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Does dietary Ca protect against toxicity of a low dietborne Cd exposure to the rainbow trout?

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ABSTRACT

We examined the toxicity of Cd, provided in a natural diet and at an environmentally relevant concentration (~12 μg g^{-1} dry wt.), to the juvenile rainbow trout (*Oncorhynchus mykiss*). In addition, we tested the protection by elevated dietary Ca against both the accumulation and toxicity of dietary Cd from this natural diet (background Ca ~1 mg g^{-1} dry wt.). Food pellets were made from blackworms (*Lumbriculus variegatus*), and spiked with Cd and either no additional Ca or elevated (~60 mg g^{-1} dry wt.) concentrations for each of the treatment diets. Survival was unaffected for trout fed diet with 12 μg g^{-1} dry wt. Cd for a month, but growth was potentially reduced. Tissue burden analysis revealed that the stomach, liver and kidney accumulated the most Cd, with concentrations progressively increasing in the liver and kidney over the whole exposure period. Cd concentrations in the plasma and red blood cells were unaffected by the different treatments, but subcellular fractionation analysis indicated that a higher concentration of Cd was associated with the metal-sensitive fractions of red blood cells of the fish that were exposed to the dietborne Cd. Dietary Cd exposure also caused potential toxicity to cells of the stomach in that they bound more Cd to heat-denaturable proteins. However, detoxification appeared to take place in the Cd-exposed fish because more Cd was bound to metallothionein-like proteins by week 4 of exposure. Elevated Ca in the Cd diet generally protected against accumulation and toxicity of dietborne Cd. The protection against Cd accumulation was almost complete at the gills, robust in the stomach and whole body (≥50% reductions), but not significant in the liver, kidney, carcass, plasma, or red blood cells. Elevated dietary Ca also reduced Cd accumulation in the organelles of the fish stomach and red blood cells. In addition, dietborne Ca not only reduced the uptake of Cd by the cells, but also altered how the cells handled Cd intracellularly. In general, our results have demonstrated the need to use diets with natural compositions for dietary toxicity studies.

1. Introduction

Concentrations of metals in natural diets can produce adverse biological effects, even though such levels are often much lower than those tested in laboratory trials. For instance, a diet of benthic invertebrates which had been acclimated to elevated metal concentrations in the field reduced feeding activity and caused histological changes in the cutthroat trout (Farag et al., 1999). A similar diet also caused scale loss and increased metal accumulation in the gut tissue of the rainbow trout (Farag et al., 1994). Effects such as these could potentially lead to reduced survival and growth of fish. Fathead minnows fed *Chironomus tentans* that had been exposed to metal mine effluent, had reduced larval hatchings and increased deformities, leading to reduced total reproductive output (Rickwood et al., 2006). Significant growth inhibition was also observed in rainbow trout fed contaminated blackworms (*Lumbriculus variegatus*) that had been cultured in metal-contaminated sediments collected from the field (Hansen et al., 2004).

Sublethal levels of Cd can affect ionoregulation and cause physiological disturbances in the fish (Pratap et al., 1989; McGeer et al., 2000; Baldisserotto et al., 2005). For example, dietary Cd at 500 μg g^{-1} dry wt. has been reported to reduce the tubular reabsorption and thereby increase the renal excretion of major ions such as Mg^{2+}, Na^{+}, Cl^{-}, K^{+}, and Ca^{2+} (Chowdhury and Wood, 2007). Fish acclimated to the Cd diet also had a lower turnover of plasma Cd, and thus greater Cd concentrations in the plasma. Besides plasma, red blood cells are also a major reservoir for metals in the blood. They can accumulate 1.8–100× more metals than the plasma (Chowdhury et al., 2004a; Alves and Wood, 2006). In addition, red blood cells are very susceptible to oxidative damage by metals (Gwozdzinski et al., 1992; Alves and Wood, 2006). For example, Cu affected antioxidant enzymatic activities of red blood cells

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of the marine fish *Dicentrarchus labrax* (Gwozdzinski et al., 1992). Therefore, exposure to dietary Cd may also cause haematological problems in the fish.

Subcellular Cd partitioning is a potential predictor for Cd toxicity in aquatic organisms (Wang and Rainbow, 2006). Metals accumulated by organisms are associated with different subcellular compartments (heat-denaturable proteins such as enzymes; heat-stable or metallothionein-like metal-binding moieties such as metallothionein and glutathione; metal-rich granules such as carbonates and phosphates; organelles such as mitochondria, lysosomes, microsomes; cellular debris such as cellular membranes, nuclei and large cellular fragments). Subcellular partitioning is metal- and organism-specific, as well as being dynamic to the environmental conditions and metal exposure. Clear correlations between metal toxicity and their subcellular metal partitioning have been demonstrated in phytoplankton (Miao and Wang, 2006, 2007), crustaceans (Wallace et al., 2000) and bivalves (Perceval et al., 2006). In our previous study, subcellular Cd distribution was examined in the gut of the rainbow trout, which had been fed Cd-contaminated worms (Ng and Wood, 2008). Cd distribution was shifted from the metal-sensitive heat-denaturable proteins to the metal-detoxified metallothionein-like proteins. This demonstrated an efficient Cd detoxification occurring in the gut of the trout. Therefore subcellular fractionation of metal-binding in tissues allows us to understand the mechanisms for metal toxicity (e.g., metal bound to metal-sensitive compartments), and how the organisms detoxify metals (e.g., metal bound to metallothionein-like proteins).

It has been well demonstrated that Ca in the water and diet protects fish against the uptake of Cd (Wood et al., 2006). Cd is taken up at the gills by Ca transporters, and elevated waterborne Ca competes with Cd for these transporters (Niyogi and Wood, 2004). Elevated dietborne Ca down-regulates the Ca transporters on the gills (Galvez and Wood, 2007) and reduces the subsequent waterborne Cd uptake (Zohouri et al., 2001; Baldisserotto et al., 2004, 2005; Franklin et al., 2005). Elevated dietary Ca, when tested in commercial diets, also decreases Cd uptake through the gastrointestinal tract (Baldisserotto et al., 2005; Franklin et al., 2005). Indeed, whole body Cd uptake from the diet and internal organ-specific Cd burdens of the trout over 28 d can be reduced 40–50% by a 2.5–3-fold elevation of Ca in commercial diets (Baldisserotto et al., 2005; Franklin et al., 2005). Both *in vivo* and *in vitro* studies suggest that Ca and Cd interactions may primarily occur in the stomach (Franklin et al., 2005). All the evidence above has been obtained from high Cd dose treatments applied to commercial pellets; there still remains little knowledge on the potential protection by Ca against low dietborne Cd concentrations in natural food.

The objectives of this study were to examine the toxicity of dietary Cd at an environmentally relevant concentration to rainbow trout (*Oncorhynchus mykiss*) in a natural diet. In addition, we tested the potential protection by elevated dietary Ca against the accumulation and toxicity of this relatively low dietary Cd burden. *L. variegatus*, commonly known as blackworms, were used for feeding the trout. *L. variegatus* is a natural food source for the rainbow trout that can support normal growth and survival of several fish species (Mount et al., 2006). We made artificial pellets from the worms, and spiked them with Cd and Ca for the treatment diets. Survival and individual specific growth rates of the fish were monitored. Similar to our previous study (Ng and Wood, 2008), subcellular fractionation was also applied to the stomach tissue of the fish to understand how this organ handled Cd. To our knowledge, this is the first study to have used subcellular fractionation on red blood cells to understand Cd toxicity on haematology in fish.

2. Materials and methods

2.1. Diet preparation using the worms

Oligochaetes (*L. variegatus*) were purchased from Aquatic Foods Ltd., CA, USA. They were held at 12 ± 1 °C in an aerated flow-through system of dechlorinated Hamilton city tapwater (approximate ionic composition in mmol L^{-1}: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8–8.0, dissolved organic carbon ~3 mg C L^{-1}, hardness ~140 mg L^{-1} as $CaCO_3$, 0.06 μg L^{-1} Cd) for at least 48 h to remove the surface-bound mucus, then rinsed three times with water that had been treated by reverse osmosis. Then the worms were blotted dry with filter paper, oven-dried at 65 °C for 24 h (or until dry), ground to powder using a commercial blender, weighed, and finally rehydrated with 25% (v/w) NANOpure II water (Sybron/Barnstead, Boston, MA). For treatment diets with appropriate amounts of additional Cd and/or Ca, aliquots of $Cd(NO_3)_2{\cdot}4H_2O$ and $CaCO_3$ were dissolved and suspended in the NANOpure II water before addition to the ground worm powder. The resulting paste was mixed thoroughly and allowed to "thicken" with the aid of a hair dryer ("cool" setting) for about 30 min. The paste was then extruded into thin (~2 mm) strands through a pasta maker, air-dried overnight and cut into small pellets by a scalpel. Worm pellets were stored at −20 °C until use.

We employed two control diets, worm pellets with background Cd and Ca (Control Low) and worm pellets with background Cd and nominal Ca concentration of 60 mg g^{-1} dry wt. (Control High). Control High treatment allowed us to evaluate the effects of high Ca alone on the fish. The treatment diets had nominal concentrations of 12 μg Cd g^{-1} dry wt., with additional Ca (High Ca + Cd: nominal 60 mg Ca g^{-1} dry wt.) or without additional Ca (Cd only). We made pellets with nominal 60 mg Ca g^{-1} dry wt. since this level of Ca showed significant protection against Cd toxicity in trout in earlier studies with much higher dietary Cd concentrations (Baldisserotto et al., 2005; Franklin et al., 2005). The level of Cd (12 μg g^{-1} dry wt.) was selected because this is a relevant Cd concentration in the freshwater benthic invertebrates in Ontario lakes of Canada (Kraemer et al., 2006).

About 0.13 g pellets from each diet were digested in 2 mL 1 N nitric acid at 60 °C for 2 d in order to validate the Cd and Ca concentrations. The digest was diluted and analysed for Cd by a graphite furnace (GFAAS, Varian, Mulgrave, Vic., Australia) and for Ca by a flame atomic absorption spectrometer (FAAS, Varian Spectra AA220, Mississauga, ON, Canada). Certified reference material (TM15, National Water Research Institute, Environment Canada) was used for the quality control of the metal analysis. Agreement was within 10%. Measured concentrations of Cd and Ca are presented in Table 1.

Table 1
Measured Cd and Ca concentrations in the worm pellets. Mean ± standard deviation (n = 2 replicates of about 0.13 g each). The non-parametric Kruskal–Wallis test was used to test for significant differences (P < 0.05) among diets followed by Dunnett's T3 *post hoc* test to identify the difference between diets (P < 0.05). Means not sharing the same letter indicate significant differences between diets. Control Low: pellets without additional Cd or Ca; Control High: pellets with nominal 60 mg Ca g^{-1} dry wt.; Cd only: pellets with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: pellets with nominal 60 mg Ca g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt.

Diet	Ca (mg g^{-1} dry wt.)	Cd (μg g^{-1} dry wt.)
Control Low	0.6 ± 0.03[a]	0.2 ± 0.1[a]
Control High	65.8 ± 0.9[b]	0.2 ± 0.0[a]
Cd only	0.8 ± 0.4[a]	11.6 ± 2.2[b]
High Ca + Cd	76.2 ± 17.1[b]	9.6 ± 0.8[b]

2.2. Fish feeding

Juvenile rainbow trout (*O. mykiss*) (12–15 g) were purchased from Humber Springs Hatchery, Orangeville, ON, Canada. They were held in dechlorinated Hamilton tapwater (12 ± 1 °C; composition as above) in flow-through 200 L tanks with aeration, and acclimated to the Control Low worm pellet diet (with background Cd and Ca concentrations) at a 0.7% body wt. daily ration for 1 week. The fish were then starved for 2 d and anaesthetized using tricaine methane sulphonate. They were weighed individually and a 12 mm Passive Integrated Transponder tag (PIT, TX1400L-125 kHz Destron Fearing, St. Paul, MN, USA) was implanted into the peritoneal cavity of each fish. After implantation, the tagged fish were put back into the tanks for recovery. Due to a limited number of the PIT tags, only ~80% fish were tagged. Bulk weight of the untagged fish was also measured for estimating the amount of food to be provided to each tank. Tagged and untagged fish were randomly and evenly distributed among the exposure tanks. There were 2 replicate tanks for each dietary exposure (Control Low, Control High, Cd only, High Ca + Cd) each containing 23 fish.

Fish in each tank were fed a 0.7% body weight ration throughout the experiment. Water samples were also taken from the tanks for testing the potential leaching of Cd from the pellets at various times after feeding. No significant leaching was found. Tank standpipes were removed every day to drain any fecal material that had settled at the bottom of the tanks. At the end of every week, individual weights of the tagged fish, as well as bulk weight of all the fish were measured in order to calculate the specific amount of food needed for each tank for the following week. A subset of 5 fish from each tank (10 fish from each treatment) was then transferred to separate 20-L flow-through tanks for Cd gut clearance. These fish were fed 0.7% body weight ration of Control Low worm pellets (with background Cd and Ca concentrations) daily for 5 d. Tests demonstrated that this procedure ensured complete Cd clearance of gastrointestinal contents while maintaining the Cd concentration in the fish bodies essentially unchanged. The entire dietary exposure lasted for 4 weeks. The daily doses of Cd fed to the fish were $1.1 \pm 0.0\ ng\ g^{-1}\ d^{-1}$ (Control Low), $1.6 \pm 0.0\ ng\ g^{-1}\ d^{-1}$ (Control High), $69.2 \pm 0.0\ ng\ g^{-1}\ d^{-1}$ (Cd only) and $66.0 \pm 0.0\ ng\ g^{-1}\ d^{-1}$ (High Ca + Cd).

2.3. Growth and mortality of fish

Mortality of the fish from all tanks was recorded during the dietary exposure period. Specific growth rates of the fish were monitored by weighing the individual tagged fish every week and individual specific growth rate was calculated as described by Franklin et al. (2005).

2.4. Tissue-specific Cd concentrations in the fish

After the 5 d gut clearance from every week's dietary exposure, 10 fish from each treatment were euthanized with an overdose of tricaine methane sulphonate, blood-sampled (see below), and dissected. Brain, gill, stomach, anterior intestine, mid-intestine, posterior intestine, kidney and carcass were collected and weighed. Gastrointestinal contents (negligible except for mucus) were manually cleared from the gut which was divided into its four distinct sections mentioned above. The gastrointestinal tract, as well as the gills, were rinsed in 0.9% sodium chloride to remove any surface-bound metals. All tissues were stored at −20 °C until metal analysis.

The tissues were digested in a similar manner as used for the worm pellets in 1 N nitric acid for 2 d, except for the intestinal tissues which were allowed to digest for 4 d to allow them to completely dissolve. The Cd concentrations in all tissues were measured by GFAAS after dilution. Whole body Cd concentration was calculated by multiplying the tissue-specific Cd concentration by the individual tissue weight, adding the fractions to yield a total Cd content per fish, and then dividing this by the total weight of the fish.

2.5. Metal concentrations in fish blood

Simultaneously with the tissue dissection at the end of the dietary experiment (4 weeks), blood was also collected immediately by caudal puncture of each fish with a 1-mL syringe pre-rinsed with lithium heparin ($20\ i.u.\ mL^{-1}$) in modified Cortland saline (Wolf, 1963). Mannitol (analytical reagent, BDH Chemicals) was used to replace $CaCl_2$ in the saline. The blood samples were then centrifuged at $18{,}000 \times g$ for 15 min at 4 °C to separate the plasma from the red blood cells. Both samples were stored at 4 °C until further analysis.

Since previous studies demonstrated an increase of metal concentrations in the blood of the fish after dietary exposure (Chowdhury et al., 2004a; Alves and Wood, 2006; Chowdhury and Wood, 2007), the Cd concentration was measured in the red blood cells; the plasma was also analysed for Cd, as well as for Ca. Fish plasma was acidified and appropriately diluted with 1% nitric acid, then Ca concentrations were measured by FAAS and Cd concentration was measured by GFAAS. Red blood cells were digested and measured as for the intestinal tissue.

2.6. Subcellular fractionation of stomach and red blood cells

The stomach is the largest tissue of the gut, and the one suspected to be both a major site of Cd absorption and of Ca/Cd interactions (see Section 1). Thus, the stomach tissues of 1-week and 4-week exposed fish were subcellularly fractionated, in order to understand potential Cd toxicity and detoxification in the gut at times close to the start and at the end of the dietary exposure. The total Cd concentration in the stomach at weeks 1 and 4 was the sum of Cd concentrations in the subcellular fractions, whereas the values obtained from the digestion of total stomach tissues were used for weeks 2 and 3. Since the recovery of Cd in the subcellular fractionation was essentially complete (see below), discrepancies between the two methods of analyses were considered to be negligible.

Stomach tissues from two fish of each treatment were combined for subcellular fractionation ($n = 5$ per treatment at each time). Stomach tissues were sliced into small pieces and homogenized in 3 mL of 25 mM Tris–base buffer (pH: 7.13, with 0.2 mM phenylmethane-sulphonylfluoride and 2 mM 2-mercaptoethanol). The fractionation procedures used have been established for a diverse range of aquatic organisms (Wallace et al., 2003). Before fractionation, portions of homogenate from 4 randomly selected samples were taken for a Cd recovery test. In this study, we used the same protocol as used for rainbow trout by Ng and Wood (2008). The resultant subcellular fractions were cellular debris (CD), metal-rich granules (MRG), organelles (ORG) and soluble cytosolic proteins such as metallothionein-like proteins (MTLP) and heat-denaturable proteins (HDP). The fractions that can store, and potentially detoxify metals, are MTLP + MRG defined as the biologically detoxified metal fractions (BDM), whereas the fractions where metals can potentially be toxic to fish (ORG + HDP), are defined as metal-sensitive fractions (MSF).

For digestion of the subcellular fractions, 1 mL 1 N nitric acid was added to the MRG, ORG, HDP and the total homogenate. The samples were then heated at 80 °C for 2 d to facilitate the digestion of the compressed cellular material. Since the CD and MTLP were in liquid form, they were directly acidified with 1% nitric acid. All samples were spun at $5000 \times g$ for 15 min and the supernatant

was subsequently collected or diluted for Cd analysis by GFAAS. Recovery of Cd (sum of Cd from subcellular fractions/Cd in the total homogenate) was 100.8 ± 24.0% (mean ± SD).

Subcellular fractionation was first applied to the red blood cells of the fish to investigate how the red blood cells handle Cd from their diet. Blood was collected from each fish as mentioned above, after 1 and 4 weeks of dietary exposure. Due to the shortage of collected volume, the blood samples from both of the control treatment groups were combined to calculate the subcellular Cd concentrations for control fish. Subcellular Cd concentrations were measured only in the week 4 red blood cells. Three samples of red blood cells were pooled together to form three replicates from each treatment for subcellular fractionation. About 2 mL Tris–base buffer was added into each pooled red blood cell sample and sonicated with a Microson™ Ultrasonic Cell Disruptor for 2 min with output power setting 9 in iced condition. Remaining fractionation procedures were similar to those used above for the stomach tissue. Recovery of Cd in the red blood cells was 96.2 ± 10.0%.

2.7. Statistical analysis

Data were expressed as means ± 1 standard deviation, and tested for equal variance and normal distribution before analysis. For the time series results (growth, tissue-specific concentration, subcellular concentrations and distribution in the stomach), effects of diets, time and interaction between diets and time were tested by two-way ANOVA. Other results were tested by one-way ANOVA (parametric data) or Kruskal–Wallis test (non-parametric data), for the effect of diets only. Differences between groups were tested by Tukey's Multiple Comparison (parametric data) or Dunnett's T3 *post hoc* tests (non-parametric data). The Student's *t*-test (two-tailed, unpaired) was used for comparisons of two means where appropriate. Significance of all tests was taken at $P < 0.05$.

3. Results

3.1. Growth and mortality of fish

Dietary Cd or Ca did not affect survival of the fish. Individual specific growth rates of the fish ranged between 0.05 and 0.94% d^{-1} (Fig. 1) and showed no statistically significant overall effect of diets ($P = 0.28$). However, there was an overall effect of time such that all fish grew slower in the later period of the exposure ($P < 0.01$). For example, in week 4, average growth rate of the control fish was only half of that in week 1. The reduction in growth was particularly obvious in the fish fed Cd only diet, and only in this group was the growth rate significantly lower in weeks 3–4 relative to those in the preceding weeks ($P < 0.05$). However, there were no interactive effects of diets and time ($P = 0.18$).

3.2. Tissue-specific Cd concentrations in the fish

Cd concentrations in the fish brains were comparable in all the fish. Cd was only slightly detectable in half of the brain samples (data not shown, detection limit of Cd: 0.05 ng g^{-1}). Concentrations varied from 0.01 ± 0.03 ng g^{-1} wet wt. (Control High after week 4) to 0.5 ± 1.4 ng g^{-1} wet wt. (Control Low after week 1).

Cd concentrations in the gills of the fish fed the Cd only diet were significantly higher (25–100×) than the fish fed the control diets ($P < 0.01$) (Table 2). The Cd diet that had the addition of 60 mg Ca g^{-1} dry wt. (High Ca + Cd diet) significantly reduced the accumulation of Cd in the gills back to the levels of the control fish in all weeks ($P < 0.05$), except at week 4. At this time, fish fed High Ca + Cd diet had 4× higher Cd concentration in the gills than that in the fish fed Control High diet ($P = 0.045$), although it was still greatly depressed relative to the Cd only diet.

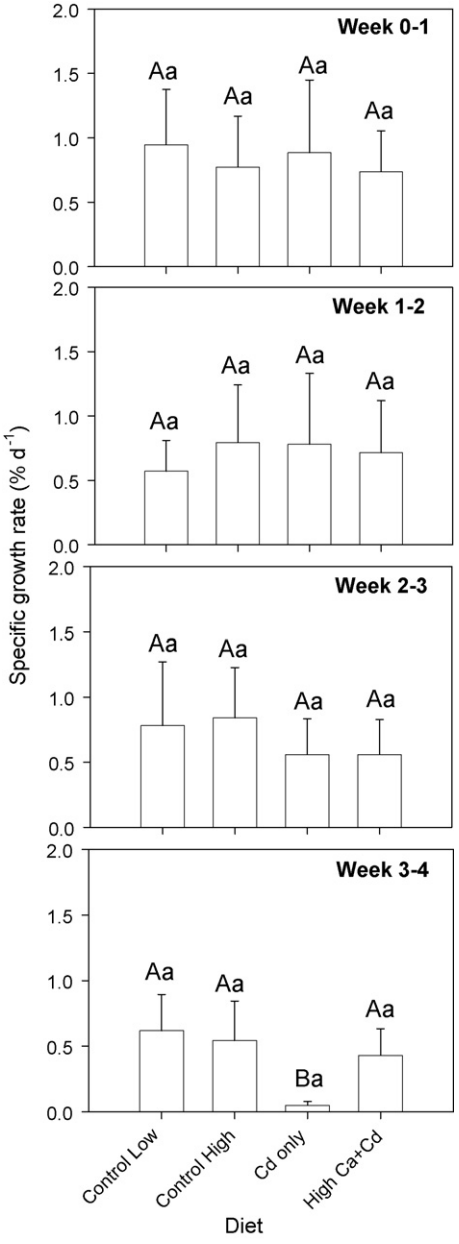

Fig. 1. Individual specific growth rate (SGR, % d^{-1}) of rainbow trout fed worm pellets with different concentrations of Cd and Ca, for 4 weeks. Mean ± standard deviation (n = approximately 36, 31, 26, 21 per treatment for week 0–1, 1–2, 2–3, 3–4, respectively). Two-way ANOVA was used to test for significant effects of diets and time, also interaction between diets and time ($P < 0.05$). Tukey's Multiple Comparison *post hoc* test was then used when a significant difference was found in the two-way ANOVA to identify the differences ($P < 0.05$) between groups. Means not sharing the same letters in the same case indicate significant differences between diets in the same week (lower case), and significant differences between weeks within the same diet (upper case). Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt.; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt.

The results for Cd concentrations in the anterior, mid and posterior intestine are presented in another publication (Klinck et al., submitted for publication). Among all the tissues, stomach, liver and kidney accumulated the most Cd, with kidney containing the highest Cd concentration (Table 2). Cd concentrations in stomach of

Table 2
Cd concentrations in the organs of rainbow trout that were fed worm pellets with different Cd and Ca concentrations for 4 weeks. Mean ± standard deviation (n = 10). Two-way ANOVA was used to test for effects of diets and time, also interaction between diets and time (P < 0.05). Dunnett's T3 *post hoc* test was then used to identify the differences between groups (P < 0.05) when a significance difference was found from the Two-way ANOVA. Means not sharing the same letter in the same case indicate significant difference between diets in the same week (lower case) or between weeks in the same diet (upper case). Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt.; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt.

Organs	Treatment	Cd (ng g^{-1} wet wt.)			
		Week 1	Week 2	Week 3	Week 4
Gill	Control Low	0.5 ± 0.3^{Aa}	0.8 ± 0.3^{Aa}	0.6 ± 0.2^{Aa}	2.2 ± 0.4^{Aa}
	Control High	0.8 ± 0.3^{Aa}	0.3 ± 0.2^{Aa}	0.3 ± 0.2^{Aa}	1.1 ± 0.6^{Ab}
	Cd only	24.8 ± 7.1^{Ab}	39.8 ± 11.5^{Ab}	104.9 ± 36.8^{Ab}	50.8 ± 19.5^{Ac}
	High Ca + Cd	1.0 ± 0.3^{Aa}	1.0 ± 0.5^{Aa}	0.9 ± 0.3^{Aa}	4.4 ± 1.5^{Aa}
Stomach	Control Low	14.6 ± 6.3^{ABab}	5.8 ± 2.3^{Aa}	15.2 ± 3.5^{Ba}	5.6 ± 0.7^{Aa}
	Control High	9.8 ± 3.9^{ABa}	5.6 ± 2.0^{Aa}	10.3 ± 3.9^{Ba}	7.5 ± 1.5^{ABa}
	Cd only	47.8 ± 15.9^{Ac}	64.6 ± 18.7^{Ab}	127.7 ± 33.3^{Bb}	95.8 ± 30.8^{ABb}
	High Ca + Cd	27.2 ± 3.2^{Ab}	36.3 ± 9.4^{Ab}	35.0 ± 10.3^{Ac}	43.5 ± 5.6^{Ab}
Liver	Control Low	2.2 ± 1.2^{Aa}	2.5 ± 0.9^{Aa}	1.8 ± 0.7^{Aa}	9.1 ± 3.2^{Ba}
	Control High	2.5 ± 1.1^{Aa}	1.7 ± 0.7^{Aa}	11.2 ± 3.0^{Bb}	9.4 ± 7.1^{ABa}
	Cd only	36.4 ± 10.9^{Ab}	102.7 ± 47.4^{Bb}	102.5 ± 49.0^{Bc}	170.3 ± 49.3^{Bb}
	High Ca + Cd	36.2 ± 9.2^{Ab}	31.6 ± 16.5^{Ab}	77.4 ± 17.5^{Bc}	144.7 ± 18.5^{Cb}
Kidney	Control Low	33.1 ± 11.8^{Aa}	50.7 ± 12.9^{Aa}	28.7 ± 9.3^{Aa}	29.5 ± 8.9^{Aa}
	Control High	34.7 ± 7.2^{Aa}	73.1 ± 27.1^{Aa}	33.7 ± 9.0^{Aa}	26.8 ± 8.0^{Aa}
	Cd only	169.4 ± 90.8^{Aa}	213.6 ± 48.8^{Ab}	394.2 ± 123.9^{Ab}	386.4 ± 130.9^{Ab}
	High Ca + Cd	297.0 ± 135.0^{Ab}	237.6 ± 68.6^{Ab}	235.2 ± 103.5^{Ab}	310.8 ± 110.9^{Ab}
Carcass	Control Low	0.02 ± 0.05^{Aa}	0.08 ± 0.24^{Aa}	0.001 ± 0.004^{Aa}	0.8 ± 0.4^{Ba}
	Control High	0.0004 ± 0.0011^{Aa}	0.07 ± 0.03^{Ba}	0.006 ± 0.018^{Aa}	0.7 ± 0.4^{Ca}
	Cd only	0.2 ± 0.1^{ACa}	0.06 ± 0.05^{Ca}	0.2 ± 0.1^{Aa}	2.8 ± 1.0^{Ba}
	High Ca + Cd	0.1 ± 0.1^{Aa}	0.2 ± 0.1^{Aa}	0.2 ± 0.1^{Aa}	1.1 ± 0.1^{Ba}
Whole body	Control Low	1.1 ± 0.4^{ABa}	0.8 ± 0.2^{Aa}	0.8 ± 0.1^{Ab}	1.7 ± 0.7^{Ba}
	Control High	0.8 ± 0.2^{Aa}	0.9 ± 0.5^{ABa}	1.1 ± 0.3^{ABa}	1.5 ± 0.4^{Ba}
	Cd only	10.9 ± 3.0^{Ab}	15.0 ± 2.9^{ABb}	20.3 ± 5.4^{Bb}	25.4 ± 10.0^{Bb}
	High Ca + Cd	8.1 ± 2.4^{Ab}	10.7 ± 5.0^{Ab}	7.6 ± 2.9^{Ac}	11.1 ± 3.8^{Ac}

fish changed over time (P = 0.01), with an interactive effect (P = 0.03) of increase in fish fed Cd diets and reduction in fish fed control diets (P = 0.01). Cd only treated fish had 3–20× higher Cd concentrations in the stomach than the control (P < 0.05), and this concentration increased 2–3-fold over the dietary exposure period (P < 0.05). Fish fed the High Ca + Cd diet had significantly lower Cd concentrations in the stomach than the fish fed the Cd only diet on weeks 1 and 3 (P < 0.05), but these concentrations were still higher than in the control fish (P < 0.05). Overall, Ca reduced the Cd levels in the stomach by 50–75% compared to fish fed dietary Cd alone (Table 2).

There was about a 4–4.7-fold increase of Cd concentration in the liver of the fish over time (P < 0.01, Table 2) with the most obvious increases at the end of the exposure (by weeks 3 or 4) (P < 0.05). Fish fed the Cd diet had 18–50× higher Cd concentrations in the liver compared to the control fish (P < 0.01). The Cd-only-treated fish also increased Cd accumulation in the liver from week 2 (P < 0.05). Although the average Cd concentration in the liver of the fish exposed to the High Ca + Cd diet was lower than in those exposed to Cd only diet from week 2 onwards, there was not a significant difference (P > 0.05).

Fish fed different diets generally maintained a constant Cd concentration in the kidney over time (P = 0.59) (Table 2). Cd-treated fish had 5–13-fold higher Cd concentrations in the kidney compared to the control fish (P < 0.01), but this effect was not significant in the first week in the fish fed Cd only diet, compared with the controls (Cd *vs* Control Low, P = 0.12; Cd *vs* Control High, P = 0.12), due to high variation among the replicates. In general, kidneys of fish exposed to the High Ca + Cd diet had Cd concentrations comparable with those of fish exposed to the Cd only diet (P > 0.05).

The fish carcasses exhibited very low Cd concentrations which fluctuated over time (Table 2). In general, Cd concentration in the fish carcass was comparable among different treatments (P = 0.38), and it increased over time in all treatments (P < 0.01). Cd concentration in the carcass increased in the first two weeks, and dropped at week 3, except for the Cd treated fish. Then, Cd concentration increased again and reached the highest values at week 4.

Whole bodies of fish fed the control diet had Cd concentrations ranging from 0.8 to 1.7 ng g^{-1} wet wt. (Table 2). Dietary Cd increased the Cd concentration in the whole bodies 10–20× (P < 0.05) and the fish exposed to the Cd only diet exhibited more than a 2-fold increase of Cd concentration over the weeks (P < 0.05). The High Ca + Cd diet significantly depressed the increase of Cd in the whole bodies by about 50% starting from week 3 (P < 0.05), but the Cd levels in the fish were still higher than the controls (P < 0.05) (Table 2).

3.3. Metal concentrations in fish blood

After 4 weeks of exposure to dietary Cd, Ca concentrations in the plasma of the fish exposed to Control High and Cd only diets were lower than in the fish fed Control Low diet (P < 0.05; Table 3). Cd concentrations in the plasma were comparable in all fish (P = 0.34). Red blood cells of the fish had 13–40× higher Cd concentrations (Fig. 2) than the plasma (Table 3). This erythrocyte Cd concentration (12.7–15.9 ng Cd g^{-1} wet wt.) was comparable in fish fed different diets (P = 0.48) (Fig. 2).

3.4. Subcellular Cd concentrations and distribution in the red blood cells

The subcellular Cd distribution in the red blood cells followed this order in the control fish: CD > MRG > MTLP > HDP > ORG (Table 4). Fish fed the Cd only diet had a higher Cd concentration in the MSF (i.e., ORG + HDP), compared to fish fed the control diet (P < 0.01) (Fig. 2). This may be attributed to a higher percentage of Cd

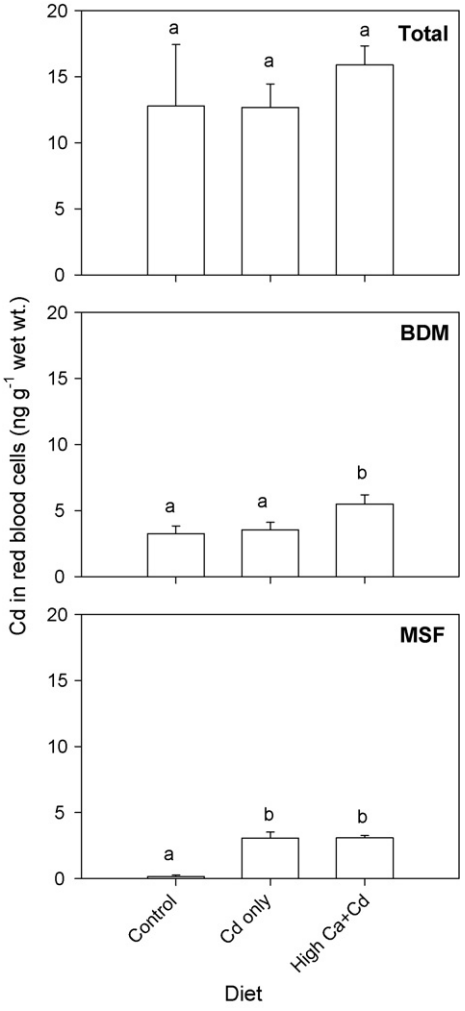

Fig. 2. Cd concentrations in red blood cells (ng g^{-1} wet wt.) of rainbow trout that were fed worm pellets containing different concentrations of Cd and Ca, for 4 weeks. Mean ± standard deviation (n = 3, with 3 blood samples combining to form 1 replicate). Cd concentrations in the total red blood cells were calculated from the sum of Cd concentrations in all subcellular fractions. One-way ANOVA was used to test for significant differences (P < 0.05) among treatments, followed by Tukey's Multiple Comparison *post hoc* test for identifying the significant differences (P < 0.05) between treatments. Means not sharing the same letters indicate significant differences between treatments. Control: fish fed diet without additional Cd or Ca and diet with nominal 60 mg Ca g^{-1}; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt. BDM: biologically detoxified metal fractions (MTLP + MRG); MSF: metal-sensitive fractions (HDP + ORG).

Table 3
Ca and Cd concentrations in the plasma of rainbow trout on week 4, after being fed worm pellets with different Cd and Ca concentrations. Mean ± standard deviation (n = 10). One-way ANOVA was used to test for the significant differences (P < 0.05) among diets, followed by Tukey's Multiple Comparison *post hoc* test to identify differences between diets (P < 0.05). Means not sharing the same letter indicate significant difference between diets. Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt.; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt.

Treatment	Ca (mM)	Cd (nM)
Control Low	2.7 ± 0.8[a]	5.4 ± 1.1[a]
Control High	1.2 ± 0.4[b]	3.5 ± 0.5[a]
Cd only	1.4 ± 0.7[b]	4.8 ± 1.3[a]
High Ca + Cd	2.3 ± 0.7[ab]	8.8 ± 2.5[a]

distributed in the ORG (16–20-fold) (P < 0.01) and HDP (3–4-fold) (P = 0.01) (Table 4). The High Ca + Cd diet significantly increased the Cd concentration in the BDM (i.e., MTLP + MRG) of the fish red blood cells (P = 0.01), compared to the Cd diet alone, but did not reduce the Cd concentration in the MSF (P = 0.98) (Fig. 2). The High Ca + Cd diet also produced slight, but not significant protective effects by increasing the percentage of Cd in MRG (P = 0.26), and decreasing the distribution in ORG (P = 0.51), thus reducing the total Cd distribution in MSF (P = 0.62) and increasing it in BDM (P = 0.18) (Table 4).

3.5. Subcellular Cd concentrations and distribution in the stomach

The distribution of Cd (as a percentage or concentration) in the subcellular fractions of the stomach of the fish fed control diets in week 1 was MTLP > ORG > MRG > CD > HDP (Table 5; Fig. 3). Fish fed the Cd only diet increased both the concentration and percentage of Cd distributed, in the metal-detoxified concretions (MRG) (P < 0.05) after the first week, however, the concentration of Cd was not changed in the metal-detoxified protein (MTLP), compared to the control (P = 0.43). Fish fed the Cd only diet had 6-fold (P < 0.01) higher Cd concentrations and 4–6-fold (P < 0.01) higher percentage distributions respectively in the HDP. Meanwhile, the concentration (P = 0.01) and percentage of Cd (P < 0.01) also increased in CD of the fish fed the Cd only diet after 1 week. The High Ca + Cd diet did not affect the Cd distribution in the ORG and HDP of the Cd-exposed fish (P > 0.05). The results from week 1 generally showed potential toxicity of Cd (elevated Cd percentage in HDP) but a detoxification (elevated Cd percentage in MRG) of Cd started to take place in the stomach.

Subcellular Cd distribution changed in the control fish over 4 weeks (Table 5; Fig. 3). Percentage Cd distribution increased in the HDP (P < 0.01) whereas it decreased in the MTLP (P < 0.01) and ORG (P = 0.02) of the control fish (fed Control Low/Control High diets), compared to week 1 (Fig. 3). The concentration and percentage of Cd distribution in the MTLP were also higher than the control

Table 4
Percentages of Cd in subcellular fractions of red blood cells in rainbow trout on week 4, after being fed worm pellets with different Cd and Ca concentrations. Mean ± standard deviation (n = 3, with three blood samples combining to form one replicate).

Treatment	MTLP %	MRG %	ORG %	HDP %	CD %	BDM %	MSF %
Control	5.5 ± 1.8[a]	16.5 ± 1.9[a]	0.5 ± 0.1[a]	1.0 ± 0.1[a]	82.4 ± 11.1[a]	22.0 ± 0.6[a]	1.4 ± 0.3[a]
Cd only	4.4 ± 1.1[a]	24.0 ± 4.0[ab]	20.5 ± 4.6[b]	3.8 ± 1.7[b]	57.1 ± 8.6[b]	27.1 ± 6.0[ab]	24.2 ± 5.1[b]
High Ca + Cd	4.5 ± 1.4[a]	30.1 ± 4.0[b]	16.1 ± 0.9[b]	3.4 ± 0.2[b]	46.0 ± 3.4[b]	34.5 ± 2.7[b]	19.5 ± 1.2[b]

Percentage data were arcsine transformed and tested for significant difference (P < 0.05) among diets by one-way ANOVA, followed by the Tukey's Multiple Comparison *post hoc* test to identify specific differences between diets (P < 0.05). Means not sharing the same letter indicate significant differences between diets. Control: fish fed diet without additional Cd or Ca and diet with nominal 60 mg Ca g^{-1} dry wt.; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt. MTLP: metallothionein-like proteins; MRG: metal-rich granules; ORG: organelles; HDP: heat-denaturable proteins; CD: cellular debris; BDM: biologically detoxified metal fractions (MTLP + MRG); MSF: metal-sensitive fractions (ORG + HDP).

Table 5
Percentages of Cd in subcellular fractions of stomach in rainbow trout on weeks 1 and 4, after being fed worm pellets with different concentrations of Cd and Ca. Mean ± standard deviation ($n = 5$, with two stomach tissues combined to form one replicate). Percentage data were arcsine transformed and tested for significant effects of diets and time, also interaction between diets and time by two-way ANOVA ($P < 0.05$). Tukey's Multiple Comparison *post hoc* test or Student's *t*-test were then used to identify specific differences between diets in the same week or differences between weeks within the same diet ($P < 0.05$), when a significance difference was found from the two-way ANOVA. Means not sharing the same letter indicate significant differences between diets in the same week. Significant difference ($P < 0.05$) between weeks in the same diet is indicated by "+". Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt.; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: fish fed diet with nominal 60 mg Ca g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt. MTLP: metallothionein-like proteins; MRG: metal-rich granules; ORG: organelles; HDP: heat-denaturable proteins; CD: cellular debris; BDM: biologically detoxified metal fractions (MTLP + MRG); MSF: metal-sensitive fractions (HDP + ORG).

Treatment	MTLP %	MRG %	ORG %	HDP %	CD %	BDM %	MSF %
Week 1							
Control Low	62.2 ± 12.3^{a}	9.0 ± 4.6^{a}	31.6 ± 14.4^{a}	1.2 ± 0.3^{a}	3.9 ± 4.7^{a}	71.2 ± 16.8^{a}	32.7 ± 14.1^{a}
Control High	52.4 ± 14.0^{ab}	19.8 ± 10.0^{ab}	24.5 ± 7.1^{a}	2.0 ± 0.8^{a}	1.7 ± 3.4^{a}	72.2 ± 7.9^{a}	26.1 ± 7.9^{a}
Cd only	39.9 ± 9.6^{b}	24.3 ± 10.0^{b}	8.8 ± 1.4^{a}	6.6 ± 1.0^{b}	27.2 ± 4.0^{b}	64.2 ± 12.6^{a}	14.6 ± 1.0^{a}
High Ca + Cd	37.7 ± 6.7^{b}	35.9 ± 7.5^{b}	11.5 ± 2.6^{a}	9.0 ± 2.5^{b}	5.8 ± 3.0^{a}	73.6 ± 3.9^{a}	21.8 ± 2.6^{a}
Week 4							
Control Low	9.2 ± 4.6^{a+}	30.8 ± 3.4^{a}	7.6 ± 3.6^{a+}	3.8 ± 0.8^{a+}	54.5 ± 3.0^{ab}	36.4 ± 4.7^{a+}	9.1 ± 4.6^{a+}
Control High	5.5 ± 2.1^{a+}	15.9 ± 4.0^{b}	2.6 ± 2.7^{a+}	3.9 ± 1.9^{a}	76.1 ± 7.9^{a}	20.0 ± 6.3^{a+}	2.3 ± 1.7^{a+}
Cd only	44.8 ± 16.0^{b}	13.5 ± 5.4^{b}	5.0 ± 1.2^{a+}	6.8 ± 1.5^{a}	42.0 ± 13.0^{bc}	55.6 ± 23.1^{ab}	10.8 ± 1.4^{a+}
High Ca + Cd	46.4 ± 4.0^{b}	36.6 ± 9.9^{a}	1.9 ± 0.8^{a+}	9.3 ± 2.1^{a}	10.8 ± 1.7^{c}	83.0 ± 7.2^{b}	11.4 ± 2.2^{a+}

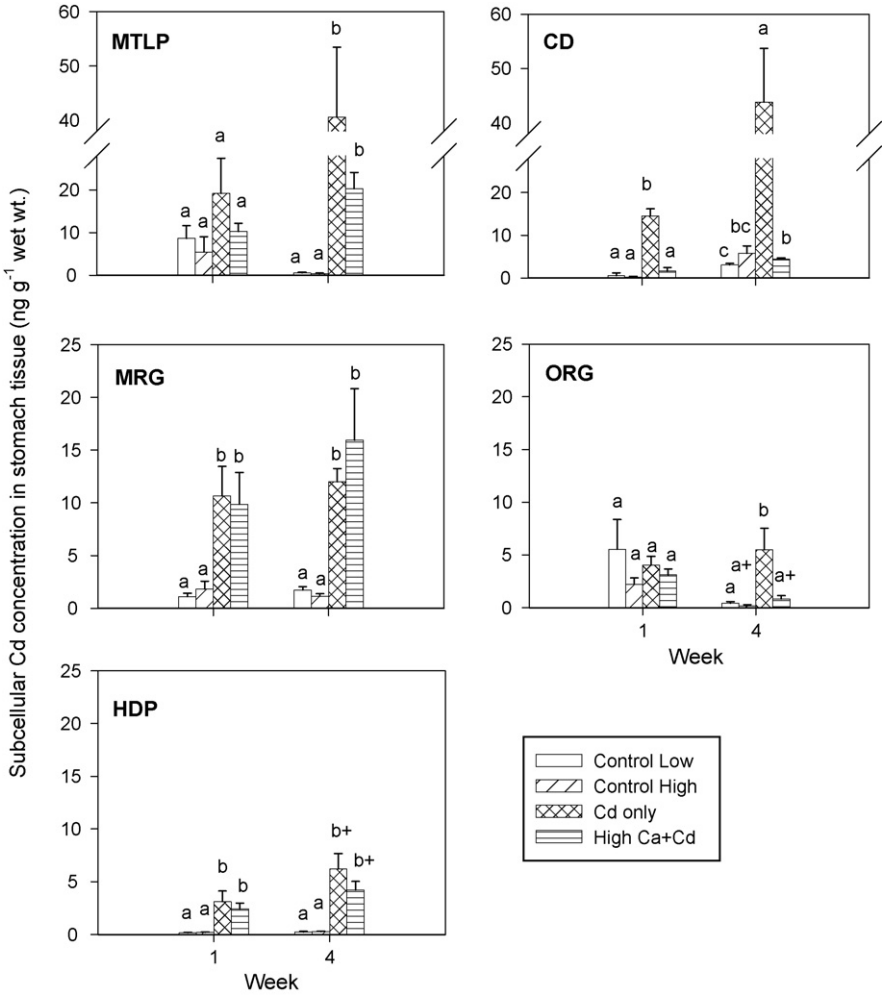

Fig. 3. Cd concentrations (ng g^{-1} wet wt.) in subcellular fractions of stomach tissue in rainbow trout on weeks 1 and 4, after being fed worm pellets with different concentrations of Cd and Ca. Mean ± standard deviation ($n = 10$ for whole tissue; 5 for subcellular fractions with 2 stomach tissues combined to form 1 replicate). Two-way ANOVA was used to test for significant effects of diets and time, also interaction between diets ($P < 0.05$). Dunnett's T3 *post hoc* test or Mann–Whitney *U* test was then used to identify specific differences between diets in the same week or differences between weeks within the same diet ($P < 0.05$) when a significance difference was found from the two-way ANOVA. Means not sharing the same letters indicate significant differences between diets in the same week. Significant differences ($P < 0.05$) between weeks within the same diet are indicated by "+". Control Low: fish fed diet without additional Cd or Ca; Control High: fish fed diet with nominal 60 mg Ca g^{-1} dry wt.; Cd only: fish fed diet with nominal 12 μg Cd g^{-1} dry wt.; High Ca + Cd: fish fed diet with nominal 60 Ca mg g^{-1} dry wt. and 12 μg Cd g^{-1} dry wt. MTLP: metallothionein-like proteins; MRG: metal-rich granules; HDP: heat-denaturable proteins; CD: cellular debris; ORG: organelles.

($P < 0.05$) at week 4. The Cd concentration in the MRG was higher in the Cd-exposed fish than the control fish ($P < 0.01$), and was similar to that of week 1 concentration ($P = 0.42$). Cd concentrations were also higher in the organelles of the fish exposed to the Cd only diet, compared to those of control fish ($P < 0.05$). On the other hand, the Cd concentration increased in the HDP of the Cd-exposed fish in week 4, compared to week 1 ($P = 0.01$) and it was again higher than that of the control fish ($P < 0.01$). Dietary supplementation with Ca reduced the concentration of Cd in ORG by 5-fold ($P = 0.02$) to a level comparable to that of the control fish ($P = 0.99$). However, it did not affect the concentration in the HDP significantly ($P = 0.32$). Overall, the detoxification of Cd was more obviously observed in the stomach after exposure to Cd for 4 weeks (elevated concentrations in MTLP and MRG). A higher percentage of Cd was associated with the BDM fraction of the stomach of the Cd-exposed fish, although not significantly, compared to the control fish ($P = 0.55$). In addition, fish fed High Ca + Cd diet had comparable Cd distributions in the BDM fraction as the fish fed the Cd only diet ($P = 0.24$).

4. Discussion

4.1. Do our treatment diet concentrations of Cd and Ca match naturally found concentrations?

The measured Cd and Ca concentrations in our worm pellets fell into the natural range of the environment. The lowest Cd concentration 0.18–0.24 $\mu g\,g^{-1}$ dry wt. in the control worm pellets was slightly lower or comparable to that in the control live worms found in our previous study (Ng and Wood, 2008) (0.1 $\mu g\,g^{-1}$ wet wt., assuming 85% water in the worm bodies according to Mount et al. (2006)) and the benthic invertebrates collected from clean sites in north eastern Ontario, Canada (Klinck et al., 2007). The Cd concentration in benthic invertebrates and zooplankton collected from contaminated freshwater systems in North America generally ranged between 1 and 29 $\mu g\,g^{-1}$ dry wt. (Farag et al., 1994, 1999; Kraemer et al., 2006). Cd concentrations in the diets we used (~12 $\mu g\,g^{-1}$ dry wt.) fell in the mid-point of this "natural" range and were 25–42-fold lower than those used in many previous dietary studies (300 and 500 $\mu g\,g^{-1}$ dry wt.; Baldisserotto et al., 2005; Franklin et al., 2005). Ca concentrations in the control worm pellets were also comparable to natural levels (1 $mg\,g^{-1}$ dry wt.) (Hansen et al., 2004; Klinck et al., 2007). The worm pellet diet containing the elevated Ca level is comparable to the concentration used in previous investigations (60 $mg\,g^{-1}$ dry wt.) (Baldisserotto et al., 2005; Franklin et al., 2005), which allowed us to compare the effects of dietary Ca between the studies.

Using live food is an alternative for understanding the effects of natural dietary Ca on Cd uptake by the fish, but may be difficult to implement experimentally. Xie et al. (2008) have successfully manipulated Ca concentration in *L. variegatus* by exposing the worms to water with different water hardness, although the change was relatively small. They found a difference of about 0.1 mg Ca g^{-1} wet wt. in worms that had been kept in very hard and very soft water for 10 d. A longer duration of exposure may be necessary to increase the variation of Ca concentration in the worms for studying the effect of Ca on trophic transfer of Cd to the fish.

4.2. Does a low dietary Cd exposure reduce the survival and growth of fish? Does a Cd diet supplemented with elevated Ca reduce these toxic effects?

The survival rate of fish fed diets containing environmental concentrations of Cd was unaffected, but their growth may be reduced in the long-term since a significant reduction in growth at weeks 3–4 relative to previous weeks was observed only in the Cd-treated fish. Field and laboratory studies exposing the fish to diets with low (Farag et al., 1994, 1999; Hansen et al., 2004; Ng and Wood, 2008) to high concentrations of Cd (Chowdhury et al., 2004a; Baldisserotto et al., 2005; Franklin et al., 2005; Chowdhury and Wood, 2007) generally did not report increased mortality, but effects on growth rates were variable among the studies. No significant impact on growth was observed in dietary studies exposing the rainbow trout to 300–500 $\mu g\,Cd\,g^{-1}$ dry wt. commercial food for 30–52 d (Chowdhury et al., 2004a; Baldisserotto et al., 2005; Franklin et al., 2005; Chowdhury and Wood, 2007), although elevated mortality and reduced growth were seen when massive levels (1500–2200 $\mu g\,Cd\,g^{-1}$ dry wt. commercial food) were used (Szebedinszky et al., 2001). Commercial trout food typically contains a high concentration of Ca (~20 $mg\,g^{-1}$), which may reduce the toxicity of dietary Cd to the fish. However, the worm pellets in our study (without additional spike of Ca) contained naturally low concentrations of Ca (only 0.6–0.8 mg g^{-1}). These more than 20-fold lower dietary Ca concentrations may explain the potential negative effect of dietary Cd on growth in this study.

In contrast to commercial diet, exposing the fish to a natural diet with a much lower Cd concentration may reduce their growth. It has been shown that cutthroat trout (*Oncorhynchus clarki*) grew 3–8-fold slower than the control fish after being fed benthic invertebrates that were collected from metal-contaminated sites in the Coeur d'Alene River, ID, USA, for 40–90 d (Farag et al., 1999). The reduction in growth started to occur from 44 d of the dietary exposure. The invertebrates from these field sites not only contained about 29 $\mu g\,Cd\,g^{-1}$ dry wt., but also elevated concentrations of other metals. In addition, juvenile rainbow trout fed *L. variegatus* that were cultured in metal-contaminated sediments for 14–67 d, exhibited reductions in growth (Hansen et al., 2004) after 2 weeks. These adverse effects on growth may be attributed to metals other than Cd in the field and/or to higher bioavailability of Cd from the natural food than the commercial food. A high percentage of Cd is often bound to the proteins of natural food (~70%, due to strong sulfydryl bonds) (Harrison and Curtis, 1992). This may result in higher bioavailability than for metals that may only be loosely bound when added to commercial pellet food. Higher Cd bioavailability may lead to higher toxicity to the fish, even when the Cd dose is low. For example, a 50% reduction in growth after 3 weeks of dietary exposure was observed when the rainbow trout were fed worms at a Cd daily dose 6–8-fold lower (Ng and Wood, 2008) than other dietary studies that used commercial food. This reinforces the conclusion that utilizing food with natural diet composition is important for metal bio-monitoring and dietary studies because Cd may interact with other moieties in the diet, altering Cd uptake and toxicity. The delayed effect on growth may be explained by the redistribution of energy reserve from growth to metal detoxification when metal accumulation reaches a toxic level.

4.3. What is the Cd tissue-specific distribution in the fish and does the supplement of Ca to the Cd diet change the accumulation pattern?

Exposure to low dietary Cd did not cause significant accumulation of Cd in the brains of rainbow trout. This is in agreement with the study of Szebedinszky et al. (2001) where massive levels of dietary Cd (up to 1500 $\mu g\,g^{-1}$ dry wt. commercial food) failed to elevate brain Cd concentration. Many reports have demonstrated that metals in the blood are restricted by the blood–brain barrier, and thus generally do not accumulate in the brains (Melgar et al., 1997). However in fish, waterborne metals may be taken up via water-exposed receptor cells of sensory nerves and subsequently

transferred toward the brain by axonal transport (Arvidson, 1988), for example, waterborne Hg accumulated in the olfactory system and optical nerve of the trout (Rouleau, 1999). Thus it is possible for metals to be potentially transferred to the brains. Woo et al. (1993) demonstrated that blue tilapia exposed to high concentrations of waterborne Cd had increased Cd accumulation in their brains. In addition, Sloman et al. (2005) demonstrated significant Cd and Pb accumulation in the brain tissue of the rainbow trout after exposure to waterborne 7 $\mu g\,L^{-1}$ Cd and 325 $\mu g\,L^{-1}$ Pb for 48 h. However, this was not observed in trout that were exposed to 2 $\mu g\,L^{-1}$ Cd for 7 d (Scott et al., 2003). To our knowledge, no studies have investigated metal accumulation in the brains from a dietary metal exposure. Our study found a few fish brains with Cd levels higher than background. More investigations are necessary to conclude whether dietary Cd can circumvent the blood–brain barrier.

Rainbow trout can still accumulate significant levels of Cd in their organs even when they are exposed to a low Cd diet. In this study, the Cd concentrations in fish organs or their whole bodies was 15–30-fold lower than in other studies that exposed fish to corresponding higher Cd diets (25–42× higher) (Baldisserotto et al., 2005; Franklin et al., 2005). Similar to those studies, the liver, kidney and stomach accumulated the most Cd and average concentrations progressively increased over the dietary exposure period in the liver and kidney. In general, the independence of Cd tissue-specific distribution from the Cd dose in the diet may imply that fish handle Cd in a similar manner before dietary Cd causes any disruption to the metal metabolism.

Dietary Cd also caused an increase in Cd accumulation in the gills. This was probably not caused by leaching of Cd from the diet to the water because our monitoring studies found negligible Cd concentrations in the water after the food was provided to the fish. In accordance with Szebedinszky et al. (2001), we speculate that Cd accumulation in the gills was caused by Cd taken up from the gastro-intestinal tract, transported through the bloodstream, and entering through the basolateral membranes of gill cells. The physiological impact of a gill Cd load obtained in this manner appears to be much less than when the same load is obtained directly from the water through apical pathways (Szebedinszky et al., 2001; Niyogi and Wood, 2003).

In the present study, the degree of protection by elevated dietary Ca against Cd accumulation in the fish varied depending on the specific organ. Partial reduction in accumulation (≥50%) was observed in the stomach, and whole body, which is comparable to the previous study conducted by Franklin et al. (2005). Until recently, the stomach was not believed to be important for metal ion absorption in mammals or in fish (Fields et al., 1986; Clearwater et al., 2000), but it now has an emerging role, at least for fish (Wood et al., 2006). Our present report and previous dietary Cd studies have revealed a high concentration of Cd in the stomach tissue (Baldisserotto et al., 2005; Franklin et al., 2005), which is reduced by elevated dietary Ca. *In vitro*, elevated luminal Ca inhibits Cd absorption in the stomach, but not in other parts of the gastro-intestinal tract (Ojo and Wood, 2008). *In vivo*, the stomach is exposed to up to 10-fold higher dissolved concentrations of Cd and Ca in acidic gastric chyme than seen in slightly alkaline intestinal chyme (Baldisserotto et al., 2005; Bucking and Wood, 2007, 2008). Indeed, the stomach has been found to display the highest net uptake for Ca in trout 2 h after the ingestion of a single meal (Bucking and Wood, 2007). In addition, Chowdhury et al. (2004a) reported a rapid absorption of gastrically infused ^{109}Cd into the plasma of rainbow trout by 0.5 h, indicating that Cd absorption may start in stomach and continue along the intestinal tract. The above evidence may explain the interaction of Ca and Cd uptake at the stomach. Dietary Ca in this study almost completely (close to 100% inhibition) protected the gills from Cd accumulation, and strongly protected (≥50% inhibition) the stomach, and whole body. This result was also consistent with the strong protective effects observed in gills (70%), stomach (~50–70%), and whole body (~75%) of rainbow trout by Franklin et al. (2005).

The protective effects of dietary Ca against Cd accumulation in the liver and kidney were either insignificant or remained inconclusive. Indeed, variable protective effects on liver and kidney were also observed in previous studies. Franklin et al. (2005) showed that dietary Ca kept the liver Cd levels to less than half of that of fish fed dietary Cd alone and had no significant effect on accumulation in the kidney, whereas Baldisserotto et al. (2005) detected only minor protective effects in liver and kidney of fish exposed to a similar diet as used by Franklin et al. (2005). The contradictory protective effects observed in the stomach *versus* liver/kidney may be explained by the fact that a large portion of Cd absorption may in fact occur in other segments of the gut such as the intestine (Baldisserotto et al., 2006; Ojo and Wood, 2007, 2008) through non-Ca transporters such as DMT1 (Wood et al., 2006). Therefore, Cd accumulation in liver and kidney may not be reflective of the Cd accumulation in the stomach where most interaction of Ca and Cd probably occurs.

4.4. Does low dietary Cd exposure affect metal levels in the fish blood? Does a Cd diet supplemented with elevated Ca reduce these effects?

Low dietary Cd, with or without additional Ca, did not alter the levels of Cd in the plasma of the trout. The concentrations of plasma Cd and Ca in our control fish were in agreement with previous studies (Chowdhury et al., 2004a; Franklin et al., 2005). Chowdhury et al. (2004a) and Chowdhury and Wood (2007) found remarkable increases of Cd levels (28–49-fold) in the plasma after the fish were acclimated to a diet with 500 $\mu g\,Cd\,g^{-1}$ dry wt. for 30–52 d. The lack of effect on Cd in the present study may be explained by our significantly lower exposure Cd concentrations in the worm pellets (25–42-fold). In addition, the uptake and clearance of Cd in the plasma generally both occur within a day in the rainbow trout after ingestion of a single meal containing elevated Cd. The rate of uptake is the highest within 4 h of ingestion and Cd is cleared within a day (biological half-life: 44–54 min) (Chowdhury et al., 2004a). We measured the plasma ion concentrations only at the end of the experiment; therefore, we cannot comment on any possible transient changes in metal concentrations in the plasma. In contrast to our study, dietary Ca or Cd generally did not affect the plasma Ca concentration of the rainbow trout (Baldisserotto et al., 2005; Franklin et al., 2005). A possible explanation for this discrepancy is the higher background dietary Ca or Cd concentration used in other studies, so any potential effects of Ca may be overlooked. A time course study has shown that plasma Ca levels can peak within several hours of the first feeding of a high Ca meal (Baldisserotto et al., 2004), but then return to control levels once homeostatic mechanisms have been initiated. One such mechanism is the mobilization of stanniocalcin, a calcitropic hormone released into the blood (Wagner et al., 1989). The secretion of stanniocalcin is positively regulated by physiological levels of ionic Ca^{2+}. In the present study, an opposite trend was observed with respect to Ca levels in the plasma. This may be due to the down-regulation of Ca levels by the stanniocalcin.

Red blood cells appear to act as the reservoirs for metals in the blood of fish. They play an important role in binding metals in fish, as they also do in mammals, regardless of routes of exposure. For example, rats chronically exposed to Cd via their drinking water had a 6.8-fold greater loading of Cd in red blood cells in comparison to plasma (Crowe and Morgan, 1997). Similarly, red blood cells accumulated 1.8–9-fold (Chowdhury et al., 2004a), 3-fold (Franklin

et al., 2005) and 13–40-fold (this study) higher Cd than plasma in rainbow trout that had been exposed to dietary Cd. However, in our study, low dietary Cd did not raise Cd levels in the red blood cells, and elevated dietary Ca had no protective effect against Cd accumulation in the erythrocytes. This was inconsistent with the reported significant elevations of Cd in the red blood cells of Cd-acclimated fish in other studies (Chowdhury et al., 2004b; Franklin et al., 2005) that used a much higher Cd dose.

4.5. Can toxicity of low dietary Cd be revealed in the subcellular partitioning of the fish? Does elevated Ca in the Cd diet change the partitioning?

Subcellular metal partitioning can provide information on how the cells interact with metals, thus casting light on potential metal toxicity at cellular levels. Wallace et al. (2003) first used subcellular metal partitioning to predict toxicity of waterborne Cd and Zn based on compartmentalization using two species of clams, *Macoma balthica* and *Potamocorbula amurensis*. Subcellular metal partitioning can also relate the toxicity of metals expressed at the organismal and the population levels of bivalves (Perceval et al., 2004, 2006; Campbell et al., 2005). It has been found that the yellow perch (*Perca flavescens*) stored more Cd in all subcellular fractions, including the MSF, as total hepatic Cd increased (Campbell et al., 2005). This may link to the endocrine and metabolic perturbations observed in fish. Our previous study on rainbow trout (Ng and Wood, 2008), also indicated an efficient detoxification of dietary Cd by subcellular metal analysis of the fish gut. To our knowledge, the present study is the first to investigate subcellular metal partitioning in the red blood cells of fish.

In this study, we simultaneously compared the results on Cd concentration and percentage distribution in subcellular fractions. Concentration of Cd in subcellular fractions indicates the absolute accumulation in different compartments after the exposure, also possible toxicity to the cells, whereas percentage distribution of Cd indicates whether there is a change of strategy to handle metals in the fish. For example, fish may detoxify metals by increasing the percentage of Cd associated with MTLP, even though Cd concentration in MSF may still be high.

Previous reports have shown that red blood cells are sensitive to metals. The most abundant protein in red blood cells is haemoglobin, an iron-binding metalloprotein that transports oxygen. Metalloproteins have a high affinity for metals and high concentrations of metals can cause deleterious effects on haemoglobin function (Khangarot et al., 1999; Alves and Wood, 2006; Chiesa et al., 2006). Red blood cell counts may be reduced by the toxic effects of metals on haemoglobin synthesis and function (Chiesa et al., 2006). In addition, metals can produce oxidative stress on red blood cells of fish (Gwozdzinski et al., 1992). In our study, fish exposed to the Cd diet compared to the control fish had a higher concentration of Cd in the MSF of the red blood cells. This may relate to the high affinity binding of Cd to haemoglobin (a heat-denaturable protein). The Cd diet supplemented with Ca changed the Cd subcellular concentration in the red blood cells, although the total Cd level was unaffected. Higher concentrations of Cd were associated with the metal-detoxified fractions of the fish fed the High Ca + Cd diet, relative to the fish fed the Cd diet only. This may have been caused by the re-distribution of Cd from ORG to MRG, which could imply that there has been a reduction of Cd toxicity. However, the mechanism of how dietary Ca changed the subcellular Cd concentration needs further investigation. In other studies, dietary Ca supplementation has been reported to reduce total Cd (Franklin et al., 2005) and Pb accumulation (Alves and Wood, 2006) in the red blood cells of rainbow trout. Overall, the present study demonstrated that subcellular metal partitioning can predict potential toxicity of Cd and protection by Ca against Cd toxicity at a cellular level in the red blood cells, even when total Cd levels are unaffected by dietary Cd and Ca.

Subcellular Cd partitioning in the fish stomach is dynamic, irrespective of metal exposure. Cd apparently shifted from associating with the soluble fractions (e.g., MTLP) to the insoluble fractions (e.g., increase of average Cd distribution in CD and MRG over weeks) in the stomach of the control fish during the dietary exposure period. This may reflect a developmental change in metal metabolism in the fish. Our previous study also demonstrated a shift of Cd from the soluble to insoluble fractions (MTLP to MRG) in the gut of the unexposed trout (Ng and Wood, 2008). In fact, subcellular metal distribution varies depending on the specific organ (Perceval et al., 2006), species (Zhang and Wang, 2006), size of the body, as well as with the season (Wallace et al., 2003). It is also a time-dependent process inasmuch as metals may shift from associating with temporary fractions to longer term storage fractions (Ng and Wang, 2005). Cd-exposed fish in our study had a different Cd subcellular distribution in the stomach compared to the whole gut of the trout that were exposed to oligochaete worms with similar Cd concentrations (Ng and Wood, 2008). More Cd was distributed in the MTLP (10% higher) and CD (40% higher) of the fish stomach in our study. This difference may be related to the different portions of gut taken for subcellular analysis (previous study: whole gut; this study: stomach only).

Stomach Cd subcellular partitioning indicated that, although the low dose of dietborne Cd caused toxicity in the cells by binding Cd to the HDP, detoxification took place and became more effective over 4 weeks of exposure. Higher percentage of Cd was bound to MTLP, a widely recognized protein for detoxifying Cd (Hollis et al., 2001; Chowdhury et al., 2005) in the stomach of rainbow trout. Similarly, our previous study on rainbow trout, also reported a shift in Cd from the HDP to MTLP in the fish gut after feeding of Cd contaminated worms for 1 month (Ng and Wood, 2008). Ca supplementation of the Cd diet altered the subcellular Cd partitioning in the fish stomach, in a manner similar to that seen in the red blood cells, inasmuch as it reduced the Cd accumulation in the ORG fraction. Elevated Ca in the diet may increase binding of intracellular Ca on the organelles, resulting in less Cd actually taken up by the organelles due to competition for specific transporters or binding sites. Alternately, dietary Ca may activate particular Ca-dependent enzymes, which may mobilize the transport of Cd from the metal-sensitive organelles to other subcellular compartments.

5. Conclusions

Our study has demonstrated the need to use diets with natural compositions for the dietary toxicity studies. Worm pellets with natural levels of Ca allowed us to determine a potential toxicity of low dietborne Cd on the growth, and Ca regulation in the rainbow trout when under chronic exposure. Our results imply that previous studies using commercial food may underestimate the toxicity of dietary Cd. The toxicity of dietary Cd was also revealed by the subcellular Cd storage in the stomach and red blood cells. Cd was most associated with the metal-sensitive proteins or organelles of the red blood cells, which may predict a toxic effect on haematology of the fish. Elevated Ca generally protected against accumulation and toxicity of dietborne Cd. The degree of protection was greatest at the gills, robust in the stomach, and whole body, but not significant in the carcass, liver, kidney, blood plasma, or red cells. The protective action of dietary Ca against Cd toxicity was further supported by our subcellular fractionation analysis showing a reduction in Cd distribution in the organelles of the fish stomach and red blood cells. Therefore, dietborne Ca not only reduced the Cd uptake by the cells, but also altered how the cells handled Cd intracellularly.

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