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A Study of the Waterborne and Dietary Toxicityof Cadmium to *Hydra attenuata* and *Daphnia plux* in Soft Waters and the Development of Biotic Ligand Models to predict such toxicity

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by

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AUTHOR'S DECLARATION

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Chapter 1

General Summary

The goal of this thesis was to compare the effects of waterborne Cd exposures and dietary Cd exposures to *Hydra attenuata*. Before the Hydra could be exposed to dietborne exposures of Cd, the effects of waterborne exposures had to be understood. This was done by looking at the sensitivity of *D. pulex* to Cd and then understanding the accumulation of Cd. After bioaccumulation patterns of Cd to *D. pulex* were known, we could then feed the prey to the Hydra and compare the effects to waterborne effects of Cd to Hydra.

To understand the waterborne effects of Cd to *D. pulex* 48 h acute toxicity tests were done using a lethal endpoint. It was found that Cd toxicity was most strongly affected by Ca^{2+} activity followed by Mg^{2+} activity. *D. pulex* in soft waters were more sensitive to the effects of Ca and Mg relative to *C. dubia* in hard waters. Na^{+}, K^{+}, H^{+}, and Cl^{-} did not have any significant effects on Cd toxicity. Nordic Reservoir and Suwannee River NOM did not differ in their effects on Cd toxicity, however both sources were able to complex Cd, rendering Cd un-bioavailable to *D. pulex.*

The waterborne effects of Cd to *H. attenuata* were similar to what was seen in waterborne exposures of Cd to *D. pulex*. From this study, Ca proved to have a protective effect on Cd toxicity, while the other ions manipulated in toxicity tests (Mg, Na, K, Cl) did not. DOC also did not show any signs of decreasing Cd toxicity in the concentrations tested. When Cd toxicity was assessed by lethality, LC50s varied from 0.39-1.53μM, and when Cd toxicity was assessed using the sub-lethal end

point of clubbed tentacles, the EC50s ranged from 0.18-0.79μM. The latter concentrations may not be associated with immediate death, however the impairment of feeding capabilities would ultimately affect the population of *H. attenuata*.

From the above tests on waterborne Cd toxicity to *D. pulex* and *H. attenuata* it was found that Hydra are more sensitive and thus Daphnia can be used as a vector for dietborne exposures. To quantify the effects of dietary Cd to *H. attenuata* we chose an exposure concentration of Cd with a known toxicological effect: the EC50, which is marked by the presence of clubbed tentacles. In hardness' of 40 and 140 mg $CaCO_3$/l the EC50 for Hydra has been determined to be approximately 35 and 90 μg/l, respectively. The percentage of surviving Hydra after being exposed to dietborne exposures will allow for a comparison to the waterborne effects and determine which pathway is more sensitive. Waterborne and dietary exposures can also be combined into a co-exposure that will mimic the exposures in a natural setting where the food is exposed to the same concentration and the Hydra.

After determining that Hydra are more sensitive to Cd than Daphnia, we were able to use Daphnia as the dietborne exposure vector. When we compared the waterborne and dietborne exposures of Cd to Hydra, it was clear that when the exposure concentrations of the Hydra and the prey were consistent the dietborne effects were not significantly affecting the Hydra relative to the waterborne exposures.

Chapter 2

General Introduction

2.1 General Overview

The impact of metals on aquatic organisms is strongly influenced by the geochemistry of their environments (Campbell, 1995). Variations in ion concentrations, pH and organic matter can alter the bioavailability of dissolved metal and thus the uptake and degree of physiological disruption induced (Di Toro et al., 2001). In recent years, progress has been made on understanding geochemical influences on metal speciation and bioavailability. However, the relationship between bioavailabile metal and the toxicity of such metal remains an area that needs to be better understood. The bioavailability and thus toxicity of waterborne metals is reduced by two factors, anionic complexation of metals, and the competition between free ion forms of the metal in solution with other cationic elements (Campbell, 1995; Di Toro et al., 2001). In addition to waterborne exposures of metals, organisms can be exposed to metals through diet. While waterborne exposure to metals primarily results in the disruption of ionoregulation, dietary exposures represent a different route of exposure and different tissues being exposed. Direct effects on the digestive tract are possible and the uptake and bioaccumulation to tissues will differ compared to waterborne exposures (Goulet et al., 2007). In contaminated ecosystems, organisms are exposed via both water and diet and therefore understanding both vectors of exposure and the relative toxic effects (both acute and chronic) is essential for metals risk assessment.

2.2 *Hydra* spp.:

Hydra (Cnidaria: Hydrazoa) are freshwater organisms that are commonly found in slow moving rivers and streams (Beach and Pascoe, 1998). Such organisms have a simple anatomy consisting of two major divisions: the column and the hydranth (Trottier et al., 1997). The column can be subdivided into four sections, starting from the basal disk, which attaches the organism to a substrate followed by the peduncle, the budding region, and the gastric region (Trottier et al., 1997). The hydranth contains the hypostome where the tentacles articulate, and also the mouth (Trottier et al., 1997). The gross anatomy of Hydra is completely diplobastic (Karntanut and Pascoe, 2000), meaning that the organism consists of two layers of cells. This means that all of the cells of the organism are in contact with the aquatic environment and, therefore directly exposed to any contaminants that are present.

Because of its sensitivity to metals, Hydra is a good organism to be used in laboratory toxicity tests (Holdway et al., 2001). Hydra are also relatively small and multiply rapidly, therefore they can be tested in high numbers. Hydra reproduce asexually via budding, which significantly decreases the genetic variability in populations cultured in laboratories. Reduced genetic variability is assumed to reduce the variability of responses in tests. This allows for a stronger correlation between exposure concentrations and the responses observed (Holdway et al., 2001). Finally, the expression of toxicity in Hydra is easily assessed. Toxicity results in morphological changes; tentacles shrink and

completely retract and this is followed by the complete disintegration of the organism (Trottier et al., 1997).

2.3 Daphnia spp.:

Daphnia pulex is a small planktonic crustation that can be found in many freshwater ecosystems. A carapace covers the soft-tissues of the body and provides protection for the organism. *Daphnia* spp. are filter feeders that swim using two antenna on either side of their carapace. Near the antenna lies the opening of the digestive tract. The digestive tract runs through the body and ends at the anus, which is located near the postabdominal claw. On the ventral side of the organism are gills, which sweep water in a ciliary movement. The heart and brood pouch are located on the dorsal side of the organism. Broods can consist of 5-30 neonates, depending on the species.

The cladoceran *D. pulex* was also chosen to be a test organism in these experiments because of its ability to be reared in soft waters (Muyssen et al., 2006) and its sensitivity to metals. From a practical perspective, *Daphnia* spp. are an ideal organism because they can be easily cultured in the lab and, with a short life cycle, can produce large numbers of offspring (Muyssen et al., 2006). From an ecological perspective, *Daphnia* spp. are very low on the food chain, and therefore their response to environmental stressors may be considered as an early bio-indicator of impacts on the biota in the ecosystem (Muyssen et al.,

2006). In comparison to other *Daphnia* spp., *D. pulex* has been shown to have a higher sensitivity to metals, and thus, is believed to be a better bio-indicator to the effects of metals in the environment (Shaw et al., 2006).

2.4 Biotic Ligand Model:

Biotic Ligand Models (BLMs) predict the toxicity mitigating effects of anionic complexation, cationic competition by dissolved ions such as Ca, Mg and Na and organic matter complexation of metals to different organisms (Villavicencio et al., 2005). In order to develop a BLM, many acute toxicity tests are performed and in each parameters such as ionic composition, dissolved organic matter (DOC) concentration, and pH are varied to determine their effects, if any, on toxicity. As a result, a matrix of test results are generated and the trends in toxicity for each parameter can be observed and compared relative to other parameters. Observed trends among the parameters can be used to calculate equilibrium binding constants (Log K values). These binding constants are then incorporated into the model to integrate all of the variables that will affect the toxicity of the metals.

Niyogi and Wood (2004) discussed three principles on which the BLM is founded. These principles are integrated with an equilibrium modeling approach, which uses speciation prediction equations to determine the complexation of metal in the water and thus calculate the amount available to bind to the ligand.

The first principle is that it is the free ion form of metal that is taken up by an organism and causes toxicity; this is the direct interaction of the organism with the free ion form of the metal, and is categorized as physiology in figure 1. When free metal ions are complexed in solution they are not taken up and thus do not contribute to toxicity. The second principle is that the water chemistry will affect the speciation of the metal in solution and specifically the amount of metal in free ion form. In figure 1, these chemical interactions between the metal and inorgancs or organic matter are categorized as chemistry. The third principle is that dissolved cations in a solution can compete for the binding sites on the organism; this will result in decreased metal binding to the organism and thus decrease the toxicity of the metal (Niyogi and Wood, 2004). The competition is diagramed in figure 1 at the gill or biotic ligand. The toxicity predictions of the BLM are reported as a lethal concentration value (LC50); this value is representative of the concentration of metal that will result in a 50% mortality of the population exposed to the specific water chemistry during a defined period of time.

The primary advantage of the BLM is that as a computer model that predicts toxicity the need for laborious and expensive toxicity tests is reduced. Thus, time and resources can be better directed towards understanding environmental impacts and, if necessary, treatment and/or remediation. The BLM also serves as a tool that can be used to account for site-specific water chemistry conditions and thus has a potential role in improving water quality

guidelines and/or criteria, facilitating the derivation of site-specific guidelines/criteria. The BLM is also useful in the process of risk assessment.

2.5 Modes of Toxicity

Dissolved metals are generally considered the most bioavailable to the organism and particulate forms are not available for uptake (Niyogi and Wood, 2004). Metal can potentially have toxic effects at both the gills and, if ingested, the gastrointestinal tract. When examining the gills as a site of toxicity, it is important to understand the high level of ion uptake taking place in this tissue. Specialized channels specific for sodium, chloride, and calcium allow the ions to enter the gill (Figure 2). These ions are exchanged for other ions, in order to maintain an electrochemical balance (Paquin et al., 2002). Chloride ions are exchanged from the external environment for the similarly charged bicarbonate ion while sodium ions enter the cell in exchange for a proton or ammonium (Paquin et al., 2002). Once in the gill epithelium, sodium ions are forced into the blood by a Na^+/K^+ ATPase; this creates an electrochemical gradient that pulls chloride ions into to the blood (Paquin et al., 2002). The gill epithelium also has calcium channels that allow calcium ions to pass through the epithelium and enter the blood stream.

Ion regulation in the gut involves the same ion channels, however, there are two main differences. The first is that the lumen of the gut is not subjected to

the same changes in water chemistry as the external environment. The second difference is the epithelial tissue has different affinities for the ions. The ionic demands of the gastrointestinal tissue are not the same as that of the gill (Macklin and Josephson, 1971). Although these two tissues have differences, they are both susceptible to the effects of metal (Goulet et al., 2007).

The mechanisms for waterborne toxicity have been thoroughly examined and models such as the BLM and FIAM have been developed to predict such toxicity in many different water chemistries. The BLM incorporates competition of different cations, in both soft and hard waters and complexation of the metal with both inorganics (hydroxides, chlorides, carbonates, etc.) as well as organic matter (Niyogi and Wood, 2004). However this model does not apply to dietary exposures. Goulet et al., (2007) explain that accumulation of metal via digestion is dependant on other factors. Speciation may differ in gut resulting in different competing factors and also affect the availability of the metal. Organisms also have certain detoxification mechanisms that must be overwhelmed before toxicity is observed. Dynamic multipathway bioaccumulation models (DYMBAM) have been proposed to predict bioaccumulation in organisms. DYMBAM incorporates the rate of uptake of metals as well as the rate of elimination (Goulet et al., 2007). Few of the published bioaccumulation models predict toxicity.

2.6 Objectives:

The broad objective of this research was to understand the toxicity of Cd on the freshwater invertebrates *Hydra attenuata* and *Daphnia pulex*. This involved experiments to understand the influence of water chemistry during waterborne Cd exposures as well as the dietary toxicity of cadmium to Hydra. Ultimately, the goal was to compare the relative impacts of waterborne and dietary exposure routes to *H. attenuta*.

The first phase of the research was to develop and understand the waterborne acute toxicity of Cd and the influence of water chemistry. This work was done using *D. pulex* and water chemistry manipulations that included Ca, Mg, Na, K, Cl, pH, and natural organic matter (NOM). Waterborne toxicity of cadmium to *Hydra attenuta* used similar manipulations of the test water chemistry as mentioned above and was assessed by both lethal and sub-lethal endpoints. In both invertebrate species, the observed toxicity was compared to existing prediction models in order to determine their validity and also derive new models to more accurately predict the observed toxicity in soft waters. To address the dietary toxicity of Cd, the second phase of the research involved trophic transfer studies with *H. attenuata*, using *D. pulex* as the exposure vector to deliver Cd. The third phase was the combination of both waterborne and dietary exposures to *H. attenuata* to determine if there were any compounding effects.

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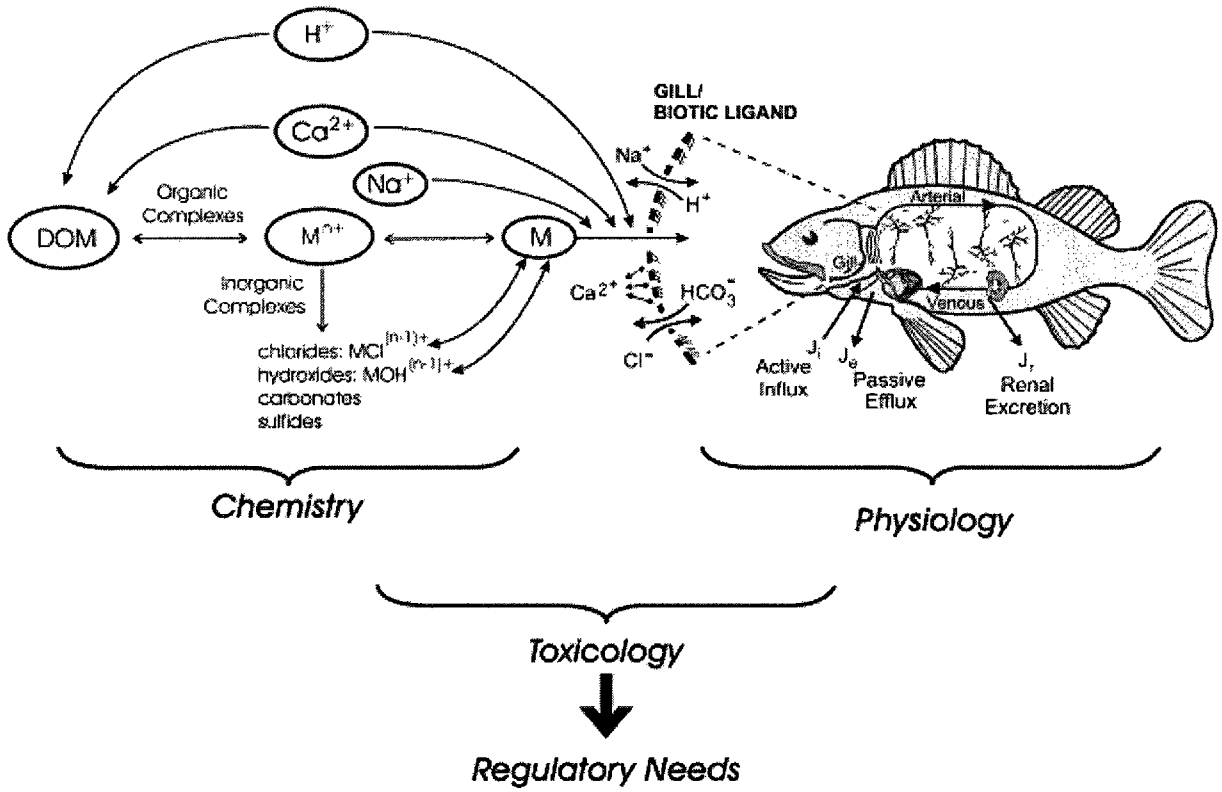
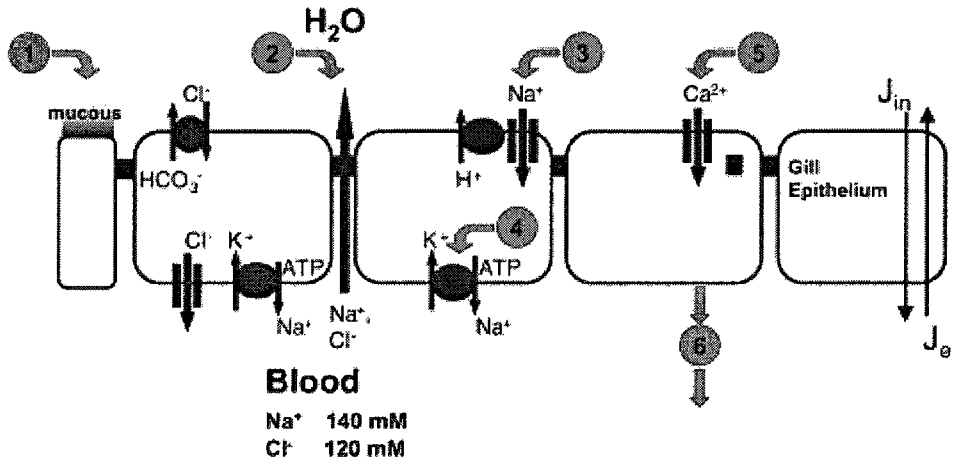
Chapter 2: Figure 1. A schematic representation of the biotic ligand model (Paquin et al., 2002).


H+
Ca2+
Na+
DOM
Organic Complexes
Mn+
M
Inorganic Complexes
chlorides: MCl(n-1)+
hydroxides: MOH(n-1)+
carbonates
sulfides
GILL/ BIOTIC LIGAND
Na+
H+
Ca2+
HCO3-
Cl-
Arterial
Gill
Venous
Ji
Active Influx
Je
Passive Efflux
Jr
Renal Excretion
Chemistry
Physiology
Toxicology
Regulatory Needs

Chapter 2: Figure 2. Schematic diagram of ion channel on gill epithelium (Paquin et al., 2002).



1- adsorption of metals resulting in gas transfer problems (increased mucous/diffusion distance)
2- metals that affect tight junctions altering efflux/ion loss (J_o)
3- metals that block Na^+ uptake (J_{in}) directly at transport site
4- metals that block Na^+ uptake (J_{in}) indirectly by inhibiting Na^+/K^+ ATPase
5- metals that block Ca^{2+} uptake (J_{in}) directly at transport site

Chapter 3

Development of a Biotic Ligand Model to predict the Acute Toxicity of Cadmium to *Daphnia pulex* in Soft Water.

This chapter is in manuscript form, and will be submitted to Environmental Toxicology and Chemitry. This manusciprt is currently under review by industrial funding partners.

Development of a biotic ligand model to predict the acute toxicity of cadmuim to *Daphnia pulex* in soft water.

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3.1 ABSTRACT

The aim of this study was to develop a biotic ligand model (BLM) that can predict the acute toxicity of cadmium to *Daphnia pulex* in soft water. Standard 48 h acute toxicity tests were used to determine EC50s in various water chemistries where the effects of Ca^{2+}, Na^{+}, Mg^{2+}, Cl^{-}, K^{+}, pH, and dissolved organic carbon (DOC) were tested. Increases in Ca^{2+} resulted in higher EC50s, indicating that Cd^{2+} competes with Ca^{2+} for uptake at the biotic ligand. Similar cation competition effects were seen when Mg^{2+} was varied but with a less pronounced protective effect relative to Ca^{2+}. Changes in Na^{+} and K^{+} concentrations had no significant effect on Cd toxicity. EC50 values increased when pH was adjusted over a range of 8.0 to 6.1. A previously published BLM (HydroQual BLM ver 2.2.3) was tested for its ability to estimate acute Cd toxicity to *D. pulex* in soft waters. While the protective effect of Ca could be predicted reasonably well, predictions for other test chemistry series did not match with measured EC50s. The existing model was modified by altering binding constants for competitive cations and the LA50 (or critical value). This modified model was able to predict Cd toxicity to *D. pulex* in soft water except for the protection provided by DOC, which was overestimated.

Keywords: BLM; Cd; modelling; cladocerans; water hardness; bioavailability; metal; water quality; risk assessment.

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3.2 INTRODUCTION

Cadmium is a non-essential element that is present in freshwater aquatic systems at trace levels but that can also be toxic if concentrations increase sufficiently, for example as a result of mining activities. The degree of toxicity in contaminated ecosystems depends not only on the concentration of metal, but also on the entire water chemistry. Bioavailability of metal in aquatic environments is dependant on geochemical speciation with the free ion form generally associated with toxicity to organisms (Campbell, 1995). Complexation of free ions reduces the potential for toxic impacts, as does cationic competition for uptake sites on the biotic ligand (Di Toro et al., 2001). Complexation and competition capacity are reduced and therefore the potential for metal toxicity is enhanced in soft waters, such as those of the many lakes on the Canadian Shield (David et al., 1997; Jeziorski and Yan, 2006).

The influence of complexation and competition on Cd toxicity was demonstrated previously in many organisms. For example the importance of complexation, and the relationship between Cd^{2+} and toxicity in grass shrimp (*Palaemonetes pugio*) was shown by Sunda et al., (1978). Niyogi et al., (2008) documented competition between Ca and Cd and complexation by dissolved organic carbon (DOC) in gill binding and acute toxicity studies to rainbow trout (*Oncorhynchus mykiss*). As waterborne Ca concentration was increased from 0.1 to 3.0 mM there was an increase in the 96 h LC50 from 1.4 to 15.4 μg Cd/L. Competition of Ca on Cd uptake into *Daphnia magna* has been shown by Tan

and Wang (2008), who compared accumulation of Cd at 0.5 and 50 mg Ca/L. Increased Ca resulted in decreased whole organism Cd (Tang and Wang, 2008).

Current water quality criteria/guidelines for Cd only take into account the modifying effect of hardness on toxicity (e.g. EPA, 2001; CCME, 1999) although there is recognition of the importance of aquatic geochemistry in assessing the potential for impacts (e.g. CCME, 2009; EPA, 2007a). Biotic ligand models (BLMs) have been successfully developed for a number of metals, including Cd, as tools to predict metal toxicity and incorporate the influence of water chemistry on bioavailability (McGeer et al., 2009). The premise of the model is that the toxicity arises from threshold accumulations of bioavailable metal (generally the free ion form) on the biotic ligand and both cationic competition and complexation are accounted for (Di Toro et al., 2001). Accumulation thresholds associated with LC50 exposure concentration are termed the LA50 (Villavicencio et al., 2005). The site(s) of accumulation associated metal toxicity are typically respiratory surfaces (Santore et al., 2002; Playle, 2004). The practical application of the BLM is to provide site-specific toxicity predictions and this approach has recently been adopted for a revised derivation of the water quality criteria for Cu in the United States of America (US EPA, 2007b).

The BLM uses the Chemical Equilibrium of Species and Surfaces (CHESS) and Windemere Humic Aqueous Model (WHAM, ver 5) for calculating speciation including organic matter complexation to predict free ion concentrations leading to estimates of toxicity (Tipping, 1994). For the BLM to accurately predict toxicity the relative equilibrium binding affinity of metal ions

(Log K values) must be known (Niyogi et al., 2004b) and these constants can be derived through gill-binding experiments or estimated from toxicity data (Villavicencio et al., 2005; Santore et al., 2002; Playle and Dixon, 1993). The final input for the BLM, the critical value (accumulation associated with 50% lethality or the LA50), can be determined from accumulation data or also developed by calibrating it to toxicity endpoints using the BLM (McGeer et al., 2009). Most of the BLMs that have been developed characterize conditions for waters of medium to high hardness and this is the case for Cd (McGeer et. al., 2009; Niyogi et al., 2008). Recent studies have illustrated that for Ni and Zn, BLMs developed for hard water conditions require modifications in order to predict acute toxicity in very soft waters (Clifford and McGeer, 2009; Kozlova et al., 2009).

The goal of the study was to understand the influence of cationic competition and complexation on the toxicity of Cd to *Daphnia pulex* in soft water and to test the predictive ability of an existing hard water BLM under these low hardness conditions. *Daphnia pulex* was chosen as the test species because of it's high sensitivity to metals and its ability to thrive in soft waters (Bury et al., 2002; Shaw et al., 2006; Environment Canada, 1996).

3.3 MATERIALS AND METHODS

D. pulex were obtained from a commercial supplier (Aquatic Research Organisms Inc. Hampton, NH) and cultured in 1.5 L glass beakers at a

temperature or 21 ± 1°C. Adults were transferred to new media daily and fed algae (70% *Pseudokirchneriella subcapitata* and 30% *Chlorella vulgaris*) and a yeast, cerrophyl and trout feed mixture (YCT, Aquatic Research Organisms NH) at the rates recommended by Environment Canada (1996). The culture medium for *D. pulex* was reconstituted soft (RS) water based on additions of $CaSO_4$, $MgSO_4$, $NaHCO_3$, and KCl (all Sigma-Aldrich Inc. St. Louis, MO) as described in (Clifford and McGeer, 2009). RS water was based on the standardized soft water media recommended by Environment Canada and the US EPA (Environmental Canada, 1996; EPA, 2002) modified to reduce the Mg content (Kozlova et al., 2009). The final measured concentrations of Ca, Mg, Na, Cl, K, SO_4^-, and HCO_3^- were 170, 140, 570, 30, 30, 310 and 570μM, respectively and 20L batches of media were made as needed and aerated prior to use. The pH was 7.8 and the dissolved organic carbon (DOC) content was measured to be 1.53 mg C/L.

Test solutions were prepared to understand the potential effects of Ca, Mg, Na, K, Cl, pH, and natural organic matter (NOM) on acute Cd toxicity. For Ca, Mg, Na, K and Cl tests RS, water was prepared as described above except for the parameter under test, which was added to give the desired concentrations. When examining the effects of pH on Cd toxicity, the methods outlined by De Schamphelaere et al., (2004), using the non-complexing buffer 3-(n-morpholino)-propanesulphonic acid (MOPS) was used at a concentration of 3.58×10^{-3} M in RS water. Additions of HCl or NaOH were used to set the solution pH, which was maintained at ±0.1 pH units. The protective effects of two

sources of NOM were also tested; Nordic Reservoir NOM (International Humic Substances Society no. 1R108N) and Suwannee River NOM (no. 1R101N). A complete outline of the test water chemistries is detailed in Table 1.

Acute lethality tests followed the Environment Canada test methodology guidelines (Environment Canada, 1996). Each toxicity test consisted of one control (soft water test medium with no added Cd) and 7 different concentrations of Cd. Exposure concentrations ranged from 0.09-4.4 μM Cd depending on the factor being tested and were made by additions of stock solution that was prepared from reference standard (Inorganic Ventures Inc., Lakewood, NJ). Each test was done in duplicate using 250 ml plastic beakers containing 100ml of test solution. To ensure uniform concentrations, 2L batches of base test medium (without Cd) were prepared and then 250 ml aliquots were spiked with the appropriate volume of Cd stock solution to create the test exposures, which were then split into the individual test replicates. Ten neonate *D. pulex* (age <24 h) were placed in each test beaker without food. After 48h, the neonates were assessed based on the endpoint of immobility. Tests with more than 10% mortality in controls were not considered valid (Environment Canada, 1996).

Water samples (10 ml) were collected prior to addition of neonates as well as at test end to assess the consistency of exposure conditions over the 48h test duration. At sampling times pH was measured (Radiometer PHM240 meter with pHC2701-8 electrode). Samples were acidified to 1% (volume with 16N trace metals grade HNO_3, Fisher Scientific, Nepean, ON) then measured by atomic absorption spectrophotometry (SpectAA-880, Varian Inc, Palo Alto, CA) for Cd.

The manipulated ion under test (either Ca, Mg, Na or K) was similarly measured on three randomly chosen samples per test series. Samples for Cd analysis were not filtered and this was based on preliminary sample collections and a comparison of 0.45 μm filtered (see below) and unfiltered samples where dissolved Cd was 96.3% (range 92.4% to 98.9%) of total (unfiltered sample) Cd. Samples for measurement of DOC concentrations were also collected, and filtered (0.45 μm Acrodisc syringe filter, HT Tuffryn) prior to analysis (5050A TOC Analyzer, Shimadzu, Columbia, MD). When NOM was the parameter under test, DOC characterization was done on all test solutions while for other trials DOC analysis was done on 3 random samples from each test.

EC50 values for total Cd were calculated using the software program PROBIT (Ver. 1.5) using the measured water chemistry and the Hydroqual BLM (ver. 2.2.3; downloaded from www.hydroqual.com/BLM) speciation of Cd in the different test media was done to derive Cd^{2+} concentrations. EC50 values for Cd^{2+} were calculated as described above. Speciation data was also used to derive stability constants for the competitive interaction of cations (Ca^{2+} and Mg^{2+}) on Cd^{2+} toxicity, calculated according to the method described by De Schamphelaere and Janssen (2002). In brief, linear regression analysis of free cation activities, Ca^{2+} on Cd^{2+} EC50, in the presence of constant Mg^{2+} was used to generate the slope and intercept variables that were used to develop the matrix equations to derive estimates of Log K_{CaBL} (De Schamphelaere and Janssen, 2002). Similarly, regression variables for the toxicity mitigating effect of Mg^{2+} activity on Cd^{2+} activity at the EC50 concentration in the presence of

constant Ca^{2+} were used to estimate the Log K_{MgBL}. The conditional equilibrium constant describing the toxic interaction of Cd^{2+} on the biotic ligand (Log K_{CdBL}) was calculated from the average of the intercepts of the individual regression relationships of Cd^{2+} on Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, H^{+}, SRNOM and NRNOM.

Predictions of acute Cd toxicity were developed using the HydroQual BLM. A *D. pulex* BLM is not available therefore the input files for *Ceriodaphnia dubia* were used and adjusted to develop a soft water BLM. Measured water chemistry values were used as model inputs and DOC was assumed to be 10% humic acid (BLM default value). Predictions were done using the model without adjustment (as downloaded from the HydroQual website) and then with adjustments to the LA50 value (adjustments made to achieve the best fit between predicted and measured). A third round of predictions was made, this time adjusting Log K constants within the input parameter file, replacing them with the calculated values for *D. pulex* in soft water. The Log Ks were calculated using methods outlined by De Schamphelaere and Janssen (2002). This soft water BLM was refined by making adjustments to the Log K values as well as the LA50 value to achieve improved correlation between measured and predicted toxicity.

3.4 RESULTS

A significant decrease in the acute Cd toxicity was observed when the Ca was increased (Table 1, Figure 1A). The concentrations of tested, Ca ranged

from 0.03 mM to 1.61 mM and over this range EC50 values increased 9 fold, from 0.15 to 1.38 μM Cd. Mg also reduced Cd toxicity, however compared to Ca the effect was modest, only a 2.6 fold increase in EC50 over the range of 0.01 mM to 1.40 mM Mg (Table 1, Figure 1B). The slopes of the linear regression lines for the effect of Ca^{2+} and Mg^{2+} on EC50 for Cd^{2+} (Table 2) confirmed that the protective effect of Mg^{2+} was about 30% that of Ca^{2+}. It is noteworthy that decreases in Mg test concentrations below those present in the culture media did not result in any decreases in EC50 below that for the culture medium as had been observed at low Ca concentrations in the Ca test series (Figure 1A and 1B, Table 1). Across a Na range of 0.54 to 1.65 mM, changes in Na did not alter Cd toxicity (Table 1, Figure 1C). The slope of the lines of regression describing the relationship between Na^{+} and Cd^{2+} toxicity does not significantly differ from zero (Table 2). Na concentrations were adjusted using NaCl and K concentrations were altered using KCl, therefore since no effect was observed on Cd toxicity, it can be concluded that in addition to Na and K, Cl also plays no role in modifying Cd toxicity (Table 1).

A linear relationship between pH and Cd toxicity was observed over a pH range of 6.10 to 8.02 (Table 1) with EC50 showing a trend toward higher values as pH decreased. This data is also shown as a function of H^{+} in Figure 2A and in spite of the trend, the slope of the linear regression of H^{+} activity and Cd^{2+} activity was not significant.

The relationships between DOC concentration and the EC50 demonstrated that NOM provided protection against toxicity (Table 1, Figure 2B).

When toxicity was expressed as a function of calculated Cd^{2+} concentrations (generated by WHAM 5, within the Hydrqual BLM), the results illustrated that toxicity mitigation was due to complexation. In other words the EC50 for Cd^{2+} was relatively constant (Fig 2C, Table 3). Both Nordic Reservoir and Suwannee River NOM sources had similar protective effects. The regression of DOC (mg/L) vs EC50 (total Cd in solution) is EC50 = 0.04 [DOC] + 0.35 (r^2 = 1.00) and EC50 = 0.04[DOC] + 0.33 (r^2 = 0.96) for Nordic Reservoir NOM and Suwannee River NOM, respectively.

Estimates of the conditional equilibrium constants for Ca^{2+}, Mg^{2+} and H^+ on Cd^{2+} toxicity were derived using geochemical speciation modeling from measured water chemistries, producing a calculated Log K_{CaBL} of 4.08, Log K_{MgBL} of 3.71 and a Log K_{HBL} of 6.13. The calculated value for the interaction of Cd^{2+} on the biotic ligand (Log K_{CdBL}) was 6.97. The HydroQual BLM for the acute effects of Cd on *C. dubia* generally under-estimated Cd toxicity to *D. pulex* in soft water (Figure 3A). When the critical values (LA50 threshold for lethal accumulation) was adjusted from 7.53 down to 7.1 nmol/g, the model delivered reasonable predictions for NOM and Ca test solutions but over-estimated toxicity in other test solutions (Figure 3B). With calculated Log K values from this study (for Ca, Mg and H) as parameter file inputs and with Log K_{NaBL} removed, the best fit between measured and predicted toxicity was achieved with a LA50 values of 2.5 nmol/g (Figure 3C). However the protective effects of DOC were over-estimated and those for Ca and Mg were under-estimated. As well, the prediction of toxicity at pH 6.1 was dramatically incorrect at 2.9 µM (point off

scale and not visible in Figure 3C) compared to the actual measured EC50 of 0.65 µM. In the final modelling series the strength of the binding affinities of both Ca and Mg was increased to 3.8 and 3.7 respectively, with a concomitant decrease in LA50 to 0.6 nmol/g (Figure 3D) in order to improve predictions. The protective effect of NOM in this model was still somewhat over-estimated (Figure 3D).

3.5 DISCUSSION

Ca had a significant protective effect on Cd toxicity (Figure 1A, Table 2) and this has been reported previously for Cd (Santore et al., 2002) and other metals including Cu (De Shamphelaere and Janssen, 2002), Zn (Clifford and McGeer, 2009) and Ni (Kozolva et al., 2009). Verbost et al., (1989) showed that Cd^{2+} uptake and Ca^{2+} uptake are functionally linked, sharing the same uptake site on the gills of fish and interacting competitively for those influx sites. Evidence that Cd^{2+} competes for uptake at Ca^{2+} uptake sites in *D. magna* is strongly supported by the recent study by Tan and Wang (2008), who looked at the effects of different Ca concentrations of the accumulation of Cd and Zn in *D. magna*. Tan and Wang (2008) found the amount of Cd accumulated was inversely proportional to the amount of Ca in solution thus clearly illustrating the competitive interaction that we also observed through toxicity mitigation.

Mg has an effect similar to that of Ca, however the protection was relatively less prominent (Figures 1A and 1B). At the highest Mg concentration a

reduced EC50 for Cd was evident (Table 2, Figure 1B) indicating that elevated Mg may be causing a physiological effect that sensitizes *Daphnia pulex* to Cd. This is rather speculative, however it is noteworthy that transferring *Ceriodaphnia dubia* from low to higher concentrations of Mg resulted in sublethal effects (temporary inhibition of reproduction; Schwartz et al., 2007). The relatively weaker protective effect of Mg compared to Ca could arise through interactions that Mg is known to have on Ca uptake mechanisms (Markich and Jerrfee, 1994; Pattnaik et al., 2007). Markich and Jeffree (1994) highlighted the permissiveness of the Ca uptake mechanisms thus allowing other divalent cations to also be taken up. Although both Ca and Mg can bind to the receptor, Ca has a higher binding affinity because its ionic radius is more suited for the ligand (Markich and Jeffree, 1994). Following a similar mechanism of Ca and Mg, other divalent metals (in this study Cd) can compete for the Ca ligand. This hypothesis has been strengthened by Pattnaik et al., (2007), who characterized Ca^{2+}-ATPase activity at varying concentrations of Ca and Mg. It was evident that the Ca^{2+}-ATPase could be induced by the presence of both Ca and Mg. These data are in agreement with the finding in our study in that although Mg can have competitive effect on the Ca ligand, it will be less pronounced due to a relatively weaker affinity for Mg compared to Ca.

Na manipulations suggest that Cd toxicity is independent of the Na concentration in the system (Figure 1C) and Niyogi et al., (2008) found similar results for Cd uptake in rainbow trout. The transport of Na across the respiratory epithelium of fish is known to be a two-part system that involves a Na transport

channels and H^+ ATPase pumps on the apical surface of the membrane, and Na^+/K^+-ATPase pumps on the basolateral side (Grosell et al., 2002). Neither of these steps in Na up-take would allow for Cd^{2+} or other divalent ions to enter the organism and therefore our results, showing no effect of Na^+ on Cd^{2+} toxicity was in agreement with Grosell, et al., (2002).

In toxicity tests where pH was varied there was a general trend toward increased EC50 values when pH decreased from 8.02 to 6.10. This change (38% increase but not significant) suggests the possibility of a modest competition between H^+ and Cd^{2+} (Figure 2A). Playle et al., (2004) found a similar competitive interaction, reducing the binding of Cd to the gills of fathead minnows (*Pimephales promelas*) as pH was decreased to 4.8. The results are contrary to those of Niyogi et al., (2008) who noted no change in acute toxicity of Cd to rainbow trout across a range of pH values from 5.8 to 8.8 or in gill binding from 4.8 to 9.4. In these tests MOPS, a buffering agent that does not complex metals (De Schamphelaere et al., 2004), was used to fix the different exposure pH values whereas Niyogi et al., (2008) co-varied pH and alkalinity.

The effects of two standard NOM sources, Nordic Reservoir and Suwannee River were examined at concentrations that typically occur in soft Canadian Shield waters (David et al., 1997). Increases in both sources of NOM resulted in higher EC50s when toxicity was considered on a total Cd basis (Figure 2B). The toxicity of Cd as a function of Cd^{2+} activity was relatively constant (Figure 2C) illustrating both the complexation of Cd by DOC and that the free ion form of Cd is associated with acute lethality. Our results therefore

are consistent with the free ion activity model (Campbell, 1995) and the results of Niyogi et al., (2008) and Playle et al., (2003) demonstrating that NOM binds Cd^{2+} in solution and the Cd-DOC complexes that are formed are not bioavailable. Cd has been shown to bind to humic acid (HA), fulvic acid (FA) and also less abundant functional groups like thiols and amines (Cao et al., 2006; Karlsson et al., 2006). Both sources of NOM, Nordic Reservoir and Suwannee River, had similar protective effects on Cd toxicity indicating that they contain similar binding characteristics with regards to Cd complexation. This result is similar to those of Clifford and McGeer (2009) with Zn but differ from those of Kozlova et al., (2009) who found that Suwannee River and Nordic Reservoir NOMs provided much different levels of protection against Ni toxicity.

In order to integrate the soft water Cd toxicity data into a biotic ligand model a step-wise approach was used. The first step was to test the capabilities of a previously published BLM, calibrated for predicting the acute impact of Cd to *Ceriodaphnia dubia* in relatively hard waters. This model was chosen, as it is the only BLM available for the effects of Cd in invertebrates. Toxicity predictions were made using the test water chemistries (Table 1) and demonstrated that *D. pulex* in soft water appears to be generally more sensitive than *C. dubia* (Figure 3A). As well, the protective effects of DOC, Ca and Mg are over-estimated by the BLM (Figure 3A). With adjustments of the LA50 to reflect the elevated sensitivity it was possible to achieve reasonable predictions for the protective effects of Ca and NOM (Figure 3B). However this sensitivity correction did not capture the effects of pH, Mg and Na test series where predicted toxicity was

greater than measured (Figure 3B).

Development of a model specific for the toxicity to *D. pulex* in soft water followed as a third step, one where the calculated equilibrium constants (Table 3) were tested. Once the LA50 value was adjusted (2.5 nmol/g) it was obvious that the protective effects of NOM were over-estimated while those of Ca and Mg were underestimated (Figure 3C). As well, the effects of H^+ were dramatically overestimated at low pH. It was possible to account for these by increasing the constants for Ca and Mg (Figure 3D and Table 3). The final soft water BLM predicted that DOC would have a stronger protective effect than it actually did (Figure 3D) and this was also observed in the negative slope of the free ion EC50 relationships (see Table 2). Unfortunately, the DOC-Cd interaction is not one that can be manipulated within the WHAM V/HydroQual BLM interface.

A comparison of the soft water BLM for acute Cd effects on *Daphnia pulex* and the hard water BLM for acute Cd effects on *Ceriodaphnia dubia* illustrated features of Cd toxicity as well as BLM modelling. The Log K_{CdBL} for *Daphnia pulex* in our study (7.0) was lower than the 8.6 reported by Playle et al., (2003) and is used in the HydroQual BLM. However it agrees reasonably well with other published values such as the 7.5 reported by Niyogi et al., (2008) and the 7.3 reported by both Niyogi et al., (2004a) as well as Hollis et al., (2000) in soft water. The protective effects of Ca^{2+} on Cd toxicity to *D. pulex* in soft water were similar to those for *C. dubia* in the HydroQual BLM however those for Mg^{2+} and H^+ were less than observed (Figure 3B). Niyogi et al., (2008) found no protective effect of Mg on Cd gill binding in rainbow trout (*Oncorhynchus mykiss*) however

Cd exposure concentrations were well above the acute LC50 concentration. The enhanced protective response to Mg^{2+} that we observed may be due to species differences or may be related to physiological differences that may occur in organisms acclimated to soft water. In a recent study with Zn, Clifford and McGeer (2009) postulated that acclimation to soft water in *Daphnia pulex* up-regulate Ca and Mg uptake mechanisms and this created conditions whereby the toxicity of divalent cationic metals that mimic Ca is enhanced in very soft water. The upregulated uptake may also explain the increased protective effect of waterborne Ca and Mg in soft waters compared to hard water.

In conclusion, our studies indicate that the acute toxicity of Cd to *Daphnia pulex* is significantly increased by Ca and to a lesser extent Mg. Tests with varying concentrations of Na and K (as chloride salts) in the exposure water did not result in changes in EC50 values. Two sources of NOM were tested and as concentrations increased toxicity decreased. The results of these experiments are consistent with FIAM and BLM principles (Campbell, 1995; Di Toro et al., 2001) and also with Cd^{2+} as a mimic of Ca^{2+} (Verbost et al., 1994). A Cd BLM, developed primarily on data from hard and moderately hard water, the HydroQual BLM was tested for its ability to predict toxicity to *Daphnia pulex* in soft water. Once adjusted for the sensitivity of *Daphnia pulex* (via calibration of LA50 value) the HydroQual BLM provided reasonably accurate predictions of the protective effects of Ca and also the NOMs. However other test data was underestimated. Using soft water specific BLM parameters an alternative BLM was developed and with adjustment, this model provided reasonable estimates of

Cd toxicity although the effects of NOM and elevated Mg were predicted to provide more protection than actually occurred.

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Chapter 3: Table 1. Cd EC50 concentrations and associated water chemistry from 48 h toxicity tests with *D. pulex*. Measured concentrations are given and Cd EC50 values which were calculated from measured total Cd values. The units are mM except for Cd (μM), DOC (mg/L) and pH and EC50 values tagged with the same letter are not significantly different.

	Ca	Mg	Na	Cl	SO_4	K	HCO_3	pH	DOC	Cd	95% CI	
										EC50	lower	upper
Ca	**0.03**	0.14	0.54	0.03	0.17	0.03	0.54	7.85	1.5	0.15[a]	0.1	0.18
	0.10	0.14	0.54	0.03	0.24	0.03	0.54	7.85	1.5	0.21[a]	0.16	0.28
	0.16	0.14	0.54	0.03	0.30	0.03	0.54	7.85	1.5	0.41[b]	0.37	0.45
	0.33	0.14	0.54	0.03	0.47	0.03	0.54	7.85	1.5	0.22[a]	0.15	0.27
	0.53	0.14	0.54	0.03	0.67	0.03	0.54	7.85	1.5	0.64[c]	0.55	0.72
	1.05	0.14	0.54	0.03	1.19	0.03	0.54	7.85	1.5	1.04[d]	0.99	1.08
	1.61	0.14	0.54	0.03	1.75	0.03	0.54	7.85	1.5	1.38[e]	1.33	1.42
Mg	0.18	**0.01**	0.54	0.03	0.19	0.03	0.54	7.85	1.5	0.24[a]	0.17	0.3
	0.18	**0.14**	0.54	0.03	0.32	0.03	0.54	7.85	1.5	0.41[b]	0.37	0.45
	0.18	**0.49**	0.54	0.03	0.67	0.03	0.54	7.85	1.5	0.63[c]	0.49	0.76
	0.18	**0.94**	0.54	0.03	1.12	0.03	0.54	7.85	1.5	0.80[c]	0.47	1.05
	0.18	**1.40**	0.54	0.03	1.58	0.03	0.54	7.85	1.5	0.61[c]	0.59	0.63

Table 1. continued.

	Ca	Mg	Na	Cl	SO_4	K	HCO_3	pH	DOC	Cd EC50	95% CI lower	Upper
Na	0.18	0.14	**0.54**	0.03	0.32	0.03	0.54	7.85	1.5	0.41[a]	0.37	0.45
	0.18	0.14	**1.08**	0.57	0.32	0.03	0.54	7.85	1.5	0.30[a]	0.11	0.53
	0.18	0.14	**1.65**	1.14	0.32	0.03	0.54	7.85	1.5	0.38[a]	0.33	0.41
K	0.18	0.14	0.54	0.05	0.32	**0.05**	0.54	7.85	1.5	0.41[a]	0.37	0.45
	0.18	0.14	0.54	0.48	0.32	**0.48**	0.54	7.85	1.5	0.47[a]	0.45	0.49
	0.18	0.14	0.54	0.96	0.32	**0.96**	0.54	7.85	1.5	0.39[a]	0.21	0.86
	0.18	0.14	0.54	1.43	0.32	**1.43**	0.54	7.85	1.5	0.43[a]	0.24	0.56
pH	0.18	0.14	0.54	0.03	0.32	0.03	0.54	**6.10**	1.5	0.65[a]	0.52	0.91
	0.18	0.14	0.54	0.03	0.32	0.03	0.54	**7.08**	1.5	0.56[a]	0.54	0.58
	0.18	0.14	0.54	0.03	0.32	0.03	0.54	**8.02**	1.5	0.47[a]	0.34	0.55
Nordic	0.18	0.14	0.54	0.03	0.32	0.03	0.54	7.85	**9.09**	0.7[a]	0.58	0.72
Reservoir	0.18	0.14	0.54	0.03	0.32	0.03	0.54	7.85	**12.32**	0.85[a]	0.72	0.98
Suwannee	0.18	0.14	0.54	0.03	0.32	0.03	0.54	7.85	**11.80**	0.72[a]	0.6	1.33
River NOM	0.18	0.14	0.54	0.03	0.32	0.03	0.54	7.85	**16.45**	1.02[b]	0.94	1.66

Chapter 3: Table 2. Regression coefficients for the linear regression of Ca^{2+}, Mg^{2+}, K^{+}, H^{+} and Na^{+} on the acute toxicity of Cd^{2+}. Where significant effects were observed, the Log K values were calculated. The units for slope are ΔEC50 (Cd^{2+} µM) per mM change in cation activity, except for NOM which is per mg C/L of DOC. The intercept is Cd^{2+} µM.

	slope	intercept	r^2	Calculated Log K value
Ca^{2+}	0.708	0.106	0.95	4.08
Mg^{2+}	0.260	0.283	0.55	3.71
Na^{+}	0.035	0.315	0.18	n.a.
K^{+}	-0.001	0.226	1.00	n.a.
H^{+}	0.227	0.416	0.72	6.13
*NR NOM	-0.018	0.339	0.88	n.a.
#SR NOM	-0.014	0.328	0.88	n.a.

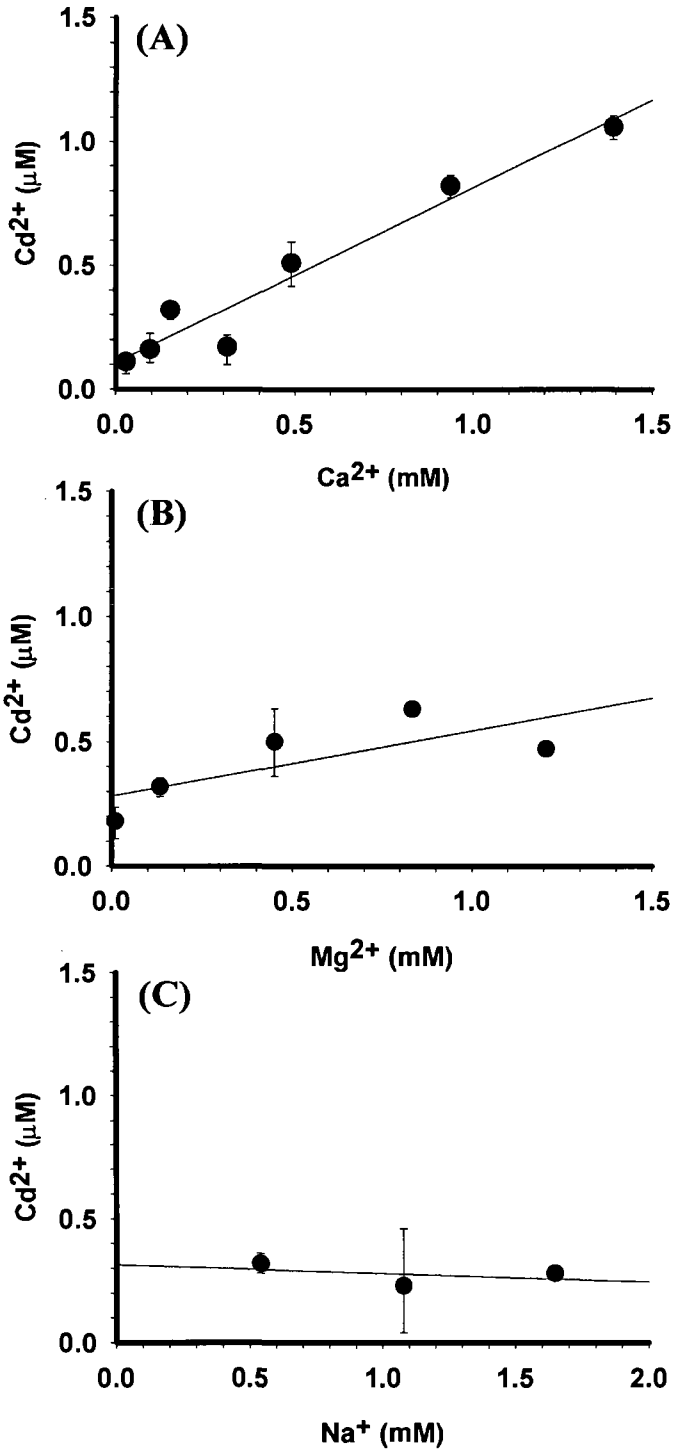
* NR NOM – Nordic Reservoir Natural Organic Matter.

SR NOM – Suwannee River Natural Organic Matter.

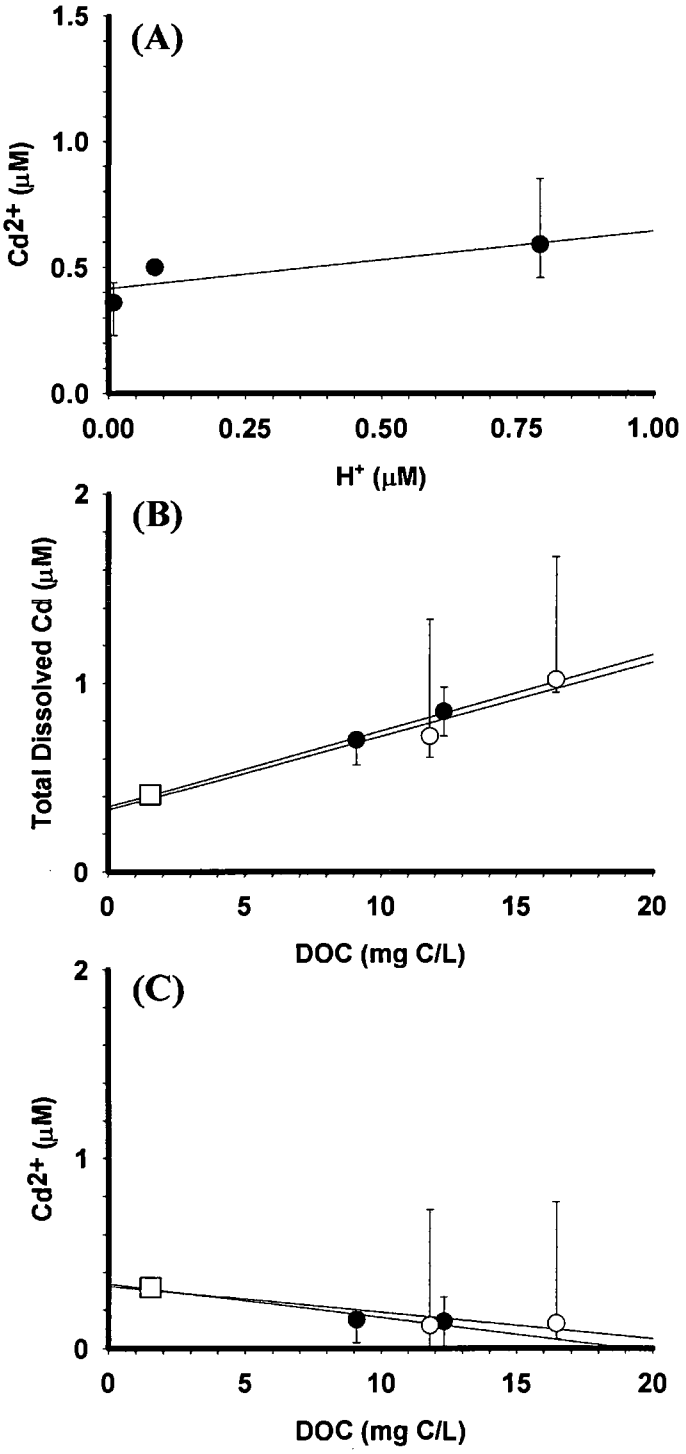
Chapter 3: Table 3. BLM input parameters for the *C. dubia* in hard water model of Santore et al., (2002) and for the modified BLM for *D. pulex* in soft waters. See Figure 3A, B, C and D for additional detail.

	Santore et al., 2002	Modified BLM
Cd-BL	8.6	7.0
Ca-BL	4.5	4.9
H-BL	6.7	7.7
Mg-BL	3.5	4.8
Na-BL	3.0	n.a.
Critical Value	7.53	0.6

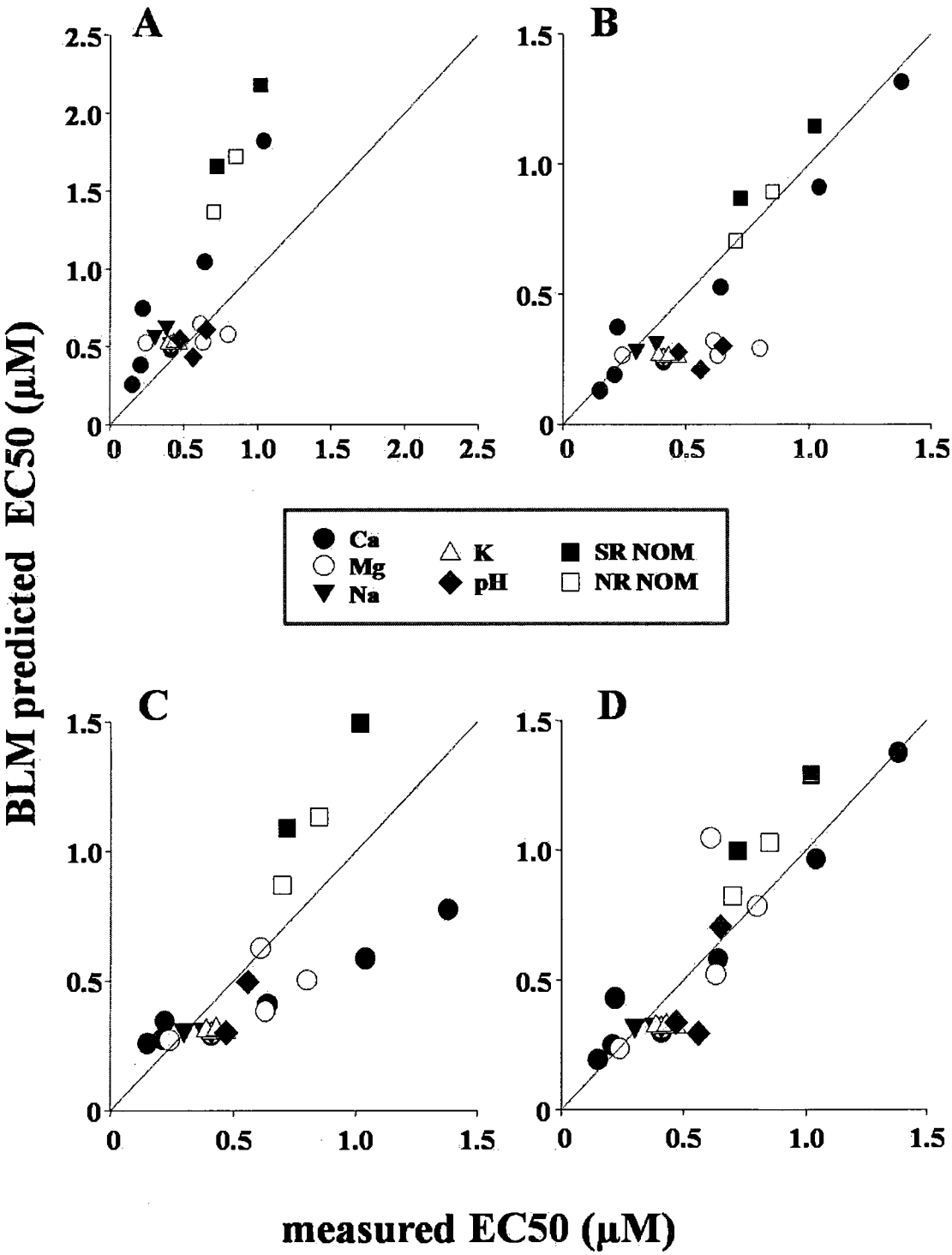
Chapter 3: Figure 1. Measured EC50s (with 95% confidence interval) for the effects of Ca^{2+} (panel A), Mg^{2+} (panel B) and Na^{+} (panel C) on Cd^{2+} to *D. pulex*. In each case the linear regression line is shown (also see Table 2).


(A)
1.5
1.0
0.5
0.0
Cd2+ (μM)
0.0
0.5
1.0
1.5
Ca2+ (mM)
(B)
1.5
1.0
0.5
0.0
Cd2+ (μM)
0.0
0.5
1.0
1.5
Mg2+ (mM)
(C)
1.5
1.0
0.5
0.0
Cd2+ (μM)
0.0
0.5
1.0
1.5
2.0
Na+ (mM)

Chapter 3: Figure 2. Measured EC50s (with 95% confidence intervals) for *D. pulex* in soft water at different H^+ concentrations (panel A) and DOC concentrations (panel B and C). Panels A and C give EC50 values on a Cd^{2+} basis while panel B shows the effect of DOC on total Cd concentration basis. For each panel, the linear regression line of best fit is shown (also see Table 2). In DOC experiments (B and C), two sources of NOM were used, Nordic Reservoir (closed circles) and Suwannee River (open circles) and the open square shows EC50 values at no added NOM (control water).


(A)
1.5
1.0
0.5
0.0
Cd2+ (μM)
0.00
0.25
0.50
0.75
1.00
H+ (μM)
(B)
2
1
0
Total Dissolved Cd (μM)
0
5
10
15
20
DOC (mg C/L)
(C)
2
1
0
Cd2+ (μM)
0
5
10
15
20
DOC (mg C/L)

Chapter 3: Figure 3. Ability of BLM models to predict measured EC50 concentrations in soft water. Panels A and B show the HydroQual BLM predictions where the model predictions are done with an unadjusted model (Panel A) and then adjustments are made to the critical value (LA50) to provide the best fit possible (Panel B). In Panel B the adjusted critical value was 7.1 nmol/g. Panel C shows the predicted EC50 where the model used Log K values that were calculated based on the toxicity data (Table 2). The final adjusted version of the modified BLM for *D. pulex* in soft water is shown in Panel D (see also Table 3). Symbols show different experimental test series and the solid diagonal lines in each panel represent the 1:1 line of perfect prediction.


A
B
C
D
Ca
Mg
Na
K
pH
SR NOM
NR NOM
BLM predicted EC50 (µM)
measured EC50 (µM)

Chapter 4

The Effects of Water Chemistry on Lethal and Sub-lethal Cadmium Toxicity to *Hydra attenuata* in Soft Water.

This chapter is in manuscript form, and will be submitted to Comparative Biochemistry and Physiology. This manusciprt is currently under review by industrial funding partners.

The Effects of Water Chemistry on Lethal and Sub-lethal Cadmuim Toxicity to *Hydra attenuata* in Soft Water.

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4.1 ABSTRACT

The primary objective of this study was to develop toxicity prediction models in soft water for *Hydra attenuata*. The toxicity of cadmium to *H. attenuata* was assessed with lethal and sub-lethal toxicity tests. Acute toxicity tests were conducted with adult hydra over a 96-hour period to generate LC50s and EC50s. Complete disintegration of the organism was used as a lethal end-point to generate LC50s, and clubbing the tentacles as a sub-lethal end point to generate EC50. For both series, water chemistry parameters of Ca^{2+}, Na^{+}, Mg^{2+}, and Cl^{-} were all altered to determine the effects, if any, on the toxicity of cadmium. Two sources of dissolved organic matter (DOM) were also tested while assessing the lethal endpoint. For both lethal and sub-lethal endpoints, increases in Ca^{2+} activity showed a protective effect, indicating a competition for Ca^{2+} and Cd^{2+} with biological ligand. Surprisingly, cadmium toxicity acted independently of Mg^{2+} activity. Altering the concentrations of Na^{+}, K^{+}, and Cl^{-} did not have any effects on cadmium toxicity when assessing either endpoint. EC50s were approximately one third of the LC50s. In lethal assays, DOM had a small protective effect against Cd toxicity but this was less than would be expected based on modeled DOC-Cd complexation. The BLM was used to estimate sublethal toxicity, however the protective effects of Ca were overestimated as was the complexation effects of natural organic matter. This would suggest that using a BLM to predict Cd toxicity to *H. attenuata* would not be advantageous over using a simple hardness equation.

Keywords: Cd; Hydra; water hardness; bioavailability; metal risk assessment.

ACKNOWLEDGEMENTS

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4.2 INTRODUCTION

Hydra (Cnidaria: Hydrazoa) are freshwater organisms that are commonly found in slow moving rivers and streams (Beach and Pascoe, 1998). They have a relatively simple anatomy consisting of two major divisions: the column and the hydranth (Trottier et al., 1997). Anatomically, Hydra are completely diplobastic (Karntanut and Pascoe, 2000), which allows all of the cells of the organism to be in contact with the aquatic environment and, therefore directly exposed to any contaminants that are present. Hydra have been shown to be sensitive to contaminants such as insecticides (Kalafatic, 1997), pharmaceuticals (Quinn et al., 2007) and metals (Holdway et al., 2001; Karntanut and Pascoe, 2002). In addition to its sensitivity to environmental stressors Hydra can be maintained and cultured in a laboratory setting, they reproduce rapidly and exhibit clear and distinctive morphological changes during stress and therefore are suited to toxicity studies (Karntanut and Pascoe, 2000; Trottier et al., 1997). Morphological changes as expressions of toxicity can be easily assessed in Hydra: tentacles shrink and then completely retract followed by a gradual regression, loss of structure and then disintegration of the organism (Trottier et al., 1997).

Understanding the effects of metals in aquatic environments requires knowledge on the interactions between the metal and the water geochemistry that influence bioavailability (Di Toro et al., 2001; De Schamphelaere and Janssen, 2002). The free ion is the most bioavailable form in solution (Campbell,

1995) and water chemistry variables that complex metal, such as natural organic matter (NOM), decrease the bioavailability to the organism (Niyogi et al., 2008; De Schamphelaere and Janssen, 2002, Di Toro et al., 2001). Additionally, cations such as Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and H^{+} may compete with metal ions for uptake into organisms (Niyogi and Wood, 2004; Di Toro et al., 2001). Competition for uptake explains, at least in part, the protective effect that hardness has, particularly for divalent metals such as Cd, Pb, and Zn (Clifford and McGeer, 2009a,b).

The combined effect that complexation and competition can have on metal toxicity to aquatic organisms have been integrated into a geochemical equilibrium modeling framework, the biotic ligand model (BLM; Di Toro et al., 2001). BLMs have been developed to provide water chemistry specific predictions for the effects of metals including Cd, Cu, Zn, Ag and Pb (Niyogi and Wood, 2004; McGeer et al., 2009). The available BLM software for Cd (HydroQual BLM available at www.hydroqual.com/blm) was developed based on the gill binding studies of Playle et al., (1993). Two recent studies have produced BLM modeling parameters for the effects of Cd on rainbow trout (Niyogi et al., 2008) and *Daphnia pulex* (Clifford and McGeer, 2009a). In general the development of BLM approaches for Cd have not been studied as extensively as other metals such as Cu, Zn or Ni (McGeer et al., 2009). For example, with the exception of Clifford and McGeer (2009a) Cd BLM studies have been done in waters of intermediate to high hardness.

The role of cationic competition and anionic complexation in reducing bioavailability and toxicity results in increased susceptibility for metal toxicity to organisms in soft water (ion depleted) environments. These environments are highly relevant in the Canadian context as many of the lakes on the Canadian Shield have very low hardness. David et al., (1997) completed a survey of 100 lakes on the Canadian Shield and recorded an average hardness of 14 mg/L as $CaCO_3$, and average Ca and Mg concentrations of 100 μM and 40 μM, respectively. The performance of BLMs in these very soft waters where toxicity may be enhanced has been identified as a gap in the understanding of ecological risks of metals (McGeer et al., 2009).

This study aims at increasing the knowledge of Cd toxicity to a less frequently studied organism, *Hydra attenuata*. Our goal was to investigate the effects of water geochemistry on Cd toxicity by manipulating water ions (e.g. Ca, Mg, Na, K, and Cl), pH and dissolved organic matter (DOM). The capacity of the existing BLM to estimate toxicity of Cd to Hydra was tested with a view to developing a BLM for Hydra in soft water. Both lethal and sub-lethal morphological endpoints were used to determine the effects of Cd in various water chemistries.

4.3 MATERIALS AND METHODS

Hydra attenuata were obtained from C. Blaise (Centre Saint-Laurent, Environment Canada, Montreal). Hydra cultures were fed brine shrimp and

maintained according to the procedures outlined in Trottier et al., (1997). In brief, cultures were maintained in 1.5 L glass crystallizing dishes, filled with 500ml of culture media and at a temperature of 21°C (± 2°C). Hydra were fed daily with rinsed *Artemis nauplii* (Ocean Star International Marine Lab Inc., Snowville UT), which had been hatched no more than 24h before in a 30 g/L NaCl at 29°C. Hydra were allowed to feed for at least 30 min before the dishes were cleaned and the culture media was replaced.

The culture media for *H. attenuata* was reconstituted soft water (RSW), modified to have a pH of 7.0 (Clifford and McGeer, 2009a). RSW used $CaSO_4$, $MgSO_4$, $NaHCO_3$, and KCl (all Sigma-Aldrich Inc. St. Louis, MO) additions to deionized water to achieve final measured concentrations of Ca, Mg, Na, Cl, K, SO_4^-, and HCO_3^- of 170, 140, 140, 30, 30, 310 and 140 μM, respectively. RSW was modified from the standardized soft water recommended by Environment Canada and US EPA to reduce the Mg content (Kozlova et al., 2009). The dissolved organic carbon (DOC) content was measured to be 1.53 mg C/L.

Test media was prepared as a 2L aliquot of culture media but lacking the chemistry parameter under test, which was added separately. The parameters that were varied in different test series were Ca, Mg, Na, K and Cl and these were added (as $CaSO_4$, $MgSO_4$, NaCl and KCl) to achieve the concentrations required for each test. Additionally, the potential protective effect of NOM on Cd toxicity was tested in RSW using two sources; Nordic Reservoir NOM (1R108N) and Suwannee River NOM (1R101N), both obtained from the International Humic Substances Society. The effect of pH on Cd toxicity was also tested in RSW. In

these tests the buffering compound 3-(n-morpholino)-propanesulphonic acid (MOPS) was used at a concentration of 750 mg/L to adjust pH from 7 (pH of RSW) to either 6.0 or 8.0 using either HNO_3 or NaOH (De Schamphelaere et al., 2004). Test solution chemistries are given in Table 2 (associated with EC50 tests) and Table 3 (LC50 tests).

Toxicity tests followed the methods given by Trottier et al., (1997) with minor modifications. In brief, tests were carried out in 12-well microplates where Hydra were exposed, in triplicate, to one of the eight exposure concentrations (each test consisted of a control and 7 Cd exposure concentrations). Appropriate volumes of Cd, from a 0.89 mM stock solution, were added to the 25 ml of test medium (exposure media without Cd as described in the previous paragraph) and 5 ml added to each of the 3 microwell plates. The remaining 10 ml was saved for subsequent analysis. Ten Hydra with either no buds or with slightly developed buds were selected and inoculated into each microplate well to start a test, which lasted for 96 h. Results from toxicity assays were considered acceptable if the mortality of the controls was less than 10% (Trottier et al., 1997).

The effects of Cd on Hydra were assessed as morphological changes to the whole organism. Morphological endpoints assessed either lethal or sublethal endpoints and were based on the 10 point system developed by Wilby (1988, as reported in Karntanut and Pascoe, 2000) where a normal healthy organism score as a 10 and the complete disintegration scores as a 0 (see Table 1). The sublethal endpoint (96 h EC50) for morphological change was clubbed and

shortened tentacles (score of 8, Table 2, Figure 1B), and for the lethal endpoint (96 h LC50) the morphological endpoint was 'tulip' phase (score of 2, see Table 1, Figure 1D). Independent test series for the two endpoints were used to assess the effects of Cd on *H. attenuata* in varying water chemistries. Images of representative morphological responses to waterborne Cd were taken using a Nikon Zoom Steriomicroscope SMZ 1500 (Kikon Canada Inc., Mississauga, ON) with a Fiber-Lite M1-150 high intensity illuminator (Dolan-Jenner Industries, Boxborough, MA). The stereomicroscope was fitted with a Paxcam digital camera and the images were captured using the Pax-it Image Management Software (Paxcam, Villa Park, IL).

Samples (10 ml) were collected for each test solution at the beginning of each test and acidified with a 1% volume of 16N HNO_3 (trace metal grade, Fisher Scientific, Nepean, ON). Total (unfiltered) Cd concentrations in solution were measured following a comparison of unfiltered and filtered (0.45 μm Acrodisc syringe filter, HT Tuffryn) samples that demonstrated that dissolved Cd was 96.3% (range 92.36% to 98.87%, n = 10) of the total Cd in solution. For each test series, three samples (randomly chosen from the 8 collected) were analyzed for Ca, Mg, Na, and K. Cation concentrations were measured by atomic absorption spectrophotometry (SpectAA-880, Varian Inc, Palo Alto, CA). The pH of test solutions was measured using a PHM240 meter with pHC2701-8 electrode (Radiometer). DOC was also measured on samples from each test series. Samples for DOC were filtered (0.45 μm Acrodisc syringe filter, HT Tuffryn) and

measurements done on a total organic carbon analyzer (5050A TOC Analyzer, Shimadzu, Columbia, MD).

96 h EC50 and LC50 concentrations for total dissolved Cd were calculated by using the software program PROBIT (Ver. 1.5). The measured water chemistry and the calculated total dissolved Cd concentrations associated with the EC50 or the LC50 were entered into the HydroQual BLM (ver. 2.2.3) to generate geochemical speciation estimates of Cd^{2+} concentrations in solution. The HydroQual BLM was also used to develop toxicity predictions for the protective effects of Ca (EC50 and LC50s) and NOM (LC50s) on Hydra. Other test solutions were not compared because no other protective effects were evident from test results. In both cases the sensitivity parameter or critical value (the LA50 or lethal accumulation (in nmol/g) associated with the EC50 or LC50) was adjusted to provide the best fit between predicted and measured. Similarly, the predicted protective effect of Ca from the US EPA "hardness equation" associated with the Criteria Maximum Concentration (CMC: http://www.epa.gov/waterscience/criteria/wqctable/index.html#K) was applied with the calculated hardness values for both Ca test series (EC50 and LC50). In each of these series the hardness adjusted CMC values was multiplied by the appropriate constant to bring the values into the range of Cd toxicity to Hydra.

4.4 RESULTS

H. attenuata responded to waterborne Cd in a dose dependent manner for both lethal (LC50) and sublethal endpoints (EC50) with the latter being about 3 fold lower (Table 2 compared to 3). Cd toxicity was significantly decreased when the waterborne Ca concentration was increased and this occurred for both lethal and sublethal endpoints (Figure 3,4). In the sub-lethal data set Ca was tested over a range of 0.2-1.21mM resulting in a 4-fold reduction in EC50 values, from 0.18 to 0.79 μM (Table 2, Figure 2A). In the lethal endpoint study Ca concentrations ranged from 0.13 to 1.31 mM and the LC50 values increased by approximately 3 fold (from 0.62 to 1.53 μM, Table 3, Figure 4A). The relationships for Ca^{2+} on sub-lethal and lethal Cd^{2+} EC50 and LC50 free ion activity based toxicity is shown in Figure 2A and 4A with regression variables given in Table 4. The slopes of these two lines of regression were similar, indicating that the effect of Ca is consistent across lethal and sublethal endpoints (Table 4). The intercept of the line of regression for the sub-lethal end point was 9 times lower than that of the regression for the lethal end point indicating a clear difference between the two endpoints. These intercepts were used to estimate the Log K_{CdBL}, the binding of Cd to the biotic ligand in the absence of competitive interactions, yielding values of 6.4 for the lethal endpoint and 7.3 for the sublethal endpoint.

In sub-lethal and lethal data sets, the concentrations of Mg were tested over 0.18-0.58 mM and 0.14-1.12mM, respectively and in each data set there was no significant effects of waterborne Mg (Table 2 and 3). The lack of

protective effect of Mg^{2+} on Cd^{2+} toxicity is also evident from the regression data (Figure 2B and 4B; Table 4).

Testing with Na and K demonstrated little protective effect on Cd toxicity. There were no significant changes in EC50 or LC50 for Na tests (Table 2, 3, Figure 3A, 5A). Similarly there was no decrease in Cd toxicity during LC50 tests with K. In fact, elevated K concentrations resulted in increased Cd toxicity (decreased LC50, Table 2). This produced a negative slope to the relationship of K^+ on Cd^{2+} toxicity (slope not significantly different from 0). In the case of the sublethal toxicity test series there was a small but none-the-less significant increase in EC50 at the highest K concentrations (Table 2) although the slope of the regression line for the effect of K^+ on Cd^{2+} toxicity was not significant (Table 4). Adjusting of Na and K concentrations was done with NaCl and KCl and because there were no consistent effects it can be concluded that Cl binding has no significant effect on Cd toxicity.

The effect of NOM and pH on Cd toxicity was assessed for the lethal endpoint only and for both sources there was a small (approx 20% increase) but significant increase in LC50 (Table 3). These differences were not sufficient to produce a significant slope to the regression relationship between NOM concentration and total dissolved Cd (Figure 5B). The BLM was used to estimate Cd complexation in solution with added NOM; the Cd^{2+} associated with the LC50 concentration with no added NOM (total Cd of 0.62 µM and DOC of 1.5 mg C/L) 0.49 µM. The modeled free ion concentrations associated with the NOM treatments that provided the highest protective effect were: Suwannee River

NOM (EC50 of 0.74 Cd_{Tot} µM at 6 mg C/L DOC) was 0.38 uM Cd^{2+} (22% reduction from the baseline EC50 of 0.49 µM Cd^{2+}), and for Nordic Reservoir NOM (EC50 of 0.75 Cd_{Tot} µM at 12 mg C/L DOC) it was 0.25 uM Cd^{2+} (nearly a 50% reduction). The effects of pH could not be tested because Hydra proved to be very sensitive to pH changes. Increasing the pH to 8, or decreasing it to 6.1, resulted in elevated mortality in the controls invalidating these tests (data not shown).

The HydroQual BLM was used to develop predictions of Cd toxicity to Hydra. There is no Hydra specific BLM and therefore the one developed for *Ceriodaphnia dubia* was used. Following adjustments of the LA50 value (the predicted accumulation associated with 50% effect, also known as the critical value) to 6.95 nmol Cd/g a good match between measured and predicted EC50 was evident (Figure 6A). In the case of LC50s the LA50 value in the model was adjusted up to 7.74 nmol/g to give a good match between measured and predicted at low Ca levels (Figure 6B). The predicted toxicity at high Ca levels showed that the BLM over-estimated Cd LC50s (Figure 6B). The actual protective effect of NOM on acute lethality was also much less than predicted by the BLM (Figure 5B). The EPA CMC hardness equation was also tested for its ability to predict the protective effect of Ca. The CMC is designed for extremely sensitive organisms and it was necessary to multiply hardness adjusted CMC values by constants of 28 and 132 (EC50 and LC50 series respectively) to bring estimates up to a level where trends in Ca protection could be compared. In both

cases the estimates provided by the adjusted CMC Cd hardness values were remarkably close to BLM prediction values (Figure 6A and 6B).

4.5 DISCUSSION

The goal of this study was to understand the toxic effects of Cd on *H. attenuata* in terms of lethal and sublethal endpoints as well as the effect of water chemistry. When exposed to Cd, the morphology of Hydra can change dramatically. We successfully applied pre-defined sublethal and lethal endpoints to evaluate the effects of Cd over 96 h. In soft water the sublethal endpoint was about three fold more sensitive than the lethal endpoint. In terms of the potential protective effects of aquatic geochemistry on the impact of Cd, this study shows that Ca strongly influences toxicity but other cations do not and neither does NOM.

Healthy *H. attenuata* will have an elongated body with 4-6 tentacles and will be responsive to stimuli (Pascoe et al., 2003). The morphological changes in response to stress have been noted as changes in tentacle size, shape and number as well as changes in body size and shape (Quinn et al., 2009; Pascoe et al., 2003; Karntanut and Pascoe, 2000; Trottier et al., 1997). Wilby (1988, as reported by Karntanut and Pascoe, 2000) assigned a scoring system to the morphological characteristics in order to quantify the effects of a toxicant on an organism (Table 1). Assessing toxicity through a progression of morphological damage allows for a more detailed understanding of the response to stressors

(Karntanut and Pascoe, 2000). Some of the stages in the ten stage scoring system can be somewhat ambiguous and it has been accepted that specific morphological endpoints are less subjective to assess toxicant effects on Hydra. Pascoe et al., (2003) found that feeding (ingestion of *Artemia*) decreases were associated with tentacle regression and clubbing (scores of 8 and less on the 10 point scale). While there is evidence of organisms surviving and being able to recover from toxicant exposure from the clubbed tentacle endpoint it is clear that feeding is affected. Inhibition of feeding can affect the long-term fitness of the population and therefore is a relevant sublethal endpoint. Based on the study of Quinn et al., (2007) we used the tulip phase of morphological regression (scale value of 2) as the lethal endpoint.

In these studies, sub-lethal and lethal Cd toxicity was reduced by the presence of Ca, illustrating a strong competitive interaction between Ca^{2+} and Cd^{2+} (Figure 2A, 4A; Table 2, 3). Cd^{2+} is a well-known analogue for Ca^{2+} up-take channels (Verbost et al., 1989). Similar trends in Cd toxicity have been shown in *Daphnia* spp. (Shaw et al., 2006; Yim et al., 2006), and with rainbow trout (*O. mykiss*) in the presence of Ca (Birceanu et al., 2008; Niyogi and Wood, 2004). Tan and Wang (2008), who studied the effects of Ca on Cd and Zn toxicity, hypothesized that since Cd is not an essential metal there is likely no direct uptake channel and it must therefore be taken up through other ion channels, such as the Ca up take channel.

The competitive effects of Ca on Cd toxicity were seen when assessing both sub-lethal and lethal end points and the strength of the protection offered by

Ca was generally similar (slopes of regressions only differed by a factor of 1.5; Table 4). The intercepts for the regressions of Ca^{2+} on lethal and sublethal Cd^{2+} endpoints, which represent the effects of free ionic Cd in the absence of protective factors, differ by over 9 fold (Table 4) and this illustrates the relative sensitivity of the sublethal endpoint. These concentrations were used to estimate binding characteristics for Cd^{2+} onto the biotic ligand and yielded Log K_{CdBL} of 6.3 and 7.4 for the lethality and tentacle regression endpoints respectively. The values generally agree with those of previous studies; 7.0 for *Daphnia pulex* in soft water (Clifford and McGeer, 2009b) and 7.5, 7.3 and 7.3 reported for rainbow trout by Niyogi et al., (2008), Niyogi et al., (2004) and Hollis et al., (2000) respectively but it is considerably lower than the 8.6 for fathead minnow reported by Playle et al. (1993).

The protective effects of Ca^{2+} on metal toxicity is frequently linked with associated protective effects from Mg^{2+} as well as the general protection that water hardness offers (Niyogi and Wood, 2004; Santore et al., 2002; Di Toro et al., 2001). Independent protective effects of Mg^{2+} on Cd^{2+} toxicity has been shown in fish species (Di Toro et al., 2001; Pascoe et al., 1986; Playle et al., 1993) but the mechanisms of this competitive protective effect are not well understood and may result from direct interactions at Mg^{2+} uptake sites and/or interactions occurring at Ca^{2+} uptake sites that Mg^{2+} and Cd^{2+} both have an affinity for (Markich and Jeffree, 1994). In this study there was no effect of Mg on Cd toxicities (Figure 2B, 4B; Table 2 and 3) and this similar to the study of Niyogi

et al., (2008) who found no protection associated with Mg in acute Cd toxicity tests with rainbow trout.

Increased waterborne Na also had no significant protective effect on Cd toxicity (Figure 3A, 5A; Table 4). Na up-take on the gills of fish occurs via a H^+ ATPase linked Na channel that has not been associated with the uptake of divalent metal ions, such as Cd^{2+} (Grossell et al., 2002). Therefore our finding that there is no competitive effect between Na^+ uptake and Cd^{2+} uptake is data is consistent with previous studies and mechanisms of Cd toxicity (Niyogi and Wood, 2004). Similarly, K also had no effects on Cd toxicity to *H, attenuata* (Figure 3B, 5A; Table 4), as expected. These results are in agreement with previous work done on Cd toxicity in rainbow trout (Birceanu et al., 2008; Niyogi et al., 2008) and *D. pulex* (Clifford et al., 2009b).

The effects of pH could not be studied because of the high sensitivity of *H. attenuata* to small variations in pH. Toxicity tests where pH was manipulated consistently resulted in mortalities greater than the acceptable 10% in the controls (data not shown). In order to understand the effect that pH could have on mitigating Cd toxicity it would be necessary to pre-acclimate cultures to the desired pH. Hyne et al., (1992) successfully studied the effects of different pH values on uranium toxicity to *H. viridissima* and *H. vulgaris.* In that study, they adjusted pH in the range of 6-9 with bicarbonate, sodium bicarbonate, tris (hydroxymethyl) methylamine, or acetic acid, although it was not clear if cultures were pre-acclimated to the test pH. In our study, pH manipulations were made with the buffer MOPS, following the protocol outlined by De Schamphelaere et

al., (2004). MOPS has been shown to have no significant effect on *Daphnia* and algae (Clifford et al., 2009a; Kozlova et al., 2009; De Schamphelaere et al., 2004), however there is no available data on the potential effects of MOPS to Hydra. Therefore, it could be that the *H. attenuata* in our study were affected by MOPS or, alternatively they are a species that is more sensitive to abrupt pH changes compared to *H. viridissima* and *H. vulgaris*.

The two sources of NOM, Nordic Reservoir and Suwannee River, both decreased Cd toxicity to Hydra (Table 3) but this protective effect was weak. These were unexpected results as the free ion activity model (Campbell, 1995) principles underlying the BLM (Di Toro et al., 2001) indicate that an increase in waterborne DOC would result in complexation of Cd^{2+} in solution and a corresponding increases in LC50 values. Under this theory the LC50 concentrations would increase on a total Cd in solution basis but on a free ion basis the concentration of Cd^{2+} would be relatively constant and this has illustrated recently with *Daphnia pulex* (Clifford et al., 2009b). In this study, when we used the BLM (HydroQual ver. 2.2.3) to estimate the LC50s on a Cd^{2+} concentration basis a decrease of Cd^{2+} was noted for the LC50s at higher DOC concentrations (reduced by up to 50%). The relatively weaker than expected (from BLM speciation predictions) effect of NOM on Cd toxicity suggests that the affinity of Cd uptake sites in Hydra is strong relative to DOC complexation of Cd. Alternatively, Cd-DOC complexes, in addition to Cd^{2+}, may be bioavailable to *H attenuata*. The results of Niyogi et al., (2008) offer some evidence on the equivocal nature of Cd-DOC interactions. In that study, there was no effect of

DOC on the short term binding of Cd to rainbow trout gills in test solutions up to 10 mg C/L, however in toxicity tests the 96 h LC50 values increased by about 60% as DOC increased from 3 to 10 mg C/L.

The development of Cd toxicity predictions illustrated that the BLM could be used to estimate the protective effect of Ca on the sublethal effects of Cd on Hydra attenuata but not LC50s (Figure 6A and 6B). The hardness equation within the EPA acute water quality criteria value (CMC) for Cd, once adjusted for the sensitivity of *H. attenuata*, proved to be very similar to the BLM based predictions and this may illustrate an overlap in some of the data sets used to generate these bioavailability adjustment procedures. Within BLM predictions of EC50s and LC50s the protective effect of Ca was the same (Log K value was not changed), and only the LA50 value had to be changed to achieve reasonable predictions. However, the measured protective effect of Ca on the lethal endpoint was weaker than it was on the sublethal endpoint and therefore the predicted toxicity matched to EC50 values (Figure 6A) it was expected that the model would underestimate LC50 toxicity (Figure 6B). The reason for a stronger protective effect of Ca at the low concentrations associated with the tentacle clubbing endpoint than at the higher (and acutely lethal) concentrations is unknown but may be related to the relative affinity of Ca vs Cd at different populations of uptake sites. These results suggest some potentially interesting features of Hydra physiology and are deserving of further study.

This study demonstrates that Ca provides a protective effect on Cd toxicity but that other cations (Mg, Na, K, Cl) do not. NOM showed minor protective

effect against Cd lethality and this was less than would be expected based on modeled DOC-Cd complexation. On a total Cd concentration basis EC50 values were approximately one third that of LC50 values and on a Cd^{2+} basis the maximum difference between lethal and sublethal endpoints was 9 fold. The BLM was successfully applied to estimate the sublethal toxicity endpoint but it overestimated the protective effect of Ca on acute lethality. While the BLM was partially successful in estimating the mitigation of Cd toxicity by Ca, it was not successful for NOM and the general lack of Cd toxicity mitigation through complexation and cationic competition suggests that application of this approach does not provided added benefits. Simple hardness adjustment approaches appeared to work equally well for sublethal toxicity and equally ineffectively for lethal toxicity.

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Chapter 4: Table 1. Scoring key for morphological traits associated with contaminant stress in Hydra. The scores corresponding to the lethal and sublethal endpoints used in this study were 2 and 8, respectively.

Score	Morphology
10	Extended tentacles and body reactive
9	Partially contracted, slow reactions
8	Clubbed tentacles, body slightly contracted
7	Shortened tentacles, body slightly contracted
6	Tentacles and body shortened
5	Totally contracted, tentacles visible
4	Totally contracted, no visible tentacles
3	Expanded, tentacles visible
2	Expanded, no visible tentacles
1	Dead but intact
0	Disintegrated

Chapter 4: Table 2. Calculated EC50 values for Cd and mean of measured water chemistry from 96 h toxicity tests assessing lethality in *H. attenuata*. Measured concentrations were used in EC50 calculations, the upper and lower bounds of the 95% confidence interval are given and for each test series the concentration of the variable manipulated is shown in bold. Concentrations are given as mM except for Cd (μM), DOC (mg C/L) and pH. A n/a indicates that the confidence interval was not generated and within each test series, EC50 values tagged with the same superscripted letter are not significantly different.

Test series	Ca	Mg	Na	Cl	SO_4^-	K	HCO^-	pH	DOC	Cd EC50	95% C.I.	
											Lower	Upper
Ca	**0.20**	0.17	0.15	0.04	0.37	0.04	0.15	7.00	1.53	0.18[a]	0.14	0.21
	0.32	0.17	0.15	0.04	0.49	0.04	0.15	7.00	1.53	0.30[b]	0.28	0.33
	0.53	0.17	0.15	0.04	0.70	0.04	0.15	7.00	1.53	0.32[b]	0.31	0.33
	0.77	0.17	0.15	0.04	0.94	0.04	0.15	7.00	1.53	0.58[c]	0.52	0.71
	1.21	0.17	0.15	0.04	1.38	0.04	0.15	7.00	1.53	0.79[c]	0.67	0.87
Mg	0.20	**0.18**	0.15	0.04	0.38	0.04	0.15	7.00	1.53	0.17[a]	0.16	0.19
	0.20	**0.62**	0.15	0.04	0.82	0.04	0.15	7.00	1.53	0.19[a]	0.14	0.23
	0.20	**0.58**	0.15	0.04	0.78	0.04	0.15	7.00	1.53	0.23[a]	0.18	0.27
Na	0.20	0.17	**0.15**	0.19	0.37	0.04	0.15	7.00	1.53	0.17[a]	0.16	0.19
	0.20	0.17	**0.64**	0.68	0.37	0.04	0.15	7.00	1.53	0.20[a]	0.06	0.62
	0.20	0.17	**1.36**	1.40	0.37	0.04	0.15	7.00	1.53	0.17[a]	0.07	0.28
	0.20	0.17	**1.45**	1.49	0.37	0.04	0.15	7.00	1.53	0.17[a]	0.09	0.27

K	0.20	0.17	0.15	0.04	0.37	**0.04**	0.15	7.00	1.53	0.17[a]	0.16	0.19
	0.20	0.17	0.15	0.55	0.37	**0.55**	0.15	7.00	1.53	0.19[ab]	0.16	0.22
	0.20	0.17	0.15	0.95	0.37	**0.95**	0.15	7.00	1.53	0.17[a]	0.14	0.20
	0.20	0.17	0.15	1.47	0.37	**1.47**	0.15	7.00	1.53	0.23[b]	0.21	0.24

Chapter 4: Table 3. Calculated LC50 values for Cd and mean of measured water chemistry from 96 h toxicity tests assessing lethality in *H. attenuata*. Measured concentrations were used in LC50 calculations, the upper and lower bounds of the 95% confidence interval are given and for each test series the concentration of the variable manipulated is shown in bold. Concentrations are given as mM except for Cd (μM), DOC (mg C/L) and pH. A n/a indicates that the confidence interval was not generated and within each test series, LC50 values tagged with the same superscripted letter are not significantly different.

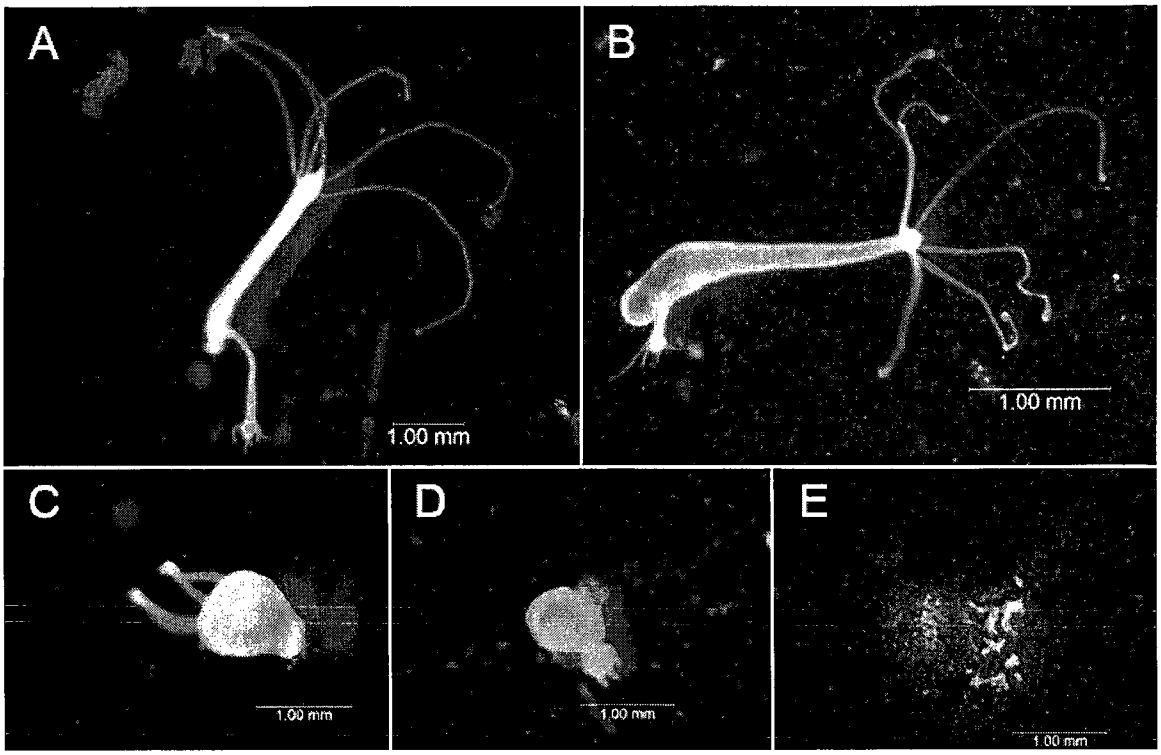
Test series	Ca	Mg	Na	Cl	SO_4^-	K	HCO^-	pH	DOC	Cd LC50	95% C.I.	
											Lower	Upper
Ca	**0.13**	0.14	0.15	0.09	0.27	0.09	0.15	7.00	1.53	0.62[a]	0.61	0.63
	0.71	0.14	0.15	0.09	0.85	0.09	0.15	7.00	1.53	1.14[b]	1.00	1.32
	1.31	0.14	0.15	0.09	1.45	0.09	0.15	7.00	1.53	1.53[b]	1.24	1.69
Mg	0.13	**0.14**	0.15	0.09	0.27	0.09	0.15	7.00	1.53	0.62[a]	0.61	0.63
	0.13	**0.61**	0.15	0.09	0.74	0.09	0.15	7.00	1.53	0.74[a]	0.27	1.05
	0.13	**1.12**	0.15	0.09	1.25	0.09	0.15	7.00	1.53	0.68[a]	0.37	0.80
Na	0.13	0.14	**0.15**	0.24	0.27	0.09	0.15	7.00	1.53	0.62	0.61	0.63
	0.13	0.14	**0.65**	0.74	0.27	0.09	0.15	7.00	1.53	0.55	n/a	n/a
	0.13	0.14	**1.13**	1.22	0.27	0.09	0.15	7.00	1.53	0.75	n/a	n/a
	0.13	0.14	**1.58**	1.67	0.27	0.09	0.15	7.00	1.53	0.59	n/a	n/a
K	0.13	0.14	0.15	0.24	0.27	**0.09**	0.15	7.00	1.53	0.62[a]	0.61	0.63
	0.13	0.14	0.15	0.75	0.27	**0.60**	0.15	7.00	1.53	0.52[b]	0.51	0.54

	0.13	0.14	0.15	1.29	0.27	**1.14**	0.15	7.00	1.53	0.39[c]	0.37	0.40
	0.13	0.14	0.15	1.80	0.27	**1.65**	0.15	7.00	1.53	0.51[b]	0.48	0.55
	0.13	0.14	0.15	0.09	0.27	0.09	0.15	7.00	**1.53**	0.62[a]	0.61	0.63
NR NOM	0.13	0.14	0.15	0.09	0.27	0.09	0.15	7.00	**7.00**	0.65[ab]	0.41	0.78
	0.13	0.14	0.15	0.09	0.27	0.09	0.15	7.00	**12.00**	0.75[b]	0.74	0.76
	0.13	0.14	0.15	0.09	0.27	0.09	0.15	7.00	**1.53**	0.62[a]	0.61	0.63
SR NOM	0.13	0.14	0.15	0.09	0.27	0.09	0.15	7.00	**6.00**	0.74[b]	0.71	0.77
	0.13	0.14	0.15	0.09	0.27	0.09	0.15	7.00	**13.00**	0.70	n/a	n/a

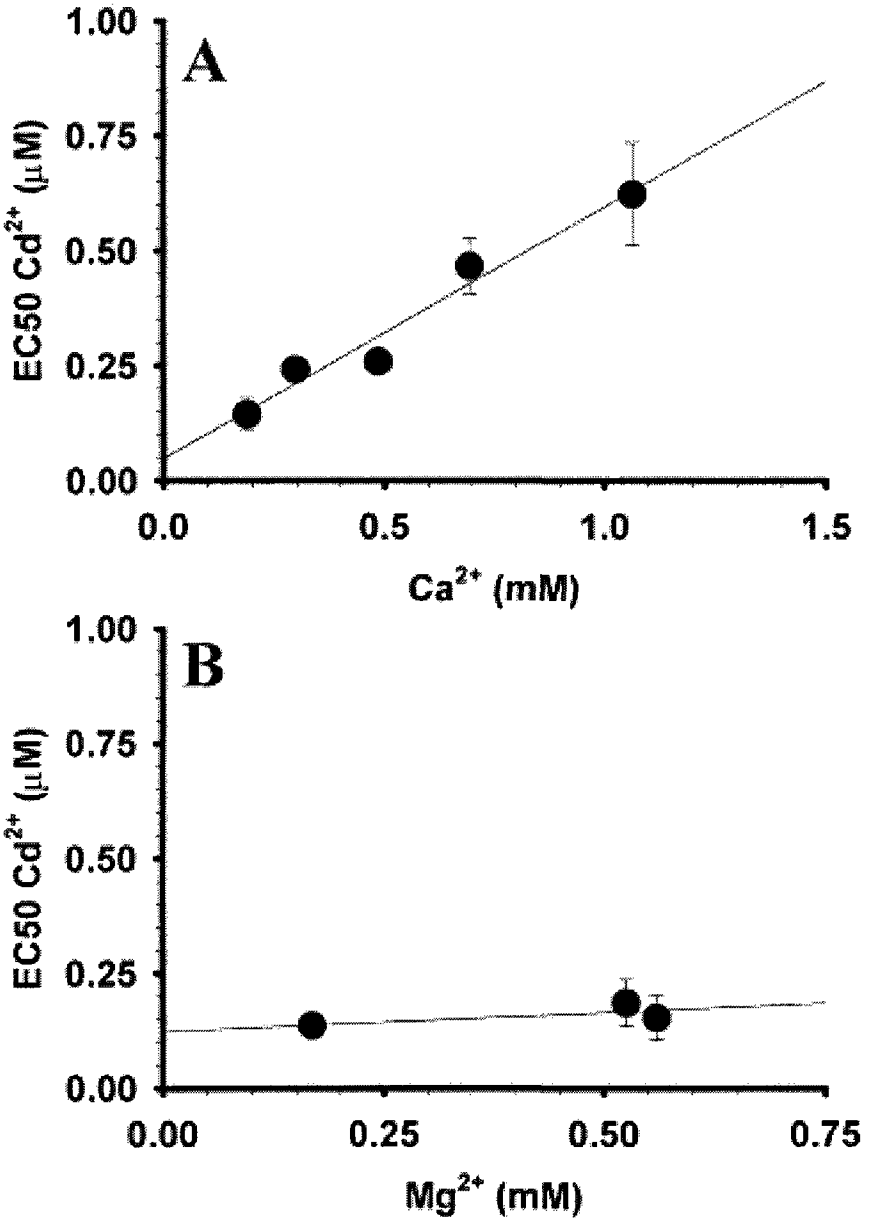
Chapter 4: Table 4. Regression coefficients for the linear regression of Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} on the sub-lethal (EC50 for clubbed tentacles) and lethal toxicity (LC50 for mortality) of Cd^{2+}. The units for slope are ΔEC50 (μmol Cd^{2+}/L) or ΔLC50 (μmol Cd^{2+}/L) per mmol/L change in cation activity except for NOM, which is per mg/L of DOC. The intercept is μmol Cd^{2+}/L. Slopes that were significantly different from 0 are denoted by *.

		Slope	Intercept	R^2
Sub-lethal	Ca	0.5471*	0.0477	0.96
	Mg	0.0827	0.1234	0.51
	Na	-0.0109	0.1458	0.27
	K	0.0207	0.1310	0.45
Lethal	Ca	0.6985*	0.4224	0.99
	Mg	0.0602	0.5064	0.21
	Na	-0.0001	0.4738	0.00
	K	-0.0840	0.4579	0.51
	Nordic Reservoir NOM	0.0124	0.5889	0.86
	Suwannee River NOM	0.0060	0.6475	0.33

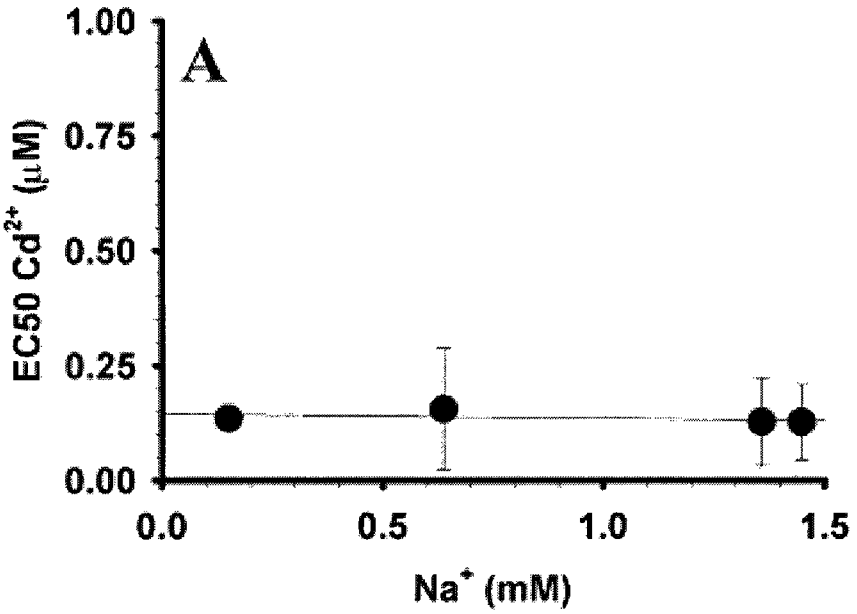
Chapter 4: Figure 1. Changes in morphological appearance associated with stress in *H. attenuata*. Panel A shows a normal responsive Hydra with a budding offspring and this corresponds to a score of 10 (see Table 1). Panel B shows a contracted body and clubbed tentacles, which corresponds to a score of 8 (the sub-lethal end point). Panel C illustrates a completely contracted body and shortened tentacles while Panel D shows the 'tulip' stage, a totally contracted body and barely visible tentacles (a score of 2 and the lethal end point). Panel E shows complete disintegration of the Hydra.

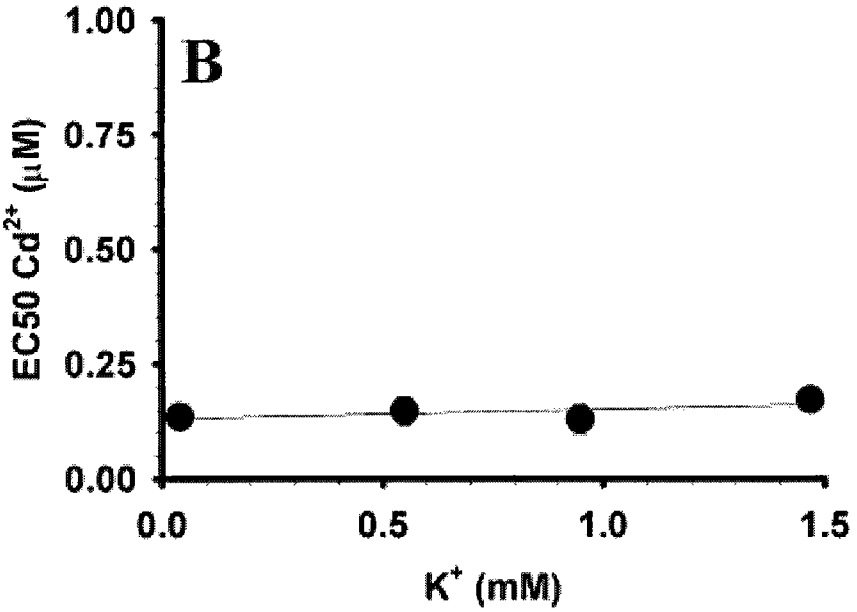

A
1.00 mm
B
1.00 mm
C
1.00 mm
D
1.00 mm
E
1.00 mm

Chapter 4: Figure 2. Measured EC50s (with 95% confidence intervals) for the effects of Ca^{2+} (panel A) and Mg^{2+} (panel B) on sub-lethal Cd^{2+} toxicity to *H. attenuata* over 96 h. The linear regression best fit line is also shown (see Table 4).

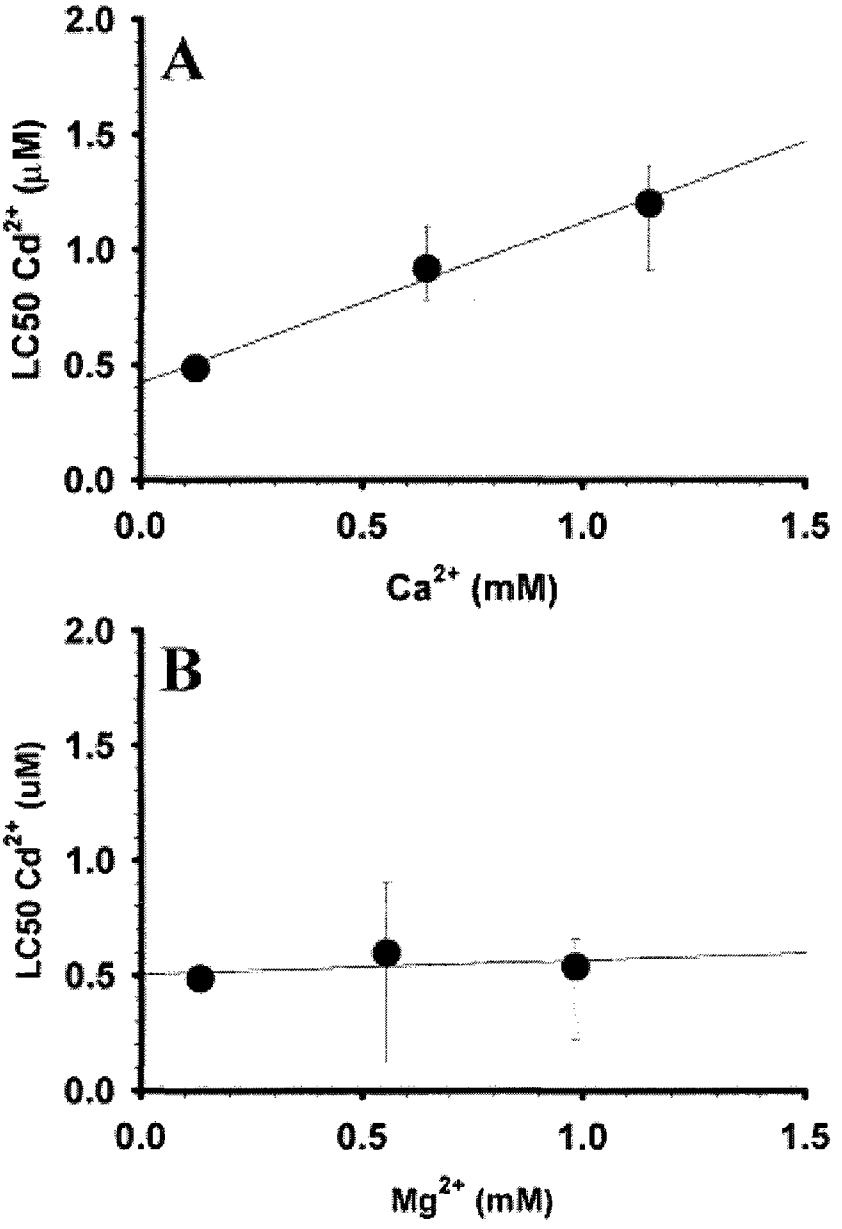

A
1.00
0.75
0.50
0.25
0.00
EC50 Cd2+ (μM)
0.0
0.5
1.0
1.5
Ca2+ (mM)
B
1.00
0.75
0.50
0.25
0.00
EC50 Cd2+ (μM)
0.00
0.25
0.50
0.75
Mg2+ (mM)

Chapter 4: Figure 3. Measured EC50s (with 95% confidence intervals) for the effects of Na^+ (panel A) and K^+ (panel B) on sub-lethal Cd^{2+} toxicity to *H. attenuata* over 96 h. Linear regression lines of best fit are shown (see Table 4).

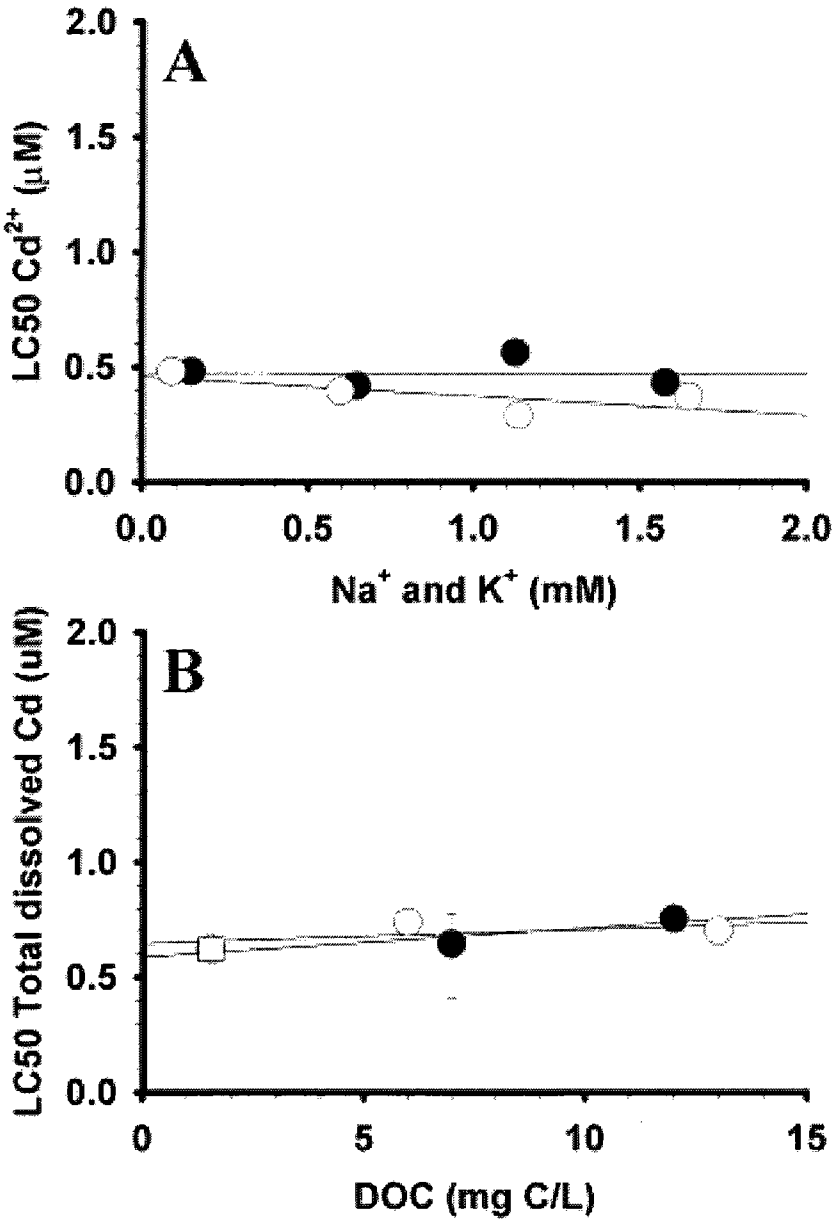

A
1.00
0.75
0.50
0.25
0.00
EC50 Cd^{2+} (μM)
0.0
0.5
1.0
1.5
Na^+ (mM)


B
1.00
0.75
0.50
0.25
0.00
EC50 Cd^{2+} (μM)
0.0
0.5
1.0
1.5
K^+ (mM)

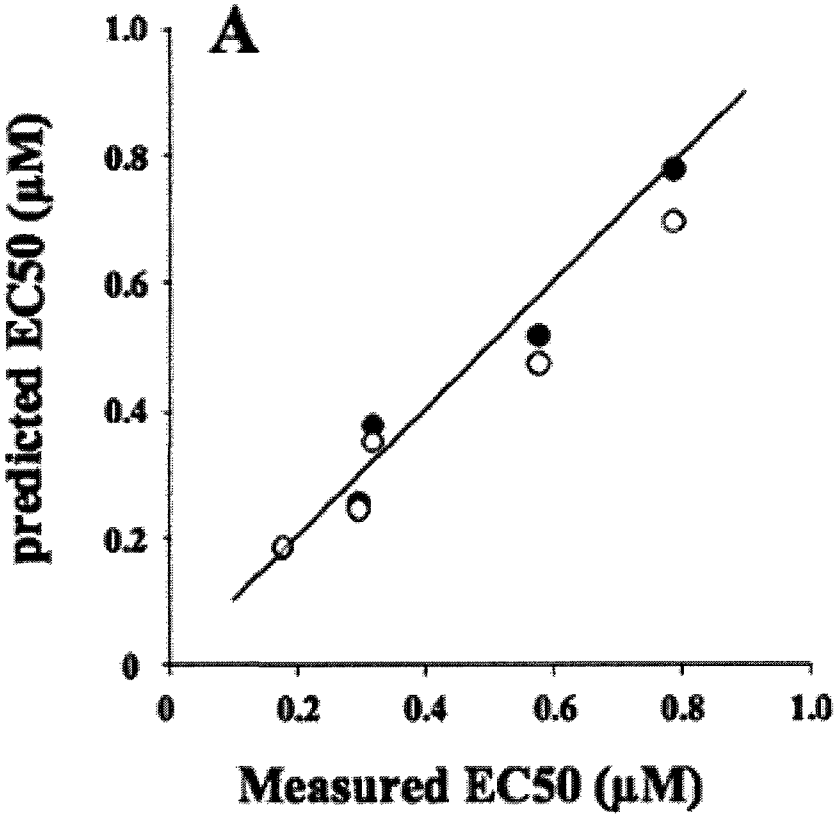
Chapter 4: Figure 4. Measured LC50s (with 95% confidence intervals) for the effects of Ca^{2+} (panel A) and Mg^{2+} (panel B) on lethal Cd^{2+} toxicity to *H. attenuata* over 96 h. Linear regression lines of best fit are shown (see Table 4).

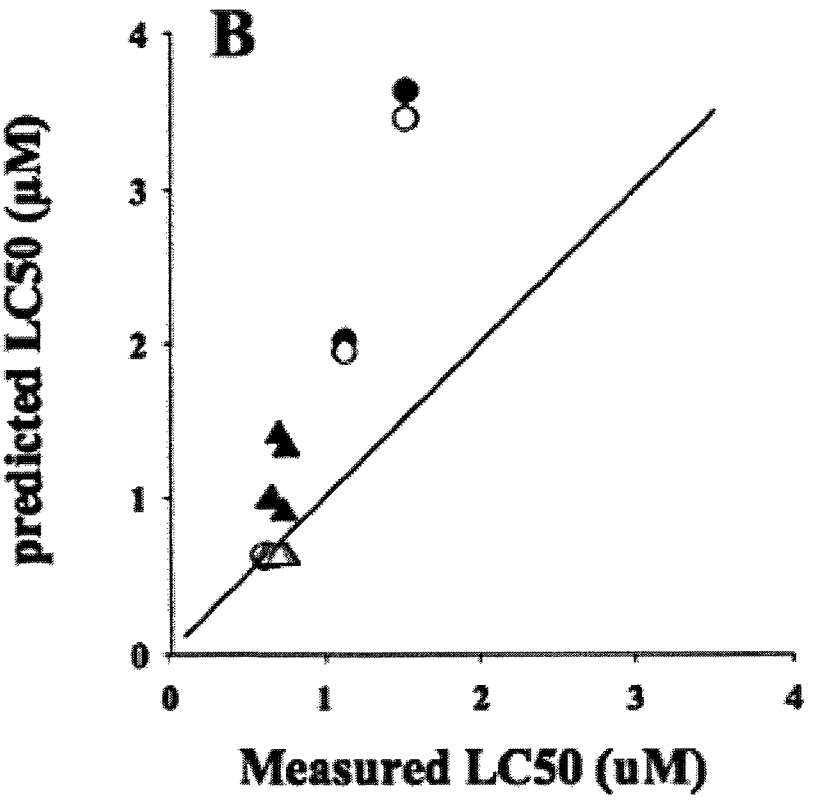

A
2.0
1.5
1.0
0.5
0.0
LC50 Cd2+ (μM)
0.0
0.5
1.0
1.5
Ca2+ (mM)
B
2.0
1.5
1.0
0.5
0.0
LC50 Cd2+ (uM)
0.0
0.5
1.0
1.5
Mg2+ (mM)

Chapter 4: Figure 5. Measured LC50s (with 95% confidence intervals) for the effects of Na^+ (panel A; closed circles), K^+ (panel A, open circles) and DOC (panel B) on Cd toxicity to *H. attenuata* over 96 h. In panel A effects are shown on a free ion basis (Cd^{2+}) while in panel B lethality is shown as total Cd. Panel B also shows the result from tests with two sources of NOM, Nordic Reservoir (closed circles) and Suwannee River (open circles) and the open square show EC50 values at no added NOM. Best-fit linear regression lines are also shown (see Table 4 for details).


A
LC50 Cd^{2+} (μM)
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
Na^+ and K^+ (mM)
B
LC50 Total dissolved Cd (uM)
2.0
1.5
1.0
0.5
0.0
0
5
10
15
DOC (mg C/L)

Chapter 4: Figure 6. Ability of modelling approaches to predict the measured sublethal toxicity (EC50s, Panel A) and lethality (LC50 Panel B) of Cd to Hydra attenuata. In both panels the prediction estimates are given for the Ca test series using the BLM (filled circles) as well as the adjusted CMC hardness equation (open circles). For LC50s (Panel B) predictions for the NOM test series is also shown (closed triangles is BLM and open is adjusted CMC hardness equation). In both panels the solid line shows where predicted values are equal to measured.


A
predicted EC50 (µM)
Measured EC50 (µM)
0
0.2
0.4
0.6
0.8
1.0


B
predicted LC50 (µM)
Measured LC50 (uM)
0
1
2
3
4

Chapter 5

Sub-lethal Waterborne and Dietary Effects of Cadmium on *Hydra attenuata*.

This chapter is in manuscript form, and will be submitted to Aquatic Toxicology. This manuscript is currently under review by industrial funding partners.

Sublethal Waterborne and Dietary Effects of Cadmium on *Hydra attenuata*.

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5.1 ABSTRACT

The objective of this study was to determine the sensitivity of *Hydra attenuata* to dietborne Cd exposure relative to waterborne exposure. The approach employed was to determine the sublethal toxicity of waterborne Cd through 96 h EC50 tests using tentacle regression and clubbing as the effect endpoint, and then to compare this to the effect of Cd delivered via the dietary route. Dietary exposures were done by loading Cd into old *Daphnia pulex* and then feeding these to *H. attenuata*. The influence of Ca on Cd bioavailability was assessed by conducting studies in soft (hardness of 40 mg/L as $CaCO_3$) and hard (140 mg/L) waters. In soft water the 96 h EC50 for Cd on *H. attenuata* was 0.30 μM while in harder waters it was 0.79 μM, illustrating the protective effect of Ca on Cd toxicity in Hydra. Preliminary studies assessing the time-course of Cd bioaccumulation in *Daphnia pulex* also showed the protective effect of Ca. The dietary toxicity of Cd to *H. attenuata* was examined by feeding them 7-8 day old *Daphnia pulex* that had accumulated Cd for 24 h (to reach whole body saturation). *D. pulex* were exposed to the Cd concentrations that were approximately equal to the EC50 concentration for waterborne toxicity to *H. attenuata*. After 96-hours, no significant tentacle clubbing was observed in dietary Cd exposures. Combined waterborne plus dietary exposures to Cd produced effects that were at a level that was similar to those induced by waterborne only exposure. The lack of effects observed during dietary exposures and the lack of additional effects in

combined water and diet exposure suggests that the dietary exposure pathway may not contribute to the impacts that Cd has on *H. attenuata*.

Keywords: Cd; Hydra; water hardness; bioavailability; dietary.

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5.2 INTRODUCTION

The biotic ligand model (BLM) has recently been developed to predict the interaction between dissolved metals and toxic effects in aquatic organisms (Di Toro et al., 2001). BLMs have been successfully developed for a variety of metals including Cu (De Schamphelaere and Janssen, 2002; Di Toro et al., 2001; Villavicencio et al., 2005), Ni (Kozlova et al., 2009) Cd (Santore et al., 2002) and Zn (Clifford and McGeer, 2009; Heijerick et al., 2005). The strength of this geochemical equilibrium approach and the robustness of it's physiological principles have led to the application of this toxicity prediction model for Cu water-quality guideline and criteria derivation (EPA, 2007). Although the BLM is being applied in regulatory contexts it has been developed for a limited number of species (McGeer et al., 2009) and it does not account for potential effects that occur via dietary exposure routes.

In fresh waters, the site of metal uptake during waterborne exposures is primarily at the respiratory epithelium (gills in fish) and effects have been well documented for a number of metals. Incorporation of dissolved metal into food represents a potentially important route of exposure for prey organisms but less is known about dietary uptake and the potential for associated impacts (reviewed Handy et al., 2005). Filter feeding aquatic organisms will also be exposed to metal bound to algae and metal bound to particulates through their diet (Geffard et al., 2008). Dietary uptake and accumulation may not always lead to toxicity, the study of Goulet et al., (2007) demonstrated that *Daphnia magna* fed Cd loaded algae

accumulated significant burdens with no effects on survival or reproduction. However, waterborne Cd exposures resulted in significant impacts with relatively low levels of Cd accumulation.

The effects of dietary and/or waterborne Cd have not been extensively studied, however, the studies that do exist appear to be inconsistent. Allen et al., (1995) and Taylor et al., (1998) found a reduction in feeding in *Daphnia magna* during dietborne exposures with Cd loaded algae. Taylor et al., (1998), Weltens et al., (2000), and Barata et al., (2002) found observed acute effects of dietary Cd on survival and feeding. Goulet et al., (2007) concluded that dietary Cd was not associated with lethal or sublethal effects, when exposed to two species (*Chlamydomonas reinhardtt* and *Pserdokirchneriella subcapita*) of Cd contaminated algae. Considering the discrepancies among studies, it is clear that there are gaps in the understanding of the potential for dietborne Cd to cause impacts in freshwater organisms.

Hydra (Cnidaria: Hydrazoa) are freshwater organisms commonly found in slow moving rivers and streams (Beach and Pascoa, 1998). Because of its sensitivity to metals, Hydra is a good organism to be used in laboratory toxicity tests (Holdway et al., 2001). Toxicity results in morphological changes; tentacles shrink, completely retract and then this is followed by the complete disintegration of the organism (Trottier et al., 1997). Hydra have been used to assess the toxicity of a variety of contaminants in freshwater (Hyne et al., 1992; Pollino and Holdway, 1999;

Quinn et al., 2008). With the exception of Karntanut and Pascoe (2007), who studied the bioaccumulation of Cu, Cd, and Zn in *Hydra vulgaris* from contaminated *Artemia nauplii*, the effects of metal-contaminated food on Hydra do not appear to have been well studied.

The goal of this study was to assess the relative toxicity of waterborne and dietary Cd to *Hydra attenuata*. *Daphnia pulex* were used as the source of dietary Cd to Hydra. To understand the role that water chemistry may have on bioaccumulation and toxicity, studies were conducted at hardnesses of 40 and 140 mg $CaCO_3$/L. This study builds on the previous works of Goulet et al., (2007) and Clifford et al., (2009a,b) to understand the relative toxicity of Cd to Daphnia and Hydra from waterborne and dietary sources.

5.3 MATERIALS AND METHODS

Cultures of *Hydra attenuata*, obtained from C. Blaise (Centre Saint-Laurent, Montreal) and maintained according to protocols adapted from Trottier et al., (1997) in glass crystallizing dishes with approximately 500ml of culture media at 22°C. Medium was renewed daily, after feeding. Hydra were fed with *Artemia salina* that had been hatched in 30 g/L NaCl at 29°C. *Artemia* were rinsed in deionized water prior to feeding and Hydra were allowed to feed for approximately 30 min before the media was replaced (Trottier et al., 1997).

Daphnia pulex were obtained from Aquatic Research Organisms Inc. (Hampton, NH). Neonate-producing cultures of approximately 30-60 adults were maintained in 1.5L glass beakers with approximately 1 L of media. Cultures followed the standardized procedures outlined by Environment Canada (1996), the medium was replaced daily at which time daphnids were fed daily with 5 ml/L of YCT (yeast, cerrophyl, and trout feed) and 10 ml/L of algal feed. Algal feed consisted of a mixture of 70% *Pseudokirchneriella subcapitata* and 30% *Chlorella vulgaris* at a concentration of 3.5×10^7 cells/ml. YCT was purchased from Aquatic Research Organisms Inc. (Hampton, NH) and algae from the Canadian Phycological Culture Centre (CPCC, formerly known as University of Toronto Culture Collection of Algae and Cyanobacteria, UTCC).

H. attenuata and *D. pulex* were maintained in reconstituted soft water (RSW), modified from the soft water guidelines recommended by the Environment Canada (1996) to reduce the Mg content (Clifford and McGeer, 2009). RSW was made by adding $CaSO_4$, $MgSO_4$, $NaHCO_3$, and KCl (Sigma-Aldrich Inc. St. Louis, MO) to deionized water to achieve concentrations of Ca, Mg, Na, Cl, K, SO_4^-, and HCO_3^- at 170, 140, 150, 30, 30, 310 and 150 μM, respectively. Solution pH was measured at 7.0 (Radiometer PHM240 meter with pHC2701-8 electrode) and the dissolved organic carbon (DOC) content was measured to be 1.53 mg C/L (5050A TOC Analyzer, Shimadzu). Two series of experimental exposures were done, one at a Ca concentration of 260 μM (corresponding to a hardness of 40 mg $CaCO_3$/L) and

the other at 1,260 µM Ca (corresponding to a hardness of 140 mg $CaCO_3$/L). Details of water chemistry are given in Table 1.

Hydra toxicity test methods followed procedures outlined in Trottier et al., (1997). 96 h toxicity tests were carried out in 12 well microplates, where each well contained 5 ml of test medium and 10 individuals with no buds or immature buds. Each test consisted of 1 unexposed control treatment along with 7 different exposure concentrations of Cd, in triplicate. Test solutions were made as 25 ml aliquots, taken from the base test media and spiked with appropriate volumes of a 0.89 mM stock solution of Cd, prepared from a purchased standard solution (Inorganic Ventures Inc., Lakewood, NJ), to achieve the desired exposure concentration. A 10 ml sample from each concentration was taken for subsequent characterization. The sub-lethal endpoint for Cd was the presence of clubbed tentacles, corresponding to a scoring of 8 on the 10 point morphological scale for contaminant effects on Hydra (see table 1 of Karntanut and Pascoe, 2000). Toxicity tests where control treatments showed more than 10% of the organisms had clubbed tentacles were not considered acceptable (Trottier et al., 1997). The 96 h EC50 concentrations for tests in water hardness' of 40 and 140 mg $CaCO_3$/L were calculated using the software program PROBIT (Ver. 1.5).

Daphnia pulex (7 to 8 d of age) were exposed to waterborne Cd to develop a dietary source for Hydra exposures. *Daphnia* were exposed in 100 ml of test solution, prepared a day prior and left to equilibrate overnight. Preliminary

experiments with *D. pulex* were done to establish appropriate exposure times to ensure that organisms had reached steady state Cd tissue burdens. In time-course tests groups of 150 *Daphnia* were exposed to either 35 μg/L or 115 μg/L of Cd depending on water hardness (40 and 140 mg/L respectively). Daphnia (n = 15) were sampled at 0, 3, 6, 12, 24, and 30 h. Sampling involved removing Daphnia from test solutions, rinsing them in MilliQ water for approximately 30 s to remove loosely bound metals and then placing individuals on a filter paper to absorb excess moisture. Individual Daphnia were weighed to the nearest 0.01 milligrams (SE2 Ultra Micro Balance, Sartorius) and then digested in 10 μl of 1N HNO_3 for subsequent analysis (adapted from Janes and Playle, 1995). The accumulation over time was plotted and curves fitted using a model that describes and exponential rise to a maximum:

$$[\, f(x) = C_S * (1-\exp^{-(\ln 2/t(½)*x)})]$$

where $f(x)$ is the Cd content at hour x, C_S describes the maximum body burden in *D. pulex* (saturation) and t(½) is the time to half C_S (McGeer et al. 2000). Modelling was done using the software package SigmaPlot (ver. 11.0).

Testing for the potential toxic effects of dietary Cd was done relative to waterborne toxic effects. In other words, the EC50 concentrations from the waterborne only exposures were used to determine appropriate exposure concentrations to produce contaminated food and in this manner a tiered strategy was applied. In the first tier Hydra were fed Daphnia that had been exposed to Cd

concentrations associated with waterborne effects in Hydra (EC50 concentration). Subsequent tier 2 dietary exposures were planned only in the case that dietary effects were evident in tier 1 (i.e. if Hydra were sensitive to the dietary exposure route relative to the waterborne exposure route). In these side-by-side tests waterborne, dietary and combined water and diet exposures were done where all Hydra were fed. Individual Hydra were exposed in a single well of a 12 well microplate which contained 5 ml of exposure medium. Hydra were fed one 7-8 day old *Daphnia* every 24 h for a period for the 96 h duration of the tests. The regurgitated remains of the *Daphnia* were removed daily with the renewal of the exposure media. Waterborne Cd exposure experiments had unexposed controls, one group that was fed uncontaminated Daphnia (n=6) and another that was not fed (n=6) for the duration of the test. The effects of waterborne exposures, dietborne exposures and co-exposures of Cd were observed at water hardness' of 40 and 140 mg $CaCO_3$/L. Treatment groups tested in water hardness of 40 mg $CaCO_3$/L and 140 mg $CaCO_3$/L had an n of 48, and 24 respectively. In all, the mean of the proportion of the individuals showing the presence of clubbed tentacles was calculated. *Daphnia* were exposed to waterborne Cd for 24 h to create Cd contaminated food for Hydra and samples for subsequent measurement of Cd burden (as described above) prior to feeding. Samples were also collected from unexposed Daphnia (uncontaminated control food).

Water samples (10 ml) were taken during each test to measure for water ion concentrations (Ca, Mg, Na, K), pH and total Cd concentration using atomic absorption spectrophotometry (SpectAA-880, Varian Inc, Palo Alto, CA) either in flame or via graphite furnace (GTA 100) mode depending on the concentration. Each sample for atomic absorption spectrophotometry was acidified to 1% volume using 16N HNO_3 (trace metal grade, Fisher Scientific, Nepean, ON).

5.4 RESULTS AND DISCUSSION

The goal of this study was to determine the relative importance of dietary Cd toxicity to *H. attenuata* in relation to waterborne effects. In waterborne only tests the EC50 for tentacle regression in hard water (140 mg/L as $CaCO_3$) was 0.79 µM Cd while in softer water it was significantly less at 0.30 µM (Table 1). The protective effect of hardness was anticipated based on recent work illustrating the protective effect of Ca on Cd sublethal impacts in Hydra (Clifford, 2009b). Protection via cationic competition of Ca^{2+} on Cd^{2+} at uptake sites for Ca^{2+} is well known and occurs in many aquatic organisms, from Hydra, Daphnia and other invertebrates to fish (Clifford and McGeer, 2009; Niyogi and Wood, 2004; Santore et al., 2002; Verbost et al., 1994).

The results from the waterborne tests establish the relative toxicity of dietary Cd in relation to waterborne Cd, and also demonstrated the protective effect of Ca.

These tests were single exposure concentrations at 0.41 μM Cd at low hardness and 1.42 μM Cd at the higher hardness (Table 2, Figure 2). The level of affected individuals was similar at these concentrations, 50% and 54% at hardness values of 40 and 140 mg/L respectively thus illustrating that more Cd was needed to produce the same effect when more Ca was present. It is noteworthy that the EC50 values (Table 1) were somewhat lower than the EC50s in these latter tests (Table 2). This difference in EC50s could have arisen because one group was fed and the other was not (in EC50 tests there was no feeding). The additional nutrition provided by the food could have facilitated this enhanced tolerance as was illustrated for acid stressed rainbow trout (D'Cruz and Wood, 1998). Additionally the presence of food (8-9 day old *Daphnia*) may have altered the bioavailability of Cd. Other differences between these tests include the fact that individuals were exposed in the dietary-waterborne comparison while in the EC50 tests it was groups of 10.

The accumulation of Cd into Daphnia at low and elevated hardness also illustrated the protective effect of Ca^{2+} on Cd^{2+} uptake. In spite of very different waterborne Cd exposure concentrations in solutions of 40 and 140 mg/L hardness, the accumulation of Cd was similar. Accumulation data was fitted to an exponential model that gave estimates of the saturation concentrations. In lower hardness an exposure of 0.29 μM Cd resulted in a modeled saturation whole body burden of 47.2 ± 4.2 nmol Cd/gram wet weight (C_S ± SEM; Figure 1A). In the higher hardness an exposure of 1.04 μM Cd the corresponding modeled whole body saturation

concentration was 47.3 ± 4.0 nmol Cd/gram wet weight (C_S ± SEM, Figure 1B). Therefore higher hardness water required a much higher exposure concentration of Cd to achieve the same body burden indicating an interaction between Ca and Cd. In fact the molar ratios of Ca:Cd were similar, 896 in soft water and 1211 in harder water. As discussed above in relation to the mitigation of impacts in Hydra, the accumulation of Cd^{2+} in *Daphnia pulex* occurs in competition with the uptake of Ca^{2+} and this has previously been illustrated in bioaccumulation studies with *Daphnia magna* (Tan and Wang, 2008) and acute toxicity test with *Daphnia pulex* (Clifford et al., 2009).

The characterization of the time-course of Cd bioaccumulation was an essential component of providing a consistent and relevant dietary exposure for Hydra. The exposure concentrations (see above) used to establish the time-course of accumulation at hardnesses of 40 and 140 (mg/L $CaCO_3$) were set at the approximate EC50 for tentacle regression in Hydra. Based on the recent study of Clifford (2009a) demonstrating the influence of water chemistry (including Ca) on the toxicity of Cd, it was known that the exposures were below the EC50 for *D. pulex* and they would survive at these concentrations. Modelling of the time-course of accumulation showing that the half-time for whole body saturation was 0.31 ± 0.09 h ($t_{½}$ ± SEM; Figure 1A) in low hardness and 0.40 ± 0.12 h (Figure 1B) in higher hardness, attesting to the very rapid initial uptake that occurred. Based on the

overall pattern of uptake we decided to use 24 h as the exposure period to prepare prey food for dietary exposures to Hydra.

Exposures to test for the relative effects of waterborne versus dietary exposures of Cd demonstrated that Hydra are unaffected by dietary Cd. Whether in hard water or in soft water, Hydra fed *Daphnia pulex* contaminated with Cd (at the Hydra EC50 concentration) showed no effects (Table 2, Figure 2). As well, Hydra exposed to waterborne Cd plus dietborne Cd showed there was no additivity as no additional tentacle clubbing occurred beyond that induced by waterborne Cd alone. This unequivocally shows that the effects of waterborne Cd are more important than those arising from dietary exposures where Hydra consume *D. pulex*.

The reasons for dietborne Cd having no effects on Hydra are either related to the bioavailability and uptake of Cd from the dietary source and/or differences in the impact that arises from accumulated Cd. During waterborne exposure the majority of cells in the organisms including those on the tentacles and the body (external epidermal as well as gasterodermal) are exposed to Cd. During dietary exposure only the gastrodermal cells will receive an exposure to Cd. Little is known about Cd uptake in Hydra but it may be that the uptake and impacts of Cd differs across cell types and gastrodermal cells are tolerant or have relatively low uptake. It is also possible that the accumulated Cd present in the *Daphnia* was sequestered by metallothioneins (MT) or other metal binding like-proteins. Work by Fraysse et al., (2006) has shown that *D. magna* can contain as much as 57% of the total

accumulated Cd bound to MTs. Daphnia have been shown to increase synthesis of these proteins in as little as 2-24 h (Amiard et al., 2006) and therefore it is also possible that the Cd accumulated by the Daphnia was bound to the MT and not available for uptake across gastrodermal cells in Hydra. It is also important to highlight that Hydra do not consume all of the Daphnia as undigested particles (e.g. the carapace) are eliminated from the gut space.

In testing the importance of dietary Cd to Hydra our experimental design was to assess effects in relation to waterborne effects in a tiered manner considering that within natural systems both waterborne and dietary exposure occur simultaneously. Dietary exposures are relevant and it is necessary to fully understand dietary exposure-effect relationships only when effects occur at and/or below concentrations that cause waterborne effects. Hydra have to be able to sustain themselves in a waterborne exposure in order to consume contaminated prey items. Therefore, as the first tier of our dietary exposure study we used *D. pulex* that had been exposed to the EC50 for tentacle regression in Hydra. When consumption of these Cd loaded daphnids did not produce effects in Hydra we concluded that, at least in the context of our exposure system, there was little potential for dietary effects of Cd in Hydra (i.e. waterborne Cd will affect populations at lower exposure concentrations than dietary based exposures).

The lack of dietary based effects at waterborne concentrations that are toxicologically relevant is consistent with findings by Goulet et al., (2007) who did not

see a contribution to chronic toxicity in *D. magna* when fed contaminated algae. In that study the direct effects of waterborne Cd occurred at relatively low concentrations and feeding of algae (both *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* were tested) that had been contaminated at very high waterborne concentrations resulted in no significant impacts on reproduction in spite of significant accumulations of Cd (Goulet et al., 2007). Mount et al., (1994) observed no effects on survival and growth in fish consuming Cd contaminated *Artemia*. Contrary to our findings Karntanut and Pascoe (2007) saw a decrease in regeneration and bud production in Hydra after feeding on Cd loaded *Artemia* (nauplii stage). However, the preparation of the dietary exposure in that study was done by exposing *Artemia* to 100 µM Cd resulting in a body burden of 2.7 µM Cd / gram dry weight, equivalent to approx 0.27 µM Cd / gram wet weight (assuming 9:1 for wet weight to dry weight ratio). The waterborne exposure used to load *Artemia* was 333 and 126 fold higher than the EC50 for Hydra exposed to waterborne Cd at hardness values of 40 and 140 (mg/L as $CaCO_3$), respectively. In our study these exposures would have been lethal to the Hydra.

In conclusion, this study illustrates the relative sensitivity of *Hydra attenuata* to waterborne and dietary Cd exposure in soft and hard water. In 96 h EC50 tests a protective effect of Ca on the toxicity of waterborne Cd was clearly evident. This Ca mitigation was also observed in Cd bioaccumulation tests with *Daphnia pulex*, which served as prey organisms in dietary exposure tests. Feeding of Hydra with Daphnia

that had been exposed to Cd concentrations similar to the waterborne EC50 concentrations did not alter toxicity. In both soft and hard water the co-exposure of both waterborne and dietary Cd resulted in no additional impacts beyond those associated with waterborne Cd only. Therefore it would appear that dietary Cd exposure could be of lesser importance compared to waterborne exposures for Hydra.

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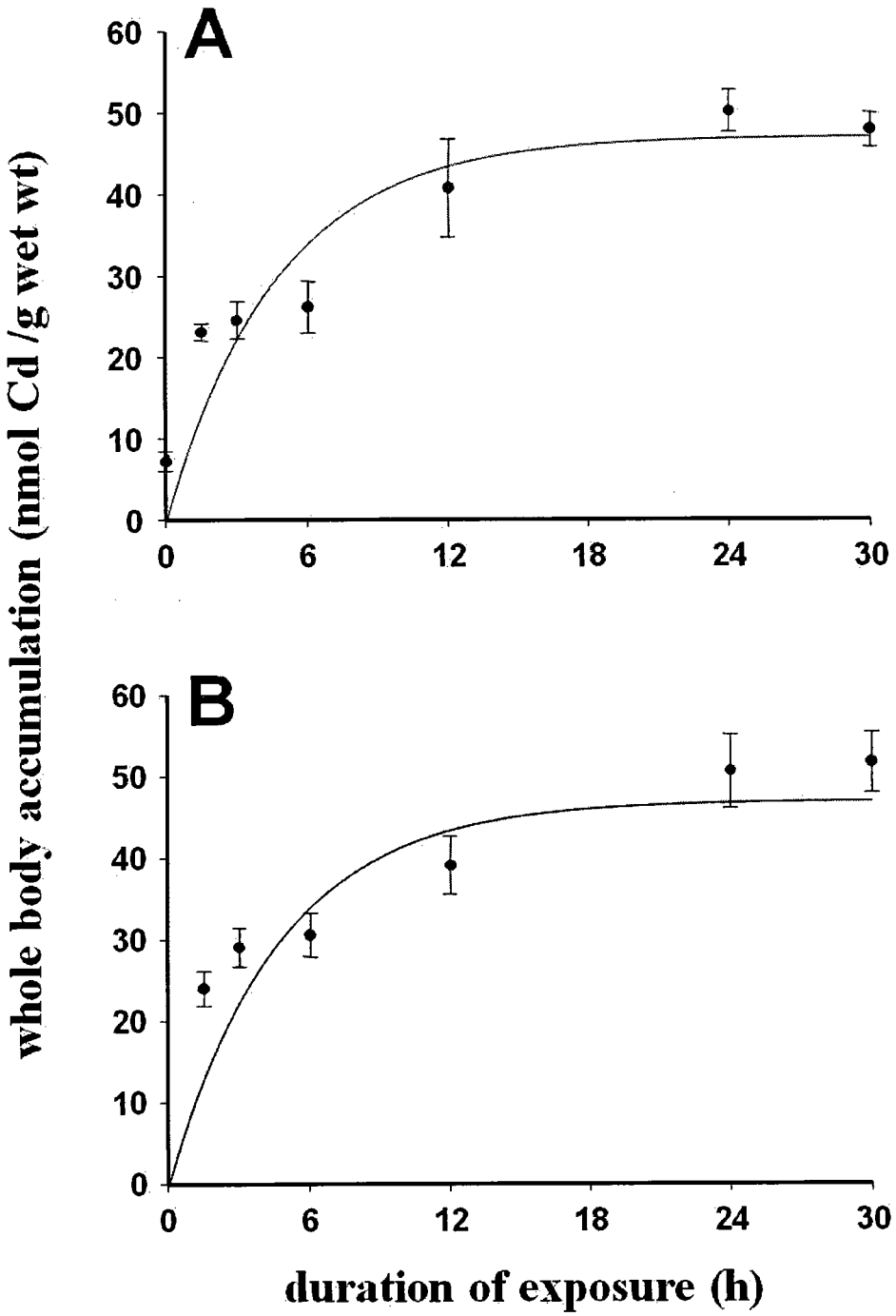
Chapter 5: Table 1. Measured concentrations for of exposure water parameters as well as the associated measured 96 h EC50 (with 95% confidence interval) for Cd in *Hydra attenuata*. The units are mM except for DOC (mg C/L), pH and EC50s (μM).

	Hardness (as mg $CaCO_3$/L)	
	40	**140**
Ca	0.26	1.26
Mg	0.14	0.14
Na	0.15	0.15
K	0.04	0.04
Cl	0.04	0.04
SO_4	0.40	1.40
pH	7.0	7.0
DOC	1.53	1.53
EC50 *H. attenuata*	0.30 (0.28-0.33)	0.79 (0.67-0.87)

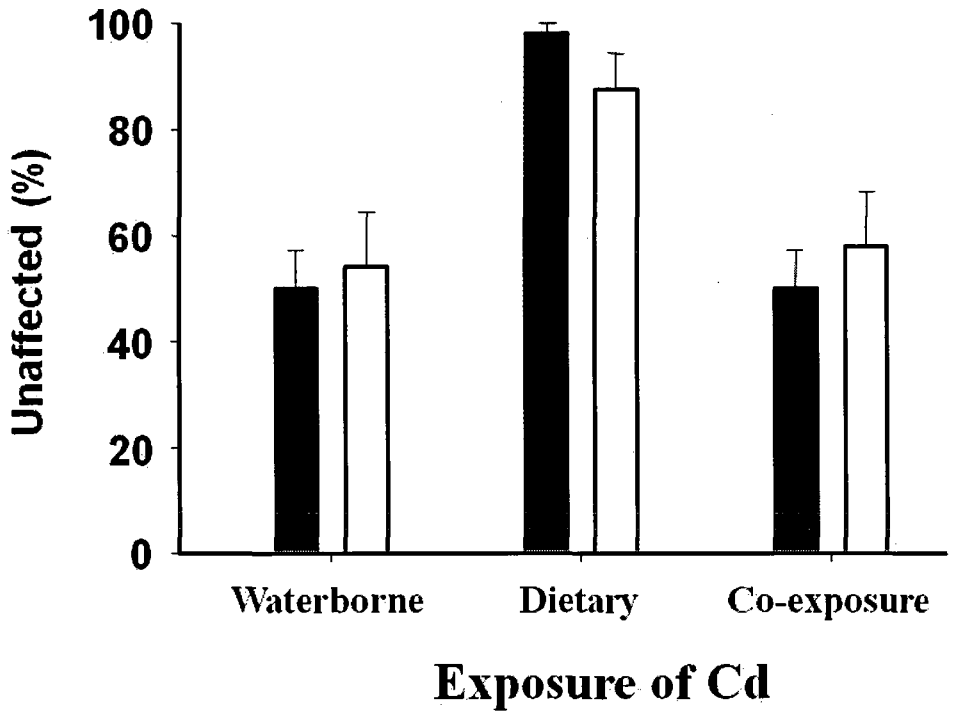
Chapter 5: Table 2. Mean (± SEM) for exposure parameters for waterborne, dietary and combined waterborne plus dietary exposures in soft and hard water. The associated effect of the treatment, as the % of Hydra showing clubbed tentacles after 96 h, is also given.

Exposure	Waterborne Cd (μM)	Dietary Cd (nmol/g w wt)	% affected	no. exposed
Soft water (40 mg $CaCO_3$)				
Control (fed)	0.003 ± 0.001	0.01 ± 0.01	0	6
Waterborne only	0.41 ± 0.004	0.01 ± 0.01	50	48
Dietary only	0.003 ± 0.001	88 ± 4.7	2	48
Waterborne + diet	0.41 ± 0.004	88 ± 4.7	50	48
Hard water (140 mg $CaCO_3$)				
Control (fed)	n.d.	0.01 ± 0.01	0	6
Waterborne only	1.42 ± 0.004	0.01 ± 0.01	46	24
Dietary only	n.d.	185 ± 7.3	12.5	24
Waterborne + diet	1.42 ± 0.004	185 ± 7.3	42	24

Chapter 5: Figure 1. Whole body bioaccumulation of Cd in 7 to 8 day old *Daphnia pulex* (mean ± SEM) over 24 h of exposure to 0.31 μM Cd at a hardness of 40 mg $CaCO_3$/L (Panel A) or 1.02 μM Cd at a hardness of 140 mg $CaCO_3$/L (Panel B). Best fit lines using an exponential model are given with r^2=0.90 of Panel A and 0.87 for Panel B, see text for other model parameter details. Each mean represents n=15 Daphnia at low hardness (A) and n=13 for high hardness (B).


A
B
60
50
40
30
20
10
0
0
6
12
18
24
30
whole body accumulation (nmol Cd /g wet wt)
duration of exposure (h)

Chapter 5: Figure 2. Percentage of Hydra displaying no clubbed tentacles (i.e. unaffected Hydra) as a result of the waterborne, dietborne, or combined water plus dietborne Cd to *H. attenuata* following a 96 h exposure. Bars show mean ± SEM percent of unaffected Hydra at exposures of either 0.41 μM Cd in solutions at a hardness of 40 mg $CaCO_3$/L (dark bars, n=48) or 1.42 μM Cd in solutions at a hardness of 140 mg $CaCO_3$/L (light bars, n=24). For dietary exposure *Daphnia pulex* were exposed to these same waterborne exposure concentrations for 24 h prior to feeding to Hydra.


100
80
60
40
20
0
Unaffected (%)
Waterborne
Dietary
Co-exposure
Exposure of Cd