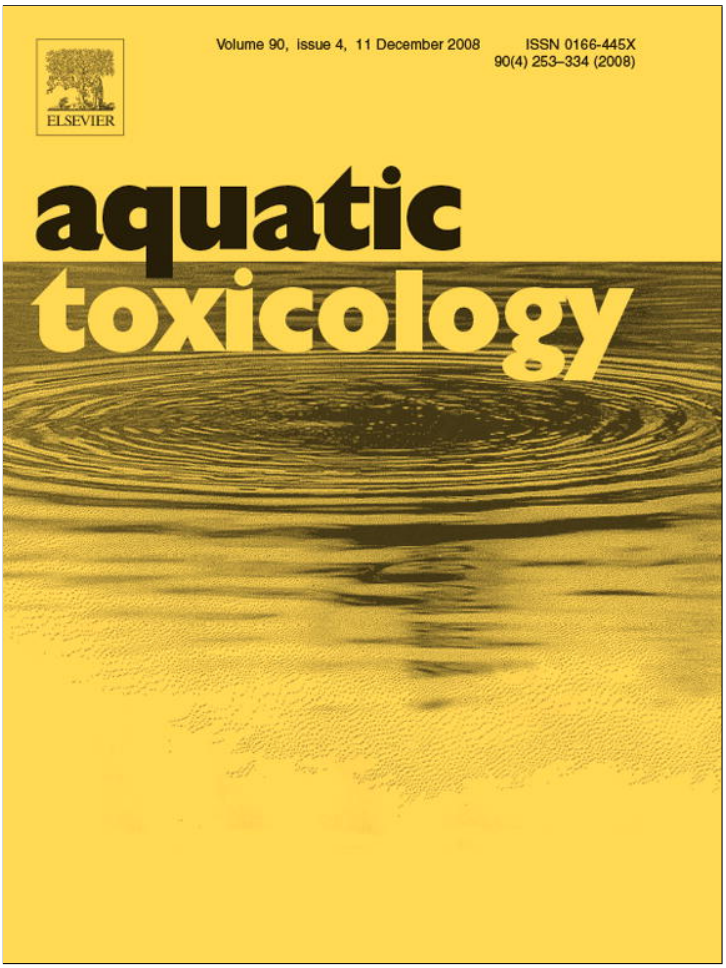

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Trophic transfer of Cd from larval chironomids (*Chironomus riparius*) exposed via sediment or waterborne routes, to zebrafish (*Danio rerio*): Tissue-specific and subcellular comparisons

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ABSTRACT

Zebrafish were fed chironomid larvae (8% wet weight daily ration) for 7 days, followed by 3 days of gut clearance in a static-renewal system. Regardless of whether the chironomids had been loaded with Cd via a waterborne exposure or sediment exposure, they had similar subcellular distributions of Cd, with the largest areas of storage being metal rich granules (MRG) > organelles (ORG) > enzymes (ENZ) except that sediment-exposed chironomids had significantly more Cd in the metallothionein-like protein (MTLP) fraction, and significantly less Cd in the cellular debris (CD) fraction. When zebrafish fed sediment-exposed chironomids (153 ± 11 μg Cd/g dry weight) were compared directly to zebrafish fed waterborne exposed chironomids (288 ± 12 μg Cd/g dry weight), identical whole-body Cd levels were observed, despite the difference in the concentration in the food source. Thus trophic transfer efficiency (TTE) of Cd was significantly greater from sediment-exposed chironomids ($2.0 \pm 0.5\%$) than from waterborne-exposed chironomids ($0.7 \pm 0.2\%$). Subsequent tests with waterborne exposed chironomids loaded to comparable Cd concentrations, as well as with Cd-spiked manufactured pellets, demonstrated that TTEs 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. However, neither trophically available metal (TAM) nor metabolically available fractions (MAF) were good predictors for the TTEs found in this study. 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 ≥71% to total body burdens on a mass-weighted basis. This study presents evidence that Cd may be acquired by fish from natural diets at levels of environmental relevance for contaminated sites, and that the exposure route of the prey influences the TTE.

1. Introduction

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 (Meyer et al., 2005). Some investigators have suggested that the metal stored in 'natural' food (i.e. typical prey items) may be in a form that is more available to predators than the manufactured food often used to investigate trophic transfer in the laboratory (Harrison and Curtis, 1992; Meyer et al., 2005; Ng and Wood, 2008). However, little is known about how the route of prey exposure (e.g. dietary or waterborne) affects the storage of metals in the predator. Liu et al. (2002) present one of the few studies where the exposure route of prey was considered. They fed *Daphnia magna* that were radiolabeled with Cd either via waterborne or via dietary exposure to zebrafish, and found no difference in the efficiency of Cd transfer between these two groups (Liu et al., 2002). 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). As Cd has no biological function, it needs to be either detoxified or excreted (Rainbow, 2002) and depending on how these mechanisms interact, there may be a potential for Cd to bioaccumulate up trophic levels. While metals are not normally considered to bioaccumulate since most of them are non-lipophilic (Timmermans et al., 1989), Croteau et al. (2005) found

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that within two trophic levels secondary poisoning occurred (from primary producers to invertebrates and fish); Cd concentrations had magnified 15-fold. This study will investigate the effect of prey exposure route on TTE of Cd from insect larvae to zebrafish, as well as determine the effect of prey exposure route on the subcellular storage of the transferred Cd.

Subcellular components like organelles (ORG) and enzymes (ENZ) are considered vulnerable to metal exposure (i.e. non-specific binding), and as such the metals bound to these components are considered metabolically available (Wallace et al., 2003). Both metallothionein-like proteins (MTLP) (Wallace and Lopez, 1997), and the debris fraction (cellular debris, CD) (Steen Redeker et al., 2007) have been suggested as available fractions which could contribute to elevated TTE. Taking these observations into consideration, we chose to use two measurements: trophically available metal (TAM) and metabolically available fraction (MAF), to quantify metal availability. TAM is the sum of the ORG, ENZ, and MTLP fractions (Wallace and Luoma, 2003), and MAF is the sum of the ORG, ENZ, and CD fractions (Steen Redeker et al., 2007).

Chironomid larvae (*Chironomus riparius*) were chosen as a model prey item because they accumulate large amounts of Cd (Yamamura et al., 1983; Seidman et al., 1986; Hare et al., 2001; Gillis and Wood, in press-a, in press-b) yet are known to be resistant to metal toxicity (Wentsel et al., 1977; Winner et al., 1980; USEPA, 2000). They are therefore well protected by the current environmental guidelines for Cd (Béchard et al., 2008). Also, since chironomid larvae are epibenthic feeders, both living in, and feeding at the sediment water interface (Pinder, 1986), they are vulnerable to piscine predators, and are potentially an important link in trophic transfer routes.

Zebrafish (*Danio rerio*) were chosen as a model predator species for several reasons. Firstly because their small size makes the issue of supplying a sufficient amount of natural food feasible, since there are mass constraints on the rate at which Cd-contaminated natural prey can be produced. Secondly, native to India, they thrive in ion-poor softwater (Boisen et al., 2003) representative of metal-sensitive waters in many parts of the tropics, as well as in North America and Europe (Wright and Henriksen, 1978; Hare and Tessier, 1998). Thirdly, since the zebrafish genome is now publically available (Lo et al., 2003), our research may provide a link to future toxico-genomic work (Neumann and Galvez, 2002).

Our main objectives were to determine the effects that route of prey exposure (waterborne or sediment/dietary), and type of prey exposure (natural food with biologically incorporated Cd, or manufactured food artificially contaminated with Cd), has on TTE. This study provides a novel contribution to the understanding of metal trophic transfer in three main ways: (1) it compares the subcellular storage of Cd in the prey organism loaded via two different exposure routes (waterborne or sediment/dietary) with the TTE of Cd to the predator zebrafish; (2) it compares the subcellular compartmentalization and tissue-specific storage of Cd in the zebrafish with respect to these different prey exposure routes; and (3) it directly compares the relative importance of a natural versus a manufactured diet in the trophic transfer of dietary Cd.

2. Materials and methods

2.1. Chironomid culture

A continuous culture of the non-biting midge *C. 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 (from Lake Ontario) as the overlying culture water: hardness 140 mg/L as $CaCO_3$ equivalents, pH 8.05, dissolved organic carbon approximately 3.0 mg L^{-1}, $[Na^+] = 0.6$, $[Cl^-] = 0.8$, $[Ca^{2+}] = 1.8$, $[K^+] = 0.4$, $[Mg^{2+}] = 0.5$, $[Cd] < 5.0 \times 10^{-7}$ mmol L^{-1}. The cultures were aerated, held at 21 ± 2 °C under a 16:8 h light:dark photoperiod regime, and fed crushed Nutrafin® fish flakes *ad libitum*. The larvae reached the 3rd instar in approximately 2 weeks. Chironomid larvae (3rd to 4th instar, 3–4 mg each) were harvested for the trophic transfer experiments using transfer pipettes and transferred to beakers containing culture water. A more detailed description of the culture methods can be found in Gillis and Wood (2008a, 2008b).

2.2. Chironomid preparation

2.2.1. Storage of chironomids

Because our culture produced 3rd to 4th instar larvae at a rate of approximately 335 per week, we collected larvae for 9 weeks to build up enough food supply for an experiment. Control chironomids were rinsed with clean culture water, blotted dry with filter paper and packaged in parchment paper for freezing (−20 °C). Each packet represented about 4% of the body weight of a typical large zebrafish. Tests were conducted to ensure that there was no loss of mass or Cd content during the period of freezer storage.

2.2.2. Preparation of chironomids exposed to waterborne 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) μg Cd/L as $Cd(NO_3)_2$ in culture water. Water samples were taken at the start and end of the exposure to verify Cd concentrations. After 48 h, larvae were rinsed with culture water, gently blotted dry, and packaged as above.

2.2.3. Preparation of chironomids exposed to sediment-borne Cd

Sediment, collected near Long Point, Lake Erie, ON (42°33′54″″N, 80°02′028″W) (composition: 60.9% silt, 33.5% clay, 5.6% sand, and 0.35% organic carbon,) was artificially contaminated (spiked) with $Cd(NO_3)_2$ in Nanopure® water, to a nominal concentration of 10.9 mg Cd/kg dry weight sediment. According to Wallace et al. (1998), this is a realistic range for a polluted environment. The Cd contaminated sediment was kneaded thoroughly by hand, daily, for 5 days, after which it was left to age for 10 months. The sediment was thoroughly re-mixed before use in experiments. A ratio of 1 L sediment to 3 L aerated culture water (composition as above) in a 6 L vessel was used, and allowed to settle for 3 days (depth 2.1–2.3 cm) before approximately 250 2nd instar larvae were added. Sediment-exposed chironomids were fed 160 mg of dry ground food (Big Al's Tropical Fish Food, Hamilton ON) three times weekly via addition of a food slurry to the overlying water. After 2 weeks, larvae (now 3rd or 4th instar) were collected from the sediment, rinsed twice with culture water, blotted dry, and packaged as above.

Overlying water and sediment samples were taken at the beginning and end of each exposure. Dialysis "minipeepers" (Doig and Liber, 2000) were used to sample the pore water, as well as the water 1–4 mm above the sediment–water interface.

2.3. Leaching test

A simple experiment was conducted with frozen chironomids (controls and those exposed to waterborne Cd) in the soft water (composition below) used in the zebrafish feeding experiments to determine if Cd was leaching out of the chironomids into the water during the tests. Test containers were set up mimicking experimental conditions. One packet of control chironomids (before exposure measuring 3.3 ± 0.1 μg Cd/g dry weight) was put into each of 3 test vessels, and one packet of Cd contaminated chironomids (282 ± 27 μg Cd/g) was put into each of 14 test vessels.

Cd-contaminated chironomids were removed from two of the vessels at 0, 0.17, 0.5, 1, 2, and 5 h. The control chironomids and remaining Cd-contaminated chironomids were removed after 24 h. Water samples were taken from each vessel at time zero, and upon removal of chironomids. Both chironomid and water samples were analyzed for Cd concentration.

2.4. Preparation of pellet food

Pellets were made by grinding up Big Al's Staple Food (tropical fish flakes) in a blender, adding Nanopure® water alone, or Nanopure® water spiked with $Cd(NO_3)_2$ and processing the resulting dough through a pasta maker. After strings of dough were dried, they were cut into pellets of approximately 2 mg each, a size that could easily be eaten by the zebrafish. Pellets were weighed out 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 ± 5, and a high Cd treatment of 312 ± 20 μg Cd/g dry weight of pellet food.

2.5. Acclimation and training of zebrafish

Zebrafish, approximately 0.5 g each, purchased from PetSmart®, Hamilton ON, were acclimated from moderately-hard Hamilton tap water to a much softer, ion-poor water containing 4% dechlorinated Hamilton tap water and 96% reverse osmosis water, hereafter referred to as soft water, over 7 days. This soft water contained a pH of 7.13 at 29 °C and (in μmol/L): $[Na^+] = 45.2$, $[Cl^-] = 43.9$, $[Ca^{2+}] = 29.0$, $[K^+] = 0.9$, $[Mg^{2+}] = 13.1$. Prior to the experiment, zebrafish were trained to eat frozen chironomids from a commercial source (Sally's Bloodworms®, Big Al's, Hamilton, ON) for 1 week. Three days before the start of an experimental series, the fish were randomly separated into individual test vessels and training feeding continued. These 1.75 L containers held 1 L of aerated very soft water and were subjected to 80% daily water changes of the static system, without air exposure of the fish.

2.6. Trophic transfer experimental designs

Zebrafish were split into treatment groups and were fed specific pre-weighed treatment diets in four separate series (described below). All fish were held in soft water in individual 1.75 L containers and fed one packet of chironomids (4% of body weight) twice daily, 8 h apart. The same ration, 4% of body weight was used for the pellet diets. This feeding regime was used for all experiments in this study and throughout the entire trial including: the last 3 days of training the 7 days of experimental food treatment, and the 3 days of gut clearance. One hour before the first feeding, each zebrafish was weighed and its fork length recorded. One hour after the second feeding of the day, 80% (800 mL) of the tank water was removed and replaced; in most cases all of the food had been consumed by this time, and all had been consumed by 24 h. A static-renewal system was used to ensure that the zebrafish ate all of the food provided, 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 just before the water change (one hour after the second feeding) and a second was taken just after the change. All water samples were analyzed for pH, Cd and ammonia concentrations. The feeding experiment continued for 7 days and was followed by 3 days of gut clearance during which the zebrafish were fed control chironomids (series 1 and 2), control pellets (series 3), or Sally's Bloodworms® (series 4) to purge any Cd-contaminated food from their guts. 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, BC) of very soft water (pH 7.2) and rinsed twice with soft water before being weighed and measured for fork length. Half of the fish from each treatment group were then sacrificed and dissected into five parts; gill, gut, liver, kidney, and carcass (all series), while the other half were frozen intact for subsequent subcellular fractionation (series 2–4). We attempted to determine the subcellular distribution of Cd in the dissected parts of the zebrafish from series 1, however the individual tissues did not provide enough mass for the analysis. Thus, there are no subcellular fractionation data for series 1.

Water samples from experimental series 1 revealed residual concentrations of Cd in the water resulting from feeding, which were not seen in the leaching test (above). To account for the waterborne Cd exposure that the treatment fish were receiving, all subsequent series of experiments incorporated a second set of fish (for all treatments) that were fed a control diet, and underwent a comparable waterborne exposure. This second set of fish, herein after referred to as '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 μm to remove any particulate waste. Echo fish were measured and sampled the same way as described above for treatment fish.

2.7. Experimental series

In series 1, zebrafish ($N = 10$ per treatment) were fed either control chironomids or high Cd chironomids (exposed to water-borne Cd). This purpose of this initial series was to determine if our methods were sensitive enough to detect the uptake of Cd in the five zebrafish tissue fractions (gill, gut, liver, kidney and carcass) in comparison to control fish.

In series 2, zebrafish ($N = 12$ per treatment) were fed either high Cd chironomids (exposed to waterborne Cd) or chironomids that had been exposed to sediment-borne Cd. Series 2 was conducted to screen for potential differences in TTE due to exposure route of prey. Controls for this series were not conducted due to prey-generation time constraints and thus series 1 control chironomids were used for comparison.

In series 3, zebrafish ($N = 12$ per treatment) were fed either a control pellet diet, or a low Cd pellet diet (matching the amount of Cd found in sediment exposed chironomids), or a high Cd pellet diet (matching the amount of Cd found in the high Cd waterborne exposed chironomids). This series was conducted in order to differentiate whether the differences in TTE observed in series 2 were due to exposure route of prey (sediment or water-borne) or concentration of Cd in the food (prey).

In series 4, zebrafish ($N = 12$ per treatment) were fed either control chironomids, or chironomids that were loaded to a low Cd concentration by waterborne exposure (matching the level of Cd found in sediment exposed chironomids, and the low Cd pellet diet), or chironomids that were loaded to a high Cd concentration by waterborne exposure. The purpose of this experiment was to determine if the results from experiment 3 (with manufactured food) could be duplicated with a natural food source (i.e. chironomids).

Series 2–4 had 'echo' treatments incorporated; the echo fish in series 2 and 4 were fed chironomids, and in series 3 were fed control pellets.

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

Lwb: Chironomids loaded to a 'low' concentration via a waterborne exposure.

Hwb: Chironomids loaded to a 'high' concentration via a waterborne 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.

2.8. Subcellular fractionation

Subcellular fractionations for both whole chironomids and whole zebrafish were completed according to Wallace et al. (2003), with a few modifications. A sucrose buffer containing in mM: sucrose (250), tris(hydroxymethyl)aminomethane (20), KCl (25), and $MgCl_2$ (5), in Nanopure™ water, adjusted to pH 7.4 with HCl was used. Also, immediately before subcellular fractionation, an anti-oxidant (2-mercaptoethanol, 2 mM) and a protease inhibitor (PMSF, 0.2 mM) were added to the 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 Tearor® (Biospec Products Inc., Dremel, Racine WI, USA). At this point in the sample preparation, a sub-sample was removed from each resultant homogenate for calculating percent recovery. The homogenate was centrifuged for 15 min at 4 °C after which the pellet (debris) was set aside and the supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C. The resultant pellet was the organelle fraction (ORG), and the supernatant was then heated at 80 °C for 10 min and spun at 30,000 × *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 × *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 series 2, 3, and 4 respectively.

2.9. Analytical techniques and calculations

All water samples were filtered through an Acrodisk® 0.45 μm in-line-syringe-tip filter, to determine dissolved metal concentration in the exposures. 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 trace metal grade HNO_3 (67–70%) was added and allowed to digest for 1 week. Following the acid digestion, 20 μL of H_2O_2 (30%) per mg of sediment was added and allowed to digest for 1 day. Chironomid tissues were digested using 100 μL/mg dry weight tissue of the same full strength HNO_3 (67–70%) for 6 days at 60 °C followed by 40 μL/mg dry weight of H_2O_2 (30%) for 1 day (per Croteau et al., 2002). Fish tissues were digested with 3-5 μL 1N HNO_3/mg wet weight for 48 h. Samples were vortexed immediately after acid was added, then again at 24 h, and at 48 h (per Chowdhury et al., 2004).

Water samples and tissue and sediment digests were analyzed for Cd using a GTA 110 Varian Graphite Furnace Atomic Absorption Spectrometer (AA) (Varian Techtron, Mulgrave, Victoria, Australia). The Cd standard was made using a Cadmium Reference Solution from Fisher Scientific (Nepean, ON). Appropriate reference materials were processed in the same manner as experimental samples in every analytical run. Percent recovery was always within ±10% for water Cd based on analytical reference material TM15 (Environment Canada, National Water Research Institute, Burlington, ON), and within ±15% for digests based on analytical reference material 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 of the latter reference materials from the Institute for National Measurement Standards, National Research Council of Canada, Ottawa, ON). Data are reported as recovered metal and are not corrected for percent recovery.

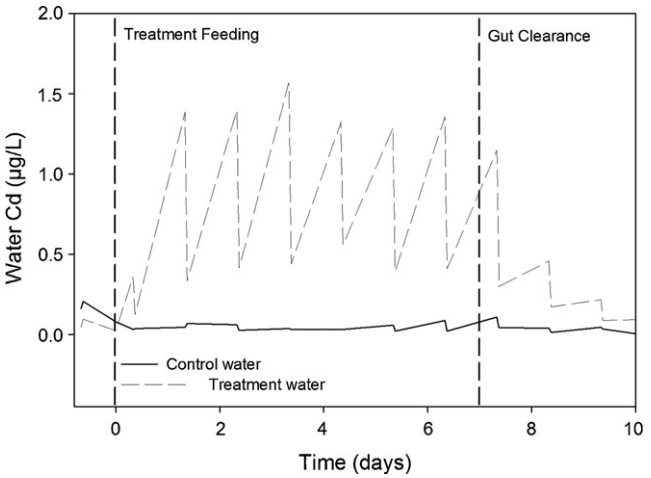

Fig. 1. Average water Cd concentrations (N = 10) over the duration of a zebrafish feeding experiment. The zebrafish were fed for 7 days with treatment food (either a control diet or high waterborne Cd exposed chironomids), followed by 3 days of gut clearance.

Water samples from the trophic transfer experiments were also monitored for pH (PHM82 Standard pH meter, Radiometer, Copenhagen, Denmark) and ammonia (salicylate hypochlorite method of Verdouw et al., 1978) and are reported in Fig. 1.

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). Figs. 2–5 report these mass-adjusted values for zebrafish tissue fractions (i.e. the fractions add up to the whole-body burden per

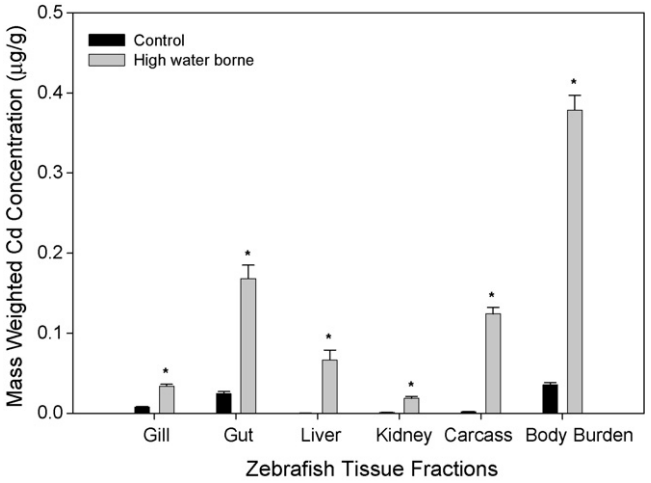

Fig. 2. Mass-weighed Cd concentrations in *Danio rerio* tissue fractions for series 1. Zebrafish were fed either control, or high Cd waterborne exposed chironomids for 7 days. The body burden is the sum of the individual tissue fractions. Error bars represent standard error (N = 10). An asterisk represents significant difference among treatments within each tissue fraction.

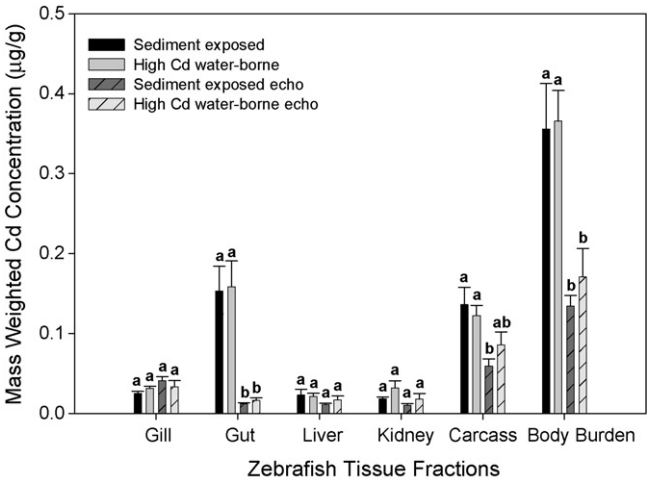

Fig. 3. Mass-weighed Cd concentrations in *D. rerio* tissue fractions for series 2. Zebrafish were fed either sediment-exposed or high Cd waterborne exposed chironomids for 7 days, followed by 3 days of gut clearance. The body burden is the sum of the individual tissue fractions. Error bars represent standard error ($N = 6$). Different letters indicate significant differences among treatments within each tissue fraction.

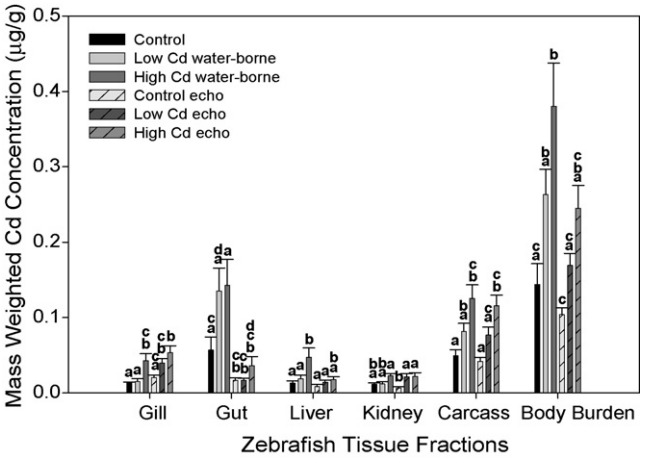

Fig. 5. Mass-weighed Cd concentrations in *D. rerio* tissue fractions for series 4. Zebrafish were fed either: control, low Cd, or high Cd waterborne exposed chironomids for 7 days, followed by 3 days of gut clearance. The body burden is the sum of the individual tissue fractions. Error bars represent standard error ($N = 6$, for all except 'high Cd waterborne' treatment in which $N = 5$). Different letters indicate significant differences among treatments within each tissue fraction.

gram whole fish), while Table 2 reports the actual Cd concentrations in individual tissues (per gram tissue).

In the subcellular fractionation measurements of chironomids, supernatant subcellular products (CD, MTLP) were digested over 48 h using the same method as for fish tissues (1N HNO_3), while MRG, ORG, and ENZ fractions were first dried completely (and weighed) before digesting with 8–12 µL 1N HNO_3/mg dry weight over 48 h at 60 °C. Samples were vortexed immediately after acid was added, and at 24 h and 48 h. Data were calculated by first reporting concentrations as µg Cd/g dry weight (MRG, ORG, and ENZ) or µg Cd/L supernatant fraction (CD and MT), after which they were converted to percents of total recovered Cd. Trophically available metal (TAM) was calculated as the sum of the ORG, ENZ, and MTLP fractions (Wallace and Luoma, 2003) and metabolically available fraction (MAF) as the sum of ORG, ENZ, and CD fractions (Steen Redeker et al., 2007).

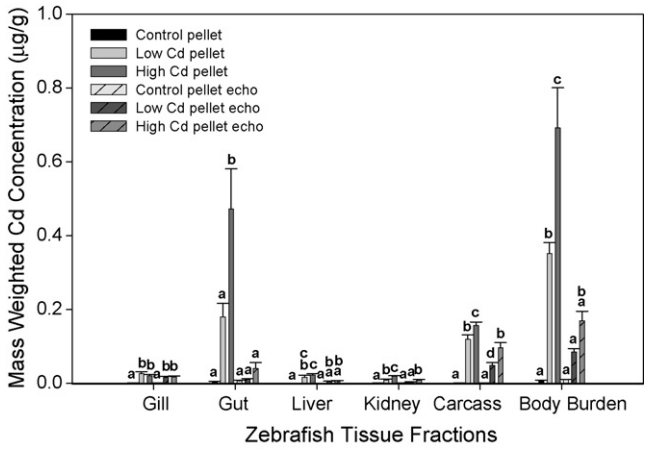

Fig. 4. Mass-weighed Cd concentrations in *D. rerio* tissue fractions for series 3. Zebrafish were fed either: control, low Cd, or high Cd pellets (commercially manufactured food spiked with $Cd(NO_3)_2$ and subsequently pelletized) for 7 days, followed by 3 days of gut clearance. The body burden is 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.

Condition factors (CF), specific growth rates (SGR) and TTEs were calculated for individual fish as follows:

$$\mathrm{CF} = \frac{\text{mass}}{\text{fork length}} \times 100\% \quad (1)$$

where mass is the mass of the fish in grams (g) and fork length is measured in centimeters (cm):

$$\mathrm{SGR} = \frac{\mathrm{Ln}(\text{mass}_{t=10}) - \mathrm{Ln}(\text{mass}_{t=0})}{\#\ \text{of days of experiment}} \times 100\% \quad (2)$$

where $\text{mass}_{t=10}$ is the mass of the fish at the end of the experiment (after 7 days of experimental food, and 3 days of gut clearance) and $\text{mass}_{t=0}$ is the mass of the fish at the initiation of the experiment:

$$\mathrm{TTE} = \left(\frac{\text{sum total of Cd in fish}}{\text{sum total of Cd fed to fish}}\right) \times 100\% \quad (3)$$

In Cd-contaminated food treatments, the 'sum total of Cd in fish' was corrected by subtracting the average Cd contamination in the corresponding 'echo' fish. This correction removes two variables: background levels of Cd in the diet, and Cd contamination due to the waterborne exposure. Thus only Cd gained by the fish from the Cd-contaminated 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.For consistency, the terminology used to describe zebrafish concentrations are as follows:

mass-weighted (tissue) = the measured concentration adjusted for mass (µg/g);
non-mass-weighted (tissue) = the measured concentration without any adjustments (µg/g);
(total) body burden = the sum of all mass-weighted tissue fractions (µg/g).

2.10. Statistical analyses

All data are presented as mean ± S.E.M. (N = number of fish) with significance taken at $p \leq 0.05$. For variance analysis among groups ANOVA was used followed by the Holm–Sidak method for pairwise multiple comparisons so as to identify individual differences. For paired comparison of two groups only, a Student's t-test was used. All statistics were completed using SigmaStat.

Table 1
The concentration of Cd in control chironomid larvae and high Cd contaminated chironomids from waterborne exposures after various durations of leaching per dry weight.

Time exposed to water (h)	Cd in chironomids (μg/g)
Control chironomids	
0	3.3 ± 0.1
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

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 ± standard error ($N = 5$ for 0 h and 24 h and $N = 3$ for intermediate times).

3. Results

3.1. Chironomid Cd concentrations

The concentrations of Cd in chironomids within identical treatments (i.e. Hwb chironomids, Lwb chironomids, etc.) across experiments (i.e. series) were not significantly different. Thus, for each treatment, the chironomid Cd concentration data were pooled, and an average treatment Cd concentration was used for TTE calculations. Chironomids exposed to 60 μg Cd/L via a waterborne exposure (Lwb) had 164.0 ± 14.5 μg Cd/g dry weight ($N = 6$ replicates of 6 chironomids each). Chironomids exposed to 260 μg Cd/L via a waterborne exposure (Hwb) had 287.9 ± 11.6 μg Cd/g dry weight ($N = 22$). Sediment-exposed chironomids (Lsed) had 153.1 ± 10.9 μg Cd/g dry weight ($N = 6$). The "training" chironomids (Sally's Bloodworms®), contained less Cd, 0.17 ± 0.09 μg/g ($N = 6$), than did controls of our cultured chironomids which had 3.41 ± 0.16 μg 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 simultaneously exposed to 5.38 ± 1.08 and 3.61 ± 0.63 μg Cd/L in the pore water and sediment-water interface water, respectively (as measured by the 'minipeepers'). The concentration of Cd in the overlying water significantly increased ($p < 0.02$) from 0.48 ± 0.10 at the start of exposure, to 1.21 ± 0.14 μg Cd/L at the end of the 15 days exposure.

3.2. Leaching test

There was no detectable Cd loss from previously frozen whole chironomids in the test water even after 24 h (Table 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 (≤0.03 μg Cd/L).

3.3. 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 0.57 ± 0.05% per day over all experiments. Condition factors were not different among experiments, and did not change from the beginning (average 1.03 ± 0.01), to end (1.03 ± 0.01) of experiments. Fish survival was 100% with the exception of series 2 (96%) and series 4 (97%).

Ammonia (12.55 ± 1.52 μmol ammonia/L) and pH levels (6.79 ± .03) in the test vessels cycled such that they were significantly higher just before an 80% daily water change than immediately after, regardless of treatment, but remained within acceptable ranges. Dissolved Cd levels (Cd treatment conditions only) similarly cycled (0.2–1.5 μg Cd/L), indicating some Cd mobilization into the water associated with zebrafish feeding that was not seen in the leaching test, and necessitating the echo treatments of series 2–4. Average dissolved Cd concentrations from fish vessels (excluding controls) were 0.58 ± 0.11, 0.70 ± 0.04, 0.73 ± 0.03, and 0.59 ± 0.03 μg Cd/L, for series 1, 2, 3, and 4, respectively. Example data from series 1, with zebrafish fed either control or high waterborne Cd exposed chironomids, are given in Fig. 1.

3.4. Trophic transfer of Cd

3.4.1. Series 1

After 7 days of feeding with 3 days of gut clearance, the mass-weighed Cd burdens were significantly higher in all tissues (gill, gut, liver, kidney, and carcass) and whole body (0.378 ± 0.021 μg Cd/g whole fish) in the zebrafish fed Hwb chironomids than in the zebrafish fed control chironomids (Fig. 2). However, there was a background amount of Cd in the control zebrafish (total body burden = 0.035 ± 0.003 μg Cd/g whole fish). In zebrafish that were fed the control chironomids, the majority of body burden was in the gut (69 ± 2%) and the gill (23 ± 2%), while in zebrafish that were fed Hwb chironomids, the body burden was more widely distributed across the five tissue fractions. On a relative basis, there was significantly less of the total Cd burden in the gill fraction ($p < 0.01$), yet, overall the gut was still the biggest contributor (40 ± 2%), followed by carcass (31 ± 2%) and liver (16 ± 3%) (Fig. 2). Non-mass-weighed Cd concentrations in individual tissues revealed that zebrafish fed control chironomids had significantly higher Cd levels in the gut than in any other tissue. Similarly, in the zebrafish fed Hwb chironomids the gut concentrations were the highest, but the kidney and liver also took up high levels of Cd (Table 2).

The TTEs were also significantly different between treatments ($p < 0.001$) with 9.20 ± 1.03% for control chironomids and 1.39 ± 0.22% for Hwb chironomids (Table 3). Note that the latter datum was corrected for the 'echo effect' using an average of Hwb echo treatments from series 2 and 4.

3.4.2. Series 2

Zebrafish fed Lsed chironomids (153.1 ± 10.9 μg Cd/g dry weight) and zebrafish fed Hwb chironomids (287.9 ± 11.6 μg Cd/g dry weight) had almost identical Cd body burdens of 0.356 ± .057 and 0.366 ± .038 μg/g whole fish, respectively, despite the different concentrations in the prey. Cd storage in these two groups of fish was similar in all tissue fractions (Fig. 3, Table 2). On a mass-weighed 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%) (Fig. 3). On a relative basis zebrafish in the echo conditions took up a significantly greater proportion of Cd in the gills than did treatment zebrafish (Fig. 3). Indeed, mass-weighed Cd tissue burdens of the echo fish were all very similar to the treatment fish in gill, liver, and kidney fractions although there were significant differences between these two groups in the gut, total body burden and between the treatment fish and 'sediment exposed echo' in the carcass (Fig. 3). The greatest Cd loads in the echo fish were in the carcass, ranging from 29 to 53%.

With respect to non-mass-weighed Cd concentrations in individual tissues, there was significantly more Cd accumulated in the gut and kidney than in gill, liver, and carcass for both zebrafish fed Lsed chironomids and zebrafish fed Hwb chironomids (Table 2). Interestingly, in both echo conditions, the kidney had much higher Cd concentrations than any other tissues (Table 2).

Table 2
The non-mass-weighed Cd concentrations in μg/g wet weight in different tissues of zebrafish after 7 days of feeding on either chironomid larvae or pellet food, followed by 3 days of gut clearance.

Zebrafish fed chironomids	Gill	Gut	Liver	Kidney	Carcass
Series 1					
Control	0.18 ± .01^{ac}	0.95 ± .11^{b}	0.02 ± .01^{a}	0.26 ± .06^{c}	0.00 ± .00^{a}
High Cd waterborne	0.86 ± .07^{a}	5.66 ± .60^{b}	2.83 ± .61^{c}	4.22 ± .54^{bc}	0.14 ± .01^{a}
Series 2					
Sediment exposed	0.64 ± .10^{a}	4.75 ± 1.04^{b}	1.31 ± .38^{a}	3.64 ± .56^{b}	0.15 ± .02^{a}
High Cd waterborne	0.68 ± .06^{a}	4.13 ± .49^{b}	1.35 ± 0.21^{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 waterborne	0.86 ± .23^{a}	0.53 ± .13^{a}	1.25 ± .24^{a}	3.39 ± .75^{b}	0.09 ± .02^{a}
Series 4					
Control	0.22 ± .02^{a}	1.26 ± .29^{bc}	0.60 ± .11^{ac}	2.01 ± .30^{b}	0.06 ± .01^{a}
Low Cd waterborne	0.26 ± .11^{a}	3.88 ± 1.58^{b}	0.93 ± .38^{ab}	2.31 ± .94^{ab}	0.09 ± .04^{a}
High Cd waterborne	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 waterborne	0.60 ± .09^{a}	0.50 ± .12^{a}	0.67 ± .12^{a}	2.82 ± .63^{b}	0.09 ± .01^{a}
Echo high Cd waterborne	0.89 ± .17^{a}	1.04 ± .21^{a}	1.11 ± .11^{a}	3.31 ± .42^{b}	0.13 ± .02^{a}
Zebrafish fed pellets	**Gill**	**Gut**	**Liver**	**Kidney**	**Carcass**
Series 3					
Control	0.03 ± .00^{a}	0.12 ± .02^{b}	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^{c}	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 ± .03^{a}	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 ± .02^{a}

Data are represented as mean ± standard error ($N = 10$ for series 1, $N = 6$ for series 2, 3, and 4, except series 4 'high Cd water-borne' treatment in which $N = 5$). Different small-case letters indicate significant differences among tissues within the same treatment.

Zebrafish fed Lsed chironomids had a significantly higher ($p < 0.03$) TTE (1.99 ± 0.46%) than zebrafish fed Hwb chironomids (0.74 ± 0.20%) (Table 3).

3.4.3. Series 3

The goal here was to determine if the TTE's of series 2 were different because of different concentrations of Cd in the prey (rather than because the chironomids were exposed by different routes). For simplicity, a pellet diet was used. Additionally, this served to clarify whether there were differences in TTE and/or tissue-specific distribution of Cd between "Cd-spiked" commercial diets *versus* natural diets (chironomids) with biologically incorporated Cd.

Zebrafish were fed control pellets (0.3 ± 0.0 μg Cd/g dry weight), low Cd pellets (Lpel; 154.4 ± 4.0 μg Cd/g dry weight) matching the amount of Cd found in Lsed chironomids, or high Cd pellets (Hpel; 291.5 ± 11.1 μg Cd/g dry weight) matching the amount of Cd found in Hwb chironomids. The mass-weighed tissue accumulations of Cd increased proportionally with concentration of Cd in the pellet food. Again, the highest overall accumulations were in the gut (49 ± 7%, 65 ± 5%) and carcass (36 ± 5%, 25 ± 4%) for zebrafish fed Lpel and Hpel food, respectively (Fig. 4). Notably, the gut fractions exceeded the carcass fractions, in contrast to previous series with chironomid diets (Figs. 2 and 3). Again, echo fish had a significantly greater percent of Cd in the gill than treatment fish, and the greatest contributor to total body burden in echo fish was the carcass ranging from 43 to 70% (Fig. 4).

With respect to non-mass-weighed Cd concentrations in individual tissues, these were markedly higher in gut than in other tissues for both Lpel and Hpel treatments, whereas concentrations in other tissues followed the patterns established in series 1 and 2. Notably, echo treatments again exhibited greater Cd accumulations in the kidney than in any other fractions (Table 2).

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). TTE's of Cd for zebrafish fed these pellet diets were comparable to those for fish fed waterborne-exposed chironomids in other series (Table 3).

Table 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.

Chironomid treatment	Series 1	Series 2	Series 4
Control	9.20 ± 1.03^{a}	–	12.22 ± 7.6^{a}
Sediment exposed	–	1.99 ± 0.46^{a}	–
Low Cd waterborne exposed	–	–	0.63 ± 0.15^{a}
High Cd waterborne exposed	1.39 ± 0.22^{b}	0.74 ± 0.20^{b}	0.86 ± 0.15^{a}
Pellet treatment			**Series 3**
Control			12.54 ± 2.12^{a}
Low Cd			0.74 ± 0.15^{b}
High Cd			0.73 ± 0.10^{b}

Treatment values were corrected for echo data (see text). Data are represented as mean ± standard error ($N = 6$ for all, except for series 1 where $N = 10$, and series 2 'sediment exposed' treatment and series 4 'high Cd waterborne' treatment in which $N = 5$). Small-case letters indicate significant differences among treatments within the same series.

3.4.4. Series 4

Series 4 mimicked series 3 except that, instead of using a pellet food, chironomids which had been exposed to waterborne Cd were used. This was to verify whether or not results from series 3, particularly the lack of dependence of TTE on Cd concentration in the pellet diet, could be duplicated with Cd-exposed chironomid larvae.

While there was a trend for Cd accumulation to be proportional to dietary load, this was significant only in liver and gill, but not in the larger mass-weighted fractions of gut and carcass, and therefore not in total body burden (Fig. 5). The biggest mass-weighed contributors to total body burden were again the gut and carcass for both zebrafish fed Lwb and Hwb chironomids (Fig. 5). Trends in mass-weighed Cd burdens in echo fish were similar to those seen in previous series.

Once again, the highest non-mass-weighed tissue concentrations of Cd were in the gut, liver, and kidney (Table 2). Non-mass-weighed levels were greatest and approximately equal in the gut tissues for zebrafish fed Lwb chironomids and Hwb chironomids, respectively (Table 2). However, in contrast to series 3 where the fish were fed pellet diets, gut tissue Cd concentrations were not markedly higher than kidney or liver concentrations. As in previous series, in echo zebrafish from all treatments, the kidney accumulated significantly higher Cd concentrations than any other tissue (Table 2).

TTEs were not significantly different between the Lwb and Hwb chironomid diets and were similar to those seen in series 3 with Lpel and Hpel pellet diets, thereby confirming that TTE is independent of dietary concentration in this range (Table 3). Again, control TTE was much higher ($p < 0.001$) than with the Cd-contaminated experimental diets (Table 3).

3.4.5. Comparisons of Cd accumulation patterns across series

In all dietary series, total accumulation of Cd was greatest on a mass-weighed basis in gut tissue followed by carcass (Figs. 2–5). 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 2). Gill Cd concentrations as well as kidney and liver in echo fish were generally comparable to those in treatment fish (Table 2), reflecting the waterborne nature of their exposure. The big differences were in carcass and especially gut fractions. Combined, the mass-adjusted gut and carcass fractions always accounted for ≥71% of the total Cd stored in zebrafish (excluding controls) (Figs. 2–5). Total accumulations of Cd in zebrafish fed Hwb chironomids were consistent, with means of 0.378 ± 0.021 μg/g whole fish for series 1, 0.366 ± 0.038 μg/g whole fish for series 2, and 0.380 ± 0.058 μg/g whole fish for series 4 (Figs. 2, 3 and 5). Furthermore, these body burdens of zebrafish fed Hwb chironomids were comparable to the body burden of zebrafish fed an Lpel diet (0.352 ± 0.030 μg/g whole fish). As well, zebrafish fed Hpel had significantly more mass-adjusted Cd in the gut (0.472 ± 0.119 μg/g whole fish), as well as a significantly greater body burden (0.692 ± 0.109 μg/g whole fish) than any other treatments in any experiment.

3.5. 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 Cd accumulated in chironomids was found in the MRG fraction (metal-rich granules), followed by the organelle (ORG) fraction, regardless of the route of exposure (Table 4). However, within the smaller fractions, chironomids that were exposed via a waterborne 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 4). This pattern was repeated in the ENZ (enzymes = heat denaturable proteins) fraction with chironomids exposed via a waterborne route having less Cd stored in the ENZ fraction than controls and Lsed chironomids, although the difference between waterborne and sediment exposed chironomids was not significant. In general, sediment exposed chironomids acted more like control chironomids than waterborne 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 (waterborne or sediment exposed chironomids). However, storage in chironomids and storage in the zebrafish of series 2 were very similar (Table 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

Table 4
Percent subcellular compartmentalization of Cd in both chironomids and zebrafish after a week long feeding experiment followed by 3 days of gut clearance.

Chironomids	MRG	ORG	ENZ	CD	MTLP
Control	38.5 ± 3.4^{a}	33.1 ± 3.0^{a}	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 waterborne	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	**MRG**	**ORG**	**ENZ**	**CD**	**MTLP**
Series 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 waterborne	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 waterborne	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}
Series 4					
Control	6.3 ± 1.4^{aA}	62.3 ± 3.9^{aA}	21.2 ± 4.9^{aA}	0.2 ± 0.1^{aA}	10.0 ± 1.7^{aA}
Low Cd waterborne	8.5 ± 1.5^{aAD}	48.6 ± 2.6^{bcAC}	37.2 ± 3.2^{bcAC}	0.3 ± 0.1^{aA}	5.4 ± 1.8^{aA}
High Cd waterborne	8.2 ± 1.1^{aAD}	45.6 ± 1.9^{bcAC}	39.3 ± 3.3^{bAC}	0.2 ± 0.2^{aA}	6.7 ± 1.7^{aA}
Echo control	9.8 ± 1.8^{aADE}	57.2 ± 4.1^{acAE}	24.0 ± 3.6^{acAD}	0.3 ± 0.1^{aA}	8.6 ± 1.8^{aA}
Echo low Cd waterborne	9.8 ± 1.3^{aADE}	52.7 ± 0.4^{acAC}	27.3 ± 2.3^{abAC}	1.3 ± 0.3^{bAC}	8.8 ± 1.0^{aA}
Echo high Cd waterborne	5.9 ± 0.8^{aA}	54.1 ± 1.9^{acADE}	29.1 ± 1.2^{abAC}	1.2 ± 0.2^{bAC}	9.7 ± 2.4^{aA}
Zebrafish fed pellets	**MRG**	**ORG**	**ENZ**	**CD**	**MTLP**
Series 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^{acB}
Low Cd	11.1 ± 2.7^{abAB}	49.9 ± 5.6^{abAC}	34.5 ± 4.5^{aAC}	4.4 ± 1.2^{abBC}	0.0 ± 0.0^{acB}
High Cd	9.0 ± 1.7^{abACD}	42.5 ± 3.7^{bBCE}	43.9 ± 5.7^{aBCD}	4.6 ± 0.8^{bBC}	0.0 ± 0.0^{acB}
Echo control	2.0 ± 0.8^{bAC}	36.8 ± 3.6^{bBC}	47.0 ± 5.9^{aBC}	5.8 ± 1.6^{bB}	6.1 ± 2.7^{bcA}
Echo low Cd	8.0 ± 1.6^{abACD}	44.1 ± 3.1^{abBCEF}	35.0 ± 7.0^{aAC}	6.0 ± 1.1^{bB}	11.6 ± 4.4^{bcA}
Echo high Cd	18.1 ± 3.2^{aBD}	38.3 ± 1.5^{bBCD}	25.0 ± 3.5^{aAC}	4.0 ± 0.9^{abBC}	16.5 ± 4.5^{bA}

Data are represented as mean ± standard error ($N = 6$ for all treatments except for series 2 'echo high Cd waterborne treatment' in which $N = 5$). Different small-case letters indicate significant differences among treatments within the same series and different capital letters indicate significant difference among treatments across series 3 and 4. Compartment categories: MRG, metal rich granules; ORG, organelles; ENZ, enzymes (or heat-denaturable proteins); CD, cellular debris; 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 series 2, 3, and 4, respectively.

zebrafish (Table 4). Furthermore, storage compartmentalization of Cd did not change with increasing Cd concentration in the zebrafish or in the chironomids, regardless of prey exposure route (sediment exposed or waterborne exposed).

Series 3 (pellet diets) and 4 (waterborne-exposed chironomid diets) were directly comparable since they had identical concentration levels in the food. However, there were no significant differences in relative Cd subcellular distributions in the zebrafish between these two series, although in the former, zebrafish fed Cd-contaminated pellets seemed to have less Cd (essentially none) in the MTLP fraction. Within series 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 the echo treatments (Table 4). On the whole, compartmentalization of Cd did not change with increasing Cd concentration in the zebrafish, regardless of food type—i.e. chironomids exposed via a waterborne 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. Metabolically available metal fractions (MAF) in chironomids (the sum ORG, ENZ, and CD fractions) were very 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. Note that the two highest points in this relationship are for control treatments.

4. Discussion

4.1. Zebrafish exposure conditions

There was no significant dissolved Cd in the water ($\leq$0.03 μg Cd/L), and frozen chironomids retained Cd even after 24 h (Table 1), thus Cd leaching into the water directly from the food was not a concern. However, there was dissolved Cd present in treatments where fish were fed Cd-contaminated food (Fig. 1) and this dissolved Cd would have contributed to the Cd accumulated in the fish during the 7-day feeding experiment. We suggest that Cd was likely released from particles (pieces of chironomid larvae or bits of food pellet) during zebrafish feeding and/or leached from the 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 μg Cd/g all had approximately 200 μg Cd/g in their fecal matter. 'Echo fish' were included in our study design to account for the waterborne exposure from leached Cd (refer to Section 2). However, it should be noted that 'echo fish' only received the residual dissolved Cd after the treatment fish were removed (1 day later). Therefore the 'echo fish' correction for accumulated Cd may not completely account for the waterborne Cd exposure in the treatment fish and thus the TTEs presented here could be slightly inflated.

We did not see a decreased growth in conjunction with Cd exposure via the diet, as overall fish growth was positive, although very small, with an average SGR of $0.57 \pm 0.05\%$ per day. Indeed, zebrafish growth rates are generally low, for example, Eaton and Farley (1974) reported a growth rate of 0.05 mm per day, which is comparable to the growth of fish in our exposure.

4.2. Does TTE depend on Cd concentration of prey?

No. Cd accumulation in zebrafish was approximately proportional to the concentration of Cd in the waterborne exposed chironomids of series 4, such that Lwb and Hwb treatments exhibited the same TTEs (Table 3, Fig. 5). The same was true for zebrafish fed with Lpel and Hpel diets in series 3 (Table 3, Fig. 4). This finding is supported by Van Campenhout et al. (2007) who also found no significant difference in the uptake of Cd by fish (carp) fed Cd loaded *C. riparius* larvae regardless if the larvae had a low (0.1 μM Cd waterborne exposed chironomids) or high (0.5 μM) internal Cd concentration.

Overall TTEs obtained in this study (due to dietary Cd) were approximately 10% for controls, and 0.5–2% for treatment conditions (Table 3). We cannot eliminate the possibility that TTEs 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 the water to inflate the calculated TTEs. Overall, our TTE values were notably lower than those from a similar trophic route in which live *C. riparius* larvae were fed to carp and reported TTEs ranged from 50 to 65% (Van Campenhout et al., 2007). On the other hand, our TTE values are similar to those found with Cd contaminated oligochaetes (*L. variegates*) (Ng and Wood, 2008) fed to trout which ranged from 0.9–6.4% after 1 week, and with *Tubifex tubifex* fed to carp which had an assimilation efficiency of 9.8% (Steen Redeker et al., 2007). TTEs 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.

Conversely, it can not be discounted that duration of prey exposure may have an effect on the TTE, as our sediment loaded chironomids which were exposed for 2 weeks time had a higher TTE than did our waterborne exposed chironomids which were exposed for 2 days. This reasoning is supported by Van Campenhout et al. (2007), who found high TTEs of Cd (50–70%) when chironomids had exposure durations of up to 1 month before being fed to carp.

4.3. Does TTE depend on the exposure route of prey?

Yes. Cd body burdens in zebrafish fed Lsed chironomids or Hwb chironomids in series 2 were identical (Fig. 3), even though sediment-exposed chironomids had only about half the amount (153 μg Cd/g dry weight) of Cd as waterborne-exposed chironomids (288 μg/g). The resulting significant difference in TTE between zebrafish fed on Lsed chironomids (~2.0%) *versus* Hwb chironomids (~0.7%; Table 3) was not a function of the concentration since we determined (above) that the TTE of zebrafish fed chironomids is not concentration-dependent. This is in direct contrast to findings of Liu et al. (2002) who reported that there was no difference between TTEs of zebrafish which were fed *Daphnia magna* radiolabeled with Cd either via waterborne exposure or via dietary exposure. However, Cd concentrations in prey were not mentioned in the study of Liu et al. (2002), so it is unclear whether they matched via the two routes of exposure. The reason for this approximately three-fold difference in TTE is unclear. Possibly it may be related to differences in Ca levels of chironomids exposed via sediment/waterborne routes since Cd uptake in chironomid larvae is affected by environmental Ca concentration (Gillis and Wood, 2008a) and in fish by the concentration of Ca in the food (Franklin et al., 2005). Also chironomids exposed via the sediment had a more 'natural' chronic (longer low level) exposure than did chironomids exposed via a waterborne route (48 h Cd in water), perhaps resulting in subtle differences in internal Cd partitioning (see below).

Although TTE was found to be dependent on route of exposure in the prey, the same is not true for relative tissue-specific Cd accumulation in the zebrafish. It appears that as long as the route of exposure is the same (e.g. dietary) then tissue-specific Cd distribution is the same. In all experiments, tissue Cd concentrations were highest in the gut and kidney > liver > gill > carcass for treatment fish fed chironomids (Table 2), and the gut and carcass accounted for the greatest fractions of the total body burdens (Figs. 2, 3 and 5).

4.4. Are responses to natural diets and manufactured diets comparable?

Yes – with some qualifications. Concentration matching of Lpel with waterborne-exposed Lsed chironomids, and Hpel with waterborne-exposed Hwb chironomids resulted in the same TTEs, and re-inforcement of the conclusion that TTE was independent of the Cd concentration in the diet (Table 3). These low TTE values ($\leq$1%) were very similar to those found by Harrison and Klaverkamp (1989) and Franklin et al. (2005) using even higher Cd concentrations in pellet diets. However a higher TTE ($\sim$2%) was obtained with sediment-exposed Lsed chironomids in the present study (Table 3). Notably, there are two reports of approximately two- to five-fold greater Cd TTEs (relative to pellets) for trout fed natural diets consisting of Cd-contaminated amphipods (Harrison and Curtis, 1992) or oligochaetes (Ng and Wood, 2008), both of which were contaminated by waterborne exposure. Clearly, the nature of the prey items, as well as the way in which the prey was contaminated, may affect the conclusion.

Tissue-specific storage of Cd in zebrafish fed the manufactured diet *versus* zebrafish fed on chironomids was also similar, but with one substantial difference. There was a more localized accumulation of Cd in the gut tissue in the pellet-fed zebrafish (Table 2) with a higher proportion of Cd in the gut relative to total body burden (Fig. 4). This is in accord with other evidence that when trout were fed pellet diets similarly spiked with $Cd(NO_3)_2$ (Chowdhury et al., 2005; Baldisserotto et al., 2005) or simply infused via the stomach with $Cd(NO_3)_2$ (Chowdhury et al., 2004), the gut wall preferentially accumulated Cd, serving as a strong barrier to the internalization. Again this provides evidence that Cd which has not been biologically incorporated may not behave identically in the predator after ingestion.

4.5. 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 the ORG fraction regardless of the exposure regime: sediment or waterborne. Yet, there were small changes in the subcellular storage of Cd in the CD and MTLP fractions. Chironomids that were exposed via a waterborne 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 ($\sim$5%) (Table 4). Waterborne-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 $\pm$ CD) fraction when it was fed Cd-contaminated algae (diet-borne), than when the polychaete was exposed via a waterborne route (Selck and Forbes, 2004). In our analyses, we did find a reduction of Cd in the CD fraction, but not in the MRG fraction.

The relative storage of Cd in the MTLP and MRG fractions is important since *C. riparius* are known to produce MTLP in response to Cd 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, Cd-resistant *Limnodrilus hoffmeisteri* (e.g. those that have evolved Cd resistance through living in contaminated sediments and water), produce both MTLPs and MRGs for Cd storage and detoxification, but non-resistant ones (e.g. from 'clean' sediment/water) only produce MTLPs in response to Cd (Wallace et al., 1998). There is, as of yet, no evidence that the same is true in chironomid larvae as subcellular studies are limited.

4.6. Is subcellular compartmentalization of Cd in zebrafish affected by route of prey exposure or type of food?

No, compartmentalization of Cd in the predator was not affected by route of prey exposure (sediment-exposed or waterborne exposed chironomids), but it was somewhat affected by type of food: chironomid or manufactured pellet. 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 significantly more Cd (4–10%) in the MTLP fraction than zebrafish fed Cd-spiked pellets (0%) (Table 4). MTLP has been shown to play a role in Cd detoxification in fish, especially in the liver, and to a lesser extent the gills (Hazma-Chaffai et al., 1995, 1997; Knapen et al., 2004). The role of MTLP in Cd binding (i.e. % Cd bound to MTLP compared to other subcellular fractions) appears to be somewhat lower in this study compared to the other investigations with fish exposed to Cd via the diet. For instance, Ng and Wood (2008) and Hazma-Chaffai et al. (1995, 1997) reported that the Cd bound to the MTLP fraction was approximately two- to eight-fold higher than reported here. However, tissue specific levels of MTLP vary significantly (i.e. liver generally has higher MTLP levels than gill as noted above). Therefore the modest levels of MTLPs reported in this study likely reflect the fact that, due to tissue constraints (see Section 2), we used whole body, rather than tissue specific subcellular compartmentalization analysis. This would result in a dilution of MTLP concentration (by inclusion of carcass) as compared to many other studies which only report tissue specific values.

Storage compartmentalization of Cd did not change with increasing concentration (e.g. low to high exposures) in chironomids or zebrafish, regardless of whether the food was chironomids exposed to waterborne Cd or Cd-spiked pellets. 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 μg/g), and is thus able to store Cd with the same relative distribution irrespective of concentration.

4.7. 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 study. If we considered only the TAM fractions of Cd in chironomids as transferable metal, our treatment TTEs would still only be approximately 2–4%—i.e. nowhere near 100% transfer. Support for a good correlation between TAM and TTE has been seen in a number of studies (Wallace and Luoma, 2003; Seebaugh and Wallace, 2004; Seebaugh et al., 2005, 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, 2008). In summary, our results indicate that for Cd-exposed chironomid larvae, TAM, and MAM should be used with caution, as neither of these was a strong predictor of TTE.

4.8. Conclusions

Route of prey exposure (dietary/sediment or waterborne) is an important factor for the transfer of Cd to predator species; the TTE of zebrafish fed on sediment-exposed chironomids was significantly higher than the TTE for zebrafish fed waterborne-exposed

chironomids. But, Cd TTEs of zebrafish fed a diet of waterborne-exposed chironomids were not significantly different from those of zebrafish fed a diet of manufactured pellet food. The biggest contributors to total body burdens in zebrafish fed Cd-contaminated food were the gut and carcass, but on non-mass weighed tissue Cd concentrations, were consistently high in the gut > kidney > liver of these 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 waterborne exposed chironomids. Subcellular storage in zebrafish did not appear to depend on the exposure route of the chironomid prey. In all zebrafish, the subcellular fractions with the most accumulation were ORG, ENZ, and MRG.

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