CHRONIC BIOACCUMULATION AND TOXICITY OF CADMIUM FROM A PERIPHYTON DIET TO *Hyalella azteca*

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada 2010

Author's Declaration

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

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ABSTRACT

Dietary cadmium (Cd) can contribute significantly to chronic bioaccumulation and toxicity in aquatic organisms. This contribution needs to be quantified so that the relative importance of waterborne and dietary cadmium exposure pathways can be incorporated into protective water quality guidelines and ecological risk assessments.

In this research, the contribution of dietary Cd from a natural periphyton diet to chronic (28 d) bioaccumulation and toxicity in the freshwater amphipod *Hyalella azteca* was quantified using a mechanistically-based saturation bioaccumulation model. Factors that influence dietary Cd bioavailability such as food type, food form, dietary Cd speciation and concentration were investigated. Assimilation efficiency, ingestion rate and the excretion rate constant of dietary Cd were determined for each of these factors. Food nutrition was also considered. Lastly, model predictions of Cd bioaccumulation and toxicity were compared to measurements of tissue concentration and survival when *H. azteca* were exposed to metal contaminated water and periphyton collected from lakes in the metal mining region of Rouyn-Noranda, Quebec, Canada.

In 28 d laboratory experiments where *H. azteca* bioaccumulated Cd from water and food, dietary Cd was estimated to contribute markedly (21 – 94 %) to bioaccumulated Cd in *H. azteca*. Effects on chronic survival were best predicted from body concentration rather than water or food exposure concentration. Assimilation efficiency of dietary Cd differed with food type likely as a result of Cd speciation, but did not differ with Cd concentration or food form. Ingestion rate differed with food form while excretion rate constants were unaffected by dietary Cd bioavailability. Predictions of chronic Cd bioaccumulation in *H. azteca* exposed to field contaminated samples were robust, however the model did not account for effects of water chemistry on Cd bioaccumulation and is thus constrained in its application. Predictions of chronic survival were over-estimated likely due to the additional toxicity caused by the low nutritional quality of the field contaminated periphyton. This research demonstrated that both waterborne and dietary Cd need to be considered in models that assess chronic risk of exposure and effects to *H. azteca*.

ACKNOWLEDGEMENTS

Grateful acknowledgement is due to the financial support of the Natural Sciences and Engineering Research Council (NSERC) through the Metals in the Human Environment (MITHE) Strategic Network. This support enabled me to perform my research, interact and gain inspiration from students and scientists across Canada and participate in international meetings. Additional acknowledgement of funding goes to the Ontario Graduate Scholarship (OGS) programme and the University of Waterloo President's Scholarship, International Doctoral Student Award, Provost Doctoral Entrance Award for Women and Graduate Entrance Award.

My sincere gratitude goes to my co-supervisors of Dr Uwe Borgmann and Dr D George Dixon who took a chance and gave me the valuable opportunity to do my PhD in Canada. Thank you both for your support, encouragement, patience and guidance and providing me with seemingly limitless freedom to explore this topic. Further thanks goes to committee members: Dr Lee Grapentine and Dr William Taylor and examiners: Dr Landis Hare and Dr Mike Stone for their advice on improving the final thesis.

Many thanks to Environment Canada, Burlington for provision of services, facilities and resources and the much appreciated advice and support from students and staff: Dr Warren Norwood, Dr Adrienne Bartlett and Dr Lara Alves; Dr Scott Brown (deceased), Maria Villella and Lisa Brown for use of radio-isotope facilities; Dr Michael Arts, Martina Rudy and Jerry Chien-Yao Chao for fatty acid analysis assistance; Benoit Lalonde for field assistance and French language translation; Dr Sue Watson and Dr Chantelle Vis for algal taxonomy advice.

Enduring appreciation goes to friends and family from North America and New Zealand who cheered me on and particularly my parents, who constantly battled the tyranny of distance to send their love, support and care packages.

My eternal gratitude is given to my wonderful husband and life-long partner in global adventure - Judd Brown. Thank you for your love and understanding.

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CHAPTER 1 GENERAL INTRODUCTION

1.1 *Relevance of dietary metals*

Aquatic organisms are exposed to metals via both dissolved and dietary pathways, yet the relative contribution of metal from these pathways to bioaccumulation and toxicity in the majority of aquatic organisms is largely unknown. Historically, research has been focused on the effects of waterborne metals, driven by the priority to regulate against acutely toxic metal exposure. The role of dietary metal in assessing and regulating chronic metal toxicity in the aquatic environment is being re-evaluated and recognition is being given to the fact that the fate and effects of metals is not solely reliant on water column chemistry but that diet is also an integral component (Meyer *et al.*, 2005). The biomagnifying effects of elements such as mercury on higher trophic consumers have been well studied (Adams *et al.*, 2005) and there is some evidence to suggest that Cd can biomagnify as well (Croteau *et al.*, 2005, Reinfelder *et al.*, 1998). However, dietary metals can also be mobilized up the food chain without magnification and it is this trophic transfer that is most relevant for the majority of dietary essential and non-essential metals (Wang, 2002). While it is clear that dietary metals can cause toxicity to aquatic biota in the laboratory and the field (Handy *et al.*, 2005; Schlekat *et al.*, 2005), the conditions under which dietary metal becomes important and the contributions of dietary metal to toxicity are less evident. There is a need to clarify the issues of dietary metal bioavailability, bioaccumulation and toxicity so that accurate protective guidelines, more complete than those currently based on "water-only" exposures, can be derived and so that models can be developed as tools for predicting effects in ecological risk assessment of metals from both dissolved and dietary pathways (Hare, *et al.*, 2003; Meyer *et al.*, 2005; Schlekat *et al.*, 2002).

1.2 *Sources of cadmium and protection of aquatic biota*

Cadmium is a borderline or Class B metal (Nieboer and Richardson, 1980) produced commercially as a by-product of Zn refining and used in electroplating, batteries, plastics, pigments and electronics (CEPA, 1994). The dominant soluble species of Cd in circum-neutral freshwaters is the free ion form though this is pH dependent, with carbonate and

hydroxide complexes becoming more important with increasing pH (Wright and Welbourn, 1994). Dissolved Cd also forms organic complexes with dissolved organic carbon and can be rapidly lost and re-mobilized within the water column by partitioning with sediment (Stephenson *et al*., 1996). Anthropogenic sources of Cd released into the aquatic environment include mining, industrial, municipal wastewater and urban stormwater discharges as well as deposition of atmospheric emissions predominantly from non-ferrous metal smelting facilities (Nriagu and Pacyna, 1988; Pacyna *et al.*, 1995). Evidence of deposition of atmospherically emitted Cd from a metal smelting facility is apparent from elevated Cd in water and sediment of lakes downwind of a copper smelter in the mining region of Rouyn-Noranda, Quebec, Canada (Borgmann *et al.*, 2004b; Telmer *et al.*, 2006). While aerial emissions from this smelter have decreased due to improved emissions scrubbing technology, the legacy of environmental Cd contamination to the lakes in the region remains (Croteau *et al.*, 2002).

Cadmium is a non-essential element but is an analogue of essential calcium. It disrupts cell function in multiple ways and has a long biological half life thereby making it highly toxic to biota (Mason and Jenkins, 1995; Simkiss and Taylor, 1995; Wright and Welbourn, 1994). The ongoing mobilization of Cd into the environment as a result of human activity and its high toxicity have earned Cd recognition as a priority substance according to a toxic risk assessment on the environment and human health conducted under the Canadian Environmental Protection Act (CEPA, 1994). Within Canada, all life-stages of aquatic biota are given long-term protection from aqueous Cd by a hardness-adjusted, no-effect, interim water quality guideline (CCME, 1999). This guideline was derived by dividing the lowest effect concentration for the most chronically sensitive species (impaired 21 d reproduction in *Daphnia magna*) by a safety factor of 10. Although dietary Cd was not explicitly considered when deriving this guideline, the chronic endpoints upon which the guideline is based will include some dietary effects as a result of feeding aquatic organisms throughout the chronic assay. The x 10 safety factor is also designed to account for unknown contributing toxic effects. However, the level of protection required to fully account for dietary Cd is not known for most aquatic organisms and this information would add to the current database from which water

quality guidelines are derived and help determine whether guidelines currently over- or under-protect aquatic life.

1.3 *Importance of H. azteca and periphyton*

Hyalella azteca is a freshwater amphipod that is part of a species complex widely distributed throughout North America. Typically, *H. azteca* inhabits the interface between surficial sediment and overlying water, burrowing into macrophyte root masses in the littoral margins of lakes (Cooper, 1965). *H. azteca* have been described as "omnivorous, general scavengers or detritus feeders" (Pennak, 1989) with high assimilation of carbon from algae and bacteria (Hargrave, 1970) – both major constituents of periphyton. In turn, *H. azteca* is a vital food source for fish and waterfowl (Anteau and Afton, 2008; Strong, 1972). *H. azteca* is also a commonly used test species in standard sediment and aqueous toxicity tests because of its ease of laboratory culture and sensitivity to contaminants (Borgmann *et al.*, 1989; Borgmann *et al.*, 2005c; Environment Canada, 1997). Of 63 metals that juvenile *H. azteca* were exposed to in a 7 d aqueous toxicity test, Cd was found to be the most lethally toxic metal (Borgmann *et al.*, 2005a). In a review of the risks to aquatic life from Cd levels approaching a low effect chronic Cd criterion (3.38 nmol/L at 50 mg/L hardness as $CaCO_3$), *H. azteca* population levels were predicted to decline and this could have large effects on predatory fish populations in situations where *H. azteca* were the dominant prey items (Mebane, 2006).

Periphyton is a complex microbial community of algae, fungi, bacteria, protozoa, Fe and Mn oxyhydroxides and sediment all bound together in an exopolymer matrix on a variety of subsurface substrates (Azim and Asaeda, 2005; Newman and McIntosh, 1989). Other terms such as phototrophic biofilms, aufwuchs, "attached algae" have been applied to the same community (Azim *et al.*, 2005). Periphyton constitutes an important food source for *H. azteca* as well as for other invertebrates and fish (Liess and Hillebrand, 2004). It also has current and potential biotechnology applications in wastewater treatment, bioremediation, agricultural fertilizers, clean-energy systems and as fish food in aquaculture (Roeselers *et al.* 2008). Periphyton has multiple binding sites and a large binding surface area which are ideal properties for extracting and accumulating metals

from the surrounding water for remediation purposes or using periphyton as a biomonitor of metal impacts (Hill *et al.*, 2000; Morin *et al.*, 2008a). However, as a primary food source, the ability of periphyton to accumulate high metal concentrations makes it a potentially toxic vector of Cd transfer to organisms of higher trophic status either directly or via multiple consumers of increasing trophic status (Xie *et al.*, 2010).

1.4 *Factors influencing cadmium bioavailability and bioaccumulation*

Bioavailable metal is that portion of total metal that is able to bind to physiologically active sites and/or pass through a biological membrane to interact with cells and their contents (Meyer *et al.*, 2005). Bioavailable metal is bioaccumulated by the organism when the influx from waterborne and dietary pathways exceeds the efflux (Meyer *et al.*, 2005).

In the Free Ion Activity Model (FIAM), it is proposed that the activity of the free ion (i.e., aquo ion) best predicts the bioavailability of the dissolved trace metal to the organism, with some exceptions (Campbell, 1995)[1]. The fish gill is used as a model to demonstrate that dissolved metal bioavailability is reduced by the formation of less bioavailable organic and inorganic complexes in the water column and by competition with other cations to binding sites on the gill membrane (Pagenkopf, 1983; Playle, 1998). Metal transport across the membrane occurs via a number of possible routes but is dominated by passive facilitated diffusion on protein carriers and via major ion channels (Simkiss and Taylor, 1995). The accumulation of the metal within the tissues of the organism then depends on the balance between metal uptake and loss processes and the abiotic and biotic factors that affect those processes such as temperature, pH, hardness, growth, number of binding sites and acclimation to the metal from previous exposure (Langston and Spence, 1995; Luoma and Rainbow, 2008).

Although *H. azteca* have gills, whole body tissue concentration is used as a surrogate for the fish gill model to explain Cd bioavailability and bioaccumulation (Borgmann, 2000).

[1] For the purposes of the current research, the use of the modelled free ion form refers to concentration rather than activity. Campbell (1995) states that "any imprecision introduced by neglecting activity coefficient variations within a given experiment is negligible in comparison with the inherent biological variability."

Calcium is the major competing ion for acute and chronic Cd bioaccumulation in *H. azteca* from the dissolved phase and Cd bioaccumulation is reduced with increasing dissolved organic carbon (10 - 20 mg/L DOC) as a result of the reduced free ion concentration (Borgmann *et al.* 2010; Schroeder, 2008; Stephenson and Mackie, 1989). Variation in H^+, Mg^{2+}, K^+, Na^+ and HCO_3^- has either a minor (in the case of H^+) or no effect on Cd bioaccumulation by *H. azteca* (Borgmann *et al.*, 2010; Schroeder 2008). Acute (1 week) Cd bioaccumulation by *H. azteca* is reduced as the number of metals (As, Co, Cr, Cu, Ni, Mn, Pb, Tl, Zn) added to the mixture is increased (Norwood *et al.*, 2007b). Cadmium is not regulated by *H. azteca* and bioaccumulation occurs readily in aqueous exposures (Schroeder, 2008).

The factors influencing the bioavailability and bioaccumulation of dietary metals have been identified for aquatic organisms but, as yet, have not been empirically defined as well as for waterborne metals. Bioavailability of dietary metals is a function of the food matrix, the intracellular and/or extracellular digestive solubilization and release of metal from the matrix, the form of that metal once released and whether the metal species is able to be transported across the gut epithelium (Campbell *et al.*, 2005; Lopez, 2005; Schlekat *et al.*, 2002). Bioavailability is most effectively represented by the assimilation efficiency of the metal rather than the free ion form in the external medium (Wang and Fisher, 1999b). Metals become concentrated in the gut fluid due to the release of metal from food, the additionally imbibed metal that entered with water via ingestion and the recirculation of fluids within the digestive system. Solubilized metals form complexes with the mass of organic ligands present in the digestive fluids and, unlike the fish gill membrane, metal-amino acid complexes can be co-transported across the gut epithelial membrane (Campbell *et al.*, 2005). In addition, the typical metal uptake mechanisms exist as for dissolved metals crossing the fish gill but it's likely that certain mechanisms, such as endocytosis, are used more within the gut (Luoma and Rainbow, 2008). Calcium channels in the stomach of rainbow trout are a dominant uptake mechanism of dietary Cd (Wood *et al.*, 2006) and a similar mechanism appears to exist in the gut of invertebrates (Craig *et al.*, 1999). As with dissolved metal exposure, bioaccumulation of dietary metal is a function of the biotic and abiotic processes affecting the metal bioavailability, uptake

and elimination such as digestive pH, ingestion rate, selective feeding behaviour, gut transit time and growth (Croteau *et al.*, 2007; Luoma and Rainbow, 2008; Wang and Fisher, 1996).

While the mechanisms of dietary Cd uptake in *H. azteca* are unknown, other features and processes affecting dietary Cd bioaccumulation have been described. *H. azteca* have a straight tube gut divided into the foregut, midgut and hindgut (Schmitz and Scherrey, 1983). The foregut and hindgut are lined with cuticle making the midgut, and most importantly the hepatopancreatic caecae that lie at the junction of the foregut and midgut, the main sites of nutrient and non-essential metal absorption. Ingested food in the foregut is triturated by the gastric mill and pressed and filtered in the pyloric stomach before entering the central receiving duct of the gland chamber. From here, finer food particles are shunted to the hepatopancreatic caecae for intensive digestion and coarse material sent through to the midgut where some digestion and absorption occurs and then the hindgut where waste is packaged for excretion (Schmitz and Scherrey, 1983). The pH of gut fluids in *H. azteca* varies along the digestive tract with pH 4.1 – 7.2 in the foregut, pH 3.8 – 4.7 in the hepatopancreatic caecae, pH 6.8 – 7.2 in the midgut and pH 7.2 – 7.7 in the hindgut (De Giusti *et al.*, 1962) suggesting that metal solubilization is most likely in the acidic environment of the hepatopancreatic caecae. Neumann *et al.* (1999) demonstrated that the gut clearance rate constant of Cd associated with sediment was approximately 0.8/h and Cd tissue excretion rate constant was 0.007/h. Stephenson and Turner (1993) measured ingestion rate of periphyton by *H. azteca* in the field to be 0.041 g/g/h with 80 % assimilation efficiency of Cd from periphyton and an uptake rate and an excretion rate constant of 2.0 nmol/g/h and 0.015/h respectively.

1.5 *Linking cadmium bioaccumulation to toxicity*

Regardless of the pathway of metal uptake, bioaccumulated metal is initially metabolically available and will participate in cellular activity or be transported to other locations where it is bioactive. Metabolically available metal that accumulates beyond a threshold concentration results in toxicity despite the organism's attempts to store or detoxify essential and non-essential metals by producing metabolically inactive metal

forms such as granules and metallothioneins (Langston and Spence, 1995; Rainbow, 2002). Toxicity has been demonstrated to be directly related to *H. azteca* body concentration for predominantly aqueous exposure to a number of metals including Cd (Borgmann, 2000). Whether this relationship holds for exposure to dietary Cd is unclear (Ball *et al.*, 2006). The mechanisms of aqueous and dietary Cd toxicity in *H. azteca* are unknown but internal partitioning of metal and excretion rate constants have been shown to differ with exposure pathway in invertebrates (Roy and Hare, 1999). Aqueous metal exposure can be acutely toxic whereas dietary metal toxicity may be avoided in the short term by food selection behaviour, and the prolonged process of digestion and uptake means that the cumulative effects of dietary metals are more likely to be expressed over chronic exposures (Schlekat *et al.*, 2002). The role of Cd in reducing the nutritional quality of the food, thereby reducing the consumer's health and ability to withstand adverse effects, also needs consideration when determining dietary Cd toxicity (Campbell *et al.*, 2005). It may also be the case that certain endpoints of toxicity are more appropriate for one exposure pathway than another or that entirely new endpoints related to digestive function need to be devised for dietary metal exposure (Handy *et al.*, 2005).

1.6 *Modelling cadmium bioaccumulation and toxicity*

Once the relationships between metal exposure, bioaccumulation and toxic effects have been clearly established for an organism, they can be coupled together mathematically using models (Landrum *et al.,* 1992). The approach consists of developing a bioaccumulation model that accounts for factors affecting metal bioavailability from both food and water pathways, then developing a second model that relates bioaccumulation to effects (Borgmann *et al.*, 2005b). In a similar way, the BLM links dissolved metal concentration to the amount of metal bound to the site of toxic action in an organism which in turn is linked to a toxic response (Paquin *et al.*, 2002). Borgmann *et al.* (2010) found that the BLM predicted chronic bioaccumulation and toxicity of dissolved Cd well for *H. azteca* but that the usually-assumed underlying mechanism of direct competition between Ca and Cd for binding was not supported by the data; bioaccumulation was best explained by anti-competitive inhibition. Currently, no model exists that links dietary Cd to chronic toxicity in *H. azteca*. The biokinetic model offers an approach to modelling

bioaccumulation which combines aqueous and dietary pathways of exposure assuming additivity of the two sources (Luoma and Rainbow, 2005). It is based on first-order kinetic rates of metal uptake and elimination as well as the physiological processes affecting dietary metal bioaccumulation, such as assimilation efficiency, ingestion rate and growth (Reinfelder *et al.*, 1998; Wang and Fisher, 1999b). However, to link bioaccumulation predicted from this model directly to toxicity, the critical body concentrations from water and dietary sources must be the same. If that proves not to be the case, a third model linking toxicity from water and dietary sources separately using a toxic unit approach may be possible (Borgmann *et al.*, 2005b).

Models have been used to demonstrate that dietary Cd is of relatively greater importance than waterborne exposure to some aquatic biota (Croteau and Luoma, 2008; Goulet *et al.*, 2007; Munger *et al.,* 1999; Orvoine *et al.,* 2006) and of lesser importance to others (Schlekat *et al.*, 2000, Wang and Rainbow, 2008). Factors that influence the relative importance of waterborne and dietary Cd pathways can be further explored using models. Some of those factors have already been discussed and include speciation of Cd in water and food, metal interactions at the site of metal binding to the membrane, ratio of Cd concentration in food relative to water, ratio of uptake and excretion rates of Cd from food relative to water, physico-chemical fluctuations in the environment, exposure duration, physiological acclimation, nutritional requirements, behavioural responses and organism growth.

1.7 *Relevant studies*

The four existing studies that investigated the relative importance of Cd in water versus food to *H. azteca*, produced conflicting results that are summarized below (Ball *et al.*, 2006; Borgmann *et al.*, 2007; Stephenson and Turner, 1993; Stewart, 2002).

Stephenson and Turner (1993) performed field transfer experiments with caged adult *H. azteca* and periphyton as part of a larger study monitoring the fate and effects of Cd at a no-effect concentration (0.8 nmol/L) at the whole-lake ecosystem level (Malley *et al.*, 1996). A previously pristine lake was spiked with stable Cd and radioactive ^{109}Cd over 5

years. Periphyton was grown on tiles placed in the Cd contaminated lake (L382) and a non-contaminated reference lake (L239) for 6 weeks. Bioaccumulation of Cd in *H. azteca* exposed to contaminated water and periphyton simultaneously was studied by caging *H. azteca* collected from L239 in L382 with periphyton from L382. Bioaccumulation of Cd in *H. azteca* exposed to contaminated periphyton only was studied by caging *H. azteca* collected from L239 in L239 with periphyton from L382. Finally, depuration of Cd from *H. azteca* was studied by caging *H. azteca* collected from L382 in L239 with periphyton from L239. Each study lasted 11 d with daily monitoring of tissue concentration by sacrificing all *H. azteca* from a replicate cage, drying and measuring ^{109}Cd. Because *H. azteca* were not depurated or washed with a metal chelating agent such as ethylenediamine tetra-acetic acid (EDTA) the final Cd measurement represented total Cd in *H. azteca* including Cd associated with periphyton in the gut and externally adsorbed Cd. This is the Cd dose that a consumer of *H. azteca* would receive but may slightly over-estimate the Cd in *H. azteca* that could relate tissue concentration to toxic effects. Based on the results of these studies, Stephenson and Turner (1993) developed a bioaccumulation model and predicted that 58 % of the Cd in *H. azteca* came from ingested periphyton and the Cd was assimilated with 80 % efficiency. This study demonstrated that, at a no-effect dissolved Cd concentration over 11 d, Cd from a natural periphyton diet was bioavailable to adult *H. azteca* and contributed markedly to body concentration although this may be slightly over-estimated due to how the tissue concentration was measured. No toxic effects on *H. azteca* were observed.

A field study by Borgmann *et al.* (2007) measured the concentrations of 27 metals in laboratory cultured adult (4 – 10 week old) *H. azteca* that were caged in variously metal contaminated sites in two rivers for 17 d. Food consisted of homogenized macrophyte and detrital material collected from each site and thus also exhibited a gradient of metal contamination (7.6 – 92 nmol/g Cd dry weight). One treatment was to feed *H. azteca* with the food collected from the same site at which they were caged. A second treatment was to transplant food collected from more contaminated sites to feed *H. azteca* caged in the least contaminated site. A third treatment was to transplant food collected from the least contaminated site to feed *H. azteca* caged in the more contaminated sites. *H. azteca* were

then depurated (24 h) in 50 µmol/L EDTA and analyzed for 27 metals. Using analysis of variance and covariance, Cd, Cu and Se in food were shown to have the greatest effect on bioaccumulation of those metals in caged *H. azteca*. A maximum of 25 % of Cd in *H. azteca* was predicted to have come from food when caged at the same site where the food was collected. There was no toxicity associated with the dietary metals. This study demonstrated that in a polymetallic environment, dietary Cd was bioavailable though contributed less than aqueous exposure to the Cd accumulated by adult *H. azteca* over 17 d, and no relationship between dietary metal and toxicity was observed.

A laboratory study by Stewart (2002) examined the relative importance of waterborne and dietary Pb and Cd to laboratory cultured adult *H. azteca* using a cultured diatom (*Navicula pelliculosa)*. The diatoms were grown for 7 d on teflon tiles in an algal growth medium containing 10 µmol/L EDTA, Pb (1.5 nmol/L) and Cd (0.32 nmol/L). Adult (6 – 19 weeks old) *H. azteca* were exposed for 6 d to Cd in water, without food, at the same EDTA, Pb and Cd concentrations that the algae were grown at. *H. azteca* were also exposed for 6 d to the same concentrations of EDTA, Pb and Cd in both water and food (as the contaminated diatom). *H. azteca* were then depurated (24 h) in 10 µmol/L EDTA and analyzed for Cd and Pb. Cadmium bioaccumulation in *H. azteca* was greater in the combined water and food exposure than in water alone, but not by a statistically significant margin. There were no effects on *H. azteca* survival attributable to metal in the diatom. This study demonstrated that over 6 d, there was no significant contribution of Cd from a fresh diatom diet to bioaccumulation in adult *H. azteca* and no lethal toxicity.

Ball *et al.* (2006) conducted laboratory exposures of cultured juvenile (0 – 1 week old) *H. azteca* to Cd in an algal diet of *Chlorella* sp. The algae were grown in a Cd spiked algal growth medium in the absence of EDTA, then washed in 50 µmol/L EDTA and dried. *H. azteca* were fed the Cd contaminated dried algal diet for 4 and 10 weeks in a non-contaminated medium spiked with 50 µmol/L EDTA. *H. azteca* were then depurated (24 h) in 50 µmol/L EDTA before Cd analysis. Bioaccumulation of Cd by *H. azteca* was either close to the detection limit or, at the two highest dietary Cd concentrations (104

and 5610 nmol/g dry weight), was 233 and 6 times, respectively, below the LBC25 of 298 nmol/L for water only exposure. Despite the lack of Cd bioaccumulation, effects on survival and growth related to Cd in algae were observed and the authors speculated that speciation of Cd in the diet reduced its bioavailability but still resulted in toxicity by indirect means (Ball *et al.*, 2006). This study showed that dietary Cd in the form of a dried algae diet could result in chronic toxicity without a strong relationship to bioaccumulation in *H. azteca*.

Together these studies demonstrate the variety of bioaccumulation and toxic responses of *H. azteca* to dietary Cd that have been recorded thus far. These experiments differed in the food types and forms (fresh versus dry), exposure conditions and duration, age and origins of *H. azteca* used, and determining tissue Cd concentration. It is clear that a standardized approach to determining dietary Cd effects on *H. azteca* is required.

1.8 *Knowledge gaps*

The present research aimed to address the following knowledge gaps that have been identified from the literature:

1. Bioavailability of Cd associated with natural versus artificial food. This has implications for how well the results from standard laboratory based dietary and chronic waterborne experiments can be extrapolated to the field.
2. Direct versus indirect toxicity of dietary Cd. This relates to determining appropriate endpoints of dietary Cd toxicity and the role that Cd has in food and *H. azteca* nutrition.
3. Linking dietary metals to bioaccumulation and toxicity. There is a need to resolve the ambiguity of whether dietary Cd is bioaccumulated by *H. azteca* and as yet, there is no evidence that links dietary Cd to chronic toxicity in *H. azteca* via bioaccumulation.
4. Relative importance of waterborne versus dietary Cd to *H. azteca*. This is particularly relevant to the derivation of water quality guidelines and conducting site-specific ecological risk assessments of Cd effects.

1.9 *Objectives*

The objectives of this research were designed to clarify the conflicting results in the literature regarding bioaccumulation and toxicity of dietary Cd in *H. azteca*, to address the knowledge gaps identified and provide a modelling tool that could be applied to assess the risk of dietary Cd to *H. azteca*. The overall objective was to determine whether dietary Cd in an ecologically relevant diet of periphyton at environmentally relevant concentrations contributed to chronic bioaccumulation and toxicity in *H. azteca* and whether this could be accurately modelled. The overall objective was broken down into four guiding objectives which will be focused on in the following chapters of this thesis:

1. model chronic Cd bioaccumulation in *H. azteca* from water and periphyton sources and determine their relative contributions (Chapter 2).
2. determine the relationship between chronic Cd bioaccumulation in *H. azteca* from periphyton and effects on survival and growth (Chapter 2).
3. determine the bioavailability of dietary Cd in periphyton to *H. azteca* and how this differs from a standard artificial diet of TetraMin® (Chapter 3).
4. compare the model predictions of chronic dietary Cd bioaccumulation and toxicity in *H. azteca* to field measurements (Chapter 4).

The approach focused initially on laboratory based chronic (28 d) exposures of cultured juvenile *H. azteca* to Cd primarily (but not exclusively) in water and periphyton separately and combined. Using measurements of Cd in water, food and *H. azteca*, a chronic Cd bioaccumulation model was developed and linked to endpoints of chronic toxicity. Factors influencing dietary Cd bioavailability were investigated using pulse-chase feeding techniques with radio-labelled ^{109}Cd. Finally, predictions from the bioaccumulation model were compared to tissue measurements of Cd in *H. azteca* collected from field contaminated sites and Cd in *H. azteca* chronically exposed under laboratory conditions, to water and periphyton collected from the same sites.

CHAPTER 2

Modelling chronic dietary cadmium bioaccumulation and toxicity from periphyton in *Hyalella azteca*

ABSTRACT

Models that estimate the separate contributions of waterborne and dietary metal to bioaccumulation in aquatic biota are important for conducting accurate risk assessments of chronic metal exposure. Bioaccumulation of non-essential metals such as cadmium can be the link between exposure of an organism to metal from multiple pathways, and toxicity. In this study, a chronic (28 d) Cd bioaccumulation model was developed, and the Cd contribution from natural periphyton to bioaccumulation was quantified and linked to toxic effects in the freshwater amphipod *Hyalella azteca*. Juvenile *H. azteca* were exposed to treatments of Cd primarily (but not exclusively) in water (3.13 – 100 nmol/L nominal) and primarily (but not exclusively) in food (389 – 26300 nmol/g ash-free dry mass), separately and combined, and Cd bioaccumulation, survival, growth and amplexus were recorded. Nutrition was measured in periphyton (biomass, chlorophyll *a*, total lipid, fatty acids, total protein) and *H. azteca* (total lipid, fatty acids, total protein) to determine interactions of food quality and metal toxicity. Dietary Cd was predicted to contribute 21 – 31 %, 59 – 94 % and 40 – 55 % to bioaccumulated Cd in *H. azteca* in primarily water, food and food+water treatments, respectively. Survival as a function of Cd body concentration (LBC50 = 679 nmol/g, 617 – 747 95 % CL) was the most robust endpoint and was independent of Cd source. Based on the LBC50, dietary Cd from food was predicted to contribute 26 %, 90 % and 46 % towards Cd in *H. azteca* and therefore contributed to toxicity in the primarily water, food and food+water treatments, respectively. *H. azteca* dry weight and amplexus declined with increasing Cd in water and periphyton, though no difference in *H. azteca* nutrition was detected. Therefore dietary Cd from periphyton contributed markedly towards bioaccumulation and warrants incorporation into models used to predict toxicity of Cd to *H. azteca*.

2.1 INTRODUCTION

The development of models based on sound mechanistic principles that predict metal toxicity to aquatic biota from both water and food is crucial to performing robust ecological risk assessments and deriving protective guidelines (Chapman *et al.*, 2003; Hare *et al.*, 2003; Meyer *et al.*, 2005). Progress has been made in the development of models that predict metal toxicity based solely on water chemistry, such as the Biotic Ligand Model (BLM) (Paquin *et al.*, 2002), and more specifically the chronic toxicity of aqueous Cd to the freshwater amphipod *Hyalella azteca* (Borgmann *et al.*, 2010; Schroeder, 2008). These equilibrium models link water chemistry to bioaccumulation and toxic effects but have yet to include an explicit contribution from dietary metal. An alternative biokinetic approach to modelling incorporates physiological rate processes derived empirically that explain bioaccumulation of metal from water and food separately and can be used to demonstrate toxicity (Croteau and Luoma, 2008; Luoma and Rainbow, 2005; Wang and Fisher, 1999a).

Bioaccumulation models have estimated that dietary Cd can contribute greater than 50 % to tissue concentration in a number of aquatic invertebrates (Croteau and Luoma, 2008; Munger and Hare, 1997; Xie *et al.*, 2010). In two field studies, Borgmann *et al.* (2007) and Stephenson and Turner (1993) predicted dietary Cd contributions to *H. azteca* of as much as 23 % and 58 % respectively. The contribution of dietary Cd to *H. azteca* needs to be verified under controlled laboratory conditions using an ecologically relevant diet. Periphyton is a natural food source for the detritivore *H. azteca* (Pennak, 1989). It is a complex community of biotic (algae, bacteria, fungi, zooplankton, detritus) and abiotic (sediment and metal oxide precipitates) components bound in an exopolymer matrix (Newman and McIntosh, 1989). Periphyton is capable of bioconcentrating Cd from the surrounding water making it important for Cd trophic transfer and potentially being toxic to the consumer (Hill *et al.*, 2000).

Chronic bioaccumulation of certain non-essential metals (Cd, Ni, Tl) in *H. azteca* from the dissolved phase has been shown to be a useful predictor of mortality (Borgmann *et*

al., 2004a, 2001, 1998, 1991). Thus, it seems plausible that chronic toxicity may be linked to dietary Cd via the contribution it makes to bioaccumulation in *H. azteca*.

Toxicity of dietary metals has sometimes been confounded by the nutritional quality of the food (Farag *et al.*, 1999; Woodward *et al.*, 1994 and 1995). Therefore nutritional composition of the food and test organism needs to be quantified over long-term exposures and suitable toxic endpoints that can detect metal effects and nutritional effects separately should be used (Campbell *et al.*, 2005). As for most invertebrates, the exact dietary nutritional requirements for *H. azteca* are unknown. However, protein is important for essential metal transport and storage processes (Simkiss and Taylor, 1995) lipid is important for growth and reproduction in amphipods (Hyne *et al.*, 2009) and specific fatty acids can only be obtained via the diet (Arts *et al.*, 2009). By comparing total protein, total lipid and fatty acids in a standardized laboratory diet such as TetraMin® with periphyton and the same measurements in *H. azteca* feeding on these diets, nutritional impacts at a coarse level can be assessed.

The objectives of this research were to: (1) develop a chronic Cd bioaccumulation model to predict total body concentration and the contributions of Cd from water and food sources that is conceptually based on a mechanistic model (2) link predictions of bioaccumulation to chronic toxic effects in *H. azteca* and (3) relate effects to nutritional quality of the food. Chronic (28 d) feeding experiments using juvenile *H. azteca* and treatments of Cd primarily (but not exclusively) in water and food separately and combined were conducted concurrently. Bioaccumulation of Cd by *H. azteca,* as well as survival, growth and amplexus (reproductive behaviour) were recorded. Periphyton was characterized using measurements of biomass and taxonomic identification. Nutritional quality of food and *H. azteca* involved measuring total protein, total lipid and fatty acids. The implications of the influence of dietary Cd on *H. azteca* were discussed in terms of water quality guidelines.

2.2 THEORY

2.2.1 *Modelling metal bioaccumulation from water and food*

Wang and Fisher (1999a) explained how bioaccumulation of metal from water and dietary sources could be modelled using a first-order kinetic equation:

$$C_{TB} = ((k_u \times C_w) / (k_e + k_g)) + ((IR \times AE \times C_f) / (k_e + k_g)) \quad (2.1)$$

where C_{TB} is the total body concentration of metal at steady state (nmol/g), k_u is the uptake rate constant from water (L/g/d), C_w is the metal concentration in the water (nmol/L), k_e is the excretion rate constant (d^{-1}), k_g is the growth rate constant (d^{-1}), IR is the ingestion rate of food (g AFDM/g *H. azteca* /d), AE is the metal assimilation efficiency from food (unitless, 0 to 1), C_f is the metal concentration in the food (nmol/g AFDM).

Model assumptions were: first-order processes of uptake, additivity of the metal from either water or food, and that steady state conditions exist. While this model has a kinetic basis, it does not account for the ability of metal binding sites and physiological rate processes to become saturated at high metal concentrations or over long exposure periods. Both of these conditions occur for organisms chronically exposed to metals at contaminated sites. A saturation model is one where a linear relationship at low concentrations or initial exposure approaches a maximum leveling off at high concentrations or long-term exposure. Bioaccumulation saturation models have been successfully used to predict metal body concentrations for *H. azteca* chronically exposed to one or more waterborne metals (Borgmann *et al.*, 2008; Borgmann *et al.*, 2004a; Norwood *et al.*, 2006) and were developed from the generalized form:

$$C_{TB} = max \times C_w / (K + C_w) \quad (2.2)$$

where max is the maximum accumulation of metal in *H. azteca* (nmol/g) and K is the half saturation constant (i.e. the concentration of C_w at which the bioaccumulation of Cd in *H. azteca* is half the theoretical maximum accumulation at infinite C_w) (nmol/L). Combining

saturation kinetics with the biokinetic approach in Eq. (2.1), provides a mechanistically based means of accurately predicting bioaccumulation of metal in *H. azteca* from a concentration gradient of metal in water and food. Saturation of k_u, IR and AE can be described by:

$$k_u = V_{umax} / (K_w + C_w) \quad (2.3)$$

$$IR = MIR_{max} / (K_i + C_f) \quad (2.4)$$

$$AE = AC_{max} / (K_a + C_f) \quad (2.5)$$

where $V_{umax} = k_{umax} \times K_w$ is the maximum total uptake rate of metal from water at high metal concentrations (nmol/g/d), k_{umax} is the maximum uptake rate constant (L/g/d) at low metal concentrations, K_w is the half saturation constant (the concentration of C_w at which k_u is half the maximum) (nmol/L), $MIR_{max} = IR_{max} \times K_i$ is the maximum metal ingestion rate at high metal concentrations (nmol/g *H. azteca*/d), IR_{max} is the maximum ingestion rate constant at low metal concentrations (g AFDM/g *H. azteca*/d), K_i is the half saturation constant (the concentration of C_f at which IR is half the maximum) (nmol/g AFDM), $AC_{max} = AE_{max} \times K_a$ is the maximum metal assimilation from food at high metal concentrations (nmol/g AFDM), AE_{max} is the maximum assimilation efficiency at low metal concentrations (unitless), K_a is the half saturation constant (the concentration of C_f at which AE is half the maximum) (nmol/g AFDM).

Substituting Eq. (2.3), (2.4) and (2.5) into Eq. (2.1) gives:

$$C_{TB} = \frac{V_{u\,max} \times C_w}{(k_e + k_g) \times (K_w + C_w)} + \frac{MIR_{max} \times AC_{max} \times C_f}{(k_e + k_g) \times (K_i + C_f) \times (K_a + C_f)} \quad (2.6)$$

At low metal concentrations $C_w << K_w$, and $C_f << K_i$ or K_a. This means that Eq. (2.6) becomes:

$$C_{TB} = \frac{k_{u\,max}\ x\ C_w}{(k_e + k_g)} + \frac{IR_{max}\ x\ AE_{max}\ x\ C_f}{(k_e + k_g)} \quad (2.7)$$

At high metal concentrations $C_w >> K_w$, and $C_f >> K_i$ or K_a. This means that Eq. (2.6) becomes:

$$C_{TB} = \frac{V_{u\,max}}{(k_e + k_g)} + \frac{MIR_{max}\ x\ (AC_{max} / C_f)}{(k_e + k_g)} \quad (2.8)$$

Because of the large number of model parameters to estimate in Eq. (2.6), they were grouped and estimated as a collection of terms such that Eq. (2.6) becomes:

$$C_{TB} = \frac{max_w\ x\ C_w}{(K_w + C_w)} + \frac{max_f\ x\ C_f}{(K_i + C_f)\ x\ (K_a + C_f)} \quad (2.9)$$

where $max_w = V_{umax} / (k_e + k_g)$ and $max_f = MIR_{max}$ x $AC_{max} / (k_e + k_g)$. In practice, K_i and K_a cannot be distinguished from the available data therefore Eq. (2.10) is used:

$$C_{TB} = \frac{max_w\ x\ C_w}{(K_w + C_w)} + \frac{max_f\ x\ C_f}{(K_f + C_f)} \quad (2.10)$$

where K_f is equal to either K_i or K_a, and the other term is incorporated into max_f under the assumption that either $C_f << K_a$ or K_i.

2.3 METHODS AND MATERIALS

2.3.1 *H. azteca culturing*

H. azteca were cultured in 1L of standard artificial medium (SAM: 1 mmol/L $CaCl_2$, 1 mmol/L $NaHCO_3$, 0.01 mmol/L NaBr, 0.05 mmol/L KCl, 0.25 mmol/L $MgSO_4$ in NANOpure® de-ionized water, pH 8.2 and hardness 125 mg/L $CaCO_3$; Borgmann, 1996) in 2 L high density polyethylene (HDPE) containers with artificial substrate (5 x 5 cm cotton gauze), 16:8 h light:dark photoperiod and 25 ^oC. Containers with approximately

100 adults each received 5 mg of ground (< 500 μm) TetraMin® (Tetra Holding (US) Inc.) fish flake diet three times per week. SAM was renewed weekly and juveniles were separated from adults.

2.3.2 *Food preparation*

Three batches of periphyton were collected (15 May, 7 June, 22 August 2008) and processed in the same manner. Batches collected on 15 May and 7 June were used in the first experiment and periphyton collected on 22 Aug was used in the repeat experiment. Non-contaminated periphyton was scraped from artificial substrates and internal surfaces of an outdoor artificial pond and centrifuged (3000 rpm for 10 min). The supernatant was replaced with SAM to a fixed volume to produce a bulk stock of periphyton that was stored in the dark at 4 ^{o}C. Periphyton was harvested the week prior to initiating an experiment to minimize storage time before experimental use. Sub-samples of the bulk stock were analyzed for ash-free dry mass (AFDM) biomass, chlorophyll *a*, total lipid, fatty acids (FA), total protein and Cd. Algal species identification was performed to genus level and taxa were ranked according to relative abundance (Biggs and Kilroy, 2000). TetraMin® is a dried commercial diet consisting of fish and shrimp meal augmented with carbohydrates, proteins, lipids and vitamins designed to optimize fish health. It is used successfully for culturing *H. azteca* as well as conducting aqueous and sediment toxicity testing with *H. azteca* (Borgmann *et al.*, 1989; Environment Canada, 1997). It was thus used as the food source in the primarily (but not exclusively) Cd in water treatment. TetraMin® was analyzed for Cd, biomass, total lipid, FA and total protein.

2.3.3 *Cd exposure of periphyton*

Periphyton was exposed to dissolved Cd that would produce dietary Cd concentrations representative of low to high Cd contaminated sites and would encompass 0 to 100 % effect levels of mortality. Cadmium exposures (nominal 0, 10, 50, 100, 500, 1000, 5000 nmol/L) in 1L SAM were gently aerated at 25 ^{o}C with 16:8 h light:dark photoperiod. Each exposure container had 189 mg and 403 mg AFDM of periphyton in the first and second experiments respectively of the harvested bulk stock of periphyton. Over 96 h,

filtered (0.45 µm polysulfone Acrodisc®) and unfiltered water samples were collected and analyzed for Cd every 24 h and the exposure solutions re-spiked with additional Cd as required to maintain the nominal concentration. At 96 h, periphyton was harvested by centrifugation (3000 rpm for 10 min), washed and made to a final volume with SAM such that there was 3.5 mg AFDM periphyton/mL of working stock from each Cd solution. Enough Cd exposed periphyton from each concentration was prepared for the entire feeding experiment by filtering 1 mL aliquots of working stock onto separate polycarbonate membranes (0.45 µm, 25 mm diameter) which were stored in sealed humidified containers in the dark at 4 ^{o}C until use in the 28 d feeding experiments. Periphyton from each exposure concentration was analyzed for Cd, biomass, chlorophyll *a*, total lipid and total protein.

2.3.4 *Feeding experiments*

The experiments were designed to have treatments of Cd in water and food separately and combined (food+water). In reality, the separate water and food treatments also had measureable levels of Cd in food and water, respectively as a result of Cd partitioning between the two exposure pathways. Therefore, throughout this chapter, the separate water and food treatments may be considered to be primarily (but not exclusively) Cd in water and primarily (but not exclusively) Cd in food, respectively.

Twenty juvenile *H. azteca* (0 – 1 week old) were exposed for 28 d to three Cd treatments (water, food, food+water) and two dietary controls (periphyton and TetraMin®) in triplicate. As in the case of Cd concentrations in periphyton in Chapter 2.3.3, dissolved Cd concentrations were representative of low to high Cd contaminated sites that would encompass 0 to 100 % effect levels of mortality. Nominal dissolved Cd in water and food+water treatments was 0, 3.13, 6.25, 12.5, 25, 50, 100 nmol/L (using 1 mmol/L $CdCl_2$ anhydrous, analytical grade in 1 % v/v HNO_3) and 0 nmol/L in the food treatment. A gradient of Cd exposed periphyton (Chapter 2.3.3) was used in the food and food+water treatments. TetraMin® was used in the water treatment thereby following the protocol of a standard chronic "aqueous" toxicity test. Experiments were conducted at 25 ^{o}C with 16:8 h light:dark photoperiod. One membrane of periphyton per replicate

container was added at each water/food renewal. TetraMin® (5 mg fresh or 3.5 mg AFDM) was dispensed into replicate containers in the water treatment using a calibrated plastic scoop. The mean ± SD food ration for both periphyton and TetraMin® was 0.063 ± 0.010 mg AFDM/amphipod/day. Test solutions (1 L SAM) were added to food and artificial substrate (3 x 3 cm 750 µm nylon mesh) in 2 L HDPE containers to facilitate mixing and then left to equilibrate for 24 h. To initiate the experiment, batches of two juvenile *H. azteca* were randomly and repeatedly collected and dispensed into cups containing 5 mL SAM until there were 20 individuals per cup. Each cup was re-counted and then randomly transferred to a treatment replicate. Static renewal of water and food occurred 3 times per week over 28 d. At 28 d, *H. azteca* were depurated in 40 mL SAM containing 50 µmol/L ethylenediamine tetra-acetic acid (EDTA), 2.5 mg fresh TetraMin® and a new piece of nylon mesh for 24 h before rinsing in SAM and obtaining wet and dry (48 h at 60 ^{o}C) weights. The mean ± SD ratio of dry:wet weight was 0.249 ± 0.028 on a per amphipod basis. *H. azteca* were stored in acid-washed cryovials at room temperature until they were analyzed for Cd, or stored at -80 ^{o}C until they were analyzed for total lipid, FA and total protein. Fatty acid analysis of *H. azteca* was performed on animals from periphyton and TetraMin® controls (2 replicates each) and pooled replicates for *H. azteca* exposed to Cd in water (12.5 nmol/L), food (exposed to 500 nmol/L) and food+water (12.5 nmol/L and food exposed to 100 nmol/L).

At each water and food change, filtered (0.45 µm polysulfone Acrodisc® and acid-washed polypropylene syringe pre-rinsed with NANOpure® de-ionized water and sample) and unfiltered water samples (1 mL) from new and old solutions were collected from one replicate of each treatment concentration and preserved (1 % v/v with 70 % Omni*Trace* Ultra™ high purity HNO_3) in acid-washed cryovials for Cd analysis. Food remnants in old solutions were collected with an acid washed disposable plastic pipette, centrifuged (3000 rpm for 5 min), rinsed with SAM, centrifuged, dried (60 ^{o}C for 48 h) and analyzed for Cd to determine change in dietary Cd over time. Ammonia, pH, conductivity and oxygen concentrations were measured in new (prior to adding the animals) and old solutions at each water renewal (mean ± SD: ammonia 0.001 ± 0.003 mmol/L, pH 8.12 ± 0.14, conductivity 413 ± 7.3 µS/cm and oxygen 7.75 ± 0.25 mg/L). Major cations, anions,

dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) were measured in one replicate of each concentration. Major cations and anions were within 4 % of nominal SAM values and DIC ranged from 9.90 to 12.05 mg/L. DOC in water treatments using TetraMin® ranged from 0.3 to 0.5 mg/L (with the exception of one TetraMin® control where DOC = 1 mg/L) and those treatments given periphyton had a lower DOC range of 0.1 to 0.3 mg/L. Major ion, DIC and DOC analyses were conducted by the National Laboratory for Environmental Testing, Environment Canada.

The experiment was performed twice and Cd in water, food and *H. azteca* were analyzed by 2-way ANOVA according to treatment and experiment and pooled for modelling and graphical representation.

2.3.5 *In situ cages*

In situ cages constructed from clear polyacrylic tubing sealed with 500 µm nylon mesh at each end (Borgmann *et al.*, 2007) were used to measure bioaccumulation of Cd in *H. azteca* from water without food for 7 d. Ten adult *H. azteca* were added to *in situ* cages which were suspended in the overlying water of 3 replicate controls, food treatments and selected water and food+water treatments receiving nominal dissolved Cd exposure of 25 nmol/L. After 7 d without food, *H. azteca* were removed, depurated and processed for Cd analysis in the same way as *H. azteca* in the feeding experiments.

2.3.6 *Nutritional analyses of food and H. azteca*

2.3.6.1 *Biomass of food*

Dry mass and AFDM of food were measured by filtering a fixed volume of bulk periphyton stock or 3.5 mg TetraMin® (AFDM) on three replicate GF/C filters (pre-ashed, pre-weighed, 25 mm diameter, stored in a desiccator) which were then dried at 105 ^{o}C for 24 h, weighed, ashed at 400 ^{o}C for 4 h and re-weighed (Biggs and Kilroy, 2000). Blank filters were included to correct for moisture associated with the filter. The difference between dried and ashed weights as a fraction of the dry weight was reported as the organic content (%) of the diet.

2.3.6.2 *Periphyton chlorophyll a*

Chlorophyll *a* was determined by filtering a fixed aliquot of periphyton onto 3 replicate GF/C filters (25 mm diameter). The filters were extracted in 90 % ethanol in a water bath at 78 ^{o}C for 5 min, stored in the dark at 4 ^{o}C overnight, centrifuged (6000 rpm for 10 min), and 2.5 mL of supernatant was read relative to a 90 % ethanol blank on a Shimadzu UV-1700 Pharmspec UV-Vis spectrophotometer at 750 nm (to correct for suspended particulates) and 665 nm for chlorophyll *a*. Extracts were then acidified with 0.0625 mL of 0.3 M HCl and re-read at 750 and 665 nm (absorbance for phaeophyton) and pre-acidified readings were adjusted for phaeophyton (Biggs and Kilroy, 2000).

2.3.6.3 *Total lipid and fatty acid analysis of food and H. azteca*

Total lipid was analyzed in oven dried *H. azteca* (1.2 – 3.3 mg) and lyophilized periphyton (4.7 – 15 mg AFDM) and TetraMin® (2.0 – 2.2 mg AFDM) stored at -80 ^{o}C using a gravimetric technique based on Folch *et al.* (1957) and Bligh and Dyer (1959). Efficiency of extraction of fatty acids (FA) was determined by adding 25 µL of 2 mg/mL 5α-cholestane (≥ 97 % GC purity in chloroform) to samples and blanks. Extraction of ground samples in 2 mL chloroform:methanol 2:1 (v/v) followed by centrifugation at 3300 rpm at 4 ^{o}C for 15 minutes to remove non-lipid material was repeated 3 times. The extract was made to volume (8 mL) with fresh chloroform:methanol. Sodium chloride (1.6 ml of 9 mg/mL) was added to the extract followed by vortexing for 10 s and centrifugation at 2800 rpm at 4 ^{o}C for 15 minutes causing a phase separation. The top methanol/NaCl layer containing waste precipitates was removed and the remaining chloroform layer was evaporated to dryness with N gas. Extracted lipid residue was brought to a final volume of 0.5 mL with chloroform:methanol. Duplicate 100 µL aliquots were dispensed into pre-weighed smooth wall tin capsules which were dried and reweighed on a Sartorius (Model ME5) microbalance with 1 µg precision. Total lipid content (%) was reported on an AFDM basis for the food and dry mass basis for *H. azteca*.

The remaining extract was transferred into a 5 mL Shimadzu vial (Sigma no. 27319U), evaporated to dryness using N gas and stored at -80 ^{o}C for analysis of fatty acid methyl

esters (FAME). Prior to derivatization, the extract was re-suspended in 1.5 mL toluene. Methylation of the extract occurred by adding 2 mL of H_2SO_4/methanol (1 %) and placing the tubes in a water bath (50 ^oC) overnight (16 h). The extract was then evaporated to dryness using N gas and re-dissolved in 2 mL hexane. A 250 µL portion of the resulting extract was used for FAME analysis.

FAME concentrations were quantified using a capillary gas chromatograph (Agilent 6890N) coupled with a flame ionization detector. Instrument configuration was described by Hebert *et al.* (2009). A 37-component FAME standard (Supelco no. 47885-U) was used to identify and quantify (four-point calibration curves) FAME in the samples. FAME was corrected for 54 ± 6 (SD) % recovery of 5α-cholestane and 97 ± 3 (SD) % efficiency of methylation. Results were reported as µg FAME/mg of dry sample (or per mg of AFDM sample in the case of periphyton).

2.3.6.4 *Total protein of food and H. azteca*

Total protein was analyzed in oven dried *H. azteca* (0.18 – 2.1 mg), lyophilized periphyton (1.2 – 2.4 mg AFDM) and TetraMin® (3.4 – 3.5 mg AFDM) stored at -80 ^oC. Protein was measured by grinding and solubilizing the sample in buffer (0.1 M NaOH and 1% Triton X-100) followed by the addition of 25 µL sample and 200 µL working solution (bicinchoninic acid (BCA) with copper sulphate) to 3 replicate microplate wells. The plate was incubated for 2 h at 24 ^oC and absorbance was read at 562 nm on a plate reader (Molecular Devices VERSA max tunable microplate reader) and protein was quantified relative to the calibrated absorbance of a standard dilution series (200 – 1000 µg/mL) of bovine serum albumin (BSA). Solubilization buffer type and incubation times were optimized in previous experiments (unpublished) and BCA reagents and standards were supplied in a kit (Sigma Aldrich BCA1). Total protein (%) was reported on an AFDM basis for the food and dry mass basis for *H. azteca*.

2.3.7 *Cd analyses*

Dried periphyton (0.028 to 2.0 mg), TetraMin® (0.096 to 1.7 mg) and *H. azteca* (0.028 to 2.3 mg) were weighed and digested according to methods based on Borgmann *et al.*

(1989) and Stephenson and Mackie (1988). Dry material was cold acid digested with 70 % ultra-pure HNO_3 (1.75 % in final digest volume) for 6 d, followed by addition of 30 % ultra-pure H_2O_2 (0.6 % in final digest volume) for 24 h at 60 ^oC then made to a final digest volume with NANOpure® de-ionized water. Final digest volumes were dependent on the range of the initial dry mass digested such that 0 to 0.749 mg, 0.750 to 1.499 mg, 1.500 to 2.249 mg and >2.249 mg ranges of initial dry mass had final digest volumes of 0.5, 1.0, 1.5, and 2.0 mL respectively. Cadmium concentration was reported on an AFDM basis for the food and dry mass basis for *H. azteca*. Certified reference materials of TORT-2 (National Research Council of Canada; lobster hepatopancreas) and CRM-482 (European Commission; lichen) were digested in each sample batch with recoveries of 98 ± 9 % and 101 ± 5 % (mean, ± SD) respectively. Cadmium analysis of water, food and *H. azteca* samples was performed on a Varian SpectrAA 400 graphite furnace atomic absorption spectrophotometer (GF-AA) with Zeeman background correction using a partition tube without modifier. In each run, calibration standards and blanks were analyzed every fifth sample to correct for drift and an external standard (CRM-TMDW, High-Purity Standards, Charleston, SC) had a recovery of 103 ± 7 % (mean ± SD). Method detection limits calculated as the upper 95 % confidence limit of the unfiltered, filtered water and digest blank samples were 0.037 nmol/L, n = 205; 0.036 nmol/L, n = 199; 0.040 nmol/g n = 67, respectively. Inter-laboratory comparisons of Cd results using polymetallic reference waters supplied by National Laboratory for Environmental Testing, Environment Canada demonstrated acceptable performance of the instrument and analytical protocol.

2.3.8 *Cd speciation*

Modelling of free Cd^{2+} concentration was performed using the Windermere Humic Aqueous Model (WHAM) version 6.0.13 (purchased from Centre for Ecology and Hydrology, UK). Model input parameters were temperature (as K), pH, major ions (Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-} mol/L), strongly competing ions (Al^{3+}, Fe^{3+}, Mn^{2+} mol/L), DIC g/L divided by 12.011 g/mol C as CO_3^{2-} mol/L and dissolved Cd mol/L (0.45 µm filtered). It was assumed that 50 % of natural organic matter was composed of carbon and 65 % of natural organic matter was active for metal binding and was 100 % fulvic acid

(Bryan, *et al.*, 2002) therefore DOC g/L was multiplied by 1.3 to give the fulvic acid g/L input value. Because of the uncertainty of the metal binding characteristics of the DOC in these solutions and consequently its influence on Cd speciation, bioaccumulation model estimates and results were based on dissolved Cd with selected comparisons made to model parameter estimates and contribution of diet to Cd in *H. azteca* based on free Cd^{2+} concentration.

2.3.9 *Data analyses*

ANOVA and non-linear regression modelling were performed with SYSTAT version 10.0. Differences between means were analyzed with 1-way and 2-way ANOVA and post-hoc analyses with Tukey's and Dunnett's tests. Assumptions of normality of distribution and homogeneity of variance were tested with visual assessment of probability density plots of non-transformed and log or square root transformed data and Levene's test on the absolute value of the residuals respectively (Environment Canada, 2005). When the assumptions were violated, Kruskall-Wallis, the non-parametric equivalent of one-way ANOVA, was used.

2.3.10 *Modelling*

2.3.10.1 *Bioaccumulation of Cd in H. azteca*

Bioaccumulation of Cd in *H. azteca* from food and water sources was modelled in SYSTAT using Eq. (2.11) which is based on Eq. (2.10). The contribution of Cd to *H. azteca* from TetraMin® in the water treatment and periphyton in the food and food+water treatments was modelled separately with the use of dummy variables. The max and inverse of K were estimated using non-linear regression. The inverse of K was used so that if K was infinite, 1/K was equal to zero. The "funpar" command was then used to calculate the max and K values with 95 % confidence limits.

$$\begin{aligned} C_{TB} = {} & max_w \times IK_w \times C_w / (1 + IK_w \times C_w) + \\ & max_{ft} \times IK_{ft} \times C_{ft}(1\text{-food}) / (1 + IK_{ft} \times C_{ft}(1\text{-food})) + \\ & max_{fp} \times IK_{fp} \times C_{fp}(food) / (1 + IK_{fp} \times C_{fp}(food)) \end{aligned} \qquad (2.11)$$

where max_w, max_{ft}, and max_{fp} are the maximum accumulation of metal in *H. azteca* from water (nmol/g), TetraMin® (nmol/g) and periphyton (nmol/g) respectively; IK_w, IK_{ft}, IK_{fp} are the inverse of the half saturation constants K_w (nmol/L), K_{ft} (nmol/g AFDM), K_{fp} (nmol/g AFDM) which are the concentration of Cd in water, TetraMin® and periphyton respectively at which C_{TB} is half the maximum. K_{ft} and K_{fp} each represent a combination of K_a and K_i from Eq. (2.9) as there were no measurements of AE or IR in this experiment. C_{ft} and C_{fp} are the concentration of Cd in TetraMin® and periphyton respectively. Food is a dummy variable that equals zero for TetraMin® and one for periphyton.

The percent contribution of Cd in *H. azteca* ($C_{TB\%,}$ %) from TetraMin® in the water treatment was modelled using:

$$C_{TB\%} = C_{TBt} / (C_{TBt} + C_{TBw}) \times 100 \quad (2.12)$$

Where C_{TBt} (nmol/g) is the total body concentration of Cd in *H. azteca* from TetraMin® = $max_{ft} \times IK_{ft} \times C_{ft}(1\text{-food}) / (1 + IK_{ft} \times C_{ft}(1\text{-food}))$ and C_{TBw} (nmol/g) is the total body concentration of Cd in *H. azteca* from water = $max_w \times IK_w \times C_w / (1 + IK_w \times C_w)$

The percent contribution of Cd in *H. azteca* from periphyton in the food and food+water treatments was modelled using:

$$C_{TB\%} = C_{TBp} / (C_{TBp} + C_{TBw}) \times 100 \quad (2.13)$$

Where C_{TBp} (nmol/g) is the total body concentration of Cd in *H. azteca* from periphyton = $max_{fp} \times IK_{fp} \times C_{fp}(food) / (1 + IK_{fp} \times C_{fp}(food))$ and C_{TBw} (nmol/g) is the total body concentration of Cd in *H. azteca* from water = $max_w \times IK_w \times C_w / (1 + IK_w \times C_w)$

2.3.10.2 *Bioaccumulation of Cd in periphyton*

The same approach of modelling bioaccumulation of Cd in *H. azteca* based on saturation kinetics was also applied to modelling bioaccumulation of Cd in the periphyton diet using:

$$C_{fp} = max_p \times C_w / (K_p + C_w) \quad (2.14)$$

Where max_p (nmol/g AFDM) is the maximum accumulation of metal in periphyton from water and K_p is the half saturation constant (the concentration of C_w at which C_{fp} is half the maximum) (nmol/L).

2.3.10.3 *Bioaccumulation of Cd in TetraMin®*

TetraMin® in the water treatment adsorbed the ambient Cd linearly without reaching saturation and was modelled as:

$$C_{ft} = (CF \times C_w) + C_{Bkt} \quad (2.15)$$

Where CF is the concentration factor (L/g) of Cd in TetraMin® with respect to Cd in the water, C_{Bkt} is the background concentration of Cd in TetraMin® = 3.21 nmol/g AFDM.

2.3.10.4 *Survival*

Survival endpoints were calculated according to Borgmann *et al.* (2004a), Borgmann *et al.* (1998) and Norwood *et al.* (2007a), on the basis of water concentration (LCX), food concentration (LFCX) and body concentration (LBCX).

Mortality rates were determined by survival in treatments at 4 weeks, and were 4^{th} root transformed to normalize the data. Saturation models were used to estimate lethal effects at the level of 50 %, 25 % and 10 % (Eq. 2.16, Eq. 2.17, Eq. 2.18). Estimates of exponent (n) which defines the sharpness of the curve, were >100 and were therefore arbitrarily fixed at 100 because they could not be estimated accurately

$$m = m' + (\ln(2)/t) \times [C\ (1/LC50 + 1/K'')/(1 + C/K'')]^{n} \quad (2.16)$$

$$LC25 = ((1/LC50 + 1/K'') / (\ln(4/3) / \ln(2))^{1/n} - 1/K'')^{-1} \quad (2.17)$$

$$LC10 = ((1/LC50 + 1/K'') / (\ln(1/0.9) / \ln(2))^{1/n} - 1/K'')^{-1} \quad (2.18)$$

where m is the total mortality rate (*H. azteca* per week), m' is the control mortality rate (*H. azteca* per week), t is exposure duration (4 weeks), C is the Cd concentration in water (nmol/L), food (nmol/g AFDM) or *H. azteca* (nmol/g) depending on the endpoint being calculated, LCX is the lethal endpoint with respect to dissolved Cd (LCX, nmol/L) but could also be with respect to Cd in food (LFCX, nmol/g AFDM) or *H. azteca* (LBX, nmol/g) depending on the choice of C, K'' is the half saturation constant (the concentration of water, food or body concentration at which m is half the maximum) (nmol/L or nmol/g AFDM) and (n) is an exponent fixed to 100. For the purposes of displaying lethal toxicity, mortality was converted to survival.

2.3.10.5 *Growth*

Growth endpoints were calculated according to Borgmann *et al.* (2004a), Borgmann *et al.* (1998) and Norwood *et al.* (2007a), on the basis of water concentration (ICX), food (IFCX) concentration and body concentration (IBCX).

H. azteca dry weight was square root transformed to normalize the data and could not be modelled on a saturation basis as with bioaccumulation and survival because the results were too variable. Therefore a general allometric growth model was used to estimate W', (a), and n and then "funpar" command was used to derive the ICX values at 50 %, 25 % and 10 % growth inhibition with 95 % confidence limits.

$$W = W'\ (1 + aC^{n})^{-1} \quad (2.19)$$

$$IC50 = (1/a)^{1/n} \quad (2.20)$$

$$IC25 = (1/3/a)^{1/n} \quad (2.21)$$

$$IC10 = (1/9/a)^{1/n} \quad (2.22)$$

Where W is final dry weight (mg/*H. azteca*) after 4 weeks, W' is the control dry weight (mg/ *H. azteca*), C is Cd in water (nmol/L), food (nmol/g AFDM) or *H. azteca* (nmol/g), (a) and n are the regression coefficient (mg/ *H. azteca*) and exponent (mg/ *H. azteca*/C) respectively. ICX is the inhibition of growth with respect to dissolved Cd (ICX, nmol/L) but could also be with respect to Cd in food (IFCX, nmol/g AFDM), or *H. azteca* (IBCX, nmol/g) depending on the choice of C. Where (a) could not be estimated using the model in Eq. (2.19), ICX, IFCX or IBCX was estimated directly in Eq.s (2.23 – 2.25) by rearranging Eq.s (2.20 – 2.22) and substituting into Eq. (2.19).

$$W = W' / (1 + (C/IC50)^n) \quad (2.23)$$

$$W = W' / (1 + 1/3(C/IC25)^n) \quad (2.24)$$

$$W = W' / (1 + 1/9(C/IC10)^n) \quad (2.25)$$

In all cases, the "funpar" command in SYSTAT was used to calculate log estimates of the endpoints and associated 95 % confidence limits. These were then back-transformed for reporting purposes.

2.4 RESULTS

2.4.1 *Food characterization*

Periphyton batches differed in % organic content, chlorophyll *a* and % protein ($P<0.05$). Due to limited biomass, % lipid was measured only in the 22 August 2008 batch and therefore could not be compared across batches. Organic content was lowest for the batch collected 7 June 2008 while periphyton collected 22 August 2008 had reduced chlorophyll *a* and % protein. Periphyton nutritional characteristics did not change with

increasing Cd (Table 2.1). Algal composition in all batches before exposure to Cd were ranked by division using the dominant genera with highest abundance of green algae (Chlorophyta: *Cladophora, Mougeotia, Ulothrix, Scenedesmus, Akistrodesmus*), followed by diatoms (Bacillariophyta: *Synedra, Navicula*) and blue green algae (Cyanobacteria: *Oscillatoria*). Relative abundance was not reassessed after 96 h Cd exposure but Cd exposure was kept short to minimize toxicity that could alter the community structure of the periphyton.

Mean ± SD organic content of control periphyton and TetraMin® was 56.9 ± 14.5 % and 100 ± 0 % respectively (Table 2.1). All Cd and nutritional measurements for periphyton and TetraMin® were reported on an AFDM basis to normalize for the variation of inorganic content. Percent total lipid was 3 times greater in TetraMin® than periphyton. Total FA was four times greater in TetraMin® than periphyton (Table 2.2). The dominant FAs in both TetraMin® and periphyton were linoleic acid, oleic acid, stearic acid and palmitic acid (A2.1). The percentages of saturated FA (SAFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) were 41, 19, 40 % and 34, 29, 37 % in TetraMin® and periphyton respectively. The ratio of ω3:ω6 was higher in periphyton than TetraMin® with the dominant ω3 FA being α-linolenic acid (ALA) and docosahexanenoic acid (DHA) in periphyton and TetraMin® respectively. The dominant ω6 FA was linoleic acid in both periphyton and TetraMin®. Percent total protein of periphyton did not differ from that of TetraMin® ($P>0.05$) (Table 2.1).

Final Cd concentrations in the periphyton prior to adding to the feeding containers ranged from 10.5 to 26300 nmol/g AFDM (Table 2.1). Significant interaction terms in the 2-way ANOVA of Cd in periphyton with categorical variables of treatment and periphyton batch indicated that, depending on the treatment, Cd in periphyton collected on 22 August 2008 was lower than that collected on 15 May 2008 but overall there were no differences in Cd with batch of periphyton. pH and DOC were not measured therefore Cd speciation was not modelled in periphyton exposure solutions. Measured dissolved (0.45 µm filtered) and total Cd ranged from 63 to 92 % and 70 to 99 % of nominal respectively after 24 h equilibration but prior to the addition of periphyton to the containers. Filtered

water samples differed from unfiltered by 11 % at most. Lower measured total Cd relative to nominal was assumed to be due to adsorption of Cd to container walls during the equilibration phase. Dissolved Cd lost from solution following the addition of periphyton was used to estimate bioaccumulation every 24 h. Subsequent uptake of Cd by periphyton was high (79 ± 18 %, mean ± SD) over the first 24 h and additional spikes of Cd were required to maintain the nominal exposure concentration (A2.2). Cadmium in periphyton as a function of increasing dissolved Cd was indicative of saturation (Figure 2.1). Modelled ($r^2 = 0.941$) estimates of the maximum concentration of Cd in periphyton and the half saturation constant using Eq. (2.14) were max_p = 28,978 (12,399 – 45,557 95 % CL) nmol/g and K_p= 809 (224 – 1395 95 % CL) nmol/L respectively. Bioconcentration factors (BCFs) calculated at each exposure concentration (Cd in food nmol/g AFDM divided by Cd in water nmol/L) decreased (400 to 9 L/g) with increasing dissolved Cd.

TetraMin® adsorbed Cd from the ambient dissolved phase during the 48 or 72 h in the water treatment of the feeding experiment. A linear accumulation of Cd in TetraMin® resulted in as much as 1200 times the initial background Cd and ranged from 144 to 3890 nmol/g (Figure 2.1, A2.3). The slope or concentration factor (Eq. 2.15) describing the total adsorption of Cd onto TetraMin® as a function of dissolved water concentration was 48.8 (46.1 – 51.6 95 % CL, $r^2 = 0.991$) L/g.

2.4.2 *Feeding experiments*

Replicate containers receiving periphyton exposed to 500 nmol/L Cd in the food and food+water treatments were erroneously fed periphyton exposed to 5000 nmol/L Cd on one of the 12 feeding occasions in the first experiment. Results relating to these six replicates were subsequently removed from the database.

2.4.2.1 *Cd in solution*

Dissolved Cd changed with treatment by -7 %, 120 % and -2 % in t = 0, 48 or 72 h water samples for water, food and food+water treatments respectively. To obtain a database of values representative of the true exposure concentration, the geometric mean of the Cd in

filtered new and old solutions was used (Figure 2.2, A2.3). In addition, values below the method detection limit (1.7 % of all filtered water samples) were included and for 33 % of all water samples where unfiltered Cd was lower than the filtered value (predominantly in control samples), the unfiltered concentration was used based on the assumption that contamination of the filter had occurred – an approach also adopted by Borgmann *et al.* (2007) with the same filter brand. Based on this database, the mean of measured dissolved Cd in the food treatment increased 2 to 97 times with increasing Cd in the periphyton relative to the control periphyton as Cd leached from the periphyton into the surrounding water. Despite having the same nominal dissolved Cd concentration in the water and food+water treatments, differences in measured relative to nominal ranged from -15 % to -20 % and -6 % to 20 % for the water and food+water treatments respectively. Dissolved Cd in the food+water treatment was overall 1.3 times higher than the water treatment. Free-ion Cd^{2+} concentration estimated using WHAM v6.0.13 was similar in each treatment with means of 73 %, 72 % and 75 % of the dissolved Cd in water, food and food+water treatments, respectively, being Cd^{2+} (A2.3). Mean dissolved Cd estimated to be bound to fulvic acid colloids was 6 %, 4 % and 2 % in the water, food and food+water treatments respectively.

2.4.2.2 *Cd in food*

Cd was released from and adsorbed by the food during the 48 h or 72 h period between food/water renewals. In the case of periphyton that was pre-exposed to Cd as described above, the geometric mean of t = 0 and t = 48 or 72 h Cd was used to represent the true dietary exposure concentration resulting in 287 to 14900 nmol/g and 430 to 14800 nmol/g in the food and food+water treatments respectively (Figure 2.2, A2.3). The final Cd concentration of TetraMin® was used since this diet was not pre-exposed to Cd and was assumed to reach steady state quickly. Cadmium released by periphyton in the food treatment resulted in 26 to 44 % loss from periphyton with increasing Cd. In the food+water treatment, Cd in periphyton increased 11 % and 5 % at nominal 3.13 and 6.25 nmol/L Cd respectively while periphyton at the higher dissolved Cd concentrations lost 5 % to 44 % Cd. TetraMin® and periphyton in the controls increased by 5 and 2 times respectively due to low background contamination of the water (0.2 nmol/L).

2.4.2.3 *Cd bioaccumulation in H. azteca in feeding experiments*

Body concentration of Cd in *H. azteca* exposed to Cd in water, food and food+water for 28 d increased as a function of increasing water concentration (Fig. 2.3) and food concentration (A2.3) in each treatment. Bioaccumulation of Cd by *H. azteca* in the water (123 – 903 nmol/g), food (104 – 819 nmol/g) and food+water (312 – 949 nmol/g) treatments overlapped even though in the case of the food treatment, the range of dissolved Cd was approximately 10 times lower than for the water and food+water treatments. Cadmium body concentration in the food+water treatment was approximately equal to the sum of Cd in *H. azteca* in separate water and food treatments with the exception of *H. azteca* exposed to nominal dissolved Cd of 12.5 nmol/L and /or periphyton exposed to 100 nmol/L. Modelled bioaccumulation of Cd (Eq. 2.11) as a function of water provided good estimates of measured values at low and high Cd concentration in the water and food+water treatments but did not pass through control values for the food treatment (Fig. 2.3) because dissolved Cd at low food concentrations was close to background. Although it was possible to fit a saturation model, full saturation of tissue with respect to Cd in water or food was not observed in measurements of body concentration due to lethality.

2.4.2.4 *Cd bioaccumulation in caged H. azteca*

Caged adult *H. azteca* were exposed solely to dissolved Cd leaching from periphyton for 7 d in the controls, and selected Cd concentrations in the water, food and food+water treatments. Cadmium in caged *H. azteca* increased with dissolved Cd for all treatments (Table 2.3). Significant increases in body concentration relative to the control occurred at dissolved Cd greater than or equal to 0.65 nmol/L or 16.9 nmol/g Cd in tissue. Direct comparison of 7 d caged *H. azteca* with 28 d fed *H. azteca* was not possible because of different exposure periods and larger body size of caged amphipods compared to those fed with periphyton. However, trends of Cd bioaccumulation across treatments within caged and non-caged *H. azteca* clearly showed that where bioaccumulation in non-caged *H. azteca* was similar for each treatment (609 nmol/g, 819 nmol/g, 949 nmol/g), bioaccumulation of caged *H. azteca* was much lower in the food treatment than the water and food+water treatments (Table 2.3). Therefore the bioaccumulation observed in non-

caged *H. azteca* in the food treatment was due to Cd in periphyton rather than dissolved Cd exposure.

2.4.3 *Modelling Cd bioaccumulation from food and water in H. azteca*

Bioaccumulation of Cd in *H. azteca* from water and separate food types could be modelled successfully using both dissolved Cd and Cd^{2+} (r^2 = 0.946 and r^2 = 0.947 respectively, n = 81, Eq.2.11). Model parameters that were estimated using Cd^{2+} concentration did not differ significantly from those estimated using dissolved Cd. Confidence limits for max and K values for all Cd sources were large and overlapped (Table 2.4). Considerable uncertainty was associated with model parameters estimated for TetraMin® due to large 95 % confidence limits. These were somewhat narrower for estimates of the initial slope (max/K). Measured and model predicted Cd in *H. azteca* for all treatments were within a factor of two of the 1:1 ratio (Figure 2.4, A2.3).

The capacity of *H. azteca* to bioaccumulate Cd was greatest from water (max_w = 1453 nmol/g) followed by TetraMin® (max_{ft} = 1130 nmol/g) and periphyton (max_{fp} = 813 nmol/g) (Table 2.4). The half saturation of Cd in periphyton (K_{fp} = 2173 nmol/g) for *H. azteca* was 2.8 fold lower than the half saturation of Cd in TetraMin® (K_{ft} = 6170 nmol/g) (Table 2.4). Half saturation parameters for water and food cannot be compared directly because of incompatible units and K varying as a function of max. The ratio max/K has narrower confidence limits than either max or K and can be compared between Cd uptake in *H. azteca* from water and food in each of the treatments when units are made compatible. By transforming max/K for Cd in *H. azteca* from water and food in each treatment into compatible units, the relative importance of the separate exposure pathways in each treatment can be assessed. In the water treatment, units of max/K for Cd uptake in *H. azteca* from TetraMin® were made compatible by multiplying max_{ft}/K_{ft} by the partitioning of Cd to TetraMin® (CF = 48.8 L/g, 46.1 – 51.7 L/g 95 % CL). In the food treatment, units of max/K for Cd uptake in *H. azteca* from periphyton were made compatible by multiplying max_{fp}/K_{fp} by the partitioning of Cd to periphyton (max_p/K_p = 2102 L/g AFDM, 1531 – 2885 L/g 95 % CL. In the food+water treatment, units of max/K for Cd uptake in *H. azteca* from periphyton were made compatible by multiplying

max_{fp}/K_{fp} by the partitioning of Cd to periphyton (max_p/K_p = 91.4 L/g AFDM, 80.8 – 103 L/g 95 % CL). The initial slope (max/K) of Cd in *H. azteca* as a function of Cd in water was 34.1 L/g (Table 2.4). The initial slope (max/K) was lower as a function of Cd in TetraMin® in the water treatment (8.94 L/g, 8.45 – 9.46 L/g 95 % CL), higher as a function of Cd in periphyton in the food treatment (786 L/g, 573 – 1079 L/g 95 % CL) and similar as a function of Cd in periphyton in the food+water treatment (34.2 L/g, 30.4 – 38.4 L/g 95 % CL).

2.4.4 *Percentage of Cd in H. azteca from food*

Using the saturation bioaccumulation model parameters (Table 2.4) based on dissolved Cd and Cd in food on an AFDM basis, the average % contribution of Cd in *H. azteca* from food ranged from 21 to 31 %, 59 to 94 % and 40 to 55 % in water (Eq. 2.12), food (Eq. 2.13) and food+water (Eq. 2.13) treatments, respectively (Figure 2.5, A2.3). When based on Cd^{2+}, the range of model predicted values were very similar resulting in 22 to 32 %, 81 to 94 % and 40 to 58 % Cd in *H. azteca* coming from food in water, food and food+water treatments respectively. The contribution of Cd in *H. azteca* from periphyton decreased quickly as dissolved Cd increased and reached a plateau of approximately 40 % at dissolved Cd >100 nmol/L (Figure 2.5). In the water treatment, the contribution of Cd in *H. azteca* from Cd adsorbed to TetraMin® increased with increasing dissolved Cd but did not contribute as much as Cd associated with periphyton. It should be noted that 100 % lethality occurred at dissolved Cd concentrations ≥ 80.2, 21.3 and 47.1 nmol/L in the water, food and food+water treatments, respectively, thus the contribution of Cd in diet to *H. azteca* was most important at dissolved Cd less than 50 nmol/L in the case of combined food and water Cd exposure. The theoretical dietary Cd contribution to bioaccumulation could still be calculated when mortality occurred since only the dissolved and dietary Cd concentrations were required as model input values.

The level of uncertainty surrounding the predicted contribution of Cd from food, increased as the dissolved Cd increased. Based on a TetraMin® diet in the water treatment, the upper and lower 95 % confidence limits of the predicted contribution of dietary Cd expanded from 7.18 - 74.9 % at 2.65 nmol/L to 1.00 – 1000 % at 80.2 nmol/L.

Based on a periphyton diet in the food and food+water treatments, the upper and lower 95 % confidence limits expanded from 77.5 – 93.6 % at 0.49 nmol/L to 17.3 – 91.2 % at 121 nmol/L. Therefore uncertainty was lower for model predictions based on periphyton rather than TetraMin® and overall, uncertainty of dietary Cd contribution was lowest at low dissolved Cd. Below 50 nmol/L, the 95 % CL were less than a factor of two wider than the estimated dietary Cd contribution for periphyton diets.

2.4.5 *Survival*

Mean ± SD control survival was 89 ± 6 % and 97 ± 4 % for *H. azteca* fed TetraMin® or periphyton for 28 d, respectively (Figure 2.6. A2.3). Survival declined with increasing Cd in water and/or food in all treatments with significant reductions ($P<0.05$) in survival relative to controls occurring at 38.6 nmol/L, 5830 nmol/g AFDM, 1050 nmol/g AFDM + 11.8 nmol/L for water, food and food+water treatments respectively (Figure 2.6. A2.3). Survival was 0 % at water and/or food Cd concentrations of 80.2 nmol/L, 14900 nmol/g AFDM, >5900 nmol/g AFDM + 47.1 nmol/L in water, food and food+water treatments respectively.

Lethal endpoints (LX10, LX25, LX50) based on dissolved Cd and free Cd^{2+} models (LCX), and Cd in food (LFCX AFDM) and *H. azteca* (LBCX) for each treatment were modelled using saturation of mortality rate (Eq.s 2.16, 2.17, 2.18) with model fits of r^2 = 0.580 to 0.877 (Tables 2.5, 2.6, 2.7). SYSTAT estimates of exponent (n) were >100 and therefore could not be determined accurately so were fixed to 100. The model parameters of K and n were correlated so that with n fixed, the shape of the curve was strongly reliant on K. Because of this relationship between K and n, not a lot of biological significance was attached to K (Borgmann *et al.* 2004a).

Lethal endpoints of treatments differed significantly at all effect levels when lethality was based on dissolved Cd or Cd^{2+} (non-overlapping 95 % confidence limits, Table 2.5). The food treatment was the most toxic though the result was strongly influenced by the contribution of Cd from food. The water treatment (LC50 28.7 nmol/L) represented the result from a standard dissolved toxicity testing approach for *H. azteca* and was least

toxic. Survival endpoints based on Cd^{2+} did not differ significantly from those based on dissolved Cd. When lethality was based on Cd in food, the food treatment was less toxic than the water and food+water treatments (Table 2.6). Again, while Cd in food was the basis for determining toxicity, dissolved Cd also influenced toxicity in the water and food+water treatments. When lethality was based on Cd in *H. azteca*, the food treatment was marginally more toxic at the 50 % effect level but generally the endpoints did not differ with treatment as indicated by the narrow but overlapping 95 % confidence limits. Consequently, LBCX (LBC10 385 nmol/g, LBC25 501 nmol/g, LBC50 679 nmol/g) were modelled using the pooled treatment (Table 2.7, Figure 2.6).

2.4.6 *Growth*

Dry weight of *H. azteca* fed with periphyton for 28 d was only 62% of the dry weight of *H. azteca* fed with the equivalent daily ration of TetraMin® ($P<0.05$, Figure 2.7, A2.3). Dry weight declined with increasing Cd in water and/or food in all treatments with significant reductions ($P<0.05$) in dry weight relative to controls occurring at Cd concentrations of 38.6 nmol/L, 5830 nmol/g AFDM, 1050 nmol/g AFDM + 11.8 nmol/L in water, food and food+water treatments respectively. *H. azteca* dry weight was 5 to 43 % lower in food+water treatments compared to food treatments though the reductions were not statistically significant.

Dry weight was used to model the inhibition of growth (IX10, IX25, IX50) as a function of dissolved Cd and Cd^{2+} (ICX), Cd in food (IFCX) and *H. azteca* (IBCX). Dry weight was less variable than wet weight. However, dry weight was still too variable to model with saturation kinetics. Therefore a generalized growth model was used (Eq.s 2.19 to 2.25). The effect of this variability was evident in the poorer model fits (r^2 = 0.445 to 0.750; Tables 2.8, 2.9 and 2.10) compared to those for mortality.

Growth endpoints of separate water and food treatments did not differ significantly (overlapping 95 % confidence limits) when modelled based on dissolved Cd or Cd^{2+} despite the food treatment having an ICX as much as 430 times lower than water or food+water treatments. Food+water was the only treatment where endpoints based on

Cd^{2+} were significantly lower than those modelled with dissolved Cd. Wide 95 % confidence limits on endpoints at all effect levels when modelled based on Cd in food meant that there were no significant differences in toxicity of treatments. When modelled on Cd body concentration, there was no significant difference in growth with treatment and therefore the endpoints were calculated based on pooled data as for survival. However, as a chronic sub-lethal endpoint, growth was no more sensitive that survival (overlapping 95 % confidence limits of pooled data endpoints) (Tables 2.7 and 2.10).

2.4.7 *Amplexus*

Amplexus is the mating behaviour displayed when the male clasps the female below him in preparation for gamete release. Although no juveniles were produced in the experiments, the number of amplexing pairs of adult *H. azteca* were affected by food type and Cd concentration. Of the control animals fed with TetraMin®, 8 amplexing pairs were recorded at 28 d while 2 pairs were recorded in the controls fed with periphyton. Amplexus decreased with increasing Cd. Twelve amplexing pairs were recorded in the water treatments (2.65 – 19.9 nmol/L) where TetraMin® was provided and 2 pairs were present in both the food (287 nmol/g AFDM and 353 nmol/g AFDM) and food+water (1050 nmol/g AFDM + 11.8 nmol/L) treatments to produce a total of 4 amplexing pairs in periphyton fed Cd treatments. Amplexus was delayed in those animals fed with periphyton. The first observation of amplexing pairs was at 21 d for TetraMin® (in water treatment 4.94 nmol/L) and 26 d for periphyton (food treatment 287 nmol/g AFDM and food+water treatment 559 nmol/g AFDM + 5.90 nmol/L) fed animals.

2.4.8 *H. azteca nutrition*

Most differences in *H. azteca* nutrition were related to food type rather than Cd effects. Percent total protein of control *H. azteca* fed on TetraMin® was 0.86 times that of control *H. azteca* fed on periphyton for 28 d ($P<0.05$) (Table 2.2). There was no effect of increasing Cd on protein in *H. azteca* in water and food+water treatments.

Percent total lipid in *H. azteca* did not differ with food type or increasing Cd in treatments ($P>0.05$). However, although not statistically significant, total FA in *H. azteca*

fed on control TetraMin® was 1.3 times higher than in *H. azteca* fed on control periphyton (Table 2.2). The same FAs (eicosapentaenoic acid (EPA), linoleic acid, oleic acid, stearic acid and palmitic acid) dominated the FA profile in *H. azteca* fed with either food (A2.1). When *H. azteca* were exposed to Cd in the water treatment, the total FA decreased slightly compared to the control animals (due to reduced oleic and linoleic acids) but was still higher than total FA in *H. azteca* exposed to Cd via periphyton in food and food+water treatments, again these differences were not statistically significant. The percent of saturated FA (SAFA), monsaturated FA (MUFA) and polyunsaturated FA (PUFA) did not change in *H. azteca* with food type or Cd treatment. Within PUFAs, *H. azteca* feeding on periphyton had a high proportion of ω3 FA (EPA) and *H. azteca* feeding on TetraMin® had a high proportion of ω6 FA (linoleic acid) irrespective of Cd concentration.

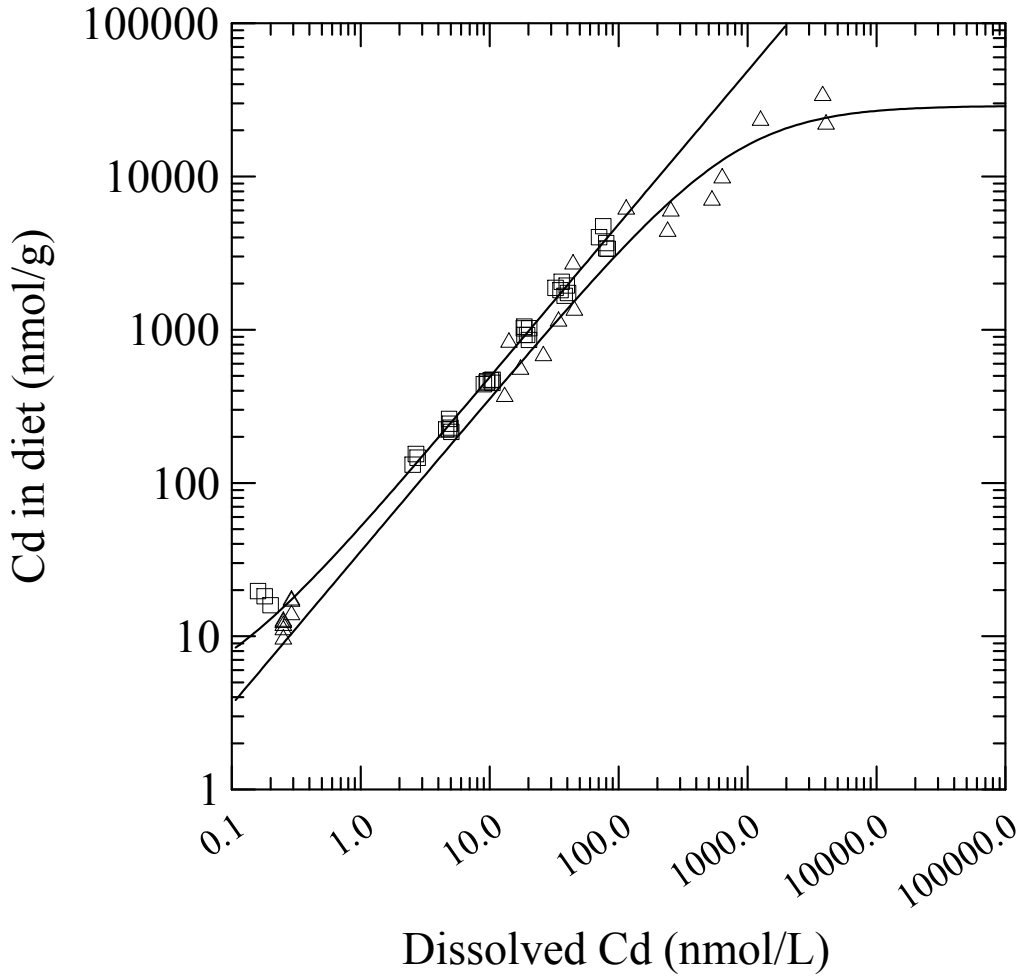


Figure 2.1 Cadmium in periphyton (triangle) and TetraMin® (square) on an AFDM basis as a function of dissolved Cd in the 4 d exposure and 28 d feeding experiment for periphyton and TetraMin®, respectively. Lines are modelled relationships for Cd in periphyton (Eq. 2.14, r^2=0.941) and TetraMin® (Eq. 2.15, r^2=0.991).

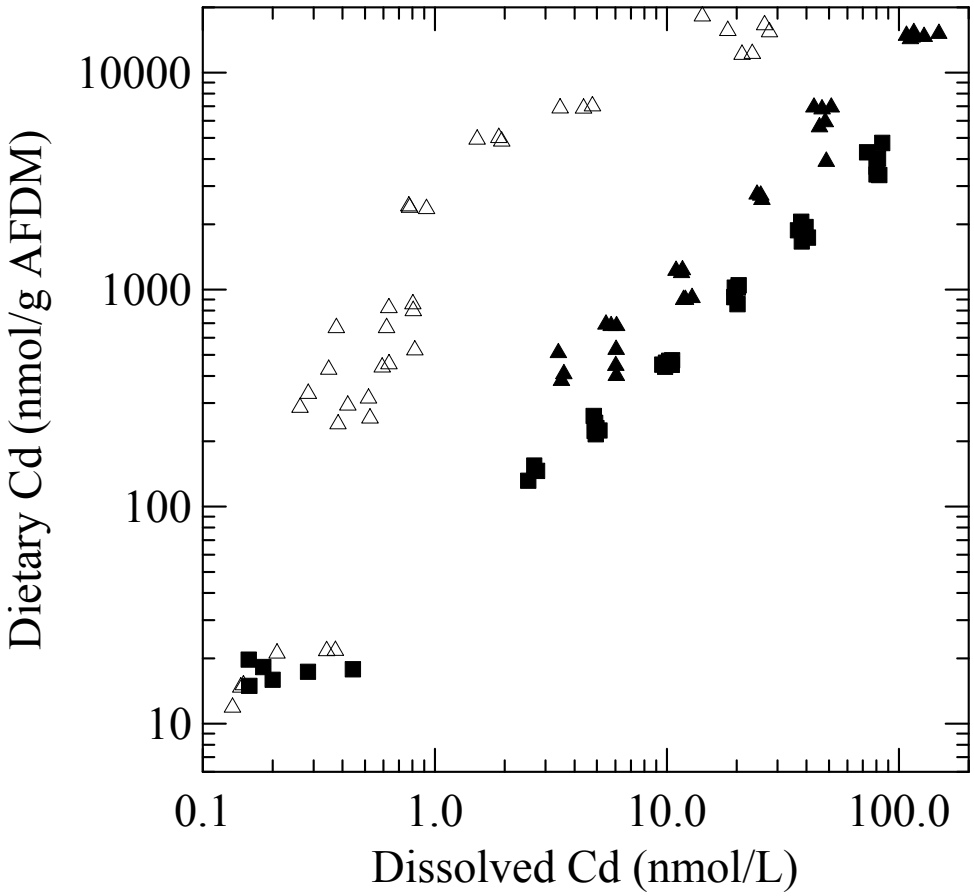


Figure 2.2 Measured Cd in diet and in water (0.45µm filtered) in 28 d feeding experiment. Food consisted of periphyton in the primarily food (open triangle) and food+water treatments (closed triangle) and TetraMin® in the primarily water treatment (closed square). Values are the geometric mean of concentrations at the beginning and end of water/food renewals.

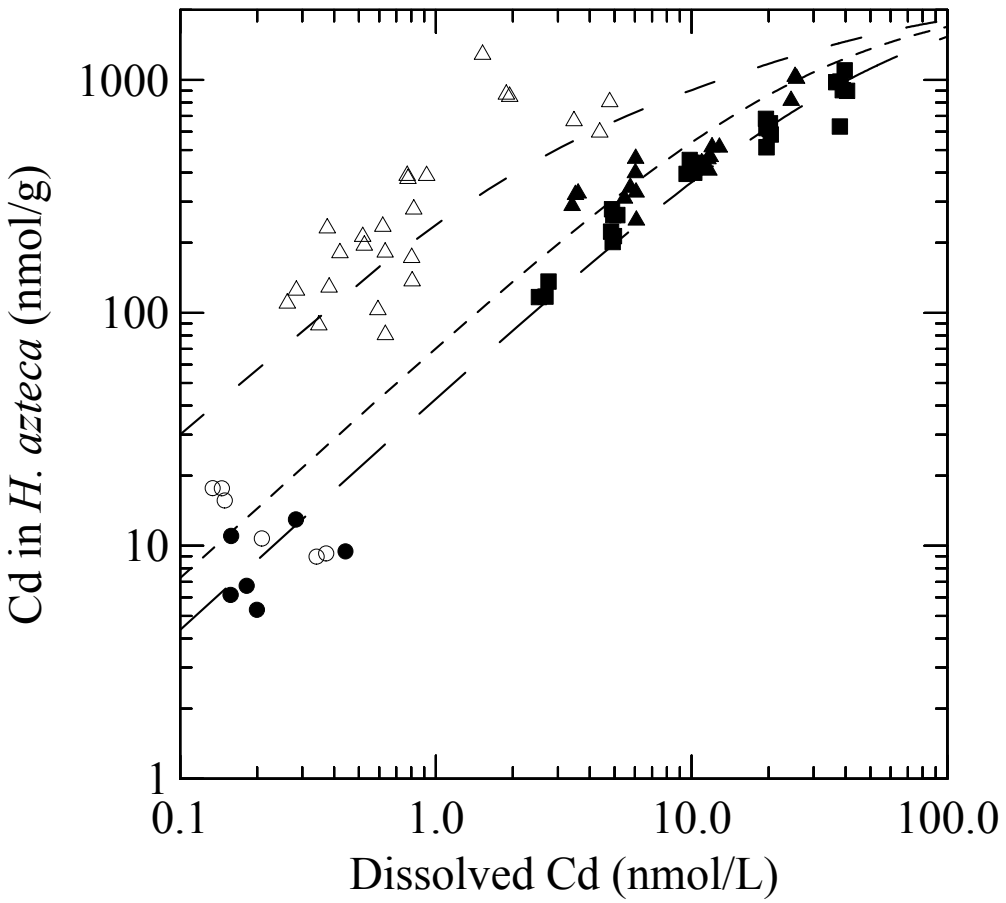


Figure 2.3 Measured 28 d Cd *H. azteca* (indicated by symbols) and modelled Cd in *H. azteca* (indicated by dashed lines, Eq. 2.11, $r^2 = 0.946$) as a function of dissolved Cd in the water treatment (closed square, large dash), food treatment (open triangle, medium dash) and food+water treatment (closed triangle, small dash). Cd in *H. azteca* feeding on control periphyton (open circle) and control TetraMin® (closed circle) is also shown.

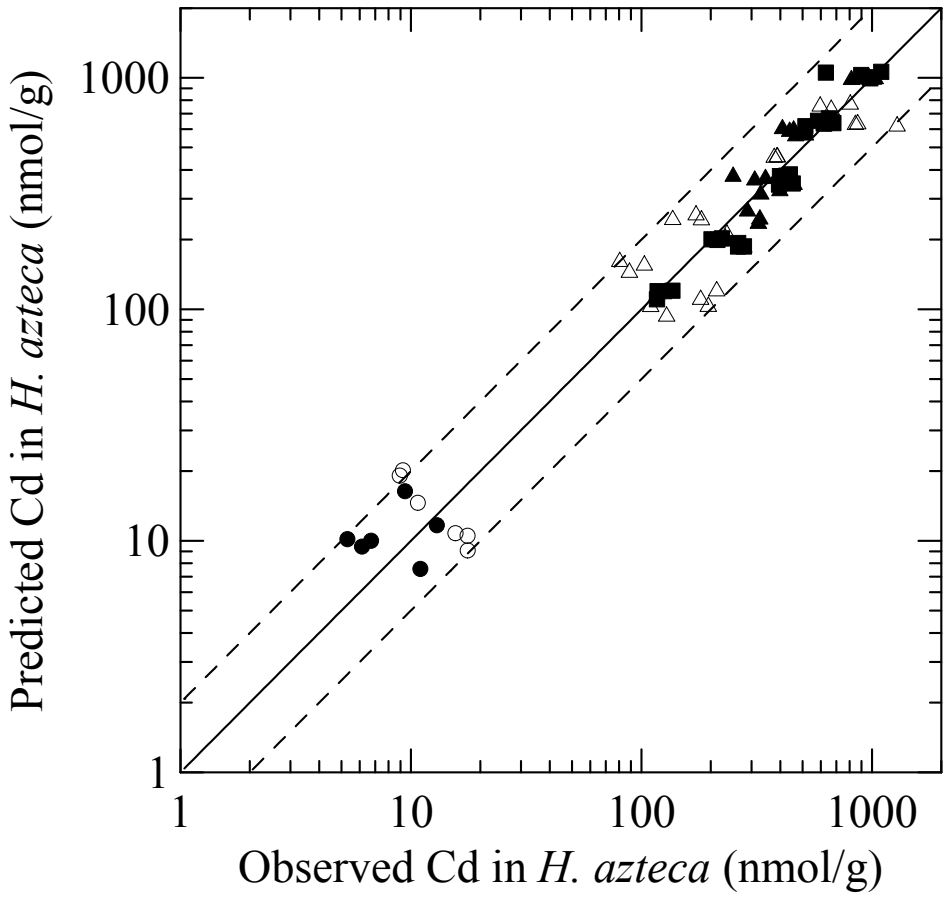


Figure 2.4 Cd in *H. azteca* predicted by the model (Eq. 2.11, $r^2 = 0.946$) as a function of the observed measured Cd in *H. azteca* after 28 d in the water treatment (closed square), food treatment (open triangle) and food+water treatment (closed triangle). Predicted versus observed Cd in *H. azteca* feeding on control periphyton (open circle) and control TetraMin® (closed circle) are also shown. Solid line is $y=x$. Dashed lines are $y=0.5x$ and $y=2x$.

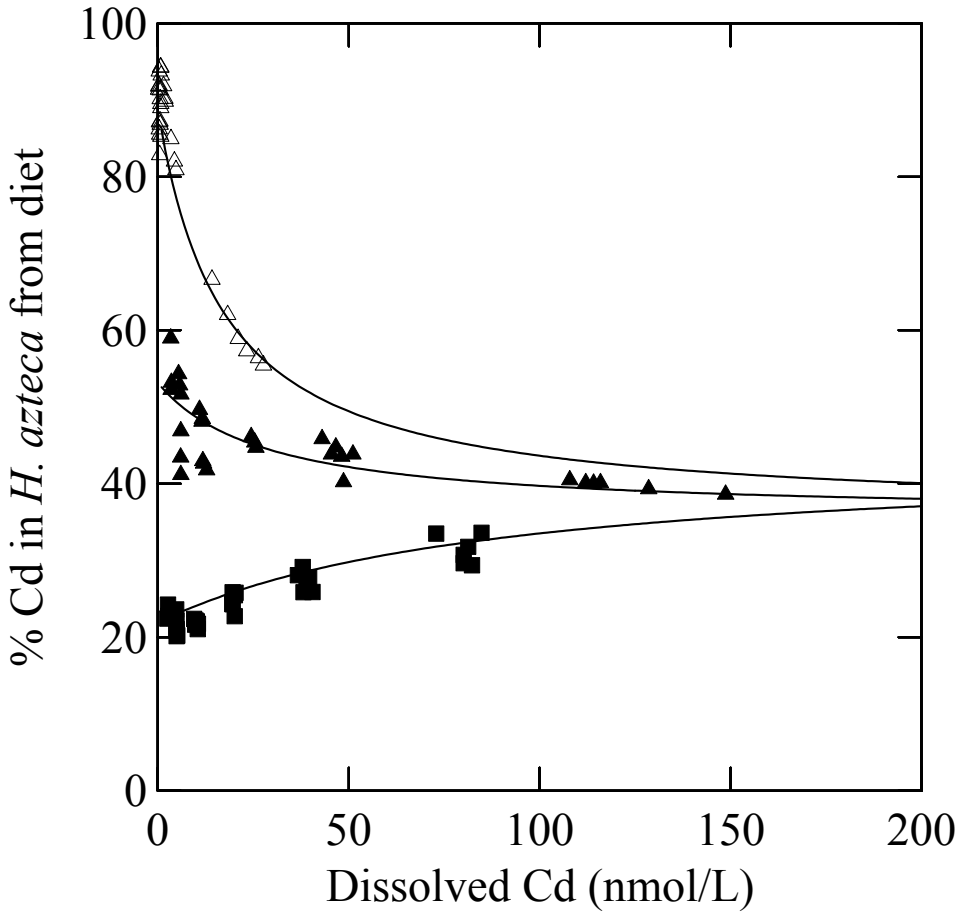


Figure 2.5 Percent Cd in *H. azteca* predicted to be from the diet after 28 d in the water treatment (closed square), food treatment (open triangle) and food+water treatment (closed triangle) as a function of dissolved Cd. Symbols are modelled (Eq. 2.12 or Eq. 2.13, $r^2 = 0.946$) values for each replicate. Solid lines are models fitted to the mean of the modelled values (Eq. 2.12 or Eq. 2.13, $r^2 = 0.946$). The theoretical dietary Cd contribution to *H. azteca* was calculated where 100 % mortality occurred, based on the measured dissolved and dietary Cd of the replicate.

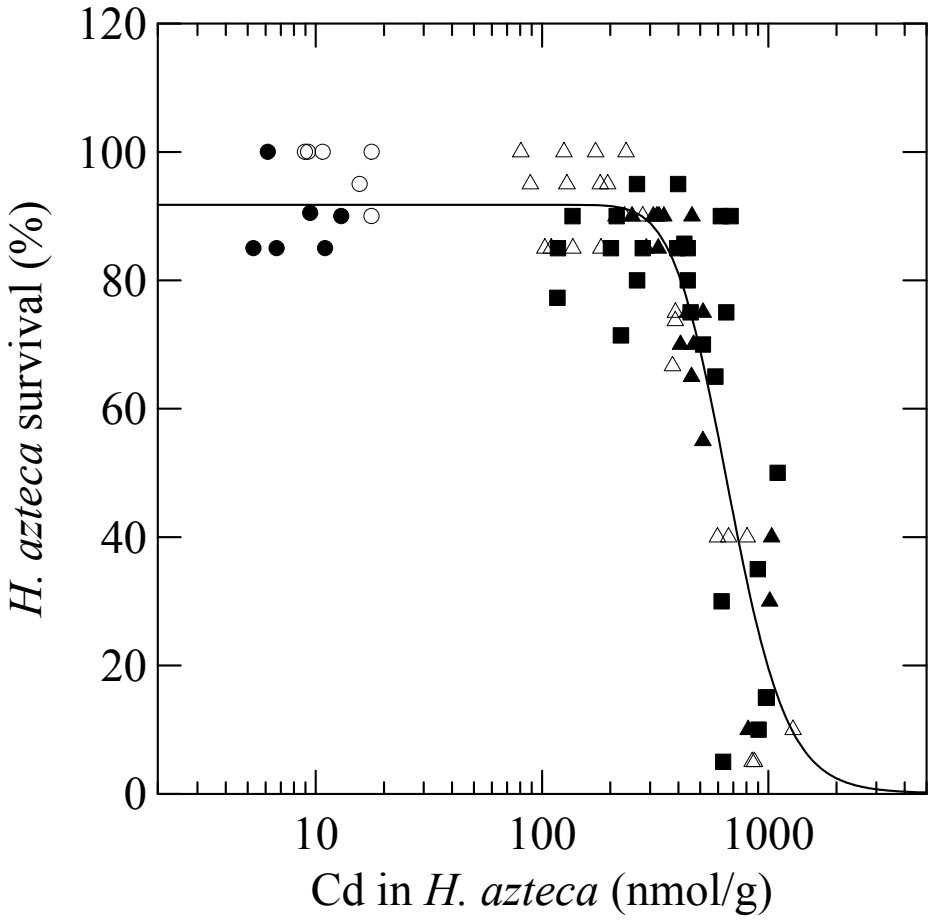


Figure 2.6 Percent survival of *H. azteca* as a function of Cd bioaccumulated by *H. azteca* after 28 d in the water treatment (closed square), food treatment (open triangle) and food+water treatment (closed triangle). Survival of *H. azteca* feeding on control periphyton (open circle) and control TetraMin® (closed circle) is also shown. Solid line is the model from Eq. 2.16 (r^2=0.710).

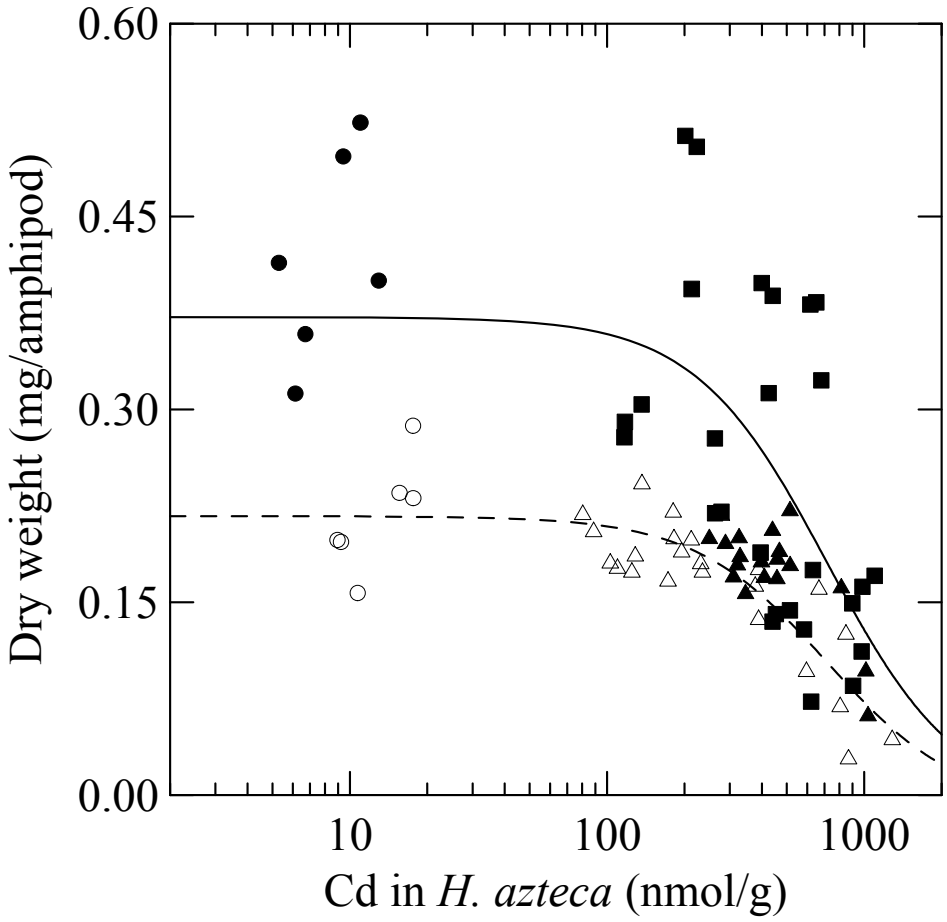


Figure 2.7 Dry weight of *H. azteca* as a function of Cd bioaccumulated by *H. azteca* after 28 d in the water treatment (closed square), food treatment (open triangle) and food+water treatment (closed triangle). Dry weight of *H. azteca* feeding on control periphyton (open circle) and control TetraMin® (closed circle) is also shown. Lines are modelled dry weight of *H. azteca* when fed on TetraMin® (Eq. 2.19, solid line, r^2=0.604) and periphyton (Eq. 2.19, dashed line, r^2=0.604).

Table 2.1 Nominal and mean measured Cd in exposure water (filtered 0.45 µm) and food and nutritional characteristics of TetraMin® and periphyton used in 28 d feeding experiment. Cd in food and food nutrition are given on an ash-free dry mass basis (standard deviations shown)

Food type[a]	Nominal Cd (nmol/L)	Measured Cd[b] (nmol/L)	Cd in food (nmol/g)	Organic content (%)	Chlorophyll *a* (mg/g)	Total lipid (%)	Total protein (%)
T	0	0.22 (0.10)	3.21 (0.26)	100[c] (0.00)	N/A	11.7 (0.41)	13.0 (0.25)
P	0	0.27 (0.03)	10.5 (1.58)	56.9 (14.5)	3.03 (0.39)	3.89 (0.17)	12.0 (4.12)
P	10	0.97[d]	389 (33.3)	69.7 (2.73)	2.33 (0.56)	4.44 (0.23)	7.00 (0.68)
P	50	18.8 (6.63)	531 (137)	55.4 (14.8)	3.14 (0.66)	4.79 (2.4)	12.5 (4.23)
P	100	31.2 (15.8)	1100 (265)	57.3 (12.8)	3.29 (0.68)	5.23[d]	12.6 (3.61)
P	500	180 (117)	4330 (1440)	58.7 (13.4)	3.61 (0.54)	3.50 (0.62)	12.1 (3.93)
P	1000	427 (276)	7620 (1670)	54.9 (13.5)	3.86 (0.70)	5.31 (0.32)	13.3 (3.47)
P	5000	3050 (1550)	26300 (5910)	59.0 (8.75)	3.69 (0.48)	4.62 (0.11)	11.1 (2.82)
Mean (SD)[e]				58.8 (5.03)	3.28 (0.52)	4.54 (0.67)	11.5 (2.10)

[a] T = TetraMin®, P = Periphyton

[b] Measured Cd (0.45 µm filtered) in exposure solution at 96 h except for Cd exposure at nominal 10 nM which was collected at 72 h. Dissolved Cd from this exposure was not reliable and was not used to calculate max and half saturation constants

[c] Dry weight and ash-free dry weight were equivalent for TetraMin®

[d] Sample size n=1 but was n=3 to 9 for other samples

[e] Mean and standard deviation of nutritional parameters in all periphyton

N/A TetraMin® was not analysed for chlorophyll *a*

To convert measurements on an ash-free dry mass basis to dry mass, multiply by the fraction of organic content

Table 2.2 Sum of fatty acid methyl ester (FAME µg/mg dry weight) composition of control TetraMin®, periphyton (on an ash-free dry mass basis) and *H. azteca* fed on control food or exposed to Cd primarily in water, food and food+water treatments for 28 d (Mean and standard deviations shown, n=2 for control food and *H. azteca* , n=1 for Cd contaminated *H. azteca*)

Fatty acid component	Control TetraMin®	Control periphyton	*H. azteca* fed on control TetraMin®	*H. azteca* fed on control periphyton	[a]*H. azteca* W12.5 fed on TetraMin®	[b]*H. azteca* F500 fed on periphyton	[c]*H. azteca* FW12.5 fed on periphyton
∑ω3	7.26 (0.55)	3.33 (0.04)	10.1 (1.07)	9.10 (1.16)	9.65	10.7	10.1
∑ω6	14.7 (1.14)	1.74 (0.07)	14.9 (1.96)	9.01 (0.79)	12.3	8.16	7.94
∑ω3/∑ω6	0.49	1.92	0.68	1.01	0.78	1.32	1.27
∑SAFA	22.7 (1.80)	4.75 (0.21)	12.8 (0.95)	11.6 (1.10)	11.7	12.1	12.4
∑MUFA	10.7 (1.30)	3.97 (0.03)	16.0 (3.14)	10.6 (1.96)	13.5	12.8	11.0
∑PUFA	22.3 (1.00)	5.16 (0.04)	27.5 (3.42)	19.8 (2.18)	24.3	20.8	19.7
Total	55.6 (5.06)	13.9 (0.22)	56.4 (7.51)	41.9 (5.23)	49.4	45.7	43.2

[a] *H. azteca* exposed to nominal dissolved Cd of 12.5 nmol/L

[b] *H. azteca* exposed to nominal dissolved Cd of 0 nmol/L and periphyton exposed to 500 nmol/L Cd

[c] *H. azteca* exposed to nominal dissolved Cd of 12.5 nmol/L and periphyton exposed to 100 nmol/L Cd

∑ω3 = sum of omega-3 fatty acids

∑ω6 = sum of omega-6 fatty acids

∑ω3/∑ω6 = sum of ratio of omega-3/omega-6 fatty acids

∑SAFA = sum of saturated fatty acids

∑MUFA = sum of mono-unsaturated fatty acids

∑PUFA = sum of poly-unsaturated fatty acids

Total = sum of individual fatty acids

Table 2.3 Mean measured Cd in water, food and *H. azteca* in feeding experiment for 28 d and mean measured Cd in adult *H. azteca* caged and unfed for 7 d in the same treatment containers (standard deviations shown, n=3 or 6)

Treatment and food type[a]	Cd in water[b] (nmol/L)	Cd in food[c] (nmol/g)	Measured Cd in *H. azteca* (nmol/g)	Measured Cd in caged *H. azteca* (nmol/g)
Control				
T	0.22 (0.11)	17.3 (1.70)	8.16 (3.03)	12.5 (0.51)
Control				
P	0.21 (0.11)	17.2 (4.31)	12.8 (4.12)	13.2 (1.52)
Cd in Water				
T	19.9 (0.39)	964 (74.6)	609 (57.8)	184 (28)
Cd in Food				
P	0.39 (0.16)	353 (89.8)	104 (19.2)	14.3 (0.87)
P	0.65 (0.17)	714 (126)	200 (51.3)	16.9 (1.23)
P	1.49 (1.03)	3270 (1139)	384 (6.60)	25.0 (2.21)
P	2.72 (1.41)	5830 (1100)	819 (241)	20.8 (2.64)
Cd in Food+Water				
P	25.8 (2.62)	3590 (1160)	949 (122)	179 (36.1)

[a] T = TetraMin®, P = Periphyton

[b] Measured dissolved (0.45µm, Acrodisc®) Cd concentration

[c] Measured Cd in food on an ash-free dry mass basis can be converted to dry mass by multiplying by organic content fraction (i.e. 1 or 0.588 for TetraMin® or periphyton, respectively)

Table 2.4 Chronic Cd saturation bioaccumulation model parameters (max and K) for *H. azteca* with 95 % confidence limits shown. Parameters were calculated for exposure to either dissolved Cd or Cd^{2+} and two food types (TetraMin® and periphyton) on an ash-free dry mass basis

Source of Cd	max[a]	95% CL	K[b]	95% CL	max/K[c]	95% CL
Dissolved Cd model[d]						
Water	1453	242 - 8740	42.6	5.07 - 357	34.1	21.3 – 54.8
TetraMin®	1130	0.010 – $1.26x10^8$	6170	0.023 – $1.63x10^9$	0.183	0.047 – 0.706
Periphyton	813	526 - 1257	2173	1143 - 4132	0.374	0.277 – 0.505
Cd^{2+} model[e]						
Water	1316	274 – 6314	27.7	4.13 – 186	47.5	29.6 – 76.4
TetraMin®	1266	0.045 – $354x10^5$	6444	0.104 – $400x10^5$	0.197	0.057 – 0.675
Periphyton	822	532 – 1272	2231	1173 - 4241	0.369	0.273 – 0.497

[a] max_w, max_{ft}, max_{fp} for water, TetraMin® and periphyton respectively in Eq. (2.11) (nmol/g *H. azteca*)

[b] K_w (nmol/L), K_{ft} (nmol/g AFDM), K_{fp} (nmol/g AFDM) half saturation constants for water, TetraMin® and periphyton in Eq. (2.11)

[c] Unit of max/K is L/g for Cd bioaccumulated from water and g AFDM food/g *H. azteca* for Cd bioaccumulated from TetraMin® or periphyton

[d] model fit r^2=0.946, n=81, P<0.001

[e] model fit r^2=0.947, n=81, P<0.001

Table 2.5 Control mortality rate (m'), half saturation constant (K"), exponent (n) was fixed to 100, sample size (N), model fit (r^2) LC10, LC25 and LC50 (with 95% CL) for *H. azteca* mortality as a function of dissolved Cd and free Cd^{2+}

Treatment	Mortality saturation model as a function of dissolved Cd and Cd^{2+}										
	m'	K" (nmol/L)	n	N	r^2	LC10 (nmol/L)	95% CL (nmol/L)	LC25 (nmol/L)	95% CL (nmol/L)	LC50 (nmol/L)	95% CL (nmol/L)
Dissolved Cd model											
Cd in water	0.035	0.871	100	33	0.738	17.4	13.4 – 22.7	22.1	17.9 – 27.1	28.7	24.6 – 33.3
Cd in food	0.011	0.024	100	28	0.680	0.685	0.499 – 0.940	0.977	0.733 – 1.30	1.55	1.12 – 2.14
Cd in food+water	0.018	0.300	100	22	0.847	8.04	6.36 – 10.2	11.1	9.25 – 13.4	16.7	14.0 – 20.0
Cd^{2+} model											
Cd in water	0.036	0.602	100	33	0.709	12.4	9.34 – 16.6	15.9	12.7 – 19.9	20.9	17.7 – 24.6
Cd in food	0.011	0.017	100	28	0.688	0.501	0.367 – 0.683	0.712	0.537 – 0.944	1.12	0.821 – 1.54
Cd in food+water	0.018	0.220	100	22	0.846	5.97	4.72 – 7.56	8.3	6.88 – 10.0	12.5	10.4 – 15.1

Table 2.6 Control mortality rate (m'), half saturation constant (K"), exponent (n) was fixed to 100, sample size (N), model fit (r^2) LFC10, LFC25 and LFC50 (with 95% CL) for *H. azteca* mortality as a function of dietary Cd on an ash-free dry mass basis

Treatment	Mortality saturation model as a function of dietary Cd on an ash-free dry mass basis										
	m'	K" (nmol/g)	n	N	r^2	LFC10 (nmol/g)	95% CL (nmol/g)	LFC25 (nmol/g)	95% CL (nmol/g)	LFC50 (nmol/g)	95% CL (nmol/g)
Cd in water	0.035	44.2	100	33	0.775	853	675 – 1080	1070	890 – 1290	1370	1200 – 1570
Cd in food	0.015	48.9	100	28	0.801	1400	837 – 2350	1990	1280 – 3090	3140	2240 - 4390
Cd in food+water	0.016	22.9	100	22	0.816	688	535 – 886	997	807 – 1230	1630	1270– 2090

Table 2.7 Control mortality rate (m'), half saturation constant (K"), exponent (n) was fixed to 100, sample size (N), model fit (r^2) LBC10, LBC25 and LBC50 (with 95% CL) for *H. azteca* mortality as a function of *H. azteca* body Cd (dry weight)

Treatment	Mortality saturation model as a function of total body Cd in *H. azteca*										
	m'	K" (nmol/g)	n	N	r^2	LBC10 (nmol/g)	95% CL (nmol/g)	LBC25 (nmol/g)	95% CL (nmol/g)	LBC50 (nmol/g)	95% CL (nmol/g)
Cd in water	0.032	22.3	100	33	0.58	459	344 – 611	585	472 – 724	768	648 – 910
Cd in food	0.014	14.0	100	28	0.877	308	250 – 380	400	335 – 478	541	468 – 626
Cd in food+water	0.010	13.2	100	22	0.815	339	288 – 399	462	404 – 527	673	567 – 798
All treatments	0.021	17.3	100	79	0.710	385	334 - 444	501	448 – 561	679	617 – 747

Table 2.8 Control dry weight (W'), coefficient (a) and exponent (n), sample size (N) model fit (r^2), IC10, IC25 and IC50 (with 95% CL) for *H. azteca* growth inhibition as a function of dissolved Cd and free Cd^{2+}

Treatment	Growth inhibition model as a function of dissolved Cd and Cd^{2+}										
	W' (mg)	a (mg)	n (nmol/L)	N	r^2	IC10 (nmol/L)	95% CL (nmol/L)	IC25 (nmol/L)	95% CL (nmol/L)	IC50 (nmol/L)	95% CL (nmol/L)
Dissolved Cd model											
Cd in water	0.412	0.079	854	33	0.445	1.49	0.080 – 27.6	5.39	0.844 – 34.5	19.5	7.47 – 51.1
Cd in food	0.284	0.912	0.725	30	0.487	0.055	0.0001 – 19.2	0.250	0.004 – 16.4	1.14	0.086 – 15.0
Cd in food+water	0.194	N/A	31.1	24	0.625	23.6	21.6 – 25.7	24.4	23.3 – 25.6	25.3	24.8 – 25.8
Cd^{2+} model											
Cd in water	0.417	0.125	0.789	33	0.425	0.861	0.031 – 24.2	3.46	0.418 – 28.7	13.9	4.71 – 41.2
Cd in food	0.277	1.09	0.764	30	0.493	0.050	0.0003 – 9.42	0.212	0.0005 – 8.62	0.894	0.100 – 8.41
Cd in food+water	0.194	N/A	31.1	24	0.625	17.8	16.3 – 19.4	18.4	17.6 – 19.4	19.1	18.7 – 19.5

N/A not applicable as constant was substituted for ICX in the model to obtain a fit

Table 2.9 Control dry weight (W'), coefficient (a) and exponent (n), sample size (N) model fit (r^2), IFC10, IFC25 and IFC50 (with 95% CL) for *H. azteca growth* inhibition as a function of dietary Cd on an ash-free dry mass basis

Treatment	Growth inhibition model as a function of dietary Cd on an ash-free dry mass basis										
	W' (mg)	a (mg)	n (nmol/g)	N	r^2	IFC10 (nmol/g)	95% CL (nmol/g)	IFC25 (nmol/g)	95% CL (nmol/g)	IFC50 (nmol/g)	95% CL (nmol/g)
Cd in water	0.421	0.004	0.828	33	0.452	61.8	2.43 – 1570	233	28.8 – 1880	878	295 – 2610
Cd in food	0.213	616	1.14	30	0.616	616	107 – 3560	1610	550 – 4710	4210	2490 – 7120
Cd in food+water	0.205	N/A	1.9	24	0.549	870	336 - 2250	1550	913 – 2640	2770	1980 - 3870

N/A not applicable as constant was substituted for IFCX in the model to obtain a fit

Table 2.10 Control dry weight (W'), coefficient (a) and exponent (n), sample size (N) model fit (r^2), IBC10, IBC25 and IBC50 (with 95% CL) for *H. azteca* growth inhibition as a function of *H. azteca* body Cd (dry weight)

Treatment	Growth inhibition model as a function of *H. azteca* body Cd (dry weight)										
	W' (mg)	a (mg)	n (nmol/g)	N	r^2	IBC10 (nmol/g)	95% CL (nmol/g)	IBC25 (nmol/g)	95% CL (nmol/g)	IBC50 (nmol/g)	95% CL (nmol/g)
Cd in water	0.409	$3.19x10^{-4}$	1.26	33	0.445	106	17.7 – 631	253	84.0 – 762	607	343 – 1073
Cd in food	0.207	0	2.25	30	0.75	251	134 – 472	409	273 – 613	668	533 – 836
Cd in food+water	0.194	N/A	9.47	24	0.682	778	580 – 1043	873	735 – 1040	981	914 - 1052
All treatments	0.282	$9.3x10^{-5}$	1.40	81	0.322	156	48.7 - 497	340	169 - 685	744	510 - 1090

N/A not applicable as constant was substituted for IBCX in the model to obtain a fit

2.5 DISCUSSION

2.5.1 *Food characterization*

Periphyton communities are dynamic and change in composition, biomass and biochemistry with environmental parameters such as temperature, light, substrate type, nutrients and grazing pressure (Vermaat, 2005; Huggins *et al.*, 2004). Nutritional quality of periphyton batches used for feeding experiments was within the range of recorded values (Azim and Asaeda, 2005; Bradac *et al.*, 2009a) and varied in % organic matter, chlorophyll *a* and % total protein. Periphyton nutritional quality in the feeding experiments was standardized to some extent by providing food rations based on organic content rather than dry mass so that there was consistency between batches and food type. Variation in the nutritional quality of the periphyton with batch did not influence the nutritional parameters or survival and growth of *H. azteca* in the controls.

Long-term metal exposure favours metal tolerant periphyton species resulting in an altered community structure and potentially altered nutritional quality (Ivorra *et al.* 1999; Real *et al.*, 2003). This effect was minimized by using a short (96 h) Cd exposure which resulted in no marked changes in % total lipid and protein with increasing Cd. Gold *et al.* (2003) exposed pre-established natural periphyton, dominated by diatoms, to Cd (89 – 890 nmol/L) for 14 d and found no significant changes in species composition. Periphyton used in the feeding experiments consisted of cosmopolitan algal species found in periphyton collected from both Cd contaminated and non-contaminated lakes within Canada (McCabe and Cyr, 2006). The algal taxonomic and nutritional composition indicates that any potential adverse effects observed in *H. azteca* feeding on Cd exposed periphyton were due to the increasing concentration of the metal and not the altered nutritional quality of the food. Nutritional quality of the TetraMin® diet used in the water treatment of the feeding experiment was comparable to periphyton in percent total protein but was of superior quality in terms of a higher concentration of total lipid and FAs.

2.5.2 *Bioconcentration of Cd in periphyton and TetraMin®*

Dissolved Cd concentrations that periphyton were pre-exposed to were designed to produce a broad range of Cd concentrations in the periphyton in order that a dose

response curve could be obtained. Final periphyton Cd concentrations were representative of periphyton growing in a gradient of chronically contaminated field sites. For example, periphyton collected from the Coeur d'Alene River, which has received mining and smeltering metal contamination since 1885, had Cd ranging from 62.3 to 7860 nmol/g dry weight (Farag *et al.*, 1998) which, if the mean % organic content of 58.8 % (Table 2.1) is used to convert to AFDM, equates to 106 to 13,400 nmol/g AFDM. This encompassed most of the Cd periphyton concentrations used in the feeding experiments. At extremely contaminated sites, such as the Riou Mort River, Cd in periphyton was as high as 16,100 nmol/g dry weight (Morin *et al.*, 2008b) or 27,400 nmol/g AFDM assuming 58.8 % organic content, which is not significantly different from the highest periphyton concentration used in these feeding experiments. In the latter example, Cd in periphyton was correlated more to particulate rather than dissolved Cd, suggesting that not all the Cd was associated with the biological component of the periphyton. Incorporation of particulates and precipitation of Fe and Mn oxides into the periphyton matrix can result in misleading estimates of metal bioavailability for trophic transfer (Newman *et al.*, 1985; Newman and McIntosh, 1989). Because the bioavailability of different forms of Cd associated with biotic and abiotic components of periphyton was unknown, a conservative approach of recording all Cd in periphyton on an AFDM, i.e. organic, basis was used here to represent the maximum bioavailable fraction of Cd in food.

The capacity of periphyton to bioconcentrate metal from the surrounding water was evident by the 4 order of magnitude increase in periphyton Cd relative to the measured water concentration at 96 h. The BCFs calculated for each concentration were representative of those found in a range of contaminated field sites (Morrin *et al.*, 2008b; Stephenson and Turner, 1993). Although equilibrium conditions may not have been achieved due to the daily spiking of the solution, uptake as measured by change in dissolved Cd every 24 h, appeared to follow the same biphasic response observed when exposing algal monocultures where equilibrium was present (Sloof *et al.* 1995). The large uptake in the first 24 h likely represented adsorption of Cd to algae, exopolymer substances and oxide surfaces and the subsequent reduced uptake suggests that external binding sites had become saturated and Cd was becoming bioincorporated into the

dominant algal component. When the highest Cd exposed periphyton was subsequently placed in non-contaminated SAM for the food treatment, 44 % of the Cd was released from the periphyton. When this same periphyton was placed in 100 nmol/L Cd in the food+water treatment, the outwards diffusion gradient would have been substantially reduced and yet still 44 % of Cd was released from the periphyton. This suggests that the initial release was due to surface-bound Cd while bioincorporated Cd was slower to release.

Model values max_p and K_p describing Cd uptake by periphyton could not be compared to the adsorption of Cd by TetraMin® because TetraMin® did not saturate with increasing dissolved Cd. However, the slopes for Cd in periphyton (40.9 slope, 19 – 88 95 % CL) and TetraMin® (48.8 slope, 46.1 – 51.6 95 % CL) with respect to dissolved Cd had overlapping 95 % CL suggesting that Cd partitioning was similar for both foods within the 10 to 100 nmol/L Cd range. However, studies show that while the amount of Cd associated with the different foods is similar, the way in which Cd is stored within the food, i.e., bioincorporation in the case of periphyton versus adsorption in the case of TetraMin®, may influence metal bioavailability to the consumer (Reinfelder and Fisher, 1991).

2.5.3 *Cd in food and water of feeding experiment*

Measurements of Cd in the food and water of the feeding experiment indicated that there was dynamic partitioning of Cd between food and water compartments. In the water treatment, Cd adsorbed to TetraMin® resulting in a secondary dietary exposure for *H. azteca* while, in the food treatment, Cd was released from periphyton resulting in an increasing gradient of secondary waterborne Cd exposure for *H. azteca*. Released Cd was shown to be bioavailable to *H. azteca* as indicated by Cd bioaccumulation in caged *H. azteca* in the food treatment. In the food+water treatment, periphyton both absorbed and released Cd but neither exposure route was significantly skewed by the influence of the other.

2.5.4 *Bioaccumulation of Cd in H. azteca*

The fact that *H. azteca* bioaccumulated Cd from periphyton is important as it verifies what was observed in the field by Stephenson and Turner (1993) and confirms that the laboratory assay is representative of the field in terms of dietary Cd bioavailability. However, secondary exposures made it difficult to irrefutably attribute Cd in *H. azteca* to a single source when comparing bioaccumulation across water, food and food+water treatments. The saturation bioaccumulation model provided a means to mathematically separate the contributions of Cd from either food or water to Cd bioaccumulated in *H. azteca*.

2.5.4.1 *Bioaccumulation model*

Using the biokinetic model (Eq. 2.1) as a foundation, physiological processes such as metal uptake, excretion, ingestion, assimilation efficiency and growth were collectively estimated using non-linear regression and were represented by the metal binding saturation terms of max and K (Eq. 2.10). The advantage of this approach was that it negated the need for empirically deriving each of the physiological processes represented in the model in order to address the ultimate objective of determining the relative contribution of Cd from water and food. However, the disadvantage of this approach was that it removed the ability to attribute the observed bioaccumulation and toxicity of Cd to a specific physiological process (Croteau and Luoma, 2008). The model assumption of steady state conditions was likely met over the whole 28 d, although Cd fluctuations occurred on shorter time scales due to the movement of Cd between food and water. The assumption of additivity of tissue concentration was generally upheld because of the mostly linear relationship between body concentration and increasing Cd in water and food.

2.5.4.2 *Model parameters*

Differences in the max and K model parameters estimated for each source of Cd (water, TetraMin®, periphyton) for *H. azteca* were not significant, as indicated by overlapping 95 % confidence limits. However, it is possible that differences in biokinetic processes such as uptake and excretion rates represented by these model terms could vary with Cd

source. For example, a high uptake rate or low excretion rate constant of Cd from water relative to food would result in a comparatively higher max_w. In addition, differences in the half saturation constant of assimilation efficiency and ingestion rate of TetraMin® as compared to periphyton could alter the K_{ft} and K_{fp}. Though few comparable bioaccumulation modelling approaches exist for freshwater invertebrates, model parameters max_{fp} and K_{fp} in the present study were found to be seven and four fold greater, respectively, than those measured using pulse/chase feeding techniques for the freshwater snail *Lymnaea stagnalis* fed Cd contaminated lettuce for 18 h (Croteau and Luoma, 2008). However, the initial slope of bioaccumulation was just 1.5 times greater for *H. azteca* feeding on periphyton compared to *L. stagnalis* feeding on lettuce. Differences in model parameters are likely to be species and diet specific and related to acute versus chronic exposures.

The initial slope (max/K) of Cd uptake by *H. azteca* was compared between water and food for each of the treatments and provided an indication of the relative importance of each Cd source to bioaccumulation. In the water treatment, where TetraMin® was in equilibrium with surrounding water, dissolved Cd had a higher initial slope and was therefore more important to bioaccumulation at low dissolved Cd than Cd associated with TetraMin®. In the food treatment, periphyton was most important, and in the food+water treatment, Cd in water and periphyton were of equal importance to bioaccumulation in *H. azteca* at low dissolved Cd. This demonstrates that bioaccumulation of Cd in *H. azteca* is not a function of waterborne exposure only and that dietary Cd can become very important especially under non-equilibrium conditions.

Model parameters based on dissolved Cd were not detectably different from those based on free Cd^{2+} due to the low level of DOC (maximum DOC=1 mg/L in a TetraMin® control) present in the feeding experiments. Parameters based on Cd^{2+} would be more applicable in natural waters where DOC is higher.

2.5.4.3 *Total body Cd concentration*

The dietary bioaccumulation model accurately predicted 28 d Cd in *H. azteca* in water, food and food+water treatments based on Cd measured in food (AFDM basis) and filtered (0.45 µm) water. Borgmann *et al.* (2010) and Schroeder (2008) developed 7 d and 28 d bioaccumulation models of dissolved Cd uptake by *H. azteca* that account for interactions of Ca^{2+} and H^{+} with Cd at binding sites. Their 28 d model (model 5) began as a 7 d bioaccumulation and was adjusted for acclimation to Ca, inhibition of acclimation to Ca by Cd, and growth dilution (Borgmann *et al.*, 2010). Because *H. azteca* were fed on TetraMin® over the 7 d, the model indirectly includes a dietary component. Predictions of body concentration from model 5 and Eq. 2.10 were compared for the water treatment and found to be within a factor of 2 of the measured body concentration. Therefore 28 d total body Cd can be predicted accurately from dissolved Cd, Ca and pH, however, model 5 does not contain an explicit diet component with which to predict the contribution of Cd in diet to total body concentration. In contrast, the 28 d diet bioaccumulation model (Eq. 2.10) can estimate the contribution of Cd in diet to total body concentration but the accuracy of the prediction is constrained by the water chemistry and the ratio of Cd in food:water of the experimental system under which the model was developed. Neither model is complete. Further research is needed to determine the relative amount of Cd accumulated from food under different water chemistry conditions before a complete model can be developed.

2.5.4.4 *Contribution of Cd in food to body concentration*

The 28 d bioaccumulation model predicted that diet could contribute 21 % to 98 % of the Cd in *H. azteca*. Therefore dietary Cd can make a significant contribution to bioaccumulation in *H. azteca*. Stephenson and Turner (1993) found 58 % of Cd in field *H. azteca* came from periphyton and Borgmann *et al.* (2007) estimated up to 25 % of Cd in caged *H. azteca* in the field came from food. Dietary Cd has also been found to contribute as much as 88 to 100 % in other freshwater invertebrates (Croteau and Luoma, 2008; Munger and Hare, 1997; Xie *et al.*, 2010), whereas in fish the contribution of Cd from diet appears to be organ specific with wild yellow perch having as much as 30 % of the Cd in the gut coming from food (Kraemer *et al.*, 2006).

The change in the percent contribution of dietary Cd to *H. azteca* as a function of dissolved Cd for each treatment was dependent on the max and K parameters of Cd bioaccumulation in *H. azteca* from water and food. In the water treatment, max was similar for water and TetraMin® but the initial slope (max/K) was lower for TetraMin® than water, therefore Cd uptake was initially greatest from the dissolved pathway but the Cd contribution from TetraMin® increased as both profiles plateaued at a similar max with increasing dissolved Cd. In the food and food+water treatments, the initial slope (max/K) for periphyton was higher than or similar to water but the max term was higher for water than for periphyton. Therefore, Cd uptake in *H. azteca* was initially greatest or equivalent from periphyton, but dissolved Cd superseded that contribution with increasing dissolved Cd.

Because the contribution of Cd from food to body concentration was estimated using the bioaccumulation model parameters, the estimates are subject to the uncertainty surrounding the model parameters which was particularly high for max and K based on TetraMin® in the water treatment. The upper and lower 95 % confidence limits on the predictions of the Cd contribution from food expanded progressively with increasing dissolved Cd. The expansion of these limits meant that even though the probability that the limits containing the true dietary Cd contribution continued to be 0.95, the accuracy of the contribution decreased with increasing dissolved Cd. Therefore care must be taken when interpreting the estimated contribution of Cd in *H. azteca* from food at water concentrations where the upper or lower limits of the estimate were greater than a factor of two. This corresponds to all dissolved Cd in the water treatment and concentrations greater than 50 nmol/L in the food+water treatment.

The similarity in the contribution of Cd from food when based on dissolved or Cd^{2+} was due to the low DOC present in test solutions therefore, contributions would be expected to differ at higher environmental DOC concentrations.

2.5.5 *Survival*

Chronic exposure of *H. azteca* to Cd in a periphyton diet significantly reduced survival at a Cd concentration of 5830 nmol/g AFDM in the food treatment with secondary dissolved Cd of 2.72 nmol/L. Furthermore, *H. azteca* tissue concentration (819 nmol/g) in the same food treatment was 7 fold higher than tissue concentration (123 nmol/g) at a similar dissolved Cd (2.65 nmol/L) in the water treatment where survival was unaffected. Therefore Cd in a natural periphyton diet was bioavailable to *H. azteca* and contributed to lethal body concentrations.

There are few studies that report dietary metal effects on survival of invertebrates or fish because most studies are focused on chronic sublethal effects (Handy *et al.*, 2005). However, it is important to verify the concentration at which diet has a significant effect on survival in order to assess whether such a scenario is likely to occur in the environment. The food treatment outlined above represents a scenario that would not be likely where equilibrium conditions exist. However, a more likely scenario of a contaminated site was observed in the food+water treatment where survival was significantly reduced at dissolved Cd of 11.8 nmol/L and dietary Cd of 1050 nmol/g AFDM. At these concentrations dietary Cd was predicted to contribute 45 % to total body concentration so both food and water could potentially contribute to toxicity.

Predicting chronic lethality has traditionally involved deriving a critical water concentration at which a specific reduction in survival occurs, i.e., LC50. The chronic LC50 derived in the water treatment based on dissolved Cd (28.7 nmol/L 24.6 – 33.3 95 % CL) compared well with that derived by Borgmann *et al.* (2004a) of 22.0 nmol/L (18.4 – 26.2 95 % CL) in SAM. However, the Cd LC50 varied depending on water chemistry or more specifically, Ca^{2+}, H^{+} and DOC (Borgmann *et al.*, 1991; Borgmann *et al.*, 2010; Schroeder, 2008). In the present research, variability in the LC50 was also observed when food became an important source of Cd uptake. For example, the LC50 in the food and food+water treatments was lower than that for the water treatment not because the dissolved Cd had become more toxic but because the toxicity of the food was being reflected by the LC50. Therefore using a guideline that does not directly account for

changes in water chemistry or the dominant food source may result in under protection of metal sensitive invertebrates such as *H. azteca*. A reliable comparison of toxicity could not be obtained when mortality was based on Cd concentration in food alone, again due to the contribution of low concentrations of dissolved Cd leaching from food.

All Cd exposure pathways were integrated when toxicity was based on body concentration. In the water treatment, the LBC50 was 768 nmol/g (648 - 910 nmol/g 95 % CL) which compared well to 847 nmol/g reported by Borgmann *et al.* (2010). The pooled data LBC50 (679 nmol/g) was slightly lower than the water treatment but 95 % confidence limits overlapped due to the influence of dietary Cd. Borgmann *et al.*, (1991) also found that endpoints based on body concentration did not vary significantly even when *H. azteca* were exposed to Cd with inorganic and organic complexing agents, Lake Ontario water, distilled water or sediment, unlike endpoints based on water concentration. Therefore, body concentration has been shown to be a robust measurement on which to base chronic toxic effects of Cd from multiple and simultaneous exposure pathways to *H. azteca*. Most importantly, because the critical body concentrations for all treatments were so similar, the route of exposure has similar toxic effects at the whole organism level. This does not imply that the mode of Cd toxicity is the same for water and dietary sources but that the effects are predictable based on body concentration.

2.5.6 *Linking contribution of Cd from food to toxicity*

Given that the bioaccumulation model successfully estimated chronic Cd body concentration and that body concentration was a robust predictor of toxicity, the model can be used to attribute source of Cd to lethal toxicity via body concentration. The LCX and LFCX concentrations from each treatment were entered into the bioaccumulation model to predict % Cd in *H. azteca* from food. At the LBC50 of 679 nmol/g, the contribution of Cd in *H. azteca* from food was predicted to be 26 %, 90 % and 46 % for the water, food and food+water treatments respectively. This contribution from diet varied little with effect level due to the steep slope of the survival versus bioaccumulation relationship. Therefore, Cd in diet made a marked contribution to the body concentration

at which survival was predicted to be reduced by 50 % and therefore also contributed markedly to chronic effects on survival.

2.5.7 *Growth*

Dry mass of *H. azteca* was significantly lower in periphyton fed animals as compared to those fed TetraMin® and is likely to be a reflection of the nutritional differences in the food. Inhibition of growth based on Cd in water, food and body concentration was evident, however, the variability of *H. azteca* dry mass meant that model fits to determine growth inhibition effects were generally poor thereby making the growth endpoints less robust than the mortality based endpoints. Borgmann *et al.* (2004a, 1993) also found *H. azteca* growth to be less sensitive than mortality for metal exposure. In contrast Ball *et al.* (2006) found that in the absence of a definitive lethal dose response relationship, *H. azteca* growth was more sensitive to Cd in a dried algal diet. Growth inhibition as a function of Cd in food (IFC50) occurred at 456 fold lower Cd in food (on equivalent dry weight basis) than the current research, possibly as a result of indirect toxicity such as food unpalatability or food avoidance. Unlike Ball *et al.* (2006), a relationship between Cd body concentration and growth did exist in the current research suggesting that growth was directly affected by dietary Cd although the mechanism of toxicity is unknown and it is likely that there is a combination of direct and indirect toxicity mechanisms.

2.5.8 *Nutritional effects*

High survival of *H. azteca* in both TetraMin® and periphyton fed controls after 28 d suggested no effect on survival due to nutritional differences in food type. However, dry weight of *H. azteca* feeding on control periphyton was significantly reduced and amplexus (mating behaviour) was delayed in those animals fed periphyton as compared to TetraMin® indicating sublethal nutritional effects. *H. azteca* dry weight and the onset of amplexus are closely related to both food nutritional quality and quantity (Hargrave, 1970; Moore and Farrar, 1996).

In general, total protein and total lipid in *H. azteca* did not reflect nutritional differences with food type. However, the lower PUFAs present in periphyton relative to TetraMin® were also lower in *H. azteca* consuming periphyton relative to TetraMin®. Certain essential PUFAs (ω-3 and ω-6 FAs) can only be obtained via the diet and are important for growth and egg production in amphipods (Hyne *et al.*, 2009). The ratio of ω-3 to ω-6 differed with food type and this was transferred to *H. azteca* signifying the importance of food source in obtaining essential PUFAs (Kainz *et al.*, 2009). Therefore it seems likely that the reduction in *H. azteca* dry weight and delay in amplexus was related to lower PUFAs in the periphyton compared to the TetraMin®. Despite the growth differences due to food type, *H. azteca* feeding on periphyton were not severely compromised nutritionally as demonstrated by the healthy survival of the control animals.

Total protein, total lipid, and PUFA concentration and profile in *H. azteca* did not differ markedly with the presence of Cd in either food type based on a single replicate sample from selected water, food and food+water treatments. Morris *et al.* (2003) observed a similar lack of response in protein and lipid content when first and second generation *H. azteca* were exposed to sublethal dissolved Cu (0, 0.055 and 0.11 µmol/L) in the presence of a biofilm diet for 27 d (in the case of first generation) and 45 – 57 d (in the case of second generation *H. azteca*). Analysis of second generation *H. azteca* for FA showed no significant changes apart from an elevation in α-linolenic acid at 0.11 µmol/L Cu. No growth effects in *H. azteca* were observed in either generation (Morris *et al.*, 2003). However, unlike Cd, Cu is an essential element regulated by *H. azteca* (Borgmann *et al.*, 1993) and therefore growth and nutrition were not likely to be affected at dissolved Cu four times lower than the LC25 (Borgmann *et al.*, 2004a). In contrast *H. azteca* growth declined significantly in response to the highest sublethal Cd treatment and yet no change in total lipid and protein was observed. This suggests that Cd is affecting growth in *H. azteca* by means other than total lipid and protein or that a more sensitive level of nutritional analysis is required (e.g., proteomic techniques). Fatty acid results are inconclusive due to the low number of replicates but did reflect growth differences in *H. azteca* based on non-contaminated food types.

2.5.9 *Implications for water quality guidelines*

The water treatment represented a standard chronic aqueous toxicity test that could be incorporated into a species sensitivity distribution (SSD) to develop a chronic water quality guideline for Cd. However, because chronic exposures must include a food source, 21 to 31 % of Cd in *H. azteca* was predicted to come from food in addition to water. Therefore, in the case of *H. azteca*, an indirect food component was already included in the calculation of toxicity endpoints based on aqueous toxicity tests and therefore a dietary component would be indirectly incorporated into a water quality guideline derived from the endpoint. However, a higher proportion of Cd may come from the diet if food is natural periphyton as compared to TetraMin®. Additional adjustment can be made to account for the maximum amount of Cd likely to come from the diet by dividing the LC10 (17.4 nmol/L) from the water treatment by the LC10 (8.04 nmol/L) from the food+water treatment. Therefore the maximum adjustment of the endpoint from a standard aqueous toxicity test to account for chronic Cd effects on *H. azteca* survival from Cd exposure to water and periphyton would be to lower that endpoint by a factor of two. This factor may be less than two if the periphyton were exposed to the same Cd concentration that *H. azteca* were exposed to. Similar adjustments could be made for other species in the SSD where research shows that diet contributes to toxicity. However it must be noted that the limitations of the bioaccumulation model, i.e. that predictions are specific to the hardness, pH, DOC and the range of diet BCFs used in the experiment, apply also to the adjusted endpoint, thus future research on the bioavailability of Cd via the diet under different water chemistries and BCFs is required for general application of this approach. A comparison of the LC10 from the food+water treatment with the hardness adjusted interim no effect chronic Canadian water quality guideline (0.36 nmol/L Cd at 125 mg/L $CaCO_3$ hardness; CCME, 1999) demonstrates a more than adequate level of protection of *H. azteca* against Cd from both aqueous and dietary sources presently exists.

2.6 CONCLUSIONS

Cadmium in a natural periphyton diet at concentrations representative of low and highly contaminated environments was bioavailable to *H. azteca* and was bioaccumulated in a manner that could be accurately modelled using principles of metal binding saturation. While secondary exposures were inevitable due to Cd partitioning between food and water, the bioaccumulation model was able to separate the contributions from water and food to total body concentration. Effects on mortality were independent of exposure pathway when based on body concentration. Therefore, bioaccumulation provided an essential link for predicting chronic Cd toxicity from food and water exposure. Growth was more variable than mortality, but declined with increasing Cd in both water and food and was lower in *H. azteca* feeding on periphyton, possibly due to the lower PUFA content of periphyton. Growth effects observed with increasing Cd were not due to a decline in *H. azteca* nutritional status as indicated by total lipid, fatty acids and total protein. Based on the bioaccumulation model developed under specific laboratory conditions, chronic dietary Cd contributed markedly to *H. azteca* body concentration and therefore to toxicity and should be an important consideration when trying to identify cause-effect relationships in an ecological risk assessment. Water quality guidelines could be further refined by lowering the chronic endpoint from a standard aqueous exposure by a maximum factor of two, in the case of *H. azteca,* to account for Cd effects from a periphyton diet.

APPENDICES

A2.1 Mean fatty acid methyl ester (FAME µg/mg dry weight) composition of control TetraMin®, periphyton (on an ash-free dry mass basis) and *H. azteca* fed on control food or exposed to Cd primarily in water, food and food+water treatments for 28 d. (standard deviations, n=2 for control food and *H. azteca* , n=1 for Cd contaminated *H. azteca*)

Molecular formula	Common Name	Control TetraMin®	Control periphyton	*H. azteca* fed on control TetraMin®	*H. azteca* fed on control periphyton	[a]*H. azteca* W12.5 fed on TetraMin®	[b]*H. azteca* F500 fed on periphyton	[c]*H. azteca* FW12.5 fed on periphyton
C15:0i	pentadecanoic acid (iso)	0	0.15 (0.01)	0.03 (0.01)	0.04 (0.02)	0	0.07	0.03
C14:1n5	myristoleic acid	0	0.04 (0.05)	0	0	0	0	0
C15:0	pentadecanoic acid	0.19 (0.06)	0.05 (0.02)	0.16 (0.00)	0.15 (0.00)	0.17	0	0.15
C15:1	cis-10-pentadecanoic acid	0	0.05 (0.08)	0	0	0	0	0
C16:0	palmitic acid	9.72 (1.09)	3.42 (0.15)	7.70 (0.71)	6.39 (0.81)	6.91	7.24	6.9
C16:1n7	palmitoleic acid	1.23 (0.16)	2.18 (0.00)	1.02 (0.05)	1.17 (0.52)	0.85	1.6	1.3
C17:0	heptadecanoic acid	0.21 (0.00)	0.04 (0.01)	0.57 (0.06)	0.43 (0.07)	0.57	0.41	0.46
C16:2n4	9,12-hexadecadienoic acid	0.10 (0.01)	0.02 (0.00)	0.92 (0.01)	0.74 (0.02)	0.9	0.64	0.63
C18:0	stearic acid	7.24 (0.41)	0.51 (0.03)	2.96 (0.12)	2.55 (0.16)	2.63	2.29	2.46
C18:1n9t	elaidic acid	0	0.04 (0.01)	0.15 (0.04)	0	0	0	0
C18:1n9c	oleic acid	8.46 (0.82)	1.53 (0.05)	13.53 (2.81)	8.34 (1.33)	11.59	10.01	8.77
C18:1n7	11-octadecenoic acid	0	0	0	0.06 (0.00)	0	0	0
C18:2n6c	linoleic acid	14.62 (1.23)	1.41 (0.07)	14.82 (1.96)	8.56 (0.61)	12.25	7.44	7.24
C20:0	arachidic acid	2.41 (0.13)	0.03 (0.04)	0.21 (0.00)	0	0	0	0
C18:3n6	γ-linolenic acid	0	0.20 (0.00)	0	0.23 (0.00)	0	0.31	0.28
C20:1n9	eicosenoic acid	0.72 (0.07)	0.09 (0.01)	1.02 (0.23)	0.76 (0.08)	0.87	0.93	0.78
C20:1n7	13-eicosenoic acid	0.08 (0.01)	0.02 (0.00)	0.17 (0.04)	0.14 (0.04)	0.15	0.26	0.19
C18:3n3	α-linolenic acid (ALA)	1.67 (0.11)	2.35 (0.02)	1.04 (0.24)	2.71 (0.74)	0.86	4.24	3.77
C21:0	heneicosanoic acid	0	0.05 (0.03)	0	0	0	0	0
C20:2	cis-11,14-eicosadienoic acid	0.40 (0.00)	0	1.47 (0.35)	0.75 (0.19)	1.4	1.01	0.88
C22:0	behenic acid	2.68 (0.12)	0.08 (0.01)	0	0	0	0	0
C20:3n6	homo-γ-linolenic acid	0	0.04 (0.00)	0	0.16 (0.02)	0	0.21	0.21
C22:1n9	erucic acid	0.16 (0.00)	0.01 (0.02)	0.12 (0.02)	0.16 (0.02)	0	0	0
C20:3n3	eicosatrienoic acid (ETA)	0	0.10 (0.04)	0.18 (0.07)	0.53 (0.12)	0.19	0.94	0.7
C20:4n6	arachidonic acid (ARA)	0	0.01 (0.02)	0	0	0	0	0
C23:0	tricosanoic acid	0.27 (0.01)	0.31 (0.00)	1.31 (0.09)	2.09 (0.16)	1.41	2.09	2.41
C22:2	cis-13,16-docosadienoic acid	0	0.07 (0.01)	0.16 (0.03)	0.19 (0.02)	0	0.25	0.23
C24:0	lignoceric acid	0	0.12 (0.01)	0	0	0	0	0
C20:5n3	eicosapentaenoic acid (EPA)	2.47 (0.19)	0.78 (0.02)	5.64 (0.56)	3.96 (0.34)	5.62	3.87	3.82
C24:1n9	nervonic acid	0.18 (0.00)	0.01 (0.02)	0	0	0	0	0
C22:4n6	7,10,13,16-docosatetraenoic acid	0	0.03 (0.00)	0.04 (0.02)	0.10 (0.03)	0	0.11	0.11
C22:5n6	4,7,10,13,16-docosapentaenoic acid	0.13 (0.00)	0.05 (0.01)	0.02 (0.00)	0.07 (0.03)	0.09	0.11	0.1
C22:5n3c	docosapentaenoic acid (DPA)	0.39 (0.03)	0	0.61 (0.05)	0.47 (0.02)	0.6	0.55	0.52
C22:6n3	docosahexaenoic acid (DHA)	2.72 (0.22)	0.10 (0.00)	2.63 (0.16)	1.44 (0.06)	2.38	1.13	1.24
∑ω3	∑ω3	7.26 (0.55)	3.33 (0.04)	10.10 (1.07)	9.10 (1.16)	9.65	10.73	10.05
∑ω6	∑ω6	14.69 (1.14)	1.74 (0.07)	14.89 (1.96)	9.01 (0.79)	12.34	8.16	7.94
∑ω3/∑ω6	∑ω3/∑ω6	0.49	1.92	0.68	1.01	0.78	1.32	1.27
∑SAFA	∑SAFA	22.73 (1.80)	4.75 (0.21)	12.84 (0.95)	11.57 (1.10)	11.69	12.11	12.41
∑MUFA	∑MUFA	10.66 (1.30)	3.97 (0.03)	16.00 (3.14)	10.60 (1.96)	13.46	12.8	11.03
∑PUFA	∑PUFA	22.25 (1.00)	5.16 (0.04)	27.52 (3.42)	19.77 (2.18)	24.29	20.79	19.73
Total	Total	55.63 (5.06)	13.88 (0.22)	56.36 (7.51)	41.94 (5.23)	49.44	45.7	43.18

[a] *H. azteca* exposed to nominal dissolved Cd of 12.5 nmol/L

[b] *H. azteca* exposed to nominal dissolved Cd of 0 nmol/L and periphyton exposed to 500 nmol/L Cd

[c] *H. azteca* exposed to nominal dissolved Cd of 12.5 nmol/L and periphyton exposed to 100 nmol/L Cd

∑ω3 = sum of omega-3 fatty acids

∑ω6 = sum of omega-6 fatty acids

∑ω3/∑ω6 = sum of ratio of omega-3/omega-6 fatty acids

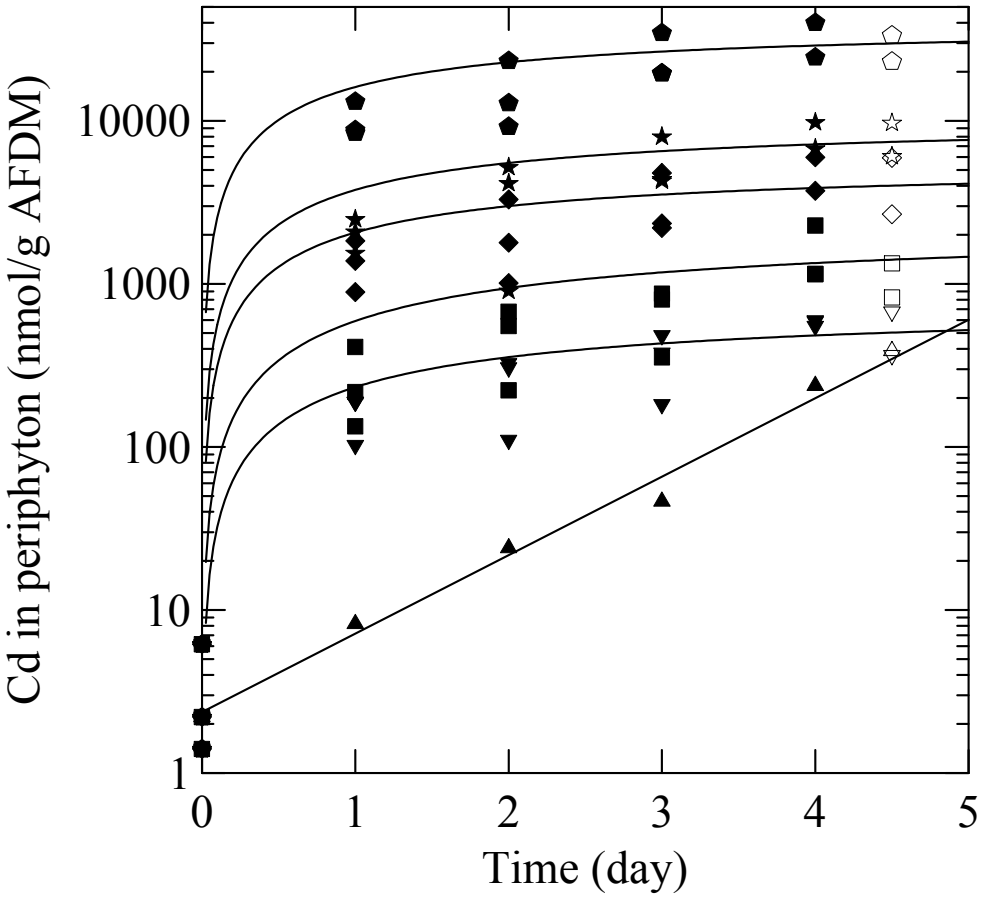
∑SAFA = sum of saturated fatty acids

∑MUFA = sum of mono-unsaturated fatty acids

∑PUFA = sum of poly-unsaturated fatty acids

Total = sum of individual fatty acids

Appendix A2.2



A2.2 Uptake of Cd by periphyton (nmol/g AFDM) exposed to 10 (▲, n=1), 50 (▼, n=3), 100 (■, n=2 or 3), 500 (◆, n=2 or 3), 1000 (★, n=2 or 3), 5000 (⬟, n=2 or 3) nmol/L as a function of time (d). Cd in periphyton was estimated from measured dissolved Cd at 24 h intervals over 4 d. Unfilled symbols are measured Cd in periphyton at 4 d at corresponding Cd exposures. Solid lines represent modelled uptake of Cd by periphyton and correspond to increasing exposure concentration.

Table A2.3 Mean measured Cd in water (dissolved and Cd^{2+}), food and *H. azteca (uncaged and caged)* , survival, dry weight, total lipid and protein after 28 d in feeding experiments. Predicted Cd in *H. azteca* and percentage of Cd from diet calculated from bioaccumulation model (standard deviations shown)

Treatment and Food type[a]	Nominal Cd in food exposure	Nominal Cd in water exposure	Cd in water[b]	Cd^{2+} in water[c]	Cd in food[d]	Measured Cd in *H. azteca*	Predicted Cd in *H. azteca*	Predicted Cd in *H. azteca* from food	Measured Cd in caged *H. azteca*	Survival	Dry weight	Total lipid	Total protein
	(nmol/L)	(nmol/L)	(nmol/L)	(%)	(nmol/g)	(nmol/g)	(nmol/g)	(%)	(nmol/g)	(%)	(mg/*H.azteca*)	(%)	(%)
Control													
T	0	0	0.22 (0.11)	68 (7)	17.3 (1.70)	8.16 (3.03)	10.8 (3.78)	29 (8)	12.5 (0.51)	89 (6)	0.411 (0.08)	11 (2)	19 (2)
Control													
P	0	0	0.21 (0.11)	72 (3)	17.2 (4.31)	12.8 (4.12)	13.5 (5.00)	47 (6)	13.2 (1.52)	97 (4)	0.214 (0.044)	10 (1)	22 (2)
Cd in Water													
T	0	3.13	2.65 (0.12)	75 (0)	144 (11.6)	123 (11.1)	111 (5.22)	23 (1)	N/A	84 (6)	0.291 (0.013)	7 (2)	19 (4)
T	0	6.25	4.94 (0.10)	72 (3)	233 (16.8)	239 (31.6)	192 (2.70)	21 (1)	N/A	84 (8)	0.333 (0.135)	10 (4)	20 (3)
T	0	12.5	10.1 (0.39)	72 (2)	457 (12.0)	425 (23.7)	356 (9.94)	22 (0.5)	N/A	84 (7)	0.236 (0.121)	9 (5)	21 (4)
T	0	25	19.9 (0.39)	73 (3)	964 (74.6)	609 (57.8)	616 (13.3)	25 (1)	184 (28)	66 (22)	0.200 (0.139)	10 (2)	22 (5)
T	0	50	38.6 (1.35)	73 (2)	1840 (141)	903 (157)	951 (17.3)	27 (1)	N/A	16 (17)	0.138 (0.036)	N/A	N/A
T	0	100	80.2 (4.00)	74 (2)	3890 (540)	N/A	1380 (39.5)	31 (2)	N/A	0 (0)	N/A	N/A	N/A
Cd in Food													
P	10	0	0.49 (0.06)	74 (0)	287 (30.3)	196 (15.8)	111 (8.67)	85 (2)	N/A	93 (3)	0.202 (0.016)	12 (1)	21 (1)
P	50	0	0.39 (0.16)	73 (1)	353 (89.8)	104 (19.2)	127 (28.8)	89 (3)	14.3 (0.87)	93 (7)	0.189 (0.018)	11 (4)	22 (1)
P	100	0	0.65 (0.17)	73 (2)	714 (126)	200 (51.3)	223 (28.3)	90 (3)	16.9 (1.23)	91 (7)	0.195 (0.029)	10 (2)	23 (1)
P	500	0	1.49 (1.03)	72 (2)	3270 (1139)	384 (6.60)	453 (1.42)	94 (4)	25.0 (2.21)	72 (4)	0.167 (0.084)	11 (0)	23 (1)
P	1000	0	2.72 (1.41)	72 (2)	5830 (1100)	819 (241)	682 (70.7)	87 (5)	20.8 (2.64)	16 (18)	0.074 (0.050)	N/A	30 (3)
P	5000	0	21.3 (5.06)	70 (2)	14900 (2400)	N/A	1190 (75.0)	59 (4)	95.1 (18.5)	0 (0)	N/A	N/A	N/A
Cd in Food+Water													
P	10	3.13	3.51 (0.10)	75 (0)	430 (68.7)	312 (20.4)	245 (15.6)	55 (4)	N/A	87 (3)	0.191 (0.011)	7 (2)	21 (1)
P	50	6.25	5.90 (0.25)	75 (2)	559 (130)	342 (72.8)	344 (27.5)	48 (5)	N/A	89 (2)	0.179 (0.015)	9 (1)	23 (2)
P	100	12.5	11.8 (0.62)	75 (2)	1050 (171)	465 (41.7)	581 (21.8)	45 (3)	N/A	68 (8)	0.188 (0.021)	12 (1)	25 (2)
P	500	25	25.8 (2.62)	74 (2)	3590 (1160)	949 (122)	990 (5.09)	45 (1)	179 (36.1)	23 (15)	0.095 (0.043)	N/A	N/A
P	1000	50	47.1 (2.79)	74 (1)	5900 (1180)	N/A	1360 (38.8)	44 (2)	N/A	0 (0)	N/A	N/A	N/A
P	5000	100	121 (15.2)	75 (3)	14800 (369)	N/A	1780 (33.4)	40 (1)	N/A	0 (0)	N/A	N/A	N/A

[a] T = TetraMin®, P = periphyton

[b] Measured filtered (0.45µm, Acrodisc®) Cd concentration

[c] Cd^{2+} concentration modelled using WHAM v6.0.13

[d] Measured Cd in food on an ash-free dry mass basis can be converted to dry mass basis by multiplying by the fraction of organic content from Table 2.1

N/A data not available

CHAPTER 3

Cadmium bioavailability to *Hyalella azteca* from a natural periphyton diet and a standardized laboratory diet and application of a biokinetic model

ABSTRACT

Differences between the bioavailability of cadmium in a natural periphyton diet and a standardized laboratory diet (TetraMin®) have important consequences for predicting bioaccumulation and toxicity in the freshwater amphipod *H. azteca*. The assimilation efficiency (AE) of Cd was compared between periphyton and TetraMin® at low (1510 and 358 nmol/g ash-free dry mass respectively) and chronically lethal (31200 and 2890 nmol/g ash-free dry mass respectively) Cd concentrations and in fresh and dry forms using a ^{109}Cd radiotracer pulse-chase feeding technique. Assimilation efficiency of Cd from periphyton (AE = 3 – 14 %) was lower than that for TetraMin® (AE = 44 – 86 %) regardless of Cd concentration or food form. Ingestion rate (IR) was lower for dry than fresh food for periphyton (0.042 and 0.16 g AFDM/g *H. azteca*/day respectively) and TetraMin® (0.19 and 0.87 AFDM/g *H. azteca*/day respectively) and the excretion rate constant (k_e) did not differ statistically with food type, form or Cd concentration (0.032 – 0.094 d^{-1}). Biokinetic models with model parameters of AE, IR and k_e were used to estimate bioaccumulation from the separate food types. These estimates were compared to those from an independent chronic Cd saturation bioaccumulation model. While the model estimates did not concur, a sensitivity analysis indicated that AE and IR were the most influential biokinetic model parameters for Cd in periphyton and TetraMin® respectively. It was hypothesized that AE was underestimated for Cd in periphyton due to a non-adapted gut enzyme system and IR was overestimated for Cd in TetraMin® due to an initial rapid ingestion phase in *H. azteca*'s feeding habits. This research demonstrated the importance of using ecologically relevant food types in laboratory experiments and acclimating the test organism to the food prior to experimentation. In addition, it is important to verify model predictions of bioaccumulation based on short-term exposures with long-term experiments to ensure accurate extrapolation to chronic field exposures.

3.1 INTRODUCTION

It has been widely demonstrated that diet is a significant pathway for metal exposure in aquatic organisms (Meyer *et al.*, 2005) and, specifically, that dietary Cd in periphyton contributes markedly to bioaccumulation in the freshwater amphipod *H. azteca* (Stephenson and Turner, 1993). Factors that influence dissolved Cd bioavailability have been well characterized and incorporated into equilibrium-based bioaccumulation models for *H. azteca* (Borgmann *et al.,* 2010; Schroeder, 2008) but less is known regarding factors influencing dietary Cd bioavailability. To date no research has addressed factors that influence Cd bioavailability in a natural periphyton diet compared to a standardized laboratory diet (TetraMin®), commonly used in assays with *H. azteca*. This comparison has important implications for interpretation of bioaccumulation and toxicity data derived from laboratory assays and for developing models that can predict bioaccumulation and ultimately toxicity of Cd from both water and food in the field.

A key parameter in determining dietary metal bioavailability is the assimilation efficiency (AE) defined as the fraction of ingested metal that is incorporated into biological tissue (Penry, 1998). Therefore AE is the net result of Cd being ingested, digested and absorbed minus the depurated fraction. Assimilation efficiency of Cd can be determined using a technique of pulse-chase feeding whereby food is uniformly labelled with the gamma-emitting radioisotope, ^{109}Cd, and fed to the organism for a period shorter than the gut passage time, to prevent recycling of dissolved Cd, followed by depuration with the same type of non-contaminated food (Griscom *et al.*, 2002b; Schlekat *et al.*, 1999, 2000; Wang and Fisher, 1999a). This method is advantageous for measuring Cd uptake and depuration in individual organisms non-destructively over time.

The chemistry (pH, redox potential, dissolved organic carbon, enzymes, surfactants) and kinetics (ingestion rate, gut retention time, excretion rate constant) of the digestive process strongly influence the bioavailability of metals in the gut of the organism (Campbell *et al.*, 2005; Griscom *et al.*, 2002a; Mayer *et al.*, 1997). However, in the context of extrapolating laboratory based model predictions to the field, the effects of

factors such as food type, form, Cd speciation and concentration on bioavailability become important as well.

Periphyton is a natural food source for *H. azteca* and other freshwater invertebrates and is a complex community of biotic (algae, zooplankton, bacteria, fungi) and abiotic (Fe and Mn oxides, fine particulate matter) components bound in an exopolymer matrix and attached to sub-surface substrates (Newman *et al.*, 1985, 1989). It has a variety of internal and external metal binding sites and is capable of bioaccumulating Cd to very high levels (Bradac *et al.*, 2009b; Hill *et al.*, 2000; Le Faucheur *et al.*, 2005). TetraMin® is a commercial fish flake diet consisting of dried fish and shrimp meal, yeast, and various carbohydrate extracts augmented with vitamins, protein and lipid, and is a standard diet for culturing and conducting assays with *H. azteca* (Borgmann, 1996; Environment Canada, 1997). These food types likely differ in their strength and capacity to bind Cd and in their distribution (internal versus externally bound) of Cd. Laboratory dietary metal experiments often employ dried food in order to standardize both the metal concentration and the food ration. However, aquatic organisms in the field are feeding on diverse, living food. The bioavailability of Cd in dry food and periphyton, is unknown. The speciation of Cd in algal food has been investigated using cellular fractionation techniques and, in general, Cd in the cytosol fraction (i.e., in soluble forms) is more bioavailable than that bound to cell walls or granules (Reinfelder and Fisher, 1991; Wallace *et al.*, 2003). However, exceptions to this relationship have been observed (Rainbow *et al.*, 2007; Schlekat *et al.*, 2000). Lastly, increasing the concentration of Cd in the food may influence bioavailability by inhibiting digestive enzymes (Mayer *et al.*, 1997) or causing direct toxicity to the organism by a mechanism as yet unknown.

The biokinetic model incorporates the bioavailability of Cd from both food and water as well as physiological parameters such as rate constants of uptake, excretion and growth in order to predict bioaccumulation of Cd in the field (Luoma and Rainbow, 2005; Reinfelder *et al.*, 1998; Wang and Rainbow, 2008). Few studies have applied this model to highly contaminated sites, i.e., in situations where metals may be having chronic toxic effects on the physiological parameters of the model (Croteau and Luoma, 2009). There

is a need to incorporate the effects of metal concentration gradients into the model and determine how this influences the model parameters in order to predict not only bioaccumulation but also toxicity.

In this study, the AE of Cd was determined for two food types (natural periphyton versus standardized TetraMin®), in two forms (fresh versus dry), and at low and high Cd concentrations representative of those causing low to high chronic toxicity. The percent cadmium either internally incorporated or externally bound to the food was also determined. Assimilation efficiency (AE), ingestion rate (IR) and excretion rate constant (k_e) were incorporated into a biokinetic model and a sensitivity analysis was performed to determine which model parameters were most influential. Model predictions based on short-term exposures were compared to predictions from an independent chronic Cd saturation bioaccumulation model. The objective of this research was to determine how factors related to food and Cd concentration influenced dietary Cd bioavailability in *H. azteca* and whether these influences can be successfully incorporated into a biokinetic model to predict bioaccumulation. The implications for conducting dietary metal experiments and extrapolating laboratory results to the field were discussed.

3.2 METHODS AND MATERIALS

3.2.1 *H. azteca culturing*

H. azteca were cultured in 1L of SAM (1 mmol/L $CaCl_2$, 1 mmol/L $NaHCO_3$, 0.01 mmol/L NaBr, 0.05 mmol/L KCl, 0.25 mmol/L $MgSO_4$ in NANOpure® de-ionized water, giving a final pH 8.2 and hardness 125 mg/L $CaCO_3$; Borgmann, 1996) in 2 L high density polyethylene (HDPE) containers with artificial substrate (3 x 3 cm 750 µm nylon mesh), 16:8 h light:dark photoperiod and 25 ^oC. Containers with approximately 100 adults each received 5 mg fresh (or 3.5 mg ash-free dry mass, AFDM) ground (< 500 µm) TetraMin® fish flake diet three times per week. SAM was renewed weekly and juveniles were separated from adults. Adults that were assigned a periphyton diet in the experiment were fed separately with 3.5 mg AFDM non-contaminated periphyton three times during the week preceding the experiment.

3.2.2 *Food preparation and Cd exposure*

Non-contaminated periphyton was scraped from artificial substrates and internal surfaces of an outdoor artificial pond and centrifuged (3000 rpm for 10 min). The supernatant was replaced with SAM to a fixed volume to produce a bulk stock of periphyton that was stored in the dark at 4 ^{o}C. TetraMin® (Tetra Holding (US) Inc.) is a commercially available diet designed to optimize fish health and used extensively in aqueous and sediment toxicity testing protocols with *H. azteca*. Sub-samples of the bulk periphyton stock and TetraMin® were analyzed for AFDM biomass (Biggs and Kilroy, 2000). Methods for the measurement of total protein, total lipid and fatty acid content in periphyton and TetraMin® and chlorophyll *a* and algal identification in periphyton were described in Chapter 2.3.6.

To achieve the low and high Cd diets, low Cd diets received aliquots of radioactive ^{109}Cd (39 MBq/mL $CdCl_2$ in 0.1 mol/L HCl) in SAM, while high Cd diets received aliquots of both radioactive and stable Cd (1 mmol/L anhydrous $CdCl_2$ ACS in 1% HNO_3) in SAM. Periphyton and TetraMin® were both exposed to 46 KBq/mL ^{109}Cd in the low Cd concentration. In the high Cd concentration, periphyton and TetraMin® were exposed to 5000 nmol/L stable Cd + 0.17 KBq/mL ^{109}Cd and 50,000 nmol/L + 39 KB/mL ^{109}Cd respectively. Following the addition of stable and/or ^{109}Cd, exposure solutions were adjusted to pH 7 with 0.1 mol/L NaOH and allowed to equilibrate for 24 h before adding food. Exposure solutions containing periphyton were maintained for 72 h at 20 ^{o}C with 16:8 h light:dark photoperiod. Exposure solutions containing TetraMin® were maintained at 4 ^{o}C for 48 h to reduce food decomposition as *H. azteca* have been observed to reject decomposed TetraMin®. Filtered (0.45 μm polysulfone Acrodisc®) and unfiltered water samples were collected from high Cd solutions every 24 h, analyzed for stable and ^{109}Cd and additional aliquots were added as necessary to maintain the nominal concentration. Mass to volume ratios ranging from 0.4 to 42 g AFDM/L for both food types, exposure concentrations and exposure times were chosen to produce similar dietary Cd concentration and activity between the two food types.

Cadmium exposed food was prepared for the pulse-chase feeding experiment by rinsing the centrifuged (3000 rpm for 10 min) pellet with SAM to remove residual Cd. A portion of each food type was lyophilized to produce the dried food treatments at low and high Cd. The remaining portion was made to a fixed volume with SAM and aliquots were filtered onto polycarbonate membranes (0.45 µm, 25 mm diameter) then stored in sealed humidified containers in the dark at 4 ^{o}C to produce the fresh diets at low and high Cd.

Periphyton and TetraMin® in the highest Cd concentration were analyzed for the amount of operationally defined internal and external Cd based on an ethylene diamine tetra-acetic acid (EDTA) extraction method modified from Franklin *et al.* (2002). Briefly, approximately 15 mg AFDM food from the highest Cd solution was centrifuged (3000 rpm for 10 min) and the supernatant was replaced with 20 mmol/L EDTA (5 mL), vortexed (20 s) and centrifuged (3000 rpm for 20 min). The EDTA supernatant was analyzed for Cd which represents the externally bound Cd, while the remaining pellet was lyophilized, weighed and analyzed for Cd which represents the internally bound plus strongly surface bound Cd in the case of periphyton and strongly bound surface Cd in the case of TetraMin®.

3.2.3 *Dietary Cd assimilation and depuration*

Food treatments consisting of fresh and dry periphyton or TetraMin® at low and high Cd concentrations were analyzed for ^{109}Cd and added (7.1 ± 2.3 mg AFDM, mean ± SD) to separate containers (2 L, high density polyethylene) with 1 L SAM. A nylon substrate (9 mm^2, 750 µm mesh size) and fifteen randomly selected adult (0.99 ± 0.24 mg dry mass, mean ± SD) *H. azteca* that had last been fed on the respective non-contaminated diet three days prior to commencing the experiment were added to a feeding container. Groups of five adult *H. azteca* were randomly assigned to cages consisting of two joined sections of 76 mm diameter clear plastic tubing with 500 µm nylon mesh covering the openings. A single cage was suspended in each feeding container. Caged *H. azteca* were not fed and were thus used to quantify possible dissolved Cd exposure as a result of Cd leaching from the food or fecal pellets. A 4 h feeding period for the uncaged *H. azteca* was chosen as a compromise between ensuring that fed *H. azteca* would have sufficient

activity for accurate ^{109}Cd counts without significant dissolved Cd excretion and recycling. Filtered (0.45µm) and non-filtered water samples were collected and analyzed for ^{109}Cd and stable Cd at the end of the feeding period.

At 4 h (t = 0 h depuration), fed and non-fed *H. azteca* were removed, rinsed in SAM first with and second without 50 µmol/L EDTA to remove loosely bound external Cd and analyzed individually and non-destructively for ^{109}Cd. Non-fed *H. azteca* were then weighed wet, frozen (-80 °C), lyophylized and weighed dry. Ten fed *H. azteca* with the highest ^{109}Cd were placed in separate depuration containers (120 mL plastic cups) with 100 mL SAM, nylon mesh substrate and non-contaminated food of the same type provided in the 4 h feeding period. At 2, 4, 6, 8, 24, 48, 72, 96 h intervals, each *H. azteca* was rinsed in SAM with and without 50 µmol/L EDTA, analyzed for ^{109}Cd and returned to renewed SAM, substrate and food. After analyzing *H. azteca* for ^{109}Cd at 96 h depuration, individuals were weighed wet, stored at -80 °C, lyophilized, weighed dry and 3 replicate individuals from each treatment were analyzed for Cd. All *H. azteca* were fed and depurated at 25 °C with 16:8 h light:dark diffuse lighting conditions. The experiment was repeated (i.e. 20 individuals per treatment in total) for all 8 food treatments except the fresh and dry low Cd TetraMin® treatments.

3.2.4 *Cd analyses*

Select samples of dried food, water and individual *H. azteca* were analyzed for Cd using a Varian SpectrAA 400 graphite furnace atomic absorption spectrophotometer (GF-AA) with Zeeman background correction. Prior to analysis, food (1.4 ± 0.18 mg) and *H. azteca* (1.4 ± 0.81 mg) (means ± SD) were cold acid digested with 70 % ultra-pure HNO_3 (1.75 % final digest volume) for 6 d, followed by addition of 30 % ultra-pure H_2O_2 (0.6 % final digest volume) for 24 h at 60 ^{o}C then made to a final digest volume (1 mL) with NANOpure® de-ionized water (Borgmann *et al.*, 1989; Stephenson and Mackie, 1988). Certified reference materials of TORT-2 (National Research Council of Canada; lobster hepatopancreas) and CRM-482 (European Commission; lichen) had digest recoveries of 101 ± 6 % and 104 ± 3 % (mean ± SD) respectively. In each run, calibration standards and blanks were analyzed every fifth sample to correct for drift and an external standard

(CRM-TMDW, High-Purity Standards, Charleston, SC) had a recovery of 103 ± 5 % (mean ± SD). Method detection limits for the unfiltered, filtered water and digest samples calculated as the upper 95 % confidence limit of the blank samples, were 0.082 nmol/L, n = 13; 0.086 nmol/L, n = 15; 0.18 nmol/g n = 76, respectively. Inter-laboratory comparisons of Cd results using polymetallic reference waters supplied by National Laboratory for Environmental Testing, Environment Canada demonstrated acceptable performance of the instrument and analytical protocol.

Food, water and live individual *H. azteca* were analyzed for ^{109}Cd using a NaI(Tl) well-type gamma detector (Perkin Elmer 1480 Wallac Wizard 3") with emissions measured at 15 – 120 keV. This emission window was optimized for counting efficiency and background using a ^{109}Cd standard curve. Sample geometry and radioactive decay were considered. Counting times (1 – 5 minutes) were adjusted so that propagated counting errors were < 5 %. ^{109}Cd in *H. azteca* was measured by gently transferring an individual into a counting tube (5 mL) containing SAM (1 mL) with a maximum counting time of 3 minutes.

3.2.5 *Data analyses*

ANOVA and non-linear regression modelling were performed with SYSTAT version 10.0. Differences between means were analyzed with 1-way ANOVA and post-hoc analyses with Tukey's test. Assumptions of normality of distribution and homogeneity of variance were tested with visual assessment of probability density plots of non-transformed and log transformed data and Levene's test on the absolute value of the residuals respectively (Environment Canada, 2005).

3.2.6 *Model parameter calculations and sensitivity analyses*

Assimilation efficiency was calculated as the y intercept estimated using non-linear regression of the percent Cd retained by *H. azteca* during the slow phase of depuration as a function of depuration time (Eq. 3.1). The corresponding slope of the regression was the excretion rate constant. The slow phase of depuration represented physiological loss of

Cd from the tissue following gut clearance of ^{109}Cd and was arbitrarily assigned by visual assessment of the depuration profiles to be $t \geq 20$ h.

$$A = AE \times e^{-k_e t} \quad (3.1)$$

Where A is the ingested ^{109}Cd remaining in *H. azteca* as a percent of the ^{109}Cd in *H. azteca* at depuration time t (d), AE is the assimilation efficiency (%), and k_e is the ^{109}Cd excretion rate constant (d^{-1}).

Ingestion rate was initially calculated by comparing the mass loss of food from containers where *H. azteca* fed for 4 h to containers with the same amount of food but where *H. azteca* were absent. This method proved to be inaccurate due to the low mass of food consumed relative to the high variability in food weight. A second approach was adopted (the results of which are presented) where IR was calculated using the ^{109}Cd in *H. azteca* following 4 h of feeding as a fraction of ^{109}Cd in the food (Eq. 3.2). Ingestion rates calculated by this method are conservative due to the potential excretion of ^{109}Cd during the 4 hr feeding period.

$$IR = A_h / A_f \times m_f / m_h / (1/6) \quad (3.2)$$

Where IR is the ingestion rate of food by *H. azteca* as measured over 4 h (g food AFDM/g *H. azteca*/d), A_h is ^{109}Cd in *H. azteca* at the beginning of depuration (cpm), A_f is the ^{109}Cd in food (cpm), m_f is the AFDM weight of the food ration (g), m_h is the dry weight of *H. azteca* (g).

The growth rate constant was calculated from an independent data set where juvenile *H. azteca* were exposed to Cd in water, periphyton and TetraMin® for 28 d (Chapter 2). It is a conditional mean growth rate constant that accounts for differences in growth related to food type and Cd concentration in the 28 d feeding experiment (Eq. 3.3).

$$k_g = \mathrm{Ln}(m_{T28}/m_{T0}) \times 1/28 \quad (3.3)$$

Where k_g is the conditional mean growth rate constant (d^{-1}), m_{T28} is the dry mass of *H. azteca* at 28 d (g), m_{T0} is the initial dry mass of juvenile *H. azteca* (g).

A biokinetic model based on the parameters calculated above was applied to the independent data set of juvenile *H. azteca* exposed to Cd in water, periphyton and TetraMin® over 28 d to calculate the amount of Cd in *H. azteca* coming from food (Eq. 3.4).

$$C_{hf} = (AE \times IR \times C_f) / (k_e + k_g) \quad (3.4)$$

Where C_{hf} is the Cd in *H. azteca* from food (nmol/g), C_f is the Cd in periphyton (nmol/g AFDM) or TetraMin® (nmol/g AFDM), AE, IR, k_e and k_g are explained above.

The Cd in *H. azteca* predicted to come from food (Eq. 3.4) was then compared to independent predictions of Cd in *H. azteca* from food using a saturation bioaccumulation model (Eq. 3.5) that was based on mechanistic principles and was fitted to the 28 d data set ($r^2 = 0.946$, Chapter 2.2) using non-linear regression.

$$C_{hf} = max_f \times C_f / (K_f + C_f) \quad (3.5)$$

Where max_f is the maximum bioaccumulation of Cd in *H. azteca* from food (nmol/g AFDM) and K_f is the half saturation constant (the concentration of C_f at which the bioaccumulation of Cd in *H. azteca* is half the maximum) (nmol/g AFDM).

The total Cd in *H. azteca* from the sum of contributions from food and water was also compared between the biokinetic model and the saturation bioaccumulation model. However, because the uptake of dissolved Cd by *H. azteca* in the absence of food had not been calculated in the biokinetic model, the same term representing Cd in *H. azteca* from water (Eq. 3.6) was used in both models and added to the independent predictions of Cd in *H. azteca* from food (Eq. 3.4 and Eq. 3.5)

$$C_{hw} = max_w \times C_w / (K_w + C_w) \quad (3.6)$$

Where max_w (nmol/g) is the maximum bioaccumulation of Cd in *H. azteca* from water (C_{hw}; nmol/g), C_w is the dissolved Cd in water (nmol/L), K_w is the half saturation constant (the concentration of C_w at which the bioaccumulation of Cd in *H. azteca* is half of the theoretical maximum bioaccumulation at infinite C_w) (nmol/L).

A sensitivity analysis was performed on the biokinetic model (Eq. 3.4) to determine which parameters have the most influence on model predictions. Individual parameter values were adjusted in turn to either the lower or upper 95 % confidence limit while holding all other parameters at the mean value. The change (%) between the mean, lower and upper predictions was calculated for each model parameter. This approach incorporated the uncertainty surrounding the mean of the model parameter into the analysis. Another approach that focused on the influence of the model parameter itself was to sequentially adjust each parameter value by 25 % while maintaining other parameters at the mean value. Again the change (%) between the mean and adjusted model outputs was calculated.

3.3 RESULTS

3.3.1 *Food characterization and Cd content*

Periphyton and TetraMin® were characterized previously (Chapter 2.5.1) and had total protein of 11.5 ± 2.10 % and 13.0 ± 0.25 %, total lipid of 4.54 ± 0.67 % and 11.7 ± 0.41 % (mean ± SD) and Σω3: Σω6 fatty acid ratios of 1.92 and 0.49, respectively. Periphyton chlorophyll *a* was 3.28 ± 0.52 (mg/g) and was dominated by Chlorophyta: *Cladophora, Mougeotia, Ulothirx, Scenedesmus and Ankistrodesmus*. Organic content of periphyton and TetraMin® were 52.1 ± 0.76 % and 100 ± 0 %, respectively (mean ± SD). To convert periphyton Cd concentrations or IRs from AFDM to dry mass multiply by 0.521.

Final Cd in periphyton was 4 times and 11 times higher than Cd in TetraMin® in low and high exposure concentrations, respectively, despite using a dissolved Cd in the highest

TetraMin® exposure that was 10 times greater than the highest periphyton exposure. Dissolved (<0.45 µm filtered) Cd in high exposure containers was 29 % and 61 % of nominal at the time of exposure completion for periphyton and TetraMin® respectively. Measured Cd in low and high exposed periphyton was 1510 ± 153 nmol/g AFDM (20.8 ± 10.3 KBq) and 31200 ± 1870 nmol/g AFDM (4.13 ± 1.89 KBq), respectively (mean ± SD). Measured Cd in low and high exposed TetraMin® was 358 ± 9.76 nmol/g AFDM (3.95 ± 4.80 KBq) and 2890 ± 384 nmol/g AFDM (12.2 ± 5.37 KBq), respectively (mean ± SD). After extracting the food in the high Cd exposures with EDTA, 50 – 63 % of Cd associated with periphyton was operationally defined as being bioincorporated or strongly surface adsorbed and 5 - 10 % of Cd associated with TetraMin® was defined as being strongly surface adsorbed.

3.3.2 *Cd bioaccumulation in H. azteca and depuration*

Cd leached from food treatments during the 4 h feeding period resulting in dissolved Cd of 0.40 ± 0.020 to 0.51 ± 0.33 nmol/L and 0.15 ± 0.010 to 3.4 ± 0.69 nmol/L in low and high Cd treatments respectively (mean ± SD). Despite the elevated dissolved Cd exposure, unfed *H. azteca* in only two treatments (high Cd in fresh TetraMin® and low Cd in dried periphyton) had activity that was significantly higher than background (1.6 times, $P<0.001$ and 1.5 times, $P<0.001$ respectively). Activity in unfed *H. azteca* was 1 - 8 % of ^{109}Cd in fed *H. azteca* therefore, the contribution of dissolved Cd to bioaccumulation was minor compared to that of dietary Cd. Nevertheless, activity of fed *H. azteca* at t = 0 h was corrected for the dissolved uptake as measured in unfed *H. azteca* for all treatments. Total Cd in *H. azteca* (estimated from the specific activity) at the beginning of depuration was 18 - 418 nmol/g and declined to 7 - 76 nmol/g after 96 h of depuration. Overall survival of *H. azteca* was 96 %.

Depuration profiles of Cd from *H. azteca* in all treatments consisted of an initial rapid loss of Cd as the gut content was purged followed by a slow release phase as Cd was lost from tissue (Figure 3.1). Beyond 20 h, the physiological loss of Cd from tissue had stabilized for both food types, therefore, 20 h was arbitrarily assigned as the point beyond which AE and k_e could be determined.

3.3.3 *Model parameters*

Overall, *H. azteca* fed with periphyton had lower (3 - 44 x) AEs of Cd than those fed with TetraMin® regardless of Cd concentration or whether the food was fresh or dry ($P<0.05$, Figure 3.2, A3.1). Within each food type, AEs were influenced differently by Cd concentration and food form. Assimilation efficiency of Cd was significantly lower for fresh periphyton exposed to low Cd as compared to high Cd, otherwise AEs for periphyton did not differ with treatment. In contrast, AEs were enhanced when *H. azteca* were fed low Cd exposed TetraMin® but the difference was only statistically significant for fresh TetraMin®.

Ingestion rates of periphyton were lower (2 - 9 x) than those of TetraMin® when comparing like forms and levels of Cd exposure (Figure 3.2, A3.1). Within each food type, IRs were 5 x ($P<0.05$) and 4 x lower for dry forms than fresh forms of periphyton and TetraMin® respectively. A positive and significant ($P<0.05$) relationship between IRs and Cd concentration in food existed with dry periphyton and fresh TetraMin®, otherwise no differences in IRs were associated with Cd in food.

Excretion rate constants (k_e) (0.0167 – 0.0958 d^{-1}) of Cd from *H. azteca* were lower when high Cd foods were consumed but did not differ significantly with food type, food form or Cd concentration ($P>0.05$, Figure 3.2, A3.1).

The conditional growth rate constant of *H. azteca* fed on periphyton (0.078 ± 0.016 d^{-1}) was 1.2 times lower than *H. azteca* fed with TetraMin® (0.093 ± 0.019 d^{-1}). For both food types, growth declined with increasing Cd exposure (Chapter 2.4.6). Therefore k_g was specific for food type and Cd concentration in the model.

3.3.4 *Model comparisons*

The biokinetic model (Eq. 3.4) was used to predict Cd in *H. azteca* from food based on concentrations of Cd in periphyton and TetraMin® measured in a 28 d dietary exposure experiment. This same 28 d experiment was previously used to create a saturation bioaccumulation model (Eq. 3.5) to predict Cd in *H. azteca* from food when exposure to

Cd was via water, periphyton and TetraMin® (Chapter 2.2.1). Using these two independent models, the predictions of Cd in *H. azteca* from food were compared (Figure 3.3A). The biokinetic model predictions were separated by food type. The model over-predicted Cd in *H. azteca* from TetraMin® by 12 – 44 times the 1:1 ratio and estimates of Cd in control animals were up to 11 times greater than measured values. The model under-predicted Cd in *H. azteca* from periphyton by 2 - 11 times the 1:1 ratio and 13 % of the predicted body concentrations were within a factor of 2 of the 1:1 ratio as compared to 0 % in the case of *H. azteca* bioaccumulating Cd from TetraMin®.

Predictions of Cd in *H. azteca* from both food and water sources using Eq. 3.4 plus 3.6 were compared to measured values from the 28 d experiment (Figure 3.3B). As for the predictions of Cd in *H. azteca* from food, the biokinetic model over-predicted body concentration for TetraMin® fed *H. azteca* and under-predicted body concentration for *H. azteca* fed with periphyton. However, there was a marked improvement in the accuracy of the predictions with TetraMin® fed *H. azteca* being within 3 – 14 times of the 1:1 ratio and 56 % of the predicted body concentrations for periphyton fed *H. azteca* being within a factor of 2 of the 1:1 ratio.

3.3.5 *Sensitivity analyses*

Sensitivity analyses of the biokinetic models for fresh periphyton and TetraMin® were used to determine the influence of individual model parameters on the overall outcome (Figure 3.4). Based on the approach where the model parameters were adjusted according to 95 % confidence limits, AE (± 24 % change) for fresh periphyton and IR (± 18 % change) for TetraMin® had the greatest effect on the model output. Changes in the model parameter of *H. azteca* growth rate constant had the least effect on the model output for both periphyton and TetraMin® (± 4-5 % change). When model parameters were adjusted by 25 %, both AE and IR had the most effect while k_e had the least effect in the case of both food types (A3.2).

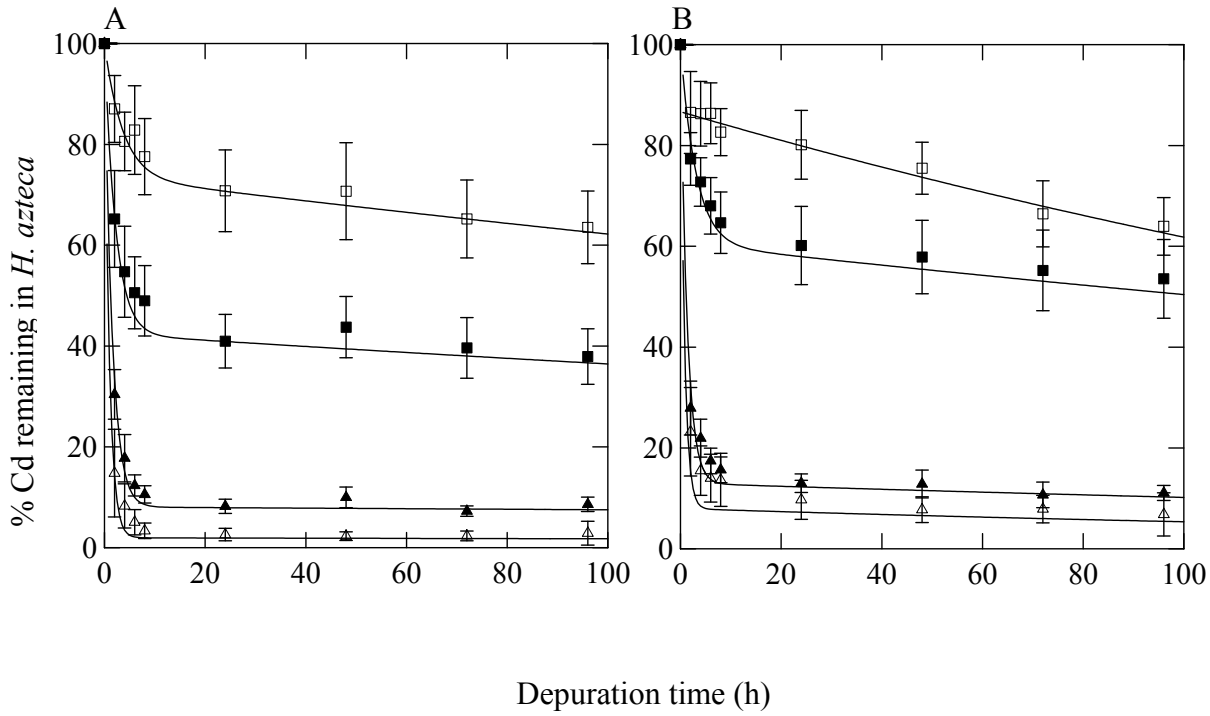


Figure 3.1 Cadmium remaining in *H. azteca* over 96 h of depuration after being fed for 4 h on Cd exposed periphyton (triangle) and TetraMin® (squares) at low (open) and high (closed) Cd concentrations in (A) fresh and (B) dry food forms. Cadmium remaining in *H. azteca* is expressed as a percent of initial body activity; data points denote means of individual *H. azteca* (n = 9 – 20) with 95% confidence limits shown.

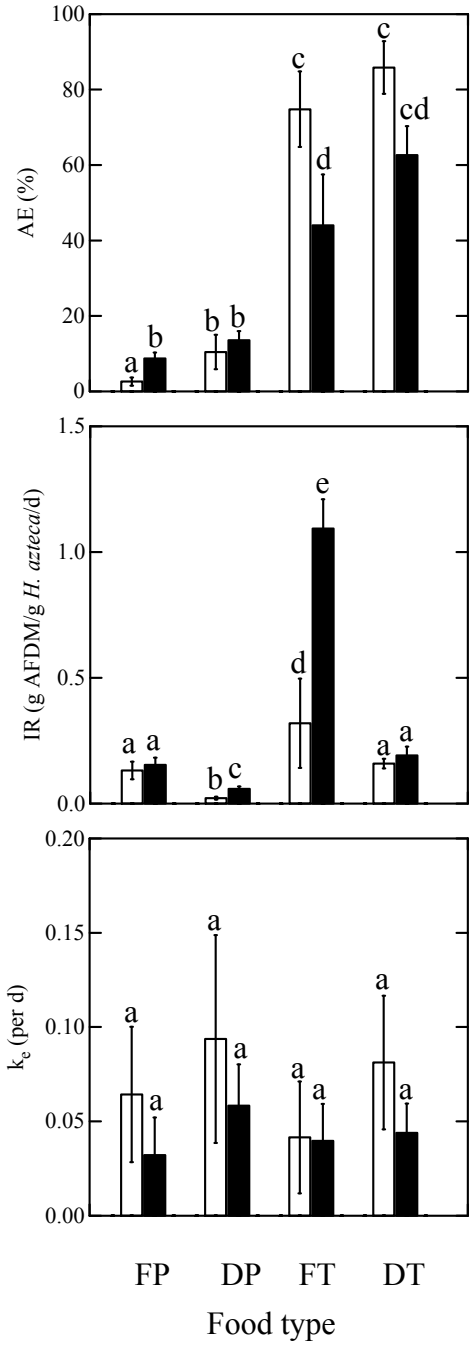


Figure 3.2 Mean assimilation efficiency (AE), ingestion rate (IR) and excretion rate constant (k_e) when *H. azteca* were fed Cd in fresh and dry periphyton (FP and DP respectively), fresh and dry TetraMin® (FT and DT respectively) at low Cd (white bars) and high Cd (black bars) for 4 h (95 % confidence limits shown). Different letters indicate statistical differences between food types ($P<0.05$).

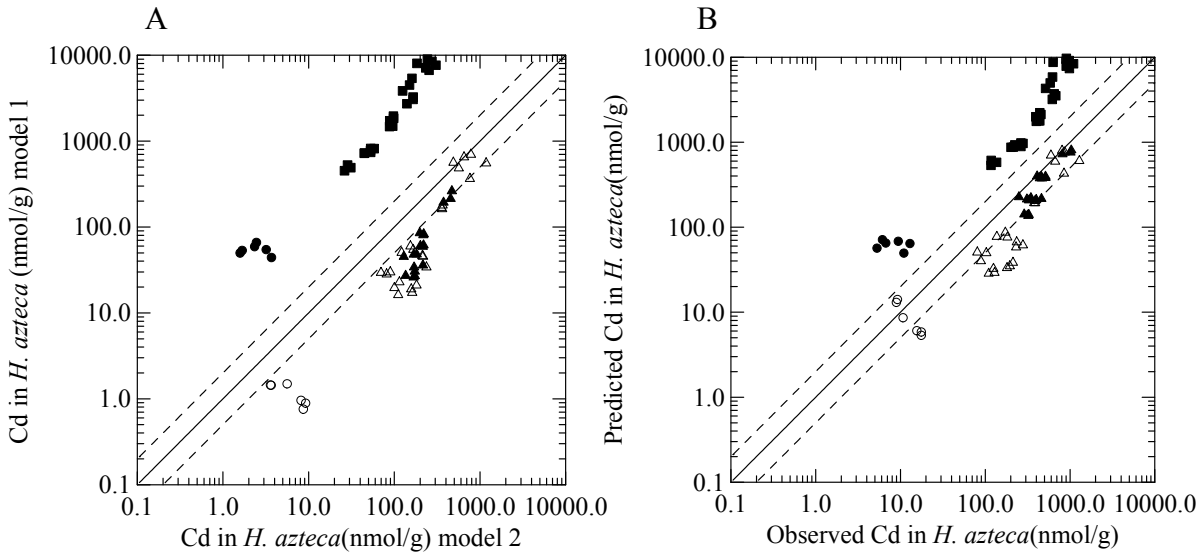


Figure 3.3 (A) Predicted Cd in *H. azteca* from food only using a biokinetic model (Eq. 3.4, model 1) compared to predicted Cd in *H. azteca* from food only using an independent chronic saturation bioaccumulation model (Eq. 3.5 model 2). (B) Predicted Cd in *H. azteca* from food and water exposure (Eq. 3.4 + Eq. 3.6) compared to measured Cd in *H. azteca* exposed to Cd in food and water over 28 d. Symbols represent Cd in *H. azteca* exposed for 28 d to Cd primarily in water (closed square), primarily in periphyton (open triangle) and in both periphyton and water (closed triangle). Predicted versus observed Cd in *H. azteca* feeding on control periphyton (open circles) and control TetraMin® (close circles) is also shown. Solid line is $y=x$. Dashed lines are $y=0.5x$ and $y=2x$.

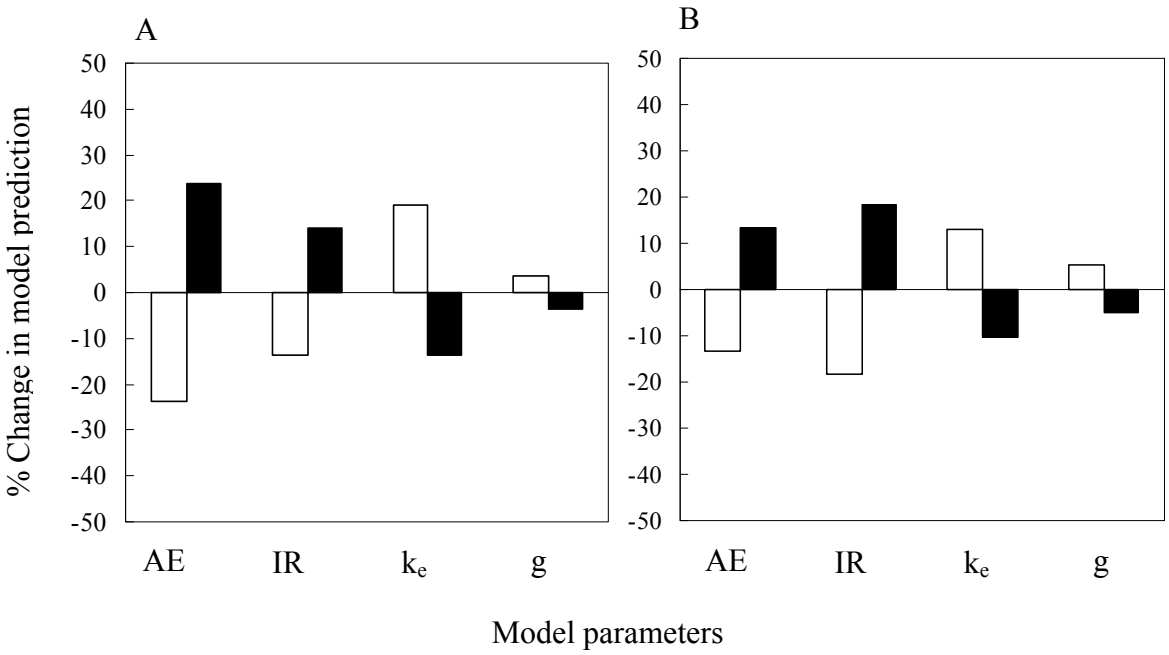


Figure 3.4 Sensitivity analysis showing the percent change in model predicted Cd in *H. azteca* when fed Cd in fresh periphyton (A) and fresh TetraMin® (B). The lower 95% CL (white bars) and upper 95% CL (black bars) values for each model parameter of assimilation efficiency (AE), ingestion rate (IR), excretion rate constant (k_e) and growth rate constant (k_g) were used in turn while the mean was used for the remaining parameters.

Table 3.1 Mean assimilation efficiency (AE), ingestion rate (IR) and excretion rate constant (k_e) averaged over low and high Cd concentrations when *H. azteca* were pulse fed with Cd contaminated periphyton or TetraMin® in fresh or dry forms (upper and lower 95% confidence limits shown)

Food type	Food form	AE (%)	IR (g AFDM/g *H. azteca*/d)	k_e (d^{-1})
Periphyton	fresh[1]	6 (4 - 7)	0.16 (0.14 – 0.18)	0.048 (0.028 – 0.068)
Periphyton	dry	12 (9 - 14)	0.042 (0.034 – 0.050)	0.077 (0.047 – 0.107)
TetraMin®	fresh[1]	54 (47 - 61)	0.87 (0.71 – 1.0)	0.040 (0.025 – 0.056)
TetraMin®	dry	70 (63 - 77)	0.19 (0.16 – 0.21)	0.056 (0.040 – 0.072)

[1] Mean estimates and upper and lower 95% confidence limits of model parameters for fresh forms of periphyton and TetraMin® were used in model comparisons and sensitivity analyses.

3.4 DISCUSSION

The biokinetic model provides insight into how dietary and waterborne metals interact with physiological mechanisms such as AE, IR and k_e that consequently influence metal bioaccumulation (Wang and Rainbow, 2008). To accurately predict bioaccumulation in the field, it is important to determine how factors such as food type, form and metal concentration influence these physiological mechanisms.

3.4.1 *Assimilation efficiency*

There was a strong dependence of Cd AE on food type. This same observation was made for the estuarine amphipod *Leptocheirus plumulosus* when pulse-fed with ^{109}Cd exposed algae, sediments and particles with different organic and iron oxide coatings (Schlekat *et al.,* 2000). Significant differences in AE were observed for Cd associated with bacterial exopolymeric coatings (27 %) and two algal species (11 and 3 %). King *et al.* (2005) similarly measured differences in Cd AE in the marine amphipod *Melita plumulosa* when pulse-fed ^{109}Cd in sediments (22 %) and a diatom (56 %).

Variability in AE with food type is a function of the chemical properties (pH, redox potential, DOC, enzymes, surfactants) of the digestive fluids that enable metal solubilization from the food matrix and the subsequent speciation that facilitates metal transport across the gut epithelial membrane (Schlekat *et al.,* 2002).

The differences in Cd AE with periphyton and TetraMin® can be explained in terms of metal solubilization and speciation within the gut of *H. azteca*. Digestive enzymes play a crucial role in initial metal solubilization and enzyme activity adapts specifically to the chemical composition of ingested food and the feeding history of the organism (Campbell *et al.*, 2005; Johnston *et al.*, 2005). Therefore *H. azteca* cultured on TetraMin® likely had an enzyme system adapted to solubilizing that food type whereas *H. azteca* that were switched to feeding on periphyton one week prior to the experiment may not have been able to adapt their enzyme system sufficiently to solubilize the very different chemical composition of periphyton. Only solubilized Cd can reach the primary sites of absorption in the hepatopancreatic caecae (Schmitz and Scherrey, 1983) therefore Cd associated

with non-solubilized periphyton would be excreted and AE reduced. Incomplete digestion of algal cells was observed for the estuarine amphipod (*Leptocheirus plumulosus*) and related to a reduced AE of Cd (Schlekat *et al.*, 2000).

Cadmium speciation differences in periphyton and TetraMin® could have resulted in different fractions of dietary Cd being solubilized. The chemical surfactant EDTA, which crudely mimics the solubilization process, extracted up to 95 % of Cd from surface adsorption sites of TetraMin® whereas only up to 50 % was extracted from periphyton with the remaining Cd being either strongly adsorbed externally to algal cell walls and inorganic components of the matrix (sulfides, oxides, recalcitrant minerals) or stored within algal cells. The lower fraction of Cd extracted from periphyton due to differences in speciation would also result in a lower AE compared to TetraMin®. Another component of Cd speciation to consider is the form resulting from the solubilization process. Since it's likely that TetraMin® solubilized more readily than periphyton, the additional amino acid ligands resulting from that process may have further assisted Cd assimilation in the gut. Cadmium readily forms complexes with amino acids which can then be carried across the epithelial membrane by a wide array of protein transporters involved in nutrient absorption (Ahearn, 1988; Campbell *et al.*, 2005). The formation of Zn-cysteine and histidine amino acid complexes was found to stimulate absorption of Zn across the gut epithelium of rainbow trout (Glover and Hogstrand, 2002). Thus the presence of these amino acid ligands and the potential for Cd to be assimilated as an amino acid complex is a function of the chemical composition and degree of solubilization of the two foods.

Therefore the lower AE of Cd from periphyton as compared to TetraMin® could be explained by the reduced capacity of the gut enzyme system to solubilize periphyton, the reduced fraction of Cd bound externally to periphyton and perhaps the reduced bioavailability of Cd species solubilized from periphyton. Some of these factors also assist in explaining why the experimentally determined Cd AE in *H. azteca* was lower from periphyton in this study (6 % AE) compared to estimated Cd AE in native *H. azteca* feeding on periphyton in the field (80 % AE; Stephenson and Turner, 1993). Native *H.*

azteca would have a digestive enzyme system adapted to the periphyton used in the field experiment hence greater solubilization and release of Cd to be available for assimilation. Also the Cd exposure concentrations were targeted for sub-lethal effects and consequently were 6000 fold lower than those used in the current study. Having such low dissolved Cd could have altered the external and internal distribution of Cd in algal cells and bioincorporated Cd could have been in a more bioavailable form.

Assimilation efficiency of Cd was not strongly dependent on food form since Cd AE in fresh food was generally not statistically significantly different from AE of Cd in dry food regardless of food type. Therefore bioaccumulation of Cd would be expected from dry food in contrast to the lack of bioaccumulation observed when Ball *et al.* (2006) fed *H. azteca* with an algal diet washed with EDTA and oven-dried. This suggests that the removal of externally bound Cd by EDTA rather than the fact that the algae was dry may have resulted in the absence of Cd bioaccumulation in *H. azteca* in Ball *et al.* (2006).

Assimilation efficiency of Cd in TetraMin® decreased with increasing Cd concentration (though only statistically significant for fresh TetraMin®). This may be an indication of dietary metal toxicity via the inhibition of digestive enzymes which would lower the AE. Chen *et al.* (2002) found a wide range of digestive enzyme activities in 35 species of marine invertebrates to be inhibited by a threshold concentration of copper. Although the same decrease in AE with increasing dietary Cd was not observed for periphyton, this may be because the solubilization of dietary Cd was already impaired by a maladapted enzyme system.

3.4.2 *Ingestion rate*

Ingestion rates proved difficult to accurately quantify over the 4 h feeding period, however while the estimates from this experiment may be conservative due to the method of calculation, on a dry mass basis they do fall within the range of measured IRs of *H. azteca* feeding on non-contaminated periphyton in the field (0.98 g/g/d from Stephenson and Turner (1993)) and sediments amended with bacteria, diatoms, green algae and blue-green algae (0.17 – 1.02 g/g/d from Hargrave (1970)). The lower IRs of dry forms of each

diet type suggested that *H. azteca* preferred fresh food forms which may be related to dry forms being less soft and palatable and more difficult to process. Increasing dietary Cd produced inconsistent effects on IR for each food type. It was expected that with increasing dietary Cd there would be a decrease in IR as was observed in a freshwater snail *Lymnaea stagnalis* (Croteau and Luoma, 2008) in the case of dietary Cd, and freshwater amphipods (*Gammarus pulex* and *Echinogammarus meridionalis)* in the case of dissolved Cd exposure and inadvertent dietary exposure (Felten *et al.*, 2008; Pestana *et al.*, 2007). These authors measured IR from 18 h to 7 d, therefore acute (4 h) effects on IR measured in this study likely do not reflect chronic dietary Cd exposure effects.

3.4.3 *Excretion rate constant*

Excretion rate constants were independent of food type, food form and dietary Cd concentration. Excretion rate constants (k_e = 0.032 – 0.064 d^{-1}) of Cd from laboratory cultured *H. azteca* fed contaminated fresh periphyton and TetraMin® were similar to that measured for native *H. azteca* exposed to Cd in periphyton and water for 11 d and depurated in a reference lake for 11 d (k_e = 0.092 d^{-1}; Stephenson and Turner, 1993). However, the authors suggested that excretion rate constants were related to exposure concentration as estimates of k_e were higher (0.29 – 0.36 d^{-1}) when fitting a model for Cd bioaccumulation in contaminated water rather than measuring k_e in non-contaminated water. Higher excretion rate constants were also estimated for *H. azteca* exposed for 7 d to Cd in separate sediment (k_e = 0.17 d^{-1}) and aqueous exposures (k_e = 0.24 d^{-1}) although depuration was conducted in non-contaminated water as in the case of the present study (Neumann *et al.,* 1999). Therefore in the context of these results, it's possible that k_e measured in this study was conditional for food type, Cd concentration and exposure duration.

3.4.4 *Model comparisons*

Validation of the biokinetic model was conducted by comparing the predicted Cd in *H. azteca* from food using the biokinetic model (Eq. 3.4) with the predicted Cd in *H. azteca* from food using a chronic saturation bioaccumulation model (Eq. 3.5) in which the model was fitted to an independent database of Cd bioaccumulation in *H. azteca* fed on

periphyton or TetraMin® for 28 d (Chapter 2.2.1). Predictions from the biokinetic model did not match those from the chronic saturation bioaccumulation model. Sensitivity analysis of the biokinetic model revealed that AE and IR were responsible for driving model predictions in the case of periphyton and TetraMin® respectively. This provided a starting point for formulating hypotheses as to why the biokinetic model differed in predicting Cd in *H. azteca* from food.

Under-prediction of the biokinetic model for those animals fed with periphyton may have been due to an underestimation of AE as a result of *H. azteca* not having the digestive capabilities to solubilize metals in periphyton as mentioned previously. *H. azteca* used in the present study were adults reared on TetraMin® and fed with periphyton one week prior to the experiment whereas *H. azteca* used in the independent bioaccumulation experiment were fed from the age of 3 – 7 d for 28 d on periphyton and thus had a digestive system that was adapted to solubilizing periphyton. Using non-linear regression, the AE required to reconcile the biokinetic model with the expected Cd body concentration from Cd contaminated periphyton when fed for 28 d was estimated to be 20 % (± 3.5 % 95 % CI). Therefore, even if *H. azteca* in the present study had been fed on periphyton from birth, the AE would be 2.7 times lower for periphyton than for TetraMin® and would be a quarter of the AE estimated for native *H. azteca* feeding on Cd contaminated periphyton in the field (Stephenson and Turner, 1993). Reasons for these discrepancies have been discussed previously as being related to differences in dietary Cd bioavailability as a result of how Cd is partitioned and speciated between all diets used.

The biokinetic model over-predicted Cd in *H. azteca* that had consumed TetraMin®. This may have been due to an over-estimation of IR during the 4 h feeding period used to develop the biokinetic model. Observations of *H. azteca* during feeding demonstrated rapid ingestion of TetraMin® during the first few hours followed by reduced feeding and finally rejection of the same food after three days as the TetraMin® aged. This gradual decline in IR was captured in the chronic bioaccumulation model by using long-term average values while the biokinetic model captured only the initial high ingestion rate thereby overestimating IR on a chronic exposure basis. To reconcile the biokinetic model

to the chronic saturation bioaccumulation model predictions of chronic body concentration, a non-linear regression estimate for IR of 0.04 g AFDM/g/d (0.005 g AFDM/g/d 95 % CI) was obtained. This IR may be skewed low by averaging over the range of Cd concentrations used in the independent database. Therefore the hypothesis of an over-estimated IR may only partly explain the over-prediction of the biokinetic model with further work on the effects of increasing dietary Cd on IR being required.

The complete biokinetic model also considers Cd bioaccumulation from water. Cadmium uptake and excretion rate constants from dissolved exposure only were not determined experimentally but were incorporated into the biokinetic model from the chronic bioaccumulation model (Eq. 3.6). Inclusion of Cd bioaccumulation from water improved the fit of the biokinetic model predictions to measured bioaccumulation over 28 d. However, predictions of bioaccumulation in *H. azteca* fed with TetraMin® were still over-predicted by a factor greater than two suggesting model parameters determined over acute exposures may not accurately represent changes to physiological processes over chronic exposure periods. The fact that the biokinetic model was over-predicting bioaccumulation was advantageous by erring towards a more conservative level of protection.

3.4.5 *Implications for dietary Cd experiments and modelling*

The choice of food type and form for use in dietary experiments is dependent on the objective of the research (Campbell *et al.*, 2005). Based on the research presented here, AE was not affected by whether periphyton was in fresh or lyophilized forms and therefore the dry form of periphyton may be more convenient to use in mechanistic studies of dietary Cd uptake for *H. azteca*. However, ingestion rate differed between fresh and dry forms of the same food type and there were differences in the AE of the two foods therefore, a fresh periphyton diet would be recommended when developing a bioaccumulation model for extrapolation to the field.

Once the choice of food type has been matched to the research objectives, the organism should be fed on the experimental diet long enough to have developed an enzyme system

capable of solubilizing it. In the case of *H. azteca*, it's possible that one week feeding on periphyton was insufficient.

This research also has implications for the contribution of dietary Cd in standard chronic aqueous Cd toxicity tests with *H. azteca* where artificial diets such as TetraMin® are commonly used. While dissolved Cd may be the main exposure pathway being investigated, at least 54 % of the Cd associated with TetraMin® could be assimilated making a contribution to *H. azteca* body concentration ranging from 21 – 31 % (Chapter 2). Therefore the final aqueous Cd endpoint will represent toxicity associated with both aqueous and dietary exposures and yet because of the food type and speciation this may not truly represent the contribution of dietary Cd to toxicity in the field.

Predictions of bioaccumulation from the biokinetic model have been successfully validated in the field with a variety of marine and freshwater invertebrates (Luoma and Rainbow 2005). In these studies, physiological model parameters were measured using invertebrates collected from the same field locations in which metal in water and food had been measured and data from highly contaminated sites were excluded due to data unavailability (Luoma and Rainbow 2005). This approach resulted in biokinetic models specific to the organism, metal and exposure concentration of the field site studied. In contrast, the approach taken in the present research was to use laboratory reared animals and measure the model parameters over a range of dietary Cd concentrations known to result in low to high chronic lethality. Again the biokinetic model developed from this approach was specific to the organism, metal and exposure concentration. However when we compared predictions from this model to estimates from an independent model of chronic Cd bioaccumulation, the predictions varied by a factor greater than two for those *H. azteca* fed with TetraMin®. A major difference between these two approaches was that the current research was conducted at dietary concentrations that were potentially chronic lethal and therefore model parameters determined empirically with acute exposures at high concentrations likely did not accurately extrapolate to chronic exposures. This has implications for determining effects of chronic toxicity on biokinetic model parameters that are measured over short time frames. One way to address this may be to conduct

chronic exposures with stable Cd and at certain time intervals, perform the pulsed feeding exposures to ^{109}Cd. The relationship between the model parameter and chronic Cd concentration could then be incorporated into the biokinetic model to account for toxic effects with increasing Cd over long exposures.

3.5 CONCLUSIONS

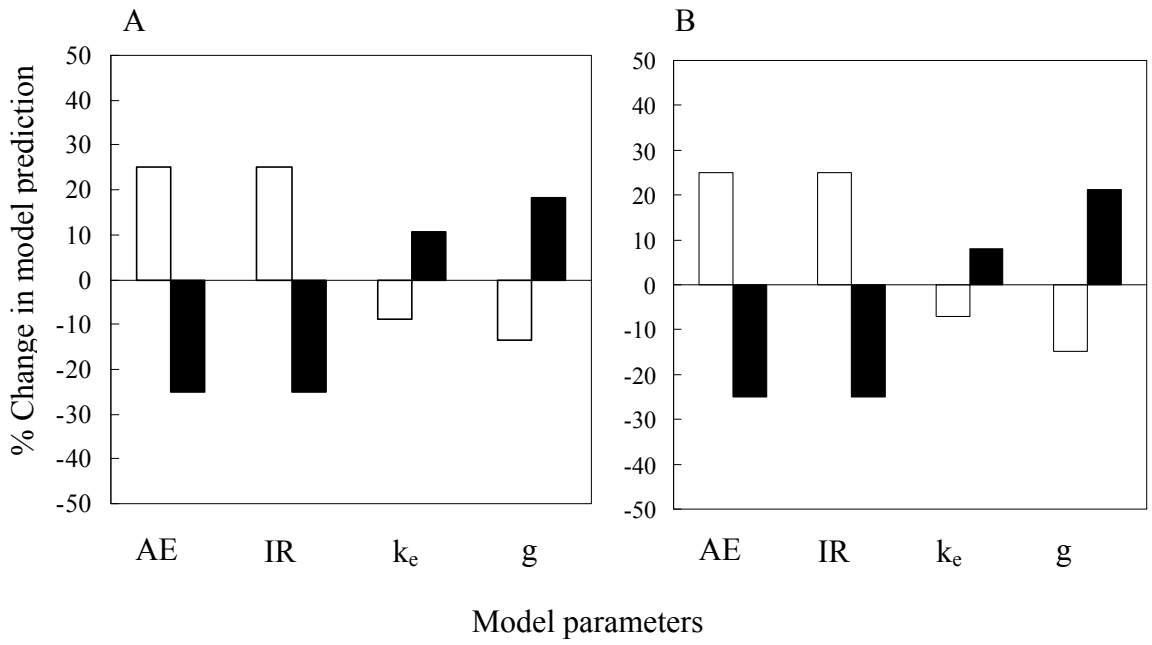
This research demonstrated that dietary Cd bioavailability was dependent on food type and Cd speciation associated with the chemical composition of the food. Assimilation efficiency of Cd from periphyton was lower than that from TetraMin® though it may be that this was partly due to the gut enzyme system of laboratory cultured *H. azteca* not being sufficiently adapted to a periphyton diet. Predictions of bioaccumulation from the biokinetic model did not concur with predictions from an independent chronic Cd saturation bioaccumulation model. An advantage of using a mechanistic biokinetic modelling approach was that hypotheses for model discrepancies could be formulated based on discrete physiological model parameters (AE and IR) and could therefore guide future research. This research had implications for both performing dietary metal experiments to achieve ecologically realistic results and enhancing the application of the biokinetic model to predict chronic bioaccumulation over a range of sublethal to chronically lethal Cd concentrations.

APPENDICES

A3.1 Mean measured Cd in fresh and dry periphyton and TetraMin® on an ash-free dry mass basis with estimated mean values of Cd assimilation efficiency (AE), ingestion rate (IR), excretion rate constant of Cd (k_e) from tissue and excretion rate constant of Cd from gut contents (k_{egut}) in *H. azteca* (± 95 % CL with sample size in parentheses)

Food type	Cd in food [1] (nmol/g AFDM)		AE (%)		IR [1] (g AFDM/g *H. azteca* /day)		k_e (d^{-1})		k_{egut} (d^{-1})	
Fresh Periphyton	1510 ± 244	(4)	3 ± 1	(20)	0.13 ± 0.035	(20)	0.064 ± 0.035	(20)	13 ± 1.1	(100)
	31200 ± 4640	(3)	9 ± 2	(20)	0.15 ± 0.029	(20)	0.032 ± 0.020	(20)	8.3 ± 0.54	(100)
Dry Periphyton	1510 ± 244	(4)	10 ± 5	(20)	0.021 ± 0.0059	(20)	0.094 ± 0.055	(20)	8.6 ± 0.84	(100)
	31200 ± 4640	(3)	14 ± 2	(18)	0.059 ± 0.009	(18)	0.058 ± 0.022	(18)	7.0 ± 0.52	(100)
Fresh TetraMin®	358 ± 88	(2)	75 ± 10	(10)	0.32 ± 0.18	(10)	0.042 ± 0.030	(10)	0.89 ± 0.17	(50)
	2890 ± 955	(3)	44 ± 14	(20)	1.1 ± 0.12	(20)	0.040 ± 0.020	(20)	2.8 ± 0.33	(100)
Dry TetraMin®	358 ± 88	(2)	86 ± 7	(9)	0.16 ± 0.02	(9)	0.081 ± 0.035	(9)	0.67 ± 0.15	(50)
	2890 ± 955	(3)	63 ± 8	(19)	0.19 ± 0.035	(19)	0.044 ± 0.016	(19)	1.6 ± 0.17	(100)

[1] Convert Cd in food or IR from an ash-free dry mass basis to dry mass basis by multiplying by the fraction of organic content which is 0.521 or 1 for periphyton or TetraMin® respectively



A 3.2 Sensitivity analysis showing the percent change in model predicted Cd in *H. azteca* when fed Cd in fresh periphyton (A) and fresh TetraMin® (B). Parameter values were increased by 25 % (white bars) or decreased by 25 % (black bars) for each model parameter of assimilation efficiency (AE), ingestion rate (IR), excretion rate constant (k_e) and growth rate constant (k_g) in turn while the mean was used for the remaining parameters.

CHAPTER 4
Validation of a chronic dietary cadmium bioaccumulation and toxicity model for *Hyalella azteca* exposed to field contaminated periphyton and lake water

ABSTRACT

A model previously developed to predict chronic bioaccumulation of cadmium (Cd) in *Hyalella azteca* from natural periphyton was validated by comparing predictions to independent measurements of Cd in *H. azteca* exposed to field contaminated water and periphyton for 28 d, and in *H. azteca* collected from the same contaminated field sites. In both cases, model predictions were shown to be robust, however, effects on Cd bioaccumulation from complexation with dissolved organic carbon (DOC) and inhibition of Cd bioaccumulation by Ca^{2+} need to be incorporated into the model to permit its wider application. The model predicted that 80 – 84 % of Cd in *H. azteca* came from periphyton when *H. azteca* were chronically exposed to Cd in lake water at 2.63 – 3.01 nmol/L (0.45 μm filtered) and periphyton at 1880 - 2630 nmol/g ash-free dry mass. Therefore, dietary Cd contributed markedly to the model predicted decrease in 28 d survival to 74 % at environmental Cd concentrations in food and water. In reality, survival decreased to 10 %. The lower than predicted survival was likely due to the higher nutritional quality of periphyton used to develop the model compared to the field collected periphyton. Overall this research demonstrated that Cd in a periphyton diet at environmental concentrations can contribute to chronic toxicity in *H. azteca* and that both dissolved and dietary exposure pathways need to be incorporated when modelling chronic Cd bioaccumulation and toxicity.

4.1 INTRODUCTION

Although dietary cadmium (Cd) in the aquatic environment has been identified as contributing to chronic bioaccumulation, trophic transfer, biomagnification and toxicity in a range of aquatic biota, quantifying this contribution has proved challenging (Croteau *et al.*, 2005; Handy *et al.*, 2005; Schlekat *et al.*, 2005). Furthermore, few studies have taken the next step in linking the contribution of dietary Cd to chronic toxic effects at environmentally relevant concentrations. Bioaccumulation modelling is a powerful tool for quantifying contributions of Cd from separate exposure pathways and providing a link between dietary Cd and chronic toxicity via body concentration (Borgmann *et al.*, 2005; Luoma and Rainbow, 2005; Rainbow, 2007).

A bioaccumulation model (Chapter 2) was developed using Cd spiked water and food in the laboratory that predicted the chronic bioaccumulation of Cd in the freshwater amphipod *Hyalella azteca* from water and a natural diet of periphyton separately and combined. In a combined food+water treatment, the model predicted that 40 – 55 % of the Cd in *H. azteca* came from the periphyton. Effects on survival as a function of body concentration were independent of exposure pathway meaning that the toxicity of Cd from periphyton was the same as that from water when based on Cd tissue concentration. This provided an important link between bioaccumulation of Cd from food and effects on survival, with the chronic LBC50 equal to 679 nmol/g (617 – 747 95 % CL).

Stephenson and Turner (1993) also developed an 11 d bioaccumulation model of Cd uptake by wild *H. azteca* from a periphyton diet based on field transfer studies of caged *H. azteca* and periphyton between a Cd spiked lake and a reference lake. Their model predicted that 58 % of the Cd in *H. azteca* came from periphyton. However, they specifically targeted a no-effect exposure concentration and thus no link between dietary Cd and chronic effects was made. Therefore the model from Chapter 2 could potentially act as an important tool in risk assessment for predicting chronic toxicity of Cd from periphyton and water. However a vital step remaining is to validate the model predictions with an independent database and to explore the extent to which the model in its current form can be applied to field conditions.

This independent database was generated by conducting 28 d laboratory exposures of *H. azteca* to lake water and periphyton collected from three lakes near Rouyn-Noranda, Quebec, Canada representing a gradient of metal contamination. A large copper smelter is an atmospheric point source of historical metal contamination for the lakes downwind of the smelter (Telmer *et al.*, 2006). Metals leaching from abandoned and active mine tailings provide aqueous point sources of contamination to the lakes in the region (Couillard *et al.*, 2004). These lakes have been studied extensively in terms of metal speciation (Fortin *et al.,* 2010; Guthrie *et al.*, 2005), effects of metals in sediments on *H. azteca* and benthic invertebrate community composition (Borgmann *et al.,* 2004b; Norwierski *et al.*, 2006), effects of dissolved metals on mussels (Perceval *et al.*, 2006) and dietary metal effects on fish (Kraemer *et al.*, 2006). To date, no study on these lakes has investigated dietary metal effects on *H. azteca*. The model was also applied to measurements of Cd in *H. azteca* collected from the lakes. In addition, laboratory cultured adult *H. azteca* were caged in these lakes for 14 d to act as further validation of extrapolating laboratory results to the field. Periphyton nutrition was measured and related to effects on *H. azteca* survival and growth.

The objectives of this research were to:

1) compare model estimates of chronic Cd bioaccumulation in *H. azteca* to independent measurements of Cd in *H. azteca* exposed to field contaminated water and periphyton in the laboratory and Cd in *H. azteca* residing in the lakes

2) quantify the contribution of Cd from periphyton to bioaccumulated Cd in *H. azteca*

3) compare model estimates of chronic toxic effects on *H. azteca* survival and growth as a result of both waterborne and dietary Cd exposure, with observed toxicity.

Together these objectives were aimed at determining whether a model developed from laboratory experiments could accurately predict how much Cd in a natural diet at environmentally relevant concentrations was bioaccumulated by *H. azteca* and whether bioaccumulation could be used to accurately predict chronic effects on survival and growth associated with dietary Cd.

4.2 METHODS AND MATERIALS

4.2.1 *Field sample collection*

Three lakes in the metal mining region of Rouyn-Noranda in NW Québec, Canada were selected for sample collection because of their circum-neutral pH and gradient of aqueous Cd concentration (Borgmann *et al.*, 2004b). Sites in Lakes Opasatica (OP) (Long. 79°17′14″, Lat. 48°05′11″), Joannès (JO) (Long. 79°40′25″, Lat. 48°11′02″) and Dufault (DT) (Long. 79°00′06″, Lat. 48°17′46″) represented low, medium and high Cd lakes respectively (Figure 4.1).

Water, sediment, periphyton and *H. azteca* samples were collected from a single site in OP, JO and DT during 17-18 July 2007 and analyzed for 27 metals. All samples were collected from ≤1 m water depth to determine Cd exposure concentrations in *H. azteca* habitat. Ambient dissolved oxygen (DO_2), pH, temperature and conductivity were measured in each lake and water samples were analyzed for major ions (Ca, Mg, Na, K, Cl, SO_4), dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC). Water samples (unfiltered and syringe filtered using 0.45 µm polysulfone Acrodisc® filters) to be analyzed for metals were stored in acid washed polypropylene bottles and acidified (1 % v/v with 70 % Omni*Trace* Ultra™ high purity HNO_3). Field blanks consisting of NANOpure® de-ionized water were processed in the same manner as the samples.

Near-shore lake sediment was collected at 1 m water depth using an Ekmann grab and the oxic surface layer (top 1 cm) was removed, dried (60 ^{o}C), sieved (< 63 µm nylon mesh sieve), digested and analyzed for 27 metals.

Periphyton was scraped from glass slides (25 mm x 75 mm) deployed as artificial substrates, cobbles and macrophytes using a stainless steel scalpel blade to form an homogenous bulk stock. Periphyton bulk stock from each lake was used as food for *in situ* caged *H. azteca*, food for *H. azteca* in 28 d laboratory-based feeding experiments and analyzed for metals, dry mass, ash-free dry mass (AFDM), chlorophyll *a*, protein, total lipid and algal species identification (Biggs and Kilroy, 2000).

Field collected *H. azteca* were depurated for 24 h in standard artificial media (SAM: 1 mmol/L $CaCl_2$, 1 mmol/L $NaHCO_3$, 0.01 mmol/L NaBr, 0.05 mmol/L KCl, 0.25 mmol/L $MgSO_4$ in NANOpure® de-ionized water, pH 8.2 and hardness 125 mg/L $CaCO_3$; Borgmann, 1996) containing 50 µmol/L ethylenediamine tetra-acetic acid (EDTA) with 2.5 mg TetraMin® (Tetra Holding (US) Inc.). *H. azteca* were then rinsed in SAM without EDTA, dried at 60 ^oC (48 h), weighed and digested for metal analysis. *H. azteca* analyzed for total protein and total lipid were stored at -80 ^oC.

4.2.2 *In situ exposures of H. azteca*

Six replicate cages constructed from two joined sections of acrylic tube (7.6 cm diameter by 7.6 cm length) with 500 µm nylon mesh sealing either end (Borgmann *et al.*, 2007) were deployed for 14 days in each lake. Each cage contained 15 adult (4 week old) *H. azteca* that had been laboratory cultured in SAM on a diet of TetraMin®. No mortality of transported *H. azteca* was observed during a period of temperature acclimation prior to addition to the cages. Cages were positioned approximately 5 cm above the sediment in 1 m or less water depth. A 30 mL aliquot (equivalent to mean (±SD) of 0.03 ± 0.02 mg AFDM/amphipod/day) of homogenized periphyton stock collected from the same lake was added to each cage as well as a glass microscope slide (25 x 75 mm) and nylon mesh (500 µm mesh size, 9 cm^2) to provide food and substrates for supplemental periphyton growth and *H. azteca* artificial habitat. *H. azteca* survival, growth, metal bioaccumulation following 24 h depuration, total protein and total lipid were measured after 14 d exposure in the cages.

4.2.3 *Laboratory feeding experiment*

The experiments were designed to have treatments of Cd in water and food separately and combined (food+water). In reality, the separate water and food treatments also had measureable levels of Cd in food and water respectively as a result of Cd partitioning between the two exposure pathways. Therefore, throughout this chapter, the separate water and food treatments may be considered to be primarily (but not exclusively) Cd in water and primarily (but not exclusively) Cd in food, respectively.

Using the water and periphyton collected from OP, JO and DT and stored at 4°C in the dark for one week, fifteen laboratory cultured juvenile *H. azteca* (0 – 1 week old) were exposed for 28 d to three metal exposure treatments (water, food, food+water) and a control of SAM with TetraMin®. Treatments were replicated four times for each lake. Test solutions consisted of unfiltered lake water in the water and food+water treatments, and SAM in the food treatment. Lake periphyton was prepared for the food and food+water treatments by filtering an aliquot (equivalent to 3.5 mg AFDM) from the bulk periphyton stock onto 0.45 µm polycarbonate membranes that were stored in humidified containers in the dark at 4 ^{o}C until use. A standard fish flake diet of TetraMin® (5 mg fresh or 3.5 mg AFDM) was used as the food source for the water treatment. The static renewal system was composed of 200 mL test solution per replicate (450 mL high density polyethylene containers) with solutions renewed every third or fourth day (i.e. twice per week). At the time of solution renewal, *H. azteca* and artificial substrate (3 x 3 cm 750 µm nylon mesh) were transferred to new solutions and provided with a mean (±SD) food ration of 0.070 ± 0.011 mg AFDM/amphipod/day of either TetraMin® or periphyton depending on the treatment. Experiments were conducted at 25 ^{o}C with 16:8 h light:dark photoperiod. At 28 d, *H. azteca* were depurated as mentioned previously with a new piece of nylon mesh before obtaining wet and dry (48 h at 60 ^{o}C) weights. The mean (±SD) ratio of dry:wet weight was 0.237 ± 0.021 on a per amphipod basis. *H. azteca* were stored in acid-washed cryovials at room temperature until being digested and analyzed for 27 metals, or stored at -80 ^{o}C until being analyzed for total protein and lipid. *H. azteca* survival was recorded.

At each water change, temperature, DO_2, pH, conductivity and ammonia were measured in each replicate of old and fresh test solutions. On one occasion, DIC and DOC were measured in one replicate of old test solutions from each treatment. Filtered (0.45 µm polycarbonate membrane) and unfiltered water samples (1 mL) were collected from each replicate of new and old solutions and preserved with acid (1 % v/v with 70 % Omni*Trace* Ultra™ high purity HNO_3) for metal analysis. New and old test solution samples were composited separately for each replicate over 28 d and two replicates of

each treatment were analyzed for 27 metals. Final aqueous metal concentration was calculated as the geometric mean of measured metal in new and old test solutions.

4.2.4 *Partitioning of metal between food and water*

The change in metal concentration in food used in the feeding experiment was quantified in a separate experiment conducted over 4 d which was the longest exposure time of food to the test solutions. The experimental design mimicked that of the 28 d feeding experiment with the exception that no animals were present so that enough food biomass could be collected for metal analysis. Filtered (0.45µm polycarbonate membrane) water samples (1 mL, n = 2 replicates) were collected at 0, 2, 4, 8, 24, 48, 72, 96 h in the control, water, and food treatments and every 24 h for food+water treatments and analyzed for Cd. Food (n = 2 replicates) was collected every 0, 2, 4, 24, 72, 96 h for control, water and food treatments and at 0 and 96 h for food+water treatments and analyzed for 27 metals. Water temperature, DO_2, pH, conductivity and ammonia were also measured daily in one treatment replicate. Final dietary metal concentration was calculated as the geometric mean of the initial and 96 h measured concentration in the food.

4.2.5 *Sample processing and analyses*

Dried sediment (10 mg), food (0.839 to 11.540 mg) and *H. azteca* (2 to 7 individuals at 0.165 to 3.182 mg) were digested at room temperature in 70 % Omni*Trace* Ultra™ high purity HNO_3 (0.250 mL, 1.75 % in final digest volume) for 6 d followed by 30 % hydrogen peroxide ACS grade (0.200 mL, 0.6 % in final digest volume) for 24 h at 60 °C and made to final volume (10 mL) with NANOpure® de-ionized water (9.55 mL).

Analysis of samples and blanks for 27 metals, DIC/DOC and major ions was performed by the National Laboratory for Environmental Testing (NLET), Burlington, Ontario, Canada. Metals were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) with instrument detection limits of <0.0005 µg/L for U, <0.001 µg/L Ag, Be, Bi, Cd, Ga, La, Sb, Tl, <0.002 µg/L for Co, <0.005 µg/L for Cr, Pb, V, <0.01 µg/L for As, Mo, Rb, <0.02 µg/L for Cu, Ni, <0.05 µg/L for Ba, Mn, Se, Sr, Zn, <0.1 µg/L for B,

<0.2 µg/L for Al, Li, <0.5 µg/L for Fe. The use of multiple standards, drift correction, blank correction and certified reference standards during each run were part of quality control requirements. Recovery of all metals from certified reference standards ranged from 86 to 106 %. Mean (±SD) recovery of Cd from TORT-2 (lobster hepatopancreas, National Research Council of Canada) was 104 ±0.43 %. Method detection limits for each metal were calculated as the upper 95 % confidence limit of the blank samples. Food and *H. azteca* digests could not be analyzed for As or Se due to matrix interferences with ICP-MS. Dissolved inorganic and organic carbon were analyzed using a UV persulfate TOC Analyzer (Pheonix 8000^{TM}). Major cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) were analyzed using a flame atomic absorption spectrophotometer. Major anions (Cl^{-}, SO_4^{2-}) were analyzed using separation on an anion exchange resin followed by measurement of conductivity.

Water samples collected from the 96 h food exposure experiment were analyzed for Cd using a Varian SpectrAA 400 graphite furnace atomic absorption spectrophotometer (GF-AA) with Zeeman background correction. Multiple standards and blanks were used during the run and correction for drift and blank readings was made. A Cd standard used to check the accuracy of the calibration had a mean (±SD) recovery of 107 ± 5 %. Method detection limit calculated as the upper 95 % confidence limit of the filtered blank water samples was 0.017 nmol/L, n = 20.

Methods for measurement of AFDM, chlorophyll *a*, total protein and total lipid are described in detail in Chapter 2.3.6. Briefly, AFDM was measured by drying samples at 105 ^{o}C for 24 h followed by ashing at 400 ^{o}C for 4 h, chlorophyll *a* was extracted in 90 % ethanol at 78 ^{o}C and spectrophotometric readings were corrected for phaeophyton (Biggs and Kilroy, 2000). Total protein was measured spectrophotometrically with the microplate bicinchoninic assay kit (Sigma Aldrich BCA1). Total lipid was measured gravimetrically after sample extraction in chloroform:methanol 2:1 (v/v) and a NaCl wash.

4.2.6 *Statistical analysis*

Statistical analysis was performed using SYSTAT version 10.0. Of the 27 metals measured in water, food and *H. azteca* in the 28 d feeding experiment, 18 metals that were above detection limits for all sample types were used in analysis of variance (ANOVA) to find statistical differences as a function of site and exposure pathway. Specifically, ANOVAs were performed to determine differences for each metal in lake water and periphyton as a function of site and for each metal in *H. azteca* as a function of lake water or periphyton. Assumptions of normality of data distribution and homogeneity of variance were tested with visual assessment of probability density plots of non-transformed and log transformed data and Levene's test (Environment Canada, 2005).

4.2.7 *Metal speciation*

Modelling of free Cd^{2+} concentration was performed using the Windermere Humic Aqueous Model (WHAM) version 6.0.13 (purchased from Centre for Ecology and Hydrology, UK). Model input parameters were temperature (as K), pH, major ions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-} mol/L), DIC g/L divided by 12.011 g/mol C as CO_3^{2-} mol/L and dissolved Cd mol/L (0.45 μm filtered). It was assumed that 50 % of natural organic matter was composed of carbon and 65 % of natural organic matter was active for metal binding and was 100 % fulvic acid (Bryan, *et al.*, 2002) therefore DOC g/L was multiplied by 1.3 to give the fulvic acid g/L input value. Twelve (Al, Ba, Cd, Co, Cu, Fe, Mn, Ni, Pb, Sr, U, Zn) of the 27 metals analyzed were also entered as they have DOC binding constants in the WHAM database.

4.2.8 *Modelling*

4.2.8.1 *Bioaccumulation model*

A chronic Cd bioaccumulation model was developed (Chapter 2.2) that predicted Cd in *H. azteca* from water and food separately and combined:

$$C_{TB} = \frac{max_w \text{ x } C_w}{(K_w + C_w)} + \frac{max_f \text{ x } C_f}{(K_f + C_f)} \qquad \text{(Eq. 4.1)}$$

Where C_{TB} is the total body concentration of Cd in *H. azteca* at steady state (nmol/g), max_w is the maximum Cd in *H. azteca* from water (nmol/g), C_w is the measured concentration of Cd in the water (nmol/L), K_w is the half saturation constant from water i.e. the concentration of Cd in the water at which the Cd in *H. azteca* is half the maximum (nmol/L), max_f is the maximum concentration of Cd in *H. azteca* from food (nmol/g), C_f is the measured concentration of Cd in food on an AFDM basis (nmol/g AFDM),and K_f is the half saturation constant from food i.e. the concentration of Cd in food at which the Cd in *H. azteca* is half the maximum (nmol/g AFDM). The model has a separate set of parameter values for dissolved Cd and free ion Cd^{2+} (Table 2.4).

4.2.8.2 *Toxicity model*

A model that predicts *H. azteca* chronic survival when exposed to Cd in water and food separately and combined was developed using mortality rate as a function of Cd in *H. azteca* (Chapter 2.3.10.4):

$$m = m' + (\ln(2)/t) \times [C_{TB} (1/LBC50 + 1/K'') / (1 + C_{TB}/K'')]^n \quad (4.2)$$

where m is the total mortality rate (*H. azteca* per week), m' is the control mortality rate (0.021475 *H. azteca* per week), t is exposure duration (4 weeks), LBC50 is the Cd body concentration resulting in 50 % lethality (679.18 nmol/g), K'' is the half saturation constant i.e. the Cd body concentration at which m is half the maximum (17.342 nmol/g) and n is an exponent fixed to 100. Total mortality rate (m) was converted to 28 d survival (S %) using:

$$S = 100 \times e^{(-4 \times m)} \quad (4.3)$$

4.3 RESULTS

4.3.1 *ANOVA of metals in lake water, periphyton and H. azteca*

Of the 18 metals (Al, Ba, Cd, Co, Cr, Cu, Fe, Ga, Mn, Mo, Ni, Rb, Sb, Sr, Tl, U, V, Zn) that were used in ANOVA, many showed significant differences in lake water and

periphyton as a function of site and in *H. azteca* as a function of exposure pathway (Table 4.1). However, Cd was the only metal to show significant differences at the $P \leq 0.001$ level of significance across all four of the ANOVA models, suggesting that a strong gradient of Cd was present in water, periphyton and *H. azteca* as a function of Cd in water and periphyton. Research by Borgmann *et al.* (2004b) indicated that Cd was the dominant source of toxicity to *H. azteca* in DT, therefore the results presented in this research will focus on Cd. Concentrations of all 27 metals measured in lake waters, test solutions, food, sediment and *H. azteca* can be found in the appendices (A4.1 – A4.5).

4.3.2 *Physico-chemistry of lake waters and test solutions*

Standard artificial medium had higher concentrations (4 – 156 fold) of major ions and DIC compared to the lake waters with the exception of SO_4 in DT (Table 4.2). This resulted in higher conductivity, hardness and pH in SAM. Lake waters were in the circum-neutral range of pH, well oxygenated, varied in hardness (2 fold) and DOC (2 fold), and were comparable in temperature to laboratory test solutions. Major ion concentrations in lake waters were generally highest in DT as was conductivity.

Lake waters and SAM used as test solutions in the laboratory with *H. azteca* and food added had mean (±SD) water temperature of 22.8 (0.55)°C, oxygen of 7.58 ± 0.31 mg/L and ammonia of 0.02 ±0.05 mmol/L. Measurements of pH, conductivity, DIC and DOC (A4.6) did not vary markedly from those measured in the lake itself (Table 4.2).

4.3.3 *Aqueous Cd and speciation*

An increasing gradient of dissolved Cd was measured in lakes OP, JO and DT ranging from 0.06 to 3.01 nmol/L (Table 4.3). Filtered (0.45µm) Cd was 61 to 93 % of unfiltered Cd indicating most Cd was present in dissolved or colloidal forms. Dissolved Cd in the same lake waters used as laboratory test solutions in the water and food+water treatments, decreased by 11 to 46 %, probably as a result of adsorption to ambient surfaces, however the gradient of concentrations was conserved (Figure 4.2). Cadmium leached from JO and DT periphyton into the surrounding water, most notably in the case of the food treatment, resulting in mean (±SD) dissolved Cd of 0.29 ± 0.03 nmol/L and

2.4 ± 0.01 nmol/L in JO and DT food treatments respectively. Cadmium was lost from solution to TetraMin® and other ambient surfaces in the water treatment resulting in a mean (±SD) 30 ± 8 % reduction in dissolved Cd between water renewals. Therefore, throughout this chapter, the separate water and food treatments may be considered to be primarily (but not exclusively) Cd in water and primarily (but not exclusively) Cd in food, respectively.

Windermere Humic Aqueous Model predicted that 2 – 81 % of dissolved Cd was bound to colloidal fulvic acid. The percent free ion Cd^{2+} varied in lake waters (20 - 46 %) and test solutions (19 – 75 %) primarily as a function of differing DOC (Tables 4.2 and 4.3). While pH was similar in all lake waters, DOC differed (JO>OP>DT) and consequently the percent of Cd^{2+} was highest in DT>OP>JO. The percent Cd^{2+} in lake waters was conserved when lake waters were used as laboratory test solutions but increased to a mean 72 % in SAM in the food treatment where DOC was as much as 6.5 times lower than in lake waters.

4.3.4 *Cd in food*

A steep gradient in Cd concentration in lake periphyton was also observed with DT being 90 and 20 fold higher than OP and JO respectively (Table 4.3). This gradient was conserved in the 28 d laboratory feeding experiment, with periphyton used in DT treatments having 61 and 28 fold higher Cd than periphyton used in OP and JO treatments respectively (Figure 4.2B). Cadmium in periphyton used in food and food+water treatments did not change over 96 h exposure to test solutions despite the measured loss of Cd to the surrounding water. However, Cd increased 1.5, 3 and 9 fold in TetraMin® exposed to OP, JO, DT lake water respectively for 96 h (A4.7) though there was no significant difference (P=0.664) in Cd in TetraMin® with lake water when averaged over time (Figure 4.2B).

4.3.5 *Measured Cd in H. azteca*

Cadmium in field collected *H. azteca* positively reflected the gradient of Cd concentrations in aqueous and dietary exposure pathways (Table 4.3). Cadmium

measurements in field collected *H. azteca*, notably from DT, had high variability and thus did not differ significantly from Cd measured in laboratory cultured *H. azteca* that were caged for 14 days in respective lakes or *H. azteca* exposed to both lake periphyton and water for 28 d in the laboratory ($P>0.05$). The exception was the two fold lower Cd in *H. azteca* collected from the field compared to laboratory *H. azteca* exposed to water and periphyton from OP for 28 d. There were no significant differences between Cd measured in caged *H. azteca* and laboratory exposed *H. azteca* in the food+water treatment ($P>0.05$), however laboratory exposed *H. azteca* were generally higher in Cd than caged or field collected *H. azteca* (A4.9).

Within each laboratory-based experimental treatment, Cd in *H. azteca* was positively related to exposure concentration with highest bioaccumulation in DT treatments (Figure 4.2C). Across treatments for each lake, Cd in *H. azteca* in the food and food+water treatments did not differ but were as much as 4.8 times greater than Cd body concentration in the water treatment. This suggests that the dominant source of Cd to *H. azteca* was the diet. However, as noted, there were secondary sources of Cd exposure in the food and water treatments due to partitioning of Cd between those two phases. The final body concentration integrated all exposure pathways thus attributing bioaccumulation to a single source in the presence of secondary sources is problematic. To resolve the issue of source apportionment, a saturation bioaccumulation model (Eq. 4.1) was used firstly to predict total Cd in *H. azteca* using the measurements of Cd in both water and food from each treatment and secondly to predict the percent contribution of Cd in *H. azteca* from food accounting for aqueous Cd exposure.

4.3.6 *Modelled Cd in H. azteca*

A comparison of the model predicted and measured Cd in *H. azteca* (Figure 4.3A, A4.8) demonstrated that the model predictions when based on dissolved Cd model parameters were robust with all values being within a factor of two of the ideal 1:1 ratio. Applying the same model to ambient measurements of Cd in lake water and periphyton similarly demonstrated robust predictions of Cd in *H. azteca* collected from each of the lakes (Figure 4.3C). When the model was applied using parameters based on the more

bioavailable Cd^{2+} concentration, the predictions were not as accurate with 35 % and 22 % of values being greater than a factor of two different from measured Cd in laboratory and field collected *H. azteca* respectively (Figure 4.3B and D, A4.9).

Based on predictions of Cd bioaccumulation in laboratory *H. azteca*, the dominant source of Cd was periphyton in the food treatment (74 ± 2 – 87 ± 0 %; mean ±SD) and the food+water treatment (67 ± 2 – 90 ± 2 %; mean ±SD) (Figure 4.4A). Dissolved Cd was the dominant contributor to Cd in *H. azteca* in the water treatment (5 ± 0 – 29 ± 5 % from food; mean ±SD). As aqueous Cd increased, the contribution of Cd from food steadily declined in the water treatment but did not change markedly in the food and food+water treatments. The predicted contribution of Cd from food in *H. azteca* in the food+water treatment was not significantly different from that predicted in *H. azteca* collected from the lakes (Figure 4.4C). When the model was applied using parameters based on Cd^{2+} instead of dissolved Cd, predicted contributions of Cd from food increased notably in all the water treatments (1.7 – 3 fold) and the food+water treatment of JO (1.3 fold) (Figure 4.4B). For field collected *H. azteca*, the contribution of Cd from food increased notably in JO (1.4 fold) when applying the model based on Cd^{2+} (Figure 4.4D).

4.3.7 *Toxicity of Cd to H. azteca*

There was no significant effect ($P>0.05$) on chronic survival or dry weight with increasing Cd concentration in the water treatment (Figure 4.5A and B). Survival of *H. azteca* feeding on periphyton was highly variable between collection sites and treatments. Therefore despite an apparent decline in survival with increasing Cd concentration, only the food+water treatment from DT had statistically significantly lower ($P=0.014$) survival (10 ± 12 %, mean ±SD) than the food+water treatment from the low Cd site (OP). The DT food+water treatment was terminated at 21 d rather than 28 d so that sufficient tissue could be obtained for Cd analysis. Mean (±SD) control survival was 88 ± 16 %. Survival in the low Cd site (OP) food and food+water treatments, where OP periphyton was the food source, was 13 to 36 % lower than the OP water treatment where TetraMin® was used suggesting that the survival effects over treatments were related to food type as well as Cd concentration. Similarly *H. azteca* dry weight was significantly lower in those

treatments where periphyton was used as compared to TetraMin® and there was no significant change in *H. azteca* dry weight with increasing Cd concentration (Figure 4.5B). No reproductive amplexus was observed nor juveniles produced. Mean (±SD) survival of laboratory cultured adult *H. azteca* caged in OP (91 ± 10 %), JO (78 ± 14 %) and DT (84 ± 8 %), with lake specific food for 14 d did not differ significantly with increasing Cd concentration ($P>0.05$, $n = 17$). Three of the six cages in JO were dislodged while deployed but only one was not retrieved.

4.3.8 *Food and H. azteca nutrition*

Mean (±SD) organic content was significantly higher in periphyton collected from JO (30 ± 0.1 %) than OP (13 ± 4 %) or DT (10 ± 0.3 %). Mean (±SD) chlorophyll *a* of periphyton from OP (0.49 ± 0.15 mg/g AFDM), JO (0.05 ± 0.00 mg/g AFDM) and DT (0.2 ± 0.03 mg/g AFDM) was low across all lakes. Total lipid was lowest for OP periphyton but total protein of periphyton did not differ with lake (Table 4.4). Total lipid and protein of all periphyton collected was lower than that of TetraMin® (total lipid = 12 ± 0.4 %, total protein = 13 ± 0.3 %, mean ± SD %) used in the water treatments. Diatoms (*Cymbella* and *Synedra*) were a dominant component of periphyton collected from all lakes though OP contained more filamentous green algae (*Cladophora*) than either JO or DT. Periphyton from DT was visibly smothered with fine particulate material.

Total lipid and protein in *H. azteca* collected from the lakes was slightly lower than in laboratory cultured *H. azteca* but there were no marked differences between lakes or experimental treatments (Table 4.4). Due to low tissue mass available for analysis, these results should be interpreted with caution.

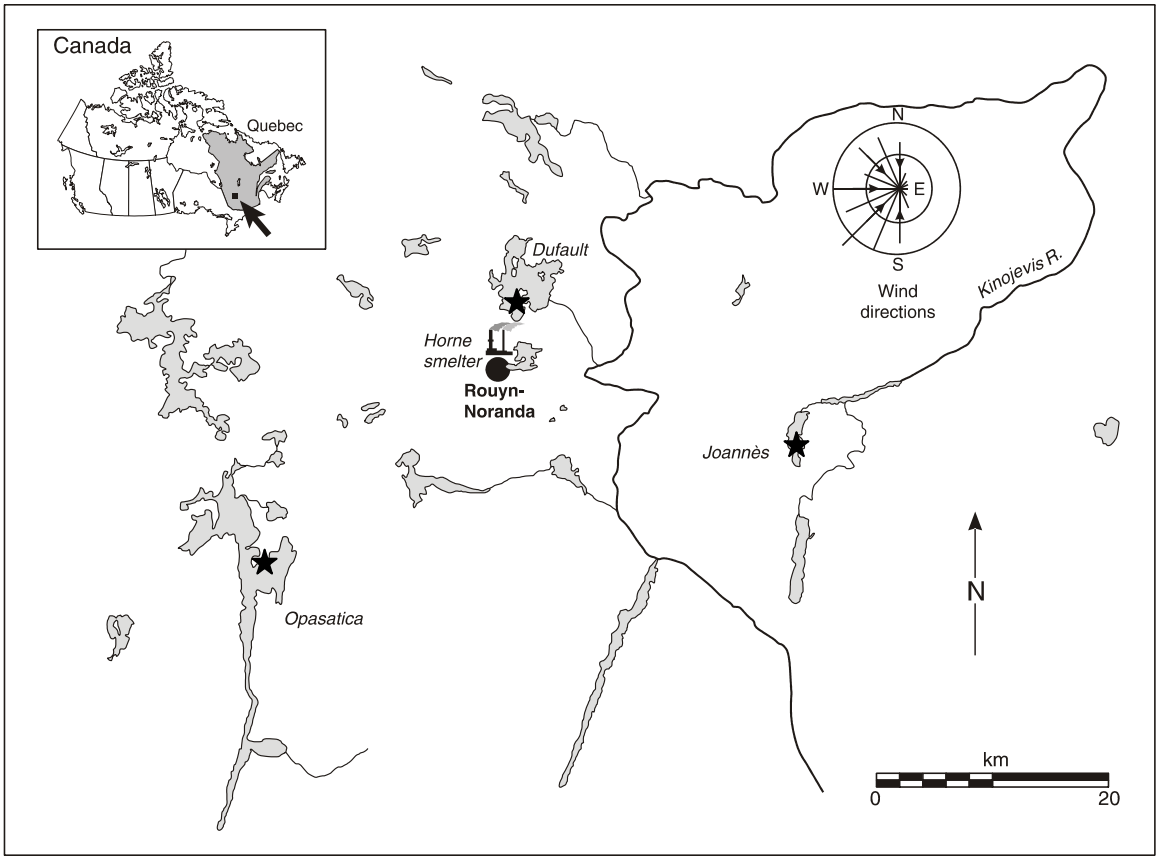


Figure 4.1 Locations of collection sites (★) in Lakes Opasatica (OP, low Cd), Joannès (JO, medium Cd) and Dufault (DT, high Cd) and the copper smelter located in Rouyn-Noranda, NW Quebec, Canada. Adapted from Borgmann *et al.* (2004b).

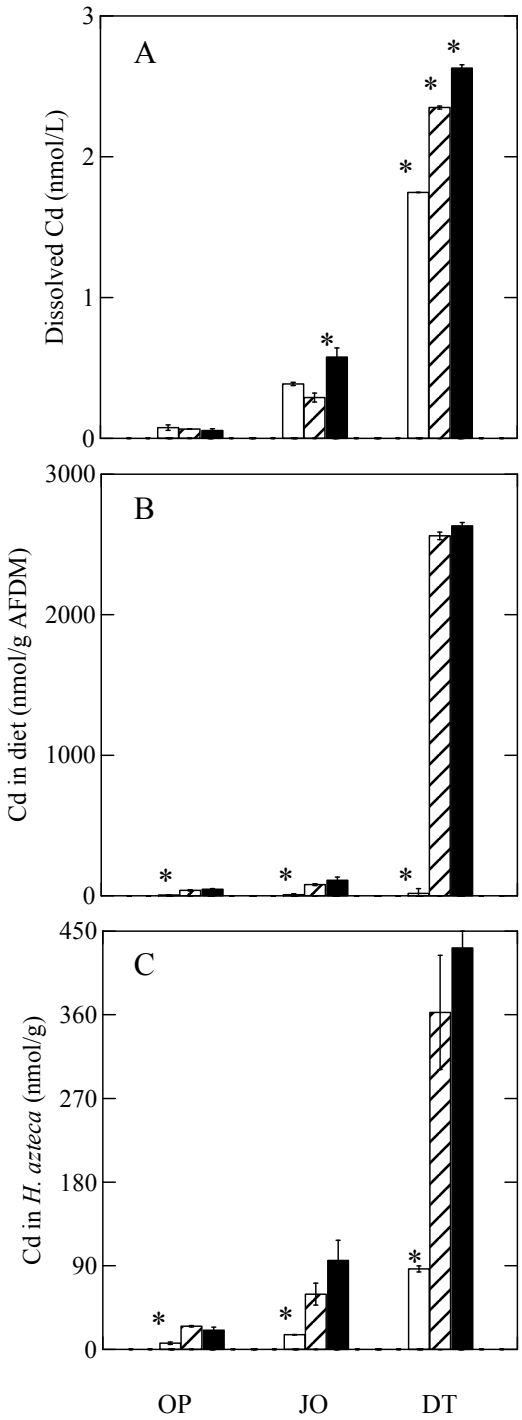


Figure 4.2 Mean ± SD (n = 2) Cd in test solutions (A), diet (on ash-free dry mass basis) (B) and *H. azteca* (C) in 28 d treatments of metals primarily in water (white bars), food (diagonal line bars) or food+water (black bars). Lake water and periphyton were collected from Lakes Opasatica (OP), Joannès (JO) and Dufault (DT). Statistically significant differences ($P<0.05$) between treatments at each collection site are shown (*).

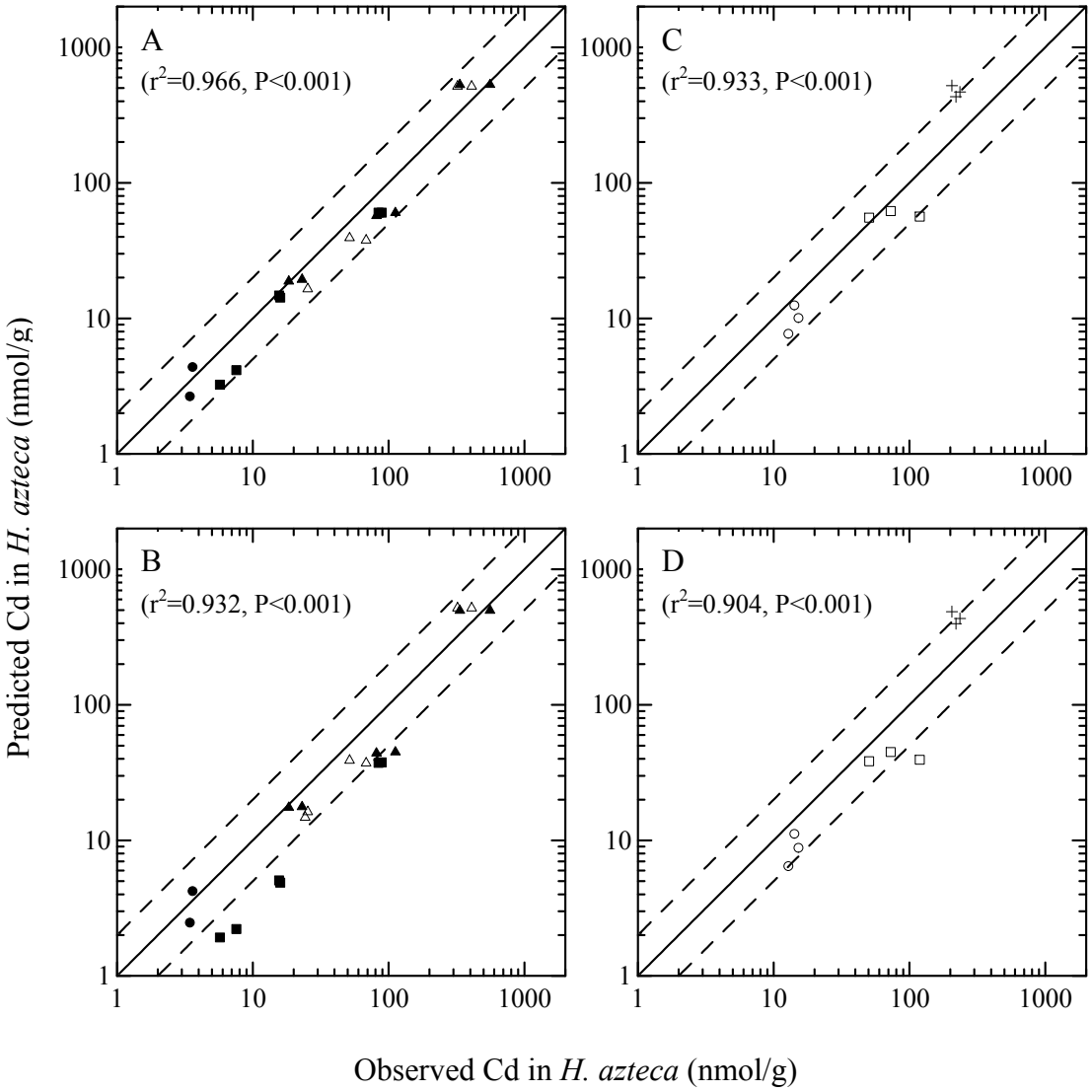


Figure 4.3 Cd in *H. azteca* predicted by the bioaccumulation saturation model based on dissolved Cd (A and C) model parameters and Cd^{2+} (B and D) model parameters versus measured Cd in *H. azteca.* Amphipods in A and B were laboratory animals exposed for 28 d to treatments of metals primarily in water (closed square), food (open triangle) or food+water (closed triangle) and controls (closed circle) using field collected samples. Amphipods in C and D were collected from Lakes Opasatica (open circle), Joannès (open square) and Dufault (cross). Solid line is $y=x$. Dashed lines are $y=0.5x$ and $y=2x$.

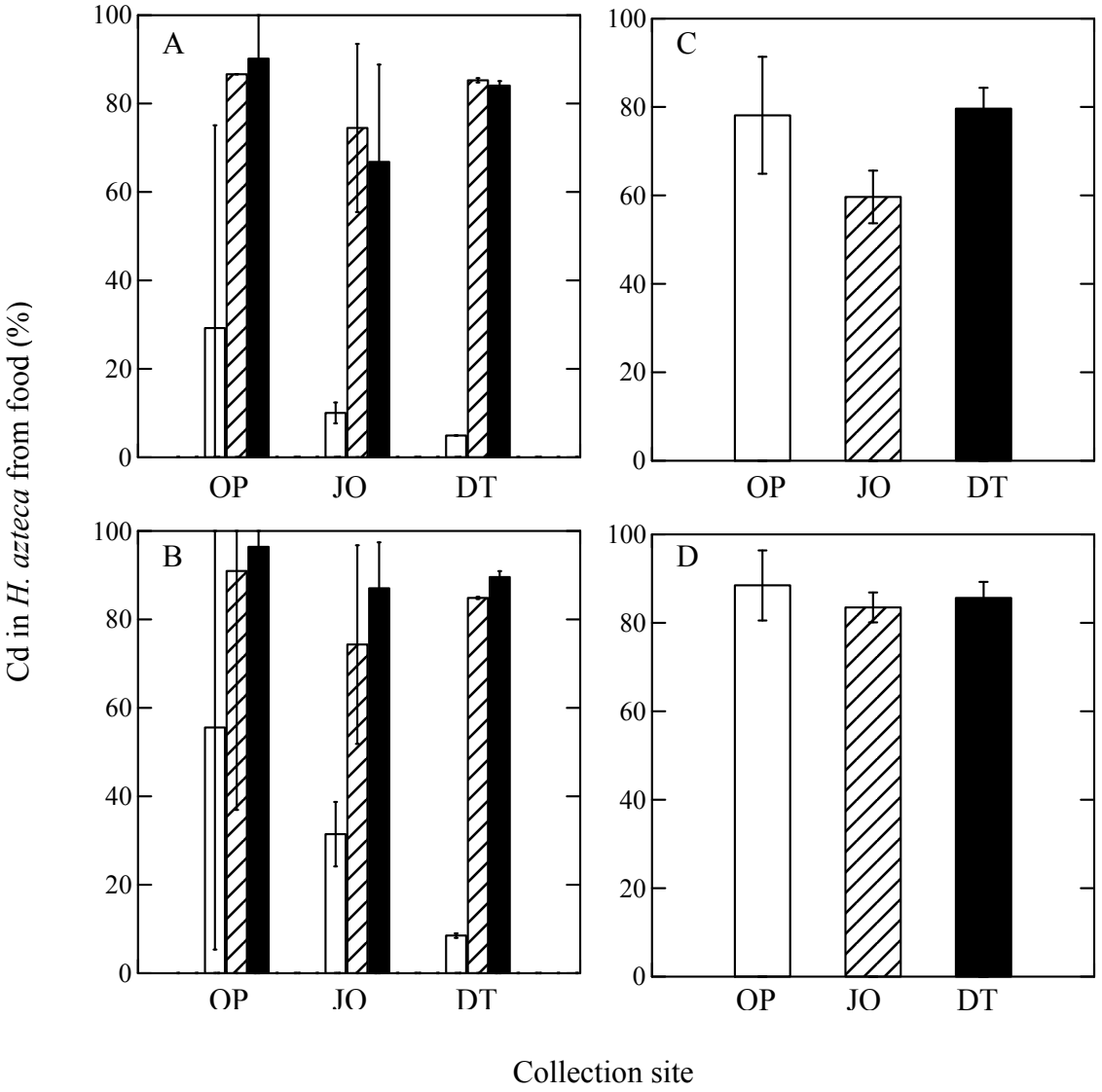


Figure 4.4 Mean (± 95 % confidence limits) percent of Cd in *H. azteca* predicted to come from food when using model parameters based on dissolved Cd (A and C) or Cd^{2+} (B and D). Amphipods in A and B were laboratory animals exposed for 28 d to treatments of metals primarily in water (white bars), food (diagonal line bars) or food+water (black bars) using field collected samples. Amphipods in C and D were collected from Lakes Opasatica (OP), Joannès (JO) and Dufault (DT).

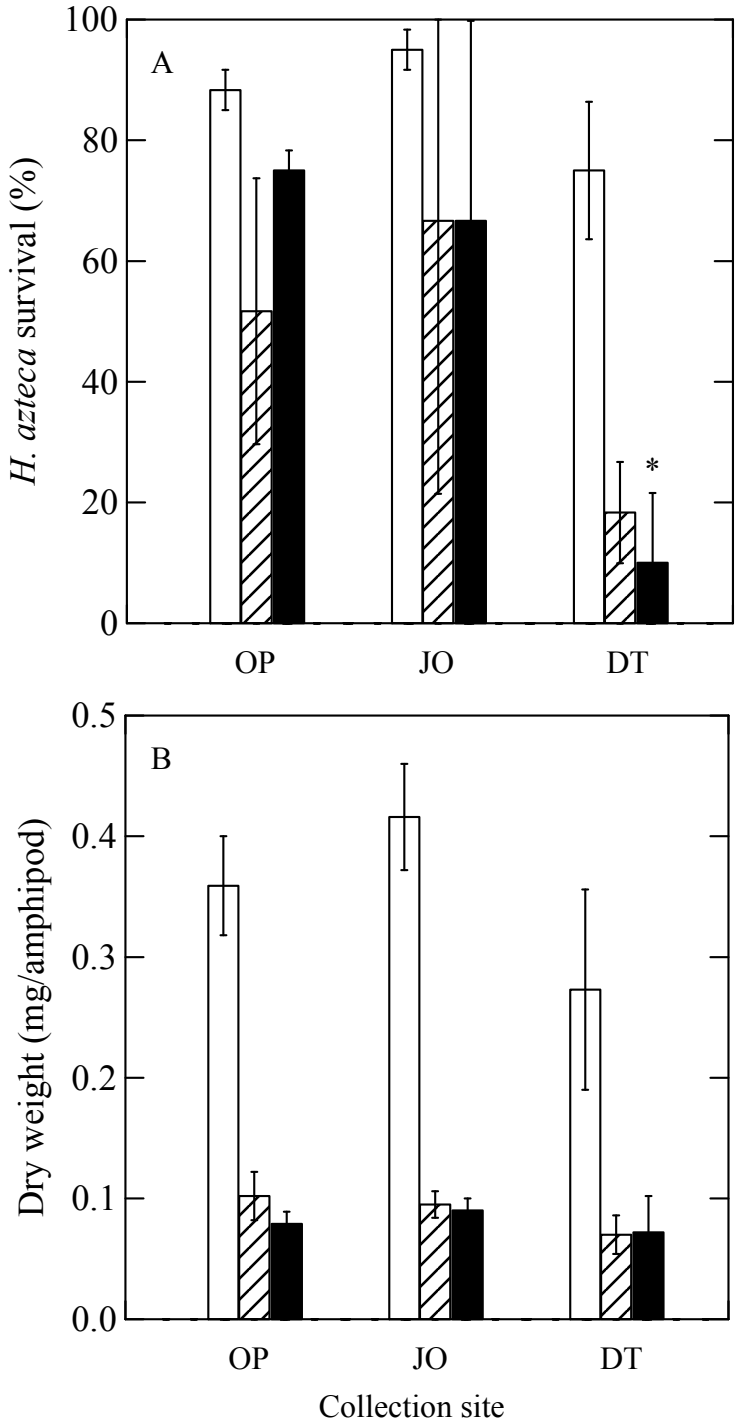


Figure 4.5 Mean ± SD (A) survival and (B) dry weight of *H. azteca* in 4 replicate 28 d treatments of metals primarily in water (white bars), food (diagonal line bars) or food+water (black bars) using field collected water and periphyton from Lakes Opasatica (OP), Joannès (JO) and Dufault (DT). Statistically significant differences ($P<0.05$) between collection sites for each treatment are shown (*).

Table 4.1 ANOVA of metals in lake water, periphyton and *H. azteca* as a function of site (for lake water and periphyton), periphyton-only (for *H. azteca*) or lake water-only (for *H.azteca*)

Metal	Lake water (site)			Periphyton (site)			*H. azteca* (periphyton-only)			*H. azteca* (lake water-only)		
	n	r^2	P	n	r^2	P	n	r^2	P	n	r^2	P
Al	6	0.999	0.000**	6	0.977	0.004*	6	0.576	0.276	6	0.545	0.307
Ba	6	0.996	0.000**	6	0.952	0.010*	6	0.904	0.030*	6	0.917	0.024*
Cd	6	0.994	0.001**	6	0.996	0.000**	6	0.994	0.000**	6	0.991	0.001**
Co	6	0.591	0.262	6	0.993	0.001**	6	0.858	0.053	6	0.896	0.033*
Cr	6	0.976	0.004*	6	0.981	0.003*	4	0.433	0.342	3	0.488	0.508
Cu	6	0.915	0.025*	6	0.997	0.000**	6	0.854	0.056	6	0.327	0.552
Fe	6	0.968	0.006*	6	0.978	0.003*	6	0.749	0.125	5	0.978	0.022*
Ga	6	0.954	0.010*	6	0.977	0.004*	6	0.113	0.835	6	0.857	0.054
Mn	6	0.974	0.004*	6	0.972	0.005*	6	0.942	0.014*	6	0.931	0.018*
Mo	6	0.998	0.000**	6	0.970	0.005*	6	0.570	0.282	6	0.618	0.236
Ni	6	0.643	0.214	6	0.992	0.001**	6	0.717	0.150	6	0.346	0.529
Rb	6	1.000	0.000**	6	0.984	0.002*	6	0.635	0.220	6	0.649	0.208
Sb	6	1.000	0.000**	6	0.991	0.001**	6	0.866	0.049*	6	0.645	0.211
Sr	6	1.000	0.000**	6	0.930	0.019*	6	0.887	0.038*	6	0.902	0.031*
Tl	6	0.980	0.003*	6	0.956	0.009*	6	0.967	0.006*	0	ND	ND
U	6	0.997	0.000**	6	0.925	0.021*	6	0.600	0.253	4	0.104	0.677
V	6	1.000	0.000**	6	0.973	0.004*	6	0.768	0.112	5	0.969	0.031*
Zn	6	0.996	0.000**	6	0.990	0.001**	6	0.486	0.368	6	0.249	0.651

n = number of samples

r^2 = coefficient of determination

*P<0.05, **P≤ 0.001

ND = no data because it was less than detection limit

Table 4.2 Mean water temperature, conductivity, pH, dissolved oxygen (DO_2), hardness, major ions dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) of water from Lakes Opasatica (OP), Joannès (JO) and Dufault (DT) and standard artificial medium (SAM) (standard deviations shown)

Parameter	n	[1]SAM	OP	JO	DT
Water temperature (°C)	2	22.8 (0.58)	20.7 (1.80)	23.6 (6.70)	20.7 (2.40)
Conductivity (µS/cm)	2	416 (11.5)	83.5 (0.71)	57.0 (2.83)	140 (2.12)
pH	2	8.17 (0.10)	7.34 (0.10)	7.71 (0.35)	7.67 (0.23)
DO_2 (mg/L)	2	7.46 (0.25)	9.57 (0.03)	9.03 (0.25)	9.09 (0.19)
[2]Hardness as $CaCO_3$ (mg/L)	1	124	32.6	26.2	55.1
Ca (µM)	1	974 (61.3)	214	190	427
Mg (µM)	1	266 (30.0)	112	72.0	124
Na (µM)	1	998 (132)	147	55.2	173
K (µM)	1	50.8 (3.78)	26.6	13.8	16.4
Cl (µM)	1	1940 (390)	92.0	12.4	119
SO_4 (µM)	1	268 (32.8)	71.8	57.5	344
DIC (mg/L)	1	13.5	5.60	3.60	4.60
[3]DOC (mg/L)	1	1.70	7.10	11.1	5.60

[1] For SAM: water temperature, conductivity, pH and DO_2 n=64; major ions n=23; DIC/DOC n=1

[2] Hardness calculated as: Hardness (mg $CaCO_3$/L) = (2.497 x [Ca]) + (4.118 x [Mg]) with [Ca] and [Mg] as mg/L

[3] DOC in SAM without animals or TetraMin® was 0.22 mg/L

n = number of samples

Table 4.3 Mean Cd measurements in water, periphyton (on an ash-free dry mass basis), sediment, field collected *H. azteca* and 14 d caged laboratory *H. azteca* in Lakes Opasatica (OP), Joannès (JO) and Dufault (DT) (standard deviations shown, n=2 or 3 or in the case of caged *H. azteca* n= 5 or 6)

Lake	Unfiltered water (nmol/L)	Filtered water[1] (nmol/L)	Cd^{2+} (nmol/L)	Periphyton (nmol/g AFDM)	Sediment (nmol/g)	*H. azteca* (nmol/g)	Caged *H. azteca* (nmol/g)
OP	0.10 (0.01)	0.06 (0.00)	0.02 (0.00)	20.8 (6.51)	25.7 (2.52)	10.4 (0.92)	15.5 (1.06)
JO	0.75 (0.03)	0.69 (0.01)	0.14 (0.00)	96.3 (10.4)	5.34 (0.00)	59.9 (35.2)	44.6 (4.36)
DT	4.10 (0.07)	3.01 (0.04)	1.38 (0.02)	1880 (433)	155 (11.3)	167 (105)	186 (5.42)

[1] 0.45µm filtered

Cd^{2+} was modelled using WHAM 6.0.13

To convert metal concentration on an ash-free dry mass to dry mass basis multiply by fraction of organic content (1, 0.13, 0.30, 0.10 for TetraMin®, OP, JO and DT periphyton respectively)

Table 4.4 Mean (SD, n=1, 2 or 3 samples[1]) total lipid and protein in periphyton, field collected *H. azteca* , 14 d caged laboratory adult *H. azteca* and juvenile *H. azteca* exposed for 28 d to treatments of metal primarily in water, food or food+water collected from Lakes Opasatica (OP), Joannès (JO) and DT (Dufault)

Sample	Collection site	Total lipid (%)	Total protein (%)
Periphyton	OP	2	9 (4)
	JO	6	10 (6)
	DT	5	10 (0.6)
Field *H. azteca*	OP	6 (2)	21
	JO	9 (0.1)	21
	DT	6 (2)	20
Caged *H. azteca*	OP	8	28 (3)
	JO	13	24 (0.2)
	DT	8*	24 (2)
28 d laboratory exposed *H. azteca*			
Control		27*	23*
Water treatment	OP	16	21
	JO	17	28*
	DT	NM	35*
Food treatment	OP	48*	34*
	JO	48*	30*
	DT	NM	35*
Food+water treatment	OP	62* (25)	30*
	JO	39*	32*
	DT	NM	NM

[1] each sample consists of 2 or 3 analytical replicates

* = dry weight sample <1mg

n = 1,2 or 3

NM = not measured due to lack of sufficient tissue mass

4.4 DISCUSSION

The experimental approach of separating Cd exposure pathways demonstrated the dynamic partitioning of Cd between water and food that occurs in a static system. Given that the separate water and food treatments in reality had primary and secondary Cd exposure pathways, it was necessary to account for the influence of these secondary exposures on Cd bioaccumulation in *H. azteca* by applying an independently derived model to mathematically separate the contributions of Cd from water and food.

4.4.1 *Predicting Cd bioaccumulation in H. azteca*

The model proved to be robust in predicting bioaccumulation of Cd in *H. azteca* exposed to field contaminated water and food for 28 days as well as Cd in *H. azteca* residing in the field contaminated sites from which the samples were collected. It is likely that the few predicted values that varied from measurements of *H. azteca* collected from JO and DT by more than a factor of two reflected the variability in field measurements rather than inaccuracies in the model predictions. However, it is known that bioaccumulation is controlled by Cd complexation with other ligands in solution and competition between the free metal ion (Cd^{2+}) and other cationic species for binding sites on membranes of the target organism (Borgmann *et al.*, 2010). These interactions form the basis of predicting metal bioaccumulation from the aqueous exposure pathway in the biotic ligand model (BLM) (Paquin *et al.*, 2002). Because the model in the present study was developed under different water chemistry than those under which the model was applied (Table 4.2), it was unknown how accurately the model predictions would match the measured values. The close agreement of predictions with measured values when the model was based on dissolved Cd can be explained by comparing two water chemistry parameters that control Cd bioaccumulation in *H. azteca* – Ca and DOC (Stephenson and Mackie, 1988). The higher Ca in SAM, with which the model was developed, as compared to the lake waters, to which the model was applied, meant that the model should have under-predicted Cd bioaccumulation as a result of competition between Ca^{2+} and Cd^{2+} for binding sites on *H. azteca*. In contrast, the lower DOC in SAM as compared to the lake waters meant that the model should have over-predicted Cd bioaccumulation due to complexation between Cd^{2+} and DOC resulting in reduced Cd^{2+} uptake at *H. azteca*

binding sites. These two effects may have cancelled each other when the model was applied using parameters based on dissolved Cd exposure. The under-prediction of Cd bioaccumulation due to Ca^{2+} competition was made obvious when the effect of DOC complexation was removed by applying the model with parameters based on Cd^{2+} exposure (Figure 4.3B and D). In the water treatment, where the effects of water chemistry were most likely to control Cd uptake, the model did indeed under-predict Cd bioaccumulation but in the food and food+water treatments where Cd contribution from food was most dominant, the effect of Ca^{2+} was not observed. While Ca in food has been shown to also reduce dietary uptake of Cd in rainbow trout (Ng *et al.* 2009; Wood *et al.*, 2006), Ca was not measured in either TetraMin® or periphyton to make such a comparison in this study.

Further investigation of how Ca^{2+} and DOC influence the bioaccumulation model was conducted by applying the model to predict bioaccumulation of Cd in *H. azteca* residing in a soft water lake (Ca = 55.4 µmol/L, Mg = 28 µmol/L) that was spiked with $CdCl_2.2H_2O$ to a sub-lethal concentration (0.801 nmol/L Cd) (Stephenson and Turner, 1993). The model under-predicted Cd in *H. azteca* by 3.7 times suggesting that the influence of very low Ca^{2+} was much stronger than the counteracting influence of Cd^{2+} complexation with DOC (7.2 mg/L). Borgmann *et al.* (2010) developed a 28 d Cd bioaccumulation model to account for anti-competitive inhibition of Cd^{2+} by Ca^{2+}, acclimation of *H. azteca* to Ca^{2+}, inhibition of acclimation by Cd^{2+} and growth dilution effects over the lifetime of *H. azteca*. When this model was applied to the Stephenson and Turner's (1993) data, the predicted Cd in *H. azteca* (198 nmol/g) was very similar to the measured Cd in *H. azteca* (205 nmol/g) demonstrating the importance of accounting for Ca competition with Cd in modelling bioaccumulation. However, the Cd contribution from food was not an explicit component of the model developed by Borgmann *et al.* (2010) and thus the model cannot be used to distinguish between the contributions of Cd from water and food separately to Cd in *H. azteca*.

4.4.2 *Percentage of Cd in H. azteca from food*

Using the conservative predictions from the bioaccumulation model based on dissolved Cd parameters, a marked contribution (67 – 90 %) of Cd in *H. azteca* was from periphyton in the food+water treatment which compared favourably with the predicted contribution (60 – 80 %) of Cd from periphyton to *H. azteca* residing in the actual lakes. The estimates of dietary Cd contribution in the food and food+water treatments as well as *H. azteca* residing in the lakes are robust given that the predictions of Cd in *H. azteca* remained mostly within a factor of two when the model based on Cd^{2+} parameters was used i.e. where Ca effects on model predictions would be most obvious. However, in the water treatment where bioaccumulation was under-predicted possibly due to the influence of Ca^{2+}, the contribution of dietary Cd may have been over-estimated. Marked contributions (40 – 55 %) of Cd from periphyton were also estimated when *H. azteca* were exposed to Cd spiked SAM and periphyton for 28 d (Chapter 2.5.4.4). Application of the model to predict the contribution of dietary Cd to *H. azteca* in Stephenson and Turner (1993) was not appropriate given that the model underestimated bioaccumulation of Cd in *H. azteca* by 3.7 times and was therefore likely to overestimate the dietary Cd contribution. However, Stephenson and Turner's (1993) own site-specific bioaccumulation model predicted 58 % of Cd in *H. azteca* came from periphyton. Cadmium in plant and detrital material fed to caged adult *H. azteca* for 17 d in two metal contaminated rivers, contributed 23 % to total body concentration in *H. azteca* (Borgmann *et al.* 2007). Together with the current study, these studies demonstrate that dietary Cd can contribute significantly to *H. azteca* body concentration. The variation in the level of contribution is likely to be related to the exposure duration and the relative concentrations of bioavailable Cd in water and food.

4.4.3 *Toxicity*

While survival decreased with increasing Cd in water and food pathways, Cd toxicity to *H. azteca* may have been confounded by the nutritional quality of the periphyton. This was most apparent when comparing *H. azteca* survival of TetraMin® fed amphipods and periphyton fed amphipods in OP. This difference widened as Cd increased indicating that Cd and possibly a diet nutrition factor were contributing to effects on survival in DT.

A model (Eq. 4.3) used to predict effects on survival from chronic exposure to Cd in food and water separately and combined based Cd in *H. azteca* was applied to bioaccumulation data from the 28 d feeding experiment. Based on the Cd measured in *H. azteca* in the OP, JO and DT food+water treatments, 92±0 %, 92±0 % and 74±18 % respective *H. azteca* mean survival (±SD, n = 2) was predicted but 75±3 %, 67±33 % and 10±12 % respective mean (±SD, n = 2) survival was observed. Therefore while the trend in survival was as predicted, the magnitude of effect was greater than predicted based on Cd in *H. azteca*. It was also observed that those animals fed with periphyton had significantly lower dry weight than those fed with TetraMin® at all Cd concentrations. In addition, adult *H. azteca* that were caged in each lake with lake specific periphyton for 14 d did not show any significant decrease in survival with increasing Cd body concentration and this was in accordance with predictions of toxicity based on tissue concentration. Because of the use of adults and the short exposure duration, any effects on caged *H. azteca* survival would have been due to Cd rather than food nutrition. The lack of effects on caged *H. azteca* survival and the reduced survival and dry weight of *H. azteca* feeding on low Cd site (OP) periphyton for 28 d, suggests that the greater than predicted mortality in 28 d laboratory exposed *H. azteca* was not due to Cd alone.

Diet nutrition has been suspected of augmenting metal toxicity in other studies (Farag *et al.*, 1999; Xie *et al.,* 2010). From comparisons of measurements of total lipid and protein in TetraMin® and field collected periphyton, TetraMin® had a higher nutritional content than periphyton. Lipid was notably lower in periphyton than TetraMin® and is vital for energy storage and reproduction in amphipods (Hyne *et al.*, 2009). Total lipid and protein measurements in *H. azteca* were found to be insensitive indicators of effects from different food types (Chapter 2.4.8) and with low tissue mass analyzed in this study, any correlation between food and *H. azteca* nutrition would be tenuous at best. However, the marked reduction in growth of *H. azteca* feeding on field collected periphyton was a strong indicator that these animals were nutritionally compromised. Differences in food nutrition also assisted in explaining why the model predicted survival was higher than that observed. While the total lipid and protein were similar between field collected periphyton and the periphyton used to develop the toxicity model, the percent organic

content was reduced by as much as 49 % in field collected periphyton. This lower organic content or conversely, higher inorganic content is likely due to sediment that was trapped within the periphyton matrix of field collected samples and thus lowered the nutritional quality of the food (Spadaro *et al.*, 2008; Wood and Armitage, 1997) from all lakes but especially so in DT treatments. Because the toxicity model was based on a more nutritious periphyton, it did not account for the lower quality of the field collected periphyton in the current study and thus over-predicted survival by 7.4 times in the food+water DT treatment and to a lesser extent in the OP and JO treatments.

The possibility of metals other than Cd contributing to toxicity was investigated by comparing Cd, Cu, Ni, Pb, Tl and Zn measured in filtered water and *H. azteca* tissue from the 28 d experimental treatments and the field collected samples with LC25 and LBC25 values calculated for 28 d dissolved exposure of *H. azteca* to Cu, Ni, Pb, Tl and Zn (Borgmann *et al.,* 2004a). *H. azteca* with Cd closest to the LBC25 (501 nmol/g) were in the food and food+water treatments for DT and were 62 % and 76 % of the LBC25 respectively. Body concentrations of Ni, Pb and Tl were not close to the LBC25 (281 nmol/g, 65 nmol/g, 364 nmol/g respectively) in any treatment or field sample. Because Cu and Zn are regulated (completely for Cu and partially for Zn, Borgmann *et al.*, (1993)) by *H. azteca*, water concentrations were compared to the LC25 (441 nmol/L and 2520 nmol/L respectively) values. Copper was 40 – 60 % of the LC25 in the food and food+water treatments of DT as well as field measurements of DT lake water. Dissolved Zn was not close to the LC25 in any treatment or lake water sample. Because no metal concentration was close to the 25 % effect level in OP treatment or field samples, these metals were not responsible for the reduced survival and dry weight in OP. However, these endpoints were based primarily on dissolved exposure therefore the contribution of Cu, Ni, Pb, Tl and Zn to dietary toxicity is unknown. Overall, based on these comparisons, Cd is the metal most likely to be contributing to the observed toxicity in DT. Borgmann *et al.* (2004b) and Nowierski *et al.* (2006) also noted the possible contribution of Cu to observed toxicity to *H. azteca* from DT sediments thus potential metal effects from Cu and other metal interactions cannot be dismissed but would be marginal in comparison to Cd.

4.4.4 *Attributing toxicity to water and food*

Previous research (Chapter 2.4.5) has shown effects of Cd on *H. azteca* survival to be independent of uptake pathway when determined as a function of body concentration. Therefore it is the fraction of Cd from water or food to body concentration that determines which pathway is contributing most to survival effects. In the present study, reduced survival of *H. azteca* was predicted in the DT food and food+water treatments based on body concentration alone but, in reality, was further reduced by the low nutritional quality of periphyton from DT. Based on the high fraction of Cd in *H. azteca* predicted to come from food versus water in these treatments, the reduced survival attributable to Cd was estimated to be due predominantly to Cd in periphyton rather than water. This is an example of dietary Cd being linked to chronic effects on *H. azteca* survival at environmentally relevant concentrations using a natural diet of periphyton. These results support evidence of dietary Cd chronic toxicity (reduced reproduction and growth) in other aquatic invertebrates and fish at environmentally realistic concentrations (Geffard *et al.,* 2007; Ng and Wood, 2008), although few studies have modelled the link between dietary Cd and toxicity.

Based on Cd body concentration of *H. azteca* collected from DT, no marked effects on *H. azteca* survival were predicted. Given the high variability of Cd measurements in *H. azteca* collected from the two Cd contaminated lakes of JO and DT, it is likely that *H. azteca* from these lakes display a range of Cd body concentrations related to their age, micro-habitat and feeding strategies. It may be difficult to collect *H. azteca* with high Cd body concentrations because they are already dead or rare. Borgmann *et al.* (2004b) found DT to have low invertebrate taxa richness and low abundance of metal sensitive invertebrates (Amphipods, sphaeriid clams, Ephemeroptera and tanytarsid midges). Reduced survival of *H. azteca* exposed to sediment from DT for 28 d in the laboratory was also observed (Borgmann *et al.*, 2004b; Norwierski *et al.*, 2006). Therefore, based on chronic laboratory exposures of *H. azteca* to water and periphyton collected from OP, JO and DT, there is the potential for effects on survival of *H. azteca* in DT due to Cd in periphyton.

4.4.5 *Model application*

The independently derived bioaccumulation model was robust in predicting chronic Cd bioaccumulation in *H. azteca* exposed to field contaminated water and periphyton in the laboratory and when compared to measurements of Cd in *H. azteca* collected from OP, JO and DT. However, the model does not account for the influence of Ca^{2+} on bioaccumulation as demonstrated when the model was applied to a soft water lake (Stephenson and Turner, 1993). Borgmann *et al.* (2010) developed a chronic bioaccumulation model to account for anti-competitive inhibition of Cd bioaccumulation by Ca^{2+} in *H. azteca* but did not explicitly include food as an exposure pathway. Therefore both of these models are incomplete in being able to predict Cd in *H. azteca* from food and water. In its present form, the model should only be applied where water chemistry conditions are similar to those under which the model was developed i.e. moderately hard water and circum-neutral pH. Integration of water chemistry and dietary Cd into one model would require a series of chronic laboratory tests where Ca is increased sequentially over a gradient of Cd concentrations in both water and periphyton and measurements of Cd in water, periphyton and *H. azteca* are made. In the long term, the concentration of Cd in food would need to be related to water concentration in order that bioaccumulation of Cd in *H. azteca* from food and water can be predicted from water chemistry alone – a complete BLM. This is indeed a challenge given the range of biological concentration factors likely to occur for different food types in various water chemistries.

Cadmium in periphyton and water were linked to chronic effects on *H. azteca* survival by Cd body concentration. The chronic survival model was able to predict the observed trend of effects on survival in the experimental treatments but was not able to account for the additional reduction in survival probably due to the comparatively lower nutritional quality of the field contaminated periphyton. This demonstrates the importance of characterizing the nutritional quality of the food and when performing site-specific risk assessment, the importance of identifying the dominant food source of *H. azteca* at the site of interest.

4.5 CONCLUSIONS

Predictions from an independent chronic Cd bioaccumulation model for *H. azteca* that accounted for both water and dietary sources of Cd, were robust when compared to measurements of Cd in *H. azteca* exposed to field contaminated samples in the laboratory and to measurements of Cd in *H. azteca* from field contaminated sites. However, because the inhibition of Cd bioaccumulation by Ca^{2+} and the complexation of Cd with DOC were not incorporated into the model, it is constrained in its application to sites with water chemistry similar to that with which the model was developed. The model also predicted that 67 – 90 % of the Cd in *H. azteca* exposed to food+water treatments from each lake was due to Cd in periphyton. Again, based on Cd body concentration, a chronic toxicity model predicted that survival of *H. azteca* exposed to water and periphyton from the highest Cd contaminated site (DT) would be 74 %. Because Cd in periphyton contributed mostly to Cd in *H. azteca* it was also contributing mostly to effects on survival. However, the nutritional quality of the periphyton likely compounded the predicted reduction in survival resulting in a 10 % observed survival. Therefore, through the use of a bioaccumulation model, Cd present at environmentally relevant concentrations in a natural periphyton diet was found to be chronically toxic to *H. azteca*. This demonstrates the need to further develop models to account for both waterborne and dietary exposure pathways when trying to predict effects from Cd on *H. azteca* in the natural environment.

APPENDICES

A4.1 Mean (SD, n=2) metal concentration (nmol/L) in test solutions (0.45µm filtered) from 28 d laboratory treatments of metal primarily in water, food and food+water using lake water and periphyton collected from Lakes Opasatica (OP), Joannès (JO), Dufault (DT) and the control consisting of standard artificial medium (SAM) and TetraMin®

Metal	Control	Water	Water	Water	Food	Food	Food	Food+Water	Food+Water	Food+Water	MDL
	SAM	OP	JO	DT	SAM	SAM	SAM	OP	JO	DT	
	TetraMin®	TetraMin®	TetraMin®	TetraMin®	OP Periphyton	JO Periphyton	DT Periphyton	OP Periphyton	JO Periphyton	DT Periphyton	
Ag	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.11
Al	241 (243)	1790 (79.0)	2710 (45.4)	280 (9.85)	103 (25.1)	402 (3.32)	306 (59.3)	2570 (102)	2670 (7.36)	398 (22.9)	36.9
As	0.70 (0.05)	7.57 (0.05)	24.7 (0.33)	21.9 (0.00)	0.98 (0.03)	1.34 (0.10)	2.60 (0.12)	8.06 (0.00)	25.8 (0.19)	20.7 (0.13)	0.13
B	ND	362 (6.29)	239 (3.12)	385 (19.6)	ND	ND	ND	382 (9.65)	250 (20.0)	385 (6.39)	9.25
Ba	2.91 (0.63)	54.1 (0.58)	34.1 (1.12)	56.0 (0.47)	14.5 (1.81)	12.9 (1.51)	25.6 (0.82)	63.8 (1.00)	42.1 (0.93)	61.9 (0.52)	0.36
Be	ND	0.29 (0.03)	0.91 (0.04)	ND	ND	ND	ND	0.38 (0.00)	1.03 (0.04)	ND	0.11
Bi	ND	ND	0.01 (0.00)	0.01 (0.00)	ND	ND	ND	ND	0.02 (0.00)	0.02 (0.00)	0.00
Cd	0.06 (0.04)	0.08 (0.02)	0.39 (0.01)	1.75 (0.00)	0.04 (0.03)	0.29 (0.03)	2.35 (0.01)	0.05 (0.01)	0.58 (0.06)	2.63 (0.02)	0.03
Cd^{2+}	0.04 (0.03)	0.02 (0.00)	0.07 (0.00)	0.74 (0.01)	0.03 (0.02)	0.21 (0.03)	1.75 (0.00)	0.01 (0.00)	0.12 (0.01)	1.13 (0.02)	
Co	0.06 (0.06)	0.23 (0.01)	0.20 (0.01)	0.28 (0.07)	0.09 (0.05)	0.36 (0.01)	1.06 (0.27)	0.37 (0.04)	0.55 (0.02)	0.81 (0.02)	0.03
Cr	1.26 (1.84)	5.18 (0.06)	9.52 (0.22)	1.19 (0.40)	0.63 (0.33)	0.67 (0.04)	0.69 (0.34)	5.88 (0.86)	12.1 (3.65)	1.45 (0.16)	0.17
Cu	24.7 (32.1)	65.5 (25.2)	68.8 (8.57)	210 (5.00)	ND	ND	38.8 (12.5)	47.8 (2.63)	72.5 (7.76)	255 (13.3)	9.96
Fe	108 (345)	424 (183)	1190 (15.4)	107 (12.3)	40.7 (15.2)	82.6 (12.5)	149 (5.70)	379 (9.82)	1520 (69.6)	198 (21.3)	22.0
Ga	0.03 (0.01)	0.16 (0.01)	0.14 (0.00)	0.11 (0.01)	ND	0.02 (0.00)	0.08 (0.00)	0.20 (0.01)	0.14 (0.01)	0.14 (0.00)	0.01
La	0.02 (0.03)	0.36 (0.02)	1.00 (0.01)	0.03 (0.01)	0.03 (0.04)	0.02 (0.00)	0.05 (0.04)	0.47 (0.00)	1.12 (0.03)	0.09 (0.01)	0.01
Li	ND	82.6 (5.33)	115 (0.00)	130 (0.00)	ND	ND	ND	72.0 (0.00)	115 (0.00)	130 (0.00)	28.8
Mn	4.34 (4.28)	7.34 (1.51)	18.6 (0.68)	5.47 (0.11)	41.0 (7.68)	61.9 (4.57)	265 (59.1)	48.1 (2.81)	94.4 (5.39)	155 (4.95)	0.91
Mo	ND	2.53 (0.03)	1.79 (0.03)	2.37 (0.11)	ND	ND	ND	2.58 (0.02)	1.87 (0.09)	2.52 (0.05)	0.10
Ni	5.63 (5.21)	17.4 (2.74)	13.3 (1.24)	11.2 (3.12)	3.86 (5.41)	3.37 (1.48)	7.06 (6.72)	16.0 (0.30)	15.6 (2.22)	8.30 (1.51)	2.28
Pb	0.85 (0.56)	0.38 (0.20)	0.59 (0.23)	0.43 (0.16)	0.55 (0.28)	0.93 (0.11)	0.96 (0.18)	ND	1.05 (0.43)	0.72 (0.18)	0.31
Rb	3.41 (0.04)	23.0 (0.12)	13.8 (0.08)	17.6 (0.13)	2.32 (0.24)	2.13 (0.17)	3.00 (0.02)	22.1 (0.21)	13.4 (0.04)	16.7 (0.04)	0.12
Sb	0.02 (0.02)	0.50 (0.01)	0.79 (0.02)	5.13 (0.00)	0.01 (0.01)	0.05 (0.00)	0.16 (0.02)	0.50 (0.01)	0.80 (0.05)	5.28 (0.01)	0.01
Se	ND	1.07 (0.00)	2.02 (0.18)	8.41 (0.02)	ND	ND	ND	1.04 (0.04)	1.90 (0.10)	8.68 (0.22)	0.63
Sr	143 (0.52)	453 (1.23)	290 (0.42)	460 (0.35)	128 (2.42)	129 (2.06)	135 (0.77)	437 (0.41)	272 (0.00)	443 (1.61)	0.57
Tl	0.04 (0.00)	0.10 (0.01)	0.03 (0.01)	0.13 (0.00)	0.04 (0.00)	0.04 (0.00)	0.05 (0.00)	0.07 (0.00)	0.02 (0.00)	0.14 (0.00)	0.00
U	0.01 (0.00)	0.15 (0.00)	0.15 (0.00)	0.05 (0.00)	0.02 (0.01)	0.01 (0.00)	0.06 (0.03)	0.16 (0.00)	0.16 (0.00)	0.06 (0.00)	0.00
V	ND	5.00 (0.03)	6.91 (0.05)	1.00 (0.01)	ND	ND	ND	5.00 (0.03)	7.12 (0.03)	1.08 (0.00)	0.49
Zn	85.8 (132)	60.3 (8.16)	60.4 (1.36)	416 (4.06)	18.7 (24.0)	37.9 (1.25)	136 (3.02)	25.8 (0.85)	68.3 (6.03)	358 (26.2)	9.36

Cd^{2+} = modelled using WHAM 6.0.13

MDL = method detection limit is the upper 95 % CL of metal in the blank samples

ND = below instrument detection limit

A4.2 Mean (SD, n=2) metal concentration (nmol/g) in TetraMin® and periphyton (on an ash-free dry mass basis) used in 28 d laboratory treatments of metal primarily in water, food and food+water using lake water and periphyton collected from Lakes Opasatica (OP), Joannès (JO), Dufault (DT) and the control consisting of standard artificial medium (SAM) and TetraMin®

Metal	Control	Water	Water	Water	Food	Food	Food	Food+Water	Food+Water	Food+Water	MDL
	SAM	OP	JO	DT	SAM	SAM	SAM	OP	JO	DT	
	TetraMin®	TetraMin®	TetraMin®	TetraMin®	OP Periphyton	JO Periphyton	DT Periphyton	OP Periphyton	JO Periphyton	DT Periphyton	
Ag	ND	ND	ND	ND	ND	ND	135 (13.2)	ND	ND	110 (15.6)	0.20
Al	79500 (25400)	83000 (49300)	92000 (57600)	82600 (51800)	4320000 (5290)	878000 (14300)	6240000 (428000)	6050000 (722000)	1150000 (304000)	5910000 (263000)	45.0
As	INT	INT	INT	INT	INT	INT	INT	INT	INT	INT	INT
B	ND (102)	ND	ND	ND	27000 (2770)	873 (41.2)	9410 (4560)	12800 (1920)	1680 (588)	13500 (10600)	11.4
Ba	124 (15.3)	232 (121)	178 (123)	214 (223)	5620 (445)	1220 (274)	8820 (1170)	8090 (1230)	1940 (621)	10500 (527)	0.11
Be	ND (1.30)	ND	ND	ND	359 (22.2)	72 (0.31)	522 (18.7)	493 (84.6)	110 (31.5)	488 (12.6)	0.08
Bi	ND (0.05)	ND	ND	ND	6.74 (0.35)	10.9 (0.12)	497 (31.9)	13.0 (1.97)	16.4 (4.99)	486 (13.9)	0.00
Cd	6.5 (1.72)	5.83 (1.48)	7.97 (6.43)	16.3 (34.6)	38.9 (4.53)	79.4 (6.05)	2560 (27.0)	47.0 (3.64)	110 (22.9)	2630 (23.5)	0.01
Co	2.4 (0.51)	2.39 (1.86)	2.84 (3.32)	2.57 (2.52)	1460 (421)	365 (6.58)	5890 (101)	1640 (107)	482 (132)	5800 (115)	0.03
Cr	ND	ND	ND	ND	6770 (444)	1560 (350)	8200 (234)	9320 (341)	1780 (528)	7750 (257)	1.76
Cu	163 (16.7)	237 (203)	264 (328)	474 (1260)	7430 (3330)	2760 (1480)	109000 (5380)	5700 (403)	2420 (362)	113000 (534)	0.58
Fe	7550 (3890)	7770 (6400)	10000 (10900)	8090 (7530)	2290000 (87800)	484000 (31800)	4800000 (396000)	3130000 (284000)	679000 (284000)	4720000 (282000)	19.5
Ga	89 (8.96)	87.7 (1.33)	74.6 (21.5)	80.1 (23.1)	1230 (18.6)	297 (16.9)	1640 (111)	1160 (269)	416 (37)	1540 (82.4)	0.59
La	0.647 (0.43)	1.30 (3.44)	5.95 (26.2)	0.71 (1.56)	741 (29.2)	370 (67.8)	1010 (80.9)	1020 (110)	403 (80.2)	995 (64.3)	0.01
Li	ND	ND	55.5 (5.58)	59.1 (10.8)	19000 (2280)	3210 (329)	27400 (3320)	29500 (2620)	3740 (1380)	26900 (692)	20.9
Mn	847 (102)	801 (142)	1020 (184)	895 (68.8)	76800 (26100)	32700 (3250)	560000 (247000)	99200 (53400)	58400 (49900)	553000 (208000)	0.23
Mo	3.02 (1.86)	3.93 (4.30)	3.42 (4.31)	3.98 (4.79)	23.4 (5.20)	4.80 (2.88)	128 (62.3)	18.4 (2.28)	8.98 (2.29)	101 (8.82)	0.03
Ni	21.2 (14.1)	ND	ND	6.41 (22.8)	7030 (1370)	909 (71.4)	6740 (113)	6940 (8.01)	1190 (322)	6450 (217)	1.00
Pb	5.51 (7.47)	2.76 (2.66)	4.21 (6.00)	3.94 (19.4)	341 (9.85)	296 (18.8)	14800 (1090)	776 (373)	403 (132)	14600 (521)	0.07
Rb	2.48 (1.40)	5.73 (11.9)	6.25 (15.2)	7.68 (23.5)	1620 (87.1)	190 (2.73)	2040 (160)	2340 (282)	251 (73.9)	2040 (17.3)	0.11
Sb	0.246 (0.28)	0.30 (0.10)	0.31 (0.122)	ND	18.0 (3.08)	7.11 (1.71)	168 (5.13)	24.2 (4.12)	8.13 (4.77)	129 (26.9)	0.03
Se	INT	INT	INT	INT	INT	INT	INT	INT	INT	INT	INT
Sr	1170 (634)	1230 (694)	1160 (678)	1140 (621)	3140 (293)	591 (147)	3640 (121)	4350 (1380)	1080 (528)	3930 (931)	0.49
Tl	ND	ND	ND	ND	3.58 (0.17)	0.23 (0.01)	7.18 (2.59)	5.38 (0.23)	0.56 (0.15)	8.27 (0.21)	0.00
U	0.3 (0.18)	0.55 (1.06)	0.35 (0.33)	0.37 (0.57)	12.9 (4.25)	2.78 (1.13)	16.0 (3.35)	22.5 (5.63)	4.42 (0.86)	19.5 (1.59)	0.00
V	13.4 (2.70)	18.2 (15.1)	31.3 (37.9)	14.7 (4.77)	4240 (225)	862 (123)	6250 (628)	5880 (428)	1200 (340)	6290 (494)	0.02
Zn	3210 (1860)	2680 (1280)	2670 (1440)	5070 (8640)	10800 (2500)	7140 (30.7)	398000 (13400)	16200 (7470)	11100 (5250)	402000 (9440)	1.08

INT = Matrix interference

MDL = method detection limit is the upper 95 % CL of metal in the blank samples x (mean digest volume / mean digest mass)

ND = below instrument detection limit

To convert metal concentration on an ash-free dry mass to dry mass basis multiply by fraction of organic content (1, 0.13, 0.30, 0.10 for TetraMin®, OP, JO and DT periphyton respectively)

A4.3 Mean (SD, n=2) metal concentration (nmol/g) in *H. azteca* exposed for 28 d to laboratory treatments of metal in primarily water, food and food+water using lake water and periphyton collected from Lakes Opasatica (OP), Joannès (JO), Dufault (DT) and the control consisting of standard artificial medium (SAM) and TetraMin®

Metal	Control	Water	Water	Water	Food	Food	Food	Food+Water	Food+Water	Food+Water	MDL
	SAM	OP	JO	DT	SAM	SAM	SAM	OP	JO	DT	
	TetraMin®	TetraMin®	TetraMin®	TetraMin®	OP Periphyton	JO Periphyton	DT Periphyton	OP Periphyton	JO Periphyton	DT Periphyton	
Ag	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.19
Al	6380 (6380)	2860 (220)	2320 (264)	2770 (376)	7590 (6990)	3390 (91.1)	9700 (5060)	7560 (1350)	5190 (4760)	9810 (7350)	43.4
As	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	INT
B	267 (46.1)	ND	ND	ND	1460 (435)	ND	ND	ND	ND	ND	11.0
Ba	10.1 (2.38)	109 (35.1)	59.6 (1.91)	40.8 (1.08)	151 (44.3)	51.2 (6.47)	123 (17.1)	418 (31.8)	310 (40.6)	197 (92.8)	0.10
Be	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08
Bi	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.00
Cd	3.53 (0.11)	6.58 (1.29)	15.8 (0.22)	86.6 (3.25)	24.8 (0.75)	59.4 (11.8)	363 (61.4)	20.6 (3.31)	95.6 (21.9)	432 (158)	0.01
Co	0.87 (0.60)	1.68 (0.05)	1.43 (0.56)	3.78 (0.64)	6.32 (0.93)	8.13 (1.06)	11.5 (0.60)	4.09 (0.98)	10.1 (5.85)	9.42 (0.73)	0.02
Cr	ND	20.9 (11.3)	13.1 (0.76)	ND	ND	9.66 (2.17)	ND	18.1 (0.61)	24.1 (12.7)	33.3 (73.3)	1.7
Cu	734 (178)	1090 (65.6)	957 (214)	1640 (39.4)	1580 (42)	1730 (8.69)	1740 (257)	1440 (4.72)	1960 (295)	1580 (61.7)	0.56
Fe	753 (3.08)	861 (148)	901 (224)	561 (13.3)	702 (39.1)	782 (62.4)	ND	1210 (270)	ND	618 (32.3)	18.8
Ga	3.69 (12.6)	42.1 (5.63)	42.5 (2.01)	32.3 (29.7)	136 (57.8)	141 (7.40)	417 (127)	236 (33.1)	356 (352)	579 (279)	0.57
La	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.01
Li	95.1 (135)	ND	ND	ND	ND	ND	ND	ND	ND	ND	20.1
Mn	119 (15.2)	142 (4.65)	97.6 (0.68)	194 (32.4)	263 (27.4)	224 (39.2)	696 (186)	324 (66.6)	264 (76.1)	1030 (406)	0.22
Mo	2.66 (1.58)	5.39 (0.23)	4.81 (0.22)	3.19 (1.55)	6.39 (0.45)	5.07 (0.10)	4.14 (1.39)	6.59 (0.19)	6.28 (0.28)	4.02 (1.58)	0.03
Ni	ND	16.7 (14.0)	23.2 (3.81)	4.85 (3.27)	32.7 (21.9)	9.67 (22.1)	30.8 (0.84)	122 (342)	46 (28.7)	81 (48.4)	0.96
Pb	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.07
Rb	26.1 (0.90)	173 (10.3)	161 (0.89)	196 (26.6)	24.4 (2.43)	19.6 (4.08)	26.8 (1.27)	121 (1.06)	154 (16.9)	237 (10.2)	0.11
Sb	ND	4.20 (3.65)	3.51 (0.64)	0.20 (0.23)	1.94 (0.58)	1.54 (0.32)	3.43 (1.72)	1.53 (0.05)	1.01 (1)	2.35 (1.05)	0.03
Se	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	INT
Sr	185 (9.21)	1660 (410)	1360 (83.9)	755 (110)	202 (12.3)	181 (0.66)	224 (7.52)	1670 (3.3)	1300 (32.2)	960 (222)	0.47
Tl	0.12 (0.01)	0.35 (0.00)	0.35 (0.02)	1.11 (0.27)	ND	ND	ND	0.20 (0.11)	ND	0.66 (0.03)	0.00
U	0.12 (0.00)	0.16 (0.00)	0.13 (0.03)	0.12 (0.02)	0.15 (0.03)	0.16 (0.03)	ND	0.21 (0.045)	0.08 (0.01)	ND	0.00
V	1.04 (0.62)	1.33 (0.16)	1.35 (0.08)	0.49 (0.32)	1.30 (0.16)	1.51 (0.12)	ND	3.37 (0.73)	1.38 (1.92)	1.05 (0.76)	0.01
Zn	1190 (106)	1330 (92.0)	1230 (0.22)	1210 (91.9)	1550 (514)	1270 (12.7)	1450 (174)	1280 (260)	1410 (213)	1870 (70.8)	1.04

INT = Matrix interference

MDL = method detection limit is the upper 95 % CL of metal in the blank samples x (mean digest volume / mean digest mass)

ND = below instrument detection limit

A4.4 Mean (SD, n=2) metal concentration (nmol/L) in lake water (0.45µm filtered), periphyton (nmol/g ash-free dry mass) and sediment (nmol/g) collected from Lakes Opasatica (OP), Joannès (JO) Dufault (DT)

Metal	Water			Periphyton[1]			Sediment[2]			Water	Periphyton
	OP	JO	DT	OP	JO	DT	OP	JO	DT	MDL	MDL
Ag	0.01 (0.00)	0.02 (0.00)	0.01 (0.00)	ND	ND	239 (102)	NM	NM	NM	0.01	0.17
Al	1560 (52.4)	4670 (26.2)	278 (5.24)	1970000 (643000)	855000 (287000)	4680000 (369000)	1510000 (47200)	526000 (107000)	993000 (5240)	7.41	39.5
As	6.47 (0.09)	23.9 (0.19)	23.9 (0.76)	ND	ND	ND	206 (28.3)	40 (0.00)	1430 (104)	0.13	INT
B	361 (0.00)	240 (0.00)	379 (0.00)	13900 (1870)	481 (6.33)	2610 (2580)	ND	ND	ND	9.25	10
Ba	55.6 (0.31)	41.6 (0.05)	57.8 (0.05)	2600 (701)	1670 (77)	11200 (660)	4460 (196)	178 (15.4)	765 (0.00)	0.36	0.09
Be	0.44 (0.00)	1.22 (0.00)	0.22 (0.00)	144 (59.7)	80.4 (23.4)	442 (18.4)	182 (11.0)	27.1 (2.35)	109 (6.28)	0.11	0.07
Bi	0.01 (0.00)	0.05 (0.00)	0.22 (0.00)	4.21 (1.85)	8.25 (2.53)	567 (21.8)	2.87 (0.00)	0.96 (0.00)	120 (8.12)	0.00	0.00
Cd	0.06 (0.00)	0.70 (0.01)	3.01 (0.04)	22.7 (4.95)	94.1 (8.76)	1850 (396)	25.7 (2.52)	5.34 (0.00)	155 (11.3)	0.01	0.01
Cd^{2+}	0.02 (0.00)	0.16 (0.00)	1.69 (0.02)								
Co	0.29 (0.00)	0.40 (0.01)	0.33 (0.01)	846 (251)	426 (36.8)	6220 (701)	653 (62.4)	96.7 (2.40)	793 (52.8)	0.03	0.02
Cr	5.09 (0.05)	10.3 (0.03)	0.87 (0.01)	18900 (905)	1140 (444)	4960 (54.5)	2400 (27.2)	1540 (571)	1400 (81.6)	0.23	1.55
Cu	38.1 (2.11)	54.6 (0.11)	219 (2.23)	2150 (716)	1920 (183)	197000 (47000)	911 (89.0)	251 (22.3)	26700 (1780)	8.62	0.51
Fe	465 (17.7)	2400 (12.7)	1110 (17.7)	1150000 (417000)	539000 (188000)	4150000 (221000)	905000 (13900)	334000 (38000)	818000 (7600)	188	17.1
Ga	0.14 (0.02)	0.17 (0.01)	0.11 (0.01)	196 (52)	180 (11.9)	895 (44.8)	209 (4.06)	92.7 (18.9)	140 (6.39)	0.01	0.52
La	0.71 (0.03)	1.61 (0.01)	0.23 (0.00)	291 (111)	252 (23.7)	691 (47.8)	330 (23.9)	152 (7.64)	201 (13.7)	0.01	0.01
Li	101 (0.00)	130 (0.00)	158 (0.00)	6890 (2180)	3860 (1010)	17800 (394)	10100 (346)	1540 (81.5)	4440 (316)	28.8	18.4
Mn	51.3 (0.26)	70.5 (0.90)	76.2 (0.90)	73700 (11700)	71000 (38200)	1000000 (209000)	1660 (64.4)	5780 (721)	17700 (180)	0.91	0.20
Mo	2.49 (0.04)	1.7 (0.08)	2.33 (0.02)	35.3 (10.3)	11.2 (0.53)	241 (34)	19.8 (22.8)	0.50 (0.00)	0.50 (0.00)	0.10	0.02
Ni	15.4 (0.36)	13.3 (0.12)	9.07 (0.12)	8070 (965)	824 (158)	4990 (455)	1480 (107)	307 (13.3)	658 (32.5)	2.13	0.88
Pb	0.27 (0.15)	1.08 (0.02)	5.02 (0.00)	183 (65.5)	239 (71.8)	16700 (366)	175 (11.3)	34.2 (2.73)	3510 (232)	0.19	0.06
Rb	19.7 (0.00)	11.4 (0.08)	14.8 (0.08)	492 (121)	344 (8.38)	1200 (58.3)	791 (3.31)	63.7 (4.14)	352 (18.2)	0.12	0.10
Sb	0.45 (0.01)	0.73 (0.017)	4.93 (0.01)	6.16 (0.83)	9.35 (8.31)	287 (46.5)	0.10 (0.00)	0.10 (0.00)	0.10 (0.00)	0.10	0.02
Se	1.33 (0.09)	2.28 (0.00)	7.54 (0.09)	ND	ND	ND	12.7 (0.00)	1.00 (0.00)	196 (8.96)	0.63	INT
Sr	414 (1.61)	254 (0.81)	414 (3.23)	2280 (622)	1120 (64.9)	3920 (202)	924 (16.1)	1490 (395)	970 (0.00)	0.57	0.43
Tl	0.10 (0.01)	0.017 (0.00)	0.16 (0.00)	1.39 (0.36)	0.62 (0.17)	7.58 (0.53)	5.90 (0.76)	0.19 (0.04)	2.00 (0.15)	0.00	0.00
U	0.20 (0.00)	0.16 (0.00)	0.06 (0.00)	6.51 (2.92)	3.03 (0.13)	13.1 (1.62)	8.65 (0.54)	2.86 (0.39)	4.70 (0.18)	0.00	0.00
V	5.12 (0.139)	8.25 (0.01)	2.1 (0.11)	2760 (949)	1020 (294)	4960 (257)	1670 (27.8)	998 (111)	1070 (13.9)	0.10	0.01
Zn	1.18 (3.24)	25.5 (1.08)	513 (8.65)	3500 (760)	6400 (624)	405000 (40300)	2420 (43.2)	657 (21.6)	183 (0.00)	12.7	0.95

Cd^{2+} = modelled using WHAM v6.0.13

INT = Matrix interference

MDL = method detection limit is the upper 95 % CL of metal in the blank water samples or digests and x (mean digest volume / mean digest mass) for periphyton

ND = below instrument detection limit

NM = not measured

[1] To convert metal concentration on an ash-free dry mass to dry mass basis multiply by fraction of organic content (0.13, 0.30, 0.10 for OP, JO and DT periphyton, respectively)

[2] Acid digestion of sediment was performed by NLET and no MDL was calculated

A4.5 Mean (SD, n=2) metal concentration (nmol/g) in *H. azteca* collected from Lakes Opasatica (OP), Joannès (JO) Dufault (DT) and in laboratory cultured adult *H. azteca* caged in the same lakes for 14 d

Metal	Field *H. azteca*			Caged *H. azteca*			*H. azteca*
	OP	JO	DT	OP	JO	DT	MDL
Ag	ND	ND	ND	ND	ND	ND	0.17
Al	1510 (44.8)	2250 (2520)	2330 (292)	2980 (708)	2910 (2.27)	3640 (1230)	39.5
As	INT	INT	INT	INT	INT	INT	INT
B	402 (17.7)	514 (376)	319 (116)	356 (61.0)	432 (51.6)	482 (89.2)	10
Ba	256 (14.4)	144 (94.4)	114 (121)	544 (231)	318 (34.2)	452 (3.16)	0.09
Be	ND	ND	ND	0.69 (0.90)	ND	ND	0.07
Bi	ND	ND	0.04 (0.02)	ND	0.03 (0.00)	ND	0.00
Cd	10.4 (0.92)	59.9 (35.2)	167 (105)	15.5 (1.06)	44.6 (4.36)	186 (5.42)	0.01
Co	4.15 (0.73)	4.32 (2.73)	5.14 (4.42)	4.13 (1.19)	5.49 (0.02)	9.04 (0.91)	0.02
Cr	14.0 (4.88)	22.5 (11.8)	ND	21.3 (24.9)	19.6 (5.64)	ND	1.55
Cu	969 (242)	1450 (535)	976 (718)	1210 (33.6)	1330 (131)	1280 (118)	0.51
Fe	904 (1070)	470 (179)	508 (21.9)	879 (409)	831 (100)	1190 (282)	17.1
Ga	ND	14.6 (5.98)	18.5 (4.75)	32.0 (4.08)	46.7 (2.16)	59.9 (1.46)	0.52
La	0.29 (0.12)	0.13 (0.14)	0.06 (0.02)	0.16 (0.01)	0.10 (0.01)	0.27 (0.07)	0.01
Li	ND	44.8 (25)	31.0 (11.3)	ND	ND	ND	18.4
Mn	1320 (509)	631 (407)	297 (228)	464 (49.3)	417 (7.38)	455 (92)	0.20
Mo	3.98 (0.16)	4.63 (2.29)	3.60 (1.84)	4.94 (0.08)	4.93 (0.38)	5.29 (0.43)	0.02
Ni	14.0 (3.96)	9.30 (8.54)	8.90 (3.77)	17.6 (4.77)	10.3 (0.87)	14.8 (10.6)	0.88
Pb	ND	0.42 (0.28)	0.94 (0.75)	ND	0.68 (1.15)	3.83 (1.88)	0.06
Rb	134 (32.4)	168 (64.6)	130 (94.3)	141 (18.3)	178 (10.6)	191 (18.8)	0.10
Sb	0.61 (0.01)	0.72 (0.40)	0.46 (0.22)	1.52 (2.06)	1.06 (0.57)	0.68 (0.07)	0.02
Se	INT	INT	INT	INT	INT	INT	INT
Sr	1320 (197)	956 (560)	481 (436)	1610 (100)	1160 (109)	1020 (93.6)	0.43
Tl	0.18 (0.05)	0.09 (0.00)	0.52 (0.28)	0.24 (0.03)	0.16 (0.03)	0.66 (0.01)	0.00
U	0.08 (0.00)	0.11 (0.06)	0.11 (0.08)	0.10 (0.00)	0.07 (0.02)	0.09 (0.01)	0.00
V	2.62 (0.13)	2.00 (0.75)	1.24 (1.09)	2.89 (0.09)	3.11 (0.45)	2.72 (0.37)	0.01
Zn	873 (199)	1120 (479)	1250 (881)	1180 (64.8)	1260 (120)	1370 (32.4)	0.95

INT = Matrix interference

MDL = method detection limit is the upper 95 % CL of metal in blank digests x (mean digest volume / mean digest mass)

ND = below instrument detection limit

A4.6 Mean (SD, n=64) water temperature, conductivity, pH, dissolved oxygen (DO_2), ammonia (NH_3), dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) of test solutions from treatments of metal primarily in water, food, food+water using lake water and periphyton collected from Lakes Opasatica (OP), Joannès (JO), Dufault (DT) and the control consisting of standard artificial medium (SAM) and TetraMin®

Parameter		Control	Water	Water	Water	Food	Food	Food	Food+Water	Food+Water	Food+Water
		SAM	OP	JO	DT	SAM	SAM	SAM	OP	JO	DT
		TetraMin®	TetraMin®	TetraMin®	TetraMin®	OP Periphyton	JO Periphyton	DT Periphyton	OP Periphyton	JO Periphyton	DT Periphyton
Temperature	(°C)	22.8 (0.58)	22.8 (0.57)	22.7 (0.56)	22.7 (0.52)	22.9 (0.58)	22.8 (0.58)	22.8 (0.57)	22.7 (0.52)	22.7 (0.52)	22.7 (0.52)
Conductivity	(mS/cm)	0.42 (0.01)	0.11 (0.03)	0.08 (0.04)	0.16 (0.04)	0.41 (0.01)	0.41 (0.02)	0.42 (0.05)	0.10 (0.02)	0.10 (0.06)	0.15 (0.07)
pH		8.17 (0.01)	7.91 (0.12)	7.75 (0.10)	7.84 (0.14)	8.26 (0.12)	8.24 (0.06)	8.24 (0.07)	7.98 (0.08)	7.82 (0.08)	7.92 (0.06)
DO_2	(mg/L)	7.46 (0.26)	7.51 (0.25)	7.51 (0.24)	7.57 (0.29)	7.59 (0.34)	7.6 (0.39)	7.62 (0.28)	7.68 (0.30)	7.62 (0.29)	7.59 (0.36)
NH_3	(mM)	0.05 (0.07)	0.05 (0.07)	0.05 (0.07)	0.04 (0.07)	0.00	0.00	0.00	0.00	0.00	0.00
DIC	(mg/L)	13.5	6.35	5.85	5.35	12.5	12.2	12.2	6.15	4.65	NM
DOC	(mg/L)	1.65	7.55	11.6	6.75	0.65	0.55	0.35	7.05	10.8	NM

NM = not measured

n=1 for DIC and DOC

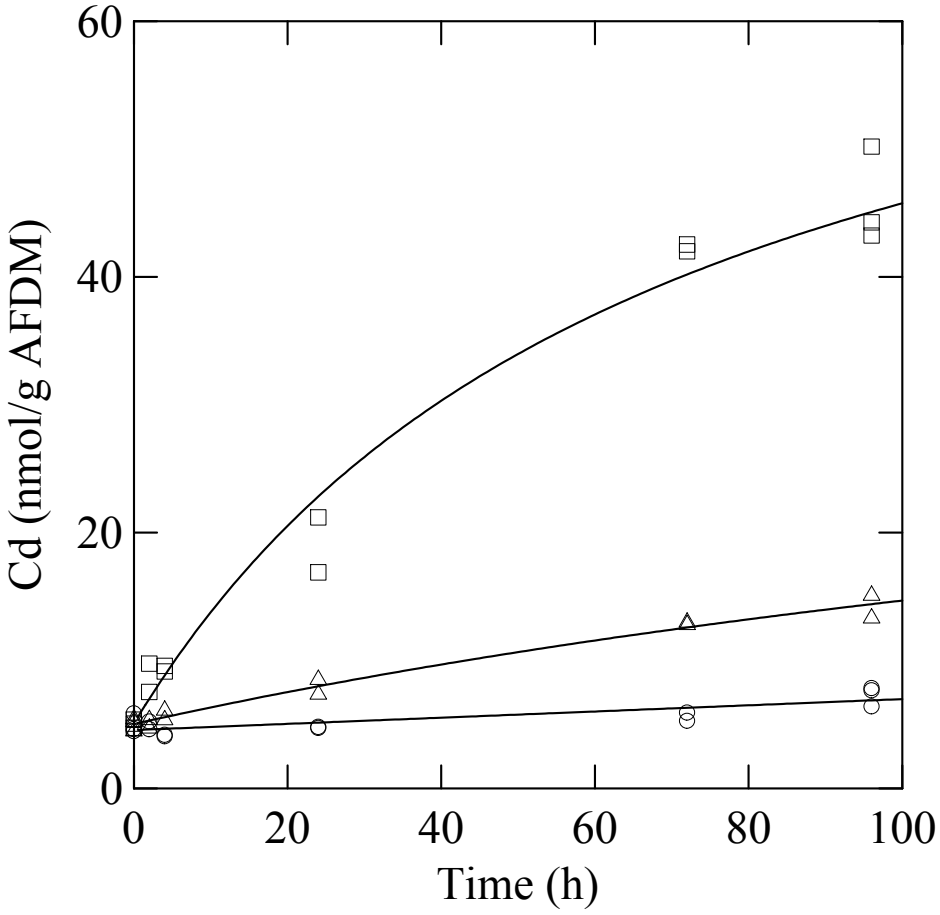


Figure A4.7 Measured (symbols) and modelled (solid lines) Cd in TetraMin® on an ash-free dry mass basis (AFDM) over 96 h when exposed to water from Lakes Opasatica (circle, r^2=0.69,n=14), Joannès (triangle, r^2=0.97, n=10) and Dufault (square, r^2=0.96, n=11).

A4.8 Mean measured Cd in water (dissolved and Cd^{2+}), food and *H. azteca* tissue as well as *H. azteca* survival and dry weight after 28 d in feeding experiments. Predicted Cd in *H. azteca,* percentage of Cd from diet and survival calculated from models (Eq.s 4.1, 4.2 and 4.3) (standard deviations shown)

Treatment and collection site[a]	Food type[b]	Cd in water[c]	Cd^{2+} in water[d]	Cd in food[e]	Measured Cd in *H. azteca*	Predicted Cd in *H. azteca*[f]	Predicted Cd in *H. azteca*[g]	Predicted Cd in *H. azteca* from food[h]	Predicted Cd in *H. azteca* from food[i]	Survival[j]	Predicted survival[k]	Dry weight
		(nmol/L)	(%)	(nmol/g)	(nmol/g)	(nmol/g)	(nmol/g)	(%)	(%)	(%)	(%)	(mg/*H.azteca*)
Control												
SAM	T	0.06 (0.04)	62 (5.9)	6.50 (1.72)	3.53 (0.11)	3.41 (1.21)	3.23 (1.24)	36 (12)	41 (15)	88 (16)	92 (0.0)	0.331 (0.082)
Metal in Water												
OP	T	0.08 (0.02)	25 (0.54)	5.83 (1.48)	6.58 (1.29)	3.67 (0.65)	2.07 (0.21)	29 (5.1)	56 (5.6)	88 (3.3)	92 (0.0)	0.359 (0.041)
JO	T	0.39 (0.01)	19 (0.16)	7.97 (6.43)	15.8 (0.22)	14.5 (0.38)	4.98 (0.13)	10 (0.26)	31 (0.81)	95 (3.3)	92 (0.0)	0.416 (0.044)
DT	T	1.75 (0.00)	42 (0.32)	16.3 (34.6)	86.6 (3.25)	60.2	37.5 (0.25)	4.9 (0.0)	8.5 (0.06)	75 (11)	92 (0.0)	0.273 (0.083)
Metal in Food												
OP	P	0.06	70	38.9 (4.53)	24.8 (0.75)	16.5	15.5	87	91	52 (22)	92 (0.0)	0.102 (0.020)
JO	P	0.29 (0.03)	71 (1.4)	79.4 (6.05)	59.4 (11.8)	38.5 (1.09)	38.0 (1.28)	74 (2.1)	74 (2.5)	67 (45)	92 (0.0)	0.095 (0.011)
DT	P	2.35 (0.01)	75 (0.18)	2560 (27.0)	363 (61.4)	516 (0.33)	518 (0.16)	85 (0.06)	85 (0.03)	18 (8.4)	84 (5.3)	0.067 (0.016)
Metal in Food+Water												
OP	P	0.05 (0.01)	24 (0.02)	47.0 (3.64)	20.6 (3.31)	19.1 (0.41)	17.6 (0.14)	90 (2.0)	96 (0.75)	75 (3.3)	92 (0.0)	0.079 (0.010)
JO	P	0.58 (0.06)	21 (0.19)	110 (22.9)	95.6 (21.9)	58.6 (2.15)	44.4 (0.59)	67 (2.4)	87 (1.2)	67 (33)	92 (0.0)	0.089 (0.010)
DT	P	2.63 (0.02)	43 (0.32)	2630 (23.5)	432 (158)	530 (0.73)	497 (0.82)	84 (0.12)	90 (0.15)	10 (12)	74 (18)	0.072 (0.030)

[a] OP=Lake Opasatica, JO=Lake Joannès, DT=Lake Dufault, SAM=standard artificial medium (Borgmann, 1996)

[b] T = TetraMin®, P = periphyton

[c] Measured filtered (0.45µm, Acrodisc®) Cd concentration

[d] Cd^{2+} concentration modelled using WHAM v6.0.13

[e] Measured Cd in food on ash-free dry mass basis can be converted to a dry mass basis by multiplying by fraction of organic content (0.13, 0.30, 0.10 for OP, JO and DT periphyton, respectively)

[f] Predicted Cd in *H. azteca* using Eq. 4.1 and parameters based on dissolved Cd

[g] Predicted Cd in *H. azteca* using Eq. 4.1 and parameters based on Cd^{2+}

[h] Predicted contribution of Cd from food using Eq. 4.1 and parameters based on dissolved Cd

[i] Predicted contribution of Cd from food using Eq. 4.1 and parameters based on Cd^{2+}

[j] Survival of *H. azteca* in DT food+water treatment was for 21 d

[k] Predicted *H. azteca* survival using Eq. 4.2 and 4.3

n=1 or 2 for Cd concentrations, dietary contributions and predicted values

n=4 replicate containers for survival and 2 to 4 replicate containers for dry weight

A4.9 Mean measured Cd in water (dissolved and Cd^{2+}), food and *H. azteca* collected from field sites and laboratory *H. azteca* caged at field sites for 14 d. Predicted Cd in *H. azteca,* percentage of Cd from diet and survival calculated from models (Eq.s 4.1, 4.2 and 4.3) (standard deviations shown)

Site[a]	Exposure[b]	Cd in water[c]	Cd^{2+} in water[d]	Cd in food[e]	Measured Cd in *H. azteca*	Predicted Cd in *H. azteca*[f]	Predicted Cd in *H. azteca*[g]	Predicted Cd in *H. azteca* from food[h]	Predicted Cd in *H. azteca* from food[i]	Survival	Predicted survival[j]	Dry weight
		(nmol/L)	(%)	(nmol/g)	(nmol/g)	(nmol/g)	(nmol/g)	(%)	(%)	(%)	(%)	(mg/*H.azteca*)
OP	Wild	0.06 (0.00)	33 (0.24)	22.7 (4.95)	10.4 (0.92)	9.89 (2.39)	8.60 (2.35)	78 (5.3)	88 (3.2)	N/C	92 (0.0)	0.107 (0.01)
JO	Wild	0.70 (0.01)	20 (0.02)	94.1 (8.76)	59.9 (35.2)	57.8 (3.56)	40.8 (3.52)	60 (2.4)	83 (1.4)	N/C	92 (0.0)	0.198 (0.10)
DT	Wild	3.01 (0.04)	46 (0.11)	1850 (396)	167 (105)	472 (44.9)	438 (45.4)	80 (1.9)	86 (1.5)	N/C	91 (0.82)	0.208 (0.19)
OP	Caged	0.06 (0.00)	33 (0.24)	22.7 (4.95)	15.5 (1.06)	N/C	N/C	N/C	N/C	91 (10)	92 (0.0)	0.307 (0.10)
JO	Caged	0.70 (0.01)	20 (0.02)	94.1 (8.76)	44.6 (4.36)	N/C	N/C	N/C	N/C	78 (14)	92 (0.0)	0.308 (0.08)
DT	Caged	3.01 (0.04)	46 (0.11)	1850 (396)	186 (5.42)	N/C	N/C	N/C	N/C	84 (8.1)	91 (0.82)	0.241 (0.02)

[a] OP=Lake Opasatica, JO=Lake Joannès, DT=Lake Dufault

[b] Wild *H. azteca* collected from each site, caged *H. azteca* are laboratory cultured adults caged for 14 d at each site

[c] Measured filtered (0.45µm, Acrodisc®) Cd concentration

[d] Cd^{2+} concentration modelled using WHAM v6.0.13

[e] Measured Cd in food on ash-free dry mass basis can be converted to a dry mass basis by multiplying by fraction of organic content (0.13, 0.30, 0.10 for OP, JO and DT periphyton, respectively)

[f] Predicted Cd in *H. azteca* using Eq. 4.1 and parameters based on dissolved Cd

[g] Predicted Cd in *H. azteca* using Eq. 4.1 and parameters based on Cd^{2+}

[h] Predicted contribution of Cd from food using Eq. 4.1 and parameters based on dissolved Cd

[i] Predicted contribution of Cd from food using Eq. 4.1 and parameters based on Cd^{2+}

[j] Predicted *H. azteca* survival using Eq. 4.2 and 4.3

n= 2 for Cd concentrations, dietary contributions and predicted values

n= 5 or 6 replicate cages for 14 d survival and 3 replicates of 5 - 7 *H. azteca* for dry weight from each site

N/C not calculated

CHAPTER 5 SUMMARY AND CONCLUSIONS

The over-arching objective of this research was to determine whether dietary Cd in an ecologically relevant diet at environmentally relevant concentrations contributed to chronic bioaccumulation and toxicity in *H. azteca* and whether this could be accurately modelled. The following conclusions address the detailed objectives as follows:

1) Model chronic bioaccumulation and the relative contributions of waterborne and dietary Cd
    - A mechanistic-based saturation bioaccumulation model was developed under laboratory conditions that produced robust (within a factor of two) predictions of chronic bioaccumulation of Cd in *H. azteca* from water and food sources separately and combined thereby accounting for secondary Cd exposures in the water and food treatments (Chapter 2).

    - Using this model, the contribution of Cd from food to *H. azteca* body concentration was estimated to be 21 – 31 % (from TetraMin® in the primarily-water treatment), 59 – 94 % (from periphyton in the primarily-food treatment) and 40 – 55 % (from periphyton in the food+water treatment). These contributions were similar to those predicted when using model parameters based on Cd^{2+} rather than dissolved Cd. Therefore dietary Cd contributed markedly to chronic bioaccumulation of Cd in *H. azteca* (Chapter 2).

    - Dietary and waterborne Cd both contributed to chronic bioaccumulation, however the relative contribution of dietary Cd from periphyton increased as the waterborne Cd concentration decreased (Chapter 2).

    - The contribution of dietary Cd to bioaccumulation in *H. azteca* was dependent on the relative concentrations of Cd in water and food and the partitioning of Cd between water, food and *H. azteca* as described by model parameters max and K (Chapter 2).

2) Connect bioaccumulation to chronic effects on survival and growth

- Effects on chronic survival were independent of exposure pathway when expressed as a function of Cd body concentration (LBC50 = 679 nmol/g, 617 – 747 95 % CL). Therefore bioaccumulation became the link between exposure pathway and effects on chronic survival (Chapter 2).

- The contribution of Cd from periphyton to LBC50 tissue concentration in the food+water treatment was predicted to be 46 % suggesting that dietary Cd contributed markedly to body concentrations resulting in a 50 % reduction in survival (Chapter 2).

- Chronic *H. azteca* growth declined with increasing Cd exposure but was less sensitive than survival as an endpoint of chronic effects and was dependent on the nutritional quality of the food. Therefore irrespective of Cd exposure, growth was lower for *H. azteca* fed with periphyton than *H. azteca* fed with TetraMin®. The lower polyunsaturated fatty acid (PUFA) content of periphyton compared to TetraMin® may have contributed to lower *H. azteca* dry weight (Chapter 2).

- No nutritional effects on *H. azteca* were observed as a result of Cd exposure though measurements of total protein, total lipid and fatty acids may have lacked sufficient sensitivity to detect effects (Chapter 2).

3) Determine bioavailability of dietary Cd in periphyton

- Bioavailability of dietary Cd as determined by assimilation efficiency was lower from periphyton (3 – 14 % AE) than TetraMin® (44 – 86 % AE). It was hypothesized that this was due in part to insufficient acclimation of the digestive enzymes in *H. azteca* to a periphyton diet (Chapter 3).

- Assimilation efficiency was likely influenced by the different speciations of Cd associated with periphyton and TetraMin®. It was not influenced by

food form (dry versus fresh) or Cd concentration. Ingestion rate was lower for dry versus fresh food and excretion rate constant was not influenced by food type, form or Cd concentration (Chapter 3).

- Predictions of dietary Cd contributions to bioaccumulation made using a biokinetic model based on the measured physiological parameters of AE, IR, k_e did not concur with predictions from the mechanistically-based Cd saturation bioaccumulation model. Sensitivity analysis of the model indicated that AE and IR were the most influential parameters of the model. Therefore short-term measurements of physiological processes may not reflect long-term bioaccumulation patterns and effects of Cd toxicity on those processes (Chapter 3).

4) Compare the predictions from models developed in the lab with field measurements
    - The chronic mechanistically-based saturation bioaccumulation model developed in the laboratory (Chapter 2) provided robust predictions of Cd bioaccumulation in *H. azteca* exposed to field contaminated water and periphyton for 28 d as well as Cd in *H. azteca* residing in the same field contaminated lakes. However, the model was constrained in its application by the fact that it didn't account for water chemistry effects of Ca and DOC on aqueous Cd bioaccumulation by *H. azteca* or periphyton (Chapter 4).

    - Comparison of tissue concentrations of multiple metals in *H. azteca* exposed to the field contaminated water and periphyton with critical body concentrations from the literature indicated that Cd was the dominant metal of concern for *H. azteca* in Lake Dufault in terms of chronic toxicity (Chapter 4).

- Cadmium from periphyton was predicted to contribute 67 – 90 % and 60 – 80 % to Cd bioaccumulation in *H. azteca* in the laboratory food+water treatments and in *H. azteca* inhabiting the field contaminated sites respectively. Therefore, dietary Cd was contributing to observed toxicity to *H. azteca* in Lake Dufault (Chapter 4).

- Using the model of chronic effects on *H. azteca* survival as a function of Cd bioaccumulation developed in the laboratory (Chapter 2), the trend of toxicity was predicted accurately. However, observed survival was much lower than predicted in Lake Dufault. Nutritional measurements of the periphyton collected from Lake Dufault suggest that the model under-predicted toxicity by not accounting for the lower nutritional quality of periphyton from Lake Dufault. Therefore, food nutrition may contribute to toxicity in addition to the effects of dietary Cd (Chapter 4).

5.1 *Implications for water quality guidelines and ecological risk assessment*

Canadian water quality guidelines currently account for some dietary Cd effects by incorporating endpoints from chronic assays in which the animals were fed and by incorporating a x 10 safety factor for undetermined effects. This research provides a more accurate means of accounting for dietary Cd toxicity by using the ratio of the LC10s from the primarily-water treatment and the food+water treatment. This indicated that a factor of x 2, would account for dietary Cd at the 10 % level of effect. Therefore, the current x 10 safety factor which ensures no effects on any life stage is protective of dietary Cd effects on *H. azteca*.

Ecological risk assessment relies on quantifying risk of exposure and effects of Cd on aquatic biota. This research demonstrated that Cd in a natural periphyton diet is bioavailable and bioaccumulated by *H. azteca* at environmentally relevant concentrations. In addition the contribution of dietary Cd was quantified and found to contribute markedly to bioaccumulation and therefore could make an important contribution to

chronic toxicity. Therefore dietary Cd needs to be considered in addition to waterborne Cd when assessing risk of chronic exposure and effects to *H. azteca*.

5.2 *Further research needed*

To further develop the chronic Cd saturation bioaccumulation model for *H. azteca* there are two main areas that require future research:

1) Modelling Cd bioaccumulation by periphyton under varying Ca, pH and DOC. In this way Cd bioaccumulation in periphyton could be modelled based on water chemistry and therefore Cd in *H. azteca* could be predicted based on water chemistry alone.
2) Modelling chronic Cd bioaccumulation by *H. azteca* from separate and combined food and water sources under varying Ca conditions and using a periphyton food source. Periphyton would be exposed to the same water chemistry conditions as *H. azteca* in order to relate Cd in periphyton and *H. azteca* to aqueous Cd.

This will then enable the model to be applied to a variety of water chemistry conditions and thus act as valuable tool for ecological risk assessment and setting site-specific guidelines.

Factors that influence dietary Cd bioavailability also require further investigation. Specifically, the hypothesis that AE of Cd from periphyton was lower than that from TetraMin® because of acclimation differences of the gut enzyme system needs to be tested. In addition, the components of periphyton (algae, bacteria, exopolymer matrix, sediment) that most influence Cd AE need to be identified as these components vary widely in periphyton.

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