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Canada

Linking Cd accumulation and effect in resistant and sensitive
freshwater invertebrates

By

Tony Straus

Bsc, Biology, WLU, 2007

Thesis

Submitted to the Department of Biology

Faculty of Science

in partial fulfilment of the requirements for the

Masters of Science in Integrative Biology

Wilfrid Laurier University

2011

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General Overview

There has been recent discussion within the field of environmental toxicology as to the viability of using the bioaccumulation of a chemical toxicant to predict toxic effects. Current literature has shown this concept to carry some merit, although there is a major flaw in that with many cases, namely those involving sensitive species, body burdens cannot be reliably linked to effects (Adams *et al.* (2010)). Bearing this issue in mind the goal of this thesis was to assess the viability of using bioaccumulation as a measure of effect in a different way.

The proposed idea utilized Cd accumulation in a resistant species the California blackworm (*Lumbriculus variegatus*), to predict effect in a sensitive species the freshwater amphipod *Hyalella azteca*. In this study *L. variegatus* accumulated cadmium in a dose dependent manner with body burdens increasing with both exposure duration and exposure concentration regardless of water hardness. In both hard (120 mg/L as CaCO₃) and soft water (22 mg/L as CaCO₃) exposures at concentrations as high as 200 µg Cd/L there were no lethal effects observed, demonstrating the ability of this organism to store and detoxify accumulated Cd within cellular debris or with metallothionein (MT) like proteins. Steady-state accumulation was not reached despite chronic exposure durations of 87 and 28 days for hard and soft water exposures respectively.

In hard water exposures *L. variegatus* suffered significant reductions in reproduction at exposures over 100 µg Cd/L, with an almost 8-fold reduction at exposure over 200 µg/L. These effects may be related to the accumulation of Cd inside sensitive organelles or the binding of Cd to important heat denaturable proteins and enzymes (Wallace *et al.*, 2003).

Hyalella azteca was sensitive to Cd exposure, resulting in 100% mortality at concentrations above 10 μ g Cd/L in hard water and 1.4 μ g Cd/L in soft water. In contrast to the resistant species, *H. azteca* was able to quickly (10 days in hard water; 14 days in soft water) accumulate Cd to a steady state body burden.

Through extrapolation of steady state concentrations and modelling, it was possible to relate lethal concentrations in the sensitive species to estimated body burdens in the resistant species. Although this study exists only as a proof of principle, this concept, if applied to contaminated fresh waters could be used to quickly determine deleterious toxic effects in sensitive members of an ecosystem. This would be accomplished through the use of a resistant species as a bioindicator. A researcher would need to go out into the field and collect samples of the indicator species from a contaminated site, then return to lab and analyze the samples for their total body burden. Based on the average of these body burdens, the researcher could predict negative effects in the ecosystem's sensitive species. However, this concept is still in its infancy and the observed relationships need to be reproduced in different water chemistries for confirmation prior to further development.

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Table of Contents

General Overview	ii
Acknowledgements	iv
Table of Contents	v
List of Figures	ix
List of Tables.....	xi
Chapter 1: Introduction	1
1.1 Cadmium	2
1.2 Cd speciation and bioavailability in freshwater.....	3
1.3 Bioaccumulation of Metals.....	4
1.4 Tissue Residue Approach (TRA)	5
1.5 Subcellular Fractionation	7
1.6 Aquatic Invertebrates.....	8
1.6.1 <i>Lumbriculus variegatus</i>	8
1.6.2 <i>Hyalella azteca</i>	9
1.7 Monitoring ecosystem health.....	10
1.8 Objectives.....	12
1.9 General Guide to Thesis Chapters	13

Chapter 2: A multiple invertebrate species test to determine if Cd accumulation in a resistant species can be used to reliably predict effects in a sensitive species in hard water	15
2.0 Abstract	16
2.1 Introduction.....	17
2.2 Materials and Methods.....	21
2.2.1 Culturing <i>L. variegatus</i> and <i>H. azteca</i>	21
2.2.2 <i>Lumbriculus variegatus</i> chronic bioaccumulation and reproduction tests	22
2.2.3 <i>H. azteca</i> chronic bioaccumulation	24
2.2.4 Sampling and Sample Processing.....	24
2.2.5 Subcellular distribution of Cd in <i>L. variegatus</i>	26
2.2.6 Analysis.....	27
2.2.7 Statistics	28
2.3 Results	28
2.3.1 Cd bioaccumulation	28
2.3.2 Reproduction and Survival.....	29
2.3.3 Weight.....	30
2.3.4 Subcellular distribution for <i>L. variegatus</i>	30
2.4 Discussion.....	31
2.5 Figures and Tables	37

Chapter 3: A multiple invertebrate species exposure to determine if Cd accumulation in a resistant species can be used to reliably predict effects in a sensitive species in soft water	50
3.0 Abstract	51
3.1 Introduction:	53
3.2 Materials and Methods.....	57
3.2.1 Culturing <i>L. variegatus</i> and <i>H. azteca</i>	57
3.2.2 Cd Exposures	57
3.2.3 Sampling and sample processing	58
3.2.4 Analysis.....	59
3.2.5 Calculations	60
3.2.6 Statistics	60
3.3 Results	61
3.3.1 Survival	61
3.3.2 Dry Weight	62
3.3.3 Cd Body Burden	62
3.3.4 Modelling steady-state and accumulation-effect relationships	62
3.4 Discussion.....	63
3.5 Figures and Tables	71
Chapter 4: General Discussion.....	85

4.1 Expanding on Chapter 3: A multiple invertebrate species exposure to determine if Cd accumulation in a resistant species can be used to reliably predict effects in a sensitive species in soft water	86
4.2 Integrating multiple fields of biology in an aquatic toxicity project.....	89
4.3 Figures.....	92
Compiled References.....	97
Appendix A: Subcellular fractionation flow diagram.....	103

List of Figures

- Figure 2.1 Cd accumulation in *L. variegatus* during a 28-day exposure to 4.6 ± 0.1, 11.6 ± 0.6, 32.4 ± 0.9, 57.4 ± 2.2, Cd/L. Values are means ± 1 SEM, n = 8 worms. * indicate significance compared to control organisms at each day (ANOVA; p < 0.05). 37
- Figure 2.2 Cd accumulation in *L. variegatus* during a 87-day exposure to 4.6 ± 0.1, 32.4 ± 0.9, 57.4 ± 2.2, 86.9 ± 0.8, 107.6 ± 2.2, 153.0 ± 2.0, 205.3 ± 3.5 µg Cd/L. Values are means ± 1 SEM, n = 6 worms. All points differ significantly from controls at each time point. * indicate significance compared to control organisms at each day (ANOVA; p < 0.05). 38
- Figure 2.3 Cd accumulation in *H. azteca* during a 21-day exposure to 1.25, 2.5, and 5 µg Cd/L in moderately hard water (140 mg/L as CaCO₃)µg Cd/L. Values are means ± 1 SEM, n = 8 amphipods. All measurements from day 1 forward are significantly different from controls (ANOVA; p < 0.05). 39
- Figure 2.4 Cumulative effect of exposure to waterborne Cd on reproduction in *L. variegatus* Results are from preliminary range finding experiments n=1. 40
- Figure 2.5 Cumulative effect of exposure to waterborne Cd on reproduction in *L. variegatus* over a chronic 28 day exposure Nominal exposure concentrations of 0, 6.25, 25, 50, 75, 100, 150, and 200 5 µg Cd/L corresponded to 4.6, 32.4, 57.4, 86.9, 107.6, 153.0, 205.3 µg Cd/L respectively. Values are means ± 1 SEM, n = 3 replicates. * indicates significance compared to control organisms at day 28 (ANOVA; p < 0.05). 41
- Figure 2.6. The effect of Cd exposure to *H. azteca* (n = 8 per exposure) growth (average dry weight) throughout the 28 day exposure to 1.25, 2.5, and 5 µg Cd/L in moderately hard water (140 mg/L as CaCO₃). * represents time point where control organisms were significantly heavier than exposed individuals (ANOVA; p < 0.05). 42
- Figure 2.7. Comparison of subcellular Cd distribution in *L. variegatus* exposed to 4.6 & 205.3 µg Cd/L (corresponding to nominal concentrations of 6.5 and 200 µg Cd/L) for 7 d. Mean (± SEM) are shown with n=3. 43
- Figure 2.8. Comparison of subcellular Cd distribution in *L. variegatus* exposed to 4.6 & 205.3 µg Cd/L (corresponding to nominal concentrations of 6.5 and 200 µg Cd/L) for 45 d. Mean (± SEM) are shown with n=3. 44
- Figure 2.9. Comparison of subcellular distribution of Cd in *L. variegatus* at days 7 and 45 of exposure to 4.6 µg Cd/L (corresponding to nominal concentration of 6.5 µg Cd/L). Mean (± SEM) are shown with n=3. 45

Figure 2.10 Comparison of subcellular distribution of Cd in <i>L. variegatus</i> at days 7 and 45 of exposure to 205.3 µg Cd/L (corresponding to nominal concentration of 200 µg Cd/L). Mean (± SEM) are shown with n=3.	46
Figure 3.1. The effect of Cd exposure to <i>H. azteca</i> survival (%) throughout 28 day exposure to Cd/L in soft water (22 mg/L as CaCO ₃).	71
Figure 3.2. The effect of Cd exposure to <i>H. azteca</i> growth throughout 28 day exposure to Cd in soft water (22 mg/L as CaCO ₃).	72
Figure 3.3 Cd accumulation in <i>L. variegatus</i> during a 28-day exposure to Cd in soft water (22 mg/L as CaCO ₃).	73
Figure 3.4 Cd accumulation in <i>L. variegatus</i> at final day of 28-day exposure to 0, 0.3, 0.5, 1.3, and 2.3 µg Cd/L. Values are means ± 1 SEM, n = 8 worms.	74
Figure 3.5. Cd accumulation in <i>H. azteca</i> during a 28-day exposure to Cd in soft water (22 mg/L as CaCO ₃).	75
Figure 3.6. Extrapolation of average body burden at steady state of accumulation in <i>L. variegatus</i> .	76
Figure 3.7. Cd accumulation in <i>L. variegatus</i> ; extrapolation of body burden at steady state of accumulation during Cd exposure.	77
Figure 3.8 Cd accumulation in <i>L. variegatus</i> ; extrapolation of body burden at steady state of accumulation during exposure to Cd. 2.3 µg Cd/L not included.	78
Figure 3.9. Linking <i>H. azteca</i> EC20 and EC50 values to specific body burdens in <i>L. variegatus</i> .	79
Figure 3.10 Body Burden vs. Dry Weight in <i>Hyalella azteca</i> exposed to Cd. Figure 3.10 Body Burden vs. Dry Weight in <i>Hyalella azteca</i> exposed to Cd. Figure 3.10 Body Burden vs. Dry Weight in <i>Hyalella azteca</i> exposed to Cd.	80
Figure 4.1 Cd accumulation in <i>Lymnaea stagnalis</i> after exposure to various Cd concentrations over 21 days.	92
Figure 4.2 Cd accumulation in <i>Lymnaea stagnalis</i> at 21 days of exposure to various Cd concentrations	93
Figure 4.3 Figure 4.3 Linking <i>H. azteca</i> EC20 and EC50 values to specific body burdens in <i>L. stagnalis</i>	94
Figure 4.4 A. Cadmium accumulation in <i>D.pulex</i> at 21 days of exposure. B. Effect of Cd exposure on reproduction after 21 days of exposure.	95

List of Tables

Table 2.1. Measured Cd body burdens ($\mu\text{g Cd/g dry wt.}$; mean \pm SEM) for <i>L. variegatus</i> over 28 days of exposure at five exposure concentrations. Mean measured Cd exposure concentrations are given, n=8.	47
Table 2.2 Measured Cd body burdens ($\mu\text{g Cd/g dry wt.}$; mean \pm SEM) for <i>H. azteca</i> over 21 days of exposure at four exposure concentrations. Mean measured Cd exposure concentrations are given n=8.	48
Table 2.3 Measured Cd subcellular fractions ($\mu\text{g Cd/g tissue}$; mean \pm SEM) for <i>L. variegatus</i> at days 7 and 45, at two different exposure concentrations. Nominal Cd exposure concentrations are given n=3.	49
Table 3.1. Cd survival effect concentrations, with upper and lower 95% confidence intervals.	81
Table 3.2. Measured Cd body burdens ($\mu\text{g Cd/g dry wt.}$; mean \pm SEM) for <i>L. variegatus</i> over 28 days of exposure at five exposure concentrations. Mean measured Cd exposure concentrations are given, n=8	82
Table 3.3. Measured Cd body burdens ($\mu\text{g Cd/g dry wt.}$; mean \pm SEM) for <i>H. azteca</i> over 28 days of exposure at five exposure concentrations. Mean measured Cd exposure concentrations are given, the N/A indicates times at which there were no surviving individuals and for each time point n=8 except for the last measurements at 1.3 $\mu\text{g Cd/L}$ where n=6 and 2.3 $\mu\text{g Cd/L}$ where n=5.	83
Table 3.4. Extrapolation of average body burden at steady state of accumulation in <i>L. variegatus</i>	84

Chapter 1: Introduction

1.1 Cadmium

Canada is one of the most prominent mining nations in the world, and as a result is home to sites with high metal loads. Cadmium is a metal which occurs naturally in the environment at trace concentrations; however, anthropogenic releases can increase these background levels to much higher concentrations (Hutton, 1983). Natural global processes including volcanic activity volcanic emissions, and inputs from erosion and weathering of rocks release an estimate of 1300 t each year (Nriagu 1989); however this value is exceeded by about 3-10 times by anthropogenic sources (Nriagu and Pacyna 1988; Yeats and Bowers 1987). Anthropogenic sources of Cd include mining and refining operations, coal combustion, electroplating processes, iron and steel production as well as pigments, batteries, fertilizers, and pesticides (Benin *et al.* 1999; Hutton, 1983; CCME, 1996).

Cd is non-essential to the majority of aquatic organisms with the exception of marine diatoms (Price and Morel 2004; Lane and Morel 2000), this means Cd is not required for any physiological function within the organism. Since Cd is a non-essential metal, most organisms lack the regulatory machinery typically associated with essential metals (Price and Morel 2004, Lane and Morel 2000).

Cd is a toxic metal, and elicits toxic effects at low exposure concentrations; for this reason the Canadian Council of Ministers of the Environment (1996) set a water quality guideline at 0.017 µg Cd/L. Even at low environmental concentrations Cd accumulates in tissues. Concentrations as high as 10-100 ppm can be found in the internal organs of mammals, as well as some species of fish, mussels, and oysters where higher concentrations are associated with those individuals caught in polluted seas

(Bernard, 2008). Different organisms will begin to show effects to Cd at varying environmental concentrations. What might be considered a lethal concentration to one species may have no effect in another. *Oncorhynchus mykiss* have $LC_{50s} < 3 \mu\text{g/L}$ Cd, while the midge fly *Chironomus riparius* has been shown to be over 10,000 times more resistant, with published LC_{50s} of 55,607 $\mu\text{g/L}$ and 96,880 $\mu\text{g/L}$ (Buhl and Hamilton, 1991; Pascoe *et al.*, 1990; Williams *et al.*, 1986; Spehar and Carlson, 1984).

1.2 Cd speciation and bioavailability in freshwater

Modifying factors such as dissolved organic carbon, pH, and water hardness can affect metal bioavailability to aquatic organisms. When considered both individually and in combination, each of these modifying factors is capable of affecting both cadmium bioavailability and toxicity. This can introduce a significant level of uncertainty when relying on total water cadmium concentrations as a measure of exposure to aquatic organisms and the prediction of potential risks (U.S. EPA, 2001; Giesy *et al.* 1977).

The free cadmium ion (Cd^{2+}) is the most common form of cadmium in most fresh waters as well as the most toxic, while precipitation by carbonate or hydroxide and formation of soluble complexes by chloride, sulfate, carbonate and hydroxide are of little importance (U.S. EPA, 2001; NRCC 1979). Particulate matter and dissolved organic material contain negatively charged functional groups that can bind a significant amount of Cd^{2+} , thereby making it unavailable to aquatic organisms and in some cases reducing the metal's toxicity (U.S. EPA, 2001; Giesy *et al.* 1977). At low environmental pH a higher percentage of the cadmium in the environment will be found in the toxic free ion

form. This occurs as cadmium bound to DOC will be forced to compete with H⁺ ions for binding sites.

Water hardness has an effect on cadmium toxicity, with higher hardness values greatly reducing toxic effects. Water hardness is essentially just a measure of the concentration of Ca²⁺ and Mg²⁺ in a solution. We can attribute its protective effects to cation competition that results in a decreased binding of Cd to uptake sites (Verbost *et al.*, 1987). Studies have shown in many organisms, for example the tubificid worms *Limnodrilus hoffmeisteri* and *Tubifex tubifex*, that hard water increases an organism's survival over a more toxic soft water exposure (Chapman *et al.*, 1982; Williams *et al.*, 1986; Reynoldson *et al.*, 1996). Since water hardness can affect toxicity it is difficult to relate total environmental cadmium to a specific effect.

1.3 Bioaccumulation of Metals

Bioaccumulation refers to accumulation of a toxicant inside an organism, and occurs when the rate of metal uptake exceeds the rate of elimination. For an organism to illicit a response to a metal, accumulation of the metal must occur. Because cadmium is non-essential, organisms lack Cd specific regulatory machinery typically associated with essential metals such as copper, or zinc and as a result are prone to experiencing toxic effects when exposed to levels above background.

An organism's ability to accumulate Cd is based on both its uptake rate, and elimination rate. Buchwalter and Luoma (2005) conducted an experiment with several aquatic insects to study their ability to take up Cd from the environment. There was a large degree of variability amongst the test species, even within the same order, with both

the lowest and highest measured rates belonging to mayflies, 0.01ng/g/h for *Ameletus sp.* and 1.30ng/g/h for *Drunella flavilinea*. Uptake rates differed by almost 2-fold amongst two members of the same genus, *Drunella grandis* and *Drunella flavilinea*, with uptake rates of 0.74 ng/g/h and 1.30 ng/g/h respectively (Buchwalter and Luoma, 2005). With such a large difference of variability within a genus, it is important to understand why there is such a large degree of difference between species. Since freshwater organisms do not need Cd, they lack Cd transporters. This means that Cd should not be able to enter the organism; however, due to ionic mimicry (the ability of a cationic form of a toxic metal to mimic an essential element) Cd^{2+} is able to enter an organism through Ca^{2+} -channels and transporters (Verboost *et al*, 1987).

1.4 Tissue Residue Approach (TRA)

A tissue residue approach (TRA) is a method of toxicity assessment which uses tissue concentrations as a measure for assessing toxic effect (McCarty, 1991; Brix *et al.*, 2005; Meador, 2006). Whole tissue (or whole body) concentration when used as a measure and linked to toxic effects, account for water borne metal speciation which affect bioavailability, as well as differences in uptake characteristics into the organism. Studies have found by expressing toxicity as a tissue residue, species variability is greatly reduced (McCarty, 1991). However, organisms are able to sequester and detoxify accumulated metal, at least to some degree, and whole tissue (or body) concentrations do not distinguish this pool from pools that can cause damage. Therefore, modifying factors operate within the exposure medium (see section 1.2 on speciation) and also within the organism in terms of Cd sequestering and detoxification. In an attempt to deal with the

issues associated with modifying factors and an organism's natural defences, tissue residue approaches have been developed which distinguish between detoxified pools of metal and metabolically active pools within tissue and cellular concentrations. These may help to establish a better dose metric for metal impacts.

The degree to which an organism can detoxify Cd and the preferred methods of detoxification can vary from one aquatic species to another. Despite the fact that organisms lack Cd-specific physiological detoxification strategies, they do possess non-specific mechanisms with which to cope with metal accumulation. For example, natural detoxification can occur through a protein called metallothionein (MT) and other metallothionein-like (MT-like) proteins. MT and MT-like proteins are able to bind metal rendering them metabolically inactive (Hammer, 1986; Roch *et al.*, 1986). Buchwalter *et al.* (2007) used a differential centrifugation technique to separate out the detoxified metal in a test that measured Cd bioaccumulation in two species of mayfly *Ephemerella infrequens* and *Rithrogena hageni*, and compared their accumulation with a caddisfly *Rhyacophila sp.* While both mayflies were able to readily detoxify by binding the accumulated Cd with MT-like proteins, with 35.8% and 69.4% being bound respectively, only 2.9% of the caddisfly's accumulated Cd was bound to MT-like proteins. Both mayflies relied heavily on MT-like proteins to detoxify Cd, while the caddisfly utilized a different technique. Instead of up-regulating MT-like proteins, the caddisfly was capable of compartmentalizing 64.4% of the Cd burden within biologically inert tissue, such as chitin (Buchwalter *et al.*, 2007). Many aquatic invertebrates utilize an additional form of metal compartmentalization, metal-rich granules. Granules represent an inert pool in which accumulated metals can be stored. Tests have shown that some organisms, such as

Chironomus riparius are capable of storing between 40-50% of its accumulated Cd in metal-rich granules (Bécharad *et al.*, 2008). Due to the ability of many organisms to detoxify accumulated Cd, it is important to know how much of the whole body metal accumulation is actually metabolically active.

In order to apply TRA's effectively, utilization of techniques which account for metal detoxification are required. Techniques like subcellular fractionation (refer to section 1.5) have been developed to separate the biologically active pools and the detoxified pools in order to obtain an accurate measure of metal concentration within each pool. These techniques place less importance on metal bioavailability in the exposure media, and account for metal detoxification in addition to any intraspecies variation in uptake rates. One downside associated with subcellular fractionation is that when working with small organisms a large sample size (pools totalling 0.2-0.3g) are needed for single data point, making this technique more viable when working with larger organisms. However, by separating the biologically active pools, these subcellular techniques are capable of providing a more accurate approximation of dose and it may be possible to determine which subcellular Cd concentration is needed to cause a specific sub-lethal response.

1.5 Subcellular Fractionation

Subcellular fractionation is a process that allows a tissue homogenate to be separated into several different operationally defined fractions (Wang and Rainbow, 2006). This protocol has been used for years within many fields of biology, dating back to Dowdall and Whittaker's (1973) work on squid synaptosomes, and recently has been

applied to understand bioaccumulation in the context of mechanisms of toxicity (Wallace *et al.*, 2003; Cain *et al.*, 2006; Buchwalter *et al.*, 2007). Wallace *et al.* (2003) used the technique to successfully separate metal-sensitive cellular components from the biologically detoxified metal in two different species of bivalves. The fractionation method separated tissue into 5 operationally defined cellular fractions: cell debris, cellular organelles, metal-rich granules, cytosolic proteins denatured by heat, and heat-stable cytosolic proteins composed primarily of metallothionein-like proteins (Wallace *et al.*, 2003). Metabolically active pools are those that are vulnerable to Cd binding (cellular organelles, heat denatured proteins), while the inactive pools are comprised of the remaining fractions: granules, heat-stable proteins and cellular debris (Cain *et al.*, 2006; Buchwalter *et al.*, 2007; Wallace *et al.*, 2003). Subcellular fractionation provides a better understanding of how accumulation at a cellular level might be linked to impacts.

1.6 Aquatic Invertebrates

Aquatic invertebrates are integral members of the aquatic macrofauna. They are often located at the base of the food chain, and many are important detritivores. Those that accumulate and are able to detoxify Cd may serve as indicators of exposure history and could potentially have a role in the trophic transfer up the food chain.

1.6.1 *Lumbriculus variegatus*

L. variegatus (California blackworm) is a freshwater oligochaete worm found in shallow marshes, ponds and swamps throughout North American and Europe (Brinkhurst and Jamieson, 1971). The blackworm is easily cultured, adults range between 4.5-6 cm in length, and they reproduce asexually through fragmentation (Brinkhurst and Jamieson,

1971). Blackworms are normally found in the sediment where they feed on microorganisms and other organic material with their posterior end exposed to the overlying water for gas exchange (Brinkhurst and Jamieson, 1971). This is important as blackworms are exposed to sediment, pore water and overlying water contaminants, and if there is Cd contamination in an ecosystem it will show up in blackworms.

The blackworm has been shown to be very resistant to pollution. In soft water studies (44-47mg/L CaCO₃) *L. variegatus* had a 10 day Cd LC₅₀ of 158 µg/L (Phipps *et al.*, 1995). This LC50 is almost 10,000x the interim guideline of 0.017 µg/L set by the CCME. Along with its high tolerance to Cd, *L. variegatus* has been shown to readily bioaccumulate Cd. In a study by Ng and Wood (2008), *L. variegatus* was shown to accumulate 30.3 µg/g over 10 days when exposed to waterborne concentrations of 200 µg/L Cd.

Subcellular studies have been conducted using the blackworm as a model. The majority of the Cd was found (average of 48%) bound to heat denaturable proteins, while all other fractions contained a significantly lower percentage (Xie *et al.*, 2007). Because the heat denaturable proteins are considered a biologically active pool, excessive accumulation within this fraction could be related to a negative effect. we should expect to see some negative effect in the test organisms.

1.6.2 *Hyaella azteca*

H. azteca is a freshwater amphipod commonly found from central Mexico to the tree line in Canada (EC 1997). Across this range *H. azteca* can be found in any freshwater body which attains a summer surface temperature above 10 °C allowing for breeding to occur (EC 1997). *H. azteca* are found most abundantly in lentic ecosystems

with vegetation providing shelter from predators. These amphipods typically graze on available aquatic plants or feed on detritus. *H. azteca* are small crustaceans with adults ranging between 6-8mm in length, with males typically being larger than females.

H. azteca are commonly used in toxicity studies. They are amenable to lab use, being cultured easily in either natural or artificial media as long as bromide ions are present (Borgmann, 2002). Lab cultures can be fed a wide variety of commonly available foods, including dried fish flakes and a slurry of yeast, cereal leaf, and trout chow (YCT). Once they reach maturity, adults reproduce easily in the laboratory environment. Small cultures of 30 adults are capable of producing between 60 and 180 offspring in a week under ideal conditions. Tests are performed on the most sensitive life-stage, and for this reason toxicity tests are performed on individuals between 2-9 days old at test start. Research has shown *H. azteca* to be sensitive to Cd exposure. Borgmann *et al.* (2005) conducted 7 day exposures resulting in LC50's of 0.57 $\mu\text{g Cd/L}$ for soft water (18 mg/L as CaCO_3) and 4.41 $\mu\text{g Cd/L}$ hard water (124 mg/L as CaCO_3). Even in hard water exposures the amphipods were more sensitive than *L. variegatus* despite the worms being exposed to a more toxic soft water exposure (*Lumbriculus* LC₅₀ of 158 $\mu\text{g/L}$; Phipps *et al.*, 1995).

1.7 Monitoring ecosystem health

Monitoring environmental health is of importance due to the input of contaminants through anthropogenic sources. In order to monitor the environment having techniques with which to quickly assess toxic effects in an aquatic ecosystem is of great importance. One method with which to observe monitor ecosystem health is biomonitoring.

Biomonitoring involves the use of organisms found within an ecosystem to provide information about the health of the ecosystem (Holt and Miller, 2011; Gadzala-Kopciuch et al., 2004).

By combining biomonitoring with an endpoint like bioaccumulation, it may be possible to find an organism that is suitable to act as an indicator of deleterious effects in many members of the ecosystem. Studies conducted by Couillard *et al.* (2008) have suggested *H. azteca* would make a good candidate for biomonitoring the environment. In their studies amphipods were transported into the field and over 17 days were left to accumulate metals from the environment. This study showed dose dependent accumulation in the amphipod and essentially proved that bioaccumulation can be used in biomonitoring. Borgmann *et al.* (2004) and Norwood *et al.* (2007) have developed an approach that relates a critical body concentration to lethality in *H. azteca*. This theory relies on determining a body burden which corresponds to lethality. While this concept has proven that it has merit and may be applicable to the field, there are issues with it. The most notable of which is that *Hyalella* is a very sensitive to metals (Borgmann *et al.*, 2005). For this reason it may not exist in heavily contaminated sites.

A resistant species may be a better choice as a biomonitor of ecosystem health. If a common freshwater species can be found that is capable of accumulating Cd reliably while suffering no significant deleterious effects, it could potentially be used as a measure of not just metal exposure but also the overall health of the ecosystem from whence it came. Since this species would be resistant to contaminants it would be present in even the most contaminated sites. This species could also be used as an indicator of whether effects would be expected in other more sensitive species within the same

ecosystem. However, in order to do so, specific body burdens in the resistant species must be able to be related to effects in other more sensitive species. The more resistant species would essentially be used as a biomonitor of metal exposure and effect in a freshwater environment. That being said, it is important to stress that although being able to relate a quantified body burden to a biological effect is of great value, an assessment of the bioavailability of Cd in an aquatic ecosystem is still very useful. This new concept of bioaccumulation-effect relationships should not be viewed as a replacement of dose-effect techniques but as an additional tool that can be used to evaluate ecosystem health.

1.8 Objectives

The research will attempt to determine whether a resistant species can be used as a biomonitor of Cd effect in an ecosystem. More specifically this research will determine whether deleterious effects in a sensitive species can be predicted based on body burdens in a resistant indicator species. This study will focus on two species: *Lumbriculus variegatus*, a resistant oligochaete, and *Hyalella azteca*, a sensitive amphipod. It is hypothesized that an effect in *H. azteca*, can be related to a Cd body burden in *L. variegatus*. To test this hypothesis the completion of three main goals is needed:

- (1) To determine the toxicological endpoints (survival, growth, reproduction) of Cd exposure in *Lumbriculus variegatus*, and *Hyalella azteca* at two different water hardness values (40 and 140 mg/L as CaCO₃).
- (2) To determine whole organism bioaccumulation patterns in both the 40 and 140 mg/L as CaCO₃ water hardness exposure scenarios.
- (3) Link whole organism bioaccumulation of Cd to toxicological endpoints.

1.9 General Guide to Thesis Chapters

The first chapter of this thesis has focused on general background and an introduction to the project. It is concluded with the objectives of this thesis. The second chapter of this thesis focuses on hard water exposures in both *L. variegatus* and *H. azteca*. Initial testing on *L. variegatus* consisted of two 28 day exposures looking at bioaccumulation and reproduction and a concentration range of 0-50 µg Cd/L. This was followed two additional *L. variegatus* exposures. The first of which was an 87 day exposure using concentrations between 0-200 µg/L which focused on bioaccumulation from both a whole organism and subcellular distribution perspectives, while the second was a 28 day reproduction test using the same exposure concentrations. These experiments were followed by testing on *H. azteca* over 21 days of exposure looking at both whole organism bioaccumulation and average dry weight throughout the test. The *H. azteca* were digested in 16N nitric acid and from this test forward all *L. variegatus* analysis was conducted in the same way to help when directly comparing the two species. The third chapter occurs in soft water and focuses on the same two species as the second chapter in this new water chemistry. Exposures used a concentration range of 0-2.3 µg/L and took place over 28 days. In this chapter rather than looking at subcellular Cd distribution as a TRA a different TRA is proposed. This approach relates Cd accumulation in a resistant species to specific effects in a sensitive species. The final chapter of this thesis expands on chapter 3 and introduces an additional sensitive and resistant species. This chapter concludes by discussing the ways in which this research is integrative.

Chapter 2: A multiple invertebrate species test to determine if Cd accumulation in a resistant species can be used to reliably predict effects in a sensitive species in hard water

2.0 Abstract

This experiment was conducted to determine bioaccumulation patterns and toxicological effects of Cd exposure in two freshwater invertebrates in order to assess their viability as bioindicators of Cd contamination in the field. Using chronic exposures of 21 to 87 days, *Lumbriculus variegatus* and *Hyaella azteca* were characterized for Cd bioaccumulation and effects (survival, growth and reproduction) in moderately hard water (140 mg/L CaCO₃).

At the highest waterborne Cd concentration (200 µg Cd/L) *L. variegatus* showed 100% survival; however, reproduction was reduced 8-fold in comparison to controls. Whole body accumulation was linear with time and exposures of 0, 6.3, 12.5, 25 and 50 µg Cd/L produced body burdens of 0.9, 8.7, 12, 17.3 and 28.8 µg Cd/g wet weight on day 28 respectively.

L. variegatus ability to detoxify metal was assessed by comparing the subcellular distribution of Cd in organisms exposed to both high and low concentrations, providing evidence to support the belief that accumulation in sensitive organelles and enzymes will lead to deleterious effects on the organism. The resistance in *Lumbriculus* was contrasted by sensitivity in *Hyaella azteca*. Initial testing using 2 to 9 day-old *H. azteca* resulted in 100% mortality at all concentrations above 10 µg Cd/L. After 21 days of exposure control organisms weighed 136 ± 37 µg; more than twice as large as surviving exposed individuals that showed no significant difference in growth between treatment concentrations. *Hyaella azteca* quickly accumulated Cd until a steady state body burden was reached, after approximately 10 days of exposure.

H. azteca was found to be a poor bioindicator species, as Cd effects are observed in *H. azteca* at environmentally relevant concentrations. *L. variegatus* in contrast was shown to be resistant beyond relevant concentrations and their ability to readily accumulate Cd make this species a candidate for use as a bioindicator.

2.1 Introduction

All fresh water ecosystems naturally contain cadmium at trace levels. Elevated levels of Cd can be reached in the environment due to anthropogenic inputs and cause adverse effects (McGeer *et al.*, 2011; Pan *et al.*, 2010). Major sources of anthropogenic input include the mining and smelting of Zn, Pb and Cu ores (Pan *et al.*, 2010). As Canada is a prominent mining nation, Cd is of particular interest.

Cd in the environment is commonly found in the free ion (Cd^{2+}) form, which is known to compete with Ca^{2+} for uptake sites on respiratory surfaces. Binding these respiratory sites leads to the uptake of Cd into an organism, where the non-essential metal can cause adverse effects including hypocalcemia (McGeer *et al.*, 2000a). The acute effects of Cd have been well studied and led to the development of Cd toxicity prediction models such as the biotic ligand model (Niyogi and Wood, 2004; Niyogi *et al.*, 2008). Despite the extensive knowledge of acute effects of Cd, little is known about its chronic toxicity. As most environmental exposures occur over prolonged periods it is important to understand the effects of chronic exposure on an organism.

Typically, in order to determine the concentration that corresponds to a specific effect a measurement of the surrounding water or sediment is done. This concentration can then be related to a specific observed effect, and be used to calculate effect concentrations. Issues with this concept arise because many of these dose-response

relationships are done in a laboratory, and these relationships may not apply to the field where numerous modifying factors can influence the exposure or the toxicity. Modifying factors such as pH, dissolved organic carbon and water hardness can have profound effects on the bioavailability of the metal and can make total environmental concentration a less than ideal measure (U.S. EPA, 2001; Giesy *et al.* 1977).

Water hardness in particular affects Cd uptake, and indirectly the accumulation of Cd and observed toxic effects. Studies have shown that for many organisms, for example the tubificid worms *Limnodrilus hoffmeisteri* and *Tubifex tubifex*, hard water significantly increases an organism's survival compared to a soft water exposure (Chapman *et al.*, 1982; Williams *et al.*, 1986; Reynoldson *et al.*, 1996). Cationic competition for uptake between Ca^{2+} and Cd^{2+} is the major reason for hard water being more protective than soft water to an organism. In hard water there are more Ca^{2+} ions to compete for binding sites and consequently less Cd^{2+} binds and is taken up by the organism. The presence of modifying factors and the complexity of water chemistry has caused researchers to look for an alternate measure of exposure that can be related to effect.

Bioaccumulation refers to the accumulation of a chemical within an organism. It typically occurs in two phases. Initially, uptake occurs and tissue concentrations increase. This occurs because the rate of chemical elimination from the organism is exceeded by the uptake into the organism. With exposure concentrations remaining constant, the uptake and elimination rates will eventually balance out and second phase, referred to as steady state, will be reached. At steady state tissue burdens remain constant. Campbell *et al.* (2008) have suggested that the bioaccumulation of a metal, such as Cd, within an

organism will continue up to and beyond specific thresholds, which correspond to specific effects. Since only the Cd that is available in the environment can be taken up into the organism, and effects are believed to be related to specific threshold concentrations, bioaccumulation (rather than environmental concentration) may serve as a better measure for assessing threshold concentrations and determining risk within an ecosystem. This experiment focuses on the chronic effects of Cd on two different freshwater invertebrates and attempts to relate accumulation to effect.

Since every ecosystem varies in its water chemistry, an understanding of bioaccumulation could lead to its use as an indicator of the dose an organism receives (independent of environmental modifying factors), as well as for predicting effects within the ecosystem. If a common freshwater species can be found that is capable of accumulating Cd reliably while suffering no significant deleterious effects, it could be used to determine Cd exposure within the environment from where it came. This species could also then be used as an indicator with which one could predict potential effects in other more sensitive species. The initial more resistant species would essentially be used as a bio-indicator of Cd effects in a freshwater environment. This is an example of a tissue residue approach (TRA). A tissue residue approach is a method of toxicity assessment that uses tissue concentrations as a character for assessing toxic effect rather than environmental concentrations (McCarty, 1991).

While the use of TRA's provides researchers a measurement that already compensates for the complexities of water chemistry, there is concern that not all of the accumulated metal is present in a toxic form. The reasoning for this is that all organisms possess some form of metal detoxification; which results in a portion of the accumulated

metal being unavailable to sensitive tissues and organelles (Wallace *et al.*, 2003). For example, despite lacking specialized machinery for Cd detoxification, organisms possess metallothionein-like proteins that are capable of binding the metal accumulated within the organism in a biologically inactive form (Wang and Rainbow, 2006; Wallace *et al.*, 2003; Kraemer *et al.*, 2005). Due to metal detoxification a TRA which accounts for only metabolically active metal is of importance.

Sub-cellular fractionation is an example of a TRA that accounts for metal detoxification. This technique looks at the Cd distribution within the cells of an organism. Using sub-cellular fractionation tissue can be split into operationally defined fractions, and the toxic metabolically active fraction (MAF) can be separated from the safe metabolically inactive fraction (MIF) (Wang and Rainbow, 2006; Wallace *et al.*, 2003). By eliminating the MIF this technique will provide a more accurate approximation of how much metal an organism is actually being exposed to. Using this technique it may be possible to deduce exactly what subcellular Cd concentration is needed to illicit a specific sublethal response.

L. variegatus (California blackworm) is a freshwater oligochaete worm found in shallow marshes, ponds and swamps throughout North American and Europe (Brinkhurst and Jamieson, 1971). The blackworm is easily cultured as adults reproduce asexually through fragmentation (Brinkhurst and Jamieson, 1971). Blackworms are an important member of the benthic community and are found in sediment where they feed on microorganisms and other organic material (Brinkhurst and Jamieson, 1971). The blackworm has been shown to be resistant to pollution. In soft water studies (44-47mg/L CaCO₃) *L. variegatus* had a 10 day Cd LC₅₀ of 158 µg Cd/L (Phipps *et al.*, 1995). Along

with its high tolerance to Cd, *L. variegatus* has been shown to readily bioaccumulate Cd. In a study by Ng and Wood (2008), *L. variegatus* was shown to accumulate 30.3 µg/g over 10 days when exposed to waterborne concentrations of 200 µg Cd/L.

H. azteca is a freshwater amphipod commonly found in all types of freshwater south of the tree line in North America (EC 1997). *H. azteca* has been widely used in both water only and sediment toxicity testing due to their amenability to lab culturing as well as their sensitivity to contaminants. Borgmann *et al.* (2005) conducted acute 7 day exposures using *H. azteca*, demonstrating their sensitivity to the metal with 7 day LC50's of 0.57 µg Cd/L for soft water (18 mg/L as CaCO₃) and 4.41 µg Cd/L for hard water (124 mg/L as CaCO₃).

The goal of this research are to determine the toxicological endpoints of Cd exposure in *Lumbriculus variegatus* and *Hyaella azteca* in a hardwater exposure using artificial Lake Ontario water (140 mg/L as CaCO₃) and to determine whether a specific response to a contaminant corresponds to a specific Cd concentration in both species. Additionally the viability of using bioaccumulation as an indicator of effect will be discussed.

2.2 Materials and Methods

2.2.1 Culturing *L. variegatus* and *H. azteca*

Lumbriculus variegatus were purchased from Aquatic Research Organisms (ARO; Hampton, NH, U.S.A.), and cultured following standard methods outlined by the US EPA (2000, EPA 600/R-99/064). Cultures were maintained in artificial Lake Ontario water consisting of 1.0 mM Ca²⁺, 0.6 mM Na⁺, 0.8 mM Cl⁻, 0.15 mM Mg²⁺, and a pH of

7.2 ±0.1. Aeration was provided and temperature was maintained at 20 ± 2 °C. Brown unbleached paper towels were utilized as a substrate and filled aquaria to a depth of 1-2 inches. Complete water and substrate changes were done once a month and at which point new cultures were started with approximately 700 adult individuals from the previous culture. Partial water changes were completed on a weekly basis. Feeding took place three times a week and consisted of a slurry of ground Tetramin® flakes (Tetra Werke, Blacksburg VA, U.S.A.) and Nutrafin® sinking complete food tablets fed *ad libitum*.

Hyaella azteca were purchased from ARO, and cultured in 1L high density polyethylene (HDPE) beakers each containing 30 adults. Cultures were maintained in artificial media containing 1.0 mM Ca²⁺, 1.0 mM Na⁺, 0.05 mM K⁺, 0.25 mM Mg²⁺ and 0.01mM Br⁻ (Sigma-Aldrich, Nepean, ON). It was essential that the bromide ion be included as *H. azteca* cannot make use of calcium without it (Borgmann, 2002). For each culture beaker a 5cm² piece of sterile cotton gauze was added for use as a substrate (Environment Canada, 1997). Temperatures in lab cultures were maintained at 20 ± 2 °C. Cultures received an equivalent of 5 mg of Tetramin® flakes (Tetra Werke, Blacksburg, VA, U.S.A.) added in a slurry 3 times a week. Culture water was replaced and neonates were separated from the adults weekly.

2.2.2 *Lumbriculus variegatus* chronic bioaccumulation and reproduction tests

Prior to starting the test 50L of artificial Lake Ontario water was mixed in a 50L large carboy, pH adjusted to 7.2 ±0.1 and left for 24 hours to equilibrate. New water was mixed throughout the test as needed. Testing followed guidelines outlined by the USEPA (2000, EPA 600/R-99/064). An initial round of range-finding exposures were conducted

in 250 mL HDPE beakers, using artificial Lake Ontario water and inert silica sand (Bell & MacKenzie Co. Ltd., Hamilton, ON, Canada) as a substrate arranged in 4 to 1 water: sediment ratio (160mL:40g) . Exposure concentrations for this round of testing were 0, 6.25, 12.5, 25, and 50 µg Cd/L. Four identical beakers were utilized for each exposure concentration and were spiked from a stock of 1.0 g/L of Cd 24 hours prior to the test start to allow for equilibration. Water renewals occurred every 2 days, with 50% of the water volume being replaced. Organisms between 2 and 2.5cm in length were selected for the test. Ten individuals were added to two of the four beakers at every concentration for reproductive observations, while 110 individuals were added to each of the other two beakers for analysis of Cd accumulation. Organisms were each fed 20µg of Tetramin® flakes in a slurry every 3 days throughout the duration of the test. Water pH, and temperature were monitored throughout the test.

A second round of testing utilized an increased exposure range of 0, 6.25, 25, 50, 75, 100, 150, 200 µg Cd/L. These tests were scaled up versions of the previous round of testing utilizing the same artificial media, and sediment in a 4:1 ratio. Feeding and water renewal schedules were identical to the initial exposure. Changes included utilizing 2000 mL HDPE beakers and ~200 individuals per beaker to account for the increased number of individuals needed to obtain an acceptable wet weight for subcellular analysis.

A subsequent 28 day test focusing on reproduction followed, and included changes similar to those in the second accumulation test: an increased concentration range (0-200 µg Cd/L) and larger 2L beakers. This exposure utilized 20 individuals as a start point as opposed to 10 from the previous reproduction test. Unlike the previous reproduction test this was completed in triplicate.

2.2.3 *H. azteca* chronic bioaccumulation

Prior to starting the test 50L of artificial Lake Ontario water was mixed in a large carboy, pH adjusted to 7.2 ± 0.1 and left for 24 hours to equilibrate, new water was mixed throughout the test as needed. An initial rangefinder test was performed using concentrations of 0, 2.5, 10, 25, 75 and 200 $\mu\text{g Cd/L}$. The test was conducted using ten 2-9 day old neonates in 250mL HDPE beakers identical to those used in the initial *L. variegatus* exposures. Tests were completed following EPS 1/RM/33 (Environment Canada, 1997) as close as possible, utilizing the same substrate (5cm² piece of cotton gauze) and an identical feeding regiment of 1mL YCT (ARO; Hampton, NH, U.S.A.) fed daily to each beaker. Water renewals followed the same schedule as the *L. variegatus* exposures, with 50% of the water volume being replaced every 2 days. Surviving individuals were counted with each water renewal and dead individuals were removed using a disposable pipette.

Due to the large number of individuals needed for a bioaccumulation test, a scaled up version of the previous exposure was adopted as a follow-up test. This exposure utilized 2000 mL HDPE beakers and 64 individuals split between 2 replicate exposures. Concentrations utilized were 0, 1.25, 2.5 and 5 $\mu\text{g Cd/L}$.

2.2.4 Sampling and Sample Processing

Water samples were taken prior to the addition of organisms to validate exposure concentrations, as well as prior to and immediately following each water renewal. With each round of sampling 10 mL of filtered (0.45 μm syringe filter; Acrodise HT tuffryn membranes, Pall Corporation, Ann Arbor, MI) and un-filtered water were taken from at least one of the replicate exposure chambers. Water samples were then acidified to 1% by

adding 100 μ L of concentrated HNO₃ (Trace Metal Grade, Fisher Scientific, Mississauga, ON).

During the initial *L. variegatus* test organisms were sampled on days 1, 7, 14, 21, and 28 for total body burden. At each time point 12 individuals were collected from each replicate exposure. Individual worms were given 6 hours for gut clearance in clean artificial Lake Ontario water before being blotted dry and three individuals were then pooled into one of 8 pre-weighed (Sartorius CP224S; Sartorius Mechatronics Corp., Bohemia, NY, U.S.A) 1.5 mL micro-centrifuge tubes. The microcentrifuge tubes were once again weighed after the addition of the worms prior to digestion. Samples were digested for 3 hours at 80 °C in a 1:5 wet weight to 1N HNO₃ ratio. Following digestion, samples were vortexed (VX-200 Vortex Mixer; Labnet, Woodbridge, NJ, U.S.A.) for 3 seconds and then centrifuged for 2 minutes at 10,000 RPM (Spectrafuge 16M; Labnet International, Edison, NJ, USA). The second round of testing utilized individual organisms rather than pools, and featured an expanded duration with sampling at days 40, 60, and 87. On days 7 and 45 of the second bioaccumulation test 200-300mg of worms were sampled for analysis of subcellular distribution. These organisms were subjected to the same 6 hour gut clearance and blot drying as those sampled for accumulation, but following weighing were frozen at -40 °C for later processing.

During both *L. variegatus* reproduction tests, organisms were counted on days 7, 14, 21, and 28. Plastic disposable pipettes were used to stir up the substrate above and around the worms. Once a worm had been dislodged from the sand they were removed into a separate beaker until all of the organisms from the test beaker had been removed, and counted. Following counting, worms were placed back into their test beaker.

Hyalella azteca were sampled on days 1, 3, 5, 7, 10, 14, and 21 for dry weight and total body burden. At each time point 4 individuals were sampled from each replicate exposure. Individual amphipods were given 6 hours for gut clearance in clean artificial Lake Ontario water before being gently blotted dry and baked in open 0.6 mL ultracentrifuge tubes for 48h at 80°C. Following drying individual *H. azteca* were weighed using a Sartorius SE2 Ultra Micro Balance (Sartorius Mechatronics Corp., Bohemia, NY, U.S.A), and their average dry weight calculated at each exposure and time point. After weighing each individual was digested in 25 uL of 16N trace-metal grade HNO₃ for 6 days at room temperature, after which 20 uL of 30% H₂O₂ was added for an additional 24 hours prior to the sample being topped up to 250 µL using MilliQ ultrapure water (Borgmann and Norwood, 1997). Following digestion samples were vortexed to ensure mixing and centrifuged (Spectrafuge 16M; Labnet International, Edison, NJ, USA). Further analysis of samples followed the same protocols as those used in the *L. variegatus* exposures.

2.2.5 Subcellular distribution of Cd in *L. variegatus*

The protocol used was adopted from Wallace *et al* (2003), and Xie *et al.* (2007) (See flow diagram in appendix A). Organisms were homogenized on ice in a 0.9% NaCl buffer solution of 1:3 (w/v) using an Omni THq digital tissue homogenizer (Omni International, Marietta, GA) at 20 RPM. Following homogenization, a 15 min 800 x g spin at 4 °C in a centrifuge (IEC-CL31R Multispeed; Thermo Electron Corp., Milford, MA) was used to separate granules and cellular debris from the supernatant. Following re-suspension of the pellet in 0.5mL of MilliQ ultrapure water, the sample was heated at 100 °C for 2 minutes. To this suspension 0.5 mL of 1N NaOH was added, followed by an

additional hour at 60°C and a 10,000 x g spin for 30 minutes at 20 °C. This final spin separated the nuclei and cellular debris (supernatant) from the granules (pellet). The initial supernatant, from the first 800 x g spin, was subjected to an organelle extraction via a spin at 4°C, 100,000 x g on an ultracentrifuge (Optima MX-IM-5; Beckman Instruments, Mississauga, Ontario, Canada). The cytosolic supernatant from the ultracentrifuge spin was heated at 80°C for 10 min, than cooled on ice for an hour, and finally ultracentrifuged at 50,000 x g for 10 min at 4°C to separate the heat sensitive proteins from the MT-like proteins. A series of 3 rinses in buffer solution and a final spin for 10 min at 10,000 x g at 4°C was completed to ensure purity of pellet fraction. Following isolation, samples were stored at -80 °C. Digestion of the pellets differed from total body burden digests, in that rather than a 3h bake, the samples were digested for 24h at room temperature. Supernatant fractions were diluted 3 fold rather than 5 fold, but were also subjected to the 24 hour incubation at room temperature. All samples were then analyzed in an identical fashion to the total body burden samples.

2.2.6 Analysis

Both water and tissues were analyzed using an atomic absorbance spectrophotometer (SpectraAA 880 GTA 100 atomizer, Varian, Mississauga, Ontario) in either flame mode (FAAS; Varian 1989) or graphite furnace mode (GFAAS; Varian 1988). Water Ca, Mg and Na were measured using FAAS. Pre-prepared certified reference material (trace metal fortified Lake Ontario water [TMDA-28.3 and TM 26.3]; National Water Research Institute, Burlington, ON, Canada) and blanks (MilliQ water and solutions used for digestion) were used as a quality control to assess accuracy, and provide reference.

Both tissue samples and subcellular fractions were analyzed using GFAAS. Following digestion samples were centrifuged and the supernatants were diluted accordingly using 1% acidified (16N HNO₃, Trace Metal grade; Sigma-Aldrich, Nepean ON, Canada) ultra-pure water. Following analysis the Cd body burden and subcellular compartment's Cd loads were calculated and expressed as µg Cd/g tissue wet weight.

2.2.7 Statistics

Data is presented as mean ± SEM unless otherwise noted. Graphs and statistical analyses were performed utilizing SigmaPlot 11.0 (Systat Software Inc.; San Jose, CA, USA). Growth data was subjected to a one-way analysis of variance (ANOVA). A Fisher LSD post hoc test was utilized to detect significance using P<0.05.

Accumulation data was subjected to a series of one way ANOVA to test significant differences from controls at each day of exposure. For bioaccumulation analysis n=8 unless otherwise noted. All accumulation data was subjected to Fisher LSD post hoc test, to detect significance using P<0.05.

2.3 Results

2.3.1 Cd bioaccumulation

In *L. variegatus* exposures target nominal concentrations of 6.25, 12.5, 25, 50, 75, 100, 150 and 200 µg Cd/L corresponded to 4.6 ± 0.1, 11.6 ± 0.6, 32.4 ± 0.9, 57.4 ± 2.2, 86.9 ± 0.8, 107.6 ± 2.2, 153.0 ± 2.0, 205.3 ± 3.5 µg Cd/L respectively (n=12).

Accumulation in *L. variegatus* was found to be dose dependent, with total body burdens increasing with both exposure concentration and duration. Initial 28 day exposures were insufficient in duration for steady state to be reached, but after 28 days of exposure total

body burdens of 0.9, 8.7, 12, 17.3 and 28.8 $\mu\text{g Cd/g}$ were obtained from 0, 4.6, 11.6, 32.4, and 57.4 $\mu\text{g Cd/L}$ respectively (Figure 2.1). At each time point, body burdens from exposed individuals were significantly higher than controls ($n=8$, $p<0.05$). A second test followed using concentrations up to 200 $\mu\text{g/L}$ and a duration of 87 days. Similar trends were observed in this experiment with total body burden increasing linearly with time, and an 87 day duration being insufficient for a steady state to be reached (Figure 2.2; Tabke 2.1).

Hyalella azteca accumulation over 21 days of exposure is presented in Figure 2.3 and Table 2.2. All exposed individuals reached a steady state of accumulation by around day 10 of exposure regardless of exposure concentration (Figure 2.3). At day 21 of exposure there was no significant difference in total body burden between organisms exposed to 1.25, 2.5 and 5 $\mu\text{g Cd/L}$.

2.3.2 Reproduction and Survival

There were no mortalities observed throughout both the 28 and 87 day Cd exposures of *L. variegatus*. Initial testing of *L. variegatus* provided no significant effect on reproduction after 28 days of exposure to concentrations as high as 50 $\mu\text{g Cd/L}$ (Fig 2.4). There appeared to be an initial inhibition of reproduction of exposed individuals between days 7 and 14. However, by the end of the exposure period, at day 28, there was no significant difference in the number of new individuals (Fig 2.4).

The second round of exposures utilized the same duration but provided an increase in sample size, and an increase in the range of exposure concentrations. The number of new organisms at test end is presented in Fig 2.5. Exposure to concentrations above 100 $\mu\text{g Cd/L}$ resulted in a significant reduction in reproduction over controls (Fig

2.5). The exposure to 100 µg Cd/L corresponded to an almost 50% reduction in reproduction, while exposures to 150 and 200 µg Cd/L corresponded to about 66 and 90% reductions respectively.

H. azteca proved to be more sensitive to Cd exposure than *L. variegatus* with 100% mortality being observed prior to day 21 at all concentrations above 10 µg Cd/L. Exposures of 5, 2.5, and 1.25 µg Cd/L corresponded to 85, 91, and 91% survival, none of which differed significantly from control organisms where a 93% survival was observed.

2.3.3 Weight

There was no significant difference in overall dry weight across Cd exposure for surviving *H. azteca* during the first 14 days of exposure (Figure 2.6). However at day 21 control organisms were significantly heavier on average than those exposed to Cd (Figure 2.6). Control organisms after 21d of exposure averaged $0.136 \pm .037$ mg, while individuals exposed averaged 0.060 ± 0.010 mg, 0.027 ± 0.008 mg, and 0.053 ± 0.023 mg for 1.25, 2.5, and 5 µg/L exposures respectively (n=8). At day 21 control organisms were significantly larger than those exposed to Cd (P<0.05).

2.3.4 Subcellular distribution for *L. variegatus*

Subcellular data is presented for the highest and lowest exposure concentrations, corresponding to concentrations resulting in no adverse effect (6.25 µg Cd/L) and a significant reduction in organism growth (200 µg Cd/L). Cd accumulated in all fractions with the exception with metal-rich granules (Table 2.3). The majority of the Cd was found in the cellular debris and heat stable proteins in all cases but day 45 at 200 µg Cd/L where a large portion of the accumulated metal was found in the heat denaturable proteins (Table 2.3). Accumulation at each time point for both exposure concentrations is

presented in Figure 2.7 for day 7 and Figure 2.8 for day 45. These figures display the difference in accumulation between the two exposure concentrations while showing similar trends in regards to Cd distribution. Figures 2.9 and 2.10 present accumulation at 6.3 µg Cd/L and 200 µg Cd/L as a percent of total Cd accumulated at each time point.

2.4 Discussion

Initial testing of *L. variegatus* revealed 100% survival at concentrations as high as 50 µg Cd/L, demonstrating the resistant nature of this species. In these exposures *L. variegatus* was able to accumulate substantial amounts of cadmium while suffering no survival effect. Initial exposures also focused on reproduction. The initial exposure using the concentration range of 50 µg Cd/L had no significant effect on reproduction. Despite this there seems to be a trend towards an initial inhibition of reproduction between days 7 and 14; however, by 28 days the organisms overcame this initial inhibition and had reproduced at a rate that was not significantly different from controls.

In the second round of exposures the concentration ranges were expanded to include concentrations up to 200 µg Cd/L; however at this concentration there was no mortality. At these exposure concentrations significant reductions in survival and reproduction were anticipated as Phipps *et al.* (1995) determined a 10 day LC50 of 158 µg Cd/L albeit in less protective soft water (44-47 mg/L as CaCO₃). However these experiments were performed without the feeding of organisms and without a suitable substrate for burrowing. The substrate provided may also have contributed to the survival of the organisms to concentrations above their anticipated range. The addition of a substrate suitable for burrowing likely provided the organisms with a less stressful

environment than the Phipps exposures, as in the field these organisms are almost never found completely exposed. At concentrations above 100 ug/L there was a significant reduction in reproduction over controls, while at 200ug/L there was an 8-fold reduction in reproduction. This indicated that although the blackworms were not dying at the highest exposure concentrations there was a deleterious effect caused by Cd exposure.

Whole-body Cd bioaccumulation in *L. variegatus* proceeded in a linear fashion (Figure 2.1), and after 28 days of exposure had not reached a steady state of accumulation. This result was unexpected as others have suggested the ability to bioaccumulate Cd to a steady state (Xie *et al.*, 2008) despite their 10-day exposure being too short in duration for it to be reached. The rate of bioaccumulation was dose-dependent with total body burdens increasing with both duration of exposure and increasing exposure concentration, similar to previous *L. variegatus* work in both spiked-water exposures (Xie *et al.*, 2008) and sediment based exposures (Piol *et al.*, 2006). Following the initial 28 day test, exposure duration and concentration range were expanded in anticipation of reaching steady state. Tests were expanded to 87 days at that point steady state accumulation was still not reached (Figure 2.2). Accumulation patterns similar to *L. variegatus* are also observed in the closely related *Tubifex tubifex* (Redeker *et al.*, 2004; Gillis *et al.*, 2004). In experiments done by Gillis *et al.* (2004) 6 weeks of exposure were needed before *T. tubifex* accumulation reached steady state; while this duration is less than half that of the completed *L. variegatus* exposure it was four times longer than *H. azteca* (Figure 2.3). The prolonged duration required for *L. variegatus* to reach steady state may be caused by the species Cd coping technique: continuous detoxification of Cd rather than increasing Cd excretion.

Cd bioaccumulation in *H. azteca* followed a more expected trend. Accumulation was characterized by increasing accumulation until a steady state of body burden was reached. At this point Cd uptake is assumed to be close to equal that of Cd excretion. This pattern was similar to that observed by Borgmann *et al.* (1991). At 140 mg/L as CaCO₃ the amphipods reached a steady state of accumulation around day 10, and total body burden trailed off around 21 days.

H. azteca growth varied greatly between exposures across all concentrations at all time points, despite all tests having been started with individuals between 2-9 days of age. There was no significant difference between controls and exposed individuals until day 21 of the 140 hardness exposure (Figure 2.6). Had this exposure had been expanded out to 28 days of duration it is possible that the effects that were observed at day 21 may not exist by day 28. As the sudden change in organism growth at around day 21 most likely coincided with a molt. This resulted in individuals significantly heavier than those in the exposed beakers (Figure 2.6). There was little difference in size between days 1 and 4, and between days 7 and 14. Day 21 showed a significant increase in growth in controls, but there was not yet a significant difference in the other exposures. At day 21 all exposed organisms were significantly smaller than control organisms, indicating that although there was no significant effect organism growth. Since there was no significant difference between body burdens at steady state and no significant difference between dry weights in exposed individuals, it was not possible to relate a specific body burden to a specific weight in these organisms. A study conducted by Nelson and Brunson (1995) found intermolt periods to be on average 5.6 days, this ranged from a low of about 4 days and a high of about 10 days. Based on these values it would be expected for a molt to

have occurred after the sampling on day 4, likely attributing to the increasing trend observed between days 4 and 7. Based on these estimates, it is possible to conclude that individuals exposed to Cd are up to 2 or 3 molts behind control organisms at day 28.

In *L. variegatus* cadmium was found in all subcellular fractions except granules. At both day 7 and at day 45 the higher exposure concentration had significantly higher Cd concentrations in all fractions (Figures 2.7 and 2.8). In both the 6.25 and 200 $\mu\text{g/L}$ exposures the majority of the accumulated Cd was found in the cellular debris and heat-stable protein (HSP) fractions (Figures 2.9 and 2.10). In the lowest exposure concentration there was a decrease over time in the proportion of Cd found in both of the metal sensitive fractions (organelles and heat denaturable proteins). This can be contrasted with the 200 $\mu\text{g/L}$ exposure where there was a decrease in the organelle fraction but an increase in the HDP fraction over time. At low exposure concentrations organisms appear to be able to detoxify Cd while at higher concentrations a higher percentage of the metal tends to accumulate in sensitive fractions. At both exposure concentrations the heat stable protein fraction contained the highest amount of bioaccumulated Cd. The observed Cd distribution differs from that of Xie *et al.* (2008). In their exposures, they found between 48 and 53% of the accumulated Cd within the heat-denaturable proteins. In their experiments only around 20% of the Cd was found bound to MT-like proteins. The biggest difference between the Xie *et al.* exposure and the one conducted in this experiment was exposure concentrations. In the Xie *et al.* experiment organisms were exposed to a low environmentally relevant concentration of 0.52 $\mu\text{g Cd/L}$. The exposures conducted in these experiments used concentrations orders of magnitude higher, and at these concentrations there were no significant effects below

100µg Cd/L. At the exposure concentrations utilized by Xie *et al.* it is possible that MT-like protein production was not induced as at these concentrations there was no threat of damage to the organism. Many researchers have proposed the use of metallothionein induction as an indicator of metal exposure (Roch *et al.*, 1986; Deeds and Klerks, 1999; Rose *et al.*, 2006).

Lumbriculus variegatus suffers negative reproductive effects from Cd exposure at concentrations as low as 105.6 µg Cd/L over 28 days. Since *L. variegatus* did not reach a steady state of accumulation it was not possible to relate the observed reduction in reproduction to a specific body burden. Regardless of this fact, the exposure concentrations required to illicit responses were well above environmentally relevant concentrations. One would anticipate exposure to Cd at these levels would result in mortality in most members of an aquatic ecosystem, thereby reducing the importance of an effect concentration in this species. *Hyaella azteca* experienced a significant reduction in size at all exposed concentrations, although there was no significant difference in weight between those organisms exposed to Cd, and no dose-response relationship. It was not possible to relate a specific body burden to mortality either as all individuals exposed to concentrations above 10 µg Cd/L died, providing no accumulation data. For this reason it is not possible to relate a specific response to a specific Cd concentration. This experiment provides little support for the use of bioaccumulation as an indicator of effect in aquatic ecosystems, as relating a specific endpoint to a specific body burden has been difficult.

Despite the findings of this experiment bioaccumulation still holds many potential benefits which should be considered before bioaccumulation is dismissed as a

bioindicator of effect. Subcellular analysis needs to be looked into over a time course.

While *L. variegatus* may not have reached steady state on a whole body basis, it is possible that one or more of the fractions may have been at steady state. If this was the case it would be possible to relate effect to a fraction burden at steady state. Despite its potential benefits, this technique is quite labour intensive and requires large pools of organism to be used effectively. These two attributes may limit the use of this technique when assessing ecosystem health.

2.5 Figures and Tables

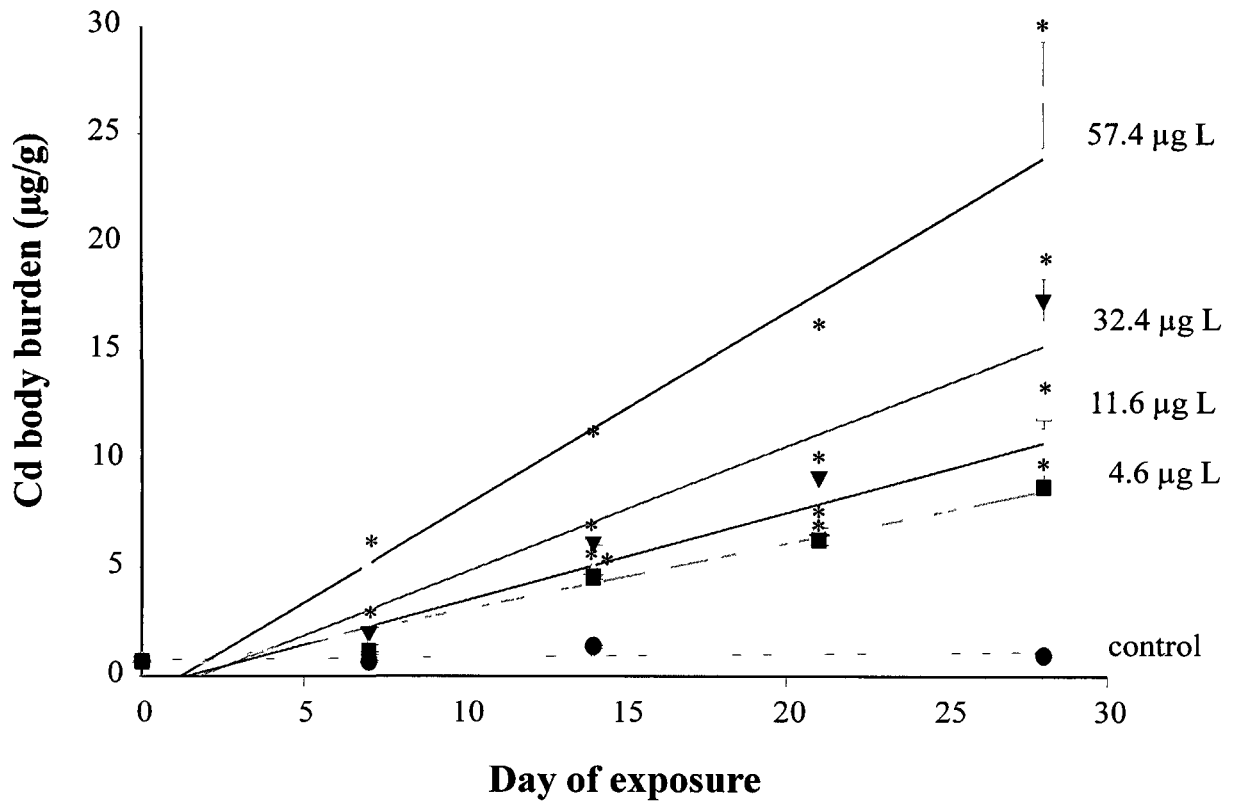


Figure 2.1 Cd accumulation in *L. variegatus* during a 28-day exposure to to 4.6 ± 0.1 , 11.6 ± 0.6 , 32.4 ± 0.9 , 57.4 ± 2.2 , Cd/L. Values are means \pm 1 SEM, n = 8 worms. * indicate significance compared to control organisms at each day (ANOVA; $p < 0.05$).

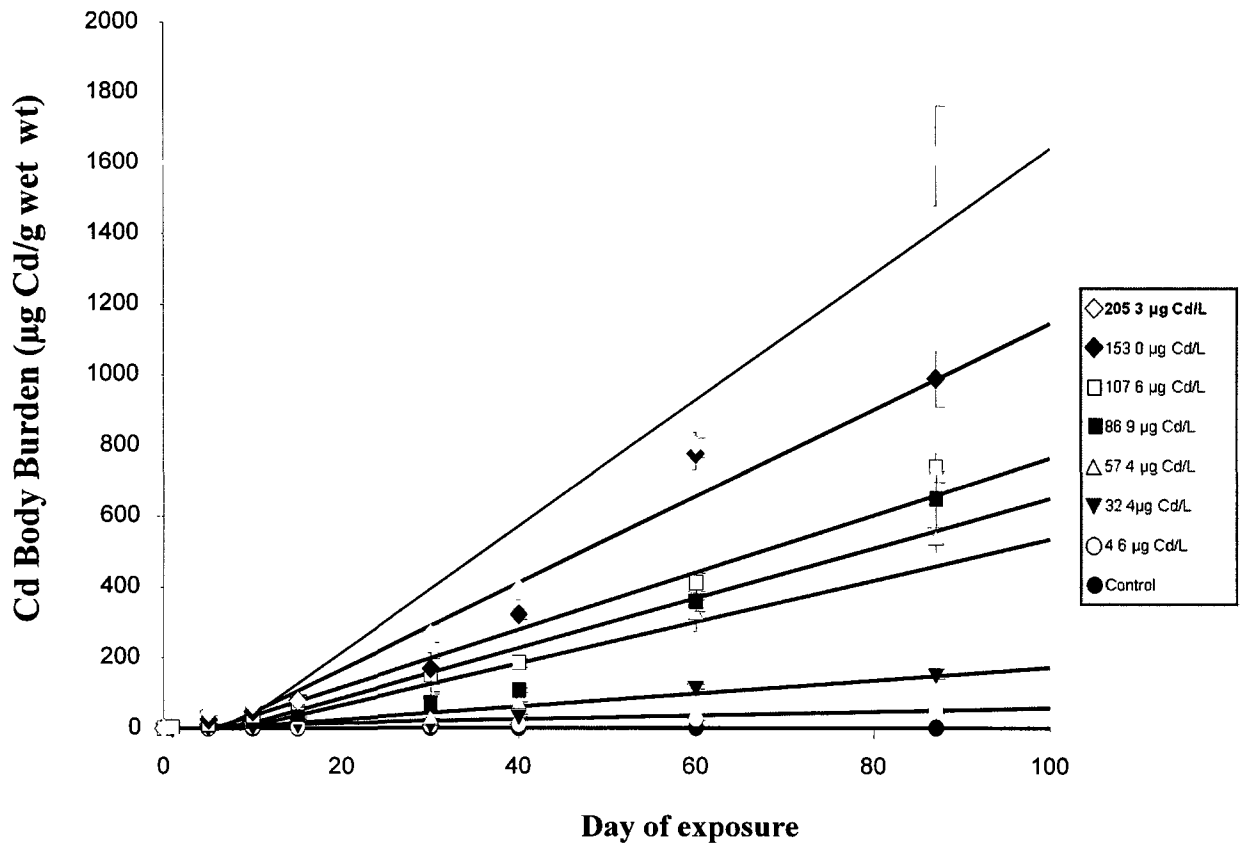


Figure 2.2 Cd accumulation in *L. variegatus* during a 87-day exposure to 4.6 ± 0.1 , 32.4 ± 0.9 , 57.4 ± 2.2 , 86.9 ± 0.8 , 107.6 ± 2.2 , 153.0 ± 2.0 , 205.3 ± 3.5 $\mu\text{g Cd/L}$. Values are means \pm 1 SEM, n = 6 worms. All points differ significantly from controls at each time point. All measurements from day 1 forward are significantly different from controls (ANOVA; $p < 0.05$).

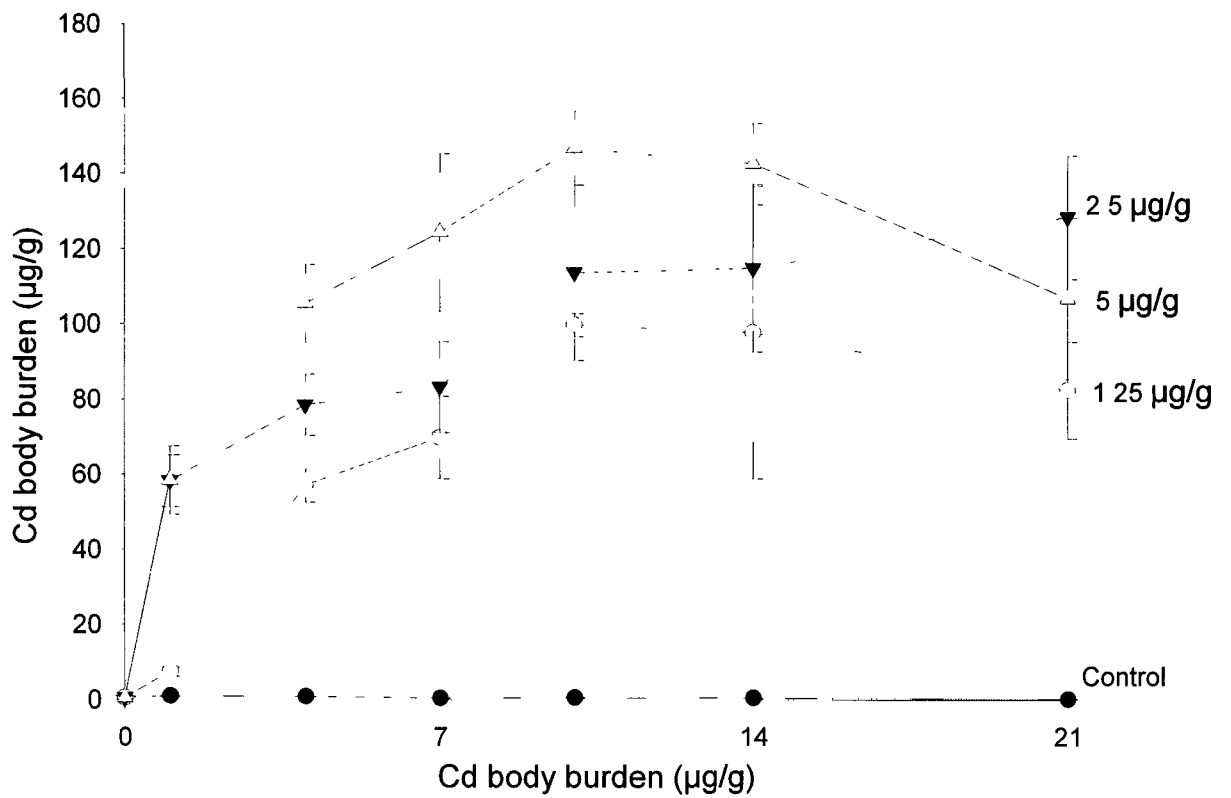


Figure 2.3 Cd accumulation in *H. azteca* during a 21-day exposure to 1.25, 2.5, and 5 µg Cd/L in moderately hard water (140 mg/L as CaCO₃)µg Cd/L. Values are means ± 1 SEM, n = 8 amphipods. All measurements from day 1 forward are significantly different from controls (ANOVA; p < 0.05).

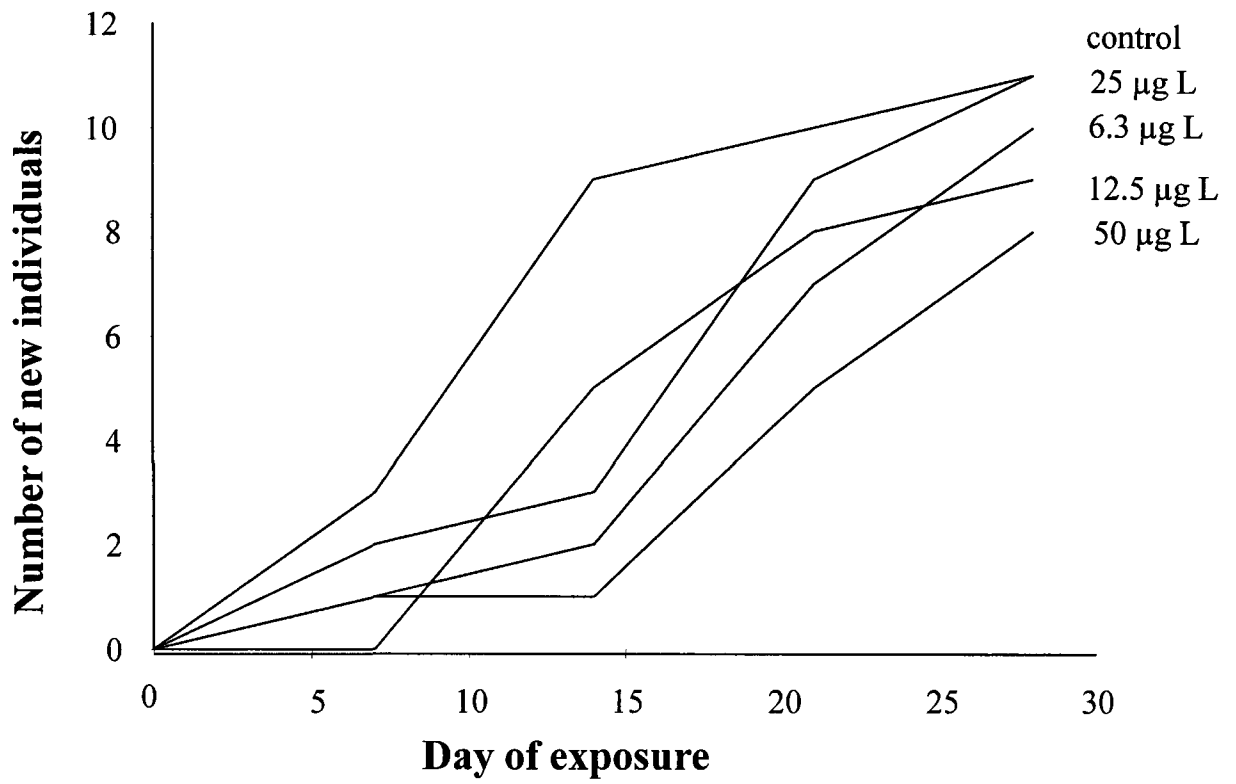


Figure 2.4 Cumulative effect of exposure to waterborne Cd on reproduction in *L. variegatus*. Results are from preliminary range finding experiments n=1.

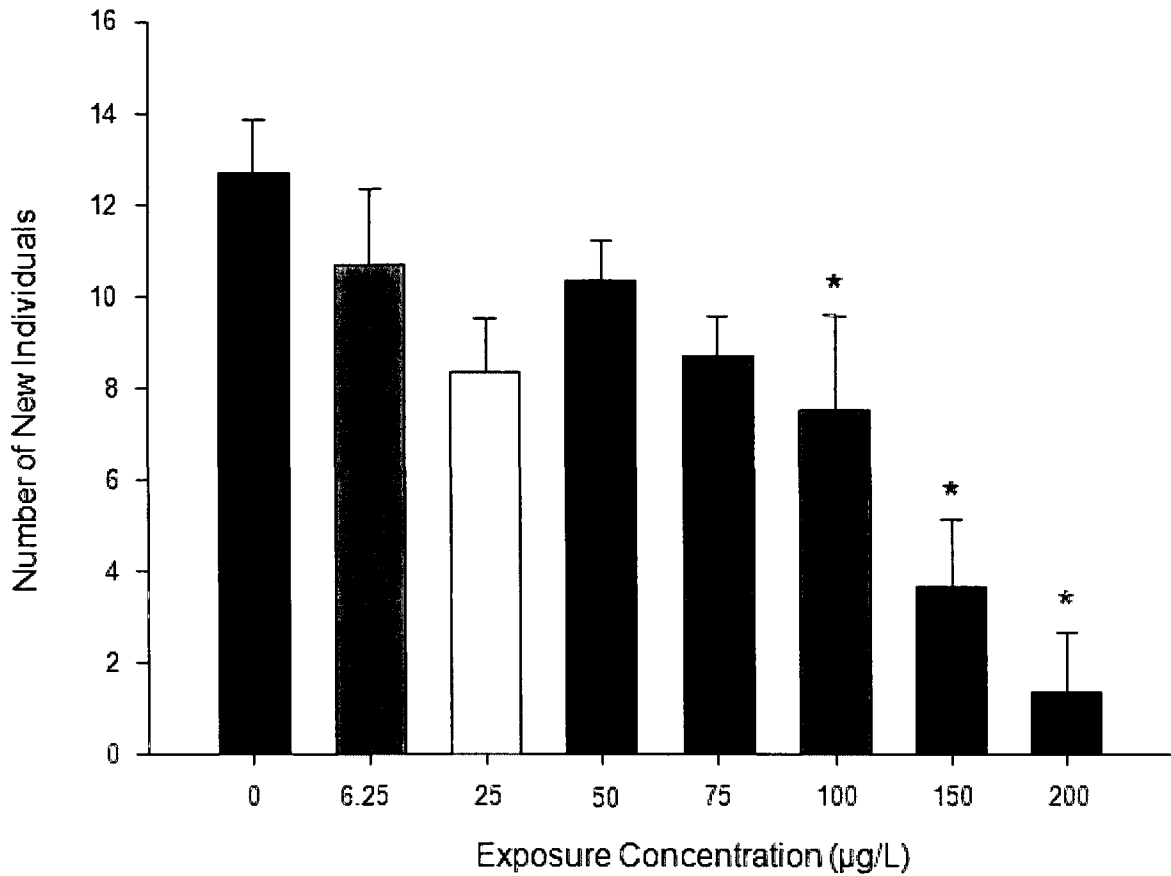


Figure 2.5 Cumulative effect of exposure to waterborne Cd on reproduction in *L. variegatus* over a chronic 28 day exposure. Nominal exposure concentrations of 0, 6.25, 25, 50, 75, 100, 150, and 200 $\mu\text{g Cd/L}$ corresponded to 4.6, 32.4, 57.4, 86.9, 107.6, 153.0, 205.3 $\mu\text{g Cd/L}$ respectively. Values are means \pm 1 SEM, n = 3 replicates. * indicates significance compared to control organisms at day 28 (ANOVA; $p < 0.05$).

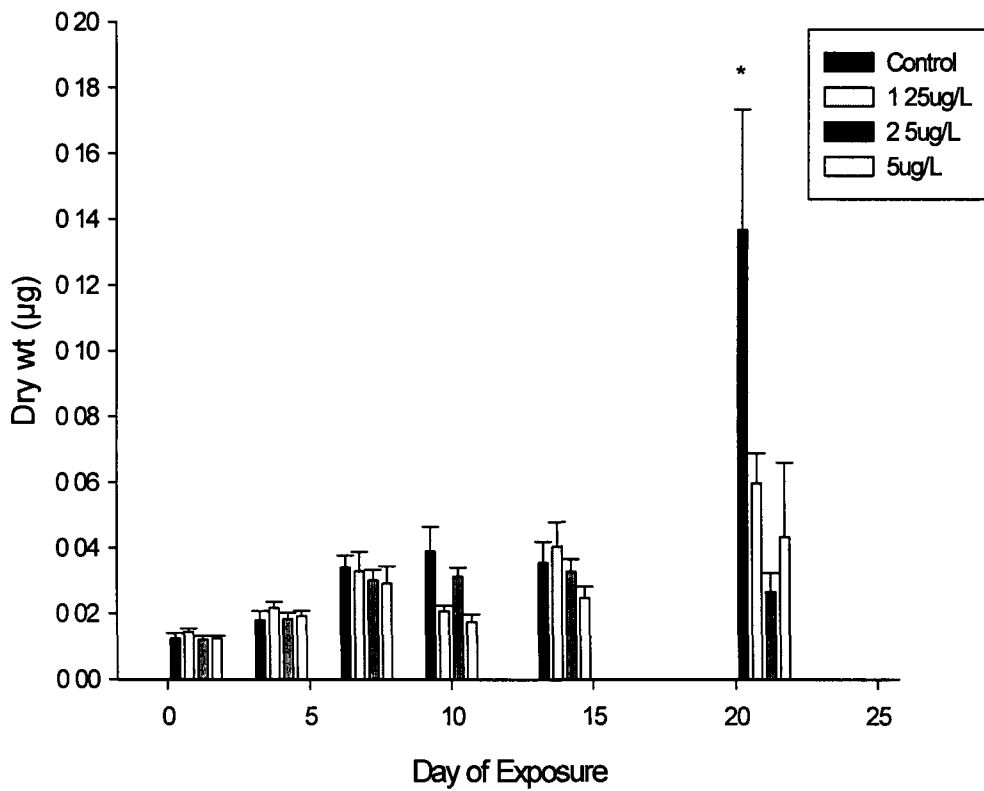


Figure 2.6. The effect of Cd exposure to *H. azteca* (n = 8 per exposure) growth (average dry weight) throughout the 28 day exposure to 1.25, 2.5, and 5 µg Cd/L in moderately hard water (140 mg/L as CaCO₃). * represents time point where control organisms were significantly heavier than exposed individuals (ANOVA; p < 0.05).

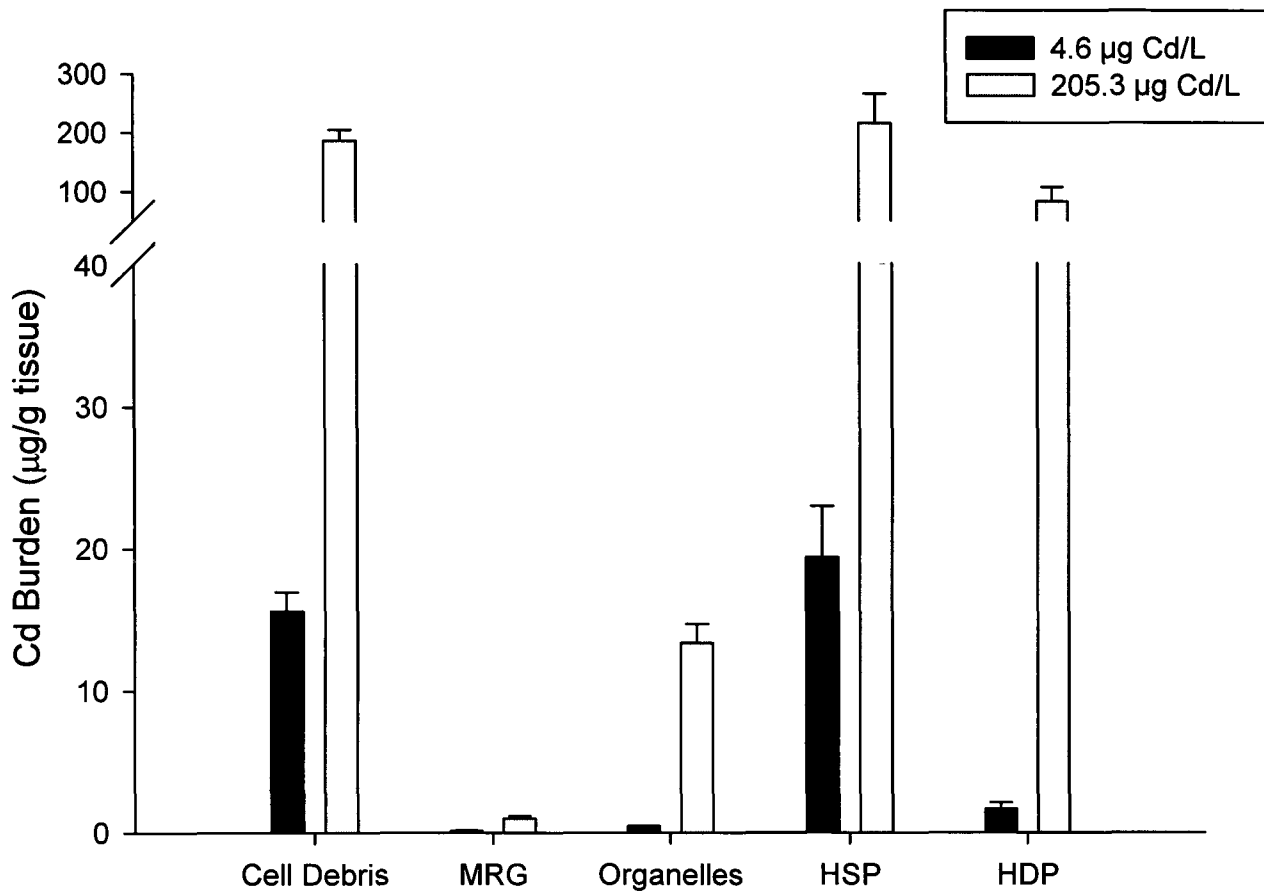


Figure 2.7. Comparison of subcellular Cd distribution in *L. variegatus* exposed to 4.6 & 205.3 µg Cd/L (corresponding to nominal concentrations of 6.5 and 200 µg Cd/L) for 7 d. Mean (\pm SEM) are shown with n=3.

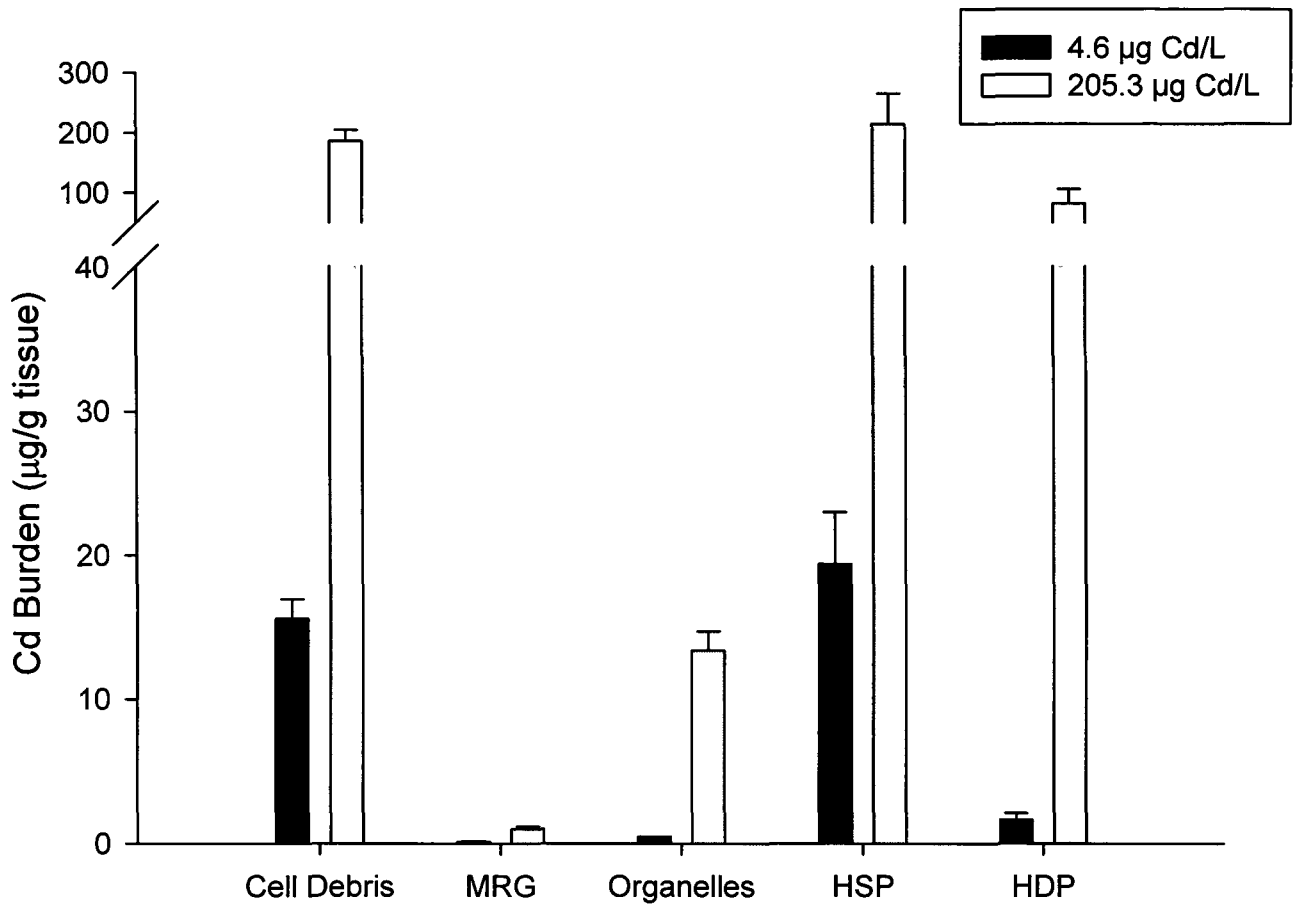


Figure 2.8. Comparison of subcellular Cd distribution in *L. variegatus* exposed to 4.6 & 205.3 µg Cd/L (corresponding to nominal concentrations of 6.5 and 200 µg Cd/L) for 45 d. Mean (\pm SEM) are shown with n=3.

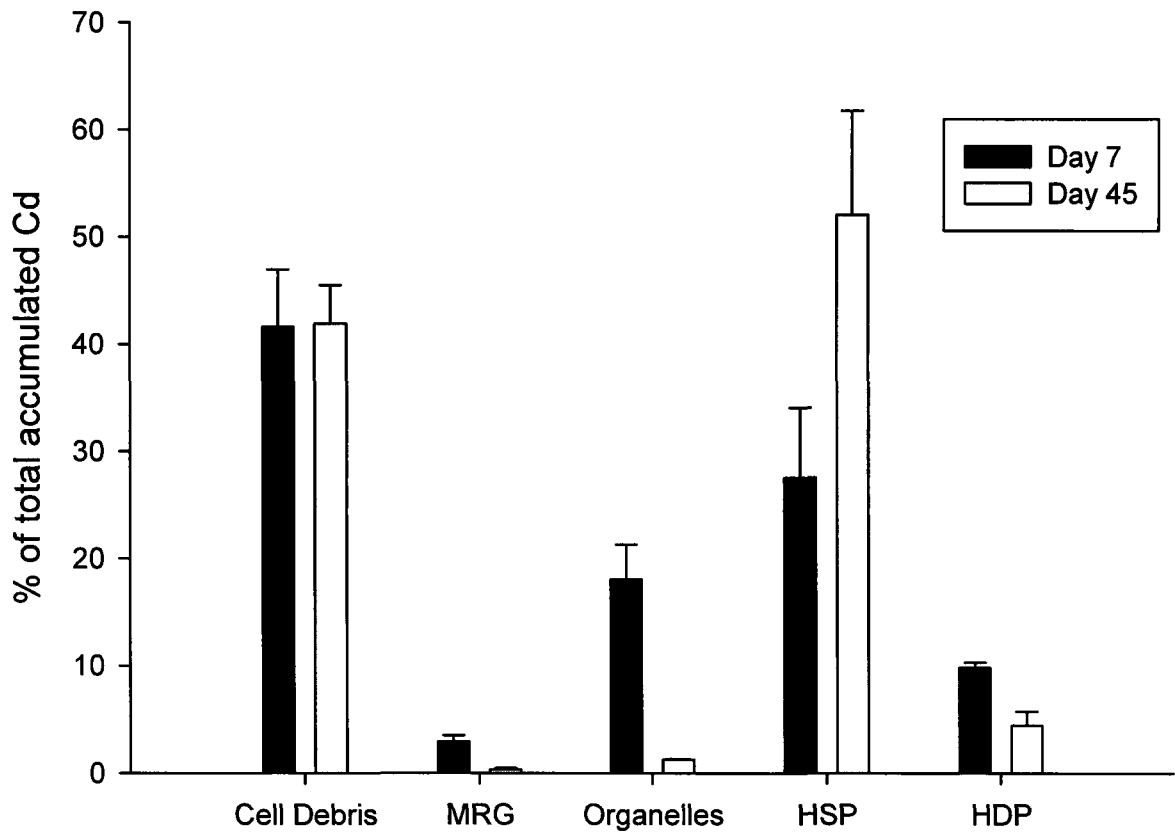


Figure 2.9. Comparison of subcellular distribution of Cd in *L. variegatus* at days 7 and 45 of exposure to 4.6 µg Cd/L (corresponding to nominal concentration of 6.5 µg Cd/L). Mean (\pm SEM) are shown with n=3.

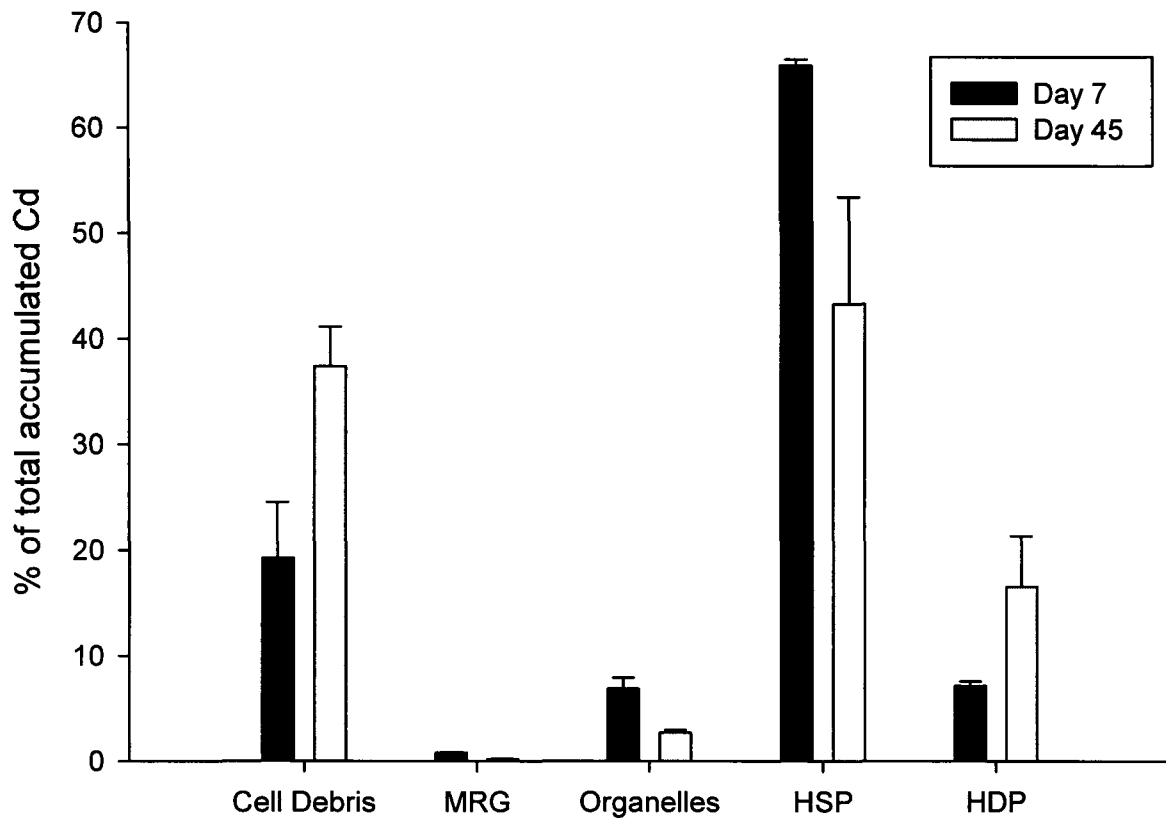


Figure 2.10 Comparison of subcellular distribution of Cd in *L. variegatus* at days 7 and 45 of exposure to 205.3 µg Cd/L (corresponding to nominal concentration of 200 µg Cd/L). Mean (\pm SEM) are shown with n=3.

Table 2.1. Measured whole-body burdens of Cd ($\mu\text{g Cd/g dry wt}$; mean \pm SEM) for *L. variegatus* over 28 days of exposure at five exposure concentrations. Mean measured Cd exposure concentrations are given, n=8. A * indicates a significant difference from controls at that time ($P<0.05$).

Day	Control	4.6 $\mu\text{g Cd/L}$	32.4 $\mu\text{g Cd/L}$	57.4 $\mu\text{g Cd/L}$	86.9 $\mu\text{g Cd/L}$	107.6 $\mu\text{g Cd/L}$	153 $\mu\text{g Cd/L}$	205.3 $\mu\text{g Cd/L}$
1	0.6 \pm 0.1	0.6 \pm 0.1	2.0 \pm 0.2	3.4 \pm 0.4	3.6 \pm 0.4	3.3 \pm 0.4	4.6 \pm 0.5	5.2 \pm 0.5
5	0.4 \pm 0.1	1.8 \pm 0.4	5.3 \pm 0.4	8.2 \pm 0.6	10.6 \pm 1.3	12.8 \pm 1.0	24.9 \pm 1.5	44.1 \pm 6.4
10	1.0 \pm 0.4	6.0 \pm 3.2	5.3 \pm 0.4	19.2 \pm 1.0	20.8 \pm 2.1	35.1 \pm 7.4	47.5 \pm 8.2	66.5 \pm 2.7
15	0.9 \pm 0.1	3.2 \pm 0.5	14.6 \pm 1.1	24.9 \pm 1.8	39.1 \pm 6.3	72.7 \pm 15.5	78.8 \pm 15.0	78.4 \pm 17.0
20	0.4 \pm 0.1	6.3 \pm 1.6	42.8 \pm 3.2	70.5 \pm 8.4	107.9 \pm 19.7	105.2 \pm 10.7	139.9 \pm 25.1	165.9 \pm 18.8
30	1.3 \pm 0.2	8.3 \pm 1.1	13.5 \pm 4.8	25.7 \pm 2.5	71.1 \pm 25.0	150.9 \pm 47.0	169.0 \pm 44.7	267.4 \pm 25.6
40	0.9 \pm 0.1	11.2 \pm 0.8	37.6 \pm 2.7	69.9 \pm 5.5	108.2 \pm 6.5	185.1 \pm 12.2	322.0 \pm 14.8	394.7 \pm 31.9
60	2.1 \pm 0.1	27.2 \pm 2.5	119.4 \pm 6.6	319.6 \pm 44.5	359.2 \pm 28.1	411.2 \pm 28.4	776.6 \pm 45.0	801.1 \pm 35.0
87	2.0 \pm 0.2	51.3 \pm 6.6	156.4 \pm 14.5	533.1 \pm 35.1	649.9 \pm 79.8	739.2 \pm 41.9	989.3 \pm 79.7	1620.6 \pm 142.6

Table 2.2 Measured whole-body burdens of Cd ($\mu\text{g Cd/g dry wt}$; mean \pm SEM) for *H. azteca* over 21 days of exposure at four exposure concentrations. Mean measured Cd exposure concentrations are given n=8. A * indicates a significant difference from controls at that time ($P<0.05$).

Day	Control	1.25 $\mu\text{g Cd/L}$	2.5 $\mu\text{g Cd/L}$	5 $\mu\text{g Cd/L}$
1	0.9 \pm 0.2	7.4 \pm 1.2*	58.1 \pm 6.9*	58.4 \pm 9.1*
4	0.7 \pm 0.1	57.0 \pm 4.4*	78.5 \pm 8.2*	105.5 \pm 10.3*
7	0.4 \pm 0.2	69.7 \pm 10.9*	83.2 \pm 12.2*	124.3 \pm 21.0*
10	0.5 \pm 0.1	99.7 \pm 3.0*	113.6 \pm 23.3*	146.8 \pm 9.9*
14	0.3 \pm 0.1	97.6 \pm 38.8*	114.7 \pm 22.2*	142.4 \pm 10.9*
21	0.1 \pm 0.0	82.4 \pm 12.9*	128.3 \pm 16.4*	106.7 \pm 21.4*

Table 2.3 Measured Cd subcellular fractions ($\mu\text{g Cd/g tissue}$; mean \pm SEM) for *L. variegatus* at days 7 and 45, at two different exposure concentrations. Nominal Cd exposure concentrations are given n=3.

Fraction	Day 7		Day 45	
	6.25 $\mu\text{g Cd/L}$	200 $\mu\text{g Cd/L}$	6.25 $\mu\text{g Cd/L}$	200 $\mu\text{g Cd/L}$
Cell Debris	1.46 \pm 0.19	10.45 \pm 2.87	7.37 \pm 8.28	185.70 \pm 18.70
MRG	0.10 \pm 0.02	0.43 \pm 0.04	0.12 \pm 0.05	1.01 \pm 0.18
Organelles	0.63 \pm 0.11	3.74 \pm 0.55	0.47 \pm 0.02	13.38 \pm 1.32
HSP	0.97 \pm 0.23	35.78 \pm 0.31	19.38 \pm 3.61	214.80 \pm 50.34
HDP	0.35 \pm 0.02	3.89 \pm 0.22	1.65 \pm 0.48	82.12 \pm 23.73

Chapter 3: A multiple invertebrate species exposure to determine if Cd accumulation in a resistant species can be used to reliably predict effects in a sensitive species in soft water

***Note: The experiments reported in this chapter are part of a larger exposure and testing experiment that was undertaken in the summer of 2010. This chapter will report on work done with *Hyaella* and *Lumbriculus* as part of that larger experiment and this represents the author's independent work. Some of the water characterization overlaps with the work of other students as we had common exposure water and shared analytical responsibilities.

3.0 Abstract

This research was conducted to assess the viability of using bioaccumulation of Cd in a resistant species to predict effects in a sensitive species. Using chronic exposures of 28 days, *Lumbriculus variegatus* and *Hyalella azteca* were characterized for Cd bioaccumulation and effects (survival and growth) in artificial soft water (40 mg/L CaCO₃). At the highest waterborne Cd concentration (2.3 µg Cd/L) *L. variegatus* showed no deleterious effects. Whole body accumulation was linear with time and 28 days of accumulation was insufficient for steady state to be reached. *H. azteca* exposed to concentrations of 1.3 and 2.3 µg Cd/L had 100% mortality after day 21 while at 28 days there was no significant reduction in survival over controls at 0.3 and 0.5 µg Cd/L, yielding a LC20 of 0.7062 µg Cd/L and LC50 of 0.9084 µg Cd/L. These findings suggest water at 140 mg/L (as CaCO₃; see Chapter 2) is 7 times more protective than those in soft water (22 mg/L as CaCO₃). Exposed *H. azteca* showed no significant reduction in size over controls, indicating in this case survival may be a more ideal endpoint. Accumulation of Cd in surviving *H. azteca* was variable and did not show any clear relationship to exposure. *L. variegatus* steady state concentrations were extrapolated and through modelling accumulation-effect relationships relating body burden in *L. variegatus* to survival effect concentrations in *H. azteca* were made. LC20 and LC50 concentrations of 0.71 and 0.91 µg Cd/L respectively corresponded to estimated body burdens of 42.6 and 50.6 µg Cd/g dry wt. *H. azteca* was found to be a less than ideal bioindicator species, as Cd effects are observed in *H. azteca* at environmentally relevant concentrations. Fresh water concentrations range from <0.1 to 122 µg/L in Canada (CCME 1999). The Cd resistance of *L. variegatus* beyond relevant concentrations and

ability of readily accumulate Cd make this species an excellent candidate species for use as a bioindicator.

3.1 Introduction:

Cadmium is a naturally occurring ubiquitous element that occurs at trace levels in the environment. Cd can result in impacts when concentrations become elevated due to anthropogenic emissions (McGeer *et al* 2011; Pan *et al* 2010). Loadings to the aquatic environment are often associated with mining and smelting of Zn, Pb and Cu ores (Pan *et al.*, 2010). Water quality criteria and guidelines for Cd are generally low and this reflects its non-essential and toxic nature. Cadmium readily bioaccumulates in aquatic organisms. Uptake across respiratory surfaces of aquatic biota (e.g. gills) occurs via Ca related processes including apical channels and basolateral Ca ATPase (Verboost *et al.*, 1988). The competitive interaction of Cd²⁺ and Ca²⁺ for uptake explains the observed protective effect that water hardness has on acute toxicity (CCME 1996) and the internal hypocalcemia that is induced during exposure (e.g. Hollis *et al.*, 1999; McGeer *et al.*, 2000a).

While the mechanisms of acute Cd toxicity (hypocalcemia) are understood and have been incorporated into prediction models such as the biotic ligand model (Niyogi and Wood 2004; Niyogi *et al.*, 2008), much less is known about chronic toxicity. Understanding the accumulation of Cd is a key aspect of understanding its chronic toxicity. During the initial phases of a chronic exposure tissue concentrations increase as the rate of uptake into the organism exceeds the rate of elimination but subsequently these rates equalize and a steady state is reached (McGeer *et al.*, 2000b). It is assumed that toxic effects occur when internal Cd concentrations reach and exceed a threshold at a specific site of toxic action (Campbell *et al.*, 2008). This experiment was designed to build on this concept and use an accumulation-effect relationship using tissue

concentrations as an alternative to a traditional exposure-response methodology using exposure concentrations. This is a tissue residue approach (TRA) and using bioaccumulation to evaluate the tissue concentrations as indicators of adverse effects (McCarty *et al.*, 2011).

The TRA relies on a clear and unambiguous burden-effect relationship. For metals (including Cd) the TRA generally cannot be applied for understanding chronic effects because of the complexities of bioaccumulation. One such complexity stems from the fact that metal, which has been taken up by an organism, may not cause a negative effect. When a metal, such as Cd, is taken up into an organism it could potentially be distributed in one of many forms, into specific organs, or into specific tissues. The metal can also be subject to metabolic conversion and elimination. Subcellular fractionation (See section 1.5) can be used to separate detoxified metals from biologically active metals. The experiments in this chapter utilized a different TRA to the subcellular fractionation used in chapter 2. Subcellular fractionation requires the pooling of thousands of organisms in order to get an acceptable n-value, and may not be amenable to field work for this reason.

Another issue with the TRA involves the difficulty associated with relating a specific body burden to a particular effect. In general metal will accumulate within an organism until an effect concentration is reached. With survival as an endpoint, as soon as that threshold is reached the organism dies, and when an organism dies it typically decays quickly (McCarty *et al.*, 2011). Due to this decay an accurate measure of the tissue residue prior to the effect would be difficult or impractical. A final complication arises from differences in uptake rates and detoxification rates. In this case differences in

the uptake rate of metals can lead to identical tissue residues (McCarty *et al.*, 2011). A slower uptake rate can allow for a portion of the accumulated metal to be detoxified, while a faster uptake may quickly overcome the organism's detoxification strategies. Due to these complications whole-body tissue burdens generally do not reflect accumulation of metals at the site of toxic action.

The recent work of Adams *et al.* (2010) proposed a modified TRA that could be applied to metals. This new conceptual model is based on the same approach inherent to the TRA (i.e., accumulation-effect relationships), but accumulation and effect are not measured in the same organism (Adams *et al.*, 2010). Accumulation is assessed in an organism that is relatively insensitive to Cd, where elevated exposure concentrations are required to produce toxic effects. Effects are measured in an organism that is sensitive to Cd and may not bioaccumulate in a consistent and predictable manner (particularly near effect thresholds). As such, the resistant accumulator organism serves as a biomonitor of metal exposure and effect in a freshwater environment.

This study was designed as a proof of principle of this modified TRA approach. The oligochaete worm *Lumbriculus variegatus* (California blackworm) was used as the resistant, accumulator organism while the amphipod *Hyaella azteca* as the chosen sensitive, effect organism. *L. variegatus* is common to fresh waters throughout North American and Europe, is easily cultured and reproduces asexually through fragmentation (Brinkhurst and Jamieson, 1971). The blackworm has been shown to be resistant to pollution. In studies in moderately soft water (44-47mg/L CaCO₃) *L. variegatus* had a 10 day LC₅₀ of 158 µg Cd/L (Phipps *et al.*, 1995). Along with its tolerance to Cd, *L. variegatus* has been shown to readily bioaccumulate Cd. In a study by Ng and Wood

(2008), *L. variegatus* accumulated 30.3 µg Cd/g tissue over 10 days when exposed to waterborne concentrations of 200 µg Cd/L. In work reported in chapter 2 of this thesis *Lumbriculus* has demonstrated its ability to accumulate Cd in a predictable dose dependent manner.

H. azteca is a freshwater amphipod that is distributed throughout North American fresh waters (EC 1997). They are amenable to lab culture and have been shown to be sensitive to metal exposure, with Cd proving to be one of the most toxic. In a study conducted by Borgmann *et al.* (2005) the 7 day LC50s were 0.57 and 4.41 µg Cd/L for soft (18 mg/L as CaCO₃) and hard (124 mg/L as CaCO₃) water respectively..

The goal of this study was to determine whether accumulation in a resistant species could be directly linked to effects in a sensitive species. In this experiment, parallel trials with different organisms (*L. variegatus* and *H. azteca*) were linked through the use of common exposure water. This served to minimize any differences between the exposure systems and to ensure that results could be compared as water chemistry can play a major role in the availability of Cd to aquatic organisms.

This experiment was conducted as part of a larger study that involved colleagues testing additional resistant (*Lymnaea stagnalis*) and sensitive (*Daphnia pulex*) organisms. *L. stagnalis*, the great pond snail, was included as a species that was more sensitive than *L. variegatus* but significantly more resistant than *H. azteca*. *D. pulex* was included as an additional sensitive species with a short lifecycle allowing for the inclusion of an additional sub-lethal endpoint, reproduction. The results associated with these additional species will be discussed in Chapter 4.

3.2 Materials and Methods

3.2.1 Culturing *L. variegatus* and *H. azteca*

Lumbriculus variegatus were purchased from Aquatic Research Organisms (ARO; Hampton, NH.), and cultured following with the standard methods outlined by the US EPA (2000, EPA 600/R-99/064). Aeration was provided and cultures were maintained in artificial Lake Ontario water containing 1.0 mM Ca²⁺, 0.6 mM Na⁺, 0.8 mM Cl⁻, 0.15 mM Mg²⁺, at a pH of 7.2 ±0.1 and temperature of 20° C. Shredded unbleached paper towels were utilized as a substrate. A portion of the water was replaced every week, and complete cleanings were done monthly. Worms were fed ground Tetramin® flakes (Tetra Werke, Blacksburg, VA, U.S.A.) administered in slurry, and Nutrafin® sinking complete food tablets fed three times a week.

Hyalella azteca were purchased from ARO, and cultured in 1L HDPE beakers each containing 30 adults. Cultures were maintained in artificial media containing 1.0 mM Ca²⁺, 1.0 mM Na⁺, 0.05 mM K⁺, 0.25 mM Mg²⁺ and 0.01mM Br⁻(purchased as salts from Sigma-Aldrich, Sigma Aldrich, Mississauga, Ontario). A 5 cm² piece of cotton gauze was utilized as a substrate (Environment Canada, 1997). Temperature was 20°C±2 and cultures were fed 5mg of dry, finely-ground flakes (Tetramin®, Tetra Werke, Blacksburg, VA, U.S.A.) 3 times a week. Culture water was replaced and neonates were separated from the adults weekly.

3.2.2 Cd Exposures

The tests utilized nominal concentrations of 0, 0.4, 0.8, 1.6, and 3.2 µg Cd/L. Dechlorinated city of Waterloo water was diluted with reverse osmosis deionized water to produce exposure water with a hardness of 22 mg/L as CaCO₃. Aliquots of this

exposure water were spiked with Cd at least 24h prior to use, with sufficient volumes made to ensure a common exposure source for all test organisms. Two L HDPE beakers were utilized for both exposures. Beakers with *L. variegatus* utilized inert silica sand (Bell & MacKenzie Co. Ltd., Hamilton, ON, Canada) as a substrate in a 4:1 water to sediment ratio, while *H. azteca* used a 5 cm x 5cm piece of sterile cotton gauze that was pre-soaked in exposure media prior to test start. Each *L. variegatus* exposure began with 30 adults, while each *H. azteca* exposure began with 35, 2-9 day old neonates. Feedings occurred every 3 days with 5mg of finely ground Tetramin® flakes (Tetra Werke, Blacksburg, VA, U.S.A.) being administered to each exposure beaker. Water renewals occurred every 2 days, with 50% of the water volume being replaced. Throughout the exposure pH was 7.30 ± 0.01 (n=9; measured by Radiometer PHM240 meter with pHC2701-8 electrode) and water temperature was $20^{\circ}\text{C} \pm 2$. Dissolved Cd was analyzed using graphite furnace atomic absorption spectroscopy (GFAAS, see section 3.1.4) and nominal concentrations of 0, 0.4, 0.8, 1.6, and 3.2 $\mu\text{g Cd/L}$ corresponded to 0.00 ± 0.00 , 0.29 ± 0.00 , 0.47 ± 0.01 , 1.33 ± 0.03 , and 2.3 ± 0.02 $\mu\text{g Cd/L}$ (n=8) respectively.

3.2.3 Sampling and sample processing

Water samples were taken prior to the addition of, as well as prior to and immediately following each water renewal. With each round of sampling 10 mL of filtered (0.45 μm syringe filter; Acrodisc HT tuffryn membranes, Pall Corporation, Ann Arbor, MI, USA) and un-filtered water were taken from at least one of the replicate exposure chambers. Water samples were then acidified to 1% by adding 100 μL of concentrated HNO_3 (Trace Metal Grade, Fisher Scientific, Mississauga ON).

Organisms were collected on days 1, 4, 7, 14, 21, and 28 and measured for dry weight and, following digestion, Cd whole-body burden. An additional sampling at day 10 was included for *H. azteca* because this time point in previous experiments coincided with the organisms reaching a steady state of accumulation. At each time point 4 individuals were sampled from each replicate exposure. Individual organisms were given 6 hours for gut clearance (Neumann *et al.*, 2009) in clean test water before being blotted dry and baked in open 0.6 mL ultracentrifuge tubes for 48h at 80°C. Following drying individual organisms were weighed using a Sartorius SE2 Ultra Micro Balance (Sartorius Mechatronics Corp., Bohemia, NY, U.S.A), and their average dry weight was calculated at each exposure and time point. After weighing individuals were digested according to the methods given by (Borgmann and Norwood, 1997) where 25 uL of 16N trace-metal grade HNO₃ was added for 6 days at room temperature, after that 20 uL of 30% H₂O₂ was added for an additional 24 hours prior to the addition of MilliQ ultrapure water bringing the sample volume up to 250 µL. Following digestion samples were mixed using a vortex and then centrifuged (Spectrafuge 16M; Labnet International, Edison, NJ, USA) at that point the supernatant was measured for Cd content.

3.2.4 Analysis

Both water and tissue were analyzed using GFAAS (Varian 1988) or flame AAS (Varian 1989) FAAS; SpectraAA 880 GTA 100 atomizer, Varian, Mississauga, Ontario). Water Ca, Mg and Na were measured using FAAS. Pre-prepared certified reference material (trace metal fortified Lake Ontario water [TMDA-28.3 and TM 26.3]; National Water Research Institute, Burlington, ON) and blanks (MilliQ water and solutions used for digestion) were used as a quality control to assess accuracy, and provide reference.

Tissue samples were analyzed for Cd using GFAAS after the digested supernatant had been appropriately diluted with 1% acidified (16N HNO₃, TraceMetal grade; Sigma-Aldrich, Nepean ON, Canada) ultra-pure water.

3.2.5 Calculations

GFAAS absorbencies were used to calculate Cd concentrations in samples. Following analysis these concentrations were utilized along with dry weights to calculate the total body burden that was expressed as µg Cd/g tissue dry weight.

H. azteca survival data and dissolved Cd concentration were used to calculate lethality endpoints (LC_x) using the software program CETIS™ (Ver. 1.6.1 revC). Utilizing survival data and running a linear interpolation (ICPIN) analysis, LC₂₀ and LC₅₀ values were calculated.

The relationship of Cd body burden and time of exposure was modelled for each exposure concentration. Steady state tissue burden and half time to saturation were derived using the following equation:

$$f(x) = C_0 + C_s * (1 - e^{(-\ln 2 / t_{1/2} * x)}) \quad \text{Equation 1}$$

Where C₀ represents background Cd concentration, C_s represents total body burden at steady state, and t_{1/2} represents time required to half reach steady state body burden (McGeer *et al* 2000b).

3.2.6 Statistics

Data is presented as mean ± SEM unless otherwise noted. Graphs and statistical analyses were performed utilizing SigmaPlot 11.0 (Systat Software Inc.; San Jose, CA, USA). Growth data was subjected to a one-way analysis of variance (ANOVA). A Fisher LSD post hoc test was utilized to detect significance using P<0.05.

Accumulation data was subjected to a series of one way ANOVAs to test significant differences from controls at each day of exposure. For bioaccumulation analysis n=8 unless otherwise noted. All accumulation data was subjected to Fisher LSD post hoc test, to detect significance using $P < 0.05$.

3.3 Results

3.3.1 Survival

L. variegatus displayed no deleterious effects from exposures as high as 2.3 µg Cd/L over 28 days. There was no significant difference in size, or reproduction from controls to exposed individuals. *H. azteca* survival was assessed by calculating the number of individuals surviving at day 28, less those sampled for total body burden during the first 21 days of exposure. Since exposures began with 35 organisms, by day 28 there were expected to be 11 surviving individuals, % survival at day 28 was then calculated by averaging the % survival across the replicate beakers. Those individuals exposed to concentrations of 1.3 and 2.3 µg Cd/L had 100% mortality after day 21 of the exposure. In the two lower exposures 0.3 and 0.5 µg Cd/L, there was no significant effect on survival when compared to the $81.8 \pm 18.2\%$ survival in controls (Figure 3.1). Those individuals exposed to 0.3 µg Cd/L had a $77.27 \pm 13.7\%$ survival while those exposed to 0.5 µg Cd/L had $81.8 \pm 9.1\%$ survival. Utilizing CETIS™ lethal effect concentrations were calculated including a LC20 of 0.71 µg Cd/L and LC50 of 0.91 µg Cd/L. Estimated EC concentrations as well as upper and lower confidence intervals are presented in Table 3.1.

3.3.2 Dry Weight

There was no significant difference in overall dry weight across Cd exposure for surviving *H. azteca* (Figure 3.2). Control organisms after 28d of exposure averaged $0.0638 \pm .008$ mg (n=18), while individuals exposed to $0.3 \mu\text{g Cd/L}$ averaged 0.0584 ± 0.0059 mg (n=17) and those exposed to $0.5\mu\text{g Cd/L}$ averaged 0.0626 ± 0.0058 mg (n=18; Figure 3.2).

3.3.3 Cd Body Burden

Accumulation in *L. variegatus* increased both with time and exposure concentration (Figure 3.3, Table 3.2). At day 28 there was a clear and linear relationship between Cd body burden and Cd exposure concentration (Figure 3.4). Accumulation of Cd in surviving *H. azteca* was variable and did not show any clear relationship to exposure, particularly over the first 2 weeks (Figure 3.5, Table 3.3). Paradoxically, on day 14 the amphipods exposed to $0.3 \mu\text{g Cd/L}$ had higher Cd body burden than those exposed to $0.5 \mu\text{g Cd/L}$ but at day 21 these burdens were virtually the same (Figure 3.5, Table 3.3). Organisms exposed to 0.3 and $0.5 \mu\text{g Cd/L}$ had an average body burden of $15.9 \pm 1.1 \mu\text{g Cd/g dry weight}$, and $21.6 \pm 3.1 \mu\text{g Cd/g dry weight}$ respectively (Table 3.3, Figure 3.5). These values were significantly different $P < 0.05$.

3.3.4 Modelling steady-state and accumulation-effect relationships

Steady state concentrations and half-times were calculated using equation 1 and the accumulation data over 28 days from Table 3.2. From these calculations *L. variegatus* body burdens at steady state for 0.5 , 1.3 and $2.3 \mu\text{g Cd/L}$ were estimated to be

22.5 ± 8.7, 63.2 ± 22.1, and 324 ± 244 µg Cd/g dry weight (Table 3.4). Estimated $t_{1/2}$ was determined to be 23, 37, and 56 days, for 0.5, 1.3 and 2.3 µg Cd/L respectively. Due to the drastic increase in body burden between day 21 and day 28 of the 0.3 µg Cd/L (Table 3.2) it was not possible to derive reasonable estimates of steady state body burdens for this concentration. With day 28 removed, a revised estimate of 6.5 ± 0.7 µg Cd/g dry weight was made and this data is presented in Figure 3.8 represented by the equation: $y = -3.5295 + 50.6x$. Modelled Cd body burdens at steady-state showed a clear relationship to exposure concentration (Figure 3.7) and over the range of exposures where toxicity to *H. azteca* was relevant, there was good correlation (Figure 3.8).

Modelled accumulation-effect relationships are presented in Figure 3.9 through plotting survival in *H. azteca*, and body burden at steady state in *L. variegatus* along the same x-axis (exposure concentration). Lines were added corresponding to LC20 and LC50 values, and their corresponding linkages to body burden in *L. variegatus* were calculated by substituting the LC concentrations into the equation of the body-burden plot. LC20 and LC50 concentrations of 0.71 and 0.91 µg Cd/L respectively, corresponded to body burdens of 42.6 and 50.6 µg Cd/g dry wt respectively.

3.4 Discussion

Throughout the exposure there were no mortalities in *L. variegatus*. This result was expected, as no survival effects were observed in Chapter 2 during 87 days of exposure at concentrations as high as 200 µg Cd/L. Cd in soft water would be expected to be more toxic than in hard water but the differences in water hardness between the two experiments were not large enough for a negative effect to be seen when the highest

exposure concentration was only 2.3 µg/L. Soft water experiments conducted by Phipps *et al.* (1995) determined a 10 day LC50 of 158 µg Cd/L and nothing from their experiment would suggest an inability to survive at the tested concentrations.

The concentration range chosen for this exposure produced variable survival in *H. azteca*. Calculated 28-day LC20 of 0.7062 µg Cd/L, and LC50 of 0.9084 µg Cd/L, are both at environmentally relevant concentrations (McGeer *et al.*, 2011) and confirm *H. azteca* as a sensitive species. Borgmann *et al.* (1991) found similar results with an LC50 of 0.72µg Cd/L in a 6-week exposure in water of a similar hardness (13 mg/L). There were no significant differences in growth or survival among unexposed amphipods (controls) and those exposed to either 0.3 or 0.5 µg Cd/L over the course of the 28 days of exposure (Figures 3.1 and 3.2). However, average weight of surviving individuals at day 28 was less than was expected based on previous experiments where at 21 days of exposure controls weighed $0.136 \pm .037$ mg (see Chapter 2, section 2.3.3). This reduced average weight may be related to the added stress of a soft water exposure.

Accumulation in *Lumbriculus* followed a similar pattern to those in previous experiments (see Chapter 2), continuously accumulating Cd in a dose dependent manner. Body burden increased with both duration of exposure and exposure concentration (Figure 3.3). Similar to experiments conducted in chapter 2, test duration was not long enough for a steady state of accumulation to be reached.

H. azteca exposed to sub-lethal Cd concentrations quickly accumulated Cd to steady state (Figure 3.5). There was a significantly higher body burden in those organisms exposed to 0.5µg Cd/L, than those exposed to 0.3 ug Cd/L at test end. A steady state of accumulation was reached between days 14 and 21. In chapter 2 a steady-

state of bioaccumulation was reached after approximately 10-14 days of exposure (see Chapter 2, Figure 2.3), the increase in duration may be caused by the difference in water chemistry, although a similar trend is clearly observed. Previous research with aquatic invertebrates have shown metal exposure in hard water has protective benefits over similar studies in softer water (Chapman *et al.*, 1982; Williams *et al.*, 1986; Reynoldson *et al.*, 1996). This research has provided similar findings in its *H. azteca* exposures in this and the second chapters of this thesis. During *H. azteca* exposures in hard water (see chapter 2) organisms were unable to survive at concentrations above 10 µg Cd/L. Soft water exposures in Chapter 3 resulted in 100% mortality at concentrations above 1.4 µg Cd/L. These findings suggest water at 140 mg/L (as CaCO₃) is 7 times more protective than those in soft water (22 mg/L as CaCO₃) SCOTT SAYS IN ABSTRACT. The observed protective effect is explained at least initially through the competitive interaction of Cd²⁺ and Ca²⁺ for uptake at the site of respiration (CCME 1996). As exposure duration increases further effects can be explained by the internal hypocalcemia that is induced during Cd exposure (Hollis *et al.*, 1999; McGeer *et al.*, 2000a). Although the acute effects of Cd are pretty well known less is understood about the chronic toxicity of Cd, and what exactly is causing the amphipod's death.

Contrary to expectations the amphipods exposed to 0.5µg Cd/L accumulated a smaller average body burden than those at the lower exposure concentration throughout the first 14 days of testing. Alves *et al.*, (2008) showed in their uranium exposed *H. azteca* experiments that there was a significant negative correlation between metal body concentration and body mass. Despite identical exposure concentrations, smaller body burdens were associated with larger organisms. Body burden was plotted vs. dry weight

to determine whether or not a similar trend could be observed in Cd exposures (Figure 3.10); this plot resulted in an R^2 of 0.111 ($P=0.029$) indicating no clear relationship between the two variables despite the fact that those exposed to 0.5 $\mu\text{g Cd/L}$ were 2.25-fold larger at day 10 and 3.13-fold larger at day 14 than those exposed to 0.3 $\mu\text{g Cd/L}$. These findings indicate there is no obvious explanation as to why the dry weights of individuals exposed to 0.5 $\mu\text{g Cd/L}$ were so much larger than individuals exposed to 0.3 $\mu\text{g Cd/L}$.

During the last 2 weeks of exposure in the two elevated exposure concentrations accumulation in *H. azteca* proved to be highly variable. Organisms accumulated Cd up until around 210-230 $\mu\text{g Cd/g}$ dry weight. No organisms survived above these concentrations, indicating a possible threshold for survival. This observation may confirm the presence of a critical body concentration (CBC) corresponding to mortality, but also may simply reflect the amount of accumulation that had occurred prior to death. The maximum accumulation at steady state in this soft water exposure was $167.0 \pm 34 \mu\text{g Cd/g dw}$, a burden quite similar to that observed at the maximum $146.8 \pm 9.9 \mu\text{g Cd/g dw}$ in the hard water exposure discussed in chapter 2 (see Chapter 2; Figure 2.3, Table 2.2). This may suggest steady state concentration will balance out to a maximum regardless of exposure concentrations.

Due to the accumulation pattern seen in Figure 3.3 using *Lumbriculus* as a biomonitor to predict effect would be difficult. The pattern of accumulation even in low exposure concentrations was linear, an observation consistent with both other spiked-water exposures (Xie *et al.*, 2008) and sediment based exposures (Piol *et al.*, 2006). Similar accumulation patterns are also observed in the closely related *Tubifex tubifex*

(Redeker *et al.*, 2004; Gillis *et al.*, 2004). In experiments done by Gillis *et al.* (2004) 6 weeks of exposure were needed before *T. tubifex* accumulation reached steady state. Xie *et al.* (2008) showed a 10 day body burden of 0.480 µg Cd/g wet weight, in their *L. variegatus* exposure. Based on values obtained from Brooke *et al.* (1996), *L. variegatus* is composed of approximately 83% water. Accumulation in spiked water exposures such as Xie *et al.* (2008) showed linear accumulation when *L. variegatus* was exposed to .56 µg Cd/L in soft water. They found a body burden of 0.48 µg Cd/g wet weight at day 10, which corresponds to 2.8 µg Cd/g dry weight as an organism is 83% water (Brooke *et al.*, 1996). At day 7 in this exposure the worms had accumulated 2 fold those of Xie's study. This difference could be attributed to a number of differences including feedings (a portion of the Cd may bind food and be ingested) and differences in water chemistry (Cd may be more available).

By plotting body burden at day 28, vs. exposure concentrations a clear dose dependent trend can be seen (Figure 3.4). This data can be compared to *H. azteca* survival data, and be used to predict survival in the amphipods, based on the body burden found in *L. variegatus*. There is however one major issue with this concept; there is a time variable that needs to be taken into consideration. The information obtained from this comparison only corresponds to exposures of 28 days. Since the blackworms are not at steady state a shorter exposure would result in a graph with lower burdens while a longer exposure would result in higher body burdens. Ideally we would get an identical graph regardless of test duration and without a steady state being reached this information we have gained is very difficult to use. Exposures in Chapter 2 proved 87 days was an insufficient amount of time for steady state to be reached, but this does not mean

blackworms will not reach steady state. It simply means that 87 days is an insufficient amount of time for it to happen. It is assumed that steady state will be reached in the field, and for this reason steady state concentrations can be extrapolated for use.

The extrapolations to steady state are presented in Figure 3.6. By taking this data and plotting body burden at steady state against exposure concentration (Figure 3.7) it is apparent that the estimated body burden and error associated with the highest exposure concentration skew the regression line. By removing this point a more precise representation of the body burdens at steady state was obtained (Figure 3.8). Since the highest concentration corresponded to 100% mortality in *H. azteca*, there is little negative in dropping this data point when performing the following modelling. The lowest exposure concentration was also omitted from Figure 3.8 as through the first 21 days of exposure there was no significant difference in body burden from controls, and in order to calculate a steady state concentration the only day that differed significantly from controls was omitted.

For this study to act as a proof of principal for this TRA *L. variegatus* must be able to be used as an indicator of Cd effect in *H. azteca*. For *L. variegatus* to act as an indicator of effect total body burden must be linked to *H. azteca* effect. The use of identical water chemistries allows for the linking of the exposures. By plotting survival in *H. azteca*, and estimated body burden at steady state in *L. variegatus* along the same x-axis (exposure concentration) we can accurately predict specific *L. variegatus* body burdens which correspond to specific effect concentrations in *H. azteca*. In Figure 3.9, *H. azteca* LC20 and LC50 values are linked to their corresponding body burdens in *L. variegatus*. These body burdens were calculated by substituting the calculated LC20 and

LC50 concentrations, 0.71 and 0.91 $\mu\text{g Cd/L}$, into the equation of the line representing *L. variegatus* body burden at steady state, yielding body burdens of 42.6 and 50.6 $\mu\text{g Cd/g}$ respectively. This essentially proves it is possible to predict effect in a sensitive species through accumulation in a resistant species.

L. variegatus as a species possesses many attributes that make it an ideal choice as a biomonitor. It possesses a holarctic distribution, including North America, Europe and Asia. *Lumbriculus* is common across its range, is easily collected from lake sediment, and is also highly resistant to Cd and other contaminants. While this species may seem like a logical choice for the designated resistant species, there are also a few negative features which need to be considered as well, including the length of time needed to reach steady state. While this may present problems in the context of a lab-based study, in a field setting at a contaminated site, where organisms will be at steady state accumulations it may be less of an issue. An accurate measurement of steady state concentrations in *L. variegatus* would surely be required for this concept to be adapted for use.

Another potential issue stems from *L. variegatus* reproducing through asexual fragmentation since it is impossible to determine an organism's age. Due to this fact an understanding of how Cd is distributed within an individual would be required to better understand what happens during fragmentation. Ideally Cd would be distributed uniformly throughout the organism, and this may very well be the case as these oligochaetes have a very basic body plan; however, it would be important in knowing for sure how Cd is distributed within an organism during fragmentation and regeneration before further pursuing this idea. Another potential issue is the recent suggestion that the species known as *L. variegatus* may actually be a group of similar organisms comprising

three separate species (Gustafsson *et al.*, 2009). In his 2009 study Gustafsson assessed the genetic variation found within *L. variegatus* from Europe, North America and Japan and found the organism to consist of at least two distinct clades, both of which occur in Europe as well as North America and a third clade from a single location in Sierra Nevada, California. Any potential differences between these clades would surely need to be determined prior to *L. variegatus* use as a bioindicator.

This experiment proved that accumulation in a resistant species could be used to predict effects in a sensitive species in this water chemistry. A more ideal indicator species, capable of quickly and reliably accumulating Cd to steady state needs to be found. As far as the proof of principle design of this experiment, it was a success; however, there are still many steps that need to be taken before this concept will be accepted as a potential tool for use in the field. The results of this experiment must be confirmed with a similar body burden at steady state corresponding to the same effect concentration in the sensitive species in different water chemistries.

3.5 Figures and Tables

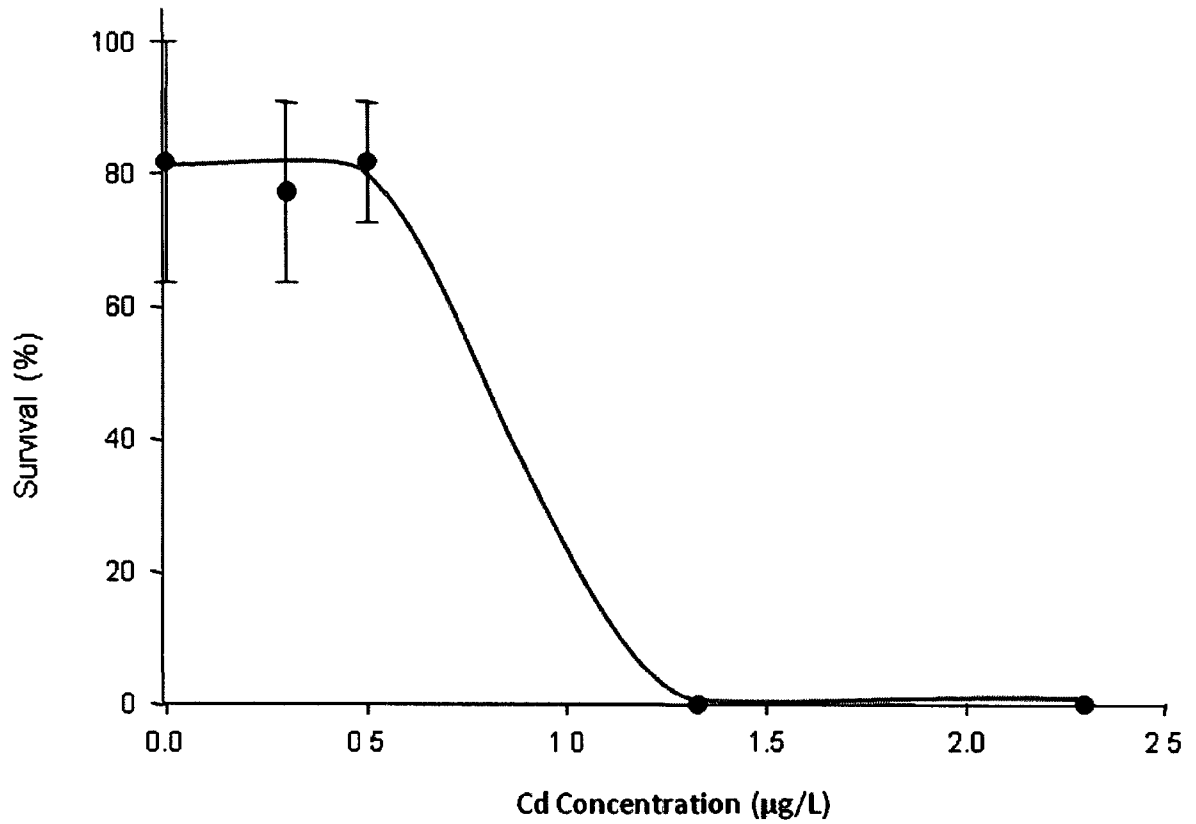


Figure 3.1. The effect of Cd exposure to *H. azteca* (n = 22 per exposure) survival (%) throughout the 28-day exposure to 0, 0.3, 0.5, 1.3, and 2.3 µg Cd/L in soft water (22 mg/L as CaCO₃).

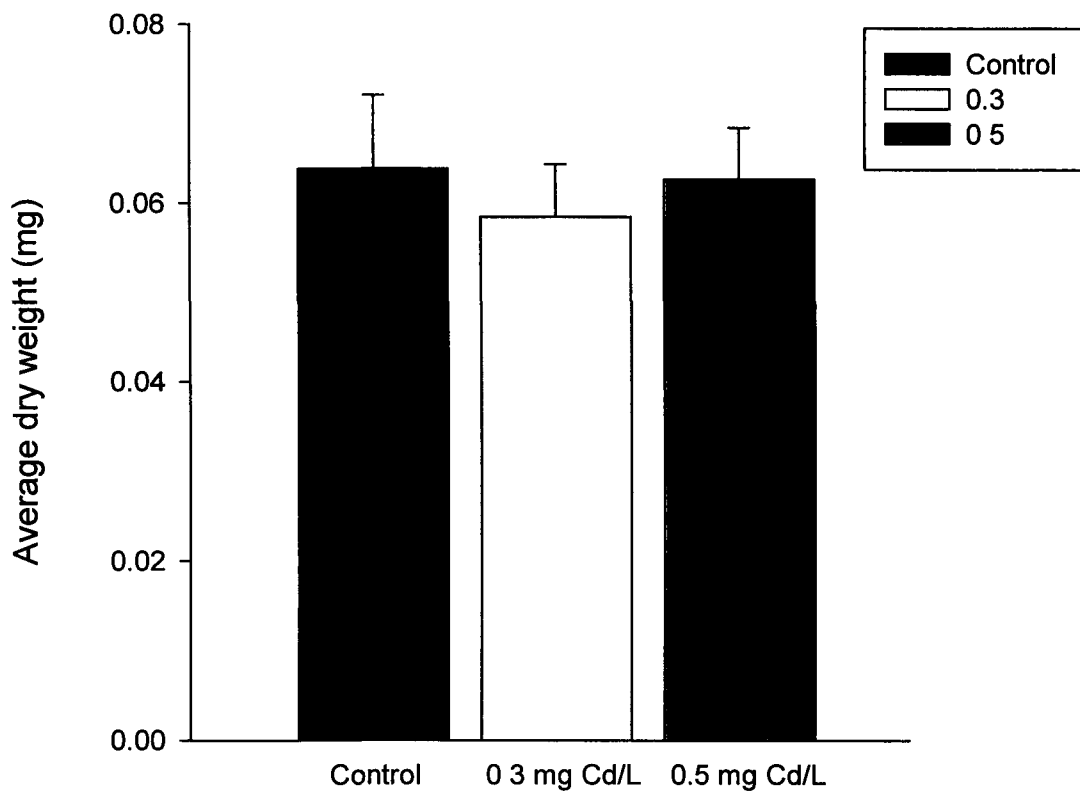


Figure 3.2. The effect of Cd exposure to *H. azteca* (n = 22 per exposure) growth (average dry weight) throughout the 28 day exposure to 0, 0.3, and 0.5 μg Cd/L in soft water (22 mg/L as CaCO_3). There were no significant differences from controls.

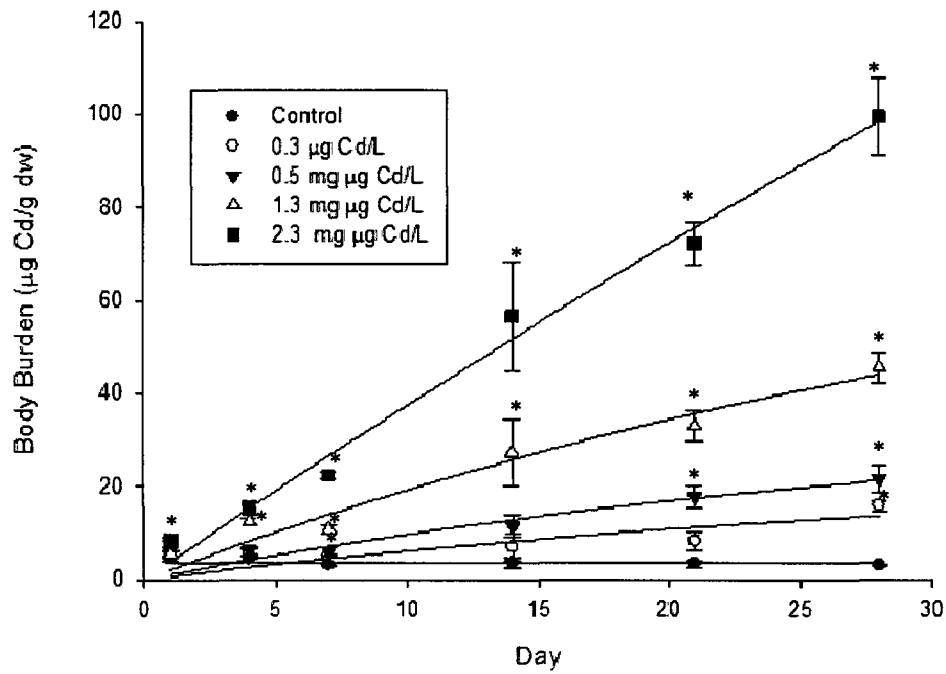


Figure 3.3 Cd accumulation in *L. variegatus* during a 28-day exposure to 0, 0.3, 0.5, 1.3, and 2.3 $\mu\text{g Cd/L}$. Values are means ± 1 SEM, $n = 8$ worms. * indicate significance compared to background Cd at each day (ANOVA; $p < 0.05$).

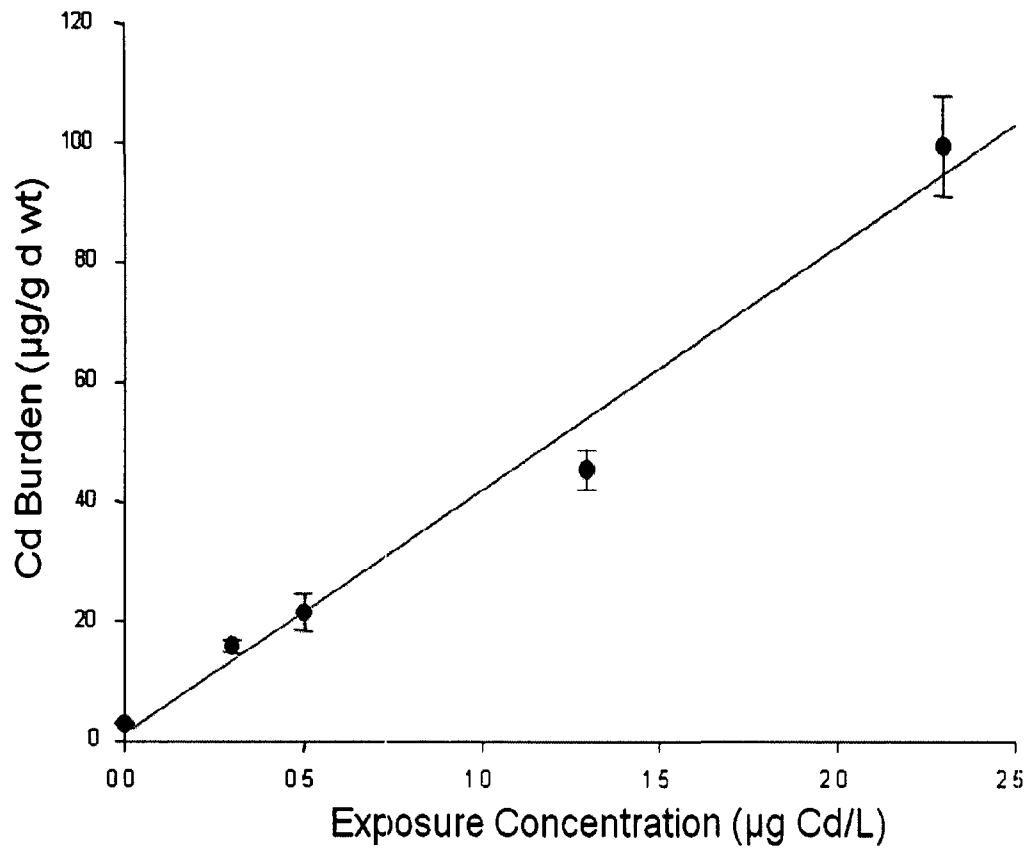


Figure 3.4. Cd accumulation in *L. variegatus* at final day of 28-day exposure to 0, 0.3, 0.5, 1.3, and 2.3 µg Cd/L. Values are means \pm 1 SEM, n = 8 worms.

Cd accumulation over 28d in *H.azteca*

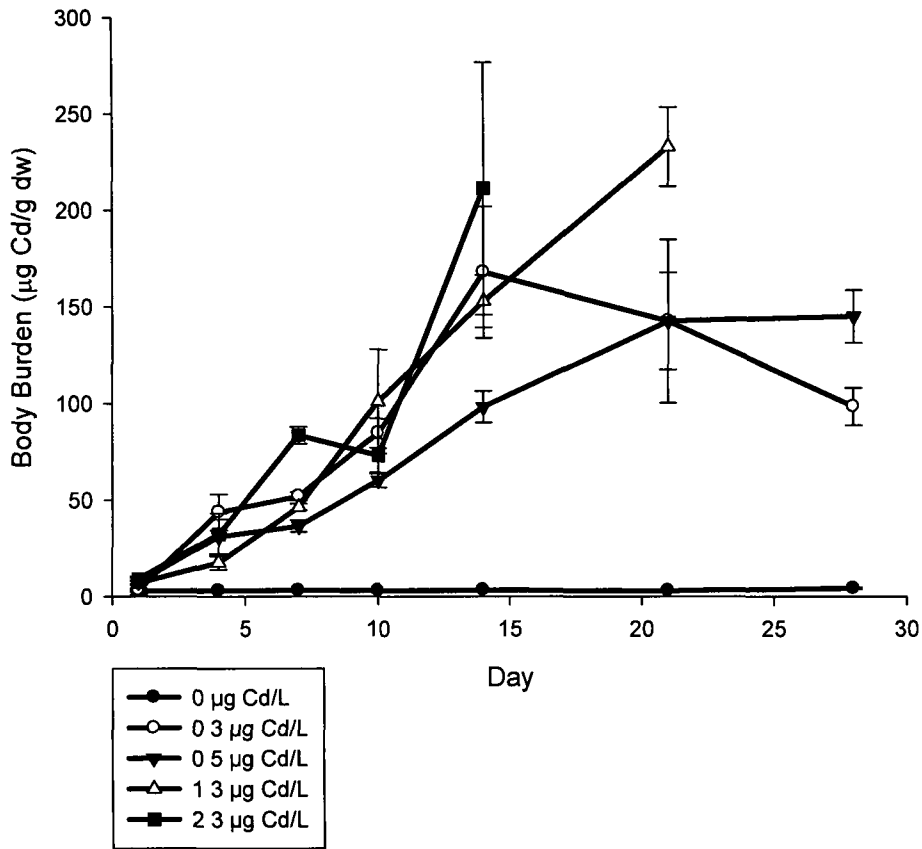


Figure 3.5 Cd accumulation in *H. azteca* during a 28-day exposure to 0, 0.3, 0.5, 1.3, and 2.3 µg Cd/L. Values are means \pm 1 SEM, n = 8 amphipods. All measurements from day 1 forward are significantly different from controls except 0.3 µg Cd/L at day 1 (ANOVA; $p < 0.05$).

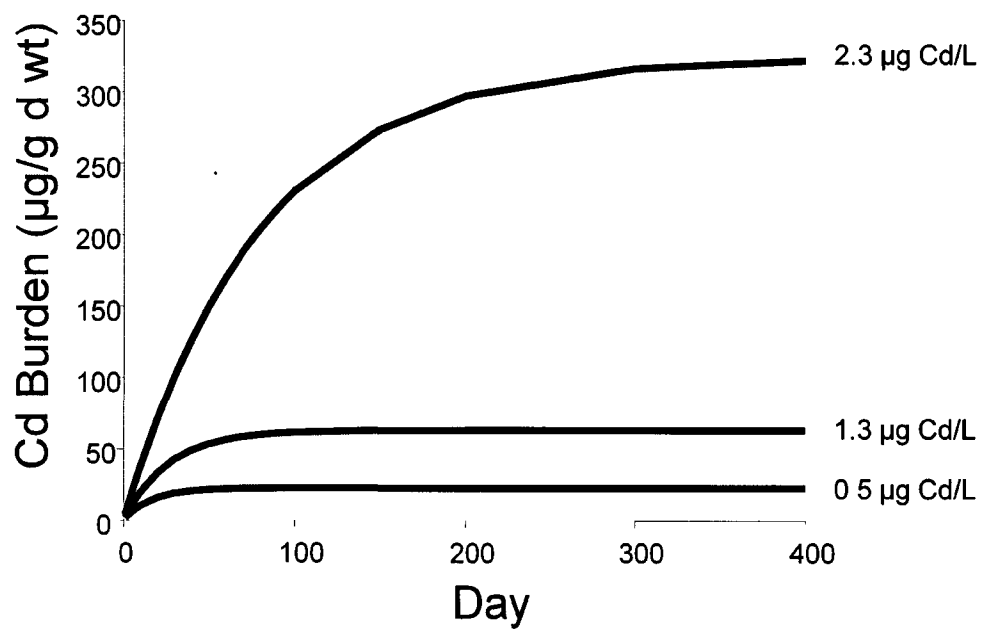


Figure 3.6. Extrapolation of average body burden at steady state of accumulation in *L. variegatus*.

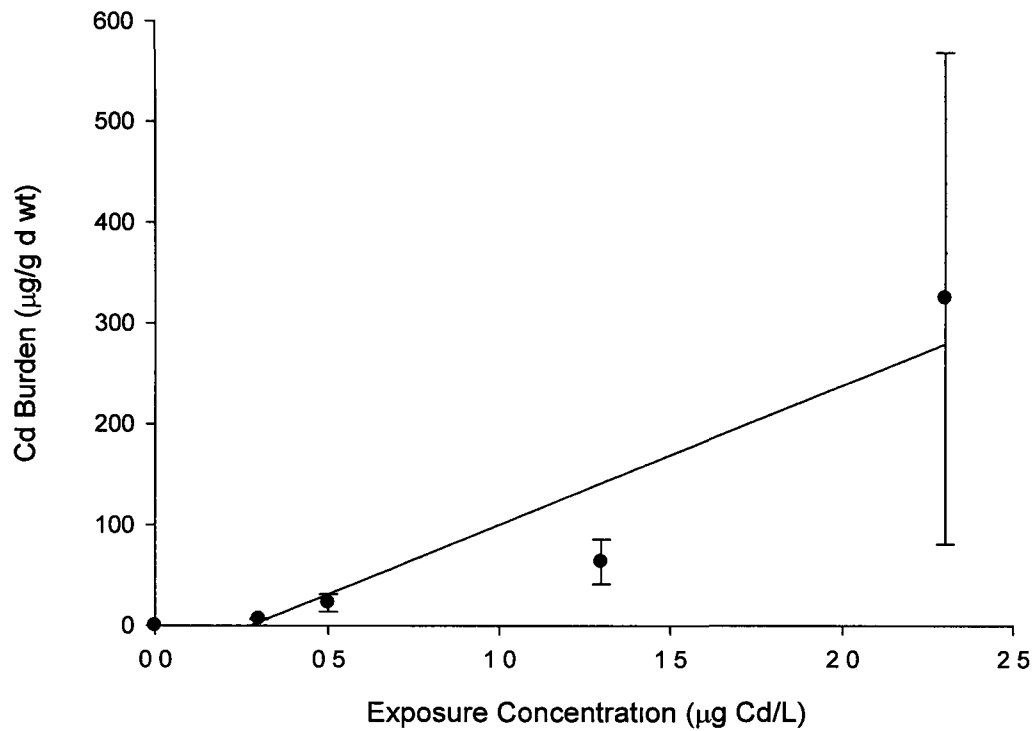


Figure 3.7. Cd accumulation in *L. variegatus*; extrapolation of body burden at steady state of accumulation during exposure to 0, 0.3, 0.5, 1.3, and 2.3 µg Cd/L. The 0.3 µg Cd/L point was calculated without data from day 28. Values are means ± 1 SEM.

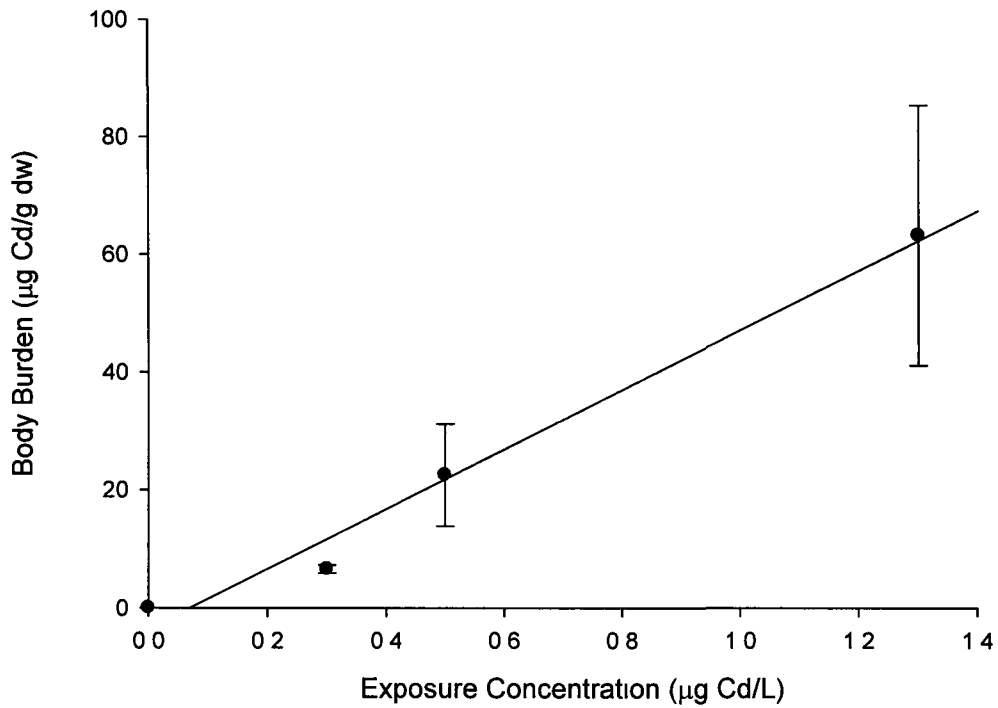


Figure 3.8 Cd accumulation in *L. variegatus*; extrapolation of body burden at steady state of accumulation during exposure to 0, 0.3, 0.5, and 1.3 µg Cd/L. The 0.3 µg Cd/L point was calculated without data from day 28. Values are means \pm 1 SEM.

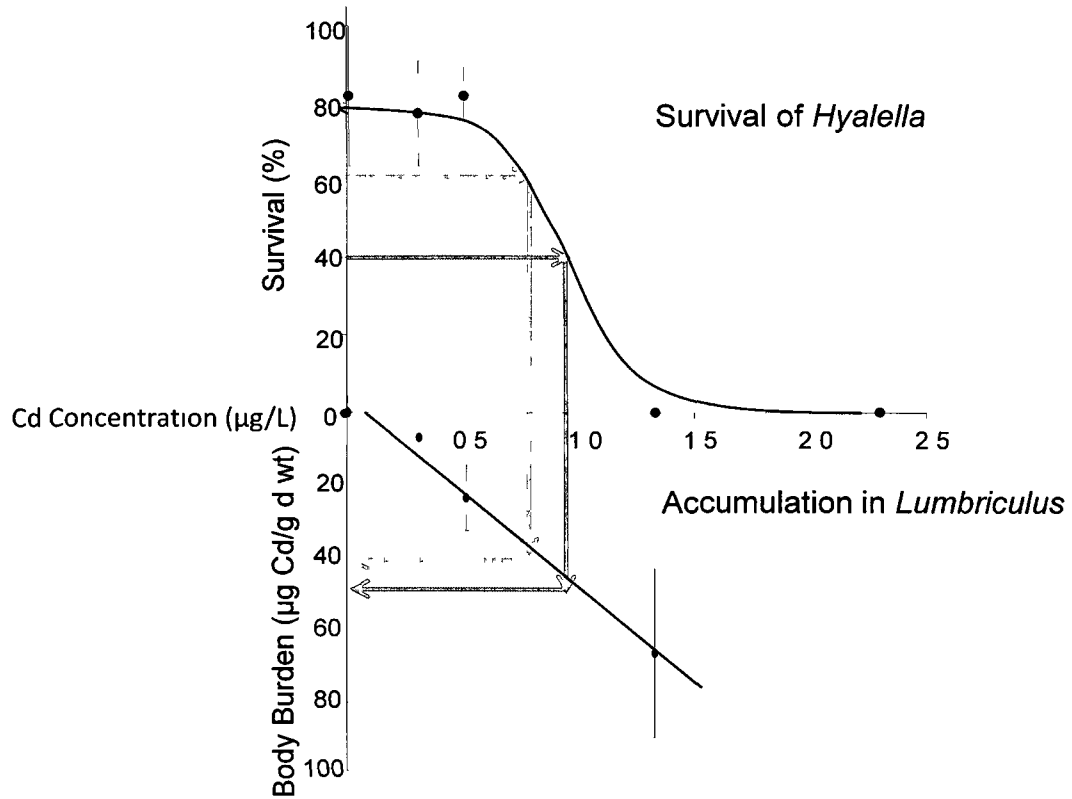


Figure 3.9. Linking *H. azteca* EC20 and EC50 values to specific body burdens in *L. variegatus*. Light grey corresponds to a LC20 of 0.71 µg Cd/L, being linked to a body burden of 42.6 µg Cd/g dry weight. Dark grey corresponds to LC50 0.91 µg Cd/L being linked to a body burden 50.6 µg Cd/g dry weight.

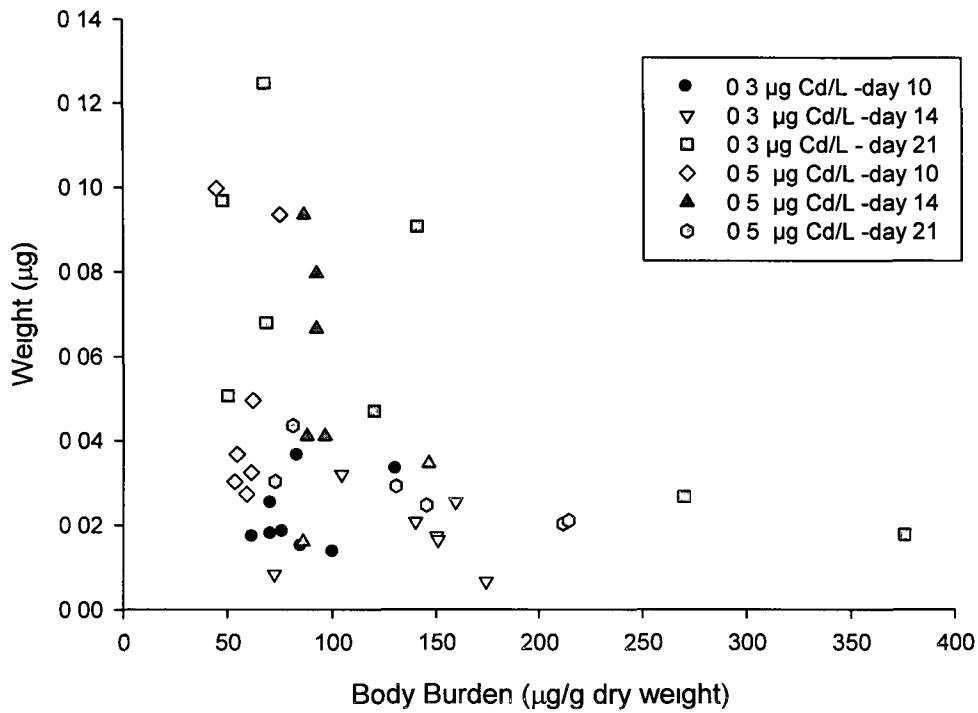


Figure 3.10 Body Burden vs Dry Weight in *Hyalella azteca* exposed to Cd. This plot shows no clear trend indicating that larger individuals accumulated a smaller body burden $R^2 = 0.111$.

Table 3.1. Cd survival effect concentrations, with upper and lower 95% confidence intervals. Calculated using CETIS 1.6.1

Effect-%	Conc-($\mu\text{g Cd/L}$)	95% LCL	95% UCL
5	0.61	N/A	0.68
10	0.64	N/A	0.71
15	0.67	N/A	0.73
20	0.71	N/A	0.76
25	0.74	N/A	0.79
50	0.91	0.44	0.95

Table 3.2. Measured Cd body burdens ($\mu\text{g Cd/g dry wt}$; mean \pm SEM) for *L. variegatus* over 28 days of exposure at five exposure concentrations. Mean measured Cd exposure concentrations are given, n=8. A * indicates a significant difference from controls at that time (P<0.05).

Day of Exposure	Control	0.3 $\mu\text{g Cd/L}$	0.5 $\mu\text{g Cd/L}$	1.3 $\mu\text{g Cd/L}$	2.3 $\mu\text{g Cd/L}$
1	4.4 \pm 0.3	5.5 \pm 0.8	6.5 \pm 0.4*	5.5 \pm 0.6	8.7 \pm 0.4*
4	4.3 \pm 0.6	5.8 \pm 0.6	6.3 \pm 0.7	12.4 \pm 0.8*	15.4 \pm 1.7*
7	3.1 \pm 0.4	5.1 \pm 0.3	6.0 \pm 0.9*	10.8 \pm 1.1*	22.3 \pm 0.7*
14	3.6 \pm 1.0	6.9 \pm 3.1	11.5 \pm 2.4	27.3 \pm 7.2*	56.7 \pm 11.7*
21	3.4 \pm 0.7	8.2 \pm 2.0	17.7 \pm 2.4*	33.0 \pm 3.4*	72.3 \pm 4.6*
28	3.0 \pm 0.1	15.9 \pm 1.1*	21.6 \pm 3.1*	45.5 \pm 3.4*	99.4 \pm 8.3*

Table 3.3. Measured Cd body burdens ($\mu\text{g Cd/g dry wt}$; mean \pm SEM) for *H. azteca* over 28 days of exposure at five exposure concentrations. Mean measured Cd exposure concentrations are given, the N/A indicates times at which there were no surviving individuals and for each time point n=8 except for the last measurements at 1.3 $\mu\text{g Cd/L}$ where n=6 and 2.3 $\mu\text{g Cd/L}$ where n=5. A * indicates a significant difference from controls at that time ($P < 0.05$).

Day of Exposure	Control	0.3 $\mu\text{g Cd/L}$	0.5 $\mu\text{g Cd/L}$	1.3 $\mu\text{g Cd/L}$	2.3 $\mu\text{g Cd/L}$
1	2.7 \pm 0.4	3.4 \pm 2.4	7.5 \pm 0.9*	7.1 \pm 2.7 *	9.1 \pm 1.2*
4	3.0 \pm 0.1	43.8 \pm 9.4*	31.1 \pm 8.9*	17.5 \pm 3.5*	32.4 \pm 10.8*
7	3.1 \pm 0.4	52.0 \pm 2.1*	36.5 \pm 3.0*	46.4 \pm 1.7*	83.6 \pm 4.3*
10	3.0 \pm 0.1	84.7 \pm 7.7*	60.2 \pm 3.4*	101.1 \pm 26.9*	73.1 \pm 8.8*
14	3.4 \pm 0.7	167.9 \pm 34.0*	98.3 \pm 8.1*	152.9 \pm 13.5*	211.3 \pm 65.5*
21	3.0 \pm 0.1	142.5 \pm 42.2*	142.6 \pm 25.0*	232.8 \pm 20.5*	N/A
28	4.3 \pm 0.6	98.4 \pm 9.7*	145.0 \pm 13.6*	N/A	N/A

Table 3.4. Extrapolation of average body burden at steady state of accumulation in *L. variegatus*.

Conc. ($\mu\text{g Cd/L}$)	Est. body burden at steady state ($\mu\text{g Cd/L}$)	Std. error	$t^{1/2}$ (days)
0.3	6.52	0.7	0.4
0.5	22.48	8.7	22.8
1.3	63.22	22.1	37.0
2.3	324.38	243.7	55.9

Chapter 4: General Discussion

4.1 Expanding on Chapter 3: A multiple invertebrate species exposure to determine if Cd accumulation in a resistant species can be used to reliably predict effects in a sensitive species in soft water

In chapter 3, an experiment was described which utilized two aquatic invertebrates in an attempt to provide a proof-of-principle in which bioaccumulation of a metal could be used to assess ecosystem health. Alongside the two exposures discussed in chapter 3 were two additional exposures which utilized an additional resistant organism as well as an additional sensitive species. The inclusion of an additional resistant species was important as although *L. variegatus* has a number of qualities that make it an excellent organism to be utilized as a biomonitor their inability to attain a steady state of accumulation during lab testing is a disadvantage. Identifying a resistant species which is able to quickly and reliably accumulate metal to a steady state would provide much needed support to this technique's viability in the field. For this concept to be applicable to the real world, body burdens in the indicator species must be related to multiple sensitive species. For these reasons *Lymnaea stagnalis* (Great Pond Snail) was included in the study as an additional resistant species, while *Daphnia pulex* was included as an additional sensitive species.

Lymnaea stagnalis is a freshwater pulmonate gastropod with a holarctic distribution, meaning it can be found in across the northern continents. *L. stagnalis* will reach a size of 3.5 – 4 cm in lab cultures, and will live for over a year in ideal conditions. Like *L. variegatus* they are easily cultured in lab environments, but are substantially

larger, and would be easier to collect in the field than the blackworms. This species does well in stagnant and polluted waters and has been shown to be resistant to Cd exposure with LC50 values in the mg/L range (Gomot 1998; Courdassier *et al.*, 2003); eclipsing those of other resistant species including *L. variegatus*. These reasons made *L. stagnalis* a good candidate species to be used in place of *L. variegatus* as the indicator species.

A colleague, Nish Pais, ran *L. stagnalis* exposures alongside those discussed in chapter 3. *L. stagnalis* accumulation over 21 days of exposure is presented in Figure 4.1. In these tests the snails attained a steady state of accumulation after as little as 4 days of Cd exposure. The ability to accumulate to steady-state so quickly eliminates the need to extrapolate body burdens and time to steady state, as was done in *L. variegatus* in chapter 3 (Fig 3.6-3.8). Figure 4.2 shows *L. stagnalis* Cd burden at steady state plotted against the experimental Cd concentration, producing a linear graph that indicates a clear dose dependent relationship. When this graph is plotted alongside *H. azteca* survival (Figure 3.1), it can be used to predict amphipod survival using the body burdens at steady state found in the snails. Mortality of 20% and 50% in *Hyaella* corresponds to 39 and 49 $\mu\text{g/g}$ dry wt in *Lymnaea* respectively. Unlike *Lumbriculus*, *Lymnaea* steady state is reached within the exposure time (Figure 4.1) and therefore there is more confidence in the modelled exposure-accumulation relationship. The snail's accumulation data provides a more reliable reference point than the blackworms. With their inability to quickly reach steady state, a specific body burden in *Lumbriculus* cannot be confidently linked to a specific exposure concentration and in turn a specific effect in a sensitive species, making *L. stagnalis* a better choice as an indicator species. Their ability to quickly accumulate to

steady state means that in the field, a researcher can assume that any snail of a reasonable size will be at steady state.

Despite their many qualities that make them an excellent choice as a bioindicator, there are some issues with using snails as the universal resistant species. Firstly, while the adults are extremely resistant to Cd, research by Gomot (1998) has shown that there may be some negative sublethal effects at much lower concentrations, with exposure causing hatching to occur 5 to 15 days later in the controls (hatched 12 to 13 days after laying). These snails also possess a lung, and unlike the other sedentary species are able to leave heavily contaminated areas providing they find a new body of water prior to desiccation.

Daphnia pulex is an abundant cladoceran commonly used in toxicity testing. *D. pulex* is a sensitive species, and as it is both the most common species of cladoceran and ubiquitous in inland fresh waters, would make for a good species for this TRA. *D. pulex* is also sensitive to many elements including Cd. During this experiment a *D. pulex* exposure was conducted by Emily-Jane Costa. The daphnids accumulated Cd in a dose dependent manner (Figure 4.4a) with average body burdens at day 21 increasing with exposure concentrations. The nature of this experiment prevented body burdens from being measured until test end, but provided insight into any reproductive effects which occurred. Figure 4.4b shows a significant reduction in reproduction in all organisms exposed to Cd. There was no significant difference between the extents of which reproduction was reduced. Therefore *D. pulex*, as sensitive organisms, does not provide a reliable tissue burden to effect relationship. An interesting note on *D. pulex*, is that although they were able to survive at higher exposure concentrations they suffered a significant deleterious effect at much lower (and environmentally relevant)

concentrations of 0.3 and 0.5 $\mu\text{g Cd/L}$. One of the benefits of this type of TRA is that it can be used with any endpoint, in this case specifically the observed reduction in reproduction related to a body burden of about 25 $\mu\text{g Cd/g dry wt.}$ in *L. stagnalis*. This negative effect concentration is well below the body burdens corresponding to survival effects in *H. azteca*. This means that although *D. pulex* is more resistant to Cd in terms of survival, it is sensitive to reproductive effects at very low exposure concentrations. This is one area where using a resistant indicator species would be very beneficial, as observed Cd body burdens can quickly be related to many endpoints in many species, providing a quick overview of ecosystem health as a whole. If relationships can be determined for multiple endpoints in multiple sensitive species this technique could become a valuable tool.

As a whole using a TRA which utilizes bioaccumulation in a metal resistant organism that strongly and reliably accumulates as an indicator of impacts in sensitive organisms hold promise. The resistant organism provides a measure of bioavailability and the toxicological endpoint could be at any level, from physiological, to organism toxicity to community indicator.

4.2 Integrating multiple fields of biology in an aquatic toxicity project

Biology as a whole has become excessively specialized. Wilfrid Laurier University (WLU) is one of the few universities to have a master's program with a focus on integrative biology. The aim of which is the training of young biologists with multiple areas of expertise, and an ability to bridge the gaps between many fields of science. The

research presented in this thesis document falls under the field of environmental toxicology, although completing it has required a broad understanding of many other specialties in biology.

In general toxicology is one of the most integrative fields within biology, and while individual studies may become very specific, this project remained quite broad in its scope and required a good understanding of many closely related fields. To fully comprehend environmental toxicology requires an expansive knowledge of the environment; first looking at the environment as a whole, then individual ecosystems within the environment, and finally the various communities and species within an ecosystem. It is because of this many toxicologists will become more specialized; potentially with a focus on specific vertebrates or invertebrates, or a specific endpoint across multiple species, or a focus on fresh waters, salt waters, etc.

This thesis has taken the broad field of environmental toxicology, and focused on a small portion of it, whether or not bioaccumulation can be used as a measure of effect. Rather than focusing on all members of an ecosystem it has focused on only a resistant and a sensitive species, and instead of applying the concept to multiple contaminants in multiple environments it has focused on Cd, in two strictly defined medias. While this may seem to be excessively specialized, this project has actually maintained a great deal of integration. An understanding of aquatic chemistry may be as important as an understanding of biology when it comes to aquatic toxicology, and was required for this project. Aquatic geochemistry is very important in this field as the Cd which is present in the environment may not even be available to an organism. If a metal is available to an organism an understanding of metal binding affinity and metal uptake is also important.

While my research was designed to bypass the outside influences of modifying factors, an understanding of why it would be ideal to do so is of great importance. Even an understanding of an organism's behaviour is important, knowing what a stressed individual looks like, and being able to recognise behavioural changes. Understanding the biology of the organisms one works with is also important. Understand the internal anatomy of an organism, and have a solid base of knowledge of how things work at both the tissue and cellular levels is integral in understanding how many toxicants work. An understanding of how an element like Cd is transported within an organism is important as well, once inside the organism it is important to understand in what tissues it will accumulate, and when there is it likely to cause damage. While many fields of biology can become overly specialized in this regard, most toxicologists will have a working knowledge with multiple different species, be they fishes or invertebrates. This research has required an understanding of multiple species and multiple exposure routes, as well as an understanding of internal Cd distribution, allowing for the integration of multiple realms of biology.

A significant portion of my project has required me to work in close association with other members of the lab. This has led to the integration of peer's work into a portion of this thesis with my own research (see section 4.1). One of the goals of this integrative biology program is to help bridge the gap between researchers, and my project has helped me in this regard. I have learned to work as a member of a team on a joint project and integrated the ideas of others along with my own, resulting in a more complete project.

4.3 Figures

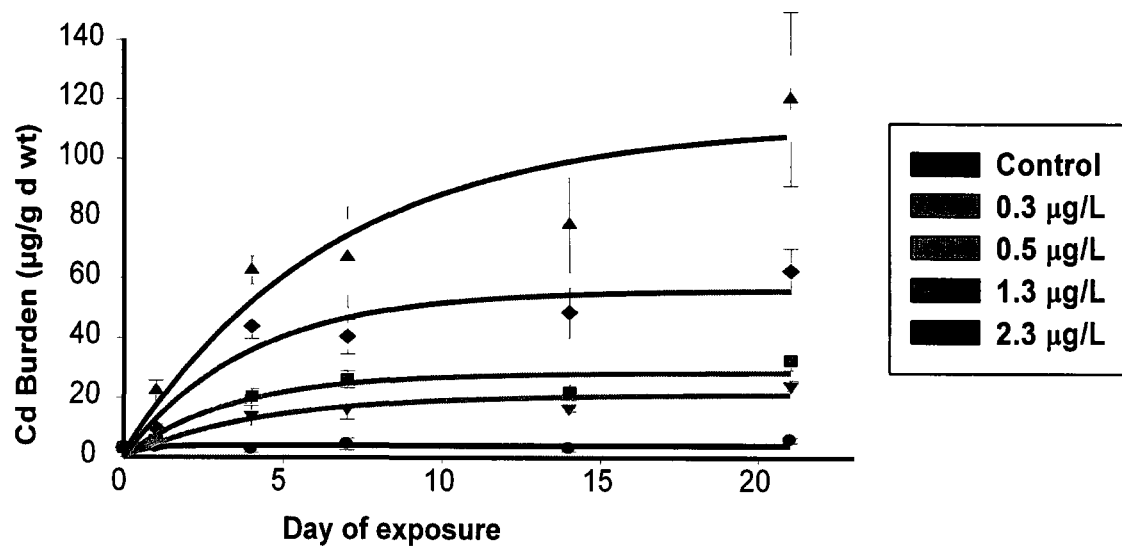


Figure 4.1 Cd accumulation in *Lymnaea stagnalis* after exposure to various Cd concentrations over 21 days.

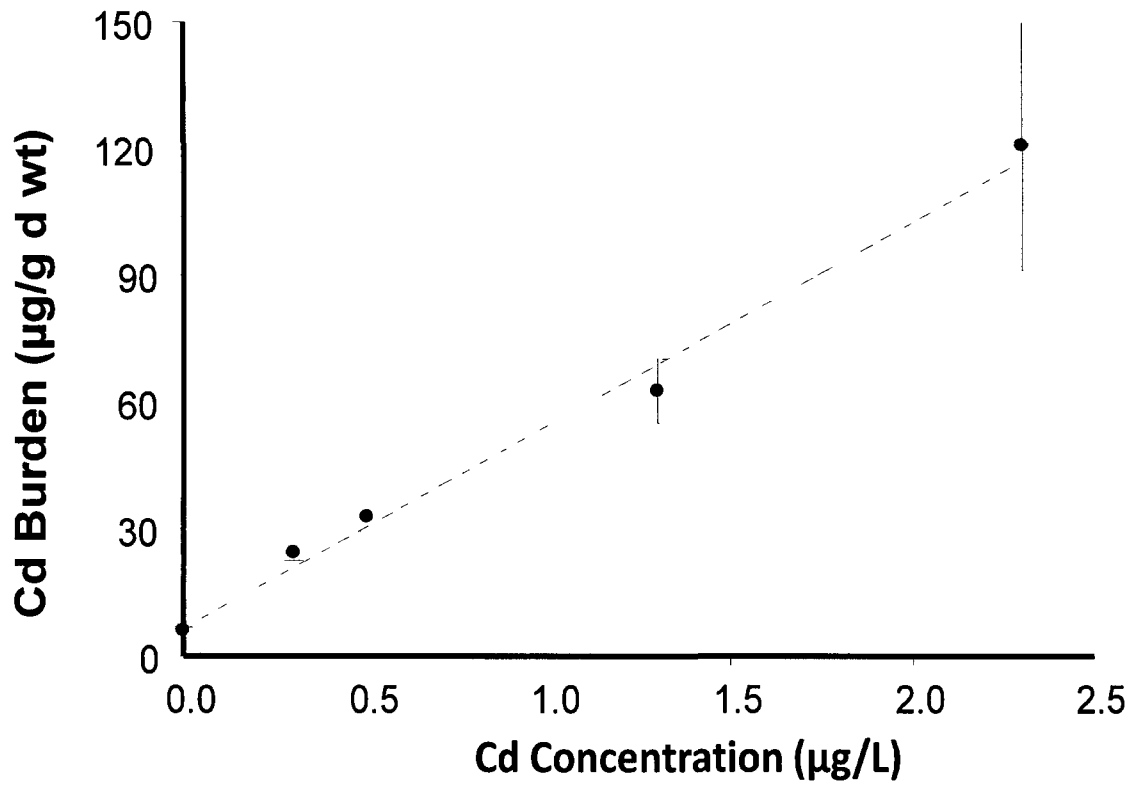


Figure 4.2 Cd accumulation in *Lymnaea stagnalis* at 21 days of exposure to various Cd concentrations.

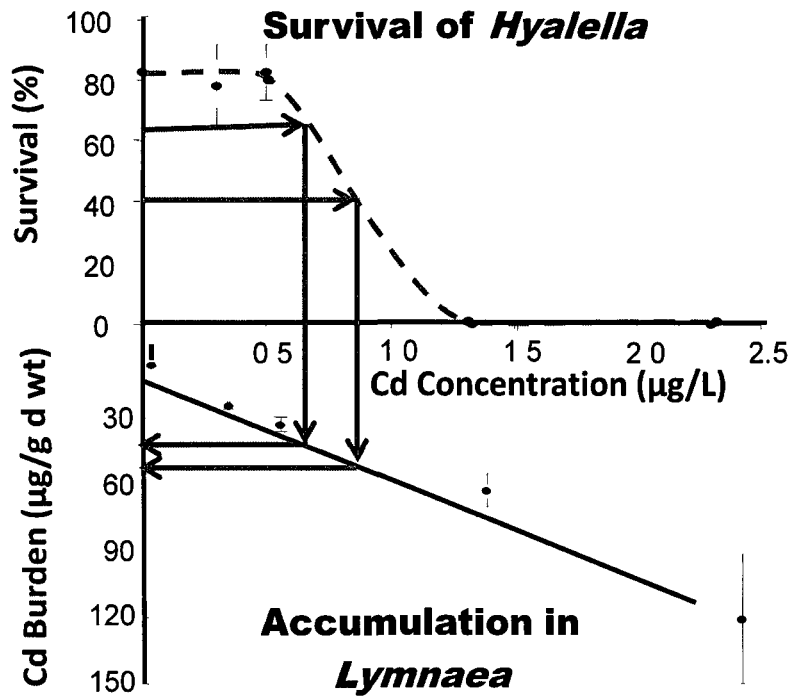


Figure 4.3 Linking *H. azteca* EC20 and EC50 values to specific body burdens in *L. stagnalis*.

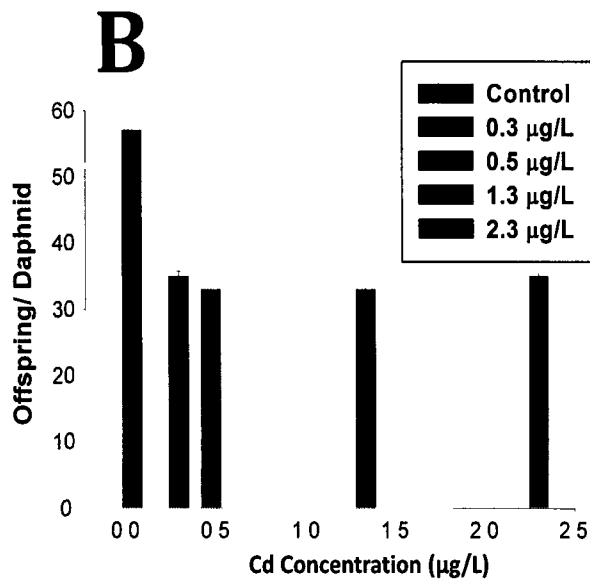
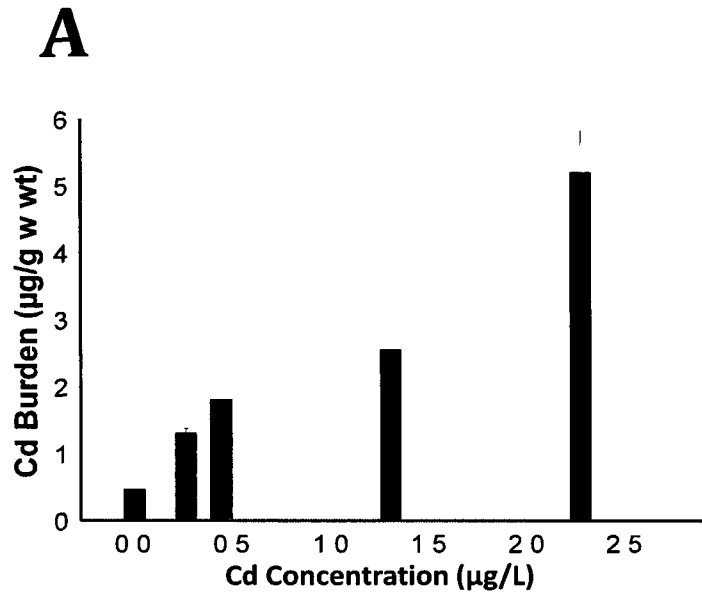


Figure 4.4 A. Cadmium accumulation in *Daphnia pulex* at 21 days of exposure. B. Effect of Cd exposure on reproduction after 21 days of exposure in *D. pulex*

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Appendix A: Subcellular fractionation flow diagram

