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**Studies on Waterborne Cadmium Exposure to  
*Lymnaea stagnalis* in Varying Water Qualities and  
the Development of a Novel Tissue Residue**

**Approach**

By

Nish Melani Pais

Honours Bachelor of Life Science, McMaster University, 2009

THESIS

Submitted to the Department of Biology

Faculty of Science

In partial fulfillment of the requirements for

Master of Science in Integrative Biology

Wilfrid Laurier University

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## Abstract

Recent studies have shown that the use of bioaccumulation (tissue residues) as a predictor for toxic effects for metals is a flawed concept. Adams *et al.* (2010) suggested a novel tissue residue approach (TRA) in which bioaccumulation in resistant organisms is related to toxic effects in sensitive organisms. The goal of this thesis was to test these assumptions in relation to Cd exposure using the great pond snail, *Lymnaea stagnalis* by developing and improving the understanding of Cd accumulation in aquatic invertebrates.

The relationship between Cd bioaccumulation and its toxicity was studied in *L. stagnalis* using acute (96h) and chronic (one month) toxicity tests in moderately hard water (140 mg/L as CaCO<sub>3</sub>). Two sizes of snails (18 or 25 mm) were tested for acute toxicity and the 96 h LC<sub>50</sub> for both sizes of snails were 350 µg Cd/L. Soft tissue accumulation reached 750 µg Cd/g dry wt. in these exposures while the shell accumulation was only 16 µg Cd/g at the highest non-lethal exposures. Three sizes of snails were tested for chronic toxicity. The 31 d LC<sub>50</sub>s for the small (5 mm), medium (10 mm) and large (15 mm) snails were 13, 50 and 46 µg Cd/L, respectively. In the smaller snails, growth was inhibited at exposure concentrations below 10 µg Cd/L.

In soft water (20 mg/L CaCO<sub>3</sub>), two chronic (28 d) tests were conducted using juvenile snails (5 mm), the first with exposures from 2.3 – 8.3 µg Cd/L and the endpoints measured were survival, bioaccumulation and growth. The 28 d LC<sub>50</sub> for the first test was 7.3 µg Cd/L. Cd accumulation within the soft tissue ranged between 7 - 300 µg Cd/g dry wt. Exposure concentrations in the second test ranged from 0.3 – 2.3 µg Cd/L and the endpoints measured were whole and sub-cellular fractionation of Cd. Cd accumulation

within the soft tissue reached levels as high as 117  $\mu\text{g Cd/g}$  dry wt. Cd accumulation was dose dependent and reached steady state tissue burden within 14 d. Sub-cellular fractionation was measured after 28 d of exposure and increasing amounts of Cd were found in the heat stable proteins and organelle fractions as Cd exposure concentration increased.

Finally, to test the novel TRA approach, a side-by-side exposure using a Cd sensitive organism (*Hyaella azteca*; based on studies by T. Straus) and a resistant organism (*L. stagnalis*) was done over 28 d using a toxicologically relevant exposure range (0.4 to 2.6  $\mu\text{g Cd/L}$ ). Endpoints were survival, growth (dry wt.) and Cd accumulation. A relationship between mortality in *Hyaella* (sensitive organism) and accumulation in *Lymnaea* (resistant organism) was determined for d 28 of exposure. The 28 d LD<sub>20</sub> and LD<sub>50</sub> in *Hyaella* were 0.42 and 0.70  $\mu\text{g Cd/L}$ , respectively. The LD<sub>20</sub> and LD<sub>50</sub> values in *Hyaella* was associated to 36 and 69  $\mu\text{g Cd/g}$  dry wt. body burden in the soft tissue of *Lymnaea*. Therefore, this novel TRA shows potential but requires more validation for it to be used in the field.

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## **Authors Declaration**

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

# Table of Contents

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Abstract .....	ii
Acknowledgements .....	iv
Authors Declaration .....	v
List of Figures .....	x
List of Tables .....	xiii
Chapter 1: .....	1
General Introduction .....	1
1.1 Cadmium .....	2
1.2 Cd Bioavailability .....	3
1.3 Tissue Residue Approach (TRA) .....	4
1.4 Bioaccumulation of Cd .....	5
1.5 Sub-cellular Fractionation .....	5
1.6 <i>Hyalella azteca</i> .....	7
1.7 <i>Lymnaea stagnalis</i> .....	7
1.8 Objectives .....	9
1.9 References .....	10
1.10 Figures .....	14
Chapter 2: .....	15
Acute and Chronic Toxicity of Waterborne Cd to <i>Lymnaea stagnalis</i> in Hard Water .....	15
2.1 Abstract .....	16
2.1 Introduction .....	17
2.3 Materials and Method .....	18
2.3.1 Snail Culture .....	18
2.3.2 General Experimental Procedure .....	19
2.3.3 Acute Test (AT) .....	21
2.3.4 Chronic Test (CT) .....	21
2.3.5 Analysis .....	21
2.3.6 Statistical Analysis and LC <sub>50</sub> Calculation .....	22
2.4 Results .....	23
2.4.1 AT .....	23

2.4.2 CT .....	24
2.5 Discussion.....	25
2.5.1 AT .....	25
2.5.2 CT .....	26
2.6 References.....	28
2.7 Figures.....	30
2.9 Tables.....	39
Chapter 3:.....	40
Tissue and Sub-cellular Accumulation of Cadmium in <i>Lymnaea stagnalis</i> during Chronic Exposure in Soft Water.....	40
3.1 Abstract.....	41
3.2 Introduction.....	42
3.3 Materials and Methods.....	45
3.3.1 Snail Culture .....	45
3.3.2 General Experimental Procedure .....	46
3.3.3 Chronic Test I (CT I) .....	47
3.3.4 Chronic Test II (CT II).....	47
3.3.5 Analysis.....	48
Water and Whole Tissue Samples .....	48
Sub-cellular Fractionation.....	48
3.3.6 Statistical Analysis and LC <sub>50</sub> Calculation.....	49
3.4 Results.....	50
3.4.1 CT I.....	50
Survival and Growth.....	50
Bioaccumulation .....	50
3.4.2 CT II.....	51
Bioaccumulation and Steady State.....	51
Sub-cellular Fractionation.....	51
3.5 Discussion.....	52
3.5.1 CT I.....	52
Survival and Growth.....	52
Bioaccumulation .....	53
3.5.2 CT II.....	54
Bioaccumulation and Steady State.....	54



Sub-cellular Fractionation.....	55
3.6 References.....	58
3.7 Figures.....	61
3.9 Tables.....	66
Chapter 4:.....	68
The Development of the Novel Tissue Residue Approach to Predict the Toxicity of Cadmium to <i>Hyalella azteca</i> using Bioaccumulation of <i>Lymnaea stagnalis</i> in Moderately Hard Water.....	68
4.1 Abstract.....	69
4.2 Introduction.....	70
4.3 Materials and Methods.....	73
4.3.1 Snail Culture .....	73
4.3.2 <i>Hyalella</i> Culture.....	74
4.3.3 General Experimental Procedure .....	74
4.3.4 Chronic Test (CT) using <i>H. azteca</i> .....	75
4.3.5 Chronic Test (CT) using <i>L. stagnalis</i> .....	75
4.3.4 Analysis.....	75
4.3.5 Statistical Analysis and LC <sub>50</sub> Calculation.....	76
4.4 Results.....	77
4.4.1 CT using <i>Hyalella</i> .....	77
Survival and Growth.....	77
Bioaccumulation .....	78
4.4.2 CT using <i>Lymnaea</i> .....	78
Bioaccumulation and Linkages.....	78
4.5 Discussion.....	79
4.5.1 CT using <i>Hyalella</i> .....	79
Survival and Growth.....	79
Bioaccumulation .....	81
4.5.2 CT using <i>Lymnaea</i> .....	82
Bioaccumulation .....	82
4.5.3 <i>Hyalella</i> and <i>Lymnaea</i> .....	82
Accumulation-effect Relationship .....	82
4.5.4 Integration .....	84
4.6 References.....	86

4.7 Figures.....	90
4.9 Tables.....	95

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## List of Figures

<b>Figure 1.1.</b> Procedure for determining the sub-cellular fractionation of metal within invertebrates.....	14
<b>Figure 2.1.</b> The effect of waterborne Cd on survival of <i>L. stagnalis</i> over 96 h of exposure to either 65, 174, 315, 628, 1301 µg Cd/L.....	30
<b>Figure 2.2.</b> Mean soft tissue (A) concentrations (µg Cd/g dry wt. ± SEM) and mean shell (B) concentration (µg Cd/g dry wt. ± SEM) for Cd in <i>L. stagnalis</i> after 96 h of exposure to waterborne Cd.....	31
<b>Figure 2.3.</b> The acute effects of waterborne Cd on soft tissue Ca <sup>2+</sup> (A) and Na <sup>+</sup> (B) concentrations in <i>Lymnaea</i> at 96 h.....	32
<b>Figure 2.4.</b> The effect of chronic exposure to waterborne Cd on the survival of <i>L. stagnalis</i> exposed to 2, 8, 26 or 94 µg Cd/L over 31 d.....	33
<b>Figure 2.5.</b> The chronic effects of waterborne Cd on the mean shell length (mm) of <i>Lymnaea</i> exposed to 2, 8, 26 or 94 µg Cd/L over 31 d for small snails (5 ± 0.02 mm; mean ± SEM; n = 10) (A), medium snails (10 ± 0.1 mm; mean ± SEM; n = 10) (B) and large snails (15 ± 0.2 mm; mean ± SEM; n = 10) (C) .....	34
<b>Figure 2.6.</b> The chronic effects of waterborne Cd on the wt. (g) of <i>Lymnaea</i> exposed to 2, 8, 26 or 94 µg Cd/L over 31 d for small snails (0.02 ± 0.004 g; mean ± SEM; n = 10) (A), medium snails (0.14 ± 0.008 g; mean ± SEM; n = 10) (B) and large snails (0.6 ± 0.09 g; mean ± SEM; n = 10) (C) .....	35
<b>Figure 2.7.</b> Soft tissue Cd accumulation of small, medium and large snails at d 31 exposed to waterborne Cd.....	36

<b>Figure 2.8.</b> Shell Cd accumulation of small, medium and large snails at d 31 exposed to waterborne Cd.....	37
<b>Figure 2.9.</b> The chronic effects of waterborne Cd on soft tissue $\text{Ca}^{2+}$ (A) and $\text{Na}^+$ (B) concentrations in <i>Lymnaea</i> at d 31.....	38
<b>Figure 3.1.</b> Chronic Test 1 (CTI):The chronic effects of waterborne Cd on the survival (A) as well as mean length (B) and mean dry wt. (C) of <i>L. stagnalis</i> over 28 d of exposure .....	61
<b>Figure 3.2.</b> CTI: Relationship between mean Cd soft tissue burden ( $\mu\text{g Cd/g dry wt.} \pm \text{SEM}$ ) and survival (A) and mean length (B) of <i>L. stagnalis</i> exposed to waterborne Cd (either 2.3, 4.5, or 8.2 $\mu\text{g Cd/L}$ ) at d 31.....	62
<b>Figure 3.3.</b> Chronic Test 2 (CTII): Mean concentration of Cd in soft tissues of <i>L. stagnalis</i> exposed to 0.3, 0.5, 1.3 or 2.3 $\mu\text{g Cd/L}$ over 28 d.....	63
<b>Figure 3.4.</b> CTII: Sub-cellular fractionation of soft tissue <i>L. stagnalis</i> exposed to 0.3, 0.5, 1.3 or 2.3 $\mu\text{g Cd/L}$ at d 28.....	64
<b>Figure 3.5.</b> CTII: Sub-cellular fractionation of soft tissue <i>L. stagnalis</i> exposed to 0.3, 0.5, 1.3 or 2.3 $\mu\text{g Cd/L}$ at d 28.....	65
<b>Figure 4.1.</b> The chronic effects of waterborne Cd on the survival of <i>H. azteca</i> exposed to 0.6, 1.3, 2.6 or 5.1 $\mu\text{g Cd/L}$ over 28 d.....	90
<b>Figure 4.2.</b> Mean ( $\pm \text{SEM}$ ) dry wt. of exposed <i>H. azteca</i> to 0.6, 1.3, 2.6 or 5.1 $\mu\text{g Cd/L}$ over 28 d. ....	91
<b>Figure 4.3.</b> Correlation between survival (A) and dry wt. (B) and whole body Cd burden in the <i>Hyaella</i> at d 28.....	92

**Figure 4.4.** Soft tissue Cd accumulation of *Lymnaea* at d 28 exposed to Cd. .... 93

**Figure 4.5.** Linkages between the chronic impact of Cd on in *H. azteca* and accumulation in *L. stagnalis*..... 94

## List of Tables

<b>Table 2.1:</b> Measured concentrations of total (unfiltered) and dissolved (0.45 µm filtered) Cd during the acute exposure.....	39
<b>Table 2.2:</b> Measured concentrations of total (unfiltered) and dissolved (0.45 µm filtered) during the chronic exposure of <i>Lymnaea</i> .....	39
<b>Table 3.1:</b> Measured concentrations of total (unfiltered) and dissolved (0.45 µm filtered) Cd during chronic test 1 (CT I).....	66
<b>Table 3.2:</b> Cd burden in the soft tissue of <i>Lymnaea</i> at d 28 of CT I.....	66
<b>Table 3.3:</b> Measured concentrations of total and dissolved Cd during (CT II)..	67
<b>Table 3.4:</b> The parameters for the exponential curve.....	67
<b>Table 4.1:</b> Measured concentrations of total and dissolved Cd during both <i>Hyaella</i> and <i>Lymnaea</i> chronic tests.....	95
<b>Table 4.2:</b> Cd burden in the whole body of <i>Hyaella</i> throughout chronic exposure.....	96

# **Chapter 1:**

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# **General Introduction**

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## 1.1 Cadmium

Metals are released to the earth's surface daily by natural processes (e.g. erosion) and from anthropogenic sources (e.g. mining and smelting operations). Canada, one of the largest mining nations in the world, produces approximately 60 minerals and metals (Pyle *et al.*, 2005) leading to the discharge of contaminants into the environment. Mining in Canada has caused elevated concentrations of metals, such as Cd, in surrounding aquatic systems (Pyle *et al.*, 2005). Cd is also released from man-made sources such as the smelting and refining of sulphide ores of zinc (Zn), lead (Pb) and copper (Cu; Norton *et al.*, 1990). The emissions from these anthropogenic processes may enter aquatic environments directly as effluents and/or through atmospheric deposition (Boudou and Ribeyre, 1997; Canadian Council of Ministers of the Environment (CCME), 1991). Cd is also used in commercial substances and therefore can be released during the lifecycle of a material or after final disposal. These sources include pigments, batteries (i.e. Ni-Cd), fertilizers, pesticides, PVC stabilizers, metal plating and also alloys (Benin *et al.*, 1999). The disposal of Cd-containing products/materials into waste streams accounts for the largest release of Cd into the environment (Nordic Council of Ministers, 2003), causing levels of Cd to increase dramatically from the naturally occurring background levels found in freshwater ecosystems (Hutton, 1983).

Cd is toxic and not required for any physiological functions of most organisms as it is a non-essential metal (Cœurdassier *et al.*, 2003). Unlike essential metals such as Cu, organisms do not have the ability to regulate Cd. At low levels of Cd exposure, adverse effects are seen on many aquatic organisms. For instance *Daphnia magna* has a lowest-observable-effect-level (LOEL) of 0.17 µg Cd/L and *D. pulex* a LOEL of 0.2 µg Cd/L



(Biesinger and Christensen, 1972 cited in CCME, 1991; Elnabarawy *et al.*, 1986 cited in CCME, 1991). These and other low-effect levels resulted in the CCME setting an interim water quality guideline for freshwater environments of 0.017 µg Cd/L (CCME, 1991).

## **1.2 Cd Bioavailability**

Metal toxicity to aquatic species depends on the amount of dissolved metal available in solution. The free ionic form ( $\text{Cd}^{2+}$ ) is the most toxic form of Cd in freshwaters, while the soluble complexes (with chloride, sulphate, carbonate or hydroxide) are less toxic (U.S. EPA, 2001). The toxicity is dependent on the bioavailability of Cd, which can be modified by the amount of dissolved organic carbon (DOC), the pH, and the hardness of water. DOCs contain negatively-charged functional groups which can bind Cd, decreasing the availability to aquatic organisms. The amount of free  $\text{Cd}^{2+}$  available to an organism is directly correlated to changes in pH and metal speciation (U.S. EPA, 2001). Of more importance to this study is water-hardness, essentially a measure of the concentration of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in solution, which has the most profound effect on Cd toxicity, with higher hardness values reducing toxic effects (U.S. EPA, 2001). Its protective effects are attributed to the competition between the two cations,  $\text{Cd}^{2+}$  and  $\text{Ca}^{2+}$ , which are similar in radius and charge (Adams and Meador, 2007). In this project, dissolved-Cd concentrations will be measured rather than total Cd concentrations as the former gives a better assessment of the potential risk in the ecosystem (Giesy *et al.*, 1977). Since water-hardness has such a profound impact on Cd

toxicity, the effects of Cd were monitored in three different water hardness values (20, 90 and 140 mg/L CaCO<sub>3</sub>) were conducted in this research project.

### **1.3 Tissue Residue Approach (TRA)**

The Tissue Residue Approach (TRA) is a complementary but newer approach than the Biotic Ligand Model (BLM; Di Toro *et al.*, 2001) for ecological risk assessment of metals as both rely on accumulation-effect relationships (as opposed to the traditional exposure-effect relationship). The former provides a true measure (internal tissue metal concentration) while the latter is a surrogate measure (external water metal concentration) of received dose at the site of toxic action. The TRA is based on the relationship between internal metal concentrations and toxicological response (Meador, 2006). Meador (2006) states there are advantages of TRA for toxicity assessment and conversion. First, critical body residues show a lower variability for many contaminants. Second, toxicodynamics (toxic potential) is more reliable for characterizing biological responses than uptake. Third, the TRA model presents a relationship between tissue residues and adverse biological effects to help in generating tissue, water, or sediment guidelines (Meador, 2006). TRA is also a powerful tool for linking the evidence from laboratory tests to data gathered from field data at potentially contaminated field sites. In general, TRA may be able to provide an improved and scientifically sound approach to understanding contaminant impacts and as a result contribute to biodiversity and ecosystem protection. Therefore, it is important to understand the patterns of metal accumulation in animals with different sensitivities and to investigate their diverse biological responses to these

metals. This will further aid in the development of a TRA model for metal risk assessment. Thus, this approach will be used in this project with identifying the toxic effects Cd has on organisms.

#### **1.4 Bioaccumulation of Cd**

Bioaccumulation varies greatly amongst freshwater organisms, since an organism's ability to accumulate Cd is based on its uptake and elimination capacity. The importance of uptake was demonstrated by Buchwalter and Luoma (2005) who conducted an experiment using several aquatic insects, studying their ability to take up Cd from the environment. Although there was a great variability amongst the tested species with respect to the rate of Cd uptake, Buchwalter and Luoma (2005) determined that the number of transporters had a greater influence on Cd uptake rate than the transporter's affinity for the metal, showing that bioaccumulation varies within aquatic organisms. Therefore, it is necessary to measure Cd concentrations in an organism's tissue to verify whether or not different concentrations of environmental Cd will have an effect on Cd bioaccumulation.

#### **1.5 Sub-cellular Fractionation**

Sub-cellular fractionation is a useful/highly characterized, widely employed technique whereby a tissue homogenate is broken up into many different functionally defined fractions (Wang and Rainbow, 2006). This method has recently been applied to

understand bioaccumulation patterns as it pertains to mechanisms of toxicity (Wallace *et al.*, 2003; Buchwalter *et al.*, 2007). It was used, for example, on two different bivalve organisms and effectively separated metal-sensitive cellular components from the biologically metal-detoxifying cellular components (Wallace *et al.*, 2003). Five cellular fractions were defined with that technique: cellular debris, cellular organelles (mitochondria, microsomes and lysosomes), metal-rich granules, cytosolic proteins denatured by heat, and heat-stable cytosolic proteins composed primarily of metallothionein-like proteins (MTLP; Wallace *et al.*, 2003). The metabolically sensitive (active) pools, consisting of the cellular organelles and heat-denatured proteins, are those that may be impaired by Cd accumulation, whereas the metabolically detoxified (inactive) pools, comprised of the remaining fractions, the granules, heat-stable proteins and cellular debris, are those that reduce the freely available Cd in cells (Buchwalter *et al.*, 2007; Wallace *et al.*, 2003). Kraemer and colleagues (2005) hypothesized about the spill-over hypothesis that proposes that when accumulated metal (i.e. Cd) designated for storage in a detoxified form exceeds the detoxified binding capacity (for example, MTLP), the metals can subsequently bind to metabolically active forms, with the potential to cause toxicity to an organism. This hypothesis predicts metal detoxification would be more efficient at low exposure concentrations, whereby metal sensitive fractions are protected. If the exposure concentration were to exceed the threshold, the binding capacity would be overwhelmed and metals would spill over into the metal sensitive fraction (Kraemer *et al.*, 2003). Therefore, sub-cellular fractionation helps in the understanding of accumulated metal distribution at a cellular level. This may help in linking accumulation to sub-lethal or lethal effects in an organism.

## **1.6 *Hyalella azteca***

*H. azteca* (Crustacea: Amphipoda) is a freshwater amphipod found in bodies of water within Canada with a temperature of 10 °C or greater, which is ideal for breeding Canada (Environment Canada, 1997). Adults can range from 6-8 mm in length. They live at the sediment-water interface and feed on detritus vegetation.

*Hyalella* are easy to culture in lab with the addition of bromide ions in the media, as it helps the organism use calcium (Borgmann, 2002). Adults reach maturity in a short time (40 d) and can reproduce in the laboratory, producing over 3 – 5 times their population. Toxicology testing is usually performed on the most sensitive life stage (2 - 9 d olds neonates). *Hyalella* are known to be sensitive to many metals including Cd in a range of water chemistries. Cd exposure tests resulted in a 7 d LC<sub>50</sub> of 0.57 µg Cd/L for soft water (18 mg/L as CaCO<sub>3</sub>; Borgmann *et al.*, 2005). The LC<sub>50</sub> increased with increasing water hardness with a 7 d LC<sub>50</sub> of 4.41 µg Cd/L in moderately hard water (124 mg/L as CaCO<sub>3</sub>; Borgmann *et al.*, 2005).

## **1.7 *Lymnaea stagnalis***

The great pond snail, *Lymnaea stagnalis* (Pulmonata: Gastropoda), is common in the freshwater environments. They are found in slow moving water in the northern hemisphere, in many countries such as Canada, United States, England and throughout Europe (Boag and Pearlstone, 1979). The great pond snail has a large head with long flattened tentacles and a rasping tongue, the radula, to feed on both plant and animal matter. In laboratory conditions, the life cycle of these snails are 14 months and reach

reproductive maturity in 4 months when at a constant temperature of 20 °C (Boag and Pearlstone, 1979). In addition, if held at a suitable temperature, these snails can reproduce continually all year around in the lab (Boag and Pearlstone, 1979). They lay large gelatinous egg-masses between three or four cm in length and that contain as many as 50 to 120 eggs. The great pond snail often surfaces to take in air into a respiratory cavity, however if there is no access to the surface, the snails are able to take in oxygen from the water through their skin. Also, their tentacles are covered with cilia which increase their surface area, thus increasing the intake of air (Boag and Pearlstone, 1979).

*L. stagnalis* are sensitive to many aquatic contaminants. Grosell and colleagues (2006) chronically exposed *Brachionus calyciflorus*, *Chironomus tentans* and *L. stagnalis* to Pb and the results showed LOEC of 284, 497 and 16 µg Pb/L, respectively. The sensitivity of the snails to Pb led to a 20 % effect concentration (EC<sub>20</sub>) of < 4 µg Pb/L. Similarly, early life stages of snails have shown to be sensitive to chronic Pb by resulting in an LC<sub>50</sub> of 19 µg Pb/L (Borgmann *et al.*, 1978).

*L. stagnalis* accumulate many metals such as Co, Cd and Cu (De Schamphelaere *et al.*, 2008; Cœurdassier *et al.*, 2003; Croteau *et al.*, 2007). Metal accumulation may cause disturbance of ionoregulatory functions and as a result, their survival and embryonic development are impacted (Grosell *et al.*, 2006). Moreover, these snails require high levels of Ca for shell formation and growth, and might be sensitive to metal exposure especially when the metal interferes with Ca homeostasis (Grosell *et al.*, 2006). De Schamphelaere *et al.* (2008) chronically exposed the snails to Co, which led to a reduction in growth at 79 µg Co/L and higher concentrations. At the end of the exposure, reduction of Ca levels in the haemolymph was seen. The metal accumulation

characteristics and sub-lethal effects (such as growth and shell development) of metals to this snail make this species an ideal model for ecological risk assessment.

## 1.8 Objectives

The goal of the project is to enhance our knowledge on the effects of Cd to the great pond snail, *L. stagnalis*. Studies are focused on developing and improving the understanding of Cd accumulation in these organisms and identifying how water chemistry influences the impacts of Cd. Finally, the overall goal is to determine the viability of bioaccumulation as an indicator of Cd effects in aquatic invertebrates. To accomplish these goals, the following objectives have been identified:

- 1) To establish the toxicological endpoints of acute and chronic waterborne Cd on *L. stagnalis*;
- 2) To compare toxicological endpoints at three water hardness values (21, 90, and 140 mg/L CaCO<sub>3</sub>);
- 3) To determine Cd bioaccumulation patterns at whole organism and sub-cellular levels in *L. stagnalis*;
- 4) To test a novel approach for bioaccumulation, using bioaccumulation in a Cd resistant organism (*L. stagnalis*) and link it to impacts encountered in a sensitive organism (*H. azteca*).

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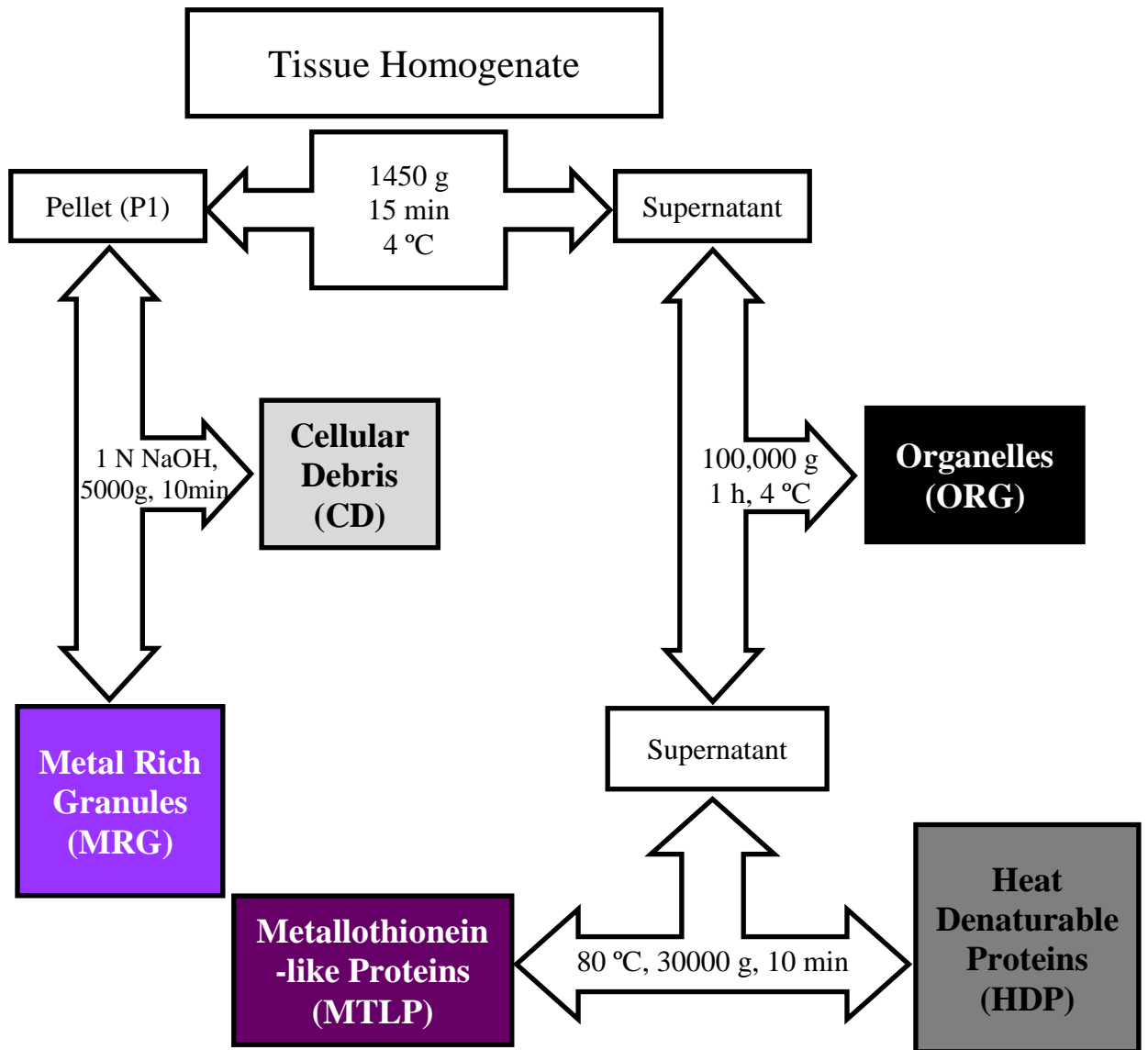


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## 1.10 Figures



**Figure 1.1.** Procedure for determining the sub-cellular fractionation of metal within invertebrates. Tissues were homogenized and centrifugation and digestion techniques were used to obtain the following sub-cellular fractions: metal rich granules, cellular debris, organelles, heat denaturable proteins and metallothionein-like proteins. The metal sensitive fractions (MSF) are in grey or black boxes, and the detoxified fractions (DF) are in purple. This schematic is adapted from Wallace *et al.*, 2003.

## **Chapter 2:**

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# **Acute and Chronic Toxicity of Waterborne Cd to *Lymnaea stagnalis* in Hard Water**

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## 2.1 Abstract

The overall goal of this study is to improve the understanding of Cd accumulation in *L. stagnalis*. Objectives of this study were to understand the relationship between bioaccumulation in an exposure and impacts of Cd exposure on the freshwater snail, *L. stagnalis*. Acute toxicity tests (96 h, static-renewal with no feeding) were conducted in moderately hard water (140 mg/L as CaCO<sub>3</sub>) using two sizes of snails (18 or 25 mm). The 96 h LC<sub>50</sub> for both sizes of snails were similar (350 µg Cd/L, 95 % CI: 148 - 590 µg Cd/L) and far above environmentally relevant concentrations. Bioaccumulation in the two sizes of snails were similar and showed to be dose dependent and accumulated linearly. However, the tissue bioaccumulation was 50 – fold higher than the shell accumulation. Ca and Na levels in the soft tissue of snails were not significantly altered compared to the controls. Subsequent chronic toxicity testing (31 d, static with daily renewal) was performed using three sizes of snails (initial shell lengths of 5, 10 or 15 mm). In these tests, growth and survival were assessed and feeding was characterized at regular intervals. Exposures of > 30 µg Cd/L resulted in 100 % mortalities in the smaller snails while medium and large snails were more resilient. Growth was inhibited at exposure concentrations greater than 10 µg Cd/L and this resulted from reduced food consumption.

## 2.1 Introduction

Pulmonate freshwater snails such as *L. stagnalis* are found in lentic systems where they play an important role in the consumption and decomposition of aquatic plants (Barnes, 1987). *Lymnaea* have not been used extensively as an aquatic test organism, and there is no standard method for testing this invertebrate. However, it has been found that *L. stagnalis* are sensitive to aquatic contaminants, such as Pb and Cu, and therefore can serve as a good model for understanding the potential for environmental impacts. *L. stagnalis* accumulate many metals such as Co, Pb, Cd and Cu (De Schamphelaere *et al.*, 2008; Cœurdassier *et al.*, 2003; Croteau *et al.*, 2007). Moreover, these snails require high levels of Ca for shell formation and growth, and might be sensitive to metal exposure especially when the metal interferes with Ca homeostasis (Grosell *et al.*, 2006).

In a comparison of the sensitivity of three species (*Brachionus calyciflorus*, *Chironomus tentans* and *L. stagnalis*) to chronic Pb exposure Grosell *et al.* (2006) showed that *Lymnaea* were much more sensitive. The EC<sub>20</sub> was less than 4 µg Pb/L. Newly hatched snails exhibited reduced growth and physiological evidence has demonstrated that Ca influx in the snails was inhibited (Grosell and Brix, 2009), resulting in lower soft tissue Ca concentrations that may in turn lead to reduced CaCO<sub>3</sub> precipitation and reduced shell formation. The high Ca uptake by the snails likely explains their hypersensitivity to Pb. This sensitivity to Pb was also been shown by Borgmann *et al.* (1978). However, *Lymnaea* do not seem to be as sensitive to cadmium, often considered to be another calcium antagonist, although high concentrations can inhibit their growth (Cd 31 d EC<sub>50</sub> of 142 µg Cd/L; Cœurdassier *et al.*, 2003).

The accumulation of many metals has been demonstrated with *Lymnaea*, for example Co, Cd and Cu (De Schamphelaere *et al.*, 2008; Cœurdassier *et al.*, 2003; Croteau *et al.*, 2007). As with Pb, metal accumulation may cause disturbance of ionoregulatory functions and as a result, their survival and embryonic development are impacted. These snails' high Cd requirement for shell formation and growth likely explains their sensitivity to metals that are known to disrupt Ca homeostasis such as Pb and Cd (Grosell *et al.*, 2006). De Schamphelaere *et al.* (2008) chronically exposed the snails to Co, which led to a reduced growth rate at exposure concentrations at and above 79 µg Co/L. This reduced growth was associated with reduced Ca in the haemolymph.

The goal of this study was to improve the understanding of waterborne Cd toxicity and accumulation in *L. stagnalis*. Acute (96 h) and chronic (31 d) static renewal toxicity tests were done in moderately hard water with survival, Cd accumulation, tissue Na and Ca content, weight and shell length as endpoints. These parameters were assessed in three different sizes of snails to determine the influence of size (age) on Cd sensitivity.

## **2.3 Materials and Method**

### **2.3.1 Snail Culture**

Adult *L. stagnalis* were very generously provided by Dr. D. Spafford of the University of Waterloo. Adults are maintained in 10 L aquaria that were in an aerated, re-circulating system (Aquatic Habitats, Apopka, FL), while the juvenile were placed in static 10 L aquaria. They were maintained in a mixture of dechlorinated Waterloo city tap



water and reverse osmosis water that gave concentrations of  $\text{Na}^+$  at  $840 \pm 36 \mu\text{M}$ ,  $\text{Ca}^{2+}$  at  $1560 \pm 122 \mu\text{M}$ ,  $\text{Mg}^{2+}$  at  $560 \pm 23 \mu\text{M}$  with a pH of  $7.3 \pm 0.1$  and temperature of  $20 \pm 1$  °C (mean  $\pm$  SEM; n = 45). They were kept under a photoperiod of 16 h light: 8 h dark and fed locally purchased romaine lettuce and Nutrafin™ Max pellets *ad libitum*. Aquaria with adults were cleaned with a siphon to remove excess feces and filters were rinsed from debris re-entering the circulating system. Once a month, egg masses were removed from adult aquaria and placed in static aquaria. To minimize the disturbance to the animals the water flow was suspended and 50 % of the culture medium renewed weekly. This produced groups of snails of similar age and size (i.e.  $5 \pm 0.02$  mm,  $10 \pm 0.1$  mm or  $15 \pm 0.2$  mm) and these were subsequently used for toxicity tests.

### 2.3.2 General Experimental Procedure

The exposure media was made with  $\text{CaSO}_4$ ,  $\text{MgSO}_4$ ,  $\text{NaHCO}_3$  and KCl (Sigma – Aldrich, Mississauga, ON) with a final concentration of  $\text{Na}^+$   $733 \pm 13 \mu\text{M}$ ,  $\text{Ca}^{2+}$   $995 \pm 45 \mu\text{M}$ ,  $\text{Mg}^{2+}$   $341 \pm 26 \mu\text{M}$ , pH  $7.3 \pm 0.05$ ,  $21 \pm 0.3$  °C (mean  $\pm$  SEM, n = 45), a hardness of 130 mg  $\text{CaCO}_3/\text{L}$  and this was designed to mimic Lake Ontario water. Snails were transferred to test conditions (without added Cd) 48 h prior to beginning tests for acclimation period and were fasted to avoid the accumulation of feces in the test waters. This was to prevent the binding of Cd to the particles. Polyethylene 1-L beakers were acid-washed in 20 % nitric acid and rinsed with deionized water and ultrapure water (18.2 M $\Omega$ , Milli-Q) multiple times before use. A control beaker was prepared with 1L of media and was left to equilibrate for 24 h. For each concentration, a primary Cd stock solution (0.1g Cd/L as  $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$ ;  $\geq 98$  % purity, VWR International, Mississauga,

ON) was diluted to 1 L with exposure media, then left in the beaker for 1 d to attain equilibrium.

To begin a test, individual snails were gently blotted with a kimwipe to remove excess moisture, weighed and then placed into exposure beakers (d 0). Exposure waters were renewed every 48 h and water samples (10 mL) were collected prior to and after renewal. Both total (non-filtered) and dissolved (0.45  $\mu\text{m}$  filtered; Acrodisc HT Tuffryn, Pall, Ann Arbor, MI) samples were collected and acidified to 1 % with  $\text{HNO}_3$  (Trace metals grade, Fisher Scientific, Nepean, ON) for subsequent Cd, Ca, Mg, and Na measurements. Survival was monitored by touching the foot of the snails with a blunt dissecting probe and the lethality endpoint was defined by a lack of response to this stimulus (Ng *et al.*, 2011; Croteau *et al.*, 2007). All surviving snails at the end of time-point were rinsed with ultrapure water for 10 minutes, gently blotted with a kimwipe to remove excess moisture and then stored at  $-80\text{ }^\circ\text{C}$ . Later, they were separated from shell and were digested separately for measurements of Cd-bioaccumulation and Ca, Na concentrations in the soft tissues.

Acute (96 h) tests were conducted with two sizes of snails,  $18 \pm 0.1$  and  $25 \pm 0.2$  mm (mean  $\pm$  SEM; n = 20) shell length. Two chronic (31 d and 28 d) tests were conducted. The first chronic test was used to determine an  $\text{LC}_{50}$  for survival and growth using 3 different snail sizes (initial shell lengths of  $5 \pm 0.2$ ,  $10 \pm 0.1$  and  $15 \pm 0.2$  mm; mean  $\pm$  SEM, n = 20). The second test was used to monitor the time course of Cd bioaccumulation throughout a chronic exposure and this was done using only  $5 \pm 0.02$  mm shell length snails.

### 2.3.3 Acute Test (AT)

An AT was run for *L. stagnalis* (96 h LC<sub>50</sub> test). This study was to determine a 96 h LC<sub>50</sub> for Cd in the snails and to understand if sensitivity varies with size (or age). The nominal concentrations of Cd used for this test were 0, 50, 100, 200, 400, 800 and 1600 µg Cd/L. Ten juvenile snails with a shell length of either 18 ± 0.1 mm or 25 ± 0.2 mm (mean ± SEM, n = 20) were exposed to each concentration. The snails were not fed during the experiment and survival was monitored every d as tissue samples were collected after 96 h as described above.

### 2.3.4 Chronic Test (CT)

The first chronic test was done with 10 snails (initial shell length either 5, 10, 15 mm) per exposure concentration (nominal: 0, 3, 10, 30, 100 µg Cd/L) for 31 d. The artificial Lake Ontario water was made by mixing well water and reverse osmosis water to establish a water hardness of 140 mg/L of CaCO<sub>3</sub>. Snails were fed lettuce *ad libitum* in each beaker every 48 h when the Cd solution was renewed. On d 10, 17, 24 and 31 d the wet wt. and length of each snail was measured. Tissues were collected from surviving snails and analyzed for Na, Ca and Cd concentrations (See below). In the second chronic exposure snails with a 5 ± 0.02 mm shell length were exposed for 28 d to determine the time-course of Cd accumulation.

### 2.3.5 Analysis

Exposed snails were thawed and soft tissue was separated from shell. Samples were dried at 80 °C for 48 h (previously determined to result in a constant dry wt.),

weighed and placed in 2.0 mL eppendorf tubes. Concentrated nitric acid (trace metals grade, Fisher Scientific, Whitby, ON) was added (1: 5, wt.:v; Janes and Playle, 1995) and samples were digested at 65 °C for 48 h. Shells were similarly digested in concentrated nitric acid (1:3, wt.: v). Samples were mixed by vortexing and then centrifuged at 5000 g for 10 min and the supernatant was subsequently collected followed by appropriate dilution in 1 % HNO<sub>3</sub> before measurement for Cd by means of atomic absorption spectrophotometry (AAS, SpectAA-880 with GTA100 graphite furnace (GF), Varian Inc., Palo Alto, CA). Water samples were also characterized for Cd content using GFAAS. Tissues and water samples were quantified for Ca, Na and Mg using AAS in flame mode. Certified reference materials (TM 28.3 and TM 26.5 National Water Research Institute, Environment Canada) were used for internal quality checks and recovery was always within 15 % of certified values.

### 2.3.6 Statistical Analysis and LC<sub>50</sub> Calculation

Data have been presented as means  $\pm$  1 standard error of the mean. For the AT and the CT the 96 h LC<sub>50</sub> and the 31 d LC<sub>50</sub> values with 95 % confidence intervals were calculated using the measured dissolved Cd concentrations and the Comprehensive Environmental Toxicity Information System (CETIS v1.6.1. rev C Tidepool Scientific Software, McKinleyville, U.S.A.) software. One-Way ANOVA was used to test effects of Cd to *Lymnaea*. Following the significant result, the Fisher LSD post hoc test was used to identify group differences. Linear regression analyses were performed to describe relationships between survival or length and Cd burden. Significance of all tests was taken at  $p < 0.05$ .

## 2.4 Results

### 2.4.1 AT

Total and dissolved Cd concentrations were measured and there was no significant difference between paired samples (Table 2.1). In moderately hard water (hardness = 140 mg/L CaCO<sub>3</sub>) the patterns of mortality were established (Fig 2.1 A & B). A 96 h LC<sub>50</sub> values for the medium snails (18 ± 0.1 mm) and large snails (25 ± 0.2 mm) were 357 (95 % CI: 159 - 590) and 347 (148 - 503) µg Cd/L, respectively. The lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC) were not significantly different for both medium and large snails (LOECs of 638 and 618 µg Cd/L and NOECs at 321 and 308 µg Cd/L, respectively). In both size snails, the two highest treatments (628 and 1301 µg Cd/L) showed 90 - 100% mortality by the end of the exposure.

At the end of 96 h, the surviving snails were measured for Cd burden. Increased levels of waterborne Cd corresponded to an increase in Cd burden in soft tissue (Fig 2.2 A). Similar increase in Cd burden was seen in both medium and large snails. Cd burden in the shells were also measured and similar patterns to the soft tissue burden were seen, however, not to the same magnitude. In similar treatments, while soft tissue Cd burdens reached as high as 750 µg Cd/g dry wt., Cd burden in shells only reached 10 µg Cd/g dry wt. (Fig 2.2 B). Further analysis of the soft tissue was conducted to evaluate ion levels in the snail (Fig 2.3 A & B). Both Ca and Na levels in the soft tissue were measured and showed no significant differences between control and exposed snails in either medium or large snails.

### 2.4.2 CT

Total and dissolved Cd concentrations were measured. For dissolved, before renewal (snails exposed for 48 h) and after renewal (not exposed to snails) measurements were taken. There were no significant differences between total and dissolved (Table 2.2). Also, no significant differences between before and after measurements, with < 20 % decrease within 48 h. In moderately hard water, the 31 d LC<sub>50</sub> values and confidence intervals (CI) for the small snails ( $5 \pm 0.02$  mm), medium snails ( $10 \pm 0.1$  mm) and large snails ( $15 \pm 0.2$  mm) were 12.8 (CI: 5 – 34), 49.7 (CI: 35 – 74) and 45.7  $\mu\text{g Cd/L}$  (CI: 38 – 54), respectively (Fig 2.4 A, B & C). This is much lower than the acute tests. For the small snails, 100% mortality was seen within 17 d of treatment in the highest concentration, however, not seen in the other two size snails until d 24, this lead to a much higher LC<sub>50</sub> for the medium and large snails.

Growth in both length and wt. was also measured during the exposure for all three size snails. With the small snails, length (Fig 2.5 A) and wt. (Fig 2.6 A) were similar to the control in the lower treatments. However, in 30  $\mu\text{g Cd/L}$  treatments, both length and wt. stayed relatively the same as when the exposure started, showing no growth during the 31 d. However, this trend was not seen in either of the medium (Fig 2.5 & 2.6 B) and/or large snails (Fig 2.5 & 2.6 C) treatments. The controls and the exposed concentrations increased in both length and wt. at the same rate in these treatments.

Moreover, at the end of 31 d, the surviving snails were measured for Cd burden. Increased levels of waterborne Cd corresponded to an increase in Cd burden in soft tissue (Fig 2.7). Shell analysis for Cd indicated an increase in Cd burden in the shells with

increasing concentrations. Similar to the 96 h test, the shell Cd burden was 10 folds less compared to that of tissue Cd burden (Fig 2.8). Furthermore, Ca and Na ion levels were also measured in the soft tissue and showed no significant change at the varying concentrations compared to the controls (Fig 2.9 A & B).

## **2.5 Discussion**

### 2.5.1 AT

The Cd 96 h LC<sub>50</sub> was seen as 356.6 (95 % CI: 159 – 590) and 346.5 µg Cd/L (CI: 148 – 503) in medium and big snails, respectively. These LC<sub>50</sub>s were not significantly different from each other, showing little difference between these two sizes in snails. Also, it demonstrates that adult pond snails are not particularly sensitive to Cd. Das and Khangarot (2010) tested the waterborne Cd effect on similar sized pond snails of the same genus, *Lymnaea*. Using the Indian pond snail *L. luteola*, the researchers found the 7 d LC<sub>50</sub> was 496 µg Cd/L, when the underlying exposed medium had a hardness of 230 mg/L CaCO<sub>3</sub>. This illustrates that the genus *Lymnaea* are resistant to the toxic effects of waterborne Cd.

The Cd burden in the soft tissue was seen as dose-dependent and there was no difference among the two sizes of snails. In both groups, the controls showed 7 µg Cd/g dry wt. of Cd, however, Cd burden reached as high as 750 – 800 µg Cd/g dry wt. in the soft tissue of the snails exposed to 628 µg Cd/L. Ca levels in the soft tissue of the exposed snails were not significantly different in the medium or large snails compared to

the controls, even though Cd is a Ca antagonist. This similar trend was seen in whole body Na concentration as well. Therefore, Cd burden in the soft tissue accumulated but did not appear to have an effect on ion homeostasis levels in the soft tissue.

The Cd burden in the shell accumulated in a similar pattern to the soft tissue burden in the snail. It increased in a dose dependent manner, however, the highest exposure concentration did not elicit as high of a Cd burden in the shell as seen in the soft tissue of the snails. The highest exposure concentration (628 µg Cd/L) showed a 10 – 12 µg Cd/g dry wt. of Cd in the shells, while the soft tissue Cd burden reached 800 µg Cd/g dry wt. at the same concentration.

### 2.5.2 CT

The Cd 31 d chronic test resulted in a LC<sub>50</sub> of 12.8 (95 % CI: 4.8 – 32), 49.7 (33 – 75) and 45.7 µg Cd/L (39 – 53) in small (5 ± 0.02 mm), medium (10 ± 0.1 mm) and big snails (15 ± 0.2 mm), respectively. The small snails displayed 100 % mortality within 17 d of exposure at the highest exposure concentration and the LC<sub>50</sub> was 4 folds lower than the medium and large snails. The medium snails had a NOEC (survival endpoint) of 30 µg Cd/L; however had 100 % mortality at the next highest exposure (100 µg Cd/L). This resulted in a similar LC<sub>50</sub> compared to the large snails. This shows that the small (younger) snails are significantly more sensitivity to Cd compared to the older ones.

In the small snails, a growth effect was seen as a reduction in shell length and body wet wt. at 30 µg Cd/L. At 17 d, there was a significant increase in growth in the small snails. The shell length increased from 5 to 15 mm, while the body wt. increased from 0.04 g to 0.3 g seen in the controls and exposed snails > 30 µg Cd/L. At the end of



the exposure, control snails were  $15 \pm 0.2$  mm in length and  $0.3 \pm 0.04$  g. However snails exposed to  $30 \mu\text{g Cd/L}$ , were much smaller at  $7 \pm 0.1$  mm in length and with a wt. of  $0.04 \pm 0.02$  g. In the medium snails, all the exposed snails grew at the same rate as the controls, reaching a shell length of  $20 \pm 0.5$  mm (initial shell length  $10 \pm 0.1$  mm). The wt. also increased similar in the exposed snails to  $0.6 \pm 0.09$  g. The large snails showed a similar trend, where controls and exposed snails both reached similar length and wt.,  $24 \pm 0.4$  mm and  $1.0 \pm 0.07$  g, respectively.

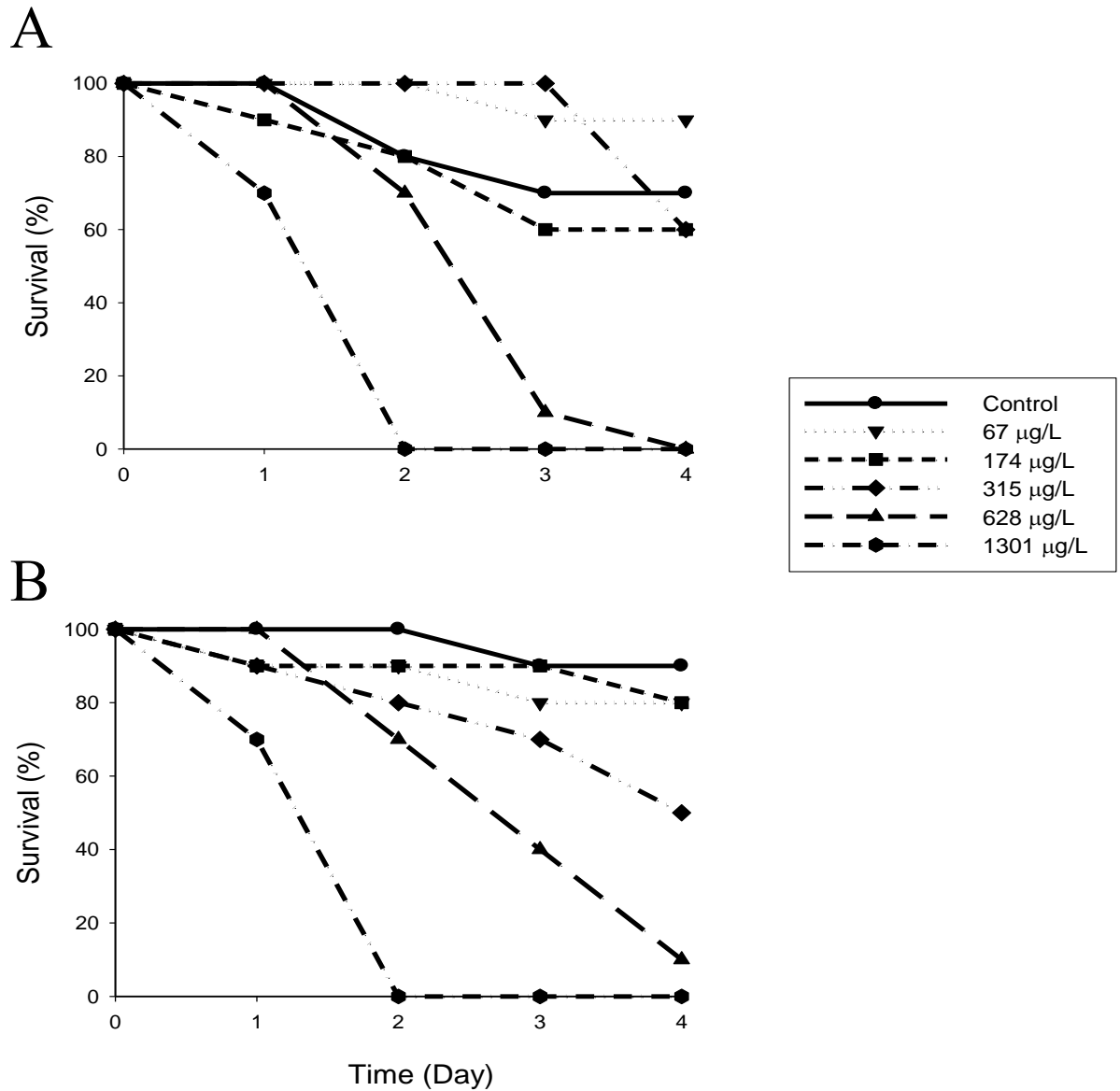
The results of this experiment demonstrated that adult snails (medium and large) are resistant to waterborne Cd exposure at the concentrations used in this study. Chronic  $\text{LC}_{50}$  values were much lower than acute values with a ratio of 7.4. The sensitivity of *Lymnaea* increases dramatically in small snails. This increased sensitivity in small snails may be due to the mechanisms of toxicity for Cd in snail, possibly influencing growth and development rather than processes such as reproduction. Energy is primarily focused on growth for the smaller snails while in the medium and large snails, where sexual maturity is approaching, the focus for energy is directed towards reproduction and (apparently) detoxification. These mechanisms of Cd toxicity in *Lymnaea* are unknown but would be interesting to study but do not appear to be related to disruption of ionic balance (e.g. Ca and Na). This is contrary to expectation for Cd, a known disruptor of Ca homeostasis (Grosell *et al.*, 2006). Only small snails showed a disruption of ion balance in soft tissue however it was an increase rather than a decrease in concentrations. This is opposite to expectations and awaits future studies for explanation.

## 2.6 References

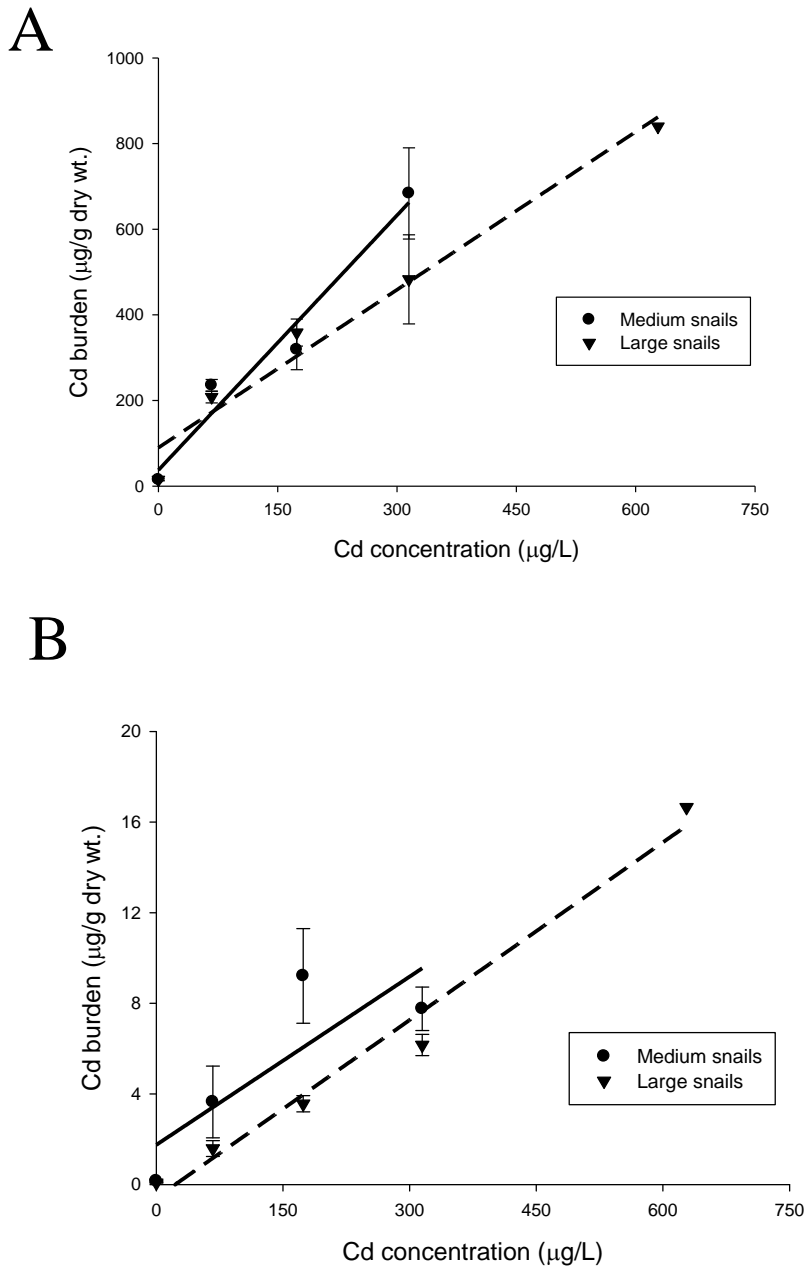
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## 2.7 Figures

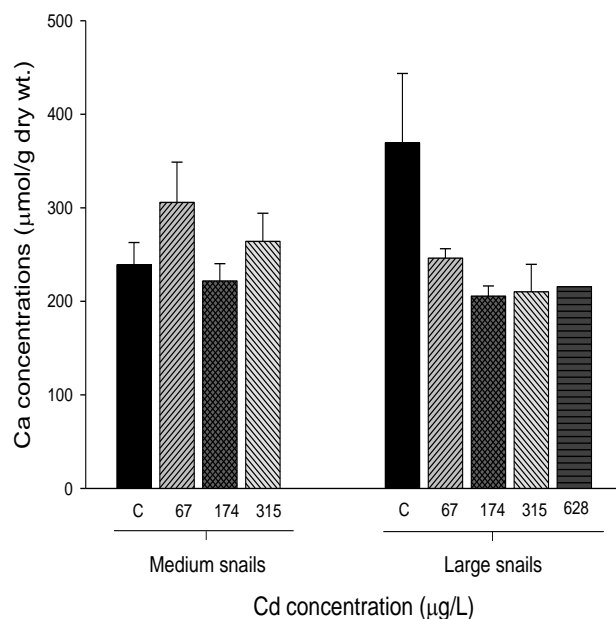


**Figure 2.1.** The effect of waterborne Cd on survival of *L. stagnalis* over 96 h of exposure to either 65, 174, 315, 628, 1301 µg Cd/L. Panel (A) show snails of  $18 \pm 0.1$  mm length (mean  $\pm$  SEM,  $n = 10$ ) and (B) shows  $25 \pm 0.2$  mm length ( $n = 10$ ). A group of unexposed snails (controls) are also included. The 96 h LC50 values were 357 (95 % CI: 159 – 590) and 347 (148 – 503) µg Cd/L for 18 mm (A) and 25 mm (B) snails, respectively.

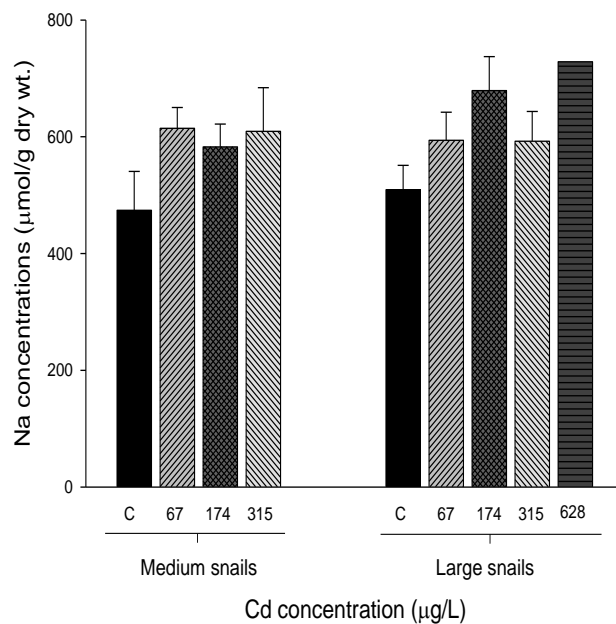


**Figure 2.2.** Mean soft tissue (A) concentrations ( $\mu\text{g Cd/g dry wt.} \pm \text{SEM}$ ) and mean shell (B) concentration ( $\mu\text{g Cd/g dry wt.} \pm \text{SEM}$ ) for Cd in *L. stagnalis* after 96 h of exposure to waterborne Cd. Exposure concentrations were 67, 174, 315 or 628  $\mu\text{g Cd/L}$  (with unexposed controls) and two groups of snails are shown, 18 mm in length (filled circles with solid line,  $18 \pm 0.1$  mm (mean  $\pm$  SEM)) and 25 mm in length (inverted triangles with dashed line,  $25 \pm 0.2$  mm). Lines show the best fit linear regression (see text for details) and for each mean  $n = 6 - 9$  except for the 315  $\mu\text{g Cd/L}$  exposed groups where for both 18 and 25 mm snails  $n = 5$ .

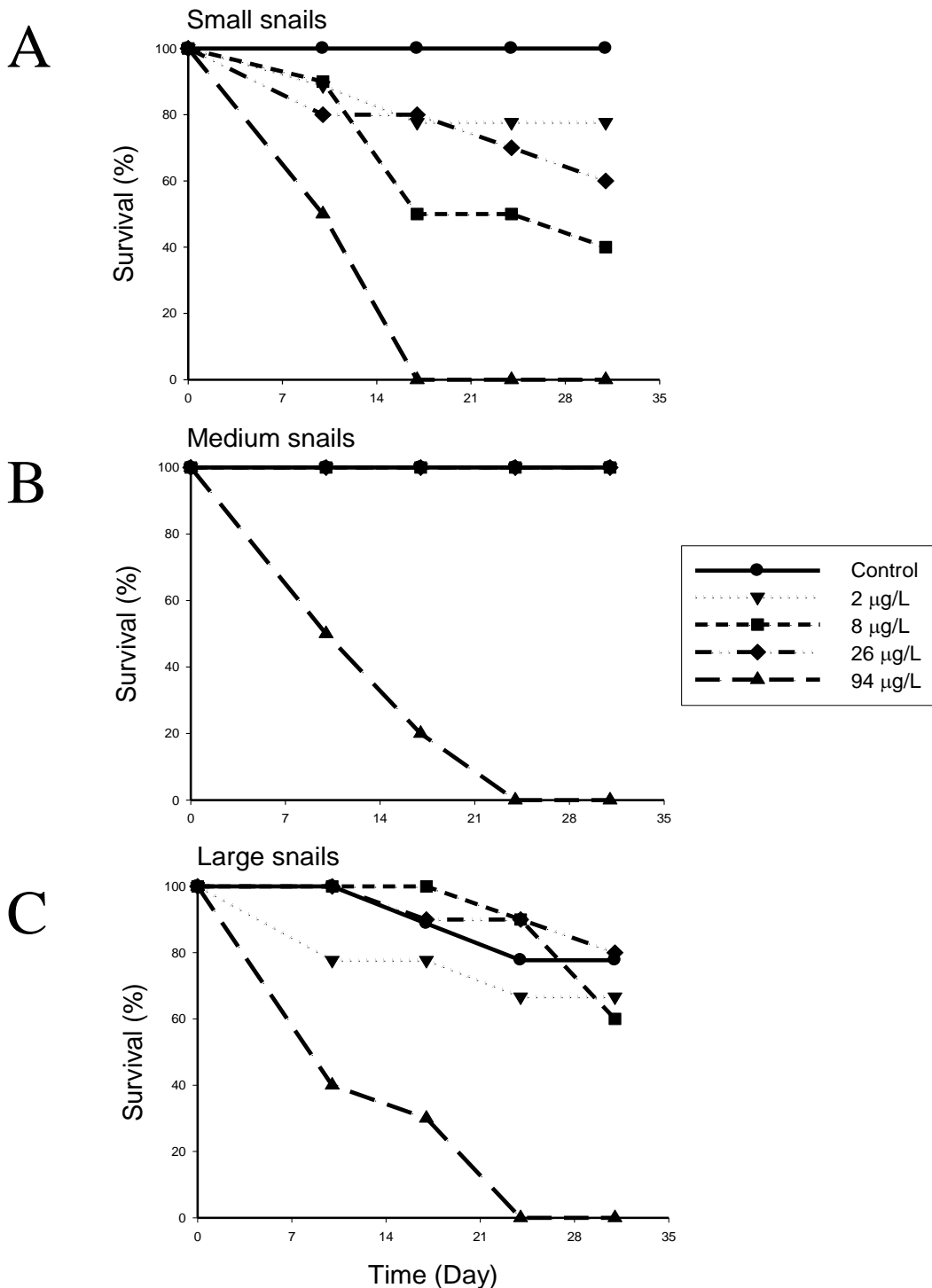
A



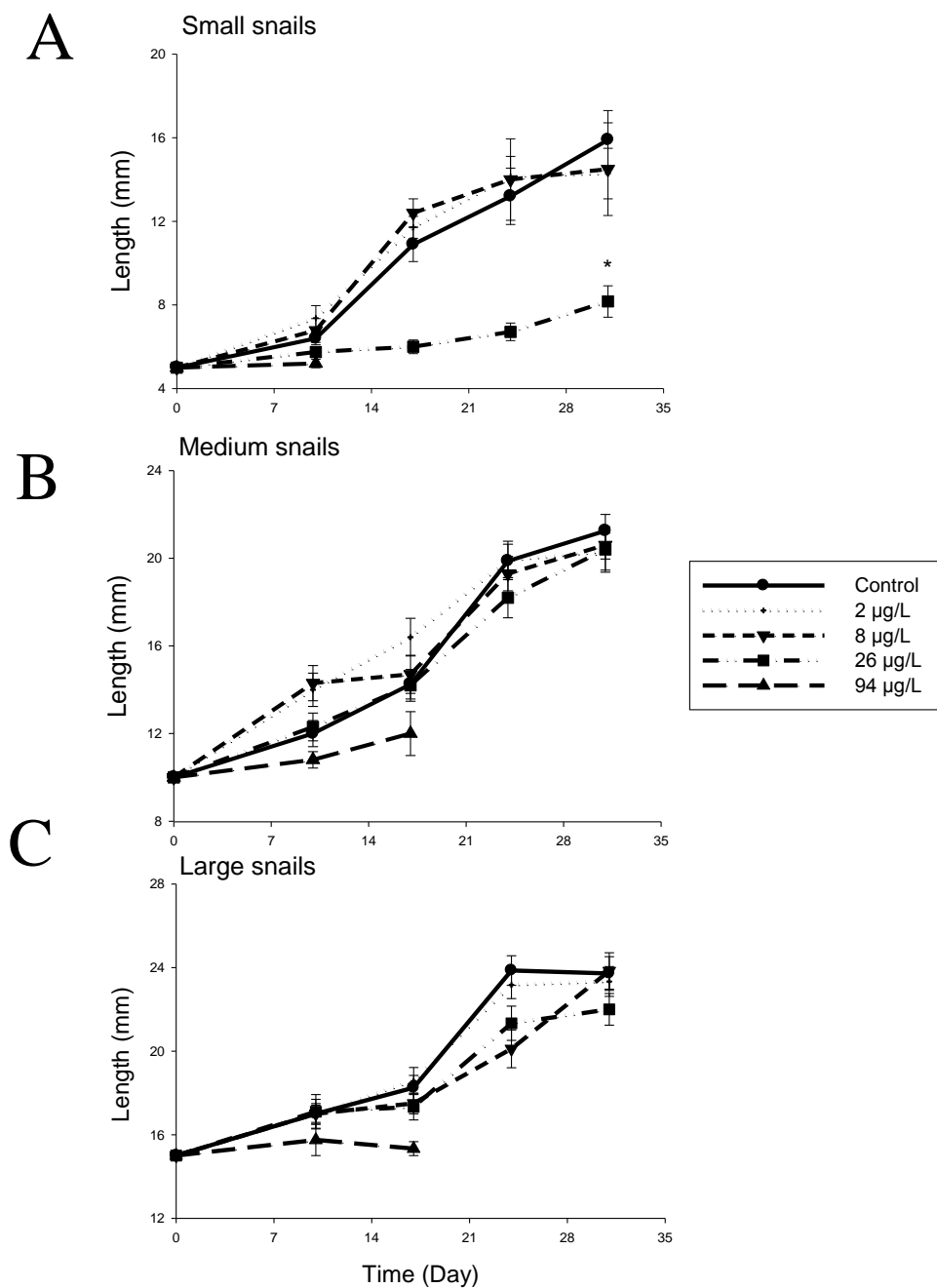
B



**Figure 2.3.** The acute effects of waterborne Cd on soft tissue  $\text{Ca}^{2+}$  (A) and  $\text{Na}^+$  (B) concentrations in *Lymnaea* at 96 h. Control groups are represented by a solid black bar and exposed Cd groups are shown with hatching gray bars. Values are mean  $\pm$  SEM ( $\mu\text{mol/g dry wt.}$ ; No SEM value for Cd concentration 628  $\mu\text{g Cd/L}$  is provided because only 1 survivor). For medium and large snails, the n values were Control n = 7, 9; 67  $\mu\text{g Cd/L}$  n = 9, 8; 174  $\mu\text{g Cd/L}$  n = 6, 8; 315  $\mu\text{g Cd/L}$  n = 5, 5; 628  $\mu\text{g Cd/L}$  n = 0, 1, respectively.

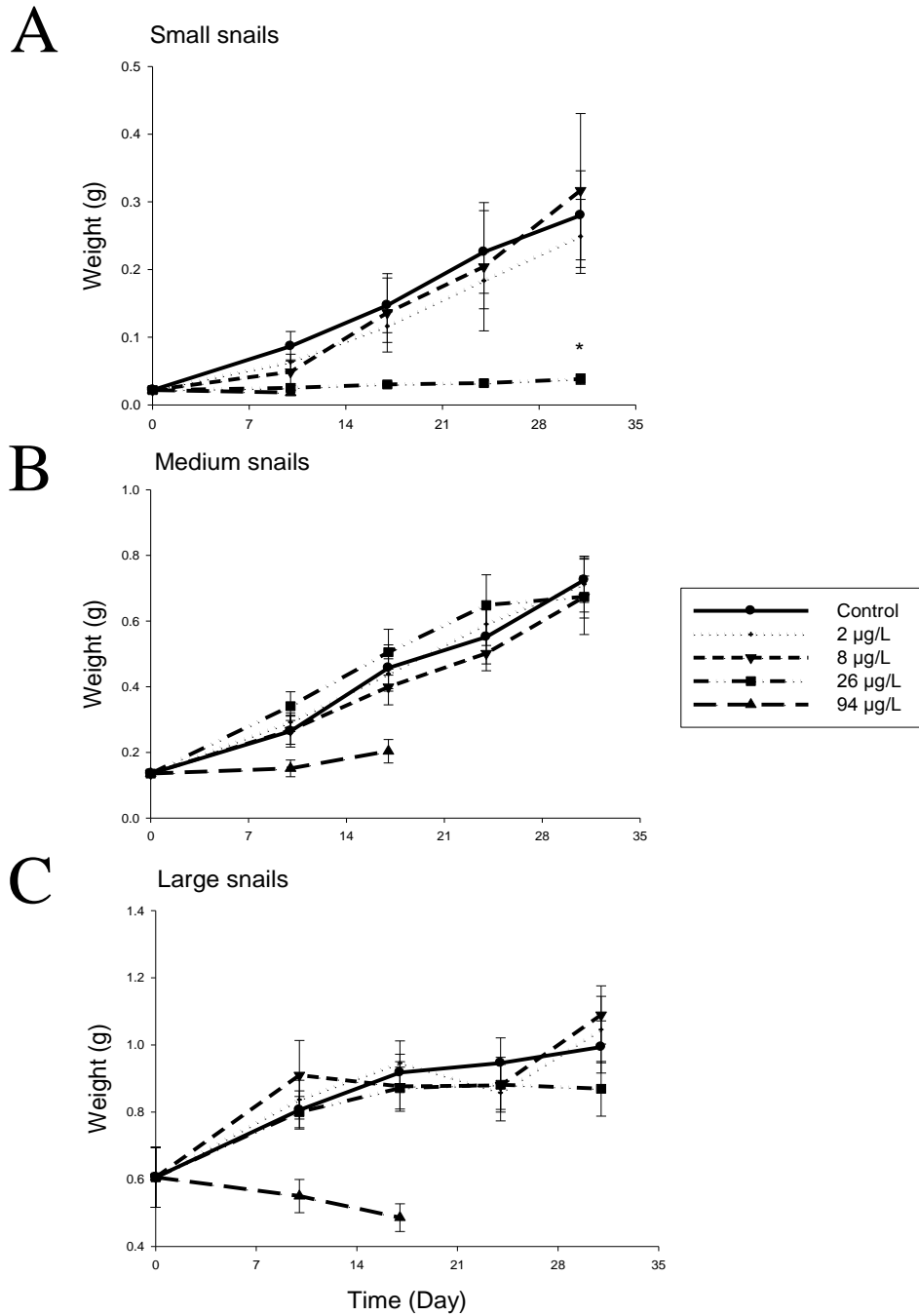


**Figure 2.4.** The effect of chronic exposure to waterborne Cd on the survival of *L. stagnalis* exposed to 2, 8, 26 or 94 µg Cd/L over 31 d. Three groups of snails are shown, panel (A) are snails with an initial length of  $5 \pm 0.02$  mm (mean  $\pm$  SEM,  $n = 10$ ), (B) length was  $10 \pm 0.1$  mm ( $n = 10$ ) and (C) length was  $15 \pm 0.2$  mm ( $n = 10$ ). In panel (B), Controls – 26 µg Cd/L had 100 % survival and had overlapping lines. A group of unexposed snails (controls) are also shown and the 31 d LC50 values were 13 (95% CI 4.8 – 32), 50 (33 – 75), and 46 (39 – 53) µg Cd/L for snails with initial starting shell lengths of 5, 10 and 15 mm respectively.

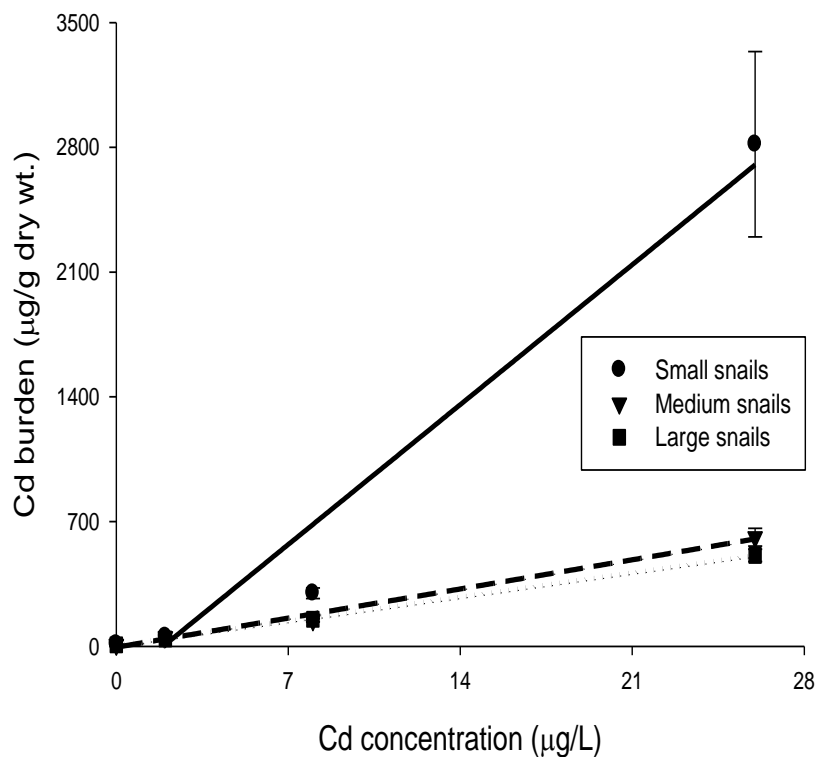


**Figure 2.5.** The chronic effects of waterborne Cd on the mean shell length (mm) of *Lymnaea* exposed to 2, 8, 26 or 94 µg Cd/L over 31 d for small snails ( $5 \pm 0.02$  mm; mean  $\pm$  SEM; n = 10) (A), medium snails ( $10 \pm 0.1$  mm; mean  $\pm$  SEM; n = 10) (B) and large snails ( $15 \pm 0.2$  mm; mean  $\pm$  SEM; n = 10) (C). Only surviving snails were measured and therefore, the n value of the exposed snails decreased from 10 to as low as 2 individuals at higher Cd treatments. A group of unexposed snails (controls) are also included and are represented as circles with a black solid line. An \* indicates significant differences from controls ( $p = 0.05$ ; Two-way ANOVA, Fisher LSD).

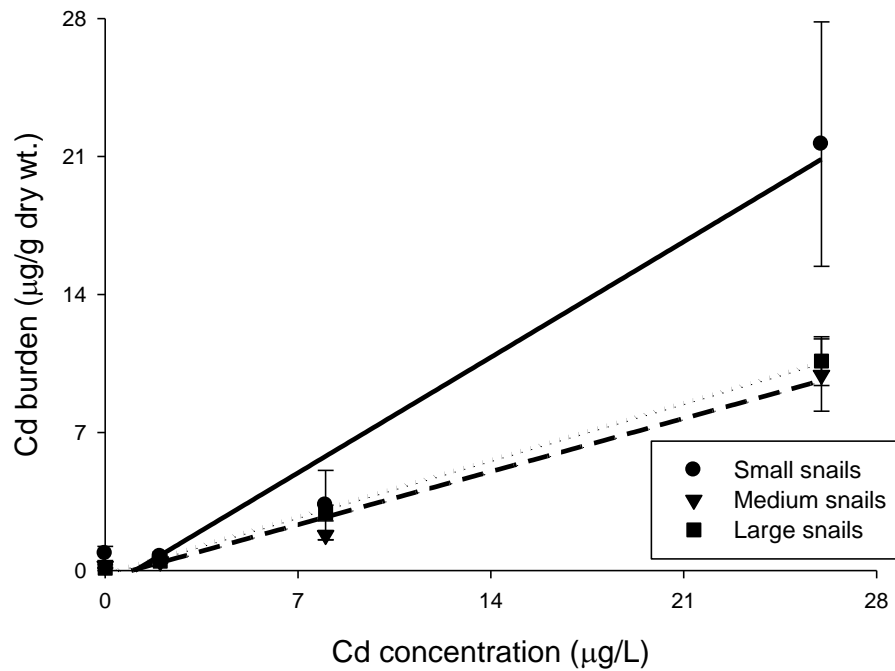




**Figure 2.6.** The chronic effects of waterborne Cd on the wt. (g) of *Lymnaea* exposed to 2, 8, 26 or 94 µg Cd/L over 31 d for small snails ( $0.02 \pm 0.004$  g; mean  $\pm$  SEM; n = 10) (A), medium snails ( $0.14 \pm 0.008$  g; mean  $\pm$  SEM; n = 10) (B) and large snails ( $0.6 \pm 0.09$  g; mean  $\pm$  SEM; n = 10) (C). Only surviving snails were measured and therefore, the n value of the exposed snails decreased from 10 to as low as 2 individuals at higher Cd treatments. A group of unexposed snails (controls) are also included and are represented as circles with a black solid line. An \* indicates significant difference from controls ( $p = 0.05$ ; Two-way ANOVA, Fisher LSD).

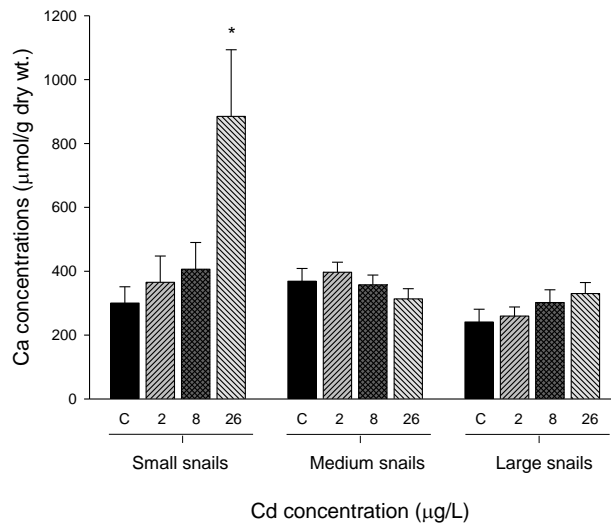


**Figure 2.7.** Soft tissue Cd accumulation of small, medium and large snails at d 31 exposed to waterborne Cd. Small snails are represented with a circle, medium snails with a triangle and large snails with a square. A linear solid line for Cd accumulation for small snails is  $y = -214 + 112x$  ( $r^2 = 0.96$ ), a linear dash line for medium snails is  $y = -5 + 23x$  ( $r^2 = 0.99$ ) and a linear dotted line for large snails is  $y = 3 + 19x$  ( $r^2 = 1$ ). Values are mean  $\pm$  SEM ( $\mu\text{g Cd/g dry wt.}$ ) For small, medium and large snails, the n values were for the control n = 10, 10, 7; for 2  $\mu\text{g Cd/L}$  n = 7, 10, 6 for 8  $\mu\text{g Cd/L}$  n = 4, 10, 6 and for 26  $\mu\text{g Cd/L}$  n = 6, 10, 8, respectively.

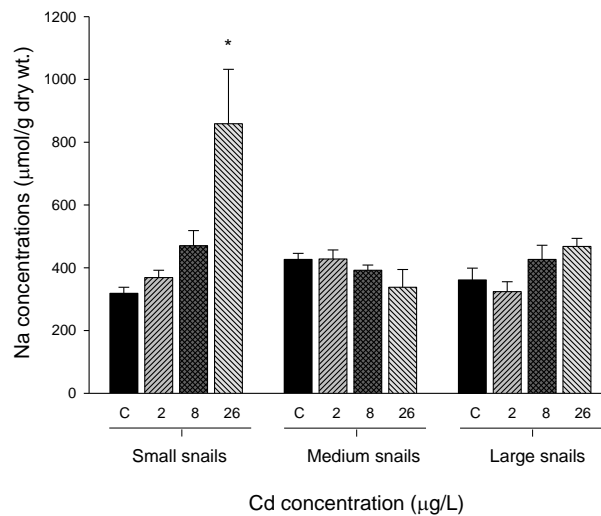


**Figure 2.8.** Shell Cd accumulation of small, medium and large snails at d 31 exposed to waterborne Cd. Small snails are represented with a circle, medium snails with a triangle and large snails with a square. A linear solid line for Cd accumulation for small snails is  $y = -0.9 + 0.8x$  ( $r^2 = 0.97$ ), a linear dash line for medium snails is  $y = -0.4 + 0.4x$  ( $r^2 = 0.98$ ) and a linear dotted line for large snails is  $y = -0.2 + 0.4x$  ( $r^2 = 1$ ). Values are mean  $\pm$  SEM ( $\mu\text{g Cd/g dry wt.}$ ) For small, medium and large snails, the n values were for the control n = 10, 10, 7; 2  $\mu\text{g Cd/L}$  n = 7, 10, 6; 8  $\mu\text{g Cd/L}$  n = 4, 10, 6; 26  $\mu\text{g Cd/L}$  n = 6, 10, 8, respectively.

A



B



**Figure 2.9.** The chronic effects of waterborne Cd on soft tissue  $\text{Ca}^{2+}$  (A) and  $\text{Na}^+$  (B) concentrations in *Lymnaea* at d 31. Control groups are represented by a solid black bar and exposed Cd groups are shown with hatching gray bars. Values are mean  $\pm$  SEM ( $\mu\text{g}$  Cd/g dry wt.). For small, medium and large snails, the n values were for the Control n = 10, 10, 7; 2  $\mu\text{g}$  Cd/L n = 7, 10, 6; 8  $\mu\text{g}$  Cd/L n = 4, 10, 6; 26  $\mu\text{g}$  Cd/L n = 6, 10, 8, respectively. An \* indicates significant differences from controls ( $p = 0.05$ ). (One-way ANOVA, Fisher LSD).

## 2.9 Tables

**Table 2.1:** Measured concentrations of total (unfiltered) and dissolved (0.45 µm filtered) Cd during the acute exposure. Temperature of exposure water ranged from 20 – 21.2 °C. Values are expressed as means ± SEM (n = 5, for each concentration).

Nominal (µg Cd/L)	Total (µg Cd/L)	Dissolved (µg Cd/L)
Control	0.03 ± 0.002	0.03 ± 0.001
100	68.1 ± 0.9	66.6 ± 1.4
200	175 ± 0.6	174 ± 2.4
400	318 ± 8.4	315 ± 6.3
800	629 ± 12	628 ± 10
1600	1305 ± 13	1301 ± 13

**Table 2.2:** Measured concentrations of total (unfiltered) and dissolved (0.45 µm filtered) during the chronic exposure of *Lymnaea*. Values are expressed as means ± SEM (n = 45, for each concentration). Dissolved Cd concentrations were measured before (fresh solutions) and after (measured at 48 h prior to solutions renewal).

Nominal (µg Cd/L)	Total (µg Cd/L)	Dissolved (µg Cd/L)	
		Before	After
Control	0.00 ± 0.01	0.01 ± 0.001	0.00 ± 0.01
3	2.50 ± 0.1	2.27 ± 0.1	1.83 ± 0.1
10	9.43 ± 0.2	8.46 ± 0.3	6.78 ± 0.2
30	28.3 ± 0.3	26.1 ± 0.8	21.7 ± 0.6
100	94.3 ± 3	85.9 ± 1.4	72.7 ± 3

## **Chapter 3:**

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# **Tissue and Sub-cellular Accumulation of Cadmium in *Lymnaea stagnalis* during Chronic Exposure in Soft Water**

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### 3.1 Abstract

The relationship between cadmium (Cd) bioaccumulation and toxic effects were examined in a freshwater gastropod, *L. stagnalis*, in soft water (20 mg/L CaCO<sub>3</sub>). Two chronic (28 d) tests were conducted using waterborne Cd concentration with juvenile snails. The exposures in the first chronic test ranged from 2.3 – 8.3 µg Cd/L and the endpoints measured were survival, bioaccumulation and growth of the snail. The LC<sub>50</sub> was 7.3 µg Cd/L. Cd accumulation within the soft tissue reached as high as 300 µg Cd/g dry wt. at exposure concentration 8.3 µg Cd/ L compared to the control at 7 µg Cd/g dry wt. Shell length was effected with higher Cd concentrations. The control shell length was 15 mm at the end of the exposure, but at the highest concentration, the shell length only reached 11 mm. A negative correlation between total Cd burden in soft tissue and survival or shell length was seen ( $r^2 = 0.95$  and  $0.96$ , respectively). The second test was conducted using environmentally relevant Cd concentrations (0.3 – 2.3 µg Cd/L) and the endpoint measured was bioaccumulation (whole and sub-cellular fractionation). Due to the low Cd concentrations, no survival effects were seen. Cd accumulation within the soft tissue ranged between 6.2 - 117 µg Cd/g dry wt., from the control to the highest concentration. Cd accumulation within the soft tissue of the snail was dose dependent and reached steady state within 14 d of exposure. Sub-cellular fractionation was performed on snails after 28 d of exposure. Proportionally, Cd burden within the heat stable proteins and organelles increased with increasing Cd exposure. On the other hand, Cd burden within the heat denaturable proteins and metal rich granules stayed relatively similar at all concentrations. Therefore, sub-cellular fractionation displays Cd disruption that may be a more sensitive endpoint to measure when determining toxicity within an organism.

### 3.2 Introduction

Bioaccumulation of metals can be used to assess the potential of toxic effects in an organism (Adams *et al.*, 2010). Aquatic organisms such as gastropods, when confronted with exposure to anthropological loadings of metals such as Cd will accumulate it in the soft tissues (Berger and Dallinger, 1989). These accumulations, if retained in the tissues, can serve as a useful tool for monitoring Cd in the environment. Among gastropods, *L. stagnalis* are widely used to investigate the effects of various toxic substances due to its size, ability to accumulate metals reliability and their wide geographical distribution (Desouky, 2006; Cœurdassier *et al.*, 2003). In addition to their wide distribution, these gastropods occur in aquatic systems with a variety of different geochemical compositions such as hardness, pH and dissolved organic carbon (DOC). These geochemical factors alter the bioavailability, and therefore the toxicity, of metals such as Cd (Di Toro *et al.*, 2001). Differences in bioavailability may also be reflected in accumulated tissue burden.

When monitoring metal bioavailability in the environment, a whole organism or various organs are usually used to measure accumulation. Toxic effects will generally occur as a result of physiological disruptions at the cellular level (Shi and Wang, 2004) but not all of the metal internalized will be associated with toxic effects (Rainbow, 2007). Accumulated metal can be separated into two classifications; the metabolically active pool and the detoxified pool (Wang and Rainbow, 2006). The metabolically active pool or metal sensitive fractions (MSF) consists of elements such as organelles (ORG) and heat denaturable proteins (HDP), which are sensitive; they damage as a result of bound bioaccumulated metal. The detoxified pools or detoxified fractions (DF) consists of the



metal rich granules (MRG) and the heat stable proteins such as metallothionein protein (MT) or metallothionein like proteins (MTLP), which are induced by metal exposure and bind to metals to prevent toxic effects (Wang and Rainbow, 2006; Wallace *et al.*, 2003). MTs and MTLPs can be occur in many aquatic invertebrates including *Lymnaea*, have important roles in the homeostasis of essential metals and also help in the detoxification of non-essential metals and excess levels of essential metals (Leung *et al.*, 2003). Measurements of the sub-cellular distribution of metals have the potential to provide a better indication of fraction of the bioaccumulated metal that can cause toxicity compared to total metal burden (Desouky, 2006).

Sub-cellular distribution of cadmium (Cd) in aquatic organisms has been shown to provide an improved understanding of the linkages among bioaccumulation and impacts. Wallace *et al.* (2003) used two marine bivalve species (*Macoma balthica* and *Poltamocorbula amurensis*) that were exposed to 3.5 µg Cd/L or 20.5 µg Zn/L. They were both found to have a greater portion of Cd than Zn in the detoxified pool, showing the greater necessity to detoxify Cd. The two species differed in the partitioning of Cd in this detoxified pool, with *M. balthica* having a larger portion in MRG and *P. amurensis* in MTLP. This shows different detoxification strategies among these two species. On the other hand, in the sensitive fractions both species had a higher portion of Cd in the HDP compared to the ORG fraction. In a dietary Cd exposure, predatory gastropods (*Thais clavigera*) were fed snails exposed to 30 or 150 µg Cd/L Cd resulting in increased Cd in the MT fraction with decreases in the MRG fractions (Cheung *et al.*, 2006). These results and others confirm the induction of MTLP in the snails; however the rate of Cd-induced MT in much slower in lower concentrations compared to higher concentrations.

*L. stagnalis* are known to accumulate high concentrations of a variety of metals such as Pb, Co, Cd and Cu (Cœurdassier *et al.*, 2003; Croteau *et al.*, 2007). Grosell *et al.* (2006) found that Pb accumulation can cause ionoregulatory disturbances and further resulted in deleterious hatching development. With the requirement of high levels of Ca for shell formation and growth, metals interfering with Ca homeostasis can be the more toxic to *L. stagnalis* (Grosell *et al.*, 2006). However, Cœurdassier *et al.* (2003) found that *L. stagnalis* were not as sensitive to Cd, compared to other Ca antagonists. High concentrations inhibited growth, fecundity and fertility in the snails, which resulted in a range of EC<sub>50</sub> of 60 – 142 µg Cd/L. Leung *et al.* (2003) discovered adult *L. stagnalis* exposed to 0.01 µg Cd/L accumulated Cd levels similar to their controls. On the other hand, exposure to high levels (1000 µg Cd/L) resulted in accumulation of 350 µg Cd/g dry wt. of Cd in the soft tissue within 10 d in hard water of 250 mg/L of CaCO<sub>3</sub>. Although, there have been studies conducted on Cd toxicity using *Lymnaea*, more chronic studies are needed on sub-cellular distribution to broaden the knowledge of chronic waterborne Cd distribution in the metabolically active fraction and the detoxified fractions of gastropods, and how these organisms are able to accumulate large amounts of Cd.

This study involves two experiments with *L. stagnalis* exposed to waterborne Cd test for 28 d. In the first exposure, the endpoints measured were survival, bioaccumulation and growth (length and wt.). The second test was conducted using environmentally relevant Cd concentrations and the endpoint measured was bioaccumulation throughout the exposure. The goals of the study were to find linkages

between bioaccumulation in whole-body and sub-cellular distribution in soft tissue with both lethal and sub-lethal effects.

### **3.3 Materials and Methods**

#### 3.3.1 Snail Culture

Adult *L. stagnalis* were provided by Dr. D. Spafford of the University of Waterloo. Adults are maintained in 10 L aquaria that were in an aerated, re-circulating system (Aquatic Habitats, Apopka, FL), while the juvenile were placed in static 10 L aquaria. The media they were maintained in was a mixture of dechlorinated Waterloo city tap water and reverse osmosis water that delivered water with concentrations of  $\text{Na}^+ = 840 \pm 36 \mu\text{M}$ ,  $\text{Ca}^{2+} = 1560 \pm 122 \mu\text{M}$ ,  $\text{Mg}^{2+} = 560 \pm 23 \mu\text{M}$  a pH that ranged from 7.2 to 7.4 and a temperature of  $20 \pm 1 \text{ }^\circ\text{C}$  (mean  $\pm$  SEM,  $n = 45$ ). They were kept under a photoperiod of 16:8, light: dark and fed locally purchased romaine lettuce *ad libitum*. Aquaria with adult snails were cleaned with a siphon to remove excess feces and filters were rinsed from debris re-entering the circulating system. Aquaria with juvenile snails had 50 % water renewal weekly. Once a month, egg masses were removed from adult aquaria and placed in static aquaria. The snails would hatch within 10 – 14 d and result in snails of similar age ( $4 \pm 2$  weeks) and size (i.e. 5 mm) for testing. Before testing, a screening test for similar size was performed and snails at the two extremes were removed ( $< 3$  or  $> 7$  mm).

### 3.3.2 General Experimental Procedure

Exposures were conducted as static renewal (48 h) using an aquatic media made by mixing reverse osmosis and dechlorinated city water to establish a water hardness of 21 mg/L of CaCO<sub>3</sub> (Mg = 74 ± 12 μM, Ca = 140 ± 26 μM and Na = 283 ± 21 μM) and pH 7.3 ± 0.1 (mean ± SEM, n = 45). Snails were placed in this medium to acclimate for 48 h. For each concentration, a concentrated Cd stock solution (as CdCl<sub>2</sub>·2½H<sub>2</sub>O; ≥ 98 % purity, VWR International, Mississauga, ON) was diluted to achieve the target concentration and then left to equilibrate for 24 h, while the control beaker only had 1L of media and was left to equilibrate for 24 h. The snails were removed from the acclimation aquaria and blotted dry with a paper towel, to remove excess water, and then were weighed to the nearest 0.001 g. Snails were then placed into each beaker to initiate the test. Prior to and after every water renewal, both filtered (0.45 μm filters with a 3 mL syringe) and unfiltered samples (no filter) were taken. Water samples were acidified for Cd, Ca, Mg, and Na measurements. Endpoints that were examined were survival, bioaccumulation (whole and sub-cellular distribution) and growth. After 28 d of exposure, the surviving snails were placed into deionized water for 10 min., excess water was removed by blotting with a paper towel and then they were stored at - 80 °C. Later, the shell and soft tissue were separated and each digested separately for measurements of Cd bioaccumulation. Two chronic (28 d) tests were run using ~ 5 mm snails. The first chronic test (CT I) was used to determine an LC<sub>50</sub> for survival and growth. The second test (CT II) was used to monitor Cd bioaccumulation throughout the exposure to compare with other experiments conducted in the lab. Sub-cellular fractionation was conducted on d 28 to determine Cd concentrations in separate fractions.

### 3.3.3 Chronic Test I (CT I)

A chronic test was run for *L. stagnalis* (28 d LC<sub>50</sub> test). This study was to determine a 28 d LC<sub>50</sub> for Cd in the snails and to understand sensitivity in soft water. The chronic toxicity calculated endpoint was the LC<sub>50</sub> value and this was based on the measured endpoint of survival. Snails were considered to have expired when a blunt dissecting probe touching the foot of the snail had no response to the stimulus. The bioaccumulation of Cd was also measured after 28 d of exposure and body burdens were calculated on a wt. adjusted basis. Also, growth was monitored during the exposure on d 7, 14, 21 and 28 by measuring the wet wt. of individuals and length of shells. A range of Cd concentrations (nominal concentrations: 0, 2.5, 5 and 10 µg Cd/L) were tested. Ten juvenile (7 mm) per concentration were placed into the containers. The snails were given fresh food every 48 h, during water renewal throughout the experiment.

### 3.3.4 Chronic Test II (CT II)

Snails were exposed to one of the following Cd concentrations (nominal: 0, 0.4, 0.8, 1.6 or 3.2 µg Cd/L) and had 3 replicates per concentration for 28 d. Twelve snails were placed in each replicate beaker and were fed lettuce *ad libitum* and replaced every 48 h, when the Cd solutions were renewed. Juvenile snails (10, 4 – 5 mm) were sampled on d 0, followed by 6 snails being sampled on d 1, 4, 7, 21 and 28 from each concentration (2 snails per replicate beaker). Only the snails collected on d 28 was used for sub-cellular distribution.

### 3.3.5 Analysis

#### Water and Whole Tissue Samples

The sampled snails from exposures were thawed and soft tissue was separated from shell. Samples were dried at 80 °C until constant wt. was obtained and placed in 2.0 mL eppendorf tubes. Concentrated (70 %) trace metal grade nitric acid (HNO<sub>3</sub>; Fisher Scientific, Nepean ON) was added for digestion. Acid was added in a 1:5 (wt.: v) ratio and placed in the oven at 65 °C in sealed eppendorf tubes. After 48 h, samples were mixed by the vortex and then centrifuged at 5000 g for 10 min. and the supernatant was subsequently collected followed by dilution in 1 % HNO<sub>3</sub> before measurement by means of atomic absorption spectrophotometry (AAS, SpectAA-880, Varian Inc., Palo Alto, CA). Water samples were measured for Ca, Mg and Na concentrations by flame AAS, and Cd was measured using graphite furnace (GTA 100) AAS. Certified reference materials (TM 28.3 and TM 26.5, National Water Research Institute, Environment Canada) were used to assess recovery of metals in water and recovery was within 15 %. Lobster hepatopancreas reference material (TORT-2, National Research Council Canada) was used to assess the recovery of metals in tissue for tissue quality checks and recovery was never less than 85 % (85 – 92.3 %).

#### Sub-cellular Fractionation

Sub-cellular distribution protocol was adapted from Wallace *et al.* (2003) and conducted on d 28 snails in the CT II. In short, 1:5 (wet wt.: v) buffer solution was added. The buffer was a 20 mM Tris-Base (pH = 8.6 with 2 mM 2-mercaptoethanol and 0.2 mM phenylmethanesulfonylfluoride). The tissue was homogenized with the buffer on ice for 7

seconds using the Omni THq digital tissue homogenizer (Omni International, Marietta, GA) at 20 RPM. One-third of the homogenate was taken for Cd metal recovery. The remaining homogenate (2/3) was centrifuged at 1450 g for 15 min. at 4 °C. The pellet (P1) was placed back in the - 80 °C freezer for later analysis. The supernatant was then decanted and re-centrifuged at 100,000 g for 1 h at 4 °C, and the pellet formed was the organelles (ORG) consisting of mitochondria, microsomes and lysosomes. The supernatant was then placed on a hot plate for 10 min at 80 °C. Then cooled for 1 h at 4°C and centrifuged at 30,000 g for 10 min at room temperature. This process separates heat denaturable proteins (HDP; pellet) and heat stable proteins such as metallothionein-like proteins (MTLP; supernatant). P1 was removed from the freezer and thawed on ice. Prior to placing on a hot plate for 1 h at 80 °C, 1 mL of 1N NaOH was added. It was left on bench top to reach room temperature (approximately 10 min.), and then was centrifuged at 5000 g for 10 min at 20°C. This separated the cellular debris and nuclei (CD; supernatant) from metal rich granules (MRG; pellet).

### 3.3.6 Statistical Analysis and LC<sub>50</sub> Calculation

Data have been presented as means  $\pm$  standard error of the mean, unless otherwise stated. For CT I, the 28 d LC<sub>50</sub> values with 95 % confidence intervals were calculated from observed mortality responses and the measured dissolved Cd concentrations using CETIS (Comprehensive Environmental Toxicity Information System Software v1.6.1. rev C Tidepool Scientific Software, McKinleyville, U.S.A.). Two-Way ANOVA was used to test effects of Cd and exposure time. Following the significant result, the Fisher LSD post hoc test was used to identify differences among means. Linear and exponential analyses were performed to describe relationships between survival or length and Cd

burden, as well as between Cd burdens at different concentrations through-out an exposure. Significance of all tests was taken at  $p < 0.05$ .

### **3.4 Results**

#### 3.4.1 CT I

##### Survival and Growth

The nominal concentrations of 2.5, 5 and 10  $\mu\text{g Cd/L}$  corresponded to  $2.27 \pm 0.1$ ,  $4.51 \pm 0.3$ , and  $8.24 \pm 0.8 \mu\text{g Cd/L}$  respectively ( $n = 45$ ; Table 1.1). Survival decreased with increasing Cd exposure which resulted in a  $LC_{50}$  value of 7.3 (95 % CI: 4.7 to 11.3)  $\mu\text{g Cd/L}$  (Fig 3.1 A). Length and wt. were reduced by the increase in Cd exposure however, only the two higher Cd concentrations (4.5 & 8.2  $\mu\text{g Cd/L}$ ) showed significant differences in length compared to the control (Fig 3.1 B and C).

##### Bioaccumulation

Accumulation in *Lymnaea* was found to increase with higher Cd concentrations, reaching  $298 \pm 36 \mu\text{g Cd/g dry wt.}$  at Cd concentration of 8.3  $\mu\text{g Cd/L}$ , compared to the control ( $6.6 \pm 0.3 \mu\text{g Cd/g dry wt.}$ ; Table 3.2). The Cd burden was compared with survival and length on d 28 and a negative correlation was shown (Fig 3.2). With increased Cd accumulation in the soft tissue, there was a decrease in survival with a linear line of  $y = 99 - 0.2 x$  ( $r^2 = 0.95$ ,  $p = 0.02$ ; Fig 3.2 A). Similarly, a reduction in



length was seen with an increase in soft tissue body burden with a linear line of  $y = 15 - 0.01x$  ( $r^2 = 0.96$ ,  $p = 0.02$ ; Fig 3.2 B).

### 3.4.2 CT II

#### Bioaccumulation and Steady State

The nominal concentrations of 0.4, 0.8, 1.6 and 3.2  $\mu\text{g Cd/L}$  corresponded to measured concentrations of  $0.3 \pm 0.02$ ,  $0.5 \pm 0.03$ ,  $1.3 \pm 0.08$  and  $2.3 \pm 0.04$   $\mu\text{g Cd/L}$  respectively ( $n = 45$ ; Table 3.3). Accumulation in *L. stagnalis* was dose dependent and all concentrations reached steady state tissue burden by d 14 of exposure (Fig 3.3). The accumulation had significantly increased throughout the exposure until d 14 where it begins level off or stay constant. The body burdens from exposed individuals were significantly higher than controls ( $n = 6$ ,  $p < 0.05$ ). The exponential curves are displayed in Table 4 with  $r^2$  values.

#### Sub-cellular Fractionation

Sub-cellular distribution was conducted on the soft tissue of snails at d 28. Cd burden increased in all parts of the cell. (Fig 3.4 A) Proportionally, compared to the total body burden, metallothionein-like proteins are the only fraction that increased in burden (Fig 3.4 B). However, the metal rich granules and organelles stayed relatively similar to the control. The cellular debris and heat denaturable proteins, decreased in Cd burden in the soft tissue. Therefore, in the control, the order of Cd burden was seen as  $\text{ORG} > \text{HDP} > \text{MRG} > \text{CD} > \text{MTLP}$ . However, at the highest Cd exposure (2.3  $\mu\text{g Cd/L}$ ), the Cd burden changed and is better seen as  $\text{ORG} > \text{MTLP} > \text{MRG} > \text{HDP} > \text{CD}$ .

Comparing only the metabolically active pool or metal sensitive fraction (MSF) (consisting of ORG and HDP) and detoxified fraction (DF; consisting of MRG and MTLP), the DF shows an increase in Cd burden with increasing Cd exposure (Fig 3.5 A). Additionally, if you look at it proportionally comparing to total soft tissue burden, the control snails have a much smaller burden in the DF than the MSF (Fig 3.5 B). However, with increasing Cd exposure concentration, there was an increase in Cd burden seen in the DF, reaching equal levels to the MSF at the highest concentration.

## **3.5 Discussion**

### 3.5.1 CT I

#### Survival and Growth

The 28 d LC<sub>50</sub> value was 7.3 µg Cd/L (95 % CI: 4.7 – 11.3) in soft water of 20 mg/L as CaCO<sub>3</sub>. Therefore, this demonstrates that the juvenile snails are moderately resistant to Cd, compared to salmonids and amphipods, even though it may compete with Ca<sup>2+</sup> ion. However, *L. stagnalis* are sensitive to other metals, such as Cu (Na<sup>+</sup> ion antagonist), Co and Pb (Ca<sup>2+</sup> ion antagonists) as indicated by other studies (Ng *et al.* 2011; De Schamphelaere *et al.*, 2008; Grosell *et al.*, 2006). Unlike other invertebrates, such as the *Daphnia magna* which are much more sensitive and experienced a 16 % decrease in reproduction within 21 d of exposure to 0.17 µg Cd/L at a water hardness of 49 mg/L (as CaCO<sub>3</sub>; Biesinger and Christensen, 1972). *H. azteca* were also more

sensitive to Cd, with a 42 d LC<sub>50</sub> of 0.53 µg Cd/L in water with a hardness value of in 130 mg/L as CaCO<sub>3</sub> (Borgmann *et al.*, 1991).

In the current study, a 27 % deduction in shell length was seen on d 28 at the highest exposure concentration compared to the control. This again demonstrates that *Lymnaea* are less sensitive as sub-lethal growth effects (11 % reduction in body wt.) were observed over 46 d in Atlantic salmon (*Salmo salar*) exposed to waterborne Cd concentrations of 0.47 µg Cd/L (Rombough and Garside, 1982). *Lymnaea* have demonstrated a reduction in growth when exposed to Pb concentrations, associated with a reduction of Ca influx (Grosell and Brix, 2009). Similarly, Ng *et al.* (2011) found that in chronic waterborne Cu exposures, *Lymnaea* resulted in a reduction in growth but this was not associated with a decrease in feeding or internal Ca balance. They hypothesized it may be due to a re-allocation of energy to detoxification of Cu toxicity. A similar theory may be used to describe the reduction in growth caused by Cd exposure as detoxification may be a priority for survival.

### Bioaccumulation

There was an increase in Cd accumulation in the soft tissue as waterborne Cd concentrations increased, showing a dose dependent response in this study. Hoang *et al.* (2008) found bioaccumulation of Cu in whole body (soft tissue + shell) of Florida apple snail, *Pomacea paludosa*, to be dose dependent as well. With Cu body burden 30, 50 and 60 µg Cu/g dry wt. exposed to Cu concentrations (6, 8.2 and 12.2 µg Cu/L, respectively) for 28 d at water hardness of 54 mg/L as CaCO<sub>3</sub>. Ng *et al.* (2011) showed similar results, reaching accumulation of  $9.3 \pm 1.3$  µg Cu/g wet wt. at the highest Cu concentration exposed (18.2 µg Cu/L). Cd burden within the soft tissue of the snails to toxic effects

were shown to be correlated. Increased Cd burden in the soft tissue was correlated to a decreased survival as well as decreased shell length.

### 3.5.2 CT II

#### Bioaccumulation and Steady State

At low (and environmentally relevant) concentrations, there was no mortality or reduction in shell length seen by the exposed snails, compared to the controls. The snails showed an increase in Cd burden concentration through the exposure at all concentrations until reaching a plateau after d 14 of approximately, 6, 25, 30, 60 and 120  $\mu\text{g Cd/g}$  at concentrations of control, 0.3, 0.5, 1.3 and 2.3  $\mu\text{g Cd/L}$ . This corresponds with the theory where initially, uptake increases tissue Cd concentration because of the rate of elimination is exceeded by uptake. With exposure concentrations remaining constant, the uptake and elimination will eventually balance, finally reaching steady state (Campbell *et al.*, 2008). Steady state tissue burden was also seen in the Florida apple snail, *P. paludosa*, when Hoang *et al.* (2008) exposed juvenile apple snails to Cu. The whole body (shell + soft tissue) Cu burden increased rapidly from the start of the exposure, eventually slowing down, until reaching steady state tissue burden after 14 d of exposure. However in this study, Cd burden on d 28 from the first test is significantly different from the second test, which may be caused by the different size of the juvenile snails at the start of the exposure. The snails in CT II were younger (4 – 5 mm), however the controls reached similar sizes at the end of exposure, as in CT I, and maybe the snails had to increase uptake of  $\text{Ca}^{2+}$  ion, and perhaps increasing the  $\text{Cd}^{2+}$  ion as well.

## Sub-cellular Fractionation

In the present study, although, the first test was able to compare Cd body burden with toxic effects seen in the snails, the Cd concentrations used were not environmentally relevant concentrations. However, when using more relevant concentrations, no toxic effects were seen on a whole body level, such as mortality or growth reduction. To try to assess whether toxic effects were related to sub-cellular fractions, analysis of distribution to pools within the cells was conducted. Sub-cellular distribution was conducted on the soft tissue of snails at d 28. Cd burden increased in all pools. There was evidence of MT or MTLP induction to detoxify accumulated Cd however the sensitive fractions also increased and only at the second highest concentration (1.3 µg Cd/L), exposure was there evidence of a protection against accumulation in sensitive fractions (Fig 3.4 and 3.5). Wallace *et al.* (2003) found similar results in a marine bivalve *P. amurensis*, exposed to 3.5 µg Cd/L, where an increase in burden of Cd in the MTLP fraction was seen as a strategy for detoxifying Cd. Similarly, Leung *et al.* (2003) exposed *L. stagnalis* to waterborne Cd of 0.01 and 1000 µg Cd/L, for 10 d. Snails exposed to 1000 µg Cd/L contained significantly higher concentrations of Cd compared to controls, a 3-fold increase of MTLP was seen in the highest concentrations of Cd. However, at the lower concentration, no significant increase in Cd burden within the MTLP fraction was seen (Leung *et al.*, 2003). With dietary Cd, the marine snail, *T. clavigera* also was found to have an increase of Cd in the MTLP fraction, with increasing time (Cheung *et al.*, 2006). MTLP increase in metal burden is seen in other metals such as Cu. For example, Ng *et al.* (2011) reported a significant increase of Cu burden within the MTLP proportion, with increased Cu exposure concentrations.

When comparing the burden of Cd seen in each proportion, in the current study, organelle and MTLP had the highest fraction, with MRG, then HDP and finally CD. However, this is not seen in *Lymnaea* exposed to other metals. For example, when *Lymnaea* are exposed to Cu, organelles have 32 – 43 % of the Cu burden with MTLP and CD in second with (20 – 33 % and 16 – 33 %, respectively; Ng *et al.*, 2011). HDP are between 2 – 6 %, and finally, the MRG with only 2 - 6 %. Although, the two highest fractions did stay similar with HDP and MTLP be approximately 30 – 45 % of the Cd (Ng *et al.*, 2011).

There was a transfer of Cd burden from the MSF to the DF. This was seen when the snails were exposed to increasing Cd concentration, showed an increase in the DF, while a decrease of burden in MSF while at the same time an increase in a DF. Wallace *et al.* (2003) hypothesized that there was a redistribution of Cd burden from MSF into DF in the bivalve *P. amurensis* with no decrease in Cd burden seen in the snails, similarly exposed, but rather a change in location of Cd burden.

In the present study, MRG did not show an increase in Cd burden. Similarly, Cheung *et al.* (2006) discovered a negative correlation between Cd proportioned in the MRG fraction and accumulation of total body burden. However, MRG is known to increase as a result of toxic effects from metals. For example, Ng *et al.* (2011) showed a significant increase in Cu levels in the MRG fraction. This may be because of different contaminants are able to induce different detoxification strategies to maintain homeostasis within an organism.

Therefore, although, toxic effects can be linked to soft tissue Cd burden, they are only seen in high concentrations. These concentrations needed to be higher than natural

occurrences because of the tolerance of *Lymnaea* to Cd, which will lead to adverse effect (lethal and sub-lethal). However, for more environmentally relevant concentrations, induction of MTLP was seen, however MTLP was not measured. However, future studies should measure the levels of MTLP induced due to Cd toxicity. Moreover, the biologically detoxified fraction was shown to increase in Cd burden while metal sensitive fraction was shown to decrease in the burden, possibly re-location of Cd burden as a detoxification mechanism.

### 3.6 References

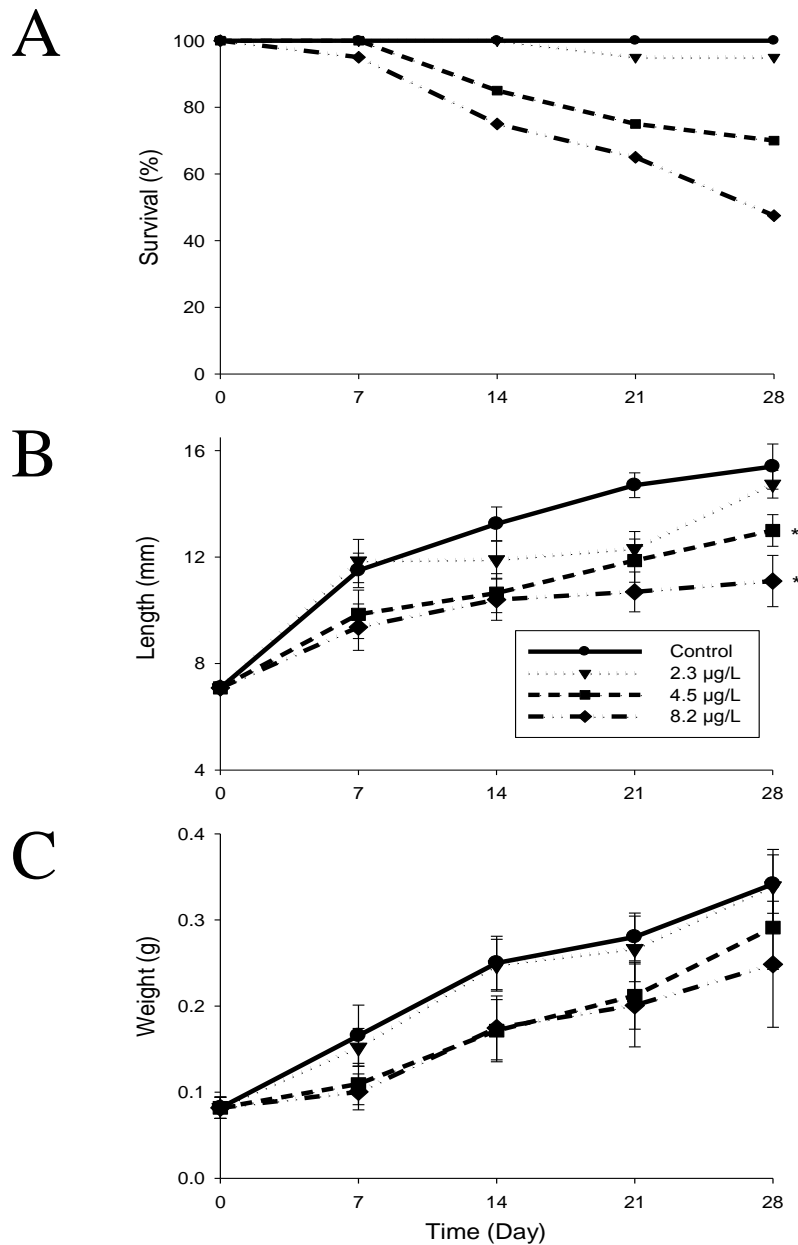
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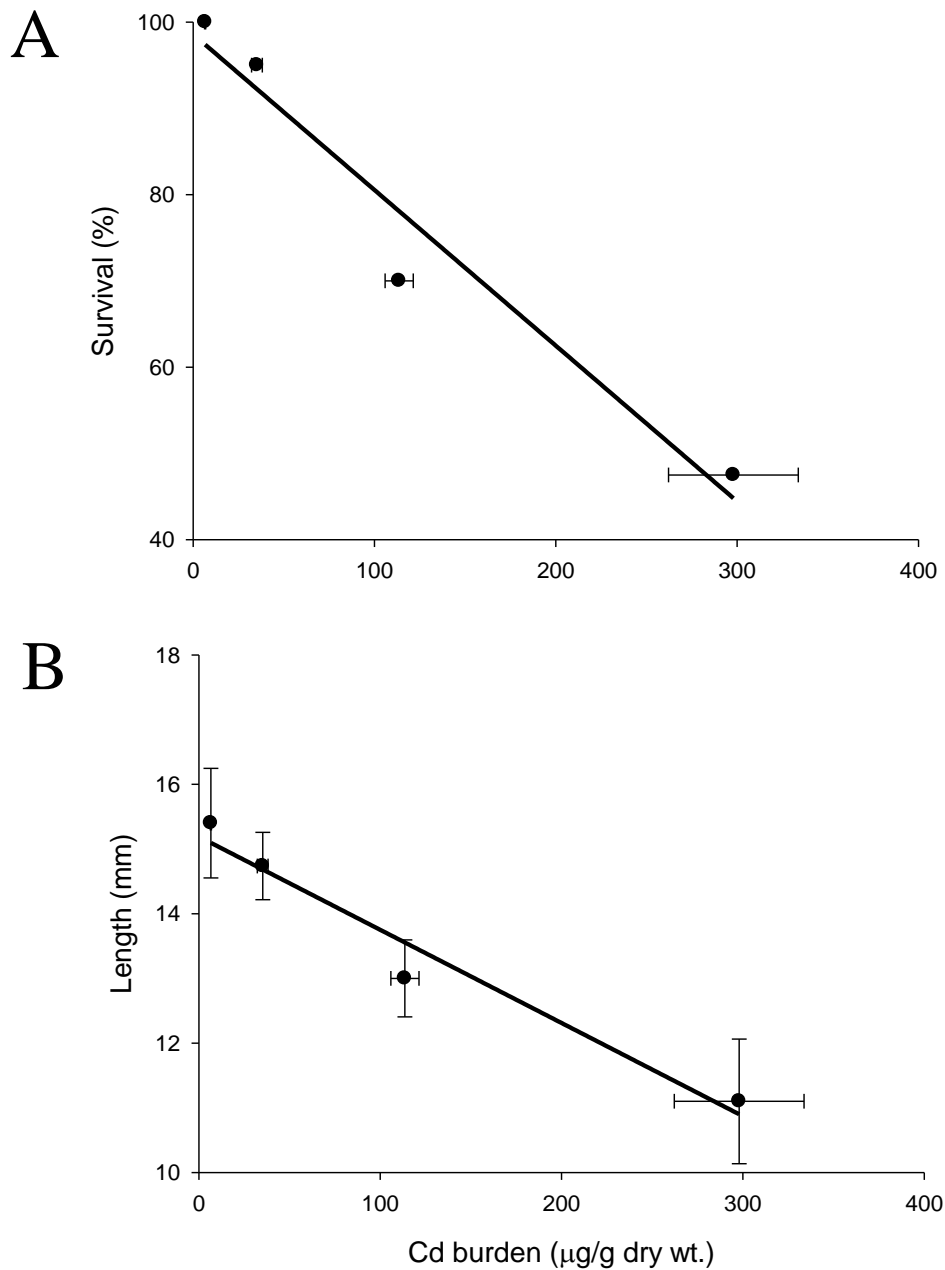
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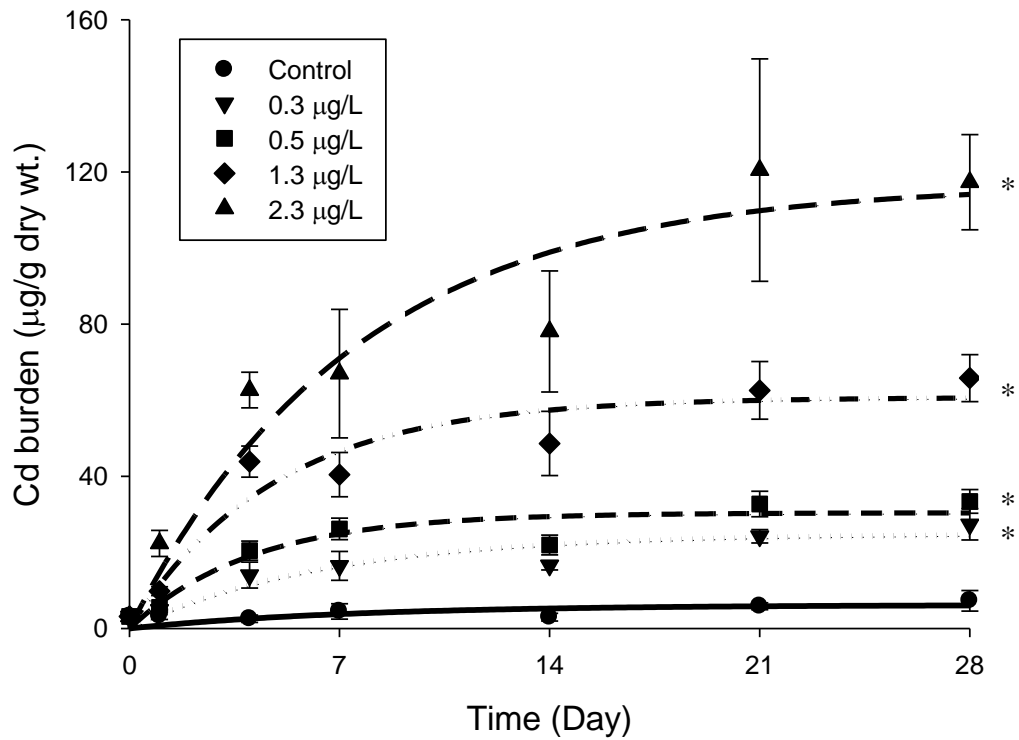
### 3.7 Figures



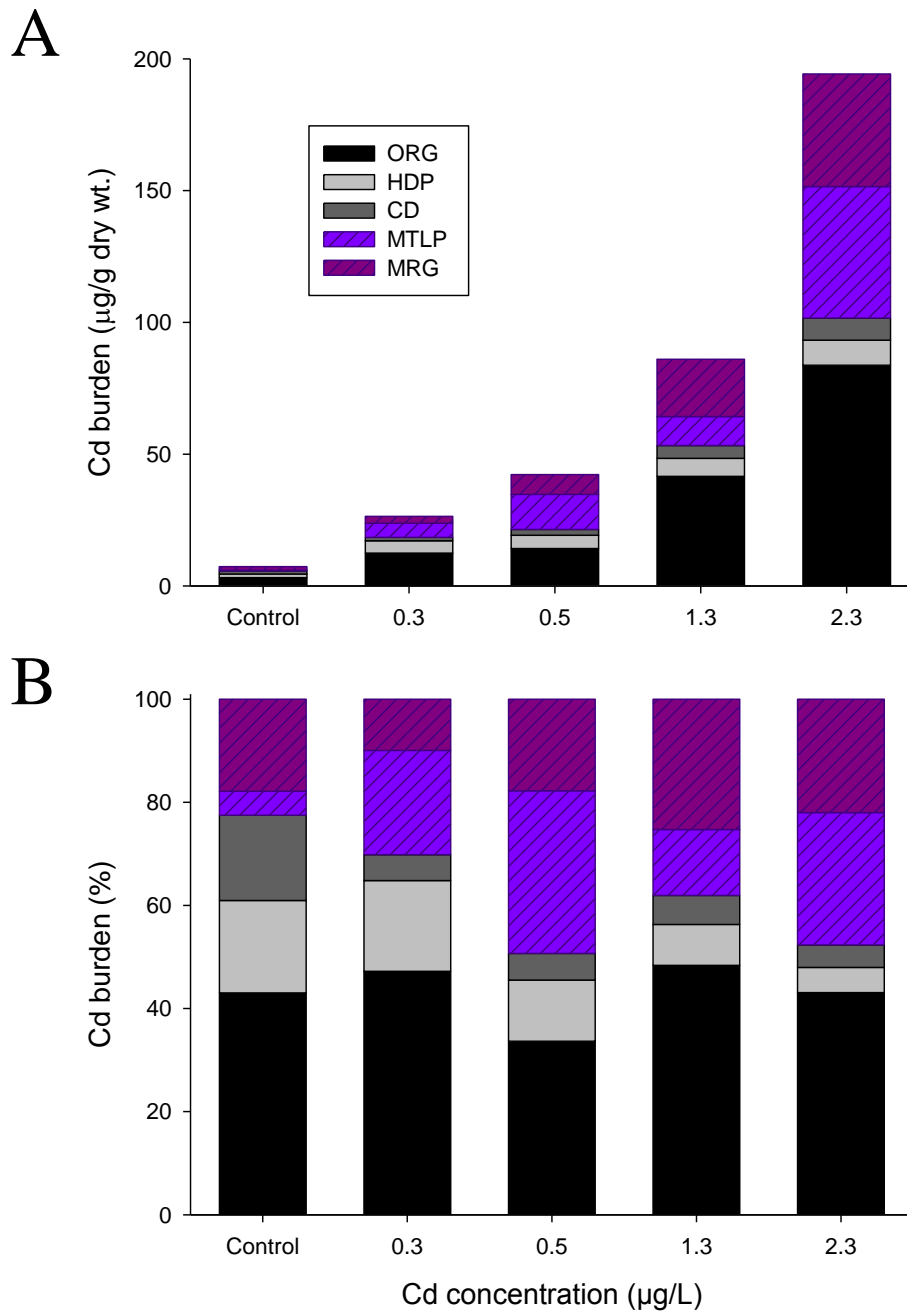
**Figure 3.1.** Chronic Test 1 (CTI):The chronic effects of waterborne Cd on the survival (A) as well as mean length (B) and mean dry wt. (C) of *L. stagnalis* over 28 d of exposure. Cd exposure concentrations are given in the legend of the graph and a group of unexposed snails (controls) are also included. In panels (A), (B) and (C), the error bars indicate SEM (for each mean  $n = 14 - 20$  except for  $8.2 \mu\text{g Cd/L}$  exposed group where  $n = 9$ ) and an \* indicates a significant difference from controls on that d ( $p < 0.05$ ). The  $\text{LC}_{50}$  value was  $7.3$  (95 % CI:  $4.7$  to  $11.3$ )  $\mu\text{g Cd/L}$ . (Two-way ANOVA, Fisher LSD).



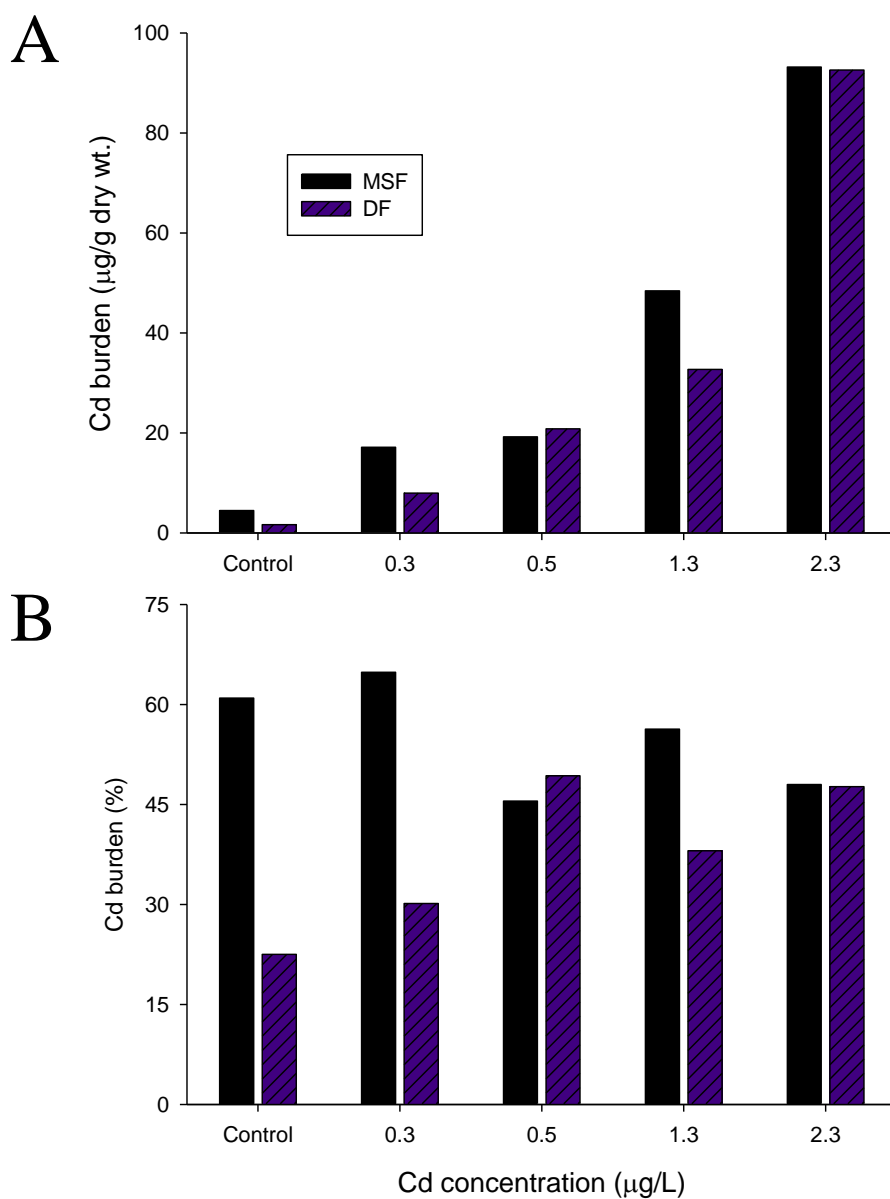
**Figure 3.2.** CTI: Relationship between mean Cd soft tissue burden ( $\mu\text{g Cd/g dry wt.} \pm \text{SEM}$ ) and survival (A) and mean length (B) of *L. stagnalis* exposed to waterborne Cd (either 2.3, 4.5, or 8.2  $\mu\text{g Cd/L}$ ) at d 31. Survival and tissue burden (A) shows a line  $y = 99 - 0.2x$  ( $r^2 = 0.95$ ,  $p = 0.02$ ) representing the correlation. Length and tissue burden (B) show a correlation where  $y = 15 - 0.01x$  ( $r^2 = 0.96$ ,  $p = 0.02$ ).



**Figure 3.3.** Chronic Test 2 (CTII): Mean concentration of Cd in soft tissues of *L. stagnalis* exposed to 0.3, 0.5, 1.3 or 2.3 µg Cd/L over 28 d. Concentrations in an unexposed control group are also included and are represented by a circle. Means are shown with SEM, n= 6 for each d and lines show the best fit of an exponential model [ $y = a * (1 - e^{-b * x})$ ], see table 4 for details. An \* indicates significant differences from the control on d 28. (Two-way ANOVA, Fisher LSD).



**Figure 3.4.** CTII: Sub-cellular fractionation of soft tissue *L. stagnalis* exposed to 0.3, 0.5, 1.3 or 2.3 µg Cd/L at d 28. A control group is also included. The fractions are stacked and show ORG: organelles, HDP: heat denatured proteins, CD: cellular debris, MTLP: metallothionein-like proteins and MRG: metal rich granules. Panel (A) is the Cd burden displayed as (µg Cd/g dry wt.), whereas panel (B) shows the Cd burden in percentage at the various fractions. Detoxified fractions are shown in purple with hatch lines. Values are mean (µg Cd/g dry wt.), n = 6.



**Figure 3.5.** CTII: Sub-cellular fractionation of soft tissue *L. stagnalis* exposed to 0.3, 0.5, 1.3 or 2.3 µg Cd/L at d 28. A control group is also included. The fractions are grouped and show MSF: metal sensitive fraction, which includes, organelles and heat denatured proteins and DF: detoxified fraction, which includes metallothionein-like proteins and metal rich granules. The MSF is shown in black, and the DF in purple. CD values are not included. Panel (A) is the Cd burden displayed as (µg Cd/g dry wt.), whereas panel (B) shows the Cd burden in percentage at the various fractions. Values are mean (µg Cd/g dry wt.), n = 6.

### 3.9 Tables

**Table 3.1:** Measured concentrations of total (unfiltered) and dissolved (0.45 µm filtered) Cd during chronic test 1 (CT I). Temperature of exposure water ranged from 21 – 21.9 °C. Values are expressed as means ± SEM (n = 45, for each concentration). Dissolved Cd concentrations were measured before (fresh solutions) and after (measured at 48 h prior to solutions renewal).

Nominal (µg Cd/L)	Total (µg Cd/L)	Dissolved (µg Cd/L)	
		Before	After
Control	0.00 ± 0.01	0.01 ± 0.001	0.00 ± 0.01
2.5	2.47 ± 0.1	2.27 ± 0.1	2.05 ± 0.1
5	4.76 ± 0.2	4.51 ± 0.3	4.02 ± 1.5
10	9.37 ± 0.3	8.24 ± 0.8	8.07 ± 0.6

**Table 3.2:** Cd burden in the soft tissue of *Lymnaea* at d 28 of CT I. Cd concentrations displayed with the burden associated (µg/g dry wt.) ± SEM with n values. An \* indicates significance from controls. (One-way ANOVA, Fisher LSD).

Concentration (µg Cd/L)	Cd burden (µg Cd/g dry wt.) ± SEM (n value)
Control	6.6 ± 0.3 (20)
2.3	35.2 ± 3 (19) *
4.5	114 ± 8 (14) *
8.2	298 ± 36 (9) *



**Table 3.3:** Measured concentrations of total and dissolved Cd during chronic test 2 (CT II). Temperature of exposure water was 20 – 20.3 °C. Values are expressed as means ± SEM (n = 45, for each concentration). Dissolved Cd concentrations were measured before (fresh solutions) and after (measured at 48 h prior to solutions renewal).

Nominal (µg Cd/L)	Total (µg Cd/L)	Dissolved (µg Cd/L)	
		Before	After
Control	0.00 ± 0.01	0.02 ± 0.001	0.01 ± 0.01
0.4	0.35 ± 0.01	0.31 ± 0.02	0.30 ± 0.04
0.8	0.53 ± 0.02	0.52 ± 0.03	0.51 ± 0.01
1.6	1.41 ± 0.07	1.34 ± 0.08	1.29 ± 0.06
3.2	2.51 ± 0.06	2.31 ± 0.04	2.21 ± 0.02

**Table 3.4:** The parameters for the exponential curve fitting from chronic exposure II (Fig 3.3). The exponential curve model was  $y = C_{max} * (1 - e^{-ax})$  and the  $r^2$  values.

Concentration (µg Cd/L)	C <sub>max</sub> Exponential Curves	a	r <sup>2</sup>
Control	6.2 ± 2	0.13 ± 0.1	0
0.3	25 ± 3	0.15 ± 0.05	0.89
0.5	30 ± 3	0.24 ± 0.08	0.9
1.3	61 ± 5	0.21 ± 0.06	0.93
2.3	117 ± 12	0.13 ± 0.04	0.93

## **Chapter 4:**

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# **The Development of the Novel Tissue Residue Approach to Predict the Toxicity of Cadmium to *Hyalella azteca* using Bioaccumulation of *Lymnaea stagnalis* in Moderately Hard Water**

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## 4.1 Abstract

Chronic cadmium toxicity research has gaps due to minimal studies focusing on chronic work. Bioaccumulation is one method of measuring Cd toxicity in an organism. The TRA uses bioaccumulation to find a potential linkage to toxic effects in an organism. Although this is an ideal method, organisms are complicated. Some organisms have the ability to prevent toxic effects from occurring, while other organisms are too sensitive and cannot tolerate accumulation of metals. Therefore, a novel TRA is suggested, where a resistant organism with the ability to accumulate Cd reliably and dose dependently is compared to a more sensitive organism that shows toxic effects at lower Cd concentrations. Accumulation-effect relationships using Cd was the goal of this study. Two chronic experiments using *Hyaella* and *Lymnaea* were conducted simultaneously. For *Hyaella*, endpoints that were monitored were survival, growth (dry wt.) and accumulation throughout the exposure. For *Lymnaea*, the endpoint measured was bioaccumulation. The relationship in *Hyaella* between Cd body burden and survival was 58.3 µg Cd/g dry wt. associated with a 50 % decrease in survival. Due to low levels of Cd, no relationship between accumulation and toxic effects were conducted in *Lymnaea*. Finally, a relationship between mortality in *Hyaella* (sensitive organism) and accumulation in *Lymnaea* (resistant organism) was determined for d 28 of exposure. An LD<sub>20</sub> and LD<sub>50</sub> in *Hyaella* was associated with a 36 and 69 µg Cd/g dry wt. Cd body burden in the soft tissue of *Lymnaea*. Therefore, this novel TRA shows potential but requires more validation for it to be used in the field.

## 4.2 Introduction

Cadmium occurs naturally in the environment, but can be elevated in aquatic environments due to anthropogenic sources such as mining or smelting of metals (Zn, Cu, Pb) (Pan *et al.*, 2010). Cd is a known Ca antagonist and is taken up across a respiratory surface of an aquatic organism through Ca channels (Verbost *et al.*, 1988). While there are many studies documenting the acute effects of Cd, there are fewer that assess the chronic toxicity of Cd.

The traditional parameter used for effects assessment in aquatic organisms is the concentration in the environmental surroundings (the aquatic medium). While measures of total, dissolved (or more recently) free ion concentrations of metal are used to express effects these are surrogates for the concentration acting at the site of toxicity. An internal measure of Cd toxicity may be a better indication because it comes closer to approximating the concentration at the site of toxic action. For relatively small invertebrates whole organism Cd bioaccumulation is one way to assess toxic effects in an organism (Adams *et al.*, 2010).

Bioaccumulation usually occurs in two stages. The initial stage is characterized by increases in tissue concentration as the rate of uptake exceeds rate of elimination of Cd into the organism. The second stage occurs when a steady state develops when elimination and uptake rates balance and tissue burden become constant (Campbell *et al.*, 2008). It is worth noting that there are exceptions to this two stage scenario for bioaccumulation as some organisms form and store metals into granules and thus never really reaches a steady state condition (Croteau and Luoma, 2008). Borgmann *et al.* (1991) examined Cd bioaccumulation in *H. azteca* and found a relationship with EC<sub>50</sub>,

where a  $0.53 \pm 0.2 \mu\text{g Cd/L}$  exposure was associated with Cd burden of  $38 \mu\text{g Cd/g}$  dry wt. Hoang *et al.* (2008) showed steady state reached by the Florida apple snail, *P. paludosa*, exposed to waterborne Cu. The whole body Cu burden rapidly increased until d 14 after which a steady state tissue burden was reached. In my studies (Chapter 3) steady state tissue burden was shown in *Lymnaea* after 14 d of exposure.

The tissue residue approach (TRA) relies on an unambiguous burden to effect relationship in an organism. Toxic effects occur when internal Cd concentrations reach and exceed a threshold at a specific site of toxic action (Campbell *et al.*, 2008). However organisms have the ability to detoxify internalized Cd, preventing it from inducing toxicity (Adams *et al.*, 2010; Wang and Rainbow, 2006; Desouky, 2006). When measuring whole organism or tissue burdens, detoxified pools of metal cannot be distinguished from concentrations that might be interacting at the site of toxic action and therefore clear burden-to-effect relationships cannot be established. In the case mentioned above, Borgmann *et al.* (1991) exposed *Hyaella* to Cd and was able to link a tissue burden to toxicity. However this was only done in one exposure condition and under other conditions (e.g. altered water hardness) may result in the accumulation associated with toxicity being very different.

Adams *et al.* (2010) suggested a novel TRA that could be applied to metals. The modified TRA concept is based on the same approach, in other words, finding relationships between bioaccumulation and toxic effects; however this is not measured in the same organism. Accumulations are assessed in a Cd resistant organism, which can accumulate Cd reliably, in a dose dependent manner and reach steady state tissue burden. While, toxic effects are measured in a Cd sensitive organism, which may not

reliably and consistently accumulate Cd but shows toxicity at low levels of Cd. Therefore, an accumulation-effect relationship was established between two organisms sharing similar freshwater environments, and the resistant organism acts as a bio-indicator of toxicity in the sensitive organisms.

*L. stagnalis* is a freshwater gastropod, known to reliably accumulate high concentrations of a variety of metals such as Pb, Co, Cd and Cu (Cœurdassier *et al.*, 2003; Croteau *et al.*, 2007; Ng *et al.*, 2011). In a study conducted by Cœurdassier *et al.* (2003), they found that *L. stagnalis* were not sensitive to Cd, compared to other organisms. If concentrations were sufficiently elevated inhibited growth, fecundity and fertility can occur and the EC<sub>50</sub> values ranged from 60 to 142 µg Cd/L. Leung *et al.* (2003) discovered adult *L. stagnalis* exposed to 0.01 µg Cd/L did not accumulate significant amounts of Cd as tissue concentrations were similar to controls. On the other hand, exposure to high levels (1000 µg Cd/L) resulted in accumulation of 350 µg Cd/g dry wt. of Cd in the soft tissue within 10 d in hard water of 250 mg/L of CaCO<sub>3</sub>. More studies on chronic Cd toxicity are needed with environmentally relevant concentrations to understand accumulation-effect relationship in *Lymnaea*.

*H. azteca* are freshwater amphipods that are found in North American freshwaters (Environment Canada, 1997). They are easily cultured in laboratory conditions and are known to be sensitive to a variety of metals, including Cd (Borgmann *et al.*, 1991; Borgmann *et al.*, 2005). Borgmann *et al.* (2005) found 7 d LC<sub>50</sub>s were 0.57 and 4.41 µg Cd/L for soft and hard water, respectively (18 and 124 mg/L as CaCO<sub>3</sub>, respectively). A previous study by Borgmann *et al.* (1991) found an EC<sub>50</sub> for Cd accumulation as 38 µg Cd/g dry wt., in hard water (130 mg/L as CaCO<sub>3</sub>).

The goal of this study was to develop a TRA using the model suggest by Adams *et al.* (2010) where potential relationships between accumulation and toxic effects are determined in two different organisms. This study involved side by side experiments with *L. stagnalis* and *H. azteca* exposed to the same concentrations of waterborne Cd for 28 d, in similar water chemistry. In the *H. azteca* exposure, the endpoints measured were survival, bioaccumulation throughout the exposure and growth (dry wt.). Relationships between Cd body burden and toxic effects were determined in *Hyaella*. In the *Lymnaea* exposure, the endpoint measured was bioaccumulation at d 28 of exposure. Due to low levels of Cd exposure and the relative resistance of *Lymnaea*, no toxic effects were anticipated in *Lymnaea*. The goal was to establish a relationship between toxic effects in *Hyaella* (sensitive organism) and accumulation in *Lymnaea* (resistant organism) determined over 28 d of exposure.

## **4.3 Materials and Methods**

### **4.3.1 Snail Culture**

*L. stagnalis* were generously provided by Dr. D. Spafford of the University of Waterloo. Adult and juvenile snails were maintained in a mixture of dechlorinated Waterloo city tap water and reverse osmosis water (see Chapter 2 for details) at  $20 \pm 1$  °C in 10 L aquaria within an Aquatic Habitats (Fisher Scientific, Whitby, ON) recirculating system where approximately 10 % of the water was renewed every second d. When adults in an aquarium laid egg masses, they were moved into new aquaria, water flow

was suspended as eggs hatched. The snails were fed romaine lettuce *ad libitum* and further culture details are provided in Chapter 2.

#### 4.3.2 *Hyalella* Culture

*H. azteca* were purchased from Aquatic Research Organisms (Hampton NH), and cultured in 1L polyethylene beakers. Cultures were maintained in artificial media containing 1.0 mM Ca<sup>2+</sup>, 1.0 mM Na<sup>+</sup>, 0.05 mM K<sup>+</sup>, 0.25 mM Mg<sup>2+</sup> and 0.01 mM Br<sup>-</sup> (Sigma-Aldrich, Nepean, ON). A 5x5 cm piece of sterile cotton gauze was added to each beaker for use as a substrate (Environment Canada, 1997). Temperatures in lab cultures were maintained at 20 ± 2 °C. Each beaker held approximate 20 organisms and received 5 mg of ground Tetramin® flakes (Tetra Werke, Blacksburg, VA, U.S.A.) 3 times a week. Culture water was replaced and neonates were separated from the adults weekly.

#### 4.3.3 General Experimental Procedure

Exposure media was made using reverse osmosis water and dechlorinated Waterloo water to establish a water hardness of 90 mg/L as CaCO<sub>3</sub> (Na = 719 ± 46 µM, Mg = 351 ± 21 µM and Ca = 557 ± 31 µM) and a pH of 7.3 ± 0.1 (mean ± SEM, n = 45). Nominal Cd exposure concentrations were 0, 0.8, 1.6, 3.2, 6.4 µg Cd/L. Cd exposure solutions were made in batches of 20 L 48 h prior to test and placed in polypropylene beakers for 24 h to equilibrate. Prior to the exposure snails were blotted to remove excess water and then *Hyalella* were placed in separate exposure containers beside each other to ensure similar conditions. Water was renewed every 48 h and 10 mL samples were taken prior to and after renewal. Samples were collected for total metal (no filtration) and dissolved metal (0.45 µm filtered) as described in Chapter 2.



#### 4.3.4 Chronic Test (CT) using *H. azteca*

Exposures followed standard method EPS 1/RM/33 (Environment Canada, 1997) and were conducted using ten 2 - 9 d old neonates in 500 mL beakers with 400 mL of exposure media. Four replicates were done, a substrate (5 x 5 cm piece of cotton gauze) was provided for each beaker and 5 mg of ground Tetramin® flakes were fed every water renewal (every 48 h). Surviving individuals were placed back in the exposure at each water renewal and dead individuals were removed using a disposable pipette. To monitor Cd bioaccumulation during the exposure, 6 individual organisms were sampled on d 0, 1, 4, 7, 14, 21 and 28. Each *Hyalella* was given 6 h the control medium to allow for clearance of gut contents before further being weighed and digested (see below).

#### 4.3.5 Chronic Test (CT) using *L. stagnalis*

The test was conducted using 10 snails with a shell length of approximately 5 mm snails, in each beaker. Lettuce was placed in each beaker after water renewal. Every week (on d 7, 14, 21 and 28 of exposure) survival was assessed and shell length of snails was measured. At the end of the exposure soft tissues were collected for Cd accumulation was characterization by removing snails from the exposure, placing them in ultrapure water for 10 min and then storing in the freezer at - 80 °C.

#### 4.3.4 Analysis

Sample analysis was as described in Chapter 2 for *Lymnaea* tissues and water samples. In brief, snails were sampled on d 28 for soft tissue Cd burden. The soft tissue was separated from the shell, weighted and then placed into 2.0 mL centrifuge tubes and

dried at 80 °C for 48 h. Following drying, individuals were weighed and then digested in 300 µL of 70 % trace-metal grade HNO<sub>3</sub> for 2 d at 65 °C. Individual amphipods were blotted dry, placed in the oven in open 0.6 mL centrifuge tubes for 48 h at 80 °C, weighed using a Sartorius SE2 Ultra Micro Balance (Sartorius Mechatronics Corp., Bohemia, NY, U.S.A) and then digested in 25 µL of concentrated nitric acid (trace-metal grade, Fisher Scientific, Whitby, ON) for 6 d, after which 20 µL of 30 % H<sub>2</sub>O<sub>2</sub> was added for an additional 24 h (Borgmann and Norwood, 1997).

Following digestion of soft tissue (snails) and whole organism (amphipods), samples were mixed by vortex and centrifuged (Spectrafuge 16M; Labnet International, Edison, NJ, USA) at 5000 g for 10 min and the supernatant was measured after appropriate diluted with 1 % HNO<sub>3</sub> (trace metal grade). Cd characterization (tissue and water samples) was done by graphite furnace atomic absorption spectrophotometry (GF-AAS, GTA 100 with SpectAA-880, Varian Inc., Palo Alto, CA). Water samples for Ca, Na and Mg were measured using AAS in flame mode. Certified reference materials (TM 28.3 and TM 26.5, National Water Research Institute, Environment Canada) were used to assess recovery of metals in water and recovery was within 15 %. Lobster hepatopancreas reference material (TORT-2, National Research Council Canada) was used to assess the recovery of metals in tissue for tissue quality checks and recovery was never less than 85 % (85 – 92.3 %).

#### 4.3.5 Statistical Analysis and LC<sub>50</sub> Calculation

Data have been presented as means ± 1 standard error of the mean, unless otherwise stated. For *Hyalella* test, the 7, 14, 21 and 28 d LC<sub>50</sub> values with 95 %

confidence intervals were calculated from observed mortality responses and the measured dissolved Cd concentrations using CETIS (Comprehensive Environmental Toxicity Information System Software v1.6.1. rev C Tidepool Scientific Software, McKinleyville, U.S.A.). One-Way ANOVA was used to test effects of Cd accumulation and exposure concentration in *Lymnaea*. Two-Way ANOVA was used to test effects of Cd toxicity and growth over the course of the exposure. Following the significant result, the Fisher LSD post hoc test was used to identify differences among means. Linear and exponential analyses were performed to describe relationships between survival or growth and Cd burden in *Hyalella*. Significance of all tests was taken at  $p < 0.05$ .

## **4.4 Results**

### 4.4.1 CT using *Hyalella*

#### Survival and Growth

Cd content of water samples from the *Hyalella* and the *Lymnaea* exposures were not significantly different and at each exposure concentration there was no significant difference between total Cd concentration and dissolved Cd concentration (Table 4.1). The measured Cd concentration in exposures (0.6, 1.3, 2.6 and 5.1  $\mu\text{g Cd/L}$ ) were lower than the nominal target values. In the intermediate hard water (hardness = 87  $\text{mg/L CaCO}_3$ ), the *Hyalella* showed a decrease in survival with increasing Cd concentration (Fig 4.1). The 7, 14, 21 and 28 d  $\text{LC}_{50}$  values for the *Hyalella* were 4.6  $\mu\text{g Cd/L}$  (95% CI: 3.0 to 7.0  $\mu\text{g Cd/L}$ ), 1.6  $\mu\text{g Cd/L}$  (1.1 – 2.2  $\mu\text{g Cd/L}$ ), 0.75  $\mu\text{g Cd/L}$  (0.51 – 1.1  $\mu\text{g Cd/L}$ )

and 0.70 µg Cd/L (0.53 – 0.9 µg Cd/L), respectively. This shows that *Hyalrella* are sensitive to Cd, where 100% mortality was seen at d 14 at the highest (5.1 µg Cd/L) exposure concentration. Dry wt. of individuals also decreased with increasing Cd concentrations, showing similar significant reduction in dry weight to all exposed individuals at d 28.

### Bioaccumulation

The bioaccumulation of Cd was also measured during the exposure. Bioaccumulation patterns showed that *Hyalrella* can accumulate Cd and reach steady state by d 7, leading once again to higher Cd accumulation at the higher Cd exposure concentrations (Table 4.2). However on d 28, there were no significant differences in accumulation at the higher concentrations, a 1.3 µg Cd/L exposure gave  $110 \pm 64$  µg Cd/g dry wt. while exposure to 2.6 µg Cd/L resulted in 145 µg Cd/g dry wt. (Table 4.2). The Cd burden was compared with survival and length on d 28 and a negative correlation was evident (Fig 4.3 A, linear regression  $y = 85 - 0.6x$  ( $r^2 = 0.8$ )). Similarly, a reduction in dry weight of *Hyalrella* was seen with an increase in tissue body burden (Fig 4.3 B, linear regression  $y = 0.1 - 0.0002x$  ( $r^2 = 0.1$ )).

#### 4.4.2 CT using *Lymnaea*

### Bioaccumulation and Linkages

The *Lymnaea* were exposed to the same water chemistry and Cd concentrations as the *Hyalrella* but showed no mortality effect (100 % survival in all exposures). Similarly shell length and tissue weight at the end of the exposure did not vary significantly among

exposure groups (Table 4.2). At the end of the 28 d exposure, the surviving snails were analyzed for Cd burden. Increased exposure to waterborne Cd corresponded to an increase in Cd burden in soft tissue (Fig 4.5). The lowest Cd concentration resulted in accumulation of  $74.4 \pm 16.9 \mu\text{g Cd/g}$  at d 28.

Accumulation (in *Lymnaea*) vs. effect (in *Hyalella*) model is presented in Fig 4.5 by plotting survival of *H. azteca*, and body burden at steady state of *L. stagnalis* along the same x-axis (Cd exposure concentration). The lines show a 20 % (purple line) and 50 % (red line) survival effect in *Hyalella* was linked to tissue burden in *Lymnaea*. The tissue concentrations in *Lymnaea* associated with impacts in *Hyalella* were 36 and 69  $\mu\text{g Cd/g}$  dry wt., respectively.

## **4.5 Discussion**

### 4.5.1 CT using *Hyalella*

#### Survival and Growth

The concentration range was chosen due to the sensitivity of *Hyalella* and was confirmed when *Hyalella* mortality increased with both increasing Cd concentration and test duration through 28 d of exposure, leading to 28 d LC<sub>50</sub> value at 0.7  $\mu\text{g Cd/L}$  (95 % CI: 0.6 – 0.9  $\mu\text{g Cd/L}$ ). This concentration confirms the sensitivity of *Hyalella* because it is an environmentally relevant concentration (McGeer *et al.*, 2011). Test duration of only 21 d may be required to accurately assess toxicity as LC<sub>50</sub> values for d 21 and 28 were similar (Fig 4.1). More acute toxicity studies have been conducted compared to chronic

toxicity of Cd using *Hyalella* therefore it is difficult to confirm the correct range of the LC<sub>50</sub>. Collyard *et al.* (1994) reported a 96 h LC<sub>50</sub> value of 6 – 13 µg Cd/L in water hardness values similar to the present study, showing similar toxicity range as in the present study. However, toxicity data reported in the present study showed higher toxicity effects than data reported by other studies. Borgmann *et al.* (1991) found a similar LC<sub>50</sub> value as the current test (0.72 µg Cd/L), in a 6 week exposure of water hardness at 13 mg/L as CaCO<sub>3</sub>, although lower water hardness values are supposed to increase *Hyalella* sensitivity to Cd (Borgmann *et al.*, 2005). Seudel *et al.* (1997) found a 14 d LC<sub>50</sub> value of 0.65 µg Cd/L (95 % CI: 0.38 – 0.92 µg Cd/L) in soft water (6 - 28 mg/L as CaCO<sub>3</sub>). All the differences in LC<sub>50</sub> values may be due to the variation in exposure condition (e.g. duration, water hardness, pH and DOC levels), therefore, may contribute to the observed differences in cadmium toxicity. In conclusion, *Hyalella* are one of the most sensitive organisms to waterborne Cd exposure.

In control beakers, the average size of *Hyalella* on d 28 was  $0.38 \pm 0.04$  mg wet wt. Borgmann *et al.* (1991) showed *Hyalella* at  $1.3 \pm 0.012$  mg wet wt. ( $\pm$  SD) after 6 weeks at water hardness value of 130 mg/L as CaCO<sub>3</sub>. Once again, this dissimilarity may be due to water chemistry differences as well as exposure duration. Growth was a more sensitive measure of Cd toxic effect as all the exposed *Hyalella*, even at the lowest concentration (0.6 µg Cd/L), had a significant reduction in dry wt. compared to the controls (Fig 4.2). However, it has been reported that survival is a more sensitive measure than growth (Seudel *et al.*, 1997; Borgmann *et al.* 1989). Seudel *et al.* (1997) conducted a 14 d test which showed no significant difference in wt. and similarly, the current study showed no differences in dry wt. of Cd exposed *Hyalella* and controls on d 14.

## Bioaccumulation

Cd bioaccumulation in *H. azteca* was seen to increase rapidly at the start of the exposure (Table 4.2). However, over time, accumulation seemed to equilibrate at which point, it is assumed that elimination and uptake are equal, reaching steady state tissue burden between d 7 and 14 of exposure (Table 4.2). Significant differences in tissue body burden were seen in the surviving *Hyalabella* at the two highest Cd concentrations (1.3 and 2.6 µg Cd/L) compared to the control on d 28, reaching levels of 110 – 145 µg Cd/g dry wt. However, due to the high variability of Cd burden in the whole body, no significant differences are seen amongst the exposed *Hyalabella*. Borgmann *et al.* (1991) discovered that hardness of water plays a minor effect on bioaccumulation. At water hardness of 13 and 130 mg/L as CaCO<sub>3</sub>, body burdens were approximately 100 and 90 µg Cd/g dry wt., respectively, at the highest concentration (1 µg Cd/L), with the lower hardness value slightly higher in burden. The body burdens are slightly lower than the ones presented in the current study, which may be a result of higher exposure concentrations in the current study.

Survival and dry wt. of *Hyalabella* were plotted against Cd accumulation to find a relationship curve (Fig 4.3). The linkage between survival and Cd burden was a strong linear relationship with an  $r^2$  value of 0.8 (Fig. 4.3 A). It was defined that 8.3 and 58.3 µg Cd/g dry wt. body burdens would cause 20 or 50 % decline in survival of *Hyalabella*. Borgmann *et al.* (1991) defined their body burden EC<sub>50</sub> for water hardness values of 130 and 13 mg/L as CaCO<sub>3</sub> to be 38 and 79 µg Cd/g dry wt. The current study showed similar range in results falling in between the hard and soft water results presented by Borgmann *et al.* (1991). However, when comparing dry wt. of the *Hyalabella* against Cd

bioaccumulation, a very weak relationship was seen with an  $r^2$  value of 0.1 (Fig 4.3 B). Other studies have shown growth to be a weaker endpoint to monitor compared to survival (Borgmann *et al.*, 1991; Borgmann *et al.*, 2005; Seudel *et al.*, 1997).

#### 4.5.2 CT using *Lymnaea*

##### Bioaccumulation

*Lymnaea* did not show any survival or growth effect in relation to the Cd concentrations exposed. The shell lengths were not significantly different from highest exposure concentration to the control (Table 4.3). The bioaccumulation of Cd showed to accumulation in a dose dependent pattern with higher Cd exposure concentrations, showing greater burden in the soft tissue of the *Lymnaea* (Fig 4.4). This resulted in a strong linear relationship between Cd exposure concentrations to Cd body burden with  $r^2$  value of 0.99. This linear relationship with accumulation and exposure concentration is seen in other metals as well with this species, such as Cu, Co and Pb (Ng *et al.*, 2011; De Schamphelaere *et al.*, 2008; Grosell *et al.*, 2006).

#### 4.5.3 *Hyalella* and *Lymnaea*

##### Accumulation-effect Relationship

Due to the high variability of bioaccumulation of *Hyalella*, it is difficult to accurately and reliably compare bioaccumulation and toxic effects (i.e. survival, growth) seen in *Hyalella* (Table 4.2). Moreover, although *Lymnaea* are more reliable with Cd accumulation, at environmentally relevant concentrations, it is difficult to identify toxic effects because they are moderately resistant to Cd (Fig. 4.4). Also, *Lymnaea* are known



to reach steady state tissue burden in metal exposures in a short time frame (See Chapter 3 above, Fig 3.3; Hoang *et al.*, 2008). Therefore, comparing an organism that can reliably accumulate Cd and reach steady state tissue burden (*Lymnaea*) to an organism with toxic effects seen in environmentally relevant concentrations (*Hyalella*) can be beneficial for identifying an accumulation-effect relationship. The use of identical water chemistries and concentrations, as well as side by side exposures allow for linking of these two exposures. Plotting 28 d survival in *Hyalella* and body burden at steady state tissue burden in *Lymnaea* against the same x-axis (Cd concentrations), accurate predictions of specific body burdens can correspond to specific effect concentrations. Fig 4.5 accurately depicts the novel TRA approach Adams *et al.* (2010) hypothesized for an accumulation-effect linkage. In the figure, *H. azteca* LD<sub>20</sub> and LD<sub>50</sub> values are 0.42 and 0.70 µg Cd/L, linked to body burdens of 36 and 69 µg Cd/g dry wt. in *L. stagnalis*.

*L. stagnalis* are the ideal organisms to be used as a resistant organism for this approach. They accumulate metals reliability and in a dose dependent manner and reach steady state tissue burden in a relatively short time (14 d; Ng *et al.*, 2011; Chapter 3 of thesis, Fig. 3.3, Hoang *et al.*, 2008). They are known to be resistant to Cd compared to most organisms (Cœurdassier *et al.*, 2003) and have a wide geographical distribution (Desouky, 2006; Cœurdassier *et al.*, 2003). Their relative size is ideal for multiple measurements on an individual organism. On the other hand, *H. azteca* is an ideal organism to be used in laboratory as a sensitive organism because they are known to be the most sensitive organism to Cd (Borgmann *et al.*, 1991; Seudel *et al.*, 1997). They show toxic effects of Cd at environmentally relevant concentrations and this response is exposure concentration dependent.

In conclusion, *H. azteca* had a 28 d LC<sub>50</sub> value of 0.7 µg Cd/L and exposed individuals had a reduction in dry wt. compared to the control. *Hyalella* accumulated Cd at the two higher concentrations between 110 to 145 µg Cd/g dry wt. Accumulation-effect relationships were plotted for *Hyalella* on d 28 and a strong correlation between survival and accumulation was seen with 50 % reduction in survival was linked to 58.3 µg Cd/g body burden. However, due to the variability of body burden in *Hyalella*, this relationship may vary. *Lymnaea* did not show any toxic effect with Cd exposure. *Lymnaea* did accumulate Cd exposure dependently and reliably. However, no accumulation-effect relationship was established using only *Lymnaea*. Therefore, a novel TRA was predicted using *Hyalella* survival and *Lymnaea* accumulation, plotted on the same x-axis, Cd concentration. This helped determine an LD<sub>50</sub> of *Hyalella* (0.7 µg Cd/L) associated with a body burden in *Lymnaea* of 69 µg Cd/g dry wt. Therefore, this novel TRA holds promise in determining accumulation-effect relationships and requires further validation.

#### 4.5.4 Integration

Cadmium is present in the aquatic environment, and at elevated levels can cause detrimental effects to organisms. Invertebrate organisms are abundant in the aquatic ecosystem and can be the first to be effected by metal contamination. *Hyalella* and *Daphnia* are among the most sensitive organisms to waterborne Cd. However, there are many invertebrates with the ability to detoxify and store Cd, without causing detrimental effects. For example, *Lymnaea* and *Lumbriculus* use MTLP which binds Cd, rendering it biologically unavailable for further toxic effects. As biologists' one aspect of a problem/project is focused on and tends to separate the "big picture," losing focus of the main

goal. However, this project tries to integrate multiple solutions to assess environmental disturbances in an ecosystem.

In the present thesis, the main focus of the research was to determine if bioaccumulation can be used as an effective measure of toxicity. The focus was on one organism, *Lymnaea*, an abundant freshwater snail, seen in a variety of aquatic environments throughout the northern hemisphere. Low Cd concentrations (environmentally relevant) were shown to have no toxicological effects seen in the whole body of *Lymnaea* due to their moderate resistance to Cd. A molecular technique focusing on the Cd distribution within a cell was used to potentially link toxic effects with accumulation. However, more work on sub-cellular fractionation needs to be completed to fully understand the patterns observed. Since one organism was not able to link accumulation with effect, a boarder picture was used. Two organisms that inhabit a common ecosystem were exposed separately to similar treatments (i.e. water chemistry, exposure concentrations). This novel approach linked toxic effects seen in one organism (sensitive to Cd) to accumulation in another organism (moderately resistant to Cd). The survival effect seen in *Hyaella* was associated to the Cd accumulation seen in *Lymnaea*. This integrates the project and unifies its goal to use bioaccumulation as a toxicological measure for assessing the environment.

Furthermore, this project integrates research performed by multiple labs, both at McMaster University as well as Wilfrid Laurier University. This project will help establish multiple accumulation-to-effect relationships for Cd, Cu and Ni using *Lymnaea*, *Hyaella*, *Daphnia*, rainbow trout and round gobies; all organisms that share a freshwater environment.

## 4.6 References

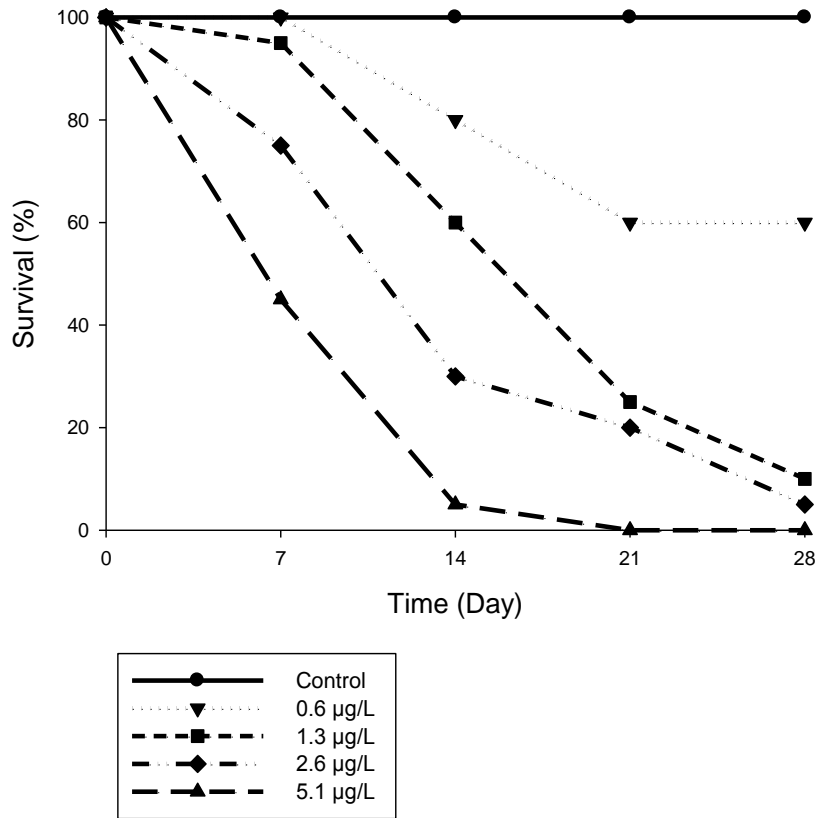
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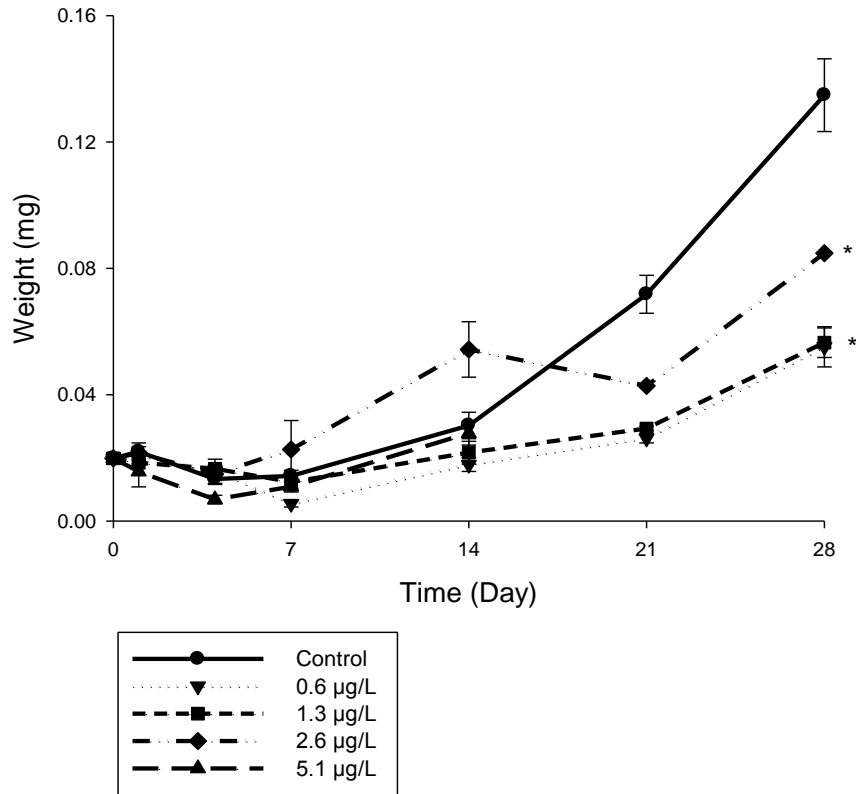
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## 4.7 Figures

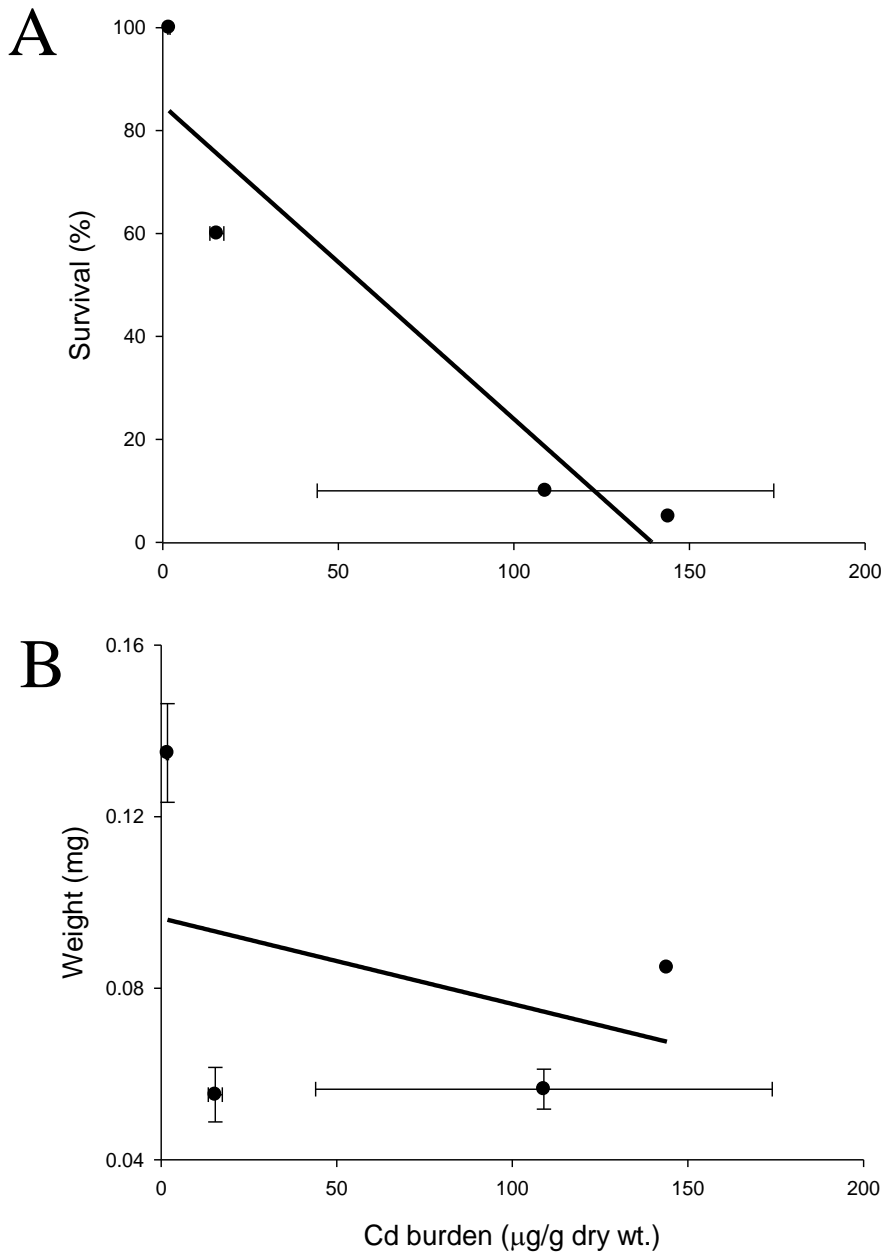


**Figure 4.1.** The chronic effects of waterborne Cd on the survival of *H. azteca* exposed to 0.6, 1.3, 2.6 or 5.1 µg Cd/L over 28 d. A group of unexposed *Hyalella* (controls) was also included. Values are in mean with n = 10. The 7, 14, 21 and 28 d LC50 values were calculated as 4.6 (95 % CI: 3.0 - 7.0), 1.6 (1.1 - 2.2), 0.75 (0.51 - 0.9) and 0.70 (0.53 - 0.9) µg Cd/L respectively.

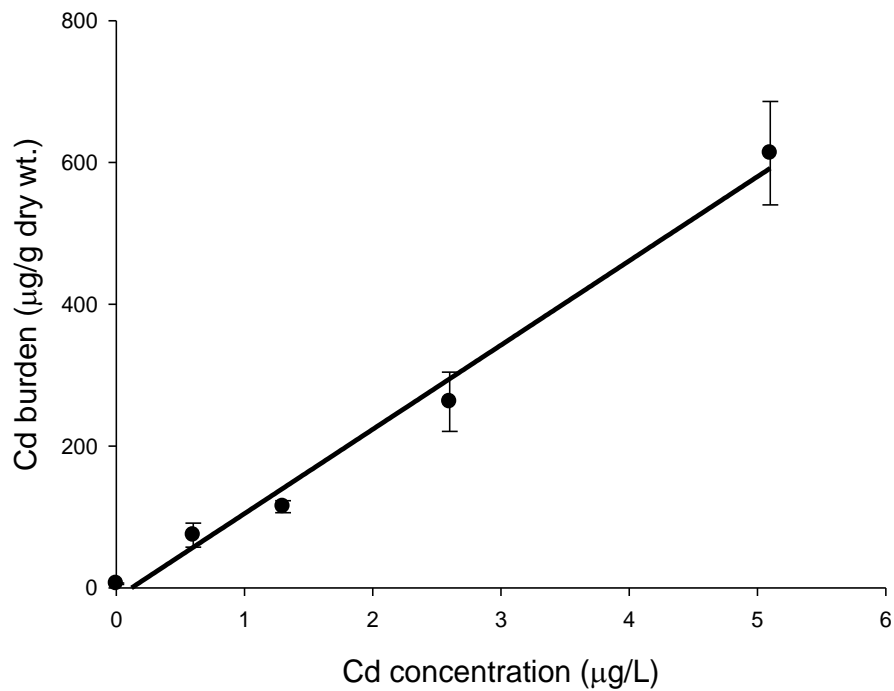




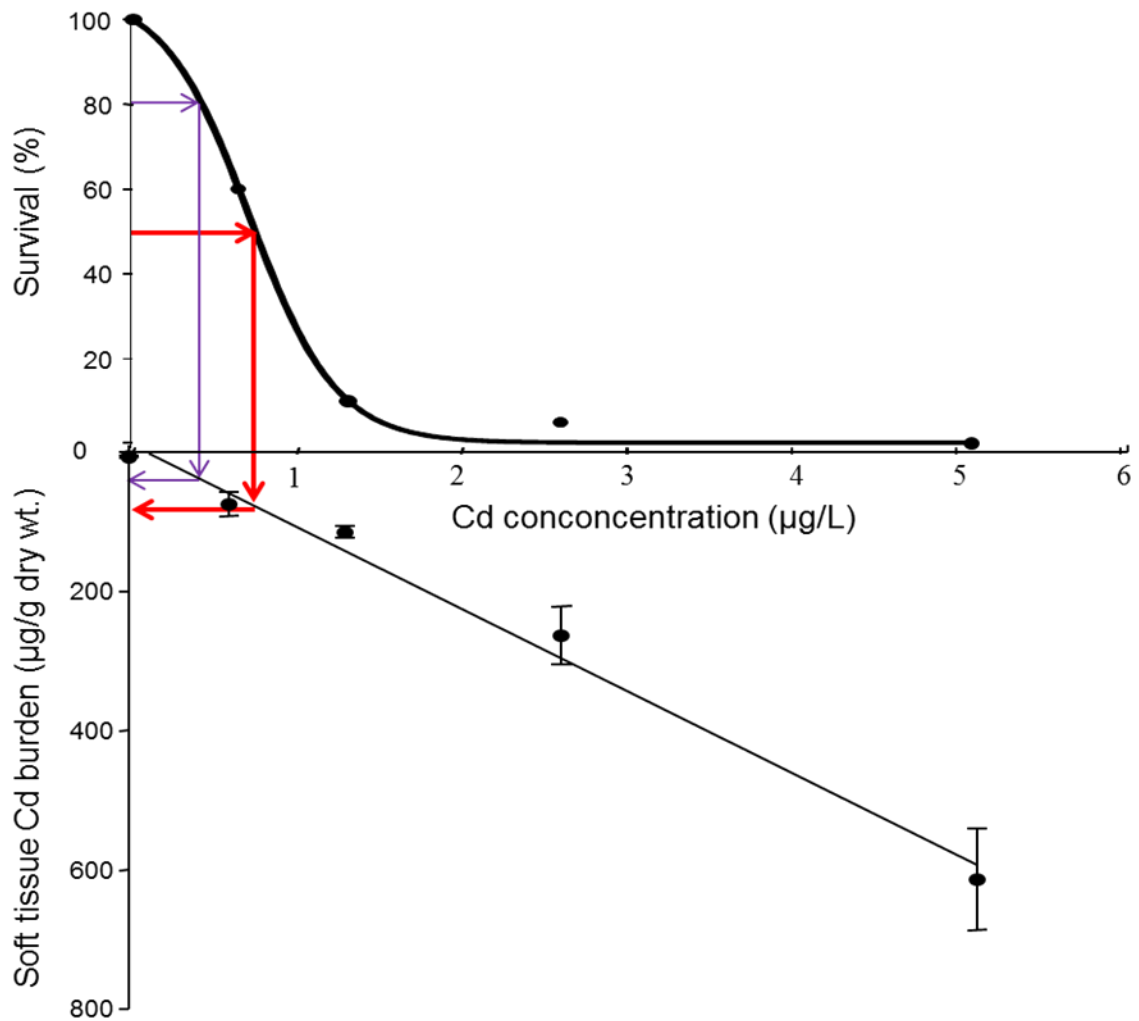
**Figure 4.2.** Mean ( $\pm$  SEM) dry wt. of exposed *H. azteca* to 0.6, 1.3, 2.6 or 5.1  $\mu\text{g Cd/L}$  over 28 d. A group of unexposed (control) *Hyalella* were included, data is shown on a whole organism basis with  $n = 6$  for each mean and an \* indicates a significant difference from control at that d ( $p < 0.05$ ; Two-way ANOVA, Fisher LSD).



**Figure 4.3.** Correlation between survival (A) and dry wt. (B) and whole body Cd burden in the *Hyalella* at d 28. Values are in mean  $\pm$  SEM. Linear lines depicting correlations for panel (A)  $y = 85 - 0.6x$  ( $r^2 = 0.8$ ,  $p = 0.05$ ) and panel (B)  $y = 0.1 - 0.0002x$  ( $r^2 = 0.1$ ,  $p = 0.6$ ). The n values are, for the control  $n = 20$ ,  $0.6 \mu\text{g Cd/L}$   $n = 12$ ,  $1.3 \mu\text{g Cd/L}$   $n = 2$  and  $2.6 \mu\text{g Cd/L}$   $n = 1$ .



**Figure 4.4.** Soft tissue Cd accumulation of *Lymnaea* at d 28 exposed to waterborne Cd. A linear solid line for Cd accumulation is shown ( $y = -14 + 119 x$ ;  $r^2 = 0.99$ ,  $p = 0.0006$ ). Values are mean  $\pm$  SEM ( $\mu\text{g Cd/g dry wt.}$ ),  $n = 10$ .



**Figure 4.5.** Linkages between the chronic impact of Cd on in *H. azteca* and accumulation in *L. stagnalis*. The top part of the figure shows the exposure-response relationship in *Hyalella*, a sigmoidal curve ( $y = 106.9 / (1 + e^{-(x-0.7)/-0.26})$ ), while the bottom half shows the matching exposure accumulation relationship (both after 28 d of exposure). Soft tissue Cd concentrations in *Lymnaea* are given as mean  $\pm$  SEM ( $\mu\text{g Cd/g dry wt.}$ ,  $n = 10$ ) and the line show the best fit linear regression ( $y = -14 + 119x$  ( $r^2 = 0.99$ ,  $p = 0.0006$ )). The purple and red lines indicates the 20 % and 50 % survival effect levels in *Hyalella*, corresponding to 0.42 and 0.7  $\mu\text{g Cd/L}$ , respectively. The body burdens in *Lymnaea* associated with these effects in *Hyalella* were 36 and 69  $\mu\text{g Cd/g d wt.}$ , respectively. Values are mean  $\pm$  SEM ( $\mu\text{g Cd/g dry wt.}$ ).

## 4.9 Tables

**Table 4.1:** Measured concentrations of total and dissolved Cd during both *Hyalella* and *Lymnaea* chronic tests. Temperature of exposure water was 21 – 21.7 °C. Values are expressed as means  $\pm$  SEM (n = 90, for each concentration). Dissolved Cd concentrations were measured before (fresh solutions) and after (measured at 48 h prior to solutions renewal).

Nominal ( $\mu\text{g Cd/L}$ )	Total ( $\mu\text{g Cd/L}$ )	Dissolved ( $\mu\text{g Cd/L}$ )	
		Before	After
Control	0.01 $\pm$ 0.001	0.01 $\pm$ 0.001	0.01 $\pm$ 0.004
0.8	0.64 $\pm$ 0.02	0.61 $\pm$ 0.02	0.59 $\pm$ 0.02
1.6	1.38 $\pm$ 0.04	1.29 $\pm$ 0.04	1.23 $\pm$ 0.03
3.2	2.65 $\pm$ 0.07	2.60 $\pm$ 0.07	2.35 $\pm$ 0.09
6.4	5.20 $\pm$ 0.08	5.12 $\pm$ 0.08	4.39 $\pm$ 0.15

**Table 4.2:** Cd burden in the whole body of *Hyaella* throughout chronic exposure. Cd concentrations displayed with the burden associated ( $\mu\text{g/g}$  dry wt.)  $\pm$  SEM with (n values). An \* indicates significance from controls. (Two-way ANOVA, Fisher LSD).

Conc. ( $\mu\text{g Cd/L}$ )	Cd burden ( $\mu\text{g Cd/g dry wt.}$ ) $\pm$ SEM (n value)						
Days	0	1	4	7	14	21	28
Control	1.7 $\pm$ 0.6 (15)	1.4 $\pm$ 0.3 (6)	2.4 $\pm$ 0.3 (6)	1.5 $\pm$ 0.3 (6)	1.0 $\pm$ 0.4 (6)	1.6 $\pm$ 0.9 (6)	1.9 $\pm$ 0.3 (20)
0.6		2.8 $\pm$ 0.6 (6)	4.6 $\pm$ 2 (6)	50 $\pm$ 27 (6)	32 $\pm$ 4 (6)	43 $\pm$ 3 (3)	15 $\pm$ 2 (12)*
1.3		6.1 $\pm$ 2.0 (6)	28 $\pm$ 5 (6)	50 $\pm$ 5 (6)	45 $\pm$ 19 (6)	56 $\pm$ 6 (2)	110 $\pm$ 64 (2)*
2.6		13 $\pm$ 2 (6)	100 $\pm$ 11 (6)	120 $\pm$ 17 (6)	154 $\pm$ 64 (6)	114 (1)	145 (1)*
5.1		53 $\pm$ 13 (6)	157 $\pm$ 25 (6)	80 $\pm$ 33 (6)	217 $\pm$ 71 (6)	N/A	N/A

**Table 4.3:** Shell length of *Lymnaea* at d 28 of exposure. Shell lengths displayed as mean (mm)  $\pm$  SEM with (n values).

Concentration ( $\mu\text{g Cd/L}$ )	Shell length (mean $\pm$ SEM, mm)
Control	9.45 $\pm$ 1.2 (6)
0.6	8.80 $\pm$ 0.7 (6)
1.3	8.65 $\pm$ 0.6 (6)
2.6	8.50 $\pm$ 1.2 (6)
5.1	7.6 $\pm$ 0.6 (6)