TOXICITY OF METALS TO CHIRONOMIDS,

CD TROPHIC TRANSFER FROM CHIRONOMIDS TO ZEBRAFISH

TOXICITY OF CD, CU, PB, NI, AND ZN TO CHIRONOMIDS, AND TROPHIC TRANSFER OF CD FROM CHIRONOMIDS TO ZEBRAFISH

By

KAREN M. BÉCHARD, B.SC.

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AUTHOR: Karen M. Béchard, B.Sc. (Queen's University)

SUPERVISORS: Professor C.M. Wood and P.L. Gillis

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Abstract

The toxicity of the metals: Cd, Cu, Pb, Ni, and Zn to the freshwater aquatic larvae of the midge fly *Chironomus riparius* was investigated using 24 h waterborne exposures. Even at the most sensitive life stage, first instar, the chironomids were extremely metal tolerant, with LC50 values for all metals being orders of magnitudes above both the CCME Canadian water quality guidelines for the protection of aquatic life, and the USEPA Water Quality Criteria. This high tolerance of *C. riparius* to metal toxicity, combined with an exceptional ability to accumulate and tolerate high internal metal burdens makes the chironomid an ideal organism to use in studies on factors affecting the trophic transfer of Cd.

Zebrafish were fed with Cd-contaminated chironomids for 7 days, followed by 3 days of gut clearance with clean chironomids. Chironomids loaded with Cd by exposure to Cd-contaminated sediments exhibited a significantly higher trophic transfer efficiency (TTE) than did zebrafish fed chironomids contaminated with Cd by waterborne exposure, although in both cases the TTE's were low (<2%). The majority of Cd transferred to zebrafish was stored in the gut and carcass, irrespective of ingestion of a natural diet (chironomids loaded with Cd), or a manufactured pellet diet of identical Cd concentrations. On a tissue concentration basis, the highest tissue accumulations in zebrafish were (in decreasing order): kidney > gut > liver > gill > carcass; this accumulation pattern was also independent of concentration of Cd in the diet or of prey exposure route.

Subcellular fractionation of chironomids found most of the Cd in the metal rich granule fraction followed by the organelle fraction. It also revealed that sedimentexposed chironomids had significantly more Cd in the metallothionein-like protein fraction, and significantly less Cd in the cellular debris fraction than water-borne exposed chironomids, although these fractions accounted for only a small percent (~7%) of the total accumulated Cd. Despite this difference in prey subcellular fractionation, the subcellular storage of Cd in zebrafish fed on sediment-exposed chironomids and zebrafish fed on water-borne exposed chironomids was the same, with the highest accumulations in the organelles, enzymes, and metal rich granules fractions. Main areas of subcellular storage in zebrafish fed on a manufactured pellet food were identical to those of zebrafish fed on chironomids. However, zebrafish fed on chironomids had significantly more Cd in the metallothionein-like protein fraction (5-10%) than did zebrafish fed on pellets (0%).

Overall, TTE's were independent of concentration, but were dependent on route of prey exposure. Tissue-specific accumulations and tissue-specific distributions in zebrafish were independent of both concentration and route of prey exposure, and the gut consistently accounted for the highest proportion of overall body burden, and had the highest accumulation of all tissues; verifying its importance in preventing the internalization of Cd. Subcellular distributions were also independent of concentration, although zebrafish fed a biological food (chironomids) had more Cd in the metallothionein-like protein fraction than zebrafish fed a manufactured food. This has biological implications for Cd detoxification.

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Thesis Format

This thesis is organized into four chapters. Chapter one is a general introduction, including a relevant literature review and the rationale behind my thesis topics. Chapters two and three contain experimental work in the form of manuscripts that is under review (chapter two) or will be submitted (chapter three) for publication in peer-reviewed scientific journals. Chapter four is a general discussion, providing a brief review of major results and conclusions that were established in the previous two chapters. References have been combined into one list, and are included at the end of the theses following the last chapter.

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Chapter 2:	Acute Toxicity of Waterborne Cd, Cu, Pb, Ni, and Zn to First Instar <i>Chironomus riparius</i> Larvae	
Authors:	K.M. Béchard, P.L. Gillis, and C.M. Wood	
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Chapter 3:	Trophic transfer of Cd from chironomids (<i>Chironomus riparius</i>) exposed via sediment or water-borne routes, to zebrafish (<i>Danio rerio</i>): tissue-specific and subcellular comparisons	
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Comments:	This study was performed by KMB under the supervision and guidance of CMW and PLG. This paper, with slight modifications, will be submitted to a peer reviewed journal.	

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CHAPTER 1

General Introduction

Metal Guidelines for the Protection of Aquatic Life

When studying the effects of metal toxicants to aquatic organisms, it is important to always keep environmental relevance in mind. To that end, Canadian Water Quality Guidelines (CCME, 2006) for the protection of aquatic life, and the U.S. Water Quality Criteria (U.S. EPA, 1996) for the metals: Cd, Cu, Pb, Ni, and Zn (those used in this thesis) adjusted to a water hardness (very soft water) of 20 mg/L (as CaCO₃ equivalents) are given in Table 1.1. The water hardness of 20 mg/L is still greater than the extremely soft waters employed in this thesis (approximately 8-10 mg/L), however, these were the lowest extrapolations recommended by the regulatory bodies. This extremely soft water was employed so as to be representative of Scandinavian and Canadian Shield waters ("sensitive waters") where metal contamination from anthropogenic activities is currently of great concern (K ε lin *et* al., 2006; Wyn *et al.*, 2007). In this thesis, values were considered to be environmentally relevant if they were within one order of magnitude of the guideline values.

The present study started by surveying the toxicity of five different metals to chironomids, but subsequently shifted to a focus on Cd. Cd comes from many sources, including anthropogenic: municipal waste water effluents, industrial point source effluents (eg. mining spoil runoff), urban runoff, landfill leachates, forestry, fuel combustion, as well as natural sources and soil/substrate disturbances (Environment Canada 2001). However, Cd has no known biological functions, except that in

phytoplankton it may substitute for Zn in specific enzymes (Lane and Morel, 2000). Thus, it needs to be either detoxified or excreted (Rainbow, 2002), and depending on how these two modes of cealing with Cd toxicity are carried out (i.e. storage or excretion mechanisms), there may be the potential for bioaccumulation up trophic levels. Indeed, Croteau *et al.* (2005) found that within two trophic levels, cadmium concentrations had biomagnified 15 folc.

Reasons for Using Study Organisms

Chironomids

Chironomids, from the Order Diptera, Family Chironomidae, are widely distributed with approximately 1090 North American species (Triplehorn and Johnson, 2005), and many others around the world. The chironomid life cycle consists of four stages; egg, larvae, pupae, and imagines (adults). The larvae stage of *C. riparius* (of which there are four instars) is aquatic, and commonly used in toxicology experiments (e.g. Craig *et al.*, 1999; Groenendijk *et al.*, 2002). Larval chironomids will swim briefly in the overlying water just above the sediment water interface, but typically they burrow within the top 7.5 cm of the sediments (Charbonneau and Hare, 1998). Even though burrowing is usually limited to soft, shallow sediment (Pinder, 1986), it still contributes to bioturbation (Gosselin and Hare, 2003), and to the potential release of compounds previously bound to the sediment particles. This study will focus on the larvae stage.

The chironomid *Chironomus riparius*, a non-biting midge, was thought to be an ideal species to use in this study for a number of reasons. Firstly, they live at the

sediment-water interface and feed from organic matter on the surface of sediments. Thus *C. riparius* are poter tially exposed to higher concentrations of metals than pelagic invertebrates. Secondly, *C. riparius* are relatively easy to culture, and have a short lifecycle of about 25-30 days (at 21°C). Chironomids live within and on the sediment, exposing them to pore water which normally has higher concentrations of metal than the overlying water since it is in close contact with the sediment. Given their close association with the sediment, and exposure to pore water, and that they are preyed upon by higher trophic organisms, they are an important organism to study in the transfer of metals from the benthic to the pelagic food web. Exposing chironomids to a range of different metal concentrations indicates how sensitive chironomids are to that particular metal. In turn, producing LC50 estimates (concentration of metal lethal to 50% of the animals in a given time period) from these data would provide a widely accepted index of species tolerance.

In the course of this research, it was found that *C. riparius* larvae were in fact exceptionally tolerant of metals (Chapter 2), in agreement with some earlier literature, as summarized below. Thus, the resultant LC50 estimates were not of environmental relevance by the above-stated criterion. However, this tolerance was associated with an exceptional ability to accumulate one metal in particular (Cd), meaning that these organisms can serve as important vehicles of trophic transfer to organisms higher in the food chain, such as fish. The focus of the research therefore shifted to this issue of trophic transfer of Cd, in light of recent intense toxicological and regulatory interest in dietary metal toxicity to fish (Meyer et al., 2005).

Zebrafish

The tropical zebrafish, *Danio rerio*, was chosen as a suitable organism to use in trophic transfer studies with North American native chironomids for three main reasons. An important factor was that zebrafish readily eat chironomids, so lack of eating would not be a major issue. Another factor is that they are soft water tolerant (Boisen *et al.*, 2003), and thus experiments can be done under lower ionic strength conditions representative of "sensitive waters". And, thirdly, zebrafish are now being used for many molecular and toxicc genomic studies (*e.g.* Neumann and Galvez, 2002; Craig *et al.*, in submission). This research hopes to provide a link to molecular studies by determining where metals that have been biologically incorporated via a dietary route are stored in different tissues (gill, gut, liver, kidney, carcass), as well as their subcellular distribution in the whole body.

Toxicity of Metals to Chironomids

Chironomids, along with other sediment dwellers such as oligochaetes are known to have considerable resistance to metal toxicity. In fact, when insect communities in metal polluted lakes are surveyed, oligochaetes which are also extremely metal tolerant, and chironomids are found to be the dominant organisms present (Wentsel *et al.*, 1977; Winner *et al.*, 1980). Furthermore, 4th instar *Chironomus riparius* were the most tolerant to Cd out of all aquatic organisms from which data were collected in the USEPA (2000) species sensitivity distribution for Cd. Chironomids are not just resistant to Cd metal toxicity, indeed, they are resistant to many metals, including but not limited to: Cu

(Taylor et al., 1991), Pb (Rao and Saxena, 1981; Qureshi et al., 1980), Ni (Powlesland and George, 1986), and Zn (Rao and Saxena, 1981).

Metal Accumulation by Chironomids: Water-borne exposure versus Sediment Exposure

Chironomids, like all aquatic invertebrates, accumulate metals in their tissues whether they are important for the metabolism or not (Rainbow, 2002). Water-borne metal is taken up by most aquatic organisms through the apical surface of epithelial membranes. This untake likely occurs through facilitated diffusion mechanisms (e.g. ion channels) since the lipid bilayer in the cell membrane has a "low permeability to dissolved hydrophilic trace metal ions" (Craig et al., 1999). In chironomids, the uptake route for Cd appears to be through apical Ca channels (Craig et al., 1999) in surface epithelia. In accordance with this theory, Gillis and Wood (2007) reported that internal Ca levels of C. riparius drop within the first hour of water-borne Cd exposure in both soft and hard waters regardless of concentration. While uptake of metal from the overlying water may be an extremely important source of metal contamination to chironomids, in burrowing, sediment- feeding taxa (such as the species being used, C. riparius) there is also the potential to accumulate 'substantial Cd' from the sediment (Warren et al., 1998). Selck and Forbes (2004) found a difference in the uptake of Cd between water-borne and feeding exposures in another deposit feeder, the polychaete (Capitella sp.). These authors reported that uptake of Cd from the water was related to surface area of the organism, and that uptake of Cd from diet was dependent on the amount of sediment passing through the gut (Selck and Forbes, 2004). In a related issue, it is important to note that for deposit feeders, and sediment dwellers, it is impossible to entirely separate

dietary contamination (through gut) and sediment uptake (through apical surface), so the simple term "sediment-exposed" is employed throughout this thesis.

Other studies have noted that on a whole-body scale, the anterior portion of the posterior-mesenteron (posterior midgut) is a strong site of Cd accumulation, holding > 80% of the total body-burden in chironomids (Seidman et al., 1986; Craig et al., 1998). Furthermore, Craig et al. (1998) went on to state that Cd accumulation occurs in this location regardless of the duration or exposure route of Cd: in food and water, versus only water. While these authors reported no difference in the main storage of accumulated Cd between food and water exposures, Warren et al.'s (1998) paper discusses that "sediment-feeding taxa" such as Chironomus "obtain substantial Cd from the sediment". In a similar species, Chironomus staegeri, Cd is known to be stored in the digestive tract for both short term waterborne exposures and longer sediment/water exposures (Craig et al., 1998). These results are unique from other species, such as epibenthic amphipods, which live in, but do not feed from, low Cd-sediments, and in a 'natural' study, were found to take up their Cd from the overlying water (Warren et al., 1998). In aquatic invertebrates, bioaccumulated Cd is commonly stored in the cytosol bound to metallothionein (Rainbow, 2002).

Mechanisms of Metal Tolerance

The fact that chironomid larvae are able to accumulate and withstand huge amounts of metal without toxicity has been proven multiple times, yet, the reason for their extreme metal tolerance is still a topic for ongoing research and debate. Timmermans and Walker (1989) determined that Zn, Cd, and Cu were all accumulated in

chironomids, and further, that there was a "simultaneous decrease in body burden of the successive developmental stages". This trend was particularly noticeable with Cd and Zn, such that body burden decreased as follows: $3^{rd}-4^{th}$ instar larvae > pupae > imagines (adult) for equivalent concentrations of metal exposure. It was found that chironomids could transfer a portion of the accumulated metal to larval skins (from molts 1, 2, and 3) and exuvae (pupae skin) which would decrease their total body concentration since they lose these during metamorphosis (Timmermans and Walker, 1989). Notably, for copper, the body burden drastically decreased after the larval stage indicating that the organisms are able to eliminate their accumulated copper somewhere during the process of metamorphosis between the 4th instar larva and pupae stage. From this study, it is very apparent that chironomids have multiple ways of eliminating metal from their systems: through larval skins and exuvae (partial elimination of Cd and Zn), and through other processes in the larval stage (explored below). Some studies have also suggested that in the aquatic larval stages, chironomids are able to detoxify large amounts of metal by the production of metallothionein-like proteins (MTLP) (Yamarura et al., 1983; Seidman et al., 1986; Gillis et al., 2002) which bind metals and play a key role in metal transport. In response to Cd exposure, this MTLP production has been found to occur in a dosedependent manner (Gillis *et al.*, 2002). It has been suggested that the role of MTLP is likely to be important for transporting metal to sites of metal-rich granule (MRG) production (Wallace *et al.*, 1998) which are also associated with metal accumulation (Klerks and Bartholomew, 1991).

Another physiological feature that may have a role in making a) toxic compounds less harmful or b) easier to excrete, may be the Malphighian tubules since they have the ability to transport a wide range of solutes (Dow and Davies, 2006). Others have suggested that chiror omids are able to excrete large quantities of metal (Timmermans and Walker, 1989; Postma et al., 1996). Groenendijk et al. (2002) found that Chironomus riparius are able to adapt to Cd- and Zn-contaminanted waters, while Klerks and Bartholomew (1991) found that oligochaetes (Limnodrilus hoffmeisteri) from a metal polluted cove (Foundry cove on the Hudson river) accumulated more Cd than did oligochaetes from a reference site, and had higher levels of metallothionein-like proteins. This phenomenon was still observed in the offspring of 'resistant' oligochaetes from the cove, implying that clevated levels of MTLP in response to Cd exposure have a genetic link (Klerks and Bartholomew, 1991). These workers proposed that these elevated protein levels contribute to the resistance of oligochaetes to metals. Postma et al. (1996) expanded upon this, finding that C. riparius that had been living in high Cd conditions in the field had both increased excretion abilities and increased storage abilities (mainly in the gut), indicating that the species has effective ways of dealing with prolonged metal exposure.

Trophic Transfer

Due to the extreme tolerance chironomids have shown to metal toxicity, and their ability to bioaccumulate (Yamamura *et al.*, 1983; Seidman *et al.*, 1986; Hare *et al*, 2001), they are an ideal candidate to study trophic transfer. As well, their lifestyle - living in, and feeding from secliment predisposes them to high metal exposure. Cd is a non-

essential toxic metal, and as such does not have a required minimum concentration within chironomids, nor a necessary function in physiological processes. Consequently, any Cd bioaccumulated by chironomids needs to be either detoxified or excreted (Rainbow, 2002). The location and physiological form of accumulated metals are very important in the process of trophic transfer. Since metals are generally not stored in fatty tissues, they are usually not biomagnified up the food chain (exception given in Croteau et al, 2005). However, bioaccumulation can still occur, and is dependent on a number of factors. As mentioned, the physiological form of the stored detoxified metals plays a role, as well as the potential for integration of that metal in the predator species (reviewed by Rainbow, 2002). Thus, if the prey has 'detoxified' the metal by binding with metallothionein-like proteins (MTLP) in the cytosol, this metal may be available to a predator; it is considered trophically available metal (TAM) (Wallace and Luoma, 2003). However if the metal was detoxified by incorporation into granules, it would not be readily available to higher trophic levels; it is considered to be trophically unavailable metal (TUM) (Wallace and Luoma, 2003). In addition, the route of metal uptake (waterborne versus dietary) by the prey species can alte: the storage of metal in that organism (Timmermans et al., 1992). All of these factors can affect the trophic transfer efficiency (TTE) of metals.

Metal in Fish Tissues

Soft Water and Hard Water Effects on Cd Uptake

In juvenile rainbow trout, it has been found that metal uptake during chronic water-borne Cd exposure (0.11 μ g/L) in soft water ([Ca] ~1mM) was time-dependent

with large increases occurring in the gill, and small increases in the liver (Hollis *et al.*, 2000a). However in a lower Ca (soft water ~0.26 mM) treatment of a similar experiment at a higher Cd concentration (3 μ g/L chronic water-borne exposures), juvenile rainbow trout were found to accumulate Cd mainly in the kidney, gills, and liver (Hollis *et al.*, 2000b). At higher Ca concentrations, the accumulation of Cd into the kidney, gills, and liver were decreased although Ca levels remained the same (Hollis *et al.*, 2000b). It was also determined that trout did not become acclimated to Cd after 30 days of water-borne exposure when in soft water (Hollis *et al.*, 2000a), although did become acclimated (>10 fold increase in LC50s) when in hard water (Hollis *et al.*, 1999). The protective effect of Ca in hard water is also evidenced through a greater mortality at comparable concentrations of Cd in the soft water (low Ca) (Hollis *et al.*, 2000b). Overall, these observations indicate that Cd contamination in soft water is more potent to fish, and the protective effect of vater hardness is well-explained by findings on the mechanism of Cd entry across the gills, as explained below.

Water-borne Versus Dietary Exposure

As mentioned above, the route of metal uptake: water-borne or dietary, can affect the storage of metals (including Cd) in an organism. In fact, some researchers have found that in predatory insects, transfer of Cd is greater from dietary sources (contaminated chiromonids fed to invertebrate predators) than from water to invertebrate predators (Timmermans *et al.*, 1992; Roy and Hare, 1999). Rouleau *et al.* (2006) found a similar relationship in coastal demersal fish. Cd exposure via the diet was associated with a higher hepatic Cd burden than via Cd water-borne exposure in natural conditions

(Rouleau et al., 2006). Kraemer et al. (2006) noted that Cd in the liver and gut of perch seemed to come from both water-borne and dietary exposures while Cd in the gills and kidney was mostly taken up from the water. Similarly, Farag et al. (1994) determined that rainbow trout that were exposed to dietary metals (contaminated invertebrates) for 21 days had scale loss and metal accumulation in the gut (adult trout). Another group of rainbow trout were exposed to metals (Cd, Cu, and Pb) for 21 days via a water-borne exposure; these fish had decreased survival, scale loss, and metal accumulation in the gill and kidney. Farag et al. (1994) went on to conclude that water-borne exposures were linked with higher accumulation of metal in the gill and kidney, while dietary exposures were linked with higher metal accumulation in the stomach and pyloric caeca. Szebedinsky et al. (2001) was in accord with these findings, noting that juvenile rainbow trout exposed to dietary Cd (contaminated commercial trout chow) accumulated the highest percentage of Cd in the gut tissue, whereas in water-borne exposed trout, the greatest percentage of Cd accumulation was in the carcass. These authors go on to specify that the highest tissue-specific accumulation occurred in the kidney for both dietary and water-borne exposed fish (Szebedinsky et al., 2001). Furthermore, Szebedinsky et al. (2001) showed that when Cd is taken up through the diet, it can affect the uptake of Cd at the gills (through the water); rainbow trout pre-exposed to a dietary Cd subsequently tock up less new Cd at the gills and exhibited increased tolerance of waterborne Cd challenge (i.e. acclimation). Comparatively, Kraal et al. (1995) noted that the gut was the biggest Cd accumulator in carp. This conclusion was true of both carp exposed to water-borne Cd for 4 weeks, and carp fed contaminated chironomids for 4

weeks, although the gill was the second biggest accumulator of Cd in the water-borne exposed fish.

More recent studies have found similar storage of Cd in fish. Franklin *et al.* (2005) found that juvenile rainbow trout accumulated Cd through both diet and water, and that the orders of Cd accumulation were gill and kidney > liver > gut > carcass for water-borne exposed fish, and gut > kidney > liver > gill > carcass > bone for dietary exposed fish. Ng and Wood (in submission) likewise report that after a 30 day chronic dietary exposure, the gut and kidney retained high concentrations of Cd. Chowdhury *et al.* (2004) similarly noted that when trout were exposed to Cd via gastrointestinal infusion, the gut was the major storage area after 24 h, and that roughly half of the Cd was lost from the fish. Relative to overall gut burden, the Cd-treated fish had the most Cd in the cecae > posterior intestine > mid intestine > stomach (Chowdhury *et al.*, 2004).

Other research shows that Cd uptake and storage can be influenced by dietary Ca (Zohouri et al., 2001; Baldisserotto *et al.*, 2004a,b, 2005, 2006; Franklin *et al.*, 2005). Baldisserotto *et al.* (2004a, b) clearly show that trout fed high Ca²⁺ diets took up less water-borne Cd, and had strongly reduced the Cd concentrations in the gill, liver, and plasma, compared to fish fed control diets. Dietary Ca has also been found to protect against dietary Cd uptake (Baldisserotto *et al.* (2005). In juvenile rainbow trout, high Ca²⁺ diets reduced dietary Cd accumulation by 70% after 15 days and 50% after 30 days (Balisserotto *et al.*, 2005). Another study performed on isolated intestinal sacs *in vitro* by Baldisserotto *et al.* (2006) explains that dietary Ca is protective because it actually prevents the uptake of dietary Cd into the intestinal tissue.

In summary, water-borne exposures are associated with higher tissue-specific Cd accumulation in the gill and kidney, and in dietary exposures, Cd is accumulated predominately in the gut. Ca can have a protective effect on both water-borne and dietary Cd accumulation.

Mechanisms of Metal Uptake

Acute Cd toxicity to fish causes hypocalcaemia in extracellular fluids, because Cd²⁺ is able to compete for and ultimately block Ca²⁺ uptake pathways which fish mainly use for Ca homeostasis (Verbost *et al.*, 1989). Cd^{2+} appears to selectively inhibit the basolateral Ca^{2+} pump (a high-affinity Ca^{2+} -ATPase) in gill ionocytes, causing an increase in cytosolic Ca^{2+} levels (Verbost *et al.*, 1989). Others have found that in trout the interactions of Ca and Cd are directly competitive (Nivogi and Wood, 2004). The internal distribution of Cd, after its uptake through the gills or alimentary canal (gut), is through the blood (Thomann *et al.*, 1997). Thus, Cd in the blood plasma binds to transport proteins and circulates throughout the body, exchanging with 'aqueous phase Cd' in the different tissues: e.g. gills, kidney, liver, other storage compartments (Thomann et al., 1997). This creates an equilibrium between the aqueous phase Cd and Cd bound to the tissue in each tissue fraction (Thomann et al., 1997). For a schematic of this relationship adapted from Thomann *et al.* (1997), see Figure 1.1. However, this model is not perfect and the authors themselves, found that the kidney did not reach a steady state (Thomann et al., 1997) suggesting that some compartments are able to continue accumulating. Chowdhury and Wood (2007) were able to add to this model (by proposing that the kidney has a role in excretion), indicating that trout exposed to dietary

Cd excrete Cd in their urine (via the kidney), although on a mass-balance basis this excretion was negligible.

It is well known that the dissolved Cd concentrations, and not total metal concentrations, that are the predictors of waterborne Cd toxicity to fish. By way of explanation, Cd in the form of the free aqueous ion Cd^{2+} is thought to enter the fish via Ca^{2+} channels at the apical membrane of the gills (Verbost *et al.*, 1989; Szebedinsky *et al.*, 2001). This is based on evidence that Cd^{2+} (indirectly) blocks apical active uptake Ca^{2+} channels, thereby inhibiting transepithelial Ca^{2+} influx at the gill (Verbost *et al.*, 1989). Thus, hard water, since it has higher Ca levels, is more protective against Cd uptake and toxicity than soft water.

When Cd is taken up through the diet, the largest portion of Cd is generally found in the gut tissues (Farag *et al.*, 1994; Kraal *et al.*, 1995; Szebedinsky *et al.*, 2001; Franklin *et al.*, 2005). In more detail, a study with 30-day acclimated, and nonacclimated rainbow trout in moderately-hard water found that when radiolabeled Cd was infused into the stomach (via catheter) and followed for 24h, the result was that the majority of accumulated Cd remained in the gut or gut-lumen for both fish treatments, and that only a very small percentage was internalized past the gut wall (Chowdhury *et al.*, 2004). This indicates that the gut accumulates Cd in its tissues, acting as a barrier to the transfer of Cd from the lumen to blood, and that in this way it contributes to the protection of the fish from dietary Cd (Chowdhury *et al.*, 2004).

Another interesting finding is that increased Ca levels in the diet can decrease Cd taken up via the gills, as well as decrease dietary Cd uptake (as summarized above)

(Baldisserotto *et al.*, 2005). Others have noted that part of this protective effect is likely due to Ca blocking the upregulation of Ca transport, and therefore by ionic mimicry blocking the upregulation of Cd transport, that would otherwise occur if Ca levels were not increased (Baldisserotto *et al.*, 2006; Wood *et al.*, 2006). It has also been noted that in trout, a high Ca^{2+} diet protects against Cd uptake in the stomach (and not in the intestinal segments) (Wood *et al.*, 2006). By way of explanation, uptake of Ca appears to occur largely in the stomach and not in the intestine; the highly acidic stomach chyme has 5-10 fold more dissolved Ca^{2+} than the intestine, and similarly more Cd^{2+} , than the circumneutral to basic intestine (reviewed by Wood *et al.*, 2006). Overall, Cd and Ca have an interactive relationship, sharing many uptake pathways.

However, there are other possible mechanisms of Cd uptake through the gut. Due to its divalent nature, Cd may be able to enter through pathways used for essential metals, such as Cu, Fe, Ni, and Zn. For example, it may be able to enter through the divalent metal transporter1, DMT1 (a transporter for Zn, Fe, Ni uptake), through the high affinity copper transporter 1. CTR1, or through the zinc transporters, ZIP and ZnT. Burke and Handy (2005) note that uptake of a divalent metal, Cu, in intestinal cells of rainbow trout exhibit CTR1-like a tributes: Na-independence, and stimulation by low pH. More support for the ability of divalent metals to use an intestinal transporter in rainbow trout is given by Nadella *et al.* (2006). These authors report that Cu uptake in both the midand posterior intestine appeared to be biphasic, with a saturable component at low concentrations indicative of a carrier-mediated process, together with some pharmacological properties typical of CTR1 or DMT1 transporters (Nadella *et al.*, 2006).

Both ZIP and ZnT zinc transporters have had their tissue specific expressions mapped in zebrafish (Feeney *et al.*, 2005), and in plants, they have been found to transport other metals including Cd, Fe, Mn, Zn (reviewed by Guerinot, 2000). However, as of yet, studies involving other metals potentially using these sites in fish are lacking.

A separate study on the effects of low Fe food and subsequent Cd accumulation in zebrafish also provides support for DMT1 as a transporter of non-essential divalent metals (Cooper *et al.*, 2006). Results indicated that fish fed the low Fe diets accumulated much higher levels cf Cd in the liver from the gastrointestinal tract than fish fed normal diets. When the authors subsequently measured DMT1 and ferroportin (an iron exporter) transcript levels in the gut and gills, they found that DMT1 was elevated in both the gill and gut, and ferroportin was elevated in the gut when fish were fed the low Fe diet. The authors suggest that these two findings combined (i.e. increased Cd accumulation, and increased DMT1 expression) when fish are fed low Fe diets, provides evidence that Cd may enter via water-borne (gills) or dietary (gut) routes through DMT1 transporters (Cooper *et al.*, 2006).

Subcellular Storage

Background and Methodology

The subcellular compartmentalization technique has been widely used in the last few years in order to explain storage of metals (Wallace and Lopez, 1997; Sokolova *et al.*, 2005) in animals and transfer of metals up the food chain (Wallace and Lopez, 1996; Cheung and Wang, 2005; Rainbow *et al.*, 2006; Steen Redeker *et al.*, 2007). One of the original techniques separated tissues into three parts: 1) tissue fragments, 2) intracellular

organelles (ORG) (e.g. nuclear, mitochondrial, and microsomal fractions), and 3) cytoplasm and proteins (Wallace and Lopez, 1996). Cheung and Wang (2005) expanded on this method in a number of manners. Firstly, they further separated the 1st component (tissue fragments) into metal rich granules (MRG) and cellular debris (CD). Secondly, they further divided the 3rd component (cytoplasm and protein) into metallothionein-like proteins (heat-stable proteins, MTLP) and the heat -sensitive proteins (Cheung and Wang, 2005). Throughout this thesis, the heat-sensitive proteins will be referred to as other enzymes (ENZ). Yet another refinement on this method was made by Bonneris et al. (2005) in the further subdivision of organelles (ORG) into mitochondria (Mi) and lysosomes and microsomes (L+M). It is possible to harvest these fractions by using different centrifugation speeds and different temperature treatments. Once divided, these six fractions can each be analyzed separately for Cd concentration. Of these six fractions, ENZ, MTLP, Mi and L+M (ORG) have been defined as the trophically available metal (TAM) while the CD and MRG fractions are considered trophically unavailable metal since they do not seem to transfer as readily (Wallace and Luoma, 2003). The soluble fractions consist of the ENZ and MTLP, which together were originally considered to comprise the cytoplasmic portion, and the insoluble fractions consist of the remainder -MRG, CD and the ORG fractions (Cheung and Wang, 2005). Furthermore, these fractions can be defined as a metabolically available component (MA) containing ORG, ENZ and CD (Steen Redeker et al., 2007), or as biologically detoxified metal (BDM) containing MRG and MTLP fractions, as well as metal-sensitive fractions (MSF) containing ORG and ENZ (Wallace et al., 2003). Given this complex array of techniques

and definitions available, the present investigation employs the method for subcellular compartmentalization given by Wallace *et al.* (2003) which separates homogenized tissue into 5 fractions: MRG, ORG, ENZ, CD, and MTLP. A schematic of this method, adapted from Wallace *et al.* (2003) is given in Figure 1.2. This method was chosen since the work of a colleague (T. Ng, personal communication) has shown that with the organisms under study, there is considerable cross-contamination between the mitochondria (Mi) and lysosome and microsome (L+M) fractions when separated. Thus the Mi and L+M fractions have not been separated out, and have been reported as one fraction: ENZ.

Subcellular Metal Storage

In polychaetes, which are deposit-feeders like chironomids, the majority of Cd was found in the debris fraction (includes both MRG and CD) irrespective of a dietary or water-borne exposure route, although some changes were noted among the other fractions (Selck and Forbes, 2004). In particular, that there was 'relatively more' Cd in the debris fraction, and less in all others when exposed to water-borne Cd versus dietary Cd (in algae) (Selck and Forbes, 2004). Wallace and Lopez (1996) further found that both Cd concentration and duration of Cd exposure had direct effects on oligochaete subcellular distribution, noting that with either increased concentration or duration, more Cd was taken up in the cytosolic fraction (including both ENZ and MTLP). This could be explained by the production of MTLP in order to bind Cd (Wallace and Lopez, 1996).

Thus, subcellular storage of Cd may be affected by route of prey exposure (i.e. waterborne, sediment-borne/ dietary).

In general, it is agreed that where metal is stored subcellularly within an organism is an important factor in determining how well it will be accumulated into a predator. Specifically, Cd, in the cytosolic proteins (ENZ fraction and MTLP fraction) of aquatic invertebrates are considered available (Wallace and Lopez, 1997; Rainbow *et al.*, 2006). However, on a physiological basis, Cd in the MTLP fraction is considered to be 'detoxified' because when it binds with metal, it is unable to bind with other proteins or enzymes (reviewed by Roesijadi, 1992). In contrast, Cd in the ENZ fraction is presumed to be toxic to the organism since it would prevent proteins from functioning in their normal metabolic capacity (reviewed by Rainbow, 2002). MTLP's are well known for binding metals, transporting metals, and playing a key role in the detoxification of metals (Olsson and Haux, 1986; Roesijadi, 1992). MRG's have been proposed to then play a role in the storage or metals (e.g. in a detoxified form) such that MTLP's and MRG's likely interact (George, 1982).

Trophically Available Metal (TAM) has been suggested to explain the transfer of metal from a prey species to a predator (Wallace and Luoma, 2003). However, Cheung and Wang (2005) found no direct link with TAM for the transfer of Cd from different prey to a predatory gastropod. More recently, Steen Redeker *et al.* (2007), and Ng and Wood (in submission) found that there was no direct link between TAM (Cd) and trophic transfer in an oligochaete prey consumed by either carp or trout respectively. In general, it seems that TAM is sometimes a useful tool for explaining metal transfer, yet should be

used with caution, as a direct link between TAM and metal transfer is not always seen. Biologically detoxified metals (MTLP+MRG), or the mutually exclusive metabolically available (MA) (ORG+ENZ+CD) fractions have also been used to try and explain trophic transfer efficiencies and metal storage (Wallace *et al.*, 2003; Steen Redeker *et al.*, 2007). In these cases, it seems that the MA fractions have been useful in explaining some aspects of toxicity (i e. there was a strong correlation between MA and metal transferred). With this background in mind, the current study addresses both TAM and MA components, as well as the 5 individual fractions.

Project Objectives

In general, this thesis was concerned with metal toxicity in chironomids, and the trophic transfer of C1 from chironomids to zebrafish. Hard water is known to be protective of Cd toxicity, likely due to the higher presence of Ca (Davies *et al.*, 1993), thus in these studies, low ionic water or 'soft water' is used to be representative of the sensitive waters found in the Canadian shield lakes which are current areas of anthropogenic metal contamination, and therefore also of research on this topic (Kalin *et al.*, 2006; Wyn *et al.*, 2007).

Previous studies have demonstrated that *C. riparius* are most sensitive to metal toxicity in the first instar life-stage (Gauss *et al.* 1985, Nebeker *et al.* 1984, Pascoe *et al.* 1989, Williams *et al.* 1986). Thus, the first goal of this thesis was to quantify the acute toxicity of the metals Cd, Cu, Pb, Ni, and Zn individually to this most sensitive life stage (first instar chironomids) in soft water, and to determine the environmental relevance of

results (i.e. in comparison to water quality guidelines). We found that for all metals tested, lethal concentrations at which 50% of the population died (LC50), were all orders of magnitude above the Water Quality Criteria put forth by both the CCME (2006) and USEPA (1996). Thus, *C. riparius* larvae are extremely tolerant to water-borne metal exposure even at its most sensitive life stage in soft water, and are well protected by current environmental guidelines. Because chironomids are so resistant to metal toxicity (Wentsel *et al.*, 1977; Winner *et al.*, 1980), and because they are able to accumulate large amounts of metal (Yamamura *et al.*, 1983; Seidman *et al.*, 1986; Hare *et al.*, 2001; Gillis and Wood, 2007), and further, since they are a link from benthic to pelagic food webs, they were an ideal organism for studying trophic transfer.

The second goal of this thesis was to determine if the Cd exposure route of chironomids, whether sediment exposed or water-borne exposed, affected: a) subcellular storage of Cd in the chironomids, b) the trophic transfer efficiency (TTE) of Cd to the zebrafish predator, c) the tissue distribution of Cd in zebrafish, and d) the subcellular compartmentalization of Cd in whole zebrafish. As well, we addressed the issue of whether Cd added directly as a Cd(NO3)₂ "spike" to a manufactured pellet diet exhibited similar uptake patterns and TTE's as did natural diets (chironomids) when fed to zebrafish.

Our specific hypotheses were as follows. Firstly, we predicted that TTE would not be affected by route of metal exposure (dietary or waterborne) in the prey (chironomids). This is based on research by Liu *et al.* (2002) who found no difference in the TTE of Cd between water-borne exposed *Daphnia magna* fed to zebrafish and dietary exposed

Daphnia magna fed to zebrafish. Secondly, we predicted higher Cd TTE's for zebrafish fed Cd-contaminatec chironomids, than zebrafish fed Cd-spiked manufactured pellet food, based on evidence from Harrison and Curtis (1992) that the TTE's of metal absorption by a predator from manufactured pellet food may be lower than TTE's from a natural food source. Furthermore, we hypothesized that Cd TTE would not be concentration-dependent, as other studies have found that TTE of Cd is independent of Cd concentration in natural prey (Van Campenhout *et al.*, 2007; Ng and Wood, in submission).

We also had additional hypotheses relating to the handling of Cd within the zebrafish predator. We anticipated that the highest accumulation of Cd in dietary exposures would be in the gut with considerable amounts in the kidney and liver as well, since in dietary exposures of Cd to other fish species this has been the commonly observed pattern (Kraal *et al.*, 1995; Szebedinszky *et al.*, 2001; Baldissirotto *et al.*, 2005; Chowdhury *et al.*, 2005; Franklin *et al.*, 2005; Ng and Wood, in submission). Furthermore, the gut has been identified as on organ which is able to both accumulate large amounts of Cd, as well as prevent the internalization of Cd into other organs (Chowdhury *et al.*, 2004; Wood *et al.*, 2006). Similarly, based on previous research (Szebedinszky *et al.*, 2001; Franklin *et al.*, 2005; Ng and Wood, in submission), we predicted that the gut should also account for the greatest proportion of overall body burden, followed by the carcass.

Our goals with respect to subcellular compartmentalization of Cd in both chironomids and zeb: afish were simply to characterize the patterns which occurred in

various exposure and dietary scenarios. It was harder to put forth hypotheses for the subcellular compartmentalization of Cd, since this has been found to vary across organisms and is often dependent upon available metal fractions (Reinfelder and Fisher, 1994; Wallace and Lopez, 1996; Wallace *et al.*, 2003; Cheung and Wang, 2005, Zhang and Wang, 2006). However, in a polychaete, most of the Cd accumulation was found in 'debris' (MRG+CD) fractions irrespective of water-borne or dietary exposure (Selck and Forbes, 2004), so a likely outcome was thought to be that a high proportion of Cd would be found in the MRG + CD fractions in the chironomids for both water-borne and sediment/dietary exposures. Regardless of the outcome, the results of these fractionation analyses were considered useful in interpreting TTE data.

Some of our hypotheses were confirmed, while others were disproven. The General Discussion brings these findings into perspective.

Table 1.1 The CCME (2006) Water Quality Criteria for the Protection of Aquatic Life and the U.S. EPA (1996) Criterion Maximum Concentrations (CMC) (1996) for Cd, Cu, Pb, Ni, and Zn adjusted for water hardness (20 mg/L CaCO₃ equivalents) and expressed as dissolved metal in the water column.

Metal	CCME (µg/L)	USEPA CMC (µg/L)
Cadmium (Cd)	0.017	0.4
Copper (Cu)	2	2.9
Lead (Pb)	1	10.8
Nickel (Ni)	25	120.0
Zinc (Zn)	30	30
Figure 1.1 A schematic of the route of Cd uptake and distribution in fish. Cd entry routes are represented with bold text and arrows, and the boxed 'S' within organs represents that storage of the metal can occur in each of these areas. Expanded from Thomann *et al.* (1997).



Figure 1.2 A schematic of the subcellular fractionation process followed in order to measure the metal concentration in 5 subcellular compartments: Metal Rich Granules (MRG), Cellular Debris (CD), Organelles (ORG), Enzymes (ENZ), and Metallothionein-like proteins (MTLP). Adapted from Wallace *et al.* (2003).



CHAPTER 2

Acute Toxicity of Waterborne Cd, Cu, Pb, Ni, and Zn to First Instar *Chironomus riparius* Larvae

K.M. Béchard, P.L. Gillis, and C.M. Wood

Abstract

The acute toxicities of waterborne Cd, Cu, Pb, Ni, and Zn were determined in the first instar larvae (generally considered to be the most sensitive) of *Chironomus riparius*, under standardized conditions. Toxicity tests were conducted in soft water (hardness 8 mg/L as CaCO₃ equivalents) in the absence of food, and were limited to 24 h to avoid control mortality associated with food deprivation. For each metal, a logarithmic range of concentrations was tested between 0 to 25 mg/L. First instar *C. riparius* are most sensitive to Pb with a 24 h LC50 of 0.61 mg/L (CI of 0.26-1.15 mg/L) and to Cu with a 24 h LC50 of 2.09 mg/L (CI of 1.57-2.96 mg/L). The LC50 for Cd was 9.38 mg/L, while the LC50s for Zn, and Ni were both greater than the highest tested concentration of 25 mg metal/L. Our results found that even first instar chironomid larvae are well protected by both the current CCME Canadian water quality guidelines for the protection of aquatic life, and the USEPA Water Quality Criteria, as LC50s ranged from 25 to >25000 times higher than the guideline concentrations.

Introduction

Assessing the toxicity of metals on aquatic life has been a long-standing practice. Metals such as cadmium (Cd), copper (Cu), lead (Pb), nickel (Ni), and zinc (Zn) have all been found in waterways, particularly from sources such as: municipal waste water effluents, industrial point source effluents (eg. mining spoil runoff), urban runoff, landfill leachates, natural sources and soil/substrate disturbances (Environment Canada 2001). Toxicity tests serve to quantify how poisonous or lethal various metals are to aquatic life. Both acute and chronic studies are necessary; however, acute exposure data are useful because they help explain toxic effects (Watts and Pascoe 2000). Many benthic freshwater invertebrates have been used for these toxicity tests, among them amphipods, oligochaetes, and chronomid larvae (USEPA 1994).

In general, chironomids appear to be exceptionally tolerant to metals. Indeed, it has been shown that chironomids are able to both acclimate (Miller and Hendricks 1996) and adapt (Groenenclijk *et al.* 2002) to metals in their environment. In this way, they have proven themselves to be a) gradually adapting to metal exposure (Groenendijk *et al.* 2002) b) efficient at storing metal, for example in the form of metallothionein-like proteins (Gillis *et al.* 2002) and c) efficient at excreting (Postma *et al.* 1996) or shedding (Groenendijk *et al.* 1999) metal.

It is generally agreed that of the four larval instars of chironomids, the first and youngest instar is the most sensitive to waterborne toxicants (Gauss *et al.* 1985, Nebeker *et al.* 1984, Pascoe *et al.* 1989, Williams *et al.* 1986). Therefore the first instar should be the most appropriate life-stage for toxicity tests aimed at environmental protection.

However, Larrain *et al.* (1997) argue that an older instar may be viewed as more appropriate when background mortality is taken into consideration. Most likely this is because the ability of first instars to survive without food for extended periods of time is relatively limited. Food deprivation is a necessity in acute waterborne tests with metals, because the presence of organic matter, such as food, will complex the metal and reduce its toxicity (De Schamphelaere *et al.* 2004).

Although many studies have examined the toxicity of metals to chironomids (Milani *et al.* 2003, Fhipps *et al.* 1995, Rao and Saxena 1981) a direct comparison of the toxicity of a suite of metals to the most sensitive life-stage of a single chironomid species is lacking. The goal of our study was to compare the acute waterborne toxicity of Cd, Cu, Pb, Ni, and Zn to first instar *C. riparius* under standardized conditions. The exposures were conducted in a simplified laboratory soft water to minimize the protective effects of water chemistry. To avoid the probable background mortality problem caused by fooddeprivation, we limited our tests to 24 h.

Materials and Methods

The *C. riparius* culture was initiated with egg masses acquired from Environment Canada (Burlington, ON., Canada). The cultures (and exposures) were held under a 16h:8h light:dark cycle. *C. riparius* egg masses were collected from culture tanks and placed in glass Petri dishes containing aerated de-chlorinated moderately-hard Hamilton tap water from Lake Ontario held at a temperature of $20\pm1^{\circ}$ C, hereafter referred to as culture water. The ionic composition of the Hamilton city tap water in mM was $[Na^+] =$

0.6, $[CI^{-}] = 0.8$, $[Ca^{2+}] = 1.8$, $[K^{+}] = 0.4$, $[Mg^{2+}]$, pH 8.05, 140 mg/L hardness as CaCO₃. In order to ensure that only newly hatched, first instar larvae were used in the toxicity tests, 48 h after collection egg ropes were transferred to a fresh Petri dish containing culture water. Toxicity tests were conducted in synthetic soft water (52.9 μ M/L Ca⁺⁺, 22.9 μ M/L Mg⁺⁺, 112.6 μ M/L Na⁺, 6.3 μ M/L K⁺, pH 6.52, 8 mg/L hardness as CaCO₃). Individual metal stock solutions were made from metal salts, Cd from Cd(NO₃)₂*4H₂0 (Fisher Scientific), Cu from CuSO₄*5H₂0 (BDH Inc), Ni from NiSO₄*6H₂0 (Sigma Aldrich), Pb from Pb(NO₃)₂ (Sigma Aldrich), and Zn from ZnCl₂ (Sigma Aldrich). All chemicals used in this study were analytical grade. The test solutions ranged logarithmically from 0-25mg metal/L of soft water.

Two hours after the final egg mass transfer (i.e. larvae were < 2 h old), ten first instar chironomids were added, under a dissecting microscope to each well of a multiwell plate containing 2 ml of exposure solution. Staggered start times by concentration were employed in order to keep 24 h exposure consistent across concentrations. Each exposure concentration had 2-3 replicates (each of 10 larvae) dependent upon the number of newly hatched first instar larvae available. After 24 h, percent survival was determined in each replicate well. Preliminary experiments found that if exposures were extended beyond 24 h significant control mortality would result, likely due to lack of food.

The larvae were assumed to be dead if they did not move in reaction to flickering light and gentle prodding. Experiments were considered valid only if control survival met a minimum of 80%. Repeat experiments were done in the same way for all metals

tested: Cd, Cu, Pb, Ni, and Zn. 1.5 ml water samples were taken from each well at the end of each experiment, and were filtered (through an AcrodiskTM 0.45 μm in-linesyringe-tip filter) and analyzed for metal concentration using a GTA 110 Varian Graphite Furnace Atomic Absorption Spectrometer (AA) (Varian Techtron, Mulgrave, Victoria, Australia). Measured water metal concentrations were all within +/- 20% of nominal values, and the recovery of each metal was +/- 15% as determined from the Analytical Reference Material TM15 (Environment Canada, National Water Research Institute). Subsequent Probit analysis yielded 24 h median lethal concentrations with 95% confidence intervals (CIs) where possible, for each metal. Probits were determined using the Environmental Protection Agency (EPA) Probit Analysis Program Version 1.5 (1992).

Results

Percent survival data of first instar *C. riparius* after 24h in exposure solutions of Cd, Cu, Pb, Ni, and Zn at each concentration are given in Figure 2.1 (a-e). Estimated LC50 values calculated for these data are also presented in Table 2.1. Note that 95% CIs could not be estimated for the Cd LC50 due to heterogeneity of the data. LC50s for Zn and Ni were greater than the highest concentration tested of 25mg metal/L.

The LC50 for first instar LC50s are compared to the CCME (2006) and USEPA (1996) Criterion Maximum Concentrations (CMCs) in Table 2.1. Note that, although the USEPA CMC values in Table 2.1 are calculated for a hardness of 20 mg/L CaCO₃

equivalents (the lowest recommended extrapolation), this hardness level (20mg/L) is still higher than the laboratory water used in this experiment (hardness approximately 8mg/L).

Discussion

The present LC50 data for first instar *C. riparius* have been compared with relevant literature data in Table 2.2. It should be noted that variability seen in the data could have come from a number of sources, including individual variation of chironomid larvae and the slight, but variable dilution of the exposure solutions when chironomid larvae were added to test vessels.

Cadmium

Our 24 h first instar LC50 of 9.38mg Cd/L, is comparable to the 24 h LC50 of 2.1 mg Cd/L for the same instar found by Williams *et al.* (1986), but is much higher than the 0.021 mg/L that Milani *et al.* (2003) reported for a first instar, 96 h exposure in hard water. Differences in LC50 values are not only observed with first instar chironomids, but are also seen with older instars and may be related to differences in test conditions. Fed third and fourth instar *C. riparius* 48h LC50s have been noted at 72 and 725 mg/L Cd respectively (Williams *et al.* 1986), while Rao and Saxena (1981) reported a 48h LC50 of 50 mg/L using third to fourth instar *C. tendipes* larvae, both in hard water.

Copper

We obtained a 24 h first instar LC50 of 2.09 mg Cu/L for *C. riparius*. In contrast, acute toxicity data for *C. tentans* in a soft water (43 mg/L CaCO₃ equivalents) indicate that unfed first instars are sensitive to copper with a 96 h EC50 of 16.7 μ g/L Cu (Gauss *et al.* 1985). First instar *C. riparius* are similarly sensitive to Cu with a 96 h LC50 of 43 μ g/L in hard water (Milani *et al.* 2003). Likewise, Nebeker *et al.* (1984) found that first instar *C. tentans* are sensitive to copper with a 96 h LC50 of 298 μ g/L. Although data from some studies (Gauss *et al.* 1985, Milani *et al.* 2003, Nebeker *et al.* 1984) place chironomids in the μ g/L range for sensitivity, others indicate somewhat greater tolerance including Taylor *et al.* (1991) who found a 48h LC50 of 1.2mg Cu/L for second instar *C. riparius* and the present study with a first instar *C. riparius* 24h LC50 for Cu in the mg/L range (2.09 mg/L). These differences in chironomid sensitivity may be explained by the duration of the exposure, since shorter 'acute' exposures are associated with higher LC50s while longer, more 'chronic' exposures are associated with lower LC50s (Watts and Pascoe 2000, Williams *et al.* 1986).

Lead

Of the five metals tested in this study, Pb was the most toxic to first instar *C. riparius*. The 24h LC50 value for first instar *C. riparius* was 0.61 mg/L Pb. Other values in the literature are substantially higher. Rao and Saxena (1981) found a 48h LC50 of 50 mg/L using third to fourth instar *C. tendipes* larvae in hard water, while Qureshi *et al.* (1980) measured a 24 h LC50 of 350 mg/L, and a 48 h LC50 of 220 mg/L with fourth instar chironomid larvae (species unidentified) in hard water. Similar sensitivity

differences have been noted when comparing literature LC50s for *C. riparius* with other metals (Milani *et al.* 2003).

Nickel

The 24h Ni LC50 for first instar *C. riparius* obtained in this study (>25mg/L) in soft water is comparable to a 48h LC50 of 79.5 mg/L obtained with first instar *C. riparius* in hard water (Powlesland and George 1986). In contrast, Milani *et al.* (2003) reported a first instar fed, 96 h LC50 of 5.25 mg/L in hard water. This difference could be due to length of exposure (Milani *et al.* used 96 h), as LC50s usually decrease with increased duration of exposure (Watts and Pascoe 2000, Williams *et al.* 1986).

Zinc

The present study found that the 24 h LC50 value for Zn in first instar *C. riparius* was greater than the highest concentration tested (25 mg/L) in soft water. In comparison, Rao and Saxena (1981) found an acute 48 h LC50 of 62.5 mg/L with third to fourth instar *C. tendipes* larvae in hard water. Chronic toxicity data for *C. tentans* indicated a 10-day Zn LC50 of 1.125 mg/L when chironomids were fed, and given a monolayer of sand substrate in natural soft Lake Superior water (Phipps *et al.* 1995).

Metals in the Environment

In this study we analyzed the toxicity of Cd, Cu, Pb, Ni, and Zn separately. However, in the natural environment, aquatic organisms are often exposed to multiple metals simultaneously. Therefore under natural conditions, there is the potential that these metals may act in an additive or even synergistic manner, although the sensitivity of chironomid larvae to multiple metals cannot be predicted by the individual metal sensitivities generated in this study.

Summary and Conclusions

Of the metals tested, first instar *C. riparius* larvae were the most sensitive to Pb, followed by Cu, Cd, then Ni, and Zn. The relatively high tolerance of chironomids to Ni and Zn is not surprising given that Zn and Ni are essential elements (Gough 1993) which likely can be regulated. A surprising finding is that Cu, which is essential, was more toxic to *C. riparius* than Cd, which is not essential. Generally, Cd is more toxic to organisms than Cu. Recent studies by Gillis and Wood (2007) suggest that Cd tolerance in late instar *C. riparius* is at least in part due to their ability to maintain internal calcium balance even with continued Cd exposure. Based on the CCME and the USEPA CMCs (Table I), we would expect Cd, followed by Cu, then Pb to be the most toxic; however, in this study, Pb was found to be the most toxic followed by Cu then Cd. In general, this study has found *C. riparius* to be more metal tolerant than indicated by other studies, although there is significant variation in the LC50s within the published literature.

It should be noted that due to the rapid transfer of chironomid larvae from hard to soft water, LC50s may be under-estimates. This is because the permeability of ions (particularly Ca⁺⁺) decreases at the on-set of soft water acclimation i.e. to prevent ion loss in low-ionic water (McDonald and Rogano 1986). There is also reason to believe that

LC50s could be exaggerated. This is because soft water tolerance is associated with higher uptake capacity and affinities for ions (at least in fish) (Boisen *et al.* 2003). This may increase the apical entry of metals through ion channels, thereby elevating the toxicity of the metal to the chironomid larvae. Also, the stress resulting from rapid transfer from hard to soft water in conjunction with the stress of metal exposure may have a synergistic effect, which would result in lower than actual LC50s. We surmise that each of these effects due to rapid transfer from hard to soft water may be occurring to a small extent, but can not have affected the main results of our study.

The LC50 values for each metal are all orders of magnitude above the USEPA water quality criteria for the same metals adjusted for water hardness (20 mg/L CaCO₃ equivalents, Table I). Thus, chironomids are well protected by the current USEPA water quality guidelines, and even better protected by the current CCME guidelines which are based on chronic exposures (and thus are even more protective) than if they were based on acute exposures (as in this study).

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Producers Environmental Research Association, the International Copper Association, the Copper Development Association, Teck-Cominco, Noranda-Falconbridge, and Inco. CMW is supported by the Canada Research Chair Program. **Table 2.1** The Criterion Maximum Concentrations (CMC) of Cd, Cu, Pb, Ni, and Zn adjusted for water hardness (20 mg/L CaCO₃ equivalents) expressed as dissolved metal in the water column, as determined by the CCME (2006) and the USEPA (1996) compared to 1st instar *C. riparius* LC50s with 95% CIs determined in the present study at a water hardness of 8 mg/L CaCO₃ equivalents.

Metal	CCME (µg/L)	CMC USEPA (µg/L)	1 st instar LC50's (μg/L) (Upper-Lower 95% CI)	
Cadmium (Cd)	0.017	0.4	9380	
Copper (Cu)	2	2.9	2090 (1570-2960)	
Lead (Pb)	1	10.8	610 (260-1150)	
Nickel (Ni)	25	120.0	>25000	
Zinc (Zn)	30	30	>25000	

Table 2.2 A comparison of LC50 values for chironomids exposed to the metals; Cd, Cu, Pb, Ni, and Zn under various conditions.

Metal	Specie	Instar	Duration (h)	LC50 (mg/L)	Substrate for building tubes	Fed	Water Hardness	Study (Year)
							(mg/L)	
Cd	riparius	1 st	24	2.1	food	Y	100-110	Williams et al. (1986)
	riparius	1st	96	0.021	silica sand	Y	120-140	Milani et al. (2003)
	riparius	lst	24	9.381	N	N	8	present study
	riparius	2nd	48	2.620	filter paper	Y	114	Watts and Pascoe (2000)
	tendipes	3rd & 4th	48	25	-	-	-	Kao and Saxena (1981)
	riparius	3rd	24	500	food	Y	100-110	Williams et al. (1986)
Cu	tentans	lst	96	0.0167*	N	N	43	Gauss et al. (1985)
	tentans	lst	96	0.298	food	Y	71-84	Nebeker et al. (1984)
	riparius	1st	96	0.043	silica sand	Y	120-140	Milani et al. (2003)
	riparius	1 st	24	2.093	N	N	8	present study
	riparius	2nd	48	1.2	cellulose mulch	Y	142-160	Taylor et al. (1991)
Pb	riparius	1 st	24	0.613	N	N	8	present study
	Chironomid sp.	-	24	350	-	-	224	Qureshi et al. (1980)
	Chironomid sp.	-	48	220	-	-	224	
	tendipes	3rd & 4th	48	50.0	-	-	-	Rao and Saxena (1981)
Ni	riparius	1st	48	79.5	N	N	"hard water"	Powlesland and George (1986)
	riparius	1st	96	5.25	silica sand	Y	120-140	Milani et al. (2003)
	riparius	1st	24	>25	N	N	8	present study
Zn	riparius	1st	24	>>25	N	N	8	present study
	tendipes	3rd & 4th	48	62.5	-	-		Rao and Saxena (1981)
	tentans	-	240	1.125	sand	Y	"hard water"	Phipps et al. (1995)

Figure 2.1 Percent survival (24 h) of first instar *C. riparius* larvae at various dissolved water concentrations of **a**) Cd, **b**) Cu, **c**) Pb, **d**) Ni, and **e**) Zn: Note logarithmic scale for water metal concentrations. The dashed lines indicate the LC50 values. For Cd, Cu n=5 replicates within 2 experiments, for Pb, Ni, n=6 replicates within 2 experiments, and for Zn n=8 replicates within 3 experiments (each with 10 larvae per replicate). A different symbol is used in each experiment. Error bars represent standard error.



CHAPTER 3

Trophic transfer of Cd from chironomids (*Chironomus riparius*) exposed via sediment or water-borne routes, to zebrafish (*Danio rerio*): tissue-specific and subcellular comparisons

K.M. Béchard, P.L. Gillis, and C.M. Wood

Abstract

Zebrafish were fed chironomids loaded with Cd either via a water-borne exposure or sediment exposure for 7 days, followed by 3 days of gut clearance in a static-renewal system. Regardless of exposure route or concentration, the chironomids had similar subcellular distributions of Cd, with the largest areas of storage being: metal rich granules (MRG) > organelles (OFG) > enzymes (ENZ), except that sediment chironomids hadsignificantly more Cd in the metallothionein-like protein (MTLP) fraction, and significantly less Cd in the cellular debris (CD) fraction than water-borne exposed chironomids. When zebrafish fed on sediment-exposed chironomids $(153\pm11 \mu g Cd/g$ dry weight) were compared directly to zebrafish fed on water-borne exposed chironomids (288±12 µg Cd/g dry weight), identical whole-body Cd levels were observed, even though the concentration in the food source was approximately double in the water-borne exposed chironomids. Thus, the trophic transfer efficiency (TTE) was significantly greater for zebrafish fed sediment-exposed chironomids $(2.0\pm0.5\%)$ than for zebrafish fed water-borne exposed chironomids $(0.7\pm0.2\%)$. Subsequent tests with water-borne exposed chironomids loaded to comparable Cd concentrations, as well as with Cd-spiked manufactured pellets, demonstrated that TTE's were concentration-independent. In all treatments, zebrafish exhibited similar subcellular storage of Cd, with the greatest uptake

occurring in the ORG fraction followed by the ENZ fraction. Tissue Cd concentrations were highest in the kidney and gut tissue, then liver, but lower in the gill, and carcass. Overall, the gut and carcass contributed \geq 71% to overall body burdens on a massweighted basis. This study indicates that the exposure route of the prey influences the TTE, and reinforces the idea that Cd may be acquired by fish from natural diets at levels of environmental relevance (for polluted sites).

Introduction

Cd is a ubiquitous non-essential metal that comes from many sources, including anthropogenic: municipal waste water effluents, industrial point source effluents (eg. mining spoil runoff), urban runoff, landfill leachates, forestry, fuel combustion, as well as natural sources and soil/substrate disturbances (Environment Canada, 2001). However, since it has no biological functions in chironomids or zebrafish, it needs to be either detoxified or excreted (Rainbow, 2002). Depending on how these mechanisms interact, there may be a potential for Cd to bioaccumulate up trophic levels. Indeed, although metals are not normally considered to biomagnify since most of them are nonlipophilic (Timmermans *et al.*, 1989), Croteau *et al.* (2005) found that within two trophic levels (from primary producers to invertebrates and fish), cadmium concentrations had biomagnified 15 fold.

The impact of metals on aquatic life has long been an important area of research, and, more recently, the dietary transfer of metal to fish has been recognized as a critical route in need of further investigation from both toxicological and regulatory standpoints (reviewed by Meyer *et cl.*, 2005). Indeed, in some trophic routes with invertebrate predators, dietary exposure has been a more significant route of transfer than water-borne

exposure (Timmermans *et al.*, 1992; Roy and Hare, 1999). A similar finding was established in a demersal fish: dietary Cd exposure was associated with a higher hepatic Cd burden than water-borne Cd exposure (Rouleau *et al.*, 2006). There are also reports that Cd acquired through natural trophic routes affects the growth of fish (Woodward *et al.*, 1995; Farag *et al.*, 1999; Ng and Wood, in submission), while other studies with Cdspiked manufactured foods have shown no such effects (Harrison and Curtis, 1992; Zohouri *et al.*, 2001). Some investigators have suggested that 'natural' food may store metals in a more available form to predators than a manufactured food (Harrison and Curtis, 1992; Meyer et a., 2005; Ng and Wood, in submission). And more recently, the "trophic availability" of the metal, determined by the relative amounts of metal in subcellular components, has been shown to affect the trophic transfer efficiency (TTE) of the metal to the predator (Wallace and Lopez, 1997; Wallace and Luoma, 2003; Vijver *et al.*, 2004; Seebaugh and Wallace, 2004; Seebaugh *et al.*, 2006).

Chironomids are well known as a resistant species to metal toxicity in general (Wentsel *et al.*, 1977; Winner *et al.*, 1980; USEPA, 2000) and are well protected by the current environmental guidelines (Béchard *et al.*, in review). More notably, they have been found to accumulate large amounts of Cd in their tissues (Yamamura *et al.*, 1983; Seidman *et al.*, 1986; Hare *et al.*, 2001; Gillis and Wood, 2007). Since they are also epibenthic feeders, both living in, and feeding from the sediment layer (Pinder, 1986), yet are vulnerable to piscine predators, they are potentially an important link in trophic transfer routes.

In the present study, zebrafish (*Danio rerio*) were chosen as a model predator species for several reasons. Firstly, they readily eat chironomids, and their small size

makes the issue of supplying a sufficient natural food tractable, since there are mass constraints on the rate at which Cd-contaminated natural prey can be produced. Secondly, they thrive in ion-poor softwater (Boisson et al. 2003) representative of metalsensitive waters in North America and Europe (Wright and Henriksen, 1978; Hare and Tessier, 1998). Since hard water is known to be protective against Cd toxicity, likely due to the higher presence of Ca (Davies *et al.*, 1993), these low ionic conditions are expected to reveal more sensitive endpoints than would a similar study in hard water. Thirdly, since the zebrafish genome is now publically available (Lo *et al.*, 2003), zebrafish are beginning to be used in metal toxico-genomic studies (Gonzalez *et al.*, 2006; Chen *et al.*, 2007; Craig *et al.*, in submission). Therefore our research may provide a link to future toxico-genomic work.

Our study focused on the effects that route of prey exposure (water-borne or sediment/dietary), and type of prey exposure (natural food with biologically incorporated Cd, or manufactured food spiked with $Cd(NO_3)_2$, has on TTE. Specifically, we hypothesized that TTE would not be affected by route of metal exposure (dietary or waterborne) in the prey (chironomids) based on evidence that TTE's were not different for *Daphnia magna*, that were either water-borne exposed and dietary exposed, and subsequently fed to zebrafish (Liu *et al.*, 2002). Secondly, we predicted that TTE's would be higher for zebrafish fed with Cd-contaminated chironomids (either water-borne or sediment exposed), than zebrafish fed with a Cd-spiked pellet food. This is based on evidence that TTE's of ¹⁰⁹Cd fed to trout were higher when the trout were fed a natural diet (amphipods) than when they were fed a comparable manufactured diet (Harrison and Curtis (1992). Due to the fact that subcellular compartmentalization of Cd, has been

found to vary across organisms and is often dependent upon available metal fractions (Reinfelder and Fisher, 1994; Wallace and Lopez, 1996; Wallace et al., 2003; Cheung and Wang, 2005, Zhang and Wang, 2006), we did not make detailed predictions on this aspect of the study. However, we did hypothesize that chironomids would have a high proportion of Cd in the MRG and CD fractions since Selck and Forbes (2004) noted that for a similar species, a deposit-feeding ploychaete, accumulation of Cd was mostly in the 'debris' (MRG + CD) fraction regardless of whether they were exposed via a water-borne or dietary exposure. However, we acknowledge that the subcellular distribution of Cd in the prey can be useful for determining the fraction of metal within the organism that could be trophically available to the predator. Thus, we measured the subcellular distribution of Cd in order to test the hypothesis that the trophically available metal fractions (TAM) (Wallace and Luoma, 2003) would be a useful predictor of TTE for the trophic transfer of Cd from chironomids to zebrafish. Subsequent distribution in the predator is also useful, as it can describe the proportion and storage of metal uptake, as well its availability to the next trophic level. This study will provide a novel contribution to the growing trophic transfer database by comparing the subcellular storage of Cd in the prey organism loaded via two different exposure routes (sediment/dietary and waterborne) with the TTE of Cd to the predator zebrafish. It will also compare the compartmentalization and tissue-specific storage of Cd in the zebrafish dependent on these different prey exposure routes. Further, the relative importance of a natural versus a manufactured diet in the transfer of dietary Cd will be directly comparable to the relative importance of subcellular compartmentalization of Cd in the prey.

Materials and Methods

Chironomid Culture

Chironomid culture conditions and methods can be found in Gillis and Wood (2007). In brief, a continuous culture of the non-biting midge *Chironomus riparius* was initiated with egg masses from the National Water Research Institute (Environment Canada), Burlington, ON. Silica sand was used as a substrate and dechlorinated Hamilton city tap water (Lake Ontario – composition in mM: $[Na^+] = 0.6$, $[Cl^-] = 0.8$, $[Ca^{2+}] = 1.8$, $[K^+] = 0.4$, $[Mg^{2+}] = 0.5$, $[Cd] < 5.0 \times 10^{-7}$) as the overlying culture water. Water hardness was approximately 140 mg/L (as CaCO₃ equivalents), pH was 8.05, and dissolved organic carbon was approximately 3.0 mg/L. The cultures were aerated, held at $21\pm2^{\circ}$ C under a 16:8 h light:dark photoperiod regime, and fed crushed NutrafinTM fish flakes *ad libitum*. The larvae reached the 3rd instar approximately two weeks after a culture tank was initiated. Chironomid larvae were harvested using transfer pipettes to holding beakers containing culture water once they had reached the 3rd to 4th instar.

Preparation of Chironomids Exposed to Water-Borne Cd

Fifteen chironomids were transferred to each of 9-15 (dependent upon number of chironomids available at the time) 250 ml beakers containing either 60 (low Cd exposure) or 260 (high Cd exposure) μ g Cd /L as Cd(NO₃)₂ in Hamilton tap water. The addition of chironomids to each beaker (start time) was staggered by 10 minutes to allow enough time for processing at the end of the exposures. After 48 hours, chironomids were transferred into rinse water (culture water) before being blotted dry on filter paper and packaged on parchment paper in groups of approximately 25-30 mg. The packets

represented about 4% (wet weight chironomids) of the body weight of a typical large zebrafish. The same process was repeated for the other beakers. Each packet of chironomids was labeled, weighed, and frozen as quickly as possible. Control chironomids were similarly blotted dry and packaged immediately after being transferred to a holding beaker containing culture water.

Preparation of Sediment

Sediment was collected near Long Point, Lake Erie, ON (42° 33' 54"" N. 80° 02' 028" W). The composition of the sediment was 0.35% organic carbon, 60.9% silt, 33.5% clay, and 5.6% sand. The sediment was artificially contaminated (spiked) with $Cd(NO_3)_2$ in NanopureTM water, to a concentration of 10.89±0.14 mg Cd/kg dry weight sediment, which is a realistic range for a polluted environment (Wallace *et al.*, 1998). After spiking, the sediment was kneaded by hand thoroughly every day for 5 days, after which it was held in the dark at 4°C for 10 months. This sediment can be considered to be 'well aged'. Thus, our 'sediment exposure' more closely represents exposure to naturally contaminated sediment than spiked sediment that hasn't reached equilibrium. The sediment was re-mixed before use in experiments, so that each exposure was equally homogenized. In a 6 L exposure vessel, a ratio of 1 L sediment to 3 L aerated water (dechlorinated Hamilton tap water, composition as above) was used. Sediment depth was approximately 2.1-2.3 cm. The exposure vessel containing both sediment and water sat for three days under the experimental temperature and light conditions before the start of exposure in order for the sediment to completely settle.

Preparation of Chironomids Exposed to Sediment-Borne Cd

Approximately 250 2nd instar larvae from our culture were added to prepared sediment exposure vessels. Chironomids were fed 160 mg of dry ground food (Big Al's Staple Food Specially Formulated for Tropical Fish, Hamilton ON) three times weekly via addition of a food slurry to the overlying water. Overlying water and sediment samples were taken at the beginning and end of each exposure. Dialysis "minipeepers" (Doig and Liber, 2007) were used in the latter 2 exposures to measure the pore water, as well as the water just above the sediment-water interface. These were placed in the sediment on the 12th day of exposure, and were removed at termination on the 15th day, as 72 h is more than sufficient time for peeper equilibrium to occur in natural sediment (Doig and Liber, 2007). At the end of the exposure, 3^{rd} or 4^{th} instar chironomid larvae were rinsed twice with culture water and were subsequently blotted dry and packaged in the same way as for chironomids exposed to water-borne Cd. Sediment was digested using the method described in Borgmann and Norwood (1997) such that for every 1 mg of sediment dry mass, 25 µL of full strength nitric acid (strength of 67-70%) was added and allowed to digest for 1 week at room temperature. Following the acid digestion, the same sample was digested with 20 μ L of peroxide (30%) for 1 day.

Comparing Fresh versus Frozen Chironomids

The effect of freezing on metal content of the larvae was addressed for all treatments of chironomids: control (directly from culture), low Cd water-borne, high Cd water-borne, and sediment-borne. Non-frozen chironomids were rinsed with dechlorinated Hamilton tap water after their respective treatments, after which they were

blotted dry, and placed in the oven for 24 h in pre-weighed tubes. Chironomids were then digested with full strength HNO₃ for 1 week and H_2O_2 for 1 day (see methods below) before being analyzed for Cd content/ dry weight. Frozen chironomids were packaged as described above, and were all frozen for >3 weeks, before being dried in the oven, and then subjected to the same digestion and Cd analysis protocol as fresh chironomids.

Leaching Test

A simple experiment was conducted with chironomids exposed to water-borne Cd in the 4% soft water (tes: water - characteristics given below) to determine if Cd was leaching out of the chironomids into the water during the zebrafish feeding experiments. Test containers were set up mimicking experimental conditions. One packet of control chironomids (before exposure measuring $3.3 \pm 0.1 \ \mu g$ Cd/g dry weight for n=5) was put into each of three test vessels, and one packet of Cd contaminated chironomids measured at $282 \pm 27 \ \mu g$ Cd/g (n=5) was put into each of fourteen test vessels. Cd-contaminated chironomids were removed from two of the vessels at 10 minutes, 30 minutes, 1 hour, 2 hours and 5 hours after being exposed to the water. The control chironomids and remaining Cd-contaminated chironomids were removed after 24 hours of exposure. Water samples were taken from each vessel at time zero, and upon removal of chironomids. Chironomids were digested as outlined above, and both chironomid and water samples were analyzed for Cd concentration.

Production of Pellet Food

Pellets were made by grinding up Big Al's Staple Food (tropical fish flakes) in a blender, adding NanopureTM water or NanopureTM water spiked with Cd(NO₃)₂ and putting the dough through a pasta maker. After strings of dough were dried, they were cut into pellets of approximately 2 mg each, a weight of food that could be easily eaten by the zebrafish. Pellets were weighed out in feeding tubes in aliquots of 5-6 mg each, which was the amount of food fed to fish (twice daily). Pellet foods for three different treatment groups were made: a control treatment of $0.27\pm.02$, a low Cd treatment of 154 \pm 5, and a high Cd treatment of $312 \pm 20 \ \mu g$ Cd/g dry weight of pellet food.

Preparation of Zebrafish

Zebrafish (approximately 0.5 g from PetSmart®, Hamilton ON) were acclimated from moderately hard Hamilton tap water to a softer, ion-poor water containing 4% dechlorinated Hamilton tap water and 96% reverse osmosis water, hereafter referred to as very soft water, over 7 days. This water contained (in μ mol/L): 29.0 Ca⁺⁺, 13.1 Mg⁺⁺, 45.2 Na⁺, 0.9 K⁺, 43.9 Cl⁻, with a pH of 7.13 at 29°C. Prior to the experiment, zebrafish were trained to eat chironomids using Sally's BloodwormsTM brand frozen chironomid larvae for 1 week. Three days before the start of the experiment, the fish were separated into individual test vessels. These 1.75 L containers held 1 L of aerated very soft water and were subjected to daily water changes of the static system (without air exposure of the animal since 200 ml of water remained in the exposure vessel at all times) in order to acclimate the zebrafish to the test procedures.

Trophic Transfer Experimental Designs

All fish were held in very soft water and fed one packet of chironomids (4% of body weight) twice daily, 8 h apart. Zebrafish were split into treatment groups and were fed specific pre-weighed treatment diets. One hour before the first feeding, each zebrafish was weighed and measured. After the second feeding of the day, 80% (800 mL) of the tank water was removed and replaced. A static-renewal system was used to ensure that the zebrafish ate all of the food provided to them, and that none would be washed away, as might occur with a flow-through system.

One day prior to, and during each day of the feeding experiment, two 15 mL water samples were taken from each tank. The first sample was taken before the water change (one hour after the second feeding) and a second was taken just after the water change. All water samples were analyzed for pH, and Cd and ammonia concentrations. Since there were no differences between experiments, pH and ammonia data were pooled into two broad categories: prior to water change, and immediately after daily water change for each day. Also, ammonia values were measured from all water samples for the first 3 experiments, however, since the results were identical each time, ammonia levels were only monitored on a few samples in the 4th experiment.

The feeding experiment continued for seven days and was followed by 3 days of gut clearance during which the zebrafish were fed control chironomids (experiments 1 and 2), Sally's Bloodworms[™] (experiment 4), or control pellets (experiment 3) to purge any Cd-contaminated food from their gut. Upon completion of gut clearance the zebrafish were anesthetized in 1 g/100 ml MS222 (tricaine methane sulfonate, Aquatic Life Sciences, Syndel Laboratories Ltd., Vancouver, B.C.) of very soft water (pH 7.2) and rinsed twice with soft water before being weighed and measured for fork length. All fish

were then sacrificed, and half were dissected into five parts; gill, gut, liver, kidney and carcass, while the other half were frozen intact for subsequent subcellular fractionation.

Upon analysis of the water samples from experiment 1 of the fish tests (below), it was clear that there were residual amounts of Cd in the water due to the static-renewal system. To account for the water-borne Cd exposure that the treatment fish were receiving, all subsequent experiments incorporated a second set of fish (for all treatments) that were fee a control diet, and underwent only a comparable water-borne exposure. This second set of fish or 'echo' fish, received the day-old water that was removed from the treatment tanks daily, after it was filtered through a coarse filter of 120 microns to remove any particulate waste. All measurements and sampling for the 'echo' fish were done the same way as described above for treatment fish.

Experiment 1: Zebrafish (n=10 per treatment) were fed either control chironomids or high Cd chironomids (which had been exposed to water-borne Cd). There were no 'echo' treatments.

Experiment 2: Zebrafish (n=12 per treatment) were fed either high Cd chironomids (which had been exposed to water-borne Cd) or chironomids which had been exposed to sediment-borne Cd. 'Echo' treatments were incorporated.

Due to the findings that trophic transfer efficiencies were different between zebrafish fed on sediment exposed chironomids and zebrafish fed on high water-borne exposed chironomids, and since the Cd concentrations of the sediment exposed

chironomids and high water-borne exposed chironomids did not match, third and fourth experiments were performed to clarify these issues.

Experiment 3: The goal of this test was to determine if the reason that trophic transfer efficiencies were different was because of different concentrations of Cd in the prey. For simplicity, a pellet diet was used. Additionally, this experiment served to clarify whether there were differences in trophic transfer efficiency and/or tissue-specific distribution of Cd between "Cd-spiked" commercial diets *versus* chironomids (water-borne exposed) with biologically incorporated Cd. Zebrafish (n=12 per treatment) were fed either a control pellet diet, a low Cd pellet diet, or a high Cd pellet diet. The low Cd pellet diet was made to match the amount of Cd found in sediment exposed chironomids, and the high Cd pellet diet was made to match the amount of Cd found in the chironomids exposed to a high Cd water-borne exposure. 'Echo' treatments were incorporated, in which the fish were fed control pellets.

Experiment 4: This trial mimicked experiment 3 except that, instead of using pellet food, chironomids which had been exposed to water-borne Cd were used. This was to verify whether or not results from experiment 3 could be duplicated with a more natural food source (chironomids). Zebrafish (n=12 per treatment) were fed either a) control chironomids (i.e. chironomids that were not loaded with Cd) or b) chironomids that were loaded to a low Cd concentration, or c) chironomids that were loaded to a high Cd concentrations (both via a water-borne exposure). The chironomids exposed to the 'low' level of water-borne Cd were loaded to approximately the same level of Cd found in

sediment exposed chironomids, and the low Cd pellet diet. 'Echo' treatments were incorporated.

For clarity, the following abbreviations are used throughout the manuscript to describe the type of food that zeb: afish were fed:

- Lwb -chironomids loaded to a 'low' concentration via a water-borne exposure.
- Hwb -chironomids loaded to a 'high' concentration via a water-borne exposure.
- Lsed -chironomids loaded to a 'low' concentration via a sediment/dietary route
- Lpel -'low' concentration Cd spiked pellet food
- Hpel -'high' concentration Cd spiked pellet food

Subcellular Fractionation

Subcellular fractionation for both whole chironomids and whole zebrafish were completed according to Wallace *et al.* (2003), with a few alterations. We used a sucrose buffer containing in mM: sucrose (250), Tris(Hydroxymethyl)Aminomethane (20), KCl (25), and MgCl₂ (5), in NanopureTM water, adjusted to pH7.4 with HCl. Immediately before subcellular fractionation, an anti-oxidant (2-mercaptoethanol, 2mM) and a protease inhibitor (PMSF, 0.2mM) were added to buffer. In accordance with Wallace *et al.* (2003), buffer was added (in a constant 1:5, tissue(g):buffer(ml) ratio) and tissue was homogenized using a Tissue TearorTM (Biospec Products Inc. Dremel, Racine WI, USA). At this point, a sub-sample was removed from each resultant homogenate for calculating percent recovery. The homogenate was then spun at 1450 x g for 15 min at 4°C after which the pellet (debris) was set aside and the supernatant was spun at 100000 x g for 1 h
at 4°C. The resultant pe let was the organelle fraction (ORG), and the supernatant was then heated at 80°C for 10 min and spun at 30000 x g. The pellet was the enzyme fraction (ENZ, heat-sensitive proteins), and the supernatant was the metallothionein–like proteins fraction (MTLP, heat-stable proteins). The pellet from the original centrifugation (debris) was re-suspended using 1N NaOH (in a constant tissue:NaOH ratio of 1:3). It was heated at 80°C for 10 min, vortexed, and then heated again for 10 more minutes in order to verify that the debris fraction was completely digested before being centrifuged at 5000 x g for 10 min. The resultant pellet was the metal rich granules fraction (MRG), and the supernatant was the cellular debris (CD). The % recovery after subcellular fractionation was 72 ± 13 (N=8) for chironomids, while the % recoveries for whole zebrafish were 88 ± 8 (N=20), 83 ± 7 (N=28), and 81 ± 6 (N=32) for experiment 2, 3, and 4 respectively.

We attempted to determine the subcellular distribution of Cd in the dissected parts of the zebrafish from experiment 1, however volume sizes of each part were too small. This is why there are no subcellular fractionation data for experiment 1.

Analytical Techniques and Calculations

All water samples were filtered through an AcrodiskTM 0.45 μ m in-line-syringetip filter, to give the dissolved metal concentration and were measured for Cd using a GTA 110 Varian Graphite Furnace Atomic Absorption Spectrometer (AA) (Varian Techtron, Mulgrave, Vic:oria, Australia). The Cd standard was made using a Cadmium Reference Solution from Fisher Scientific (Nepean, Ontario). Percent recovery was always within ± 15% as determined using analytical reference materials: DORM-2

(Dogfish Muscle Certified Reference Material for Trace Metals) for fish tissue samples, and TORT-2 (Lobster Hepatopancreas Reference Material for Trace Metals) for chironomid samples both from the Institute for National Measurement Standards, National Research Council of Canada, Ottawa, Ontario. Water concentrations were considered valid if the reference material TM15 (Environment Canada, National Water Research Institute, Burlington, Ontario) for water samples was within ±10%. Data are reported as recovered metal; and are not corrected for percent recovery. Water samples from the trophic transfer experiments were analyzed for Cd, and monitored for pH (PHM82 Standard pH meter, Radiometer Copenhagen) and ammonia (salicylate hypochlorite method of Verdouw *et al.* (1978)).

Chironomids were digested using 100 μ L/ mg dry weight tissue of full strength trace metal grade HNO₃ (67-70%) for 6 days at 60°C followed by 40 μ L/ mg dry weight of H₂O₂ (30% solution) for 1 day (from Croteau *et al.* 2002). Fish tissues were digested with 3-5 μ L 1N HNO₃ / mg wet weight for 48 h. Samples were vortexed immediately after acid was added, at 24 h, and at 48 h (from Chowdhury *et al.*, 2004).

Whole body burdens of Cd were calculated from the sum of the mass-adjusted tissue contents (i.e. Cd concentration within tissue x fraction of whole body mass that tissue represents) in each of the individual tissue fractions (gills + gut + liver + kidney + carcass). In the Results section, the Figures (3.2-3.5) of zebrafish tissue fractions illustrate these mass adjusted values (i.e. the fractions add up to the whole body burden), while Table 3.2 reports the actual Cd concentration values in individual tissues.

In the subcellular fractionation measurements, supernatant subcellular products (CD, MTLP) were digested over 48 h using the same method as for fish tissues (1N

HNO₃), while MRG, ORG, and ENZ fractions were first dried completely (and weighed) before digesting with 8-12 μ L 1N HNO₃ / mg dry weight over 48 h at 60°C, again vortexing immediately after acid was added, at 24 h and at 48 h. Data were calculated by first reporting concentrations as μ g Cd/g dry weight (MRG, ORG, and ENZ) or μ g Cd/L supernatant fraction (CD and MT), after which they were converted to percents of total recovered Cd. Trophically available metal (TAM) and metabolically available metal (MA) fractions were calculated by summing together different subcellular compartments. TAM is the sum of the CRG, ENZ, and MTLP fractions (Wallace *et al.*, 2003)

Condition factors (CF) and specific growth rates (SGR) were calculated for individual fish using the following equations:

 $CF = mass of fish/(fork length)^3 * 100$ (equation 1)

SGR = $[\ln(\text{final fish weight}) - \ln(\text{initial fish weight})] / \text{days} \times 100 \text{ (equation 2)}$ where fish weights were in g, and fish lengths were in cm.

Trophic Transfer Efficiencies (TTE) were calculated using the equation:

TTE = (sum total of Cd in fish/sum total of Cd fed to fish)*100 (equation 3) In Cd-contaminated food treatments, the 'sum total of Cd in fish' was corrected by subtracting both the average Cd contamination in a control treatment fish and the average Cd contamination in the corresponding 'echo' fish. These corrections remove two variables: background levels of Cd in the diet (from control fish), and Cd contamination due to the water-borne exposure (from 'echo' fish). Thus only Cd gained by the fish from the diet was included in the numerator. In control treatment fish, the 'sum total of Cd in fish' was corrected by subtracting the average Cd contamination in the control

'echo' fish. Note that for the sake of consistency we have reported all transfer efficiencies as TTE's, al:hough we recognize that if certain authors have reported true assimilation efficiency (AE), it may be higher than TTE. In brief, estimates of AE are usually based on short-term, single meal studies, without consideration of subsequent excretion of the metal; TTE estimates are usually based on longer term, multiple feeding studies (such as the present investigation) in which both uptake and excretion are occurring.

Statistical Analyses

All data are presented as mean \pm S.E.M. (N= number of fish). Percentage data was arcsine transformed before statistical analysis. For variance analysis among groups ANOVA was used followed by the Holm-Sidak method for pairwise multiple comparisons so as to identify individual differences. The convention used in Figures and Tables is that means which share the same letter are not significantly different. For paired comparison of two groups only, a Student's t-test was used. In all cases, significance was taken a: $p \le 0.05$.

Results

Chironomid Cd concentrations

There was no difference in the amount of Cd in fresh-dried chironomids than in frozen-dried chironomids of the same treatments. Also, the concentrations of Cd within identical treatments across experiments were not significantly different. Thus, treatment data were pooled, and an average treatment concentration was used for trophic transfer

efficiency calculations. Chironomids exposed to 60 μ g Cd/L as Cd(NO₃)₂ via a waterborne exposure (Lwb) had 164.0±14.5 μ g Cd/ g dry weight (N=6 replicates of 6 chironomids each). Chironomids exposed to 260 μ g Cd/L via a water-borne exposure (Hwb) had 287.9±11.6 μ g Cd/ g dry weight (N=22). While sediment-exposed chironomids (Lsed) had 153.1±10.9 μ g Cd/ g dry weight (N=6). The "training" chironomids, Sally's BlcodwormsTM, contained less Cd, 0.17±0.09 μ g/g (N=6), than controls which had 3.41=0.16 μ g Cd/g (N=22).

Chironomids loaded via the sediment exposure were exposed to a sediment concentration of 10.89 ± 0.14 mg Cd/kg. They were also exposed to significantly different (p<0.02, paired t-test) minipeeper water Cd concentrations of 5.38 ± 1.08 and $3.61\pm.63$ µg/L for pore water and sediment-water interface water respectively. The concentration of Cd in the overlying water was significantly increased (p<0.02) over the 15 day exposure, going from 0.48 ± 0.10 at the start of exposure, to 1.21 ± 0.14 µg/L at the end of exposure.

Leaching Test

The leaching test indicated that there was very little Cd lost from whole chironomids in the test water even after 24 hours (Table 3.1). In all cases water samples from all of the test vessels at both the start time and the end time were indistinguishable from background when measured on the AA ($\leq 0.03 \ \mu g \ Cd/L$).

Trophic Transfer Experimental Monitoring

The growth of fish over the 10 days (7 days treatment and 3 days gut clearance) was small, with SGR averaging at 0.57 ± 0.05 %/day over all experiments. Condition factors were not different among experiments, and did not change from beginning (average 1.03 ± 0.01), to end (1.03 ± 0.01) of experiments. Fish mortality was extremely low. Two fish died during tests: one was in the Hwb 'echo' treatment of experiment 2, and the other was in the Hwb treatment of experiment 4. Neither was due to experimental causes.

Ammonia, and pH in the test vessel water samples were significantly higher (p<0.01 and p<0.05 respectively) just before an 80% daily water change, than immediately after, regardless of treatment. Overall means were 12.55±1.52 µmol/L (ammonia) and 6.79±.03 (pH). Cd in water also significantly increased and decreased with water changes (P<0.01), meaning that Cd levels were lower just after a water change than just before a water change from experimental days 2-7 across all Cd experimental food treatments. Examples of these phenomena occurring in experiment 1, with zebrafish fed either control or high water-borne Cd exposed chironomids, are given in Figure 3.1a (ammonia and pH in water) and 3.1b (Cd in water). Average Cd concentrations from fish vessels (excluding controls) were 0.58 ± 0.11 , $0.70\pm.04$, $0.73\pm.03$, and $0.59\pm.03 \ \mu g \ Cd/L$, for experiment 1,2,3, and 4 respectively. The latter three represent the Cd concentrations to which the 'echo' fish were exposed in the corresponding experiments. When compared to the negligible concentration ($\leq 0.03 \ \mu g \ Cd/L$) resulting from the leaching test (above), these values indicate that the waterborne Cd resulted from processing of the food by the fish, most likely via dispersion of particles during feeding and/or production of contaminated faeces.

Experiment 1

After 7 days of feeding with 3 days of gut clearance, the mass-adjusted Cd burdens (N = 10 per treatment) were significantly higher in all tissues (gill, gut, liver, kidney, and carcass) in the zebrafish fed Hwb chironomids than in the zebrafish fed control chironomids (Figure 3.2). However, there was a residual amount of Cd in the control zebrafish (total body burden = $0.035\mu g/g$). In the zebrafish fed control chironomids, it is clear that the majority of the body burden is from the gut (69±2%) and the gill (23±2%), while in the zebrafish fed Hwb chironomids, the body burden is more widely distributed. There was significantly less Cd in the gill fraction (p<0.01), yet, the gut was still the biggest contributor (40±2%), followed by carcass (31±2%) and liver (16±3) (Figure 3.2). Actual Cd concentrations in individual tissues revealed that zebrafish fed control chironomids had significantly higher Cd levels in the gut than any other tissue, although in the zebrafish fed Hwb chironomids the gut, liver, and kidney all took up high concentrations of Cd (Table 3.2).

The TTEs were also significantly different between treatments (P<0.001) with $9.20\pm1.03\%$ for control chironomids and $1.23\pm.22\%$ for Hwb chironomids (Table 3.3). Note that the latter datum was corrected for the 'echo effect' using an average of Hwb treatments from experiments 2 and 4 (see below).

Experiment 2

In Experiment 2, zebrafish fed Lsed chironomids $(153.1\pm10.9 \ \mu g \ Cd/g \ dry$ weight) and zebrafish fed Hwb chironomids $(287.9\pm11.6 \ \mu g \ Cd/g \ dry \ weight)$ had almost identical levels of Cd uptake over 7 days of $0.356\pm.057$ and $0.366\pm.038 \mu g/g$ respectively, despite the different concentrations in the prey. Cd storage in these two groups of fish was similar in all tissue fractions, meaning that Cd tissue-specific distribution was not dependent on whether the food source (chironomids) accumulated its Cd from the sediment or via a water-borne exposure (Figure 3.3, Table 3.2). On a massadjusted basis, the main tissues contributing to body burden were the gut and carcass for both zebrafish fed Lsed (gut=42±3%, carcass=39±3%), and zebrafish fed Hwb chironomids (gut=42±5%, carcass=35±5%)(Figure 3.3). This corresponds well with uptake in zebrafish fed Ewb chironomids in experiment 1 (Figure 3.2). Like the control in experiment 1, zebrafish in the echo conditions took up a significantly greater proportion of Cd in the gills than did treatment zebrafish (which took up a greater proportion of Cd in the gut), although the greatest uptake was in the carcass, ranging from 29-53% (Figure 3.3).

When fractions were not mass-adjusted, there were significantly greater concentrations of Cd accumulated in the gut and kidney than in gill, liver, and carcass fractions for both zebrafish fed Lsed chironomids and zebrafish fed Hwb chironomids (Table 3.2). And, in both echo conditions, the kidney had much higher Cd concentrations than in any other fractions (Table 3.2). Indeed, mass-weighted Cd tissue levels of the treatment fish were all very similar to the 'echo' fish in gill, liver, and kidney fractions although there were significant differences between these two groups in the gut, whole body burden and between the treatment fish and 'sediment exposed echo' in the carcass (Figure 3.3).

Zebrafish fed Lsed chironomids had a significantly higher (P=0.026) TTE (1.99±.46% (N=5)) than zebrafish fed Hwb chironomids (0.74±.20% (N=6)) (Table 3.3).

Experiment 3

Since TTEs were different in experiment 2, this experiment was performed with pellet food to see if the TTE difference was due to different concentrations of Cd in the prey. Zebrafish were fed control pellets $(0.3\pm .0 \,\mu\text{g/g} \,d\text{ry weight} \,(\text{N=5}))$, low Cd pellets (Lpel) (154.4 \pm 4.0 µg/g dry weight (N=5)) matching the amount of Cd found in Lsed chironomids, or high Cd pellets (Hpel) (291.5±11.1µg/g dry weight (N=4)) matching the amount of Cd found in Hwb chironomids. From mass-adjusted tissue accumulations, it can be seen that zebrafish uptake of Cd was dependent on the concentration of Cd in the pellet food. This proportional uptake was most visible in the gut, kidney, carcass and overall body burden (Figure 3.4). Again, the highest accumulation of Cd was in the gut and kidney (Table 3.2). Also similar to experiment 2 results, echo conditions all had much greater Cd accumulation in the kidney than in any other fractions except in the gut of the echo zebrafish for the Hpel treatment (Table 3.2). In terms of mass-adjusted fractions, the gut, then carcass accounted for the greatest percentages of overall body burden in treatment fish with: 49 ± 7 , 65 ± 5 (gut) and 36 ± 5 , 25 ± 4 (carcass) % for zebrafish fed Lpel, and zebrafish red Hpel food respectively (Figure 3.4). Again, echo fish had a significantly greater percent of Cd in the gill than treatment fish, and the greatest contributor to overall bcdy burden in echo fish was the carcass ranging from 43-70% (Figure 3.4). TTE's were not different between zebrafish fed Lpel, and zebrafish fed

Hpel, yet both were significantly lower than in zebrafish fed control pellets (P<0.001) (Table 3.3).

Experiment 4

Given that in experiment 3, dietary Cd uptake was concentration-dependent in zebrafish fed pellet food and that TTE's were not concentration-dependent (i.e. different from the situation in experiment 2), another experiment was necessary to see if this result could be duplicated with a natural food source (chironomids). Thus, this experiment mimicked experiment 3, with zebrafish fed either: control chironomids, chironomids loaded with Cd via a low Cd water-borne exposure (Lwb) (matched levels of Cd found in Lsed chironomids, and the Lpel diet), or chironomids loaded with Cd via a high Cd water-borne exposure (Hwb). Although it appears that proportional uptake of Cd occurred when looking at tissue mass-adjusted distributions in the carcass and overall body burden, this trend was not significant (Figure 3.5). It is interesting to note that concentrations of tissue-Cd in the gut were approximately equal with values of 3.88±1.58 and $3.62\pm1.01\mu g/g$ for zebrafish fed Lwb chironomids, and zebrafish fed Hwb chironomids respectively (Table 3.2). This trend was still seen after gut fractions were mass-adjusted (Figure 3 5) indicating that even when the concentration of Cd in the zebrafish food source (chironomids) is doubled, the accumulation in the gut tissue is the same. Once again, the major sites of Cd accumulation in all zebrafish fed treatment food are the gut, liver, and kidney, although these are not always significant (Table 3.2). However, in 'echo' zebrafish from all treatments, the kidney did accumulate significantly higher Cd concentrations than any other tissue (Table 3.2). The biggest mass-adjusted

contributors to overall body-burden were again the gut and carcass for both zebrafish fed Lwb and Hwb chironomids (Figure 3.5).

In confirmation of experiment 3, TTEs were not significantly different between the experimental treatments (Table 3.3) suggesting that the difference in TTE observed in experiment 2 was caused by the different exposure media (sediment vs. water) of the chironomids, rather than by differences in chironomid Cd concentrations.

Similarities and Differences Among Experiments

In all experiments (1-4), total accumulation of Cd was greatest on a mass-adjusted basis in gut tissue followed by carcass (Figs. 3.2-3.5), and tissue-specific Cd concentrations were highest in the gut, kidney, and then liver for treatment fish, and solely in the kidney for 'echo' fish (Table 3.2). Combined, the mass-adjusted gut and carcass fractions always accounted for \geq 71% of the total Cd stored in zebrafish (excluding controls) (Figures 3.2-3.5). Uptake of Cd in zebrafish fed Hwb chironomids was consistent, with means of $0.378\pm0.019\mu g/g$ (N=10) for experiment 1, $0.366\pm0.038\mu$ g/g (N=6) for experiment 2, and $0.380\pm0.058\mu$ g/g (N=6) for experiment 4 (Figures 3.2, 3.3 and 3.5). Furthermore, the body burdens of zebrafish fed Hwb chironomids were comparable to the body burden of zebrafish fed an Lpel diet $(0.352\pm0.030\mu g/g)$. Also, Cd mass-adjusted concentrations in the liver of zebrafish fed high water-borne exposed chironomids from experiment 1 $(0.066\pm0.012\mu g/g \text{ whole fish})$, and experiment 4 (0.047 \pm 0.013 µg/g whole fish) was significantly more than the Cd levels in fish livers from any other treatments, although the actual concentrations (not mass-adjusted) were still lower than the corresponding gut and kidney concentrations

(Table 3.2). As well, zebrafish fed Hpel, had significantly more mass-adjusted Cd in the gut $(0.472\pm.119 \ \mu\text{g/g}$ whole fish), as well as a significantly greater body burden $(0.692\pm.109 \ \mu\text{g/g})$ than any other treatments in any experiment. The trophic transfer efficiencies were generally low, with values <2%, except for control transfer efficiencies which were all greater than 9% (Table 3.3). Hwb chironomids all had similar TTEs, with values of $1.23\pm.22$ (experiment 1), $0.74\pm.20$ (experiment 2) and $0.86\pm.15$ (experiment 4).

In order to compare the uptake of Cd into zebrafish from biologically stored food (contaminated chironomids) to the uptake of Cd into zebrafish from a manufactured food (contaminated pellets), the total amounts of Cd fed to fish were matched up with the total amounts of Cd accumulated in the fish (Figure 3.6). Note that 'biologically stored food' contains data for both chironomids exposed to Cd via water-borne and sediment routes. Values for Cd accumulated in the whole fish were corrected for both control Cd values, and the corresponding 'ccho' Cd values. Separate regressions for zebrafish fed biologically stored food and zebrafish fed a manufactured food, were both significant, with values of $r^2=0.63$, p=0.03 and $r^2=1.00$, p<0.01 respectively, although the slopes (TTE's) were not significantly different. When sediment exposed chironomids were excluded from the biologically stored food group, the regression coefficient became $r^2=0.76$, p=0.02.

Subcellular Compartmentalization and Trophically Available Fractions

On a relative basis (i.e. expressed as a percentage of the total Cd accumulation), the greatest amount of accumulated Cd was found in the MRG fraction (metal-rich granules), followed by the organelle (ORG) fraction, regardless of the route of exposure. However, within the smaller fractions, chironomids that were exposed via a water-borne route took up significantly more Cd in the CD (cellular debris) fraction, and significantly less Cd in the MTLP (metallothionein-like protein) fraction than controls and Lsed chironomids (Table 3.4). This pattern was repeated in the ENZ (enzymes = heat denaturable proteins) fraction with chironomids exposed via a water-borne route having less Cd stored in the ENZ fraction than controls and Lsed chironomids, although the difference between water-borne and sediment exposed chironomids was not significant. In general, sediment exposed chironomids acted more like control chironomids than water-borne exposed chironomids, particularly in the CD, MTLP, and ENZ fractions.

Subcellular storage of Cd in the zebrafish did not depend on the exposure route of the food source (water-borne or sediment exposed chironomids). However, storage in chironomids and storage in the zebrafish of experiment 2 were very similar (Table 3.4). In all cases, the CD and MTLP compartments accounted for only a small percentage of the total subcellular Cd contained in both chironomids and zebrafish (Table 3.4). Furthermore, storage compartmentalization of Cd did not change with increasing Cd concentration in the zebrafish or in the chironomids, irrespective of prey exposure route (sediment exposed or water-borne exposed), meaning that storage ratios in compartments remained constant regardless of body burden.

In experiment 3, zebrafish fed Cd-contaminated pellets and in experiment 4, zebrafish fed chironomids that were exposed to Cd through a water-borne route, were directly comparable since they had identical concentration levels in the food. However, there was no major difference in relative Cd subcellular distribution between these two experiments, except that in experiment 4, zebrafish fed Cd-contaminated pellets seemed

to have less Cd in the MTLP fraction, although this difference was not significant. Within experiment 4, both zebrafish fed Lwb, and Hwb treatments had significantly less Cd in the ORG fraction than in controls, and had significantly more Cd in the ENZ fraction than in controls, although they were not significantly different than "echo's" (Table 3.4). On the whole, compartmentalization of Cd did not change with increasing Cd concentration in the zebrafish, regardless of food type: chironomids exposed via a water-borne route, or manufactured-pellet.

Trophically available metal (TAM) in chironomids (the sum of ORG, ENZ, and MTLP fractions) was between 46.1 \pm 3.7% and 60.6 \pm 3.7% regardless of chironomid treatment (Figure 3.7). Metabolically available metal fractions (MAF) in chironomids ORG, ENZ, and CD fractions) werevery similar to TAM, ranging from 47.9 \pm 4.1% to 54.7 \pm 3.6%. These similarities are due to the low percentage of Cd in both CD and MTLP fractions. Neither TAM (p=0.14) nor MAF (p=0.21) had a strong relationship with the TTE of Cd from chironomids to zebrafish (Figure 3.7).

It is interesting to note that the % of Cd in metal rich granules (MRG) was significantly higher in all treatments of experiment 2 than in experiment 4, even in the comparable treatments of zebrafish fed Hwb chironomids with corresponding echo series. This could be related to the fact that gut clearance of zebrafish in experiment 2 was done with control chironomid₃, while gut clearance of experiment 4 was done with Sally's BloodwormsTM. When gut clearance was done with control chironomid₅, most of the Cd was found in the MRG (treatment averages of 30.9-51.5%) and organelle fractions (25.8-36.2%). When gut clearance was done with Sally's BloodwormsTM, there was still a large portion of Cd in the organelle fraction (treatment averages of 45.6-62.3%), however

there was much less Cd in the MRG fraction (5.9-9.8%), and much more in the enzyme fraction (21.2-39.3%).

Discussion

Cd Leaching from the Diet

Cd leaching was smaller from previously frozen chironomids (<1%) than from previously frozen *Lumbriculus variegatus*, an oligochaete worm (~30%) (Ng and Wood, in submission). Interestingly, Cd contaminated *C. riparius* were found to exhibit significant leaching in another study (Kraal *et al.*,1995); part of this difference could be due to the fact Kraal *et al.* (1995) reported total metal Cd concentrations whereas our study reports dissolved concentrations. Since there was no significant dissolved Cd appearing in the water (\leq 0.03 µg Cd/L), and since frozen chironomids retained Cd even after 24 h (Table 3.1), we know that Cd leaching into the water directly from the food was not a concern. However, since Cd was measurable in the water (Figure 3.1b) during the feeding experiments, we suggest that Cd was likely dispersed from particles during feeding and/or leaching from the zebrafish faeces. Leaching from zebrafish faeces is likely, as Szebedinszky *et al.* (2001) reported that juvenile rainbow trout fed Cd contaminated commercial food in the range of (15-1500 mg Cd/ kg food) all had approximately 200µg Cc/g in their fecal matter.

Growth and Water Chemistry

Overall fish growth was positive in an all experiments, although very small, with an average SGR of 0.57 ± 0.05 %/day. Thus, we did not see a decreased growth in

conjunction with Cd exposure via the diet, although decreased growth due to other waterborne contaminants has been documented in zebrafish (Ensenbach and Nagel, 1995; Van den Belt *et al.*, 2003; Njiwa *et al.*, 2004). Indeed, zebrafish growth rates are generally low, for example, Eaton and Farley (1974) found that in the 30 days prior to first spawning, growth rates were 0.05mm/day, which is comparable to the growth of fish in our exposure (e.g. over 10 days increasing by ~0.5mm).

The finding that ammonia and pH were both higher before a water change (Figure 3.1a), and lower immediately after, is logical since fish excrete ammonia as a waste product. Given that ammonia is basic, an increase in ammonia corresponds with an increase in pH. To this end, the changes in ammonia concentration ranging from 0-30 μ M/L, and pH (6.5-7.1) were not large, and did not adversely affect the experiment.

Does TTE depend on Cd concentration of prey?

No. Cd assimilation in zebrafish fed on water-borne exposed chironomids did not depend on the concentration of Cd in the chironomids (Table 3.3, Figure 3.5). In other words, Cd uptake in zebrafish was proportional to the concentration of Cd in the water-borne exposed chironomids of experiment 4. Chironomids in the Lwb, and Hwb treatments, did not have different TTEs. The same was true for zebrafish fed with Lpel and Hpel diets in experiment 3(Table 3.3, Fig. 3.4).

Overall TTE's obtained in these experiments (due to dietary Cd) were approximately 10% for controls, which had only small amounts of residual cadmium, and 0.5-2% for treatment conditions. We cannot eliminate the possibility that TTE's of the controls may be artificially high, as in the absence of substantial Cd loading from the

food, these animals may be taking up enough Cd from background concentrations in the water to inflate the calculated TTE's. Overall, these TTE values were notably lower than those from a similar trophic route in which live C. riparius larvae were fed to carp where Van Campenhout et al. (2007) reported TTE's ranging from 50-65%. On the other hand, Liu et al. (2002) found similar low Cd TTE's (3-8%) when zebrafish were fed Daphnia magna. These ranges are also similar to those found with Cd contaminated oligochaetes (Lumbriculus variegates) fed to trout which ranged from 0.9-6.4% after 1 week (Ng and Wood, in submission), and with oligochaetes (Tubifex tubifex) fed to carp which had an assimilation efficiency of 9.8% (Steen Redeker et al., 2007). So, there are plenty of studies to support either high TTE's (Roy and Hare, 1999; Wang et al., 2001; Cheung and Wang. 2005; Rainbow et al., 2006; Van Campenhout et al., 2007), or low TTE's (Liu et al., 2002; Franklin et al., 2005; Zhang and Wang, 2006; Steen Redeker et al., 2007; Ng and Wood, in submission). We conclude that TTE's seem to vary depending on the prey and predator used as Kraal et al. (1995) suggested, and do not depend on the concentration of Cd in the prey.

Does TTE depend on the exposure route of prey?

Yes. Cd body burden in zebrafish fed Lsed chironomids, and zebrafish fed Hwb chironomids in experiment 2 were identical, even though Lsed chironomids had only approximately half the amount $(153\mu g \text{ Cd/ } g \text{ dry weight})$ of Cd as Hwb chironomids $(288\mu g/ g)$. Thus, zebrafish fed Lsed chironomids had a significantly higher TTE $(\sim 2.0\%)$ than zebrafish fed Hwb chironomids $(\sim 0.7\%)$ (Table 3.3). We know that the difference in TTE between zebrafish fed on sediment-exposed chironomids and zebrafish

fed on water-borne exposed chironomids is not a function of the concentration since we determined (above) that the TTE of zebrafish fed chironomids is not concentrationdependent. So, we can report with confidence that the TTE of zebrafish did depend on the exposure route of the prey such that zebrafish fed Lsed chironomids retained more Cd than zebrafish fed Hwb chironomids. This is in direct contrast to findings of Liu et al. (2002) who reported that there was no difference between TTE's of zebrafish which were fed Daphnia magna radiolabeled with Cd either via water-borne exposure or via dietary exposure. However concentrations in prey were not mentioned in the study of Liu et al. (2002), so it is unclear whether the concentrations of these two routes of exposure matched. The reason for this approximately three fold difference in TTE is unclear, however, it may be related to a) differences in Ca levels of chironomids exposed via sediment/ water-borne routes since Cd uptake in chironomid larvae is affected by environmental Ca concentration (Gillis and Wood, 2007) or b) to the fact that chironomids exposed via the sediment had a more 'natural' chronic exposure than did chironomids exposed via a water-borne route.

Does relative tissue-specific Cd accumulation in the predator depend on route of exposure in the prey?

No, it appears that as long as the route of exposure to the zebrafish is the same (e.g. dietary) then tissue-specific Cd distribution is the same. In all experiments, Cd accumulation was highest in the gut and kidney > liver > gill > carcass for treatment fish fed chironomids (Table 3.2). This is consistent with findings from a comparable trophic transfer route; trout fed on Cd water-borne contaminated oligochaetes (*Lumbriculus*)

variegatus), which preferentially accumulated Cd in the gut and kidney (Ng and Wood, in submission). Another trophic chain (*C. riparius* to carp), found similar Cd accumulation in the tissues to be: gut > kidney > liver ~ gill > muscle (Kraal *et al.*, 1995). In experiment 4, it is interesting to note that concentrations of Cd in the gut were approximately equal for zebrafish fed Lwb chironomids, and zebrafish fed Hwb chironomids, mass-adjusted or not. This indicates that the gut may be able to take up high amounts of Cd, regardless of concentration in the food (Table 3.2, Figure 3.5). For example, even when the concentration of Cd in chironomids is doubled, the accumulation (μ g/g) in the zebrafish gut is the same (Figure 3.5). Overall, accumulation of Cd in decreasing order for all treatment fish fed chironomids were gut and kidney > liver > gill > carcass.

Moreover, Cd concentrations in zebrafish fed control chironomids had a high accumulation in the gut and kidney, and very little in any other tissue (Table 3.2experiment 1 and 4). This makes sense, as the waterborne Cd concentrations in control tanks were negligible (Figure 3.1b), so the main entry of residual Cd to control chironomids was through a dietary route.

Does tissue-specific Cd distribution in the predator depend on route of exposure in the prey?

No; like tissue-specific accumulation, tissue-specific distribution (e.g. percentage of overall body burden) did not change with exposure route of prey. In zebrafish fed control chironomids, the residual Cd was mainly stored in the gut, carcass and gill (Figures 3.2, and 3.5), while in the zebrafish fed treatment chironomids, regardless of

chironomid exposure route, the order in decreasing % was: gut > carcass > liver ~ gill ~ kidney (Figures 3.2, 3.3, 3.5). These findings are in line with Szebedinszky *et al.* (2001) who found that Cd in the carcass of trout fed a control diet accounted for most of the body burden, and those fed Cd-spiked diets had the greatest proportional uptake in the gut, followed by carcass While Ng and Wood (in submission), found that the gut accounted for a high amount (>80%) of the body burden in trout fed Cd contaminated oligochaetes for 1 week. Therefore, both tissue-specific accumulation and distribution are not dependent on route cf prey exposure, and the gut is an important factor in both (highest accumulator, and contributes the most to overall body burden).

Does tissue-specific Cd distribution in the predator depend on route of exposure in the predator (i.e., dietary or waterborne?)

Yes. This conclusion is based on a comparison of diet-exposed (treatment) fish and 'echo' fish for the same treatments. Both tissue-specific Cd distribution and Cd accumulation in zebrafish did depend on whether the exposure (to zebrafish) is dietary or water-borne. Echo fish from experiments 2, 3, and 4 had a consistently higher proportional uptake of Cd in the gill than treatment fish (Figures 3.3-3.5). They also had less Cd accumulated in the gut, and more in the kidney relative to treatment fish (Table 3.2). This difference in location of Cd uptake can be understood by the type of exposure. Fish in the echo condition were exposed mainly to water-borne Cd, while those in the treatment condition were exposed mainly to dietary Cd. These results are consistent with carp (Kraal *et al.*, 1995) and trout (Szebedinszky *et al.*, 2001) exposed to water-borne Cd, which took up proportionately more Cd in the gill fraction than fish exposed to dietary

Cd. Similarly, Farag *et al.* (1994) found that metal accumulation was highest in the gill and kidney for water-borne exposures and higher in the stomach and pyloric caeca with exposure via diet. Contrastingly, De Smet *et al.* (2001) found the highest accumulation in kidney > liver > gill for carp exposed to water-borne Cd, perhaps reflecting a species difference.

Are responses to natural diets and manufactured diets comparable?

Yes – with some qualifications. Concentration matching of Lpel $(154\mu g/g)$ with Lsed chironomids, and Hpel $(292\mu g/g)$ with Hwb chironomids determined that TTE of Cd pellet food to zebrafish was independent of the Cd concentration (Table 3.3experiment 3). This same observation; that TTE was independent of dietary Cd concentration, was noted in zebrafish fed on chironomids that were exposed via a waterborne route (above) (Table 3-experiment 4). Put another way, zebrafish which had been fed on Cd-spiked pellets (made from commercially manufactured food) accumulated Cd in a proportional manner: accumulation of Cd in the tissues was dependent on the concentration of Cd in the pellet food (Figure 3.4). TTE's of Cd in zebrafish fed Lpel and Hpel treatments in experiment 3 were the same as those for zebrafish fed on waterborne exposed chironomids in experiments 1, 2 and 4, at <1%. These values were very similar to those found by Franklin et al. (2005) using a very high Cd concentration (500 μ g Cd/g) in a pellet diet: <1% TTE when fed to trout for 28 days. Although Harrison and Curtis (1992) proposed that the efficiencies of metal absorption by a predator from manufactured pellet food may be different than from a natural food source, we did not find that in this study. In fact, when TTE's of Cd from the pellet experiment (3), were

compared to the combined TTE's of biologically stored food (from chironomids that were water-borne exposed from experiments 1, 2, and 4), they were found to be similar (Figure 3.6). This is in contrast with research by Harrison and Curtis (1992) who found that Cd TTE's were greater for fish fed a natural diet (Cd-contaminated amphipods) than those fed a commercial cliet (Cd-spiked manufactured food). However, if we consider that chironomids that were exposed chronically via the sediment exposure were contaminated in a more 'natural' way, then our results do agree with those of Harrison and Curtis (1992). TTE's were greater for zebrafish fed the more naturally contaminated sediment chironomids, than when zebrafish were fed either chironomids that were exposed via an acute water-borne route or Cd-spiked pellet food.

Tissue-specific storage of Cd in zebrafish fed the manufactured diet versus zebrafish fed on chironomids that were water-borne exposed is also similar. Our tissue-specific accumulation for zebrafish fed Hpel was highest in the gut, followed by kidney > liver > gill > carcass (Table 3.2). These results are identical to those of Franklin *et al.* (2005), such that accumulation of Cd in trout fed 500 μ g Cd/g via a Cd-spiked manufactured food after 28 days was: gut > kidney > liver > gill > carcass. While Chowdhury *et al.* (2005) found: gut > kidney > liver > gills for trout fed 420mg Cd/ kg for 30days, while Baldisserotto *et al.* (2005) found the order of accumulation in tissues to be: gill > kidney > liver > carcass (gut was not separated out) for trout fed Cd via a Cd-spiked manufactured food (300 μ g Cd/g) diet for 15 days. This concentration of Cd in pellet food is identical to our 'Hpel' food, yet our tissue specific Cd storage in zebrafish was more similar to that reported by Franklin *et al.* (2005) (above).

The one substantial difference between zebrafish fed pellet food versus zebrafish fed on chironomids that were water-borne exposed, is the more localized uptake of Cd into the gut. Zebrafish fed pellet food had both a higher accumulation of Cd in the gut relative to other tissues (Table 3.2), and a higher proportion of Cd in the gut relative to overall body burden (Figure 3.4). This matches results of Baldisserotto *et al.* (2005) who found that when total accumulated Cd is reported on a relative basis (mass-adjusted), the highest proportional upteke was in the carcass (which included the gut) by >90%. It is also supported by Chowdhury *et al.* (2004) who similarly noted that when trout were exposed to Cd (as Cd(NO₃)₂) via gastrointestinal infusion, the gut was the major storage area after 24h. They also noted that the internalization of Cd into the other non-gut tissues was extremely small (~2.4% in naïve fish) indicating that the gut acts as a strong barrier to the internalization of dietary Cd (Chowdhury *et al.*, 2004, Wood *et al.*, 2006).

Does subcellular compartmentalization of Cd in chironomids change based on route of exposure?

Yes, but only by a small proportion. Subcellular storage of Cd in the chironomid was highest in the MRG followed by ORG fraction irrespective of exposure regime: sediment or water-borne. Yet, there were small changes in the subcellular storage of Cd in the CD and MTLP fractions. Chironomids that were exposed via a water-borne route took up significantly more Cd in the CD fraction, and significantly less Cd in the MTLP fraction than did sediment-exposed and control chironomids, although these changes only accounted for small percentages overall (~5%) (Table 3.4). Water-borne exposed chironomids also had less Cd in the ENZ fractions, although this was not significant.

Our results were similar to those of a deposit feeding polychaete (*Capitella sp.*) which had relatively less Cd in the debris (MRG + CD) fraction when it was fed Cdcontaminated algae (diet-borne), than when the polychaete was exposed via a waterborne route (Selck and Forbes, 2004). In our analyses, we did not find a reduction of Cd in the MRG fraction, but we did find less Cd in the CD fraction in chironomids exposed to sediment/dietary Cd, than in chironomids exposed via a water-borne route.

C. riparius are known to produce MTLP in response to Cd metal exposure (Gillis et al., 2002), and these proteins, specifically designed for metal-binding and detoxification, are a key factor in enabling chironomids to detoxify accumulated metal. In fact, in an oligochaete specie, Limnodrilus hoffmeisteri, Cd-resistant worms (e.g. worms that have evolved Cd resistance through living in contaminated sediments and water), produce both MTLP's and MRG's for Cd storage and detoxification, but nonresistant worms (e.g. from 'clean' sediment/water) only produce MTLP's in response to Cd (Wallace *et al.*, 1998). There is, as of vet, no evidence that the same is true in chironomids as subcellular studies are limited. However, because our chironomids had a high percentage of Cd in the MRG fraction (approximately 39-50%), we can not disclude the possibility that our chironomid culture may have adapted ways to deal with Cd metal toxicity in its history. Thus exposure route of Cd to chironomids is important in terms of where Cd is stored subcellularly. For example, when purified subcellular fractions were fed to the grunt (marine f.sh), TTE's were consistently higher when the fish were fed MTLP and ENZ fractions than when they were fed 'insoluble fractions' which contained CD, MRG, and ORG (Zhang and Wang, 2006). In this case, TTE's of Cd (and Se and

Zn) were dependent on MTLP and ENZ fractions (part of TAM) irrespective of prey (copepods or mussels) (Zhang and Wang, 2006).

Is subcellular compartmentalization of Cd in zebrafish affected by route of prey exposure or type of food; natural versus manufactured?

No, compartmentalization is not affected by route of prey exposure, and yes, compartmentalization is somewhat affected by type of food: chironomid or manufactured pellet. Subcellular compartmentalization of Cd in zebrafish was not affected by the route of prey exposure (sediment-exposed or water-borne exposed chironomids); in all cases, the major storage of Cd occurred in the ORG, ENZ, and MRG fractions for all zebrafish fed on chironomids and pellet food. However, zebrafish fed Cd contaminated chironomids did have more Cd (4-10%) in the MTLP fraction than zebrafish fed pellets.

Contrastingly, a similar trophic transfer study on *Lumbriculus variegatus* (contaminated to a level of $30\mu g$ Cd/g by waterborne exposure) fed to trout, analyzed subcellular fractionation of Cd in the trout gut tissue (Ng and Wood, in submission). In the gut, relative to overall burden, the order of Cd accumulation in the subcellular fractions after 1 week was as follows: MRG > ENZ > MTLP > CD > ORG (Ng and Wood, in submission). In a field study where zooplankton were exposed to water-borne Cd and fed to perch, perch livers contained the majority of Cd in the MTLP fraction (Kraemer *et al.*, 2006). Furthermore, in trout, MTLP's have been found to increase in the gill, liver, and kidney irrespective of water-borne or dietary Cd exposure (Chowdhury *et al.*, 2005). In general, the fractions MTLP and MRG are associated with metal detoxification and storage. Thus, our finding that MTLP was higher in zebrafish fed

chironomids than zebrafish fed pellets does have a biological basis, even though the percentage of change is small (2-10%).

Storage compartmentalization of Cd did not change with increasing concentration (e.g. low to high exposures) in chironomids or zebrafish, regardless of food: chironomids that were water-borne exposed, or manufactured-pellet. This is an important finding, which may indicate that machinery to deal with Cd contamination in chironomids and zebrafish does not become saturated (at least at concentrations of up to $0.4\mu g/g$), and is thus able to store Cd with the same relative distribution irrespective of concentration. Due to this finding, analysis of the data is simplified, although comparisons can still be made. For instance, Ng and Wood (in submission), find a similar concentration independence of the subcellular Cd storage in the oligochaete *Lumbriculus variegatus* and the rainbow trout.

Are TAM or MAF useful indicators for TTE in this trophic transfer route?

No. Both trophically available metal (TAM =ORG+ENZ+MTLP) fractions and metabolically available fractions (MAF = ENZ+ORG+CD) have been used by others as indicators of the amount of metal that is available for uptake by a predator (Wallace and Luoma, 2003; Steen Redeker *et al.*, 2007). However, we found that neither TAM nor MAF accounted for the TTE's found in this experiment (Figure 3.7). If we considered only the TAM fractions of Cd in chironomids as transferable metal, our treatment TTE's would still only be approximately 2-4% - i.e. nowhere near 100% transfer. Support for a good correlation between TAM and TTE can easily be found (Wallace and Luoma, 2003; Seebaugh and Wallace, 2004; Seebaugh *et al.*, 2005; Seebaugh *et al.*, 2006), but it is just as common to find cases where TAM does not show a strong relationship to TTE

(Rainbow *et al.*, 2006; Steen Redeker *et al.*, 2007; Ng and Wood, in submission). In summary, our results indicate that for Cd exposed chironomid larvae, TAM and MAF should be used with caution, as neither of these was a strong predictor of TTE (Figure 3.7).

Interestingly, the type of food used for gut clearance of the zebrafish seemed to affect the subcellular distribution of Cd (Table 3.4). Control chironomids were used for gut clearance in experiments 1 and 2, while 'Sally's bloodworms' were used in experiments 3 and 4. When Sally's chironomids were used, rather than control chironomids, there was a transfer of Cd storage from the MRG fraction to the ENZ fraction. This change in Cd storage could be related to the 4-fold difference in Ca concentrations of the different organisms; in control chironomids, Ca^{2+} is $84.5\pm3.0 \mu M/g$ (Gillis and Wood, 2007), while in Sally's bloodworms, Ca^{2+} is $20.9\pm1.0 \mu M/g$ (M. Patel, personal communication). Dietary [Ca²⁺] is known to strongly inhibit Cd absorption in some parts of the gut (Wood et al., 2006); perhaps subcellular distribution is also affected.

Conclusions

Route of prey exposure (dietary/sediment or water-borne) is an important factor for the transfer of Cd to predator species; the TTE of zebrafish fed on the more 'natural' dietary/sediment exposed chironomids was significantly higher than the TTE for zebrafish fed on water-borne exposed chironomids. But, Cd TTE's of zebrafish fed a diet of chironomids that were water-borne exposed were not significantly different than zebrafish fed a diet of mar ufactured pellet food. The biggest contributors to overall body

burdens in zebrafish fed Cd-contaminated food (chironomids or pellets) were the gut and carcass (combined always \geq 71%), but on a tissue concentration basis, Cd accumulation was consistently high in the kidney \geq gut > liver of dietary-exposed treatment fish, and solely high in the kidney \geq or 'echo' fish. Subcellular fractionation of chironomids revealed that sediment-exposed chironomids had significantly more Cd in the MTLP fraction, and significantly less Cd in the CD fraction than water-borne exposed chironomids, although these fractions accounted for only a small percent (~7%) of the total accumulated Cd. Although differences existed in fractionation of chironomids, subcellular storage in zebrafish did not appear to depend on the exposure route of chironomids (water-borne or sediment-exposed). In all zebrafish, the subcellular fractions with the most accumulation were: ORG, ENZ, and MRG. Neither concentration of Cd in prey, or type of food (i.e. chironomid or pellet) affected the subcellular storage of Cd in zebrafish.

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Table 3.1: The concentration of Cd in control chironomids and high Cd contaminated chironomids after various durations of leaching. The previously frozen chironomids were exposed to the standard soft water used in all tests for various lengths of time. Concentrations are expressed as means \pm SEM (N = 5 for 0h and 24h and N=3 for intermediate times).

	Time exposed to	Concentration Cd in		
	water (hours)	chironomids (µg/g)		
Control	0	3.3 +/- 0.1		
chironon ids	24	6.1 +/- 1.3		
Cd- contaminated chironomids	0	281.9 +/- 26.8		
	0.17	311.8 +/- 39.7		
	0.5	268.7 +/- 42.5		
	1	321.7 +/- 13.5		
	2	278.1 +/- 2.4		
	5	304.3 +/- 25.8		
	24	369.1 +/- 22.1		

Table 3.2: Cd concentration (μ g/g) of zebrafish tissues after 7 days of feeding on either chironomids or pellet food, followed by 3 days of gut clearance. Data are represented as mean ± standard error (N=6, except experiment 4 'Hwb' treatment in which N=5). Different small-case letters indicate significant differences among tissues within the same treatment.

	Zebrafish fed Chironomids	Gill	Gut	Liver	Kidney	Carcass
Exp.1	Control	0.18±.01 ^{ac}	0.95±.11 ^D	0.02±.01 ^a	0.26±.06 ^c	0.00±.00 ^a
	High Cd water-borne	0.86±.07 ^ª	5.66±.60 ^b	2.83±.61 °	4.22±.54 ^{bc}	0.14±.01 ^ª
Exp.2	Sediment exposed	0.64±.10 ^ª	4.75±1.04 ^b	1.31±.38 ^ª	3.64±0.56 ^b	0.15±.02 ^a
	High Cd water-borne	0.68±.06 ^ª	4.13±.49 ^b	1.35±.21 ^a	4.62±.55 ^b	0.14±.01 ^a
	Echo sediment	0.89±.15 ^ª	0.43±.08 ^{ab}	0.89±.18 ^ª	2.24±.37 ^c	0.07±.01 ^b
	Echo high Cd water-borne	0.86±.23 ^ª	0.53±.13 ^a	1.25±.24 ^a	3.39±.75 ^b	0.09±.02 ^a
Exp.4	Control	0.22±.02 ^a	1.26±.29 ^{bc}	0.60±.11 ^{ac}	2.01±.30 ^b	0.06±.01 ^a
	Low Cd water-borne	0.26±.11 ^ª	3.88±1.58 ^b	$0.93 \pm .38^{ab}$	2.31±.94 ^{ab}	0.09±.04 ^ª
	High Cd water-borne	0.90±.07 ^a	3.62±1.01 ^{bc}	1.46±.48 ^{ac}	3.38±.36 ^{bc}	0.14±.02 ^a
	Echo control	0.35±.08 ^a	0.37±.05 ^ª	0.47±.05 ^a	1.37±.34 ^b	0.05±.01 ^a
	Echo low Cd water-borne	0.60±.09 ^a	0.50±.12 ^ª	0.67±.12 ^ª	2.82±.63 ^b	0.09±.01 ^ª
	Echo high Cd water-borne	0.89±.17 ^a	1.04±.21 ^a	1.11±.11 ^ª	3.31±.42 ^b	0.13±.02ª
	Zebrafish fed Pellets					
Exp.3	Control	0.03±.00 ^a	0.12±.02 ^⁵	0.03±.02 ^a	0.17±.02 ^b	0.00±.00 ^a
	Low Cd	0.68±.10 ^ª	5.48±.71 ^b	0.52±.06 ^ª	2.29±.18 [°]	0.13±.01 ^ª

High Cd	0.51±.04 ^{ac}	12.07±1.91 ^b	1.11±.18 ^{ac}	3.99±.61 ^a	0.18±.01 ^c
Echo control	0.02±.00 ^a	0.13±.02 ^b	0.01±.00 ^a	0.14±.02 ^b	0.00±.00 ^ª
Echo low Cd	0.31±.04 ^a	0.26±.03ª	0.15±.06 ^{ac}	0.58±.10 ^b	0.06±.01 ^c
Echo high Cd	0.37±.04 ^a	1.38±.59 ^{ab}	0.42±.04 ^a	1.87±.40 ^b	0.11±.02ª

Table 3.3: Trophic Transfer Efficiencies (units = % of ingested Cd accumulated over 7 days) of Cd from *C. riparius* and pellet foods to zebrafish after 7 days of treatment feeding, and 3 days of gut clearance. Treatment values were corrected for both controls and echo, except for the control treatment in experiment 1 (see text). Data are represented as mean \pm standard error (N = 6 for all, except for experiment 2 'sediment exposed' treatment in which N=5). Small case letters indicate significant differences among treatments within the same experiment.

Chironomid treatment	Exp.1	Exp.2	Exp.4	Pellet Treatment	Exp.3
Control	9.20±1.03 ^a	-	12.22±7.6 ^ª	Control	12.54±2.12 ^a
Sediment Exposed	-	1.99±.46 ^ª	-	Low Cd	0.74±.15 ^b
Low Cd Water-borne Exposed	-	-	0.63±.15 ^ª	High Cd	0.73±.10 ^b
High Cd Water-borne Exposed	1.23±.22 [♭]	0.74±.20 ^b	0.86±.15 ^ª		

Table 3.4: Percent subcellular compartmentalization of Cd in both chironomids and zebrafish after a week long feeding experiment followed by 3 days of gut clearance. Data are represented as mean := standard error (N=6 for all treatments except for experiment 2 'Echo high Cd water-borne treatment' in which n=5). Different small-case letters indicate significant differences among treatments within the same experiment, different capital letters indicate significant difference among treatments across experiments 3 and 4. Compartment categories: MRG - metal rich granules; ORG - organelles; ENZ - enzymes (or heat-denaturable proteins); CD - cellular debris; and MTLP - metallothionein-like proteins. Note that the % recovery for chironomids was 72±13 %, while the % recoveries for zebrafish were 88±8 %, 83±7 %, and 81±6 % for experiment 2, 3, and 4 respectively. Data were arc-sine transformed before one-way ANOVA and Holm-Sidak comparisons: were made. Please note that supplementary figures of this data are included in the appendix of Chapter 4.
	Chironomids	MRG	ORG	ENZ	CD	MTLP
	Control	38.5±3.4 ^a	33.1±3.0 °	20.8±0.6 ^a	0.9±0.1 ^a	6.7±0.2 ^a
	Low Cd water-borne	49.8±0.7 ^a	31.1±1.8 ª	12.6±1.8 ^b	4.1±0.5 ^b	2.4±0.1 ^b
	High Cd water-borne	46.6±1.6 ^ª	32.8±1.4 ^a	13.0±0.1 ^b	5.5±0.2 ^b	2.2±0.1 ^b
	Sediment exposed	43.3±3.9 ^ª	34.6±4.2 ^ª	16.6±0.5 ^{ab}	1.3±0.1 ^a	4.2±0.2 ^c
	Zebrafish fed Chironomids					
Exp.2	Sediment exposed	44.6±8.1 ^a	35.4±6.5 ^a	15.0±1.5 ^ª	2.1±0.5 ^a	5.4±0.5 ^a
	High Cd water-borne	43.0±5.3 ^ª	25.8±1.8 ^a	14.9±2.4 ^a	1.2±0.3 ^a	4.2±0.8 ^a
	Echo sediment	30.9±5.3 ^ª	33.6±4.0 ^{°a}	37.5±5.1 ^b	1.2±0.4 ^a	0.7±0.2 ^b
	Echo high Cd water-borne	51.5±1.9 ^ª	36.2±3.6 ^a	14.9±1.0 ª	1.5±0.4 ^ª	1.1±0.4 ^b
Exp.4	Control	6.3±1.4 ^{aAD}	62.3±3.9 ^{aA}	21.2±4.9 ^{ªA}	0.2±0.1 ^{aAD}	10.0±1.7 ^{aA}
	Low Cd water-borne	8.5±1.5 ^{aADE}	48.6±2.6 ^{bAC}	37.2±3.2 ^{bcAC}	0.3±0.1 ^{aAD}	5.4±1.8 ^{ªA}
	High Cd water-borne	8.2±1.1 ^{aADE}	45.6±1.9 ^{bAC}	39.3±3.3 ^{bAC}	0.2±0.2 ^{aAD}	6.7±1.7 ^{aA}
	Echo control	9.8±1.8 ^{aBD}	57.2±4.1 abAE	24.0±3.6 ^{acAD}	0.3±0.1 ^{aAD}	8.6±1.8 ^{ªA}
	Echo low Cd water-borne	9.8±1.3 ^{aBD}	52.7±0.4 abAC	27.3±2.3 abAC	1.3±0.3 ^{bBD}	8.8±1.0 ^{ªA}
	Echo high Cd water-borne	5.9±0.8 ^{aAD}	54.1±1.9 ^{abADE}	29.1±1.2 ^{abAC}	1.2±0.2 ^{bBD}	9.7±2.4 ^{aA}
	Zebrafish fed Pellets					
Exp.3	Control	19.3±4.5 ^{aBE}	60.3±4.4 ^{aAF}	23.5±5.2 ^{aAD}	0.0±0.0 ^{aA}	0.0±0.0 ^{aB}
	Low Cd	11.1±2.7 ^{aBD}	49.9±5.6 ^{abAC}	34.5±4.5 ^{aAC}	4.4±1.2 ^{bBC}	0.0±0.0 ^{aB}
	High Cd	9.0±1.7 ^{abABC}	42.5±3.7 bBCE	43.9±5.7 ^{aBCD}	4.6±0.8 ^{bC}	0.0±0.0 ^{aB}
	Echo control	2.0±0.8 ^{bAC}	36.8±3.6 bBC	47.0±5.9 ^{aBC}	5.8±1.6 ^{bC}	6.1±2.7 ^{bA}
	Echo low Cd	8.0±1.6 abCDF	44.1±3.1 abBCEF	35.0±7.0 ^{aAC}	6.0±1.1 ^{bC}	11.6±4.4 bcA
	Echo high Cd	18.1±3.2 ^{ªBEF}	38.3±1.5 ^{bBCD}	25.0±3.5 ^{aAC}	4.0±0.9 ^{bBC}	16.5±4.5 ^{cA}

Fig. 3.1A Average water pH and ammonia levels, and **Fig. 3.1B** average water Cd concentrations (n=10) over the duration of a zebrafish feeding experiment. The zebrafish were fed for 7 days with treatment food (either a control diet or high water-borne Cd exposed chironomids), followed by 3 days of gut clearance.

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Fig. 3.2 Mass-weighted Cd concentrations in *Danio rerio* tissue fractions for experiment 1. Zebrafish were fed either control, or high Cd water-borne exposed chironomids for 7 days. The body burden is the sum of the individual tissue fractions. Error bars represent standard error (N=6). An asterisk represents significant difference among treatments within each tissue fraction.



Fig. 3.3 Mass-weighted Cd concentrations in *Danio rerio* tissue fractions for experiment
2. Zebrafish were fed either sediment-exposed or high Cd water-borne exposed
chironomids for 7 days. The body burden is the sum of the individual tissue fractions.
Error bars represent standard error (N=6). Different letters indicate significant
differences among treatments within each tissue fraction.



Fig. 3.4 Mass-weighted Cd concentrations in *Danio rerio* tissue fractions for experiment
3. Zebrafish were fed either: control, low Cd, or high Cd pellets (commercially manufactured food spiked with Cd and subsequently pelletized) for 7 days. The body burden is the sum of th€ individual tissue fractions. Error bars represent standard error (N=6). Different letters indicate significant differences among treatments within each tissue fraction.



Fig. 3.5 Mass-weighted Cd concentrations in *Danio rerio* tissue fractions for experiment
4. Zebrafish were fed either: control, low Cd, or high Cd water-borne exposed
chironomids for 7 days. The body burden is the sum of the individual tissue fractions.
Error bars represent standard error (N=6, for all except 'high Cd water-borne' treatment
in which N=5). Different letters indicate significant differences among treatments within
each tissue fraction.



Fig. 3.6 Average Cd uptake in zebrafish from food over a 7 day period as a function of Cd concentration in the food: either *C. riparius* (both water-borne, and sediment-exposed), or a manufactured pellet food. Treatments are corrected for both controls and echo Cd contamination. Solid lines represent regressions for biologically stored food and manufactured food. The dashed line represents an alternate regression for biologically stored food and errors (N= 6 for each data point).



Fig. 3.7 Correlations of percent Cd transfer from chironomids (*C. riparius*) to zebrafish (*Danio rerio*), and trophically available metal (TAM) and metabolically available metal (MAF) fractions. Error bars represent standard error (N = 6 for each data point).



% Cd in Available Fractions of Chironomids

CHAPTER 4

Concluding Remarks

As indicated in Chapter 1, chironomids have previously been identified as extremely tolerant to metal stress (Wentsel *et al.*, 1977; Winner *et al.*, 1980; USEPA, 2000). However, direct comparisons of the toxicity of metals to 1st instar (the most sensitive life stage) *C. riparius* larvae against water quality guidelines had not been made. Our study provided these comparisons, determining that even at the most sensitive life stage, *C. riparius* are well protected by current guidelines. Thus far, the capacity of chironomids to tolerate and accumulate large amounts of metal (Yamamura *et al.*, 1983; Seidman *et al.*, 1986; Hare *et al*, 2001; Gillis and Wood, 2007) has not been exploited to any great extent. We used chironomids as a vector for Cd transfer to zebrafish because of this very ability.

Our most important finding was that TTE was dependent on route of prey exposure (water-borne or sediment/dietary), with zebrafish fed on chironomids that had been Cd-exposed through the sediment/dietary route exhibiting approximately double the TTE of zebrafish fed on water-borne exposed chironomids. This is in direct contrast to what we had originally hypothesized based on a similar experiment with *Daphnia magna* fed to zebrafish (Liu *et al.* 2002).

Our second prediction, that a more 'natural' food (chironomids) would have a higher TTE when fed to zebrafish than a manufactured pellet food, was only partially supported. In fact, TTE's of Cd were identical between zebrafish fed chironomids that had been exposed through a water-borne route, and zebrafish fed a manufactured pellet diet spiked with Cd. However, if we consider that the chironomids exposed via the 15

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day sediment/dietary route was a more 'natural' exposure, than TTE's were higher by almost 3-fold when zebrafish were fed this more 'natural' diet than when they were fed either chironomids that had been water-borne exposed, or Cd-spiked pellets. Our findings suggest that the exposure route of prey is a more important factor (i.e. how Cd is taken up in prey and how available it is to the predator) than if the food source is a biological organism or manufactured.

Zebrafish which were fed Cd-contaminated food (whether it was chironomids that were water-borne exposed, or manufactured - pellets) accumulated Cd in a manner which was proportional to Cd concentration in the food. This was in accord with previous work (Van Campenhout *et al.*, 2007; Ng and Wood, in submission), and our original prediction that TTE would not be concentration-dependent.

Tissue-specific accumulation of the zebrafish in decreasing order was: gut \sim kidney > liver > gill > carcass, regardless of whether zebrafish were fed on sedimentexposed chironomids, water-borne exposed chironomids, or pellet food. Our prediction that the highest accumulation would occur in the gut tissue proved correct. These results are consistent with research on other fish, and are likely due to the gut acting as a barrier to the internalization of Cd (Chowdhury *et al.*, 2004; Franklin *et al.*, 2005; Wood *et al.*, 2006). The high concentrations of Cd in the kidney and liver also make sense, given that they are the major detoxifying organs of the body.

On a mass-adjusted basis, tissue-specific uptake of Cd was consistent, whether zebrafish were fed chironomids or pellet food. We found that the gut accounted for the largest percentage of overall body burden of zebrafish, followed by the carcass, then liver, gill, and kidney. Again, these results are consistent with out hypotheses, and with

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those of other studies (Szebedinszky *et al.*, 2001; Franklin *et al.*, 2005; Ng and Wood, in submission).

Lastly, we found that route of exposure does influence Cd subcellular storage in chironomids, but only slightly. Of the five subcellular fractions: metal rich granules (MRG), organelles (ORCi), enzymes (ENZ), metallothionein-like proteins (MTLP), and cellular debris (CD), the main areas of storage in chironomids were consistently the MRG and ORG fractions regardless of exposure regime (Fig. 4A.1). However, we did note that chironomids that were exposed via the sediment/diet had higher amounts in the MTLP fraction, and lower amounts in the CD fraction compared with chironomids that were exposed via the water. This change was not high (2-4%), however it was consistent (Fig. 4A.1). Our hypothesis was that chironomids would have similar subcellular Cd distribution as that found for a deposit-feeding polychaete, with the majority of the Cd in the debris (MRG +CD) fraction (Selck and Forbes, 2004). Our results at least partially supported this hypothesis in that we found a high amount of Cd in the MRG fraction (40-50%). However, we found a relatively low amount in the CD fraction (1-5%). The study by Selck and Forbes (2004) on which we based our hypothesis did not separate out MRG and CD, so our hypothesis can be considered correct. In general, the greatest accumulations of Cd in cl ironomids were seen in the MRG > ORG > and ENZ fractions.

In zebrafish, subcellular storage of Cd was very similar to that of chironomids, with ORG, ENZ, and MRG accounting for ~90% of all Cd, although it was interesting to note that there was significantly less Cd in the MTLP fraction when zebrafish were fed chironomids (regardless of prey exposure) than when zebrafish were fed pellets (Figures 4A.2, 4A.3, and 4A.4).

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In conclusion, we determined that chironomids facilitate the transfer of sedimentassociated metals (in a realistic range for a polluted environment- Wallace *et al.*, 1998) to higher trophic levels, with a greater TTE than chironomids only exposed through the water. This has direct implications for environmental toxicology. For example, even though the TTE of zebrafish fed sediment-exposed chironomids is low (<2%), there is a potential for high accumulation by the predator species, particularly if their diet is chronically metal-rich as occurs in some mine-impacted rivers (Woodward *et al.*, 1995; Farag *et al.*, 1999). This thesis has also pointed the way to further research in terms of investigating the TTE's of trophic routes between other organisms, and in particular examining the subcellul ϵ r compartmentalization of subsequent Cd transfer. Future research could also address the subcellular partitioning of Cd along a time-continuum in order to determine whether the storage of Cd gradually shifts between compartments in predator or prey, as this has been noted in trout (Ng and Wood, in submission).

Appendix A

Supplementary Figures

Fig. 4A.1 The relative subcellular distribution of Cd in chironomids that were; not exposed to Cd (Control), loaded to a low level of Cd through a water-borne exposure (Lwb), loaded to a high level of Cd through a water-borne exposure (Hwb), or loaded to a low level of Cd through a sediment exposure (Lsed). Subcellular categories are: MRG - metal rich granules; ORG - organelles; ENZ - enzymes (or heat-denaturable proteins); CD - cellular debris; and MTLP - metallothionein-like proteins.



Fig. 4A.2 The relative subcellular distribution of Cd in zebrafish that were fed either chironomids that had been loaded to a level of Cd via a chronic sediment/dietary route (Lsed), or zebrafish that were fed chironomids that had been loaded to a high leveo of Cd via a water-borne route (Hwb). 'Echo' fish are those that were only exposed to the water-borne Cd from corresponding 'Treatment' fish tanks. Subcellular categories are: MRG - metal rich granules; ORG - organelles; ENZ - enzymes (or heat-denaturable proteins); CD - cellular debris; and MTLP - metallothionein-like proteins.



Fig. 4A.3 The relative subcellular distribution of Cd in zebrafish that were fed pellet food with; no Cd added (control), a low level of Cd (Lpel), or a high level of Cd (Hpel). 'Echo' fish are those that were only exposed to the water-borne Cd from corresponding 'Treatment' fish tanks. Subcellular categories are: MRG - metal rich granules; ORG organelles; ENZ - enzy nes (or heat-denaturable proteins); CD - cellular debris; and MTLP - metallothionein-like proteins.



Fig. 4A.4 The relative subcellular distribution of Cd in zebrafish that were fed; control chironomids, chironomids loaded to low levels of Cd via a water-borne exposure (Lwb), or chironomids loaded to high levels of Cd via a water-borne exposure (Hwb. 'Echo' fish are those that were only exposed to the water-borne Cd from corresponding 'Treatment' fish tanks. Subcellular categories are: MRG - metal rich granules; ORG - organelles; ENZ - enzymes (or heat-denaturable proteins); CD - cellular debris; and MTLP - metallothionein-like proteins.



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