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Tissue-Specific Cadmium and Metallothionein Levels in Rainbow Trout Chronically Acclimated to Waterborne or Dietary Cadmium

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Abstract. Rainbow trout were exposed to a sublethal concentration of waterborne Cd (0 or 3 μg/L) or dietary Cd (0 or 500 mg/kg dry wt) for 30 days to induce acclimation, and tissue Cd and metallothionein (MT) levels were examined. The greatest Cd concentrations were observed in the kidney followed by the gills and liver of the fish exposed to Cd via water, but in the gut tissues followed by the kidney, liver, and gills for dietary-exposed fish, reflecting a variation depending on the route of Cd exposure. Some MT was found in the nonacclimated naïve fish with no experience of elevated Cd exposure, and these background MT levels were quite high in the posterior intestine (480 μg/g), cecae (257 μg/g), and liver (248 μg/g) relative to other tissues (7–50 μg/g). With exposure to both waterborne and dietary Cd, MT levels rose significantly in all observed tissues. The increases relative to the control levels of MT in naïve fish were in the order: kidney (5.4 times) > gills (4.6) > liver (1.3) for the waterborne exposure group, and in the order kidney (19.3 times) >> cecae and posterior intestine (~6.5 times) > liver and stomach (~5 times) > midintestine (4.3 times) > gills (2.1 times) for the dietary exposure group. At 24 hours after an acute gastrointestinal dose of Cd (276 μg/kg) infused into the stomach of dietary exposure groups, large increases of total Cd but not MT levels were found in the gut tissues of nonacclimated fish; in the Cd-acclimated fish, the posterior intestine was greatly affected with decreases in Cd (71%), Zn (33%), Cu (70%) and MT (46%) levels, suggesting an enhanced sloughing of tissue materials after infusion. Exposure to Cd did not cause any notable decrease of Zn or Cu in any tissue, except that found in the posterior intestine. However, a molar analysis indicated that although Cd levels remained less than MT binding capacity in both waterborne and dietary exposure groups, the total metal levels (Cd + Zn + Cu) greatly exceeded MT binding capacity in all tissues of Cd-exposed fish, suggesting a potential competition of Cd with other metals for binding sites on MT and non-MT proteins in the tissues.

Cadmium (Cd) is an environmental contaminant that originates from a variety of industrial processes. In vertebrates, it has no known function and can exert significant chronic toxicity. Fish can take up Cd from both waterborne and dietary sources; Cd accumulates maximally in the kidney, gills, liver, and gut, to a lesser extent in the blood, but not significantly in the brain or muscle (Harrison and Klaverkamp 1989; Sorensen 1991; Szebedinszky *et al.* 2001; Chowdhury *et al.* 2003, 2004). During chronic exposure to Cd and other toxic metals, fish undergo acclimation with changes in physiological status and an increased resistance and/or tolerance (Roch and McCarter 1984; McDonald and Wood 1993; Stubblefield *et al.* 1999; McGeer *et al.* 2000; Wood 2001). Synthesis of the cysteine-rich metalloprotein, metallothionein (MT) for binding and storage of metals in target tissues is known to play an important role in acquired tolerance during acclimation (Roch and McCarter 1984; Kito *et al.* 1986; Hogstrand and Haux 1991; Roesijadi and Robinson 1994; Wu and Hwang 2003). The binding of toxic metals to MT represents a sequestration function that renders them unable to interact with other proteins, such as enzymes, and thereby produces protection against metal toxicity at the cellular level (Kito *et al.* 1982; Kay *et al.* 1986; Roesijadi 1992). Indeed, MT-null mouse strains show an increased sensitivity to Cd toxicity (Masters *et al.* 1994).

Although fresh water fish can be acclimated to both waterborne and dietary Cd (Hollis *et al.* 1999; Stubblefield *et al.* 1999; Szebedinszky *et al.* 2001; Wood 2001), the relationship between tissue-specific Cd and MT levels has only been studied in fish exposed to waterborne Cd (Fu *et al.* 1990; De Smet *et al.* 2001; Hollis *et al.* 2001); limited dietary studies lack quantitative information on MT levels in gut and other internal tissues of fish (Weber *et al.* 1992; Farag *et al.* 1999; Dang *et al.* 2001). Szebedinszky *et al.* (2001) showed that rainbow trout acclimated chronically to dietary Cd exhibited altered uptake dynamics for waterborne Cd at the gills, and an increased tolerance to waterborne Cd. Immunochemical localization suggests that dietary Cd taken up via the gut can also enter the gills, where it accumulates in chloride cells and stimulates MT expression (Dang *et al.* 2001). Recently we have shown that there is a significant effect of dietary Cd acclimation on gastrointestinal uptake and disposition of Cd in gut as well as nongut tissues (Chowdhury *et al.* 2004).

In the present study, the relationships between tissue-specific Cd accumulation and MT induction in rainbow trout acclimated for 30 days to either waterborne Cd (3 μL) or dietborne Cd (500

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mg/kg) were investigated. Special attention was given to the dietary acclimation treatment in order to examine MT induction in different gut regions as well as in the liver, gills, and kidney since such information is not known. The effect of short-term exposure (24 hours after Cd infusion into the stomach) to elevated gastrointestinal Cd concentration was also investigated in naïve and dietary-acclimated fish in order to examine some of the short-term responses reported by Chowdhury *et al.* (2004). Zinc (Zn) and copper (Cu) concentrations in the same tissues of these groups were also analyzed to determine whether Cd interacts with Zn and Cu for binding sites on MT, since MT is considered to be involved in Zn and Cu homeostasis in fish (Hogstrand and Haux 1991; Olsson 1996) and many toxic effects of Cd result from interactions with these essential elements (Brzoska and Moniuszko-Jakoniuk 2001). A similar analysis of Cd/Zn-Cu interaction in rainbow trout for waterborne Cd exposure has been reported by Hollis *et al.* (2001). Since MT induction as an underlying mechanism of Cd acclimation was investigated in the present study, the exposure concentrations and fish sizes were chosen based on previous studies from our laboratory that showed physiological (decreased ionoregulatory or acid-base disturbance) and toxicological (increased 96-hour LC_{50}) evidences of Cd acclimation in rainbow trout of these sizes during chronic exposure to Cd at these respective levels (Hollis *et al.* 1999; McGeer *et al.* 2000; Szebedinszky *et al.* 2001; M. J. Chowdhury, E.F. Pane, C. M. Wood, unpublished data).

Materials and Methods

Acclimation to Waterborne Cd

Rainbow trout (*Oncorhynchus mykiss*; 20–25 g) were obtained from Humber Springs Trout Hatchery (Mono Mills, Ontario, Canada) and held under laboratory conditions for at least 2 weeks before experimental use. Fish were held in 500-L tanks supplied with a minimum of 1.5 L/min flow-through of moderately hard dechlorinated Hamilton City tapwater (0.03 μg/L Cd; 2.9 μg/L Cu; 16 μg/L Zn; 1 mM Ca, 0.6 mM Na; 0.7 mM Cl). The temperature and pH of the water were kept at 12 ± 1°C and 8.0 ± 0.2, respectively, throughout the experiment. The fish were fed commercial, floating trout chow (Martin's Feed Mills, Ontario, Canada; Cd concentration ≈0.162 mg/kg) at a ration of 2 % of their body weight three times per week.

For acclimation, fish from the holding tank were transferred into two 300-L tanks (150 fish per tank) and exposed to either 3 μg/L Cd (Cd-acclimation group) or 0 μg/L Cd (control or nonacclimation group) for 30 days with a water flow-through of approximately 1.8 L/min and a food ration of 1.5% per day (dry feed/wet body wt). The Cd-acclimation tank received water from a head tank via a mixing chamber that was constantly spiked with an acidified stock solution of $Cd(NO_3)_2.4H_2O$ (48.3 Mg/L; Fisher Scientific, ON, Canada) from a Mariotte bottle so that a nominal Cd concentration of 3 μg/L or 26.7 nmol/L could be maintained. This corresponds to a waterborne exposure Cd dose of 0.864 mg/kg fish/d, calculated as the product of exposure concentration (3 μg/L) and ventilation rate of water through the buccal cavity (200 mL/kg/min; Clearwater *et al.* 2002). Water samples from the experimental tanks were taken every 2 to 5 days, filtered through 0.45-μm membranes, and analyzed for Cd after acidification with HNO_3 (1%). Actual measured Cd concentrations were 3.2 ± 0.1 μg/L (28.38 ± 1.06 nmol/L, n = 10; actual dose = 0.922 mg/kg fish/d) in the Cd-acclimation tanks and 0.21 ± 0.03 μg/L (1.85 ± 0.26 nmol/L, n = 10) in the control tank. Mortality of fish in the Cd-acclimation tank was minimal (<8%) over 30 days and the average terminal body weight was 34.5 ± 1.8 g (n = 26) for the control fish and 36.6 ± 1.3 g (n = 25) for Cd-acclimated fish.

Acclimation to Dietary Cd

Adult rainbow trout (190–280 g) were obtained from the same hatchery and held in the laboratory conditions as mentioned above but with a water flow-through of 2.5 L/min. For acclimation to dietary Cd, 40 fish in one holding tank (Cd-acclimation group) were fed a 1.5% daily ration (dry feed/wet body wt) of a prepared Cd-diet containing nominally 500 mg Cd/kg dry feed for 30 days (actual measured concentration: 419.5 ± 2.3 mg Cd/kg food or 6.29 mg Cd/kg fish/d). Another holding tank was used simultaneously as a control tank in which 40 fish (nonacclimation group) were fed a prepared control diet with no added Cd (actual measured concentration: 0.162 ± 0.003 mg Cd/kg) at the same ration. Diets were prepared by mixing a concentrated stock of $Cd(NO_3)_2.4H_2O$ (6.86 g/L; Fisher Scientific, ON, Canada; Cd diet) or only doub1e-distilled water (control diet) into the commercial trout chow, followed by pelletization and drying as described by Chowdhury *et al.* (2004). Water samples from the experimental tanks were taken every 3 to 4 days, filtered through 0.45-μm membranes, and analyzed for Cd after acidification with HNO_3 (1%). The measured Cd concentrations in water were 0.14 ± 0.01 μg/L) (1.21 ± 0.07 nmol/L) and 0.22 ± 0.01 μg/L (1.96 ± 0.11 nmol/L) in control and Cd-acclimation tanks, respectively. The fish were not fed for 48 hours prior to experimentation. No mortality was recorded during the 30-day acclimation period, and the average terminal body weight was 356 ± 14 g (n = 24) for the control group and 347 ± 21 g (n = 24) for the Cd-acclimated group.

Experimental Procedures and Sampling

To determine Cd levels and MT induction in trout exposed to waterborne Cd, six fish both from control and acclimation tanks were subsampled every 2 or 4 days and sacrificed to collect the gills, liver, and kidney. The remaining carcass was assayed for Cd content. Samples collected on days 0, 2, 6, 8, 12, 16, 20, 24, 26, and 30 were analyzed for Cd and those collected on days 0, 4, 10, 18, and 28 were analyzed for MT. Tissue samples were not large enough to perform both analyses on the same fish. Tissue samples collected for MT analysis were immediately frozen in liquid N_2 and stored at −80°C for later analysis. Tissue samples and carcasses collected for Cd content were added to approximately 3 to 5 volumes of 1 N HNO_3 and kept in an oven at 60°C for 48 hours for tissue digestion.

In the case of the dietary acclimation group, six nonacclimated and six Cd-acclimated fish were cannulated by surgically implanting a catheter (PE-50 polyethylene tubing, Intramedic, Becton Dickinson, Sparks, MD, USA; internal diameter 0.58 mm, external diameter 0.97 mm, 30 cm long) into the stomach as described in Chowdhury *et al.* (2004). Each fish was placed in an aerated experimental chamber (2.5 L) supplied with flowing water (250 mL/min) and allowed to recover from surgery for at least 36 hours. Then the fish were given a single dose of Cd ($Cd(NO_3)_2.4H_2O$) in 0.1 M glucose solution into the stomach via the catheter. The fish were infused with 1.67 mL/kg of the Cd solution (165 μg/mL) using a Hamilton syringe, resulting in a Cd dose of 276 μg/kg fish (2455 nmol/kg).

Twenty-four hours after dosing, the fish were killed immediately by an overdose of MS-222 (0.6 g/L) and dissected to obtain tissue samples. The liver, gill filaments, and kidney were obtained as whole organs. For gut tissues, the whole gastrointestinal tract was removed

from the body cavity and cut into four pieces: whole stomach (from the end of esophagus to the pyloric sphincter), anterior intestine including pyloric cecae (hereafter referred to as cecae, from the pyloric sphincter to the last pyloric cecum), midintestine (from the last pyloric cecum to the beginning of the posterior intestine), and posterior intestine (rest of the tract up to anus, distinguished by its larger diameter, darker color, and anular rings). The gut pieces were longitudinally cut to open the lumen, rinsed with distilled water to remove mucus, food fragments, and nonabsorbed Cd from the lumen, and analyzed individually. In order to determine background concentrations of Cd and MT in the tissues before dosing, six nonacclimated and six Cd-acclimated fish were sampled directly from the holding tanks, and tissue samples were obtained as above. Thus, in the dietary study, tissue Cd and MT concentrations were determined in four different fish groups: nonacclimated control, nonacclimated Cd-infused, Cd-acclimated control, and Cd-acclimated Cd-infused groups. The tissue samples were immediately frozen in liquid N_2 and preserved at −80°C until further analysis.

MT Analysis

MT concentrations in the tissue were determined using the mercury saturation assay described by Klaverkamp *et al.* (2000). In this assay, an excess of radiolabelled mercury (^{203}Hg is used to displace and replace *in situ* bound Cu, Zn, and Cd on MT, while proteins other than MT are removed by the use of heat and trichloroacetic acid (TCA).

Tissue samples (200–800 mg) were homogenized in 3 to 5 volumes of 0.9% NaCl on ice using a tissue homogenizer (Biospec Products, model: 398, Racine, WI, USA). The homogenate was heat-treated in a water bath at 95°C for 5 minutes and subsequently centrifuged at 10,000 rpm for 5 minutes after cooling the homogenate on ice for 5 minutes. Two hundred microliters of supernatant was transferred to microcentrifuge tubes (2 mL) and added with 200 μL ^{203}Hg-labelled $HgCl_2$ (specific activity: 0.01 μCi/μg Hg; Isotope Products Laboratories, CA, USA) in 20% TCA. After 10 minutes of incubation, 400 μL of 50% (wt/wt) chicken egg white solution in 0.9% NaCl was added to each assay tube and mixed by vortex to remove excess (non-MT bound) mercury. After vortex mixing, vials were centrifuged at 10,000 rpm for 3 minutes and 500 μL of the TCA supernatant was counted in the gamma counter for ^{203}Hg radioactivity. A calibration curve was constructed from five known concentrations of purified rabbit liver MT-II (SIGMA, lot #80K7013) in NaCl solution (5–100 μg MT-II/ml). The known MT-II standards were treated with radioactive Hg and egg white in the same way as mentioned for tissue supernatants. Finally, radioactive counts of TCA supernatants for both tissue and standards were used to determine MT concentrations of the tissue (μg/g w wt), by comparing the simple linear regression of the calibration curve with tissue samples.

After determining MT, the remainders of the tissue homogenates were incubated with 1 N HNO_3 in equal volume at 60°C for 24 hours and used for the measurement of Cd, Zn, and Cu concentrations after necessary dilution.

Measurements and Calculations

The radioactivity of ^{203}Hg-labelled supernatant used in the MT assay was measured in a Minaxi-γ Auto-gamma 5530 counter (Canberra Packard, Mississauga, ON, Canada). Metal concentrations were measured by atomic absorption spectrophotometry (SpectrAA-220, Varian, Mississauga, ON, Canada) with graphite furnace atomization for Cd and Cu and with flame atomization for Zn. Absorbance of the appropriately diluted unknown samples (duplicate or triplicate) was related to the absorbance of a series of known standards made from certified stock solutions of the metals (Fisher Scientific) to obtain total metal concentrations expressed on the basis of per-gram wet tissue or per-liter water.

The ratio of actual metal to theoretical maximum metal-MT was calculated for tissues in which MT was determined (gills, liver, kidney, stomach, pyloric cecae, midintestine, and posterior intestine) using the following equation (Hollis *et al.* 2001):

$$\text{Metal : Metal} - \text{MT} = \text{TissueCd}/(\text{MT} \times 7) + \text{TissueZn}/(\text{MT} \times 7) + \text{TissueCu}/(\text{MT} \times 12)$$

where "Tissue metal" is the measured metal concentrations in the tissue (nmol Cd, Zn, or Cu per gram wet tissue), and "MT" is the molar tissue concentrations (nmol MT per gram wt tissue using a molecular weight of 6000 g/mol for MT). The MT value is multiplied by 7 for Cd and Zn because 1 mole of MT binds 7 moles of these metals. The MT value is multiplied by 12 for Cu because 1 mole of MT can bind 10–12 moles of Cu. Theoretically, if the ratio is less than 1 potentially all of the metal(s) (*i.e.*, Cd alone or Cd + Zn + Cu) could be bound by available MT in the tissue.

Statistical Analysis

Data are expressed as mean ± 1 SE (n), where n is the number of fish. Student's unpaired *t*-test or Newman-Keuls test was applied to compare results in different experimental groups. Proportions were subjected to arcsine transformation before statistical analysis. All statistical tests were performed using the computer software Statistica (StatSoft, Tulsa, OK, USA) or InStat (GraphPad, San Diego, CA, USA). A fiducial limit of $p \leq 0.05$ was used throughout.

Results

Tissue Concentrations of Cd

Waterbone exposure. The background concentrations of tissue Cd in the day-0 naïve trout before waterborne Cd exposure were 5.4 ± 1.9, 6.9 ± 0.9, 5.6 ± 0.6, and 2.1 ± 0.6 ng/g (n = 6) in the liver, gills, kidney, and carcass, respectively. With exposure to 3 μg/L Cd, tissue levels increased significantly in all tissues over the 30 days, with a gradual increase from day 2 in the gills and kidney (Figure. 1). At day 30, the greatest increases in the Cd-acclimated fish were found in the order kidney (656-fold) > gills (400-fold) > liver (122-fold) > carcass (15-fold) in comparison to the naïve trout (day 0), with observed concentrations of 3.7 ± 0.3, 2.7 ± 0.6, 0.65 ± 0.1, and 0.03 ± 0.004 μg/g Cd (n = 6), respectively. The kidney level of Cd in the nonacclimated (control) fish increased slightly but significantly (1.4–6-fold), probably because of the trace amount of Cd present in the fish diet (∼0.162 mg Cd/kg).

Dietary exposure. The tissue concentrations of Cd in the dietary Cd acclimation groups are presented in Figure 2. The background concentrations of Cd in nonacclimated (naïve) trout were 10.1 ± 1.5, 5.7 ± 1.2, 51.6 ± 2.0, 10.7 ± 1.5,

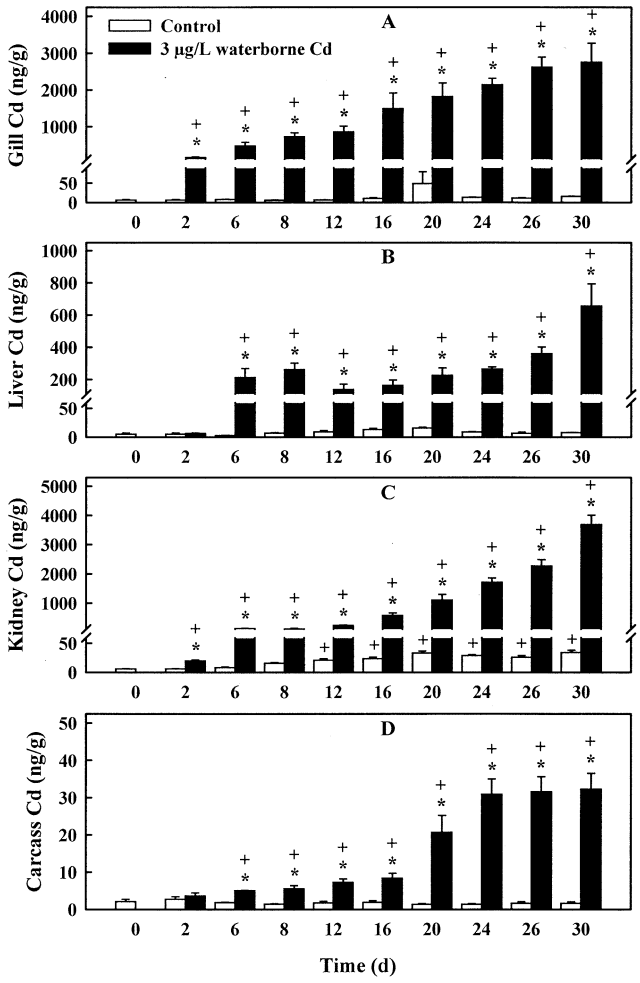

Fig. 1. Accumulation of Cd (nanograms per gram wet weight) by gills (A), liver (B), kidney (C), and carcass (D) of rainbow trout exposed to nominally 0 µg/L Cd (nonacclimated or control trout) or 3 µg/L Cd (Cd-acclimated trout) in water for 30 days. Results are presented as mean ± SE (n = 6). Bars with "*" and "+" indicate significant differences ($p < 0.05$) against control fish at each sampling day and at day 0, respectively.

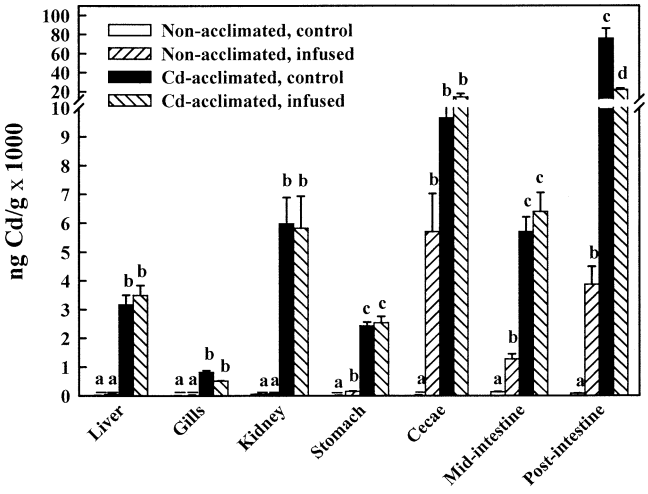

Fig. 2. Tissue accumulation of Cd (micrograms per gram wet weight) in nonacclimated control trout, in dietary Cd-acclimated control trout, and in both nonacclimated and Cd-acclimated rainbow trout 24 hours after a gastrointestinal dose of Cd (276 µg/kg w wt) infused into the stomach. The acclimated fish were preexposed to dietary Cd (nominally 500 mg/kg dry wt) for 30 d. Results are presented as mean ± SE (n = 6). Bars with different letters are significantly different ($p < 0.05$) for the same tissue.

18.1 ± 3.0, 131.8 ± 19.8, and 75.2 ± 9.1 ng/g (n = 6) in the liver, gills, kidney, stomach, pyloric cecae, midintestine, and posterior intestine, respectively. At 24 hours after gastrointestinal Cd infusion, Cd levels in the nonacclimated trout were significantly increased in all gut tissues but remained unchanged in the liver, gills, and kidney. Because of chronic exposure to dietary Cd for 30 days, gut total Cd levels in Cd-acclimated trout were substantially elevated in every tissue in comparison to naïve trout (Figure 2). The greatest relative increases were found in the order posterior intestine (~100-fold) > cecae (532-fold) > liver (315-fold) > stomach (227-fold) > gills (143-fold) > kidney (116-fold) > midintestine (43-fold), with observed concentrations of 75.5 ± 10.4, 9.6 ± 1.1, 3.2 ± 0.3, 2.4 ± 0.1, 0.8 ± 0.06, 6.0 ± 0.9, and 5.7 ± 0.9 µg/g Cd (n = 6), respectively. However, at 24 hours after gastrointestinal infusion of Cd into the Cd-acclimated fish, there were no significant increases of total Cd in any tissue. Surprisingly, the Cd burden in the posterior intestine of Cd-acclimated fish was actually reduced by 71 % at 24 hours after infusion (Figure 2).

MT Induction

Waterborne exposure. The effects of waterborne Cd exposure on MT levels in the sampled tissues are presented in Figure 3. There was always some MT in the nonacclimated control fish (naïve with no experience of elevated Cd exposure); the mean concentrations (n = 6) were much greater in the liver (148 µg/g at day 0 to 250 µg/g at day 28, Figure 3B) than those in the gills (7.4 to 13.2 µg/g, Figure 3A) and kidney (7.4 to 9.1 µg/g, Figure 3C). Indeed, liver did not show any significant differences of MT levels between nonexposed and Cd-exposed fish on any sampling day, whereas gills and kidney did from day 10 and day 18 onward, respectively. At day 28, gills, liver, and kidney MT levels in the Cd-acclimated trout were, respectively, 4.6 times (significant, Figure 3A), 1.4 times (not significant, Figure 3B) and 5.4 times (significant, Figure 3C) greater relative to the nonacclimated trout. However, relative to day 0, MT concentrations in all tissues increased during exposure to 3 µg/L Cd. The greatest increases in the Cd-acclimated fish were found in the order gills (8.2-fold) > kidney (6.6-fold) > liver (2.3-fold) at day 28, with observed concentrations of 61.0 ± 11.1, 49.1 ± 2.2, and 336.8 ± 60.4 µg/g, respectively.

Dietary exposure. Figure 4 illustrates the effects of dietary Cd exposure on MT induction in gut tissues as well as the liver, gills, and kidney. Similar to the waterborne acclimation

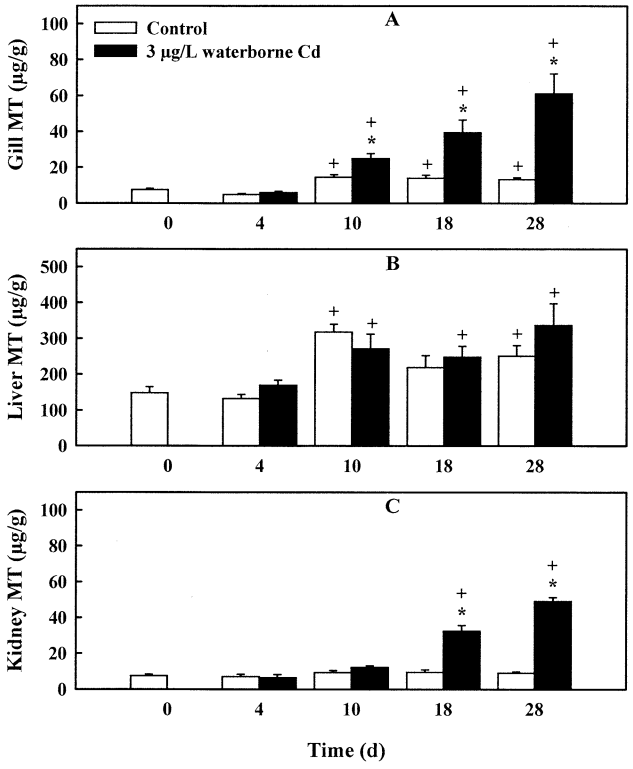

Fig. 3. Concentrations of metallothionein (µg/g w wt) in gills (A), liver (B), and kidney (C) of rainbow trout exposed to nominally 0 µg/L Cd (nonacclimated or control trout) or 3 µg/L Cd (Cd-acclimated trout) in water for 28 days. Results are presented as mean ± SE (n = 6). Bars with "*" and "+" indicate significant differences ($p < 0.05$) against control fish at each sampling day and at day 0, respectively.

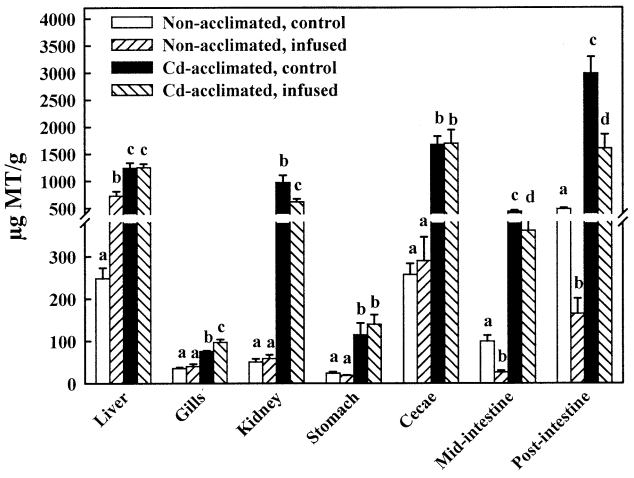

Fig. 4. Tissue concentrations of metallothionein (micrograms per gram wet weight) in nonacclimated control trout, in Cd-acclimated control trout, and in both nonacclimated and Cd-acclimated rainbow trout 24 hours after a gastrointestinal dose of Cd (276 µg/kg w wt) infused into the stomach. The acclimated fish were preexposed to dietary Cd (nominally 500 mg/kg dry wt) for 30 days. Results are presented as mean ± SE (n = 6). Bars with different letters are significantly different ($p < 0.05$) for the same tissue.

experiment, some MT was also present in the nonacclimated control fish (naïve fish) used in the dietary acclimation experiment, with highest concentrations (n = 6) being seen in posterior intestine (479.7 ± 21.3 µg/g) > cecae (256.7 ± 26.6 µg/g) > liver (247.9 ± 25.0 µg/g). MT concentrations (n = 6) in the gills, kidney, stomach, and midintestine of naïve fish were relatively low and varied between 24.9 ± 3.0 and 50.2 ± 7.9 µg/g. After the 30-day exposure to 500 mg/kg dietary Cd, MT levels in Cd-acclimated fish were elevated significantly in all tissues, with inductions in the order kidney (19.3 times) >> cecae and posterior intestine (~6.5 times) > liver and stomach (~5 times) > midintestine (4.3 times) > gills (2.1 times) relative to the background levels of MT in the same tissues of naïve fish. Similar to Cd concentrations (Figure 2), however, the greatest absolute concentrations of MT (Figure 4) were found in the posterior intestine (2984 ± 307 µg/g) and cecae (1671 ± 154 µg/g), followed by the liver (1240 ± 97), kidney (967 ± 138), midintestine (434 ± 22), stomach (114 ± 28), and gills (75 ± 2 µg/g).

With an infusion dose of 276 µg/kg Cd to nonacclimated fish, MT concentrations increased significantly in the liver over 24 hours, but decreased significantly in the midintestine and posterior intestine, whereas no significant change was observed in the gills, kidney, stomach, and cecae. In Cd-acclimated fish, MT in the gills rose slightly but significantly; in other tissues MT concentrations either did not change significantly (liver, stomach, and cecae) or decreased (kidney, midintestine, and posterior intestine) after Cd infusion (Figure 4).

Tissue Concentrations of Zn and Cu for Dietary Cd Exposure Groups

In general, tissue concentrations of Zn and Cu were much higher than those of Cd, and so are reported in micrograms per gram (Figure 5) rather than nanogram per gram (*e.g.*, Figure 2). Neither chronic preexposure to dietary Cd nor gastrointestinal Cd infusion alone, or in combination, caused any notable decrease of Zn (Figure 5A) or Cu (Figure 5B) relative to nonacclimated control fish in any tissue, except in the posterior intestine for Zn (Figure 5A). Indeed, overall tissue levels either showed an increasing trend (particularly for Cu, Figure 5B) or did not change. Zinc concentrations in the posterior intestine of nonacclimated infused fish and Cd-acclimated fish were one third to one sixth of those in naïve fish (117.5 ± 5 µg/g, n = 6), whereas in other tissues Zn levels did not change significantly because of chronic dietary Cd exposure (Figure 5A). Zn concentrations were greater in gut tissues than in non gut tissues; the highest concentrations averaged for four treatment groups were observed in the pyloric cecae (120.1 ± 15.6 µg/g, n = 24) and the lowest in the liver (2.6 ± 0.5 µg/g, n = 24).

During the 30-day exposure to dietary Cd, Cu levels in Cd-acclimated fish were elevated significantly in the kidney (5.6-fold), pyloric cecae (4.6-fold), midintestine (1.6-fold), and posterior intestine (1.8-fold) relative to nonacclimated control fish (Figure 5B). Upon Cd infusion, however, Cu levels de-

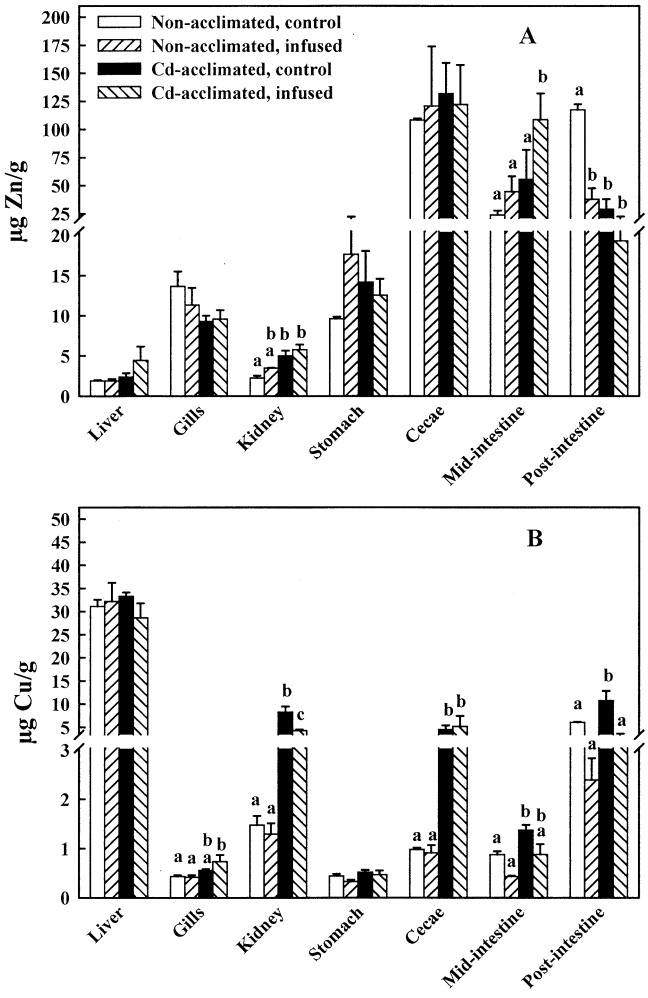

Fig. 5. Tissue concentrations of zinc (A) and copper (B) in nonacclimated control trout, in dietary Cd-acclimated control trout, and in both nonacclimated and Cd-acclimated rainbow trout 24 hours after a gastrointestinal dose of Cd (276 µg/kg w wt) infused into the stomach. The acclimated fish were preexposed to dietary Cd (nominally 500 mg/kg dry wt) for 30 days. Results are presented as mean ± SE (n = 6). Bars with different letters are significantly different ($p < 0.05$) for the same tissue. Note: metal concentrations (micrograms per gram wet weight) are from the same tissue samples used for MT assay.

creased significantly in the kidney and posterior intestine of Cd-acclimated fish. Among the four treatment groups, Cd-acclimated control fish, generally, contained the highest levels of Cu in their tissues with the trend: liver (33.2 ± 0.8 µg/g, n = 6) > kidney, cecae, and posterior-intestine (7.8 ± 0.9 µg/g, n = 18) > midintestine (1.4 ± 0.1 µg/g, n = 6) > stomach and gills (0.5 ± 0.01 µg/g, n = 12).

Tissue Metal to MT Ratios

Assuming that 1 mole of MT binds 7 moles of divalent Cd, the calculated ratios of actual Cd to the theoretical maximum Cd-MT binding sites were less then 1.0 for all tissues and all treatment groups of the waterborne and dietary experiments (Table 1, Figure 6A), meaning that there was potentially adequate MT binding capacity to complex all accumulated Cd in gut as well as nongut tissues of the naïve and Cd-exposed fish (Figures 1 and 2). Among different treatment groups, the ratios were the lowest in the naïve fish for most of the tissues (<0.008), with the exception of the kidney in waterborne control fish (0.03, Table 1). Although less than 1.0, the ratios increased significantly in all observed tissues of waterborne and dietary acclimation groups after Cd exposure (Table 1, Figure 6A). However, the ratios for nongut tissues (gills, liver, and kidney) of dietary Cd-exposed fish (<0.1, Table 1) were generally smaller than those for gut tissues in dietary exposed fish (0.04–0.5, Figure 6A), and identical to (liver) or smaller than (gills and kidney) those for nongut tissues of waterborne Cd-exposed fish (Table 1).

When tissue Cd, Zn, and Cu were considered together for dietary Cd exposure treatments (tissue Zn and Cu levels were not measured in waterborne Cd-exposed fish), the calculated actual metal to the theoretical maximum metal-MT binding sites ratios were almost equal to or well above 1.0 in both naïve and Cd-exposed fish for most of the tissues (Figure 6B), meaning that total metal levels in the tissues were greater than the total binding capacity of tissue MT. The only exceptions were for the liver, kidney and posterior intestine of Cd-acclimated fish, where the ratios were significantly below 1.0. The overall ratios for all tissues in nonacclimated control (0.9–7.7) and nonacclimated infused fish (0.3–18.8) were greater than those in Cd-acclimated control (0.2–4.2) and Cd-acclimated infused fish (0.2–5.7). This reflected a relative increase in binding capacity associated with acclimation.

Discussion

Tissue Cd Burdens

Our study demonstrates that chronic exposure to waterborne or dietary Cd results in different tissue-specific Cd accumulations. The greatest Cd concentrations were observed in the kidney and gills of the fish exposed to Cd via water (Figure 1, Table 1), but in the gut tissues followed by the kidney for dietary-exposed fish (Fig. 2). Such tissue-specific variation in Cd accumulation reflecting the exposure routes is comparable with previous studies on trout under similar experimental conditions (Szebedinszky *et al.* 2001; Hollis *et al.* 2001; Chowdhury *et al.* 2004). Among the nongut tissues (gills, kidney, liver, and carcass), kidney Cd levels were the highest in both waterborne (Figure 1 C, Table 1) and dietary (Figure 2, Table 1) acclimation groups, indicating that kidney can accumulate Cd via both routes relatively more efficiently in a chronic exposure situation. Thus, renal Cd burden can be an important indicator of chronic Cd exposure. However, earlier studies examining the distribution kinetics of Cd in chronically Cd-acclimated trout indicate that both the liver and kidney play an important role in the disposition of blood Cd; although liver is the primary site of Cd accumulation during a short-term exposure, kidney is the eventual storage site in a chronic exposure condition (Chowdhury *et al.* 2003, 2004).

The order of Cd accumulation in the gut tissues of the dietary acclimated fish (posterior intestine >> cecae > midin-

Table 1. Molar concentrations (± SE, n = 6) of Cd (nmol/g) and MT (nmol/g) and Cd/Cd-MT_{max} ratios in the gills, liver, and kidney of nonacclimated naïve fish and of the fish acclimated to waterborne Cd (3 μg/L; actual dose = 0.922 mg/kg fish/d) and dietary Cd (500 mg/kg diet; actual dose = 6.29 mg/kg fish/d) for 1 month

	Nonacclimated			Cd-acclimated		
Tissues	Cd	MT	Ratios	Cd	MT	Ratios
Waterborne						
Gills	0.12 ± 0.007	2.20 ± 0.17	∼0.008	22.77 ± 3.52[a]	10.17 ± 1.85[a]	0.36 ± 0.06[a]
Liver	0.06 ± 0.01	41.83 ± 4.96	<0.001	4.52 ± 0.60[a]	56.14 ± 10.06	0.02 ± 0.006[a]
Kidney	0.27 ± 0.02	1.52 ± 0.11	0.03 ± 0.001	26.49 ± 2.24[a]	8.18 ± 0.36[a]	0.46 ± 0.03[a]
Dietary						
Gills	0.05 ± 0.009[b]	5.91 ± 0.30[b]	∼0.001[b]	7.26 ± 0.52[a,b]	12.54 ± 0.43[a]	0.09 ± 0.01 [a,b]
Liver	0.09 ± 0.01	41.32 ± 3.40	<0.001	28.20 ± 2.73[a,b]	205.79 ± 14.77[a,b]	0.02 ± 0.003[a]
Kidney	0.46 ± 0.01[b]	8.44 ± 0.47[b]	∼0.008[b]	53.16 ± 7.37[a,b]	161.25 ± 18.83[a,b]	0.05 ± 0.007[a,b]

[a] Significant difference between nonacclimated and Cd-acclimated fish in the same row for Cd, MT, or Cd/Cd-MT_{max} ratios.
[b] Significant difference between waterborne and dietary acclimation groups in the same column for Cd, MT, or Cd/Cd-MT_{max} ratios.

testine > stomach; Figure 2) agrees with previous studies for gastrointestinal uptake of Cd in rainbow trout (Baskin 1999; Chowdhury *et al.* 2004), and is similar to that for Cu (Clearwater *et al.* 2000). At 24 hours after gastrointestinal Cd infusion, large significant increases of total Cd were found in the gut tissues of nonacclimated fish but not in the gut tissues of Cd-acclimated fish. Similar results were also reported in Chowdhury *et al.* (2004) in which an equal gastrointestinal dose of radioactive Cd (276 μg/kg) was infused into fish stomach and approximately 17% of the dose was found in the gut tissues of both groups. However, no significant increase in the Cd-acclimated fish is probably attributable to masking of new Cd by high concentrations of dietary Cd already present in the gut tissues. Indeed, in the posterior intestine of Cd-acclimated fish, Cd burden was reduced by 71% over 24 hours after infusion (Figure 2), comparable to a 78% reduction in our previous study (Chowdhury *et al.* 2004). The decrease of Cd burden in both studies, instead of an increase, suggests that Cd infusion probably caused a Cd loss by enhanced sloughing of mucosal tissue and mucus secretion in the posterior gut region, as reported previously for the gills and gut (Sorensen 1991; Handy 1996). Histopathological study also indicates that this region is relatively more affected upon exposure to dietary Cd and possesses many necrotic cells (C. Kamunde, M. J. Chowdhury, C. M. Wood, unpublished result). As also shown by Chowdhury *et al.* (2004), Cd infusion did not result in elevated Cd levels in nongut, internal tissues (liver, gills, and kidney) of either nonacclimated or acclimated fish in the present study (Figure 2). This might be due to the fact that only a small fraction of the infused Cd dose (nonacclimated: 2.4%; Cd-acclimated: 6.6%) is actually internalized across the gut wall (Chowdhury *et al.* 2004).

MT in Naïve Fish

The presence of tissue MT in the naïve fish with no experience of elevated Cd exposure in the present study suggests that there is always some MT in fish under normal conditions (Figures 3 and 4, Table 1), probably synthesized in response to background level stress, or simply for Zn and Cu homeostasis. The increase of control MT levels with time (Figure 3) and significantly greater control MT levels in the gills (5.9 versus 2.2 nmol MT/g, Table 1) and kidney (8.4 versus 1.5 nmol MT/g, Table 1) of large fish (∼350 g, dietary experiment) in comparison to small fish (∼35 g, waterborne experiment) indicates that the background levels probably rise as the fish grow. The background level of MT was found to be quite high in the liver relative to the gills and kidney of rainbow trout and carp in previous studies (De Smet *et al.* 2001; Hollis *et al.* 2001). Our findings not only support theirs, but also show that gut tissues (particularly posterior intestine and cecae) have similar or even greater levels of background MT in comparison to liver, probably reflecting their role in detoxifying the small trace of Cd that was found in our control diet (∼162 μg/kg diet).

MT Induction in Cd Exposed Fish

During exposure to elevated waterborne Cd (3 μg/L) or dietary Cd (500 mg/kg) for one month, MT levels in both gut as well as nongut tissues increased greatly in comparison to naïve fish (Figures 3 and 4), suggesting that trout can efficiently induce this binding protein as an adaptive process for the detoxification of Cd. This is in agreement with previous studies showing *de novo* synthesis of MT or elevated MT concentrations in fish tissues, particularly liver, kidney, and/or gills as a result of chronic exposure to waterborne and dietary Cd (Weber *et al.* 1992; De Smet *et al.* 2001; Hollis *et al.* 2001; Dang *et al.* 2001; Wu and Hwang 2003). The present study further demonstrates that MT levels (liver: 205.8, kidney: 161.3, gills: 12.5 nmol/g; Table 1) in the dietary acclimated fish were greater than those in the fish acclimated to waterborne Cd for 1 month (56.1, 8.2, and 10.2 nmol/g respectively; Table 1). However, for Cd levels, similar differences (but at lesser extents) hold only for the liver and kidney; gill Cd level was greater in the fish acclimated to waterborne Cd (Table 1). Actually, only in the case of kidney MT levels did the 19.7-fold difference between dietary and waterborne acclimation treatments exceed the difference (6.8-fold) in actual Cd doses used during the respective acclimations (0.92 versus 6.29 mg/kg fish/day; Table 1). In mammals, the kidney responds to Cd by both reabsorbing circulatory Cd-MT complex that has been

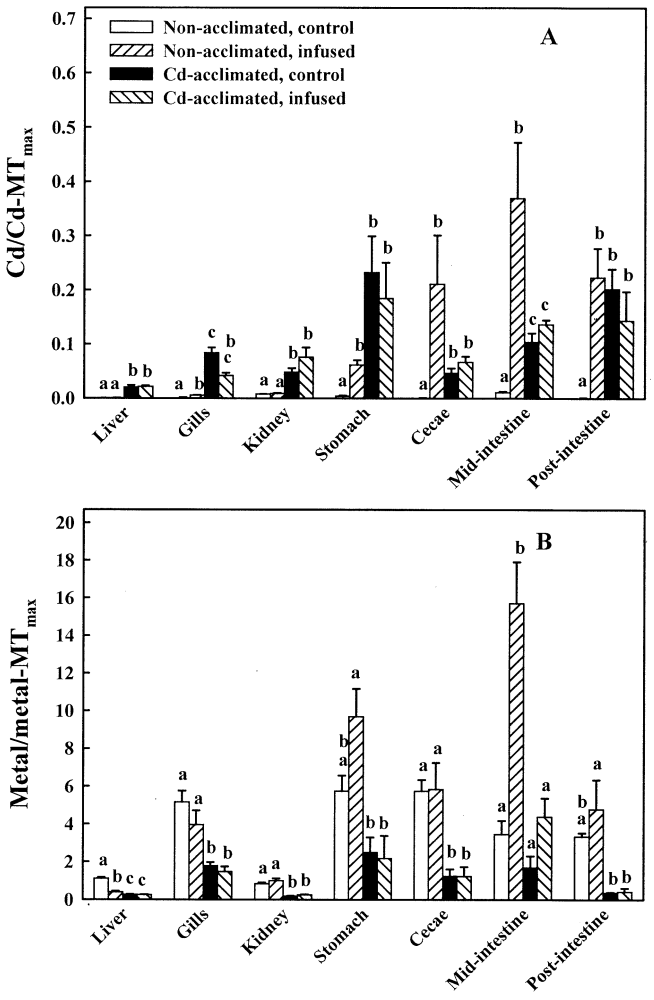

Fig. 6. A: Ratio of total Cd to theoretical maximum Cd-MT in the gut tissues (stomach, pyloric cecae, midintestine, and posterior intestine) as well as nongut tissues (liver, gills, and kidney) of nonacclimated control fish, in dietary Cd-acclimated control fish, and in both nonacclimated and Cd-acclimated rainbow trout 24 hours after a gastrointestinal dose of stable Cd (276 μg/kg w wt) infused into the stomach. B: Ratio of total metal (Cd + Zn + Cu) to theoretical maximum metal-MT in the same tissues samples (see text for details of calculation). The acclimated fish were preexposed to dietary Cd (nominally 500 mg/kg dry wt) for 30 days. Ratios are presented as mean ± SE (n = 6). Bars with different letters are significantly different ($p < 0.05$) for the same tissue.

released from the liver and gut, and filtered into the renal tubules, as well as by synthesizing renal MT for Cd storage (Zalups and Ahmad 2003). It is likely that fish also respond in a similar way and that in the dietary Cd-exposed trout, a relatively greater amount of Cd-MT complex released from extrarenal tissues was reabsorbed in the kidney and more renal MT was synthesized.

The gill MT level in the trout acclimated to waterborne Cd for 28 days was 4.6 times the level in nonacclimated fish (8.2 times the day 0 control level, Figure 3A), suggesting that gills are sensitive sites for the induction of MT even though they appear to serve as transient organs for waterborne Cd uptake in fish body. Interestingly, the gill MT level in the dietary Cd-acclimated trout was also more than double the level in nonacclimated fish and was further elevated significantly after gastrointestinal Cd infusion (Figure 4), despite the fact that gills were not exposed to waterborne Cd. MT was probably synthesized in response to basolateral uptake of dietary Cd in the gills rather than any apical uptake from water. This clearly suggests that the induction of MT in the gills, synthesized in response to either waterborne or dietary Cd, plays an important role in altering branchial Cd uptake and increased tolerance to waterborne Cd (*i.e.*, increased waterborne 96-hour LC_{50}, as shown for trout chronically acclimated to waterborne Cd (Hollis *et al.* 1999; Stubblefield *et al.* 1999) and dietary Cd (Szebedinszky *et al.* 2001).

Although elevated levels of MT in the intestine have also been reported in previous studies on dietary metal exposure of fish (Shears and Fletcher 1984; Handy *et al.* 1999; Ptashynski *et al.* 2002), the present study is the first to report quantitative data on MT induction in different regions of the gastrointestinal tract in fish exposed to dietary Cd (Figure 4). The highest MT level in the Cd-acclimated control trout (2984 μg/g), which was an increase of approximately 6.5-fold from naïve trout and 2.4 times the liver MT level in the Cd-acclimated fish, was observed in the posterior intestine, followed by the pyloric cecae and midintestine, and the lowest in the stomach. This is in accord with the regional distribution of Cd concentrations in these tissues (Figure 2). However, Cd infusion caused a 46% decrease of MT levels in the posterior intestine (Figure 4), which mimics the pattern for Cd (71% Figure 2), Zn (33% Figure 5A), and Cu (70% Figure 5B) concentrations in the same tissue, and strengthens our argument in favor of the loss of tissue material by enhanced sloughing as mentioned above.

Interactions of Cd, Cu, Zn, and MT

Although Cd has been reported to displace Zn from high molecular weight proteins, but not from endogenous MT (Sorensen 1991), Cd exposure did not significantly affect Zn levels of any tissues in this study (Figure 5A). These findings suggest a homeostatic regulation of this essential metal, in agreement with the results of Hollis *et al.* (2001) for waterborne Cd exposure. The decrease of Zn burden in the posterior intestine of Cd-exposed trout is probably attributable to the sloughing of cell materials, as discussed before.

Similar to Zn, no indication of Cu displacement by dietary Cd is evident in this study, again in agreement with the results of Hollis *et al.* (2001) for waterborne Cd exposure. Indeed, in the present study, Cu levels increased or remained unchanged, instead of showing a decrease due to displacement by Cd, in most of the tissues of Cd acclimated fish relative to naïve fish (Figure 5B). Although liver Cu level did not change in the present study, Weber *et al.* (1992) recorded an elevated liver Cu in largemouth bass exposed to dietary Cd. The elevated tissue Cu levels might be attributable to the increased binding capacity of the tissues in Cd exposed fish as a result of *de novo* synthesis of MT, and suggests that chronic Cd exposure may lead to increased loading of exogenous Cu or redistribution of endogenous Cu within the fish.

The calculated ratios of actual Cd to theoretical maximum Cd-MT for all tissues were <1.0 in naïve fish as well as in fish acclimated to waterborne and dietary Cd in the present study (Table 1, Figure 6A), suggesting that the background or induced MT was theoretically enough to bind all of the Cd taken up by the tissue in both nonexposed and Cd-exposed groups. Hollis *et al.* (2001), in a similar analysis for trout exposed to waterborne Cd, observed ratios of <1.0 in the liver, kidney, and gills of naïve fish, but in contrast to our results (Table 1), not in the gills (∼3.5) or kidney (∼1.2) of Cd-exposed fish. In fact, the ratios of the gills (0.36) and kidney (0.46) for waterborne Cd exposure are significantly greater in comparison to those for dietary exposure (0.09 and 0.05, Table 1). The preceding discussion suggests that there is probably a higher potential for toxic effect of waterborne Cd than the dietary Cd on the gills and kidney.

Practically, fish always contain substantial levels of the essential metals Cu and Zn (Figure 5). The other metals can occupy MT binding sites, rendering less MT available for Cd binding. This is particularly likely for Cu because of its higher affinity for MT relative to Cd, and was evident in the present study, as tissue Cu burden was elevated in Cd-acclimated fish (Figure 5B). In addition, considering Cd, Zn, and Cu levels together in the tissue for dietary exposure groups, the calculated actual metal to the theoretical maximum metal-MT ratios were almost equal to or well above 1.0 in both naïve and Cd-exposed fish for most of the tissues in the present study (Figure 6B). This was also the case for the gills, liver, and kidney in rainbow trout exposed to waterborne Cd (Hollis *et al.* 2001). Thus, although there was a clear induction of MT in all tissues, it was much less than the binding capacity for total metal on a molar binding site basis. This suggests that the metals are bound to other cellular proteins in addition to available MT. Obviously, this reflects the fact that Cu and Zn are essential micronutrients, associated with a vast number of enzymes and other proteins. Potentially Cd may compete with the essential metals for binding sites on non-MT proteins in this situation and may be damaging at a cellular level.

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