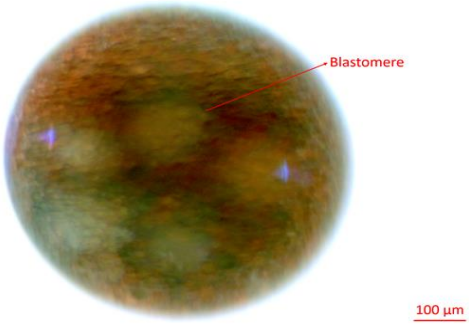

Image 1. Embryo in stage 2 of development (early cleavage) – control treatment

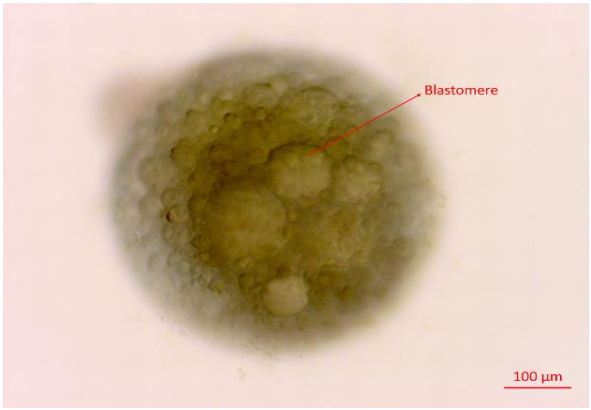

Image 2. Embryo in stage 2 of development (early cleavage) – Cd^{2+} alone treatment (Cd^{2+}-Zn^{2+} mixture)

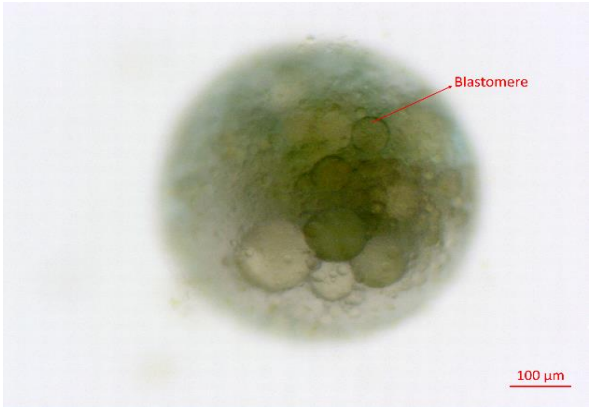

Image 3. Embryo in stage 2 of development (early cleavage) – Cd^{2+} alone treatment (Cd^{2+}-Ni^{2+} mixture)

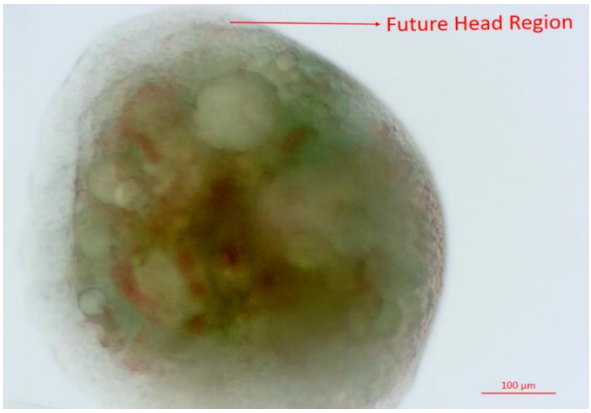

Image 4. Embryo in stage 7.3 of development (Postnaupliar segments): ventral view – control treatment

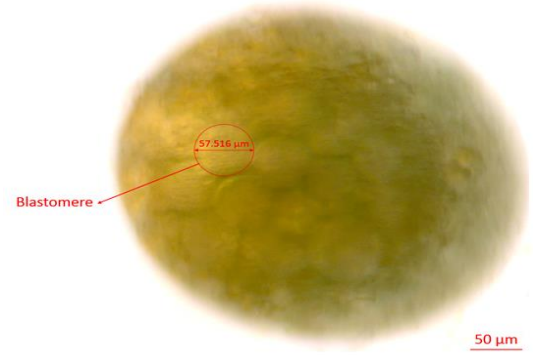

Image 5. Embryo in stage 2 of development (late cleavage): Cd^{2+}-Zn^{2+} mixture with 120µg/L Zn^{2+} and 1.5µg/L Cd^{2+}

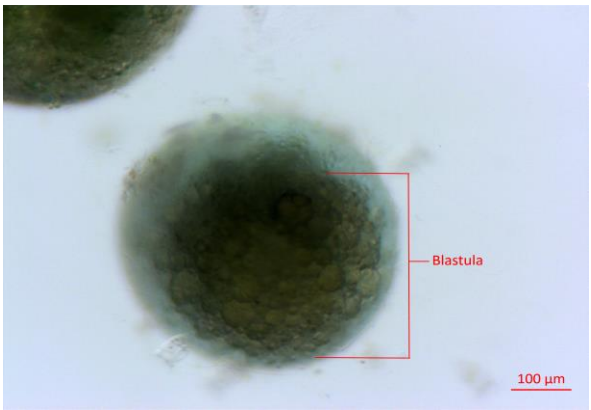

Image 6. Embryo in stage 2 of development (late cleavage) – Cd^{2+}-Zn^{2+} mixture with 140µg/L Ni^{2+} and 1.5µg/L Cd^{2+}

Image 7. Embryo in stage 9 of development (appearance of compound and nauplius eye): dorsal view – control treatment

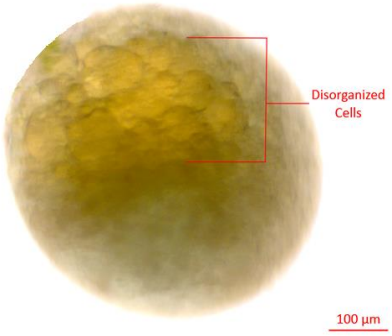

Image 8. Embryo in stage 3 of development (gastrulation) – Cd^{2+}-Zn^{2+} mixture with 20µg/L Zn^{2+} and 1.5µg/L Cd^{2+}

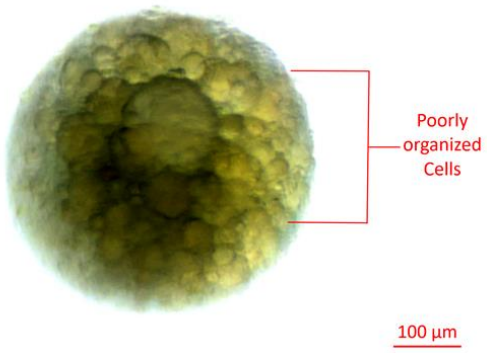

Image 9. Embryo in stage 3 of development (gastrulation) – Cd^{2+}-Ni^{2+} mixture with 120µg/L Ni^{2+} and 1.5µg/L Cd^{2+}

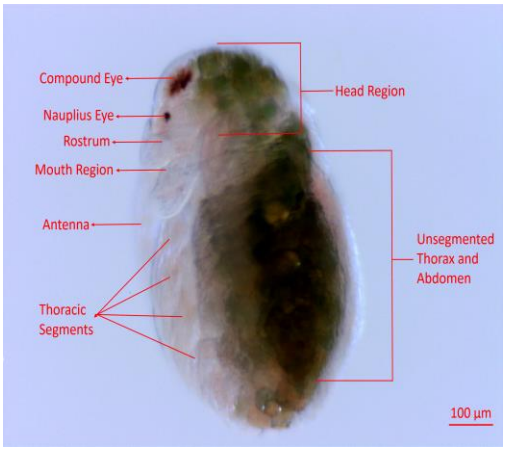

Image 10. Embryo in stage 9 of development: lateral view – control treatment

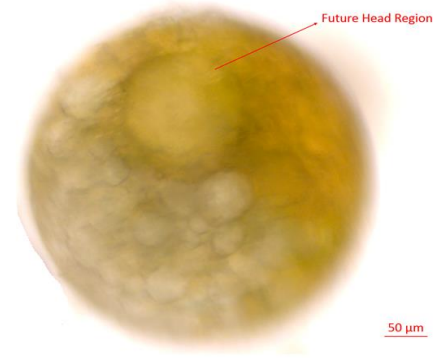

Image 11. Embryo in stage 5 of development (head formation): anterodorsal view – Cd^{2+}-Zn^{2+} mixture with 160µg/L Zn^{2+} and 1.5µg/L Cd^{2+}

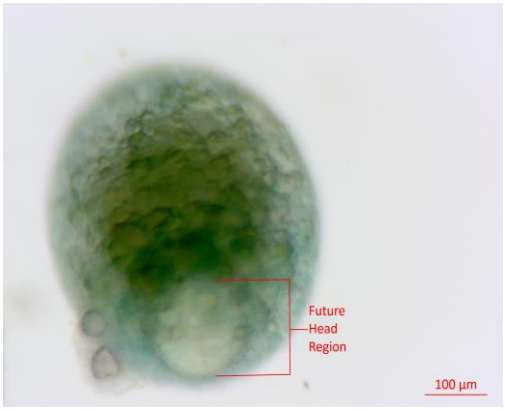

Image 12. Embryo in stage 5 of development: dorsal view – Cd^{2+}-Ni^{2+} mixture with 20µg/L Ni^{2+} and 1.5µg/L Cd^{2+}

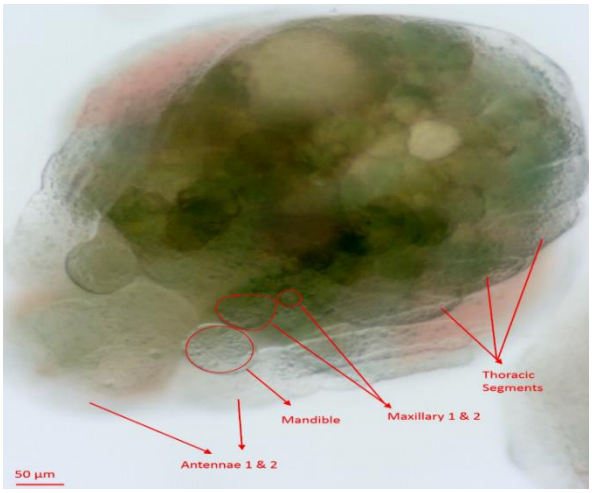

Image 13. Embryo in stage 10 of development (hook-shaped abdomen): ventral view – control treatment

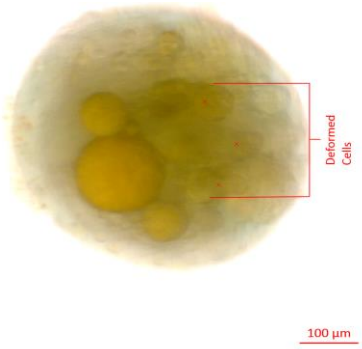

Image 14. Embryo in stage 3 of development (gastrulation) – Cd^{2+}-Zn^{2+} mixture with 40µg/L Zn^{2+} and 1.5µg/L Cd^{2+}

Image 15. Embryo in stage 3 of development (gastrulation) – Cd^{2+}-Ni^{2+} mixture with 40µg/L Ni^{2+} and 1.5µg/L Cd^{2+}

REFERENCE LIST

Adriano, D.C. 1986. “Trace Elements in the Terrestrial Environment.” Springer-Verlag, New York, NY.

Agency for Toxic Substances and Disease Registry (ATSDR). 1997. *Toxicological Profile for Nickel (Update)*. Public Health Service, U.S. Department of Health and Human Services, Altanta, GA.

Agency for Toxic Substances and Disease Registry (ATSDR). 2005. *Toxicological Profile for Nickel (Update)*. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

Agency for Toxic Substances and Disease Registry (ATSDR). 2005. *Toxicological Profile for Zinc (Update)*. Public Health Service, U.S. Department of Health and Human Services, Altanta, GA.

Agency for Toxic Substances and Disease Registry (ATSDR). 2012. *Toxicological Profile for Cadmium (Update)*. Public Health Service, U.S. Department of Health and Human Services, Altanta, GA.

Ahsanullah, M., Mobley M.C., and Rankin, P. 1988. “Individual and combined effects of zinc, cadmium, and copper on the marine amphipod, *Allorchestes compressa*.” *Australian Journal of Marine and Freshwater Research* 3: 33–37.

Alabaster, J.S. 1982. “Cadmium, in Water Quality Criteria for Freshwater Fish.” 2nd edition. Butterworth-Heinemann, pgs. 221-251. ISBN 978040810849.

American Society for Testing and Material (ASTM). 2012. “Standard guide for conducting Daphnia magna life-cycle toxicity tests.” ASTM E1193.

Amra Odobasic. 2012. “Determination and Speciation of Trace Heavy Metals in Natural Water by DPASV.” *Water Quality Monitoring and Assessment*, Dr. Voudouris (Ed.), ISBN: 978-953-51-0486-5, In Tech, Available from: http://www.intechopen.com/books/water-quality-monitoring-and assessment/determination-andspeciation-of-trace-heavy-metals-in-natural-water-by-dpasv.

Atar, D., Backx, P.H., Appel, M.M., Gao, W.D., Marban, E. 1995. "Excitation-transcription coupling mediated by zinc influx through voltage-dependent Ca^{2+} channels." *Journal of Biological Chemistry* 270: 2473-2477.

Attar, E.N. and Maly, E.J. 1982. "Acute toxicity of cadmium, zinc, and cadmium-zinc mixtures to *Daphnia magna*." *Archives of Environmental Contamination and Toxicology* 11: 291-296.

Awasthi, M. and Rai, L.C. 2004. "Adsorption of nickel, zinc, and cadmium by immobilized green algae and cyanobacteria: a comparative study." *Annals of Microbiology* 54.3: 257-267.

Babich, H., Shopsis, C. and Borenfreund, E. 1986. "Cadmium-nickel toxicity interactions towards a bacterium, filamentous fungi, and a cultured mammalian cell line." *Bulletin of Environmental Contamination and Toxicology* 37.1: 550-557.

Bannon, D. I., Abounader, R., Lees, P. S., and Bressler, J. P. 2003. "Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells." *American Journal of Physiology - Cell Physiology* 284: 44-50.

Barata, C., Markich, S.J., Baird, D.J., Taylor, G., Soares, A.M.V.M., 2002. "Genetic variability in sublethal tolerance to mixtures of cadmium and zinc in clones of *Daphnia magna Straus*." *Aquatic Toxicology* 60: 85–99.

Barbalace, K. Periodic Table of Elements –Sorted by Ionic Radius. EnvironmentalChemistry.com. 1995-2016. Accessed on line: 5/4/2016. http://environmentalchemistry.com/yogi/periodic/ionicradius.html.

Bat, L., Raffaelli, D., and Marr, I.L. 1998. "The accumulation of copper, zinc, and cadmium by the amphipod *Corophium volutator* (Pallas)." *Journal of Experimental Marine Biology and Ecology* 223: 167–184.

Biesinger, K.E., Christensen, G.M., Fiandt, J.T., 1986. "Effects of metal salt mixtures on *Daphnia magna* reproduction." *Ecotoxicology and Environmental Safety* 11: 9–14.

Block, M. and Part, P. 1986. "Increased availability of cadmium to perfused rainbow trout (*Salmo gairdneri* Rich.) gills in the presence of the complexing agents diethyldithiocarbamate, ethyl xanthate and isopropyl xanthate." *Aquatic Toxicology* 8: 295-302.

Block, M. 1991. “Uptake of cadmium in fish. Effects of xanthates and diethyldithiocarbamate.” University of Uppsala, Sweden, Faculty of Sciences (Universitatis Uppsaliensis - Comprehensive summaries of Uppsala dissertations from the Faculty of Science. No. 326).

Bodar, C.W.M., Van Leeuwen, C.J., Voogt, P.A., Zandee, D.I. 1988. “Effect of Cadmium on the reproduction strategy of *Daphnia magna*.” *Aquatic Toxicology* 12.4: 301-310.

Bodar, C.W.M., Van Der Sluis, Voogt P.A., and Zandee D.I. 1988. “Effects of Cadmium on consumption, assimilation and Biochemical parameters of *Daphnia magna*: Possible Implications for Reproduction.” *Comparative Biochemistry and Physiology* 90C.2: 341-346.

Borgmann, U. 1980. “Interactive effects of metals in mixtures on biomass production kinetics of fresh-water copepods.” *Canadian Journal of Fisheries and Aquatic Sciences* 37: 1295–1302.

Braek, G.S., Malnes, D., Jensen, A. 1980. “Heavy metal tolerance of marine phytoplankton. IV. Combined effect of zinc and cadmium on growth and uptake in some marine diatoms.” *Journal of Experimental Marine Biology and Ecology* 42: 39-54.

Calamari, D., Marchetti, R., and Vailati, G. 1980. “Influence of water hardness on cadmium toxicity to *Salmo gairdneri* rich.” *Water Research* 14.10: 1421-1426.

Cañizares-Villanueva, R.O., Martínez-Jerónimo, F., Espinosa-Chávez F. 2000. “Acute toxicity to *Daphnia magna* of effluents containing Cd, Zn, and a mixture Cd-Zn, after metal removal by *Chlorella vulgaris*.” *Environmental Toxicology* 15: 160-164.

Carroll, J.J., Ellis, S.J., and Oliver, W.S. 1979. “Influences of hardness constituents on the acute toxicity of cadmium to brook trout (*Salvelinus fontinalis*).” *Bulletin of Environmental Contamination and Toxicology* 22: 575-581.

Cempel, M. and Nikel, G. 2006. “Nickel: A review of its sources and Environmental Toxicology.” *Polish Journal of Environmental Studies* 15.3: 375-382.

Chin, D. and Means, A.R. 2000. “Calmodulin: a prototypical calcium sensor.” *Trends in Cellular Biology* 10.8: 322-328. DOI: 10.1016/S0962-8924(00)01800-6.PMID 10884684.

Chow, R.H. 1991. “Calcium block of squid calcium currents. Macroscopic data and a Kinetic Model.” *Journal of General Physiology* 98: 751-770.

Cole, R.H., R.E., Frederick, R.P. Healy, and R.G. Rolan. 1984. “Preliminary findings of the priority pollutant monitoring project of the Nationwide Urban Runoff program.” *Journal of Water Pollution Control Fed.* 56.7: 898-908. Cited in USEPA 1985j and 1986c.

Dawson, D.C. and N. Ballatori. 1995. “Membrane Transporters as Sites of Action and Routes of Entry for Toxic Metals, in Toxicology of Metals: Biochemical Aspects.” 1st edition, (eds. Goyer A.R. and George C.M.), Springer-Verlag Berlin Heidelberg, pgs. 63-73.

De Laender, F., Janssen, C.R., De Schamphelaere, K.A.C. 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.

Djekoun, M., Bensoltane, S., Bourechrouche, A., Bourechrouche, M., Berrebah, H. 2015. “In-vitro toxicity of cadmium on the development of parthenogenetic eggs of a freshwater cladoceran: *Daphnia magna*.” *Journal of Materials and Environmental Science* 6.4: 957-962.

Dobson, S. (1st draft). 1992. “Environmental Health Criteria for Cadmium - Environmental Aspects.” (eds. Gilbert M. and Jenkins P.G.). WHO task force group, Institute of Terrestrial Ecology, Monks Wood, United Kingdom.

Eisler, R. 1998-2001. “Nickel hazards to fish, wildlife, and invertebrates: a synoptic review.” *US Geological Survey, Biological Resources Division*. Biological Science Report.

Ellinor, P.T., Yang, J., Sather, W., Zhang, J-f., Tsien, R.W. 1995. “Ca^{2+} channel selectivity at a single locus for high-affinity Ca^{2+} interactions.” *Neuron* 15: 1121-1132.

Elnabaraway, M. T., Welter, A.N., Robideau, R.R. 1986. “Relative sensitivity to three daphnid species to selected organic and inorganic chemicals.” *Environmental Toxicology and Chemistry* 5.4: 393-398.

Enserink, E.L., Maas-Diepveen, J.L., Van Leeuwen, C.J. 1991. “Combined effects of metals: an ecotoxicological evaluation.” *Water Research* 25: 679-687.

Environmental Protection Agency. 2002. “Short term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms.” Washington, DC: U.S. Environmental Protection Agency, Office of Water. EPA-821-R-02-013.

Environmental Protection Agency. 1979. “Water-related fate of 129 priority pollutants.” Washington, DC: U.S. Environmental Protection Agency, Office of Water Planning and Standards. EPA 440479029a.

Environmental Protection Agency. 2016 update. “National Recommended Water Quality Criteria – Aquatic Life Criteria Table. https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table.

Evans, L.J. 1989. “Chemistry of metal retention by soils.” *Environmental Science and Technology* 23: 1046-1056.

Fern, R., Black, J.A., Ransom, B.R. and Waxman, S.G. 1996. “Cd^{2+} dependent routes to ras: mechanisms for neuronal survival, differentiation and plasticity.” *Neuron* 16: 233-236.

Fisher, N.S., Stupakoff, I., Sanudo-Wihelmy, S., Wang, W-X., Teyssie, J.L., Fowler, S.W., Crusius, J. 2000. “Trace metals in marine copepods: A field test of a bioaccumulation model coupled to laboratory uptake kinetics data.” *Marine Ecology and Progress Series* 194: 211–218.

Flik, G., Van De Winkel, J.G.J., Part, P., Wendelaar Bonga, S.E., and Lock, R.A.C. 1987. “Calmodulin mediated cadmium inhibition of phosphodiesterase activity.” *Archives of Toxicology* 59: 352-359.

Fujishiro, H., Y. yano, Y. Takada, M. Tanihara, and S. Himeno. 2012. “Roles of ZIP8, ZIP14, and DMT1 in transport of cadmium and manganese in mouse kidney proximal tubule cells.” *Metallomics* 4: 700-708

Glynn, A. W. 2001. “The influence of zinc on apical uptake of cadmium in the gills and cadmium influx to the circulatory system in zebrafish (*Danio rerio*).” *Comparative Biochemistry and Physiology: Part C* 128: 165–172.

Goto, D. and Wallace, W. 2007. “Interaction of Cd and Zn during uptake and loss in the polychaete *Capitella capitata*: Whole body and subcellular perspectives.” *Journal of Experimental Marine Biology and Ecology* 352: 65–77.

Gottofrey, J., Bjorklund, I., and Tjalve, H. 1988. "Effects of sodium isopropylxanthate, potassium amylxanthate and sodium diethyldithiocarbamate on the uptake and disposition of cadmium in the brown trout (*Salmo trutta)*." *Aquatic Toxicology* 12: 171-184.

Guy, R.D., Chakrabarti, C.L. and Schramm, L.L. 1975. "The application of a simple chemical model of natural waters to metal fixation in particulate matter." *Canadian Journal of Chemistry* 53: 661-669.

Hess, P. and Tsien, R.W. 1984. "Mechanisms of ion permeation through calcium channels." *Nature (London)* 309: 453-456.

Hess, P., Lansman, J.B. and Tsien, R.W. 1986. "Calcium channel selectivity for divalent and monovalent cations: voltage and concentration dependence of single channel current in ventricular heart cells." *Journal of General Physiology* 88: 293-319.

Hille, B. 1992. "Ionic Channels of Excitable Membranes." 2nd edition. Sinauer Associates, Sunderland, Massachusetts. DOI: 10.1016/0014-5793(92)81020-M.

Hille B. 2008. "Ion channels" *Scholarpedia* 3.10: 6051.University of Washington School of Medicine, Seattle, Washington. Reviewed by Dr. Gary Yellen, Department of Neurobiology, Harvard Medical School, Boston, MA, and Dr. Bruce Bean, Harvard Medical School, Cambridge, MA.

Hinkle, P.M. and Osborne, M.E. 1994. "Cadmium toxicity in rat pheochromocytoma cells: studies on the mechanism of uptake." *Toxicology and Applied Pharmacology* 124: 91-98.

Hogstrand, C., Wilson, R.W., Polgard, D. and Wood, C.M. 1994. "Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc." *Journal of Experimental Biology* 186: 55-73.

International Zinc Association (IZA). "Zinc in the Environment: An Introduction. 2nd edition. 1997. http://www.zinc.org/info/zinc_is_essential_for_the_environment.

Jemec, A., Drobne, D., Tišler, T., Trebše, P., Roš, M., Sepčić, K. 2007. "The applicability of acetylcholinesterase and glutathione S-transferase in *Daphnia magna* toxicity test." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 144.4: 303-309.

Jenkitkasemwong, S., Wang, C-Y, Mackenzie B., and Knutson, M.D. 2012. "Physiologic implications of metal-ion transport by ZIP14 and ZIP8." *Biometals* 25.4: 643–655.

Kadirvelu, K., Thamaraiselvi, K., Namasivayam, C. 2011. "Removal of heavy metals from industrial wastewaters by adsorption onto activated carbon prepared from an agricultural solid waste." *Bioresource Technology* 76: 63-65.

Kasprzak, K. S. and Salnikow, K. 2007. "Nickel Toxicity and Carcinogenesis, in Nickel and Its Surprising Impact in Nature." Volume 2, (eds. A. Sigel, H. Sigel and R. K. O. Sigel), John Wiley & Sons, Ltd, Chichester, UK, pgs. 621-645. DOI: 10.1002/9780470028131.ch17.

Komjarova, I. and 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.

Komjarova, I. and Blust, R. 2009. "Multi-metal interactions between Cd, Cu, Pb, and Zn uptake from water in the Zebrafish *Danio rerio*." *Environmental Science and Technology* 43: 7225-7229.

Komjarova, I. and Bury, N.R. 2014. "Evidence of common cadmium and copper uptake routes in Zebrafish *Danio rerio*." Environmental Science and Technology 48: 12946-12951.

Kraak, M.H.S., Lavy, D., Schoon, H., Toussaint, M., Peeters, W.H.M., Van Straalen, N.M. 1994. "Ecotoxicity of mixtures of metals to the zebra mussel *Dreissena ploymorpha*." *Environmental Toxicology and Chemistry* 13: 109-114.

Kwong, R.W.M. 2011. "Dietary divalent metal uptake and interactions in freshwater fish: implications for metal toxicity". A thesis submitted to the college of graduate studies and research in partial fulfillment of the requirements for the degree of doctor of philosophy in the toxicology graduate program, University of Saskatchewan, Saskatoon, Canada.

Lansman, J.B., Hess, P. and Tsien, R.W. 1986. "Blockade of current through single calcium channels by Cd^{2+}, Mg^{2+}, and Ca^{2+}." *Journal of General Physiology* 88: 321-347.

Lansman, J.B. 1990. "Blockade of current through single calcium channels by trivalent lanthanide cations: Effect of ionic radius on the rates of ion entry and exit." *Journal of General Physiology* 95: 679-696.

Lavoie, M., Fortin, C., Campbell, P.G.C. 2012a. "Extending the biotic ligand model to account for positive and negative feedback interactions between cadmium and zinc in a freshwater alga." *Environmental Science and Technology* 46.21: 12129-12136.

Lavoie, M., Fortin, C., Campbell, P.G.C. 2012b. "Influence of essential elements on cadmium uptake and toxicity in a unicellular green alga: The protective effect of trace zinc and cobalt concentrations." *Environmental Toxicology and Chemistry* 31: 1445-1452.

Long, G.J. 1997. "The effects of Cadmium on cytosolic free calcium, protein kinase C, and collagen synthesis in rat osteosarcoma (ROS 17/2.8) cells." *Toxicology and Applied Pharmacology* 143: 189-195.

Lynch, N. 2014. "Additive and non-additive mechanisms of toxicity of bi-metal mixtures to fathead minnows." Thesis submitted to the graduate school of Loyola University Chicago. Chicago, IL 60660.

Mackenzie, B., Ujwal, M. L., Chang, M. H., Romero, M. F., and Hediger, M. A. 2006. "Divalent metal-ion transporter DMT1 mediates both H+ -coupled Fe2+ transport and uncoupled fluxes." *Archives of the European Journal of Physiology* 451: 544-558.

Masnado, R.G., Geis, S.W., Sonzogni, W.C. 1995. "Comparative acute toxicity of a synthetic mine effluent to *Ceriodaphnia dubia*, larval fathead minnow, and the freshwater mussel *Anodonta imbecillis*." *Environmental Toxicology and Chemistry* 14: 1913-1920.

McComish, M.F. and Ong, J.H. 1988. "Cadmium" in Environmental inorganic chemistry: Properties, processes, and estimation methods." (eds. Bodek, I., Lyman, W.J., Reehl W.F.) New York, NY: Pergamon Press, 7.5-1 to 7.5-12.

Mebane, C.A., Dillon, F.S., and Hennessy, D.P. 2012. "Acute toxicity of cadmium, lead, zinc, and their mixtures to stream-resident fish and invertebrates." *Environmental Toxicology and Chemistry* 31: 1334-1348.

Metka, F. and Tom, Hei. 2004. "Mutagenicity of cadmium in mammalian cells: implication of oxidative DNA damage." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 546.1–2: 81-91.

Meyer, S.J., Farley, J.K., and Garman, R.E. 2015. "Metal Mixtures Modeling Evaluation Project: 1.Background." *Environmental Toxicology and Chemistry* 34.4: 726-740.

Mikala, G., Bahinski, A., Yatani, A., Tang, S., Schwartz, A. 1993. "Differential contribution by conserved glutamate residues to an ion-selectivity site in the L-type Ca^{2+} channel pore." *Federation of European Biochemical Societies Letters* 335: 265-269.

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." *EvoDevo* 5.1: 1-20.

Montvydienė, D., and Marčiulionienė, D. 2004. "Assessment of toxic interactions of heavy metals in a multicomponent mixture using *Lepidium sativum* and *Spirodela polyrrhiza*." *Environmental Toxicology* 19: 351–358.

Ntakirutimana, T., G. Du, J.S. Guo, Xu Gao, Lei Huang. 2013. "Pollution and potential ecological risk assessment of heavy metals in a lake." Polish Journal of Environmental Studies 22.4: 1129–1134.

Negilski, D.S., Ahsanullah, M., and Mobley M.C. 1981. "Toxicity of zinc, cadmium and copper to the shrimp *Callianassa australiensis*. 2. Effects of paired and triad combinations of metals." *Marine Biology* 64: 305–309.

Newman, M.C. 2015. "Fundamentals of Ecotoxicology: The Science of Pollution". 4th edition. CRC Press – Tailor and Francis Group, Boca Raton, Florida, pgs. 140-142. ISBN-978-1-4665-8229-3.

Noraho, N. and Gau, J.P. 1995. "Effects of cations, including heavy metals, on cadmium uptake by *Lemna Polyrhiza* L." *Biometals* 8.2: 95-98.

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

Nowycky, M.C., Fox, A.P. and Tsien, R.W. 1985. "Three types of neuronal calcium channels with different calcium agonist sensitivity." *Nature (London)* 316: 440-443.

Ojo, A. A. and Wood, C. M. 2008. "In vitro characterization of cadmium and zinc uptake via the gastro-intestinal tract of the rainbow trout (*Oncorhynchus mykiss*): Interactive effects and the influence of calcium." *Aquatic Toxicology* 89: 55–64.

Pagenkopf, GK. 1983. “Gill surface interaction model for trace-metal toxicity to fish. Roles of complexation, pH, and water hardness.” *Environmental Science and Technology* 17: 342–347.

Paquin, P.R., Zoltayb, V., Winfield, R.P., Wua, K.U., Mathewa, R., Santore, R.C., Di Toro, D.M. 2002. “Extension of the biotic ligand model of acute toxicity to a physiologically-based model of the survival time of rainbow trout (*Oncorhynchus mykiss*) exposed to silver.” *Comparative Biochemistry and Physiology* 133: 305–343.

Part, P., Svanberg, O., and Kiessling, A. 1985. “The availability of cadmium to perfused rainbow trout gills in different water qualities.” *Water Research* 19: 427-434.

Phillips, B.M., Nicely, P.A., Hunt, J.W., Anderson, B.S., Tjeerdema, R.S., Palmer, S.E., Palmer, F.H., Puckett, H.M. 2003. “Toxicity of cadmium, copper, nickel, zinc mixtures to larval purple sea urchins (Strongylocentrotus purpuratus).” *Bulletin of Environmental Contamination and Toxicology* 70: 592-599.

Poldoski, J.E. 1979. “Cadmium bioaccumulation assays. Their relationships to various ion equilibria in Lake Superior water.” *Environment, Science, and Technology* 13: 701-706.

Rai, D. and Zachara, J.M. 1984. “Chemical attenuation rates, coefficients, and constants in leachate migration. Volume 1: A critical Review.” Palo Alto, CA: Electric Power Research Institute.

Saijoh, K., Inoue, Y., Ratsuyama, H., Sumino, K. 1988. “The interaction of cations with activity of soluble protein kinase C from mouse brain.” *Pharmacology and Toxicology* 78: 193-199.

Sato, C., Schnoor, J.L. and McDonald, D.B. 1986. “Characterization of effects of copper, cadmium, and nickel on the growth of *Nitrosomonas europaea*.” *Environmental Toxicology and Chemistry* 5: 403-416.

Sensi, S., Canzoniero, L.M.T., Yu, S.P., Ying, H.S., Koh, J-Y., Kerchner, G.A., Choi, D.W. 1997. “Measurement of intracellular free zinc in living cortical neurons: routes of entry.” *Journal of Neuroscience* 17: 9554-9564.

Shafer, T.J. 2000. “The Role of Ion Channels in the Transport of Metals into Excitable and Nonexcitable Cells, in Molecular Biology and Toxicology of Metals.” 1st edition, (eds. Zalups R.K. and Koropatnick J.). Tailor and Francis Group, London and New York, pgs. 179-197. ISBN-0-7484-0798-7.

Shaw, J. R., Dempsey, T. D., Chen, C. Y., Hamilton, J. W., Folt, C. L. 2006. "Comparative toxicity of cadmium, zinc, and mixtures of cadmium and zinc to daphnids." *Environmental Toxicology and Chemistry* 25: 182–189.

Simkiss, K. 1998. "Mechanism of Metal Uptake, in Metal Metabolism in Aquatic Environments." Ecotoxicology Series 7, (eds. Langston, W.J and M.J. Bebianno), Chapman and Hall, London, United Kingdom. pgs. 1-17. DOI: 10.1007/978-1-4757-2761-6.

Spehar, R.L., Leonard, E.N. and DeFoe, D.L. 1978. "Chronic effects of cadmium and zinc mixtures on flagfish (*Jordanella floridae*)." *Transactions of the American Fisheries Society* 107: 354-360.

Swandulla, D. and Armstrong, C.M. 1989. "Calcium channel block by cadmium in chicken sensory neurons." *Proceedings of the National Academy of Sciences of the United States of America* 86: 1736-1740.

Taylor, W.R. 1988. "Permeation of barium and cadmium through slowly inactivating calcium channels in cat sensory neurons." *Journal of Physiology (London)* 407: 433-452.

Thornton, I. 1992. "Sources and pathways of cadmium in the environment, in Cadmium in the human environment: Toxicity and carcinogenicity." (eds. Nordberg, G.F., Herber, R.F.M., Alessio L.). IARC Scientific Publications No. 118. Lyon, France: International Agency for Research on Cancer, 149-162.

Thorpe, V.J., and Lake, P.S. 1974. "Toxicity bioassays of cadmium on selected freshwater invertebrates and the interactions of cadmium and zinc on the freshwater shrimp, *Paratya tasmaniensis* Rick." *Australian Journal of Marine and Freshwater Research* 25: 97–104.

Traudt, E. M., Ranville, J. F., Smith, S. A., Meyer, J. S. 2016. "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*. doi:10.1002/etc.3342.

Usai, C., Barberis, A., Moccagatta, L., Marchetti, C. 1997. "Pathways of cadmium uptake in excitable mammalian cells: a microspectrofluorimetric study." *European Journal of Histochemistry* 41 (Suppl. 2): 189-190.

Verriopoulos, G., and Dimas, S. 1988. "Combined toxicity of copper, cadmium, zinc, lead, nickel, and chrome to the copepod *Tisbe holothuriae*." *Bulletin of Environmental Contamination and Toxicology* 41: 378–384.

Viarengo, A. 1994. "Heavy Metal Cytotoxicity in Marine Organisms, in Advances in Comparative and Environmental Physiology." Volume 20, 1st edition, Springer-Verlag Berlin Heidelberg, pg. 88, DOI: 10.1007/978-3-642-78598-6.

Vijver, M.G., Elliot, E.G., Peijnenburg, W.J.G.M., De Snoo, G.R. 2011. "Response prediction for organisms water-exposed to metal mixtures: A meta-analysis." *Environmental Toxicology and Chemistry* 30: 1482-1487.

Wakamori, M., Strobeck, M., Niidome, T., Teremoto, T., Imoto, K. and Mori, Y. 1998. "Functional characterization of ion permeation pathway in the N-type Ca^{2+} channel." *Journal of Neurophysiology* 79: 622-634.

Waltersson, E. 1984. "Effects of enrichment reagents – distribution and spread of xanthates and xanthate derivatives during enrichment activities." Stockholm, Swedish Environmental Research Institute (Report B478) (in Swedish).

Ward, T. J. and Robinson, W. E. 2005. "Evolution of cadmium resistance in *Daphnia magna*." *Environmental Toxicology and Chemistry* 24: 2341–2349.

Weis, J.S. 1980. "Effect of zinc on regeneration in the fiddler crab *Uca pugilator* and its interactions with methylmercury and cadmium." *Marine Environmental Research* 3: 249–255.

Weiss, J.H., Hartley, D.M., Koh, J-Y., Choi, D.W. 1993. "AMPA receptor activation potentiates zinc neurotoxicity." *Neuron* 10: 43-49.

Wicklund, A. 1990. "Metabolism of cadmium and zinc in fish." University of Uppsala, Sweden, Faculty of Science (Acta Uiversitatis Uppsaliensis – Comprehensive summaries of Uppsala dissertations from the Faculty of Science, No. 250).

Wicklund Glynn, A., Haux, C. and Hogstrand, C. 1992. "Chronic toxicity and metabolism of Cd and Zn in juvenile minnows (*Phoxinus phoxinus*) exposed to a Cd and Zn mixture." *Canadian Journal of Fisheries and Aquatic Sciences* 49: 2070-2079.

Winegar, B.D. and Lansman, J.B. 1990. "Voltage-dependent block by zinc of single dihydropyridine-senstive calcium channels in mouse C2 myotubules." *Journal of Physiology (London)* 425: 563-578.

Winegar, B.D., Kelly, R. and Lansman, J.B. 1991. “Block of current through single calcium channels by Fe, Co, and Ni. Location of the transition metal binding site in the pore.” *Journal of General Physiology* 97: 351-367.

Yang, J., Ellinor, P.T., Sather, W.A., Zhang, J.F., Tsien, R.W. 1993. “Molecular determinants of Ca^{2+} selectivity and ion permeation in L-type Ca^{2+} channels.” *Nature (London)* 366: 158-161.

Yu, R.Q. and Wang, W-X. 2002. “Trace metal assimilation and release budget in *Daphnia magna*.” *Limnology and Oceanography* 47: 495–504.

Zamponi, G.W., Bourinet, E. and Snutch, T.P. 1996. “Nickel block of a family of neuronal calcium channels: subtype- and subunit-dependent action at multiple sites.” *Journal of Membrane Biology* 151: 77-90.

VITA

Mr. Pérez was born and raised in Belize City, Belize. He graduated from St. John's College Junior College in 2009 with an Associate Degree in Environmental Science. After graduation he was hired as a laboratory technician in the College's science department, a job he held for 2 ½ years. He was awarded a Council of Regent's Scholarship to Loyola University Chicago where he earned a Bachelor of Science degree majoring in Environmental Science with a concentration in Chemistry in 2014. Following this achievement, Mr. Pérez was accepted to the Master of Science program in the biology department at Loyola University Chicago where he studied Ecotoxicology and Risk Assessment in the Institute of Environmental Sustainability (IES). Upon graduation, Mr. Pérez looks forward to his new career as a Research Scientist in Aquatic Ecotoxicology at Pacific EcoRisk in Fairfield, California.