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Tissue-Specific Cadmium Accumulation, Metallothionein Induction, and Tissue Zinc and Copper Levels During Chronic Sublethal Cadmium Exposure in Juvenile Rainbow Trout

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Abstract. Juvenile rainbow trout, on 3% of body weight daily ration, were exposed to 0 (control) or 3 μg/L Cd (as $Cd(NO_3)_2 \cdot 4H_2O$) in moderately hard (140 mg/L as $CaCO_3$), alkaline (95 mg/L as $CaCO_3$, pH 8.0) water for 30 days. Particular attention focused on Cd burden in tissues (gills, liver, kidney, and whole body) and induction of metallothionein (MT) in gills, liver, and kidney during chronic Cd exposure. Mortality in Cd-exposed fish was minimal (~10%), and no growth effects occurred over the 30-day exposure. Cd accumulated in a time-dependent fashion to 9 times (gills), 3 times (liver), 20 times (kidney), 2 times (carcass), and 2 times (whole body) control levels by 30 days; absolute concentrations were in the order kidney > gill > liver > whole body > carcass. Tissue (gills, liver, and kidney) Zn and Cu burdens were not altered by chronic exposure to 3 μg/L Cd. MT concentrations in all tissues increased over the 30 days of Cd exposure, but the increases were much less than those of Cd on a molar binding site basis. Absolute MT concentrations were in the order liver > kidney > gill, but relative increases were greatest in kidney (fourfold), followed by gills (twofold) and liver (1.3-fold). MT levels were sufficient to bind all Cd in gill, liver, and kidney under control conditions, and after chronic Cd exposure remained sufficient in liver and kidney, but not in gills. Total metal levels (Cd + Zn + Cu) greatly exceeded MT binding capacity in all tissues under all conditions.

Cadmium can elicit protective mechanisms against cadmium toxicity in teleost fish during both acute and chronic exposures (reviewed in Sorensen 1991; McDonald and Wood 1993). Some of these mechanisms involve binding of Cd by specific proteins including metallothionein (MT) (Beattie and Pascoe 1979; Reichert *et al.* 1979; Brown *et al.* 1984; Thomas *et al.* 1985). MT is a low-molecular-weight, cysteine-rich protein that functions in homeostatic control of essential metals, such as Cu and Zn, and can also function in the detoxification of nonessential metals, such as Cd (Kägi and Nordberg 1979). The molecular weight of rainbow trout MT is approximately 6,000 g/mol (Olsson and Haux 1985). Because of its very high affinity for metals in groups IB and IIB of the periodic table, sequestration of such metals by MT can render them unable to interact with other proteins, such as enzymes (Winge *et al.* 1973; Brown and Parsons 1978). Benson and Birge (1985) demonstrated increased tolerance to Cd in fathead minnows, which was due in part to increased production of MT. However, others have shown that accumulation of Cd and Cu occur in both MT and high-molecular-weight cytosolic proteins (McCarter *et al.* 1982; Roch *et al.* 1982).

In the present study, we investigated the relationship between tissue-specific Cd accumulation and MT induction in juvenile rainbow trout. Cd levels and MT concentrations in gills, liver, and kidney were determined during chronic (30-day) sublethal exposure to 3 μg/L Cd (0.03 μmol/L Cd). Zinc and Cu concentrations were measured to determine whether Cd would alter these essential metals in these tissues. A particular goal was to determine whether MT buildup quantitatively tracked Cd buildup, and whether total metal levels (Cd + Zn + Cu) exceeded MT binding capacity, as metals (especially essential ones) can be also stored or incorporated into proteins and enzymes that are not associated with MT.

Materials and Methods

Fish Holding Conditions

Rainbow trout (*Oncorhynchus mykiss* [Walbaum]; 2.79 ± 0.04 g; mean ± 1 SEM [n = 4 tanks of 120 fish each]) were obtained from Rainbow Springs Hatchery in Thamesford, Ontario, and held in flowing dechlorinated Hamilton tap water (Lake Ontario water: Ca = 40

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mg/L or 1 mM, Na = 14 mg/L or 0.6 mM, Cl = 25 mg/L or 0.7 mM, dissolved organic matter [DOM] = 3 mg/L or 0.25 mM, hardness = 140 mg/L as $CaCO_3$, alkalinity = 95 mg/L as $CaCO_3$, pH 8.0, 14°C). Trout were held in 600-L aerated polyethylene tanks for 2 weeks before experimentation. Fish were fed 3% of their body weight per day (as three 1% meals per day) with Martin's Starter Food (Martin Feed Mills, Elmira, Ontario; Cd content = 1.06 ± 0.04 [n = 6] μg Cd/g wet food).

Exposure System

After 2 weeks in holding tanks, 120 fish were randomly transferred to four 200-L polyethylene exposure tanks, which were flow-through systems (flow = 1.5 L/min) with continuous aeration. Fish were fed 3% of their body weight per day (see above). An acidified Cd stock solution, with Cd added as $Cd(NO_3)_2 \cdot 4H_2O$ (Fisher Scientific. Nepean, Ontario), was delivered to a mixing head-tank via Mariotte® bottles (Mount and Brungs 1967) to achieve desired Cd concentrations in exposure tanks. Exposure tanks were spiked on the first day of Cd exposure to reach the desired Cd concentration. Water chemistry was measured weekly throughout the exposure. Fish were exposed to (1) control = nominally zero cadmium (actual measured "in-tank" value = 0.7 ± 0.5 μg/L or 0.006 ± 0.004 [12] μM Cd; mean ± 1 SEM [n = number of H_2O samples taken]) or (2) 3 μg/L Cd (actual measured "in-tank" value = 3.0 ± 0.7 μg/L or 0.03 ± 0.006 [10] μM Cd) for 30 days in dechlorinated Hamilton tap water. The two treatment conditions each had two replicates so that n = 240 fish per treatment. The sublethal exposure concentration of 3 μg/L Cd was chosen because acclimation (indicated by a 12-fold increase in the 96-h Cd LC_{50} value) was seen in trout chronically exposed to 3 μg/L Cd in our previous hard water study (Hollis *et al.* 1999).

Sampling

During the 30-day Cd exposure, 16-ml water samples were taken on a regular basis throughout the exposure, acidified with 50 μl of HNO_3 and analyzed for Na, Ca, and Cd content. Fish from each treatment tank were bulk weighed once a week. All of the fish from the tank (one tank at a time) were removed and put in a tared sieve placed inside a bucket containing water from the exposure tank. The bucket was weighed, fish were briefly removed using the sieve, and the bucket reweighed. The mass of the fish was calculated from the difference between the mass of the bucket plus sieve with and without fish.

Specific growth rates (SGRs) were determined from bulk weights from individual treatment tanks taken five times over the 30-day exposure. The best fit of these data to time was an exponential curve. SGR, as percent per day, was calculated by linear regression of ln weight versus time, using SPSS (SPSS Inc., version 8.0 for Windows, Chicago, IL) which provides mean ± 1 SE for the regression line.

Six fish from each tank were subsampled at day 0, 2, 10, 20, and 30, and gills, liver, kidney, and remaining carcass were assayed for Cd content. An additional six fish from each treatment were subsampled at day 0, 10, 20, and 30; gills, liver, and kidney were assayed for MT, Zn, and Cu content. Fish were sacrificed, and both sets of gills, the liver, and kidney were excised; gills were rinsed for 10 s in 100 ml of dechlorinated Hamilton tap water. All tissues plus remaining carcass were frozen in liquid nitrogen and stored at −70°C for later analysis of Cd, Zn, Cu, and MT content.

Tissue and Water Analyses

The concentrations of all measured parameters in tissues were expressed on a per gram wet tissue basis.

Gills, livers, kidneys, and remaining carcass to be analyzed for Cd content were analyzed according to methods described by Playle *et al.* (1993a, 1993b). Samples were thawed, weighed, and then digested in one to five times their weight of 1 N HNO_3 (TraceMetal Grade HNO_3; Fisher Scientific) for 3 h at about 80°C. Digests were shaken, left to settle for 10 min, and the supernatant diluted 10 times with deionized water (18 mgohm; Nanopure II; Sybron/Barstead, Boston, MA). Gill, liver, kidney, and carcass Cd concentrations were measured on a graphite furnace atomic absorption spectrophotometer (Varian AA-1275 with GTA-95 atomizer) against Fisher certified standards, as outlined by Hollis *et al.* (1996), using 10-μl injection volumes and N_2 gas. Operating conditions were as those described by Varian with 30-s drying time at 90°C, 12 s at 120°C, and 4 s at 1,800°C, during which Cd was read. Whole body Cd was calculated based on the data for individual fish at each sample time, as outlined by Hollis *et al.* (1999), with gills, liver, kidney, and carcass representing 3.0%, 1.5%, 0.5%, and 95.0% of the total whole body weight, respectively.

Gills, livers, and kidneys (9–260 mg) to be analyzed for Zn, Cu, and MT content were homogenized individually in 1.00 ml of 50 μM Tris-HCl, pH 8.0, at 0°C, using a glass-Teflon homogenizer. The homogenate was centrifuged at 14,000 × *g*, 4°C, for 20 min. Two hundred microliters of the supernatant was transferred to Eppendorf tubes and stored at −70°C until analyzed for MT, Zn, and Cu content. MT levels were analyzed by radioimmunoassay according to Hogstrand and Haux (1990) with the modifications described in Hogstrand *et al.* (1994). The method was a double antibody radioimmunoassay (RIA), using rabbit antiserum raised against MT from perch, *Perca fluviatilis*, as the first antibody, ^{125}I-labeled rainbow trout MT as tracer, and goat anti-rabbit lgG as the second antibody (Hogstand and Haux 1990). The MT (I and II) from rainbow trout, used as tracer, was purified according to Olsson and Haux (1986) with the modifications described by Hogstrand and Haux (1990). A 10,000-*g* supernatant prepared from the livers of Cd-injected rainbow trout was used as the MT standard. The MT content of the standard was calibrated against a standard curve prepared from purified rainbow trout MT (Hogstrand and Haux 1990). The working range of the RIA was 10 to 100 ng rainbow trout MT per assay tube, which corresponds to 0.6 to 6 μg/g liver wet weight.

The ratio of actual metal to theoretical maximum metal-MT was calculated for gills, liver, and kidney using the following equation:

$$\text{Metal: Metal-MT} = \text{Tissue Zn}/(\text{MT} \times 7) + \text{Tissue Cd}/(\text{MT} \times 7) + \text{Tissue Cu}/(\text{MT} \times 12)$$

where "Tissue Zn" is measured tissue [Zn] accumulation in gills, liver, or kidney (nmol Cd/g wet tissue) over the 30-day exposure to 3 μg/L Cd, "Tissue Cd" is measured tissue [Cd] accumulation in gills, liver, or kidney (nmol Cd/g wet tissue) over the 30-day exposure, "Tissue Cu" is measured tissue [Cu] accumulation in gills, liver, or kidney (nmol Cd/g wet tissue) over the 30-day exposure to 3 μg/L Cd, and "MT" is measured MT levels in gills, liver, or kidney (nmol MT/g; using a molecular weight of 6,000 g/mol for MT). The MT value is multiplied by seven for Zn and Cd because 1 mole of MT binds 7 moles of divalent metal. The MT value is multiplied by 12 for Cu because 10–12 moles of Cu can bind to MT in its Cu (I) oxidative state. Theoretically, if the actual metal to theoretical maximum metal-MT value is less than one, potentially all of the metal(s) (*i.e.*, Cd or Cd + Zn + Cu) could be bound by MT.

The 14,000-*g* supernatants of gills, liver, and kidney were also analyzed for Zn and Cu content. The supernatant was thawed and then diluted 2 to 100 times with deionized water (18 mgohm; Nanopure II; Sybron/Barstead) and 25 μl of concentrated 16 N HNO_3 (TraceMetal Grade HNO3; Fisher Scientific). Gill, liver, and kidney Cu concentrations were measured on a graphite furnace atomic absorption spectrophotometer (Varian AA-1275 with GTA-95 atomizer) against Fisher certified standards, as outlined by Hollis *et al.* (1996), using 10-μl

injection volumes and N_2 gas. Operating conditions were as those described by Varian with 30-s drying time at 90°C, 12 s at 120°C, and 4 s at 2,300°C, during which Cu was read. Gill, liver, and kidney Zn concentrations were analyzed by atomic absorption spectroscopy (Varian AA-1275) using an air/acetylene flame.

Water Na and Ca concentrations were measured using the Varian AA-1275 operated in standard flame absorption mode. Water Cd concentrations were measured using the methods described for tissues. Water pH was measured using a Radiometer PHM71b meter with GK2401C combination electrode. DOM was measured on the Rosemount Analytical DC-180 automated total organic carbon analyzer (Folio Instruments, Kitchener, Ontario).

Statistics

Data have been expressed as means ± 1 SE (n). An ANOVA followed by a Student-Newman-Keuls procedure was used for multiple comparisons of mean values. A fiducial limit of $p < 0.05$ was used throughout.

Results

Mortality was minimal over the 30-day exposure with 0% and 6% mortality for controls and 3 μg/L Cd exposures, respectively; mortality ceased after day 5 for the 3 μg/L Cd exposure. There were no significant differences in specific growth rate for control (2.93 ± 0.10% body weight/day) and Cd-exposed fish (2.71 ± 0.10% body weight/day) as a result of chronic Cd exposure.

Cadmium accumulation in all tissues increased significantly over the 30 days in the 3 μg/L Cd-exposed fish (Figure 1); the increases in all tissues except liver were significant by day 10. At 30 days, Cd concentrations were greatest in kidneys (Figure 1C), followed by gills (Figure 1A), livers (Figure 1B), and whole bodies (Figure 1D); carcass concentrations (data not shown) were only slightly lower than whole bodies. Gill Cd levels increased 9 times from initial (day 0) values (0.72 ± 0.29 [6] μg Cd/g wet tissue; Figure 1A); liver Cd concentrations increased 3 times from initial values (1.29 ± 1.00 [6] μg Cd/g wet tissue; Figure 1B), and kidney Cd concentrations increased 20 times from initial values (0.47 ± 0.10 [6] μg Cd/g wet tissue; Figure 1C) for Cd-exposed fish after 30 days of exposure. Remaining carcass (data not shown) and whole body (Figure 1D) Cd levels increased two times from initial values (0.51 ± 0.04 [6] μg Cd/g wet tissue) for the 3 μg/L Cd exposure. Tissue concentration factors from the water were 270, 350, 1,300, 2,100, and 3,100 times for remaining carcass, whole body, liver, gills, and kidney, respectively, after 30 days exposure to 3 μg/L Cd.

There was certainly no decrease, and perhaps a slight trend for increased tissue Zn and Cu levels with chronic Cd exposure (Table 1 and 2). Gill Zn concentration was significantly elevated 1.2-fold relative to the simultaneous control (8.96 ± 0.33 [6] μg Zn/g wet tissue) at day 10 only. Zinc concentrations averaged 10.46 ± 0.25 (42), 13.00 ± 0.62 (42), and 13.56 ± 0.49 (42) μg Zn/g wet tissue for gills, livers, and kidneys, respectively, over the 30-day exposure to 3 μg/L Cd (Table 1). Gill and kidney Cu concentrations were significantly elevated 1.7-fold relative to the simultaneous control values (0.15 ± 0.01 [6] μg Cu/g wet tissue and 1.68 ± 0.09 [6] μg Cu/g wet tissue for gill and kidney, respectively) at day 20 only. Copper concentrations averaged 0.24 ± 0.02 (42), 17.95 ± 1.03 (42), and 2.80 ± 0.17 (42) μg Cu/g wet tissue for gills, livers, and kidneys, respectively, over the 30-day exposure to 3 μg/L Cd (Table 2).

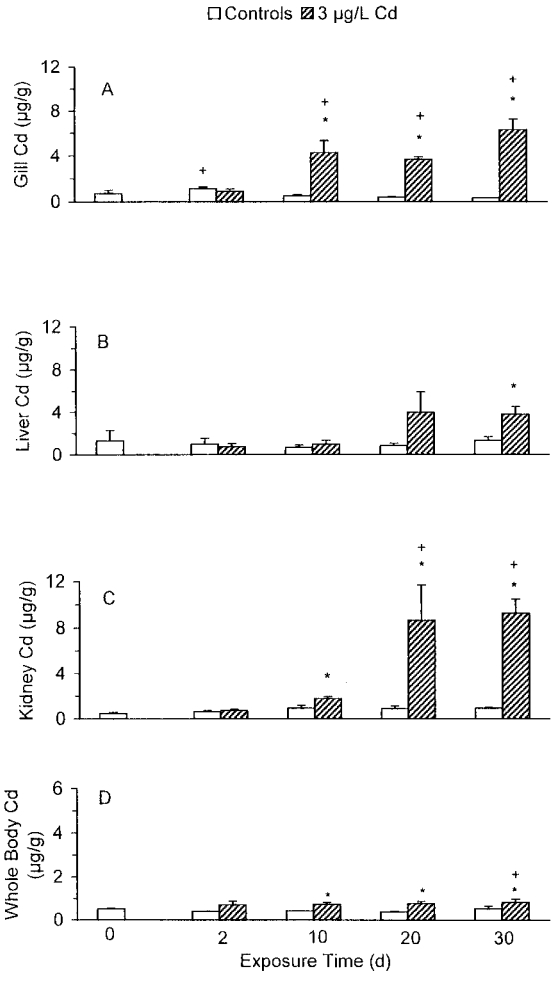

Fig. 1. Accumulation of Cd by gills (A), liver (B), kidney (C), and whole body (D) of juvenile rainbow trout exposed for 30 days to 0 μg/L Cd (clear bars) or 3 μg/L Cd (patterned bars). Means ± 1 SE (n = 6). Statistical comparisons were made against background Cd (controls) at each sampling day (*) and against background Cd at day 0 (crosses); $p < 0.05$

On an absolute basis, MT concentrations in controls were greatest in livers (Figure 2B), followed by kidneys (Figure 2C), and gills (Figure 2A), and the same was true after 30 days of Cd exposure. MT concentrations in all tissues increased over the 30 days of Cd exposure (Figure 2). On a relative basis, increases were greatest in kidney, with levels increasing fourfold from initial values (15.32 ± 1.61 [6] μg/g) after 30 days exposure to 3 μg/L Cd (Figure 2C). Over the same period, gill MT concentrations increased approximately twofold from initial (day 0) values (8.09 ± 1.66 [6] μg/g) (Figure 2A). Livers showed a trend, although not significant, of increased metallothionein concentrations (~two times from initial values of 46.58 ± 8.57 [6] μg/g) with chronic Cd exposure (Figure 2B).

Table 1. Concentration of Zn in gills, liver, and kidney of juvenile rainbow trout exposed for 30 days to 0 μg/L Cd or 3 μg/L Cd

Tissue Zn (μg/g wet tissue)	Days of Exposure			
	0	10	20	30
Gills				
Controls	10.28 ± 0.74	8.96 ± 0.33	10.44 ± 0.83	11.22 ± 0.70
3 μg/L Cd		10.58 ± 0.54*	10.91 ± 0.60	10.83 ± 0.70
Liver				
Controls	17.47 ± 1.86	14.25 ± 1.11	9.37 ± 1.05†	11.08 ± 0.85†
3 μg/L Cd		14.40 ± 0.65	10.47 ± 1.09†	13.97 ± 2.11
Kidney				
Controls	10.59 ± 2.18	13.59 ± 1.01	14.04 ± 0.62	12.51 ± 0.82
3 μg/L Cd		13.54 ± 0.88	16.16 ± 1.43	14.50 ± 0.70

Means ± 1 SE (n = 6). Statistical comparisons were made against background Zn (controls) at each sampling day (*) and against background Zn at day 0 (crosses); $p < 0.05$.

Table 2. Concentration of Cu in gills, liver, and kidney of juvenile rainbow trout exposed for 30 days to 0 μg/L Cd or 3 μg/L Cd.

Tissue Cu (μg/g wet tissue)	Days of Exposure			
	0	10	20	30
Gills				
Controls	0.43 ± 0.10	0.24 ± 0.01†	0.15 ± 0.01†	0.17 ± 0.01†
3 μg/L Cd		0.28 ± 0.04†	0.25 ± 0.03*†	0.18 ± 0.02†
Liver				
Controls	16.22 ± 2.18	12.34 ± 2.57	16.40 ± 2.04	22.65 ± 1.84†
3 μg/L Cd		16.24 ± 1.43	16.18 ± 2.03	25.61 ± 3.29†
Kidney				
Controls	3.53 ± 0.54	2.15 ± 0.24†	1.68 ± 0.09†	2.44 ± 0.31†
3 μg/L Cd		3.34 ± 0.48	2.85 ± 0.31*	3.65 ± 0.48

Means ± 1 SE (n = 6). Statistical comparisons were made against background Cu (controls) at each sampling day (*) and against background Cu at day 0 (crosses); $p < 0.05$.

However, if all fish exposed at day 10, 20, and 30 are combined and tested statistically against all controls combined, MT concentrations in livers were significantly 1.3-fold higher (66.98 ± 5.19 [18] μg/g) than controls (49.90 ± 3.12 [24] μg/g).

Calculation of the ratio of actual Cd to the theoretical maximum metal-Cd (*i.e.*, 1 mole of MT binds 7 moles of divalent Cd) under control conditions (day 0 on Figure 3) yielded values about 1.0 for gills (Figure 3A) and about 0.2 for liver (Figure 3B) and kidney (Figure 3C). These values did not change in the later control samples (data not shown). However, over 30 days' exposure to 3 μg/L Cd, the ratios increased significantly to about 4.0 (3.54 ± 0.61 [6]) in gills (Figure 3A) and 1.0 (1.19 ± 0.30 [6]) in kidney (Figure 3C), while the small rise to 0.4 (0.43 ± 0.13 [6]) in liver (Figure 3B) was not significant. Thus although MT induction did not quantitatively track Cd accumulation on a molar basis, there was adequate MT binding capacity to complex all Cd accumulated (Figure 1) in liver and kidney, whereas in the gills, this capacity was clearly exceeded by Cd accumulation.

When the calculation of actual metal to the theoretical maximum metal-MT was done for (Cd + Zn + Cu), the ratio (range = 7.0–16.0) greatly exceeded 1.0 under control conditions (day 0 in Figure 3) and did not vary significantly in later control samples (data not shown). Furthermore, despite the induction of MT, these values did not change appreciably during 30 days of exposure to 3 μg/L Cd, remaining at 13.76 ± 0.73 (42) for gills (Figure 3A), 6.16 ± 0.40 (42) for liver (Figure 3B), and 6.22 ± 0.35 (42) for kidney (Figure 3C). Thus most of the total metal in all tissues cannot be bound to MT.

Discussion

Growth During Chronic Cadmium Exposure

There are many studies indicating that growth is not affected by Cd exposure. For example, Farag *et al.* (1994) found that mixed metal solutions of 2.2 μg/L Cd with 6.4 μg/L Pb and 24 μg/L Cu had no adverse effects on growth of juvenile rainbow trout during a 21-day exposure. A similar insensitivity of growth to Cd has been seen in very long-term exposures in various water qualities—*e.g.*, for 70–178 days in rainbow trout exposed to 3–7 μg/L Cd (Kumada *et al.* 1980; Giles 1988; Davies *et al.* 1993) and for 266 days in brook trout exposed to 3.4 μg/L Cd (Benoit *et al.* 1976). However, there are also several studies showing clear negative impacts of Cd on growth (Benoit *et al.* 1976; Rombough and Garside 1982; Peterson *et al.* 1983). In the present study, there was no significant impairment of growth in trout chronically exposed to 3 μg/L Cd. Hollis *et al.* (1999) showed similar results for trout chronically exposed to 3 and 10 μg/L Cd for 30 days in the same hard water used here (140 mg/L as $CaCO_3$) and on the same dietary regime.

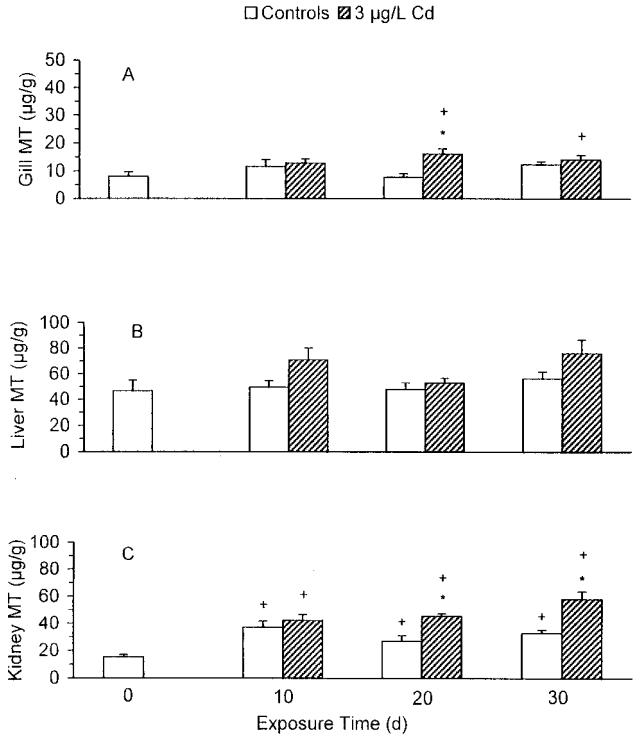

Fig. 2. MT levels in gills (A), liver (B), and kidney (C) of juvenile rainbow trout exposed for 30 days to 0 µg/L Cd (clear bars) or 3 µg/L Cd (patterned bars). Means ± 1 SE (n = 6). Statistical comparisons were made against background MT concentrations (controls) at each sampling day (*) and against background MT concentrations at day 0 (crosses); $p < 0.05$

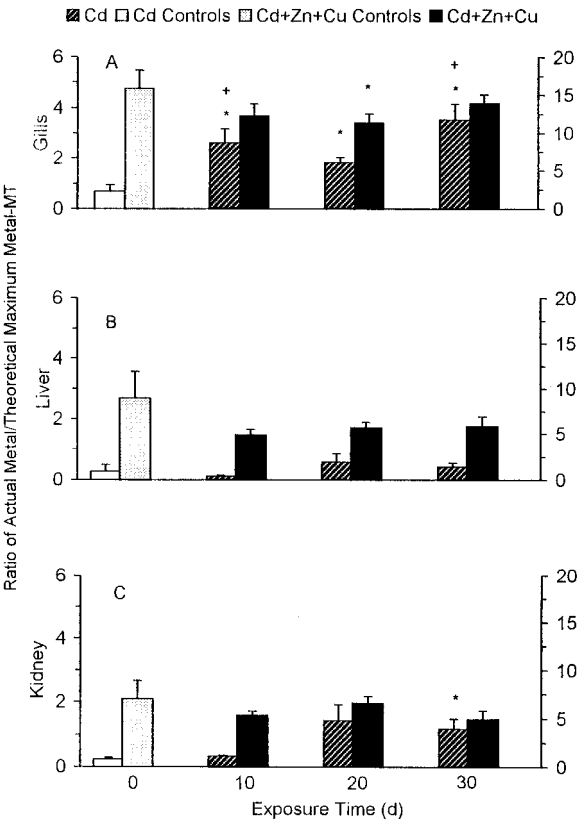

Fig. 3. Ratio of actual metal to theoretical maximum metal-MT in gills (A), liver (B), and kidney (C) of juvenile rainbow trout exposed for 30 days to 3 µg/L Cd. Ratios were calculated for Cd controls (*i.e.*, no Cd exposure; clear bars) and for Cd accumulation (striped bars); these values correspond to left-hand Y-axis. Ratios were also calculated for Cd + Zn + Cu controls (gray bars) and for Cd + Zn + Cu accumulation (black bars); these values correspond to right-hand Y-axis. Means ± 1 SE (n = 6). Statistical comparisons were made against background MT concentrations (controls) at each sampling day (*; data not shown on graph) and against background MT concentrations at day 0 (crosses); $p < 0.05$

Tissue Accumulation of Cadmium

Absolute concentrations of Cd in various tissues were comparable to those observed in our previous studies in similar water quality (Hollis *et al.* 1999, 2000). Kidneys accumulated the greatest concentration of Cd over the 30-day exposure to Cd, followed by gills, liver, whole body (and carcass) (Figure 1). Several other studies have demonstrated equal or higher concentrations of Cd (relative to gills or liver) in kidneys of trout chronically exposed to waterborne Cd, depending on uptake route. Benoit *et al.* (1976), Kumada *et al.* (1980), and Harrison and Klaverkamp (1989) observed that kidney Cd concentrations remained high even after the fish were returned to Cd-free water, indicating the importance of the kidney as an accumulator of Cd.

Tissue Zn and Cu Concentrations

Absolute levels of Cu and Zn in tissues were comparable to those measured in other recent studies on juvenile trout in similar water quality (Taylor *et al.* 2000; Alsop *et al.* 1999). There was a slight trend, significant at a few times, for increased tissue Zn and Cu levels with chronic Cd exposure (Tables 1 and 2). Clearly, there was no indication of displacement of Zn and Cu by Cd during the 30-day exposure to 3 µg/L Cd. Similarly, Olsson *et al.* (1989) found that Cu and Zn concentrations in gills, liver, and kidney of rainbow trout were not altered by exposure to 200 µg/L Cd in brackish water for 4 months. The only directly comparable study is that of Norey *et al.* (1990) who exposed rainbow trout to 3 µg/L Cd for 25 weeks in similar hard water. In that study, only duplicate (rather than mean) data were reported. Cadmium buildup appeared to occur far more slowly than in the present study, accompanied by constant Zn and Cu levels up to about week 13, and thereafter subtle decreases in gill Zn concentration and substantial increases in liver Cu concentrations on weeks 19 and 25. The significance of the latter changes was unclear. In general, the present data disagree with previous studies, which show that Cd readily displaces Zn from endogenous MT. For example, Dallinger *et al.* (1997) reported that sequestration of Cd in Arctic char occurred by displacement of Zn from MT binding sites when Cd concentrations in the liver were slightly elevated; however, at higher concentrations more MT was produced to bind excess amounts of the metal. Therefore, in the

present study, the slight increases observed in tissue Zn and Cu burdens may have been either causes or consequences of new MT induction.

MT Induction

MT concentrations in gills, liver, and kidney increased over the 30 days of 3 μg/L Cd exposure, with the greatest increase occurring in the kidneys (Figure 2). This is consistent with previous studies showing increased levels of MT and/or MT-mRNA in several internal tissues (Olsson *et al.* 1989; Norey *et al.* 1990; Wicklund Glynn *et al.* 1992) and gills (Thomas *et al.* 1983; Klaverkamp and Duncan 1987; Olsson and Hogstrand 1987; Fu *et al.* 1990; Norey *et al.* 1990) during long-term Cd exposures.

To cast light on how Cd was handled by gills, liver, and kidney relative to Cu and Zn, the actual metal to theoretical maximum metal-MT ratio was determined for Cd accumulation and Cd + Zn + Cu accumulation (Figure 3). Theoretically, if the ratio stays less than 1.0, potentially all of the metal(s) could be bound up by MT. This was clearly the case under control conditions in all tissues for Cd (Figure 3). However, once the ratio surpasses 1.0, it means that this cannot be true, and some metal must be non-MT bound, and therefore potentially more damaging. We see this clearly in gills in Cd-exposed fish (ratios > 3; Figure 3A), but certainly not in the liver (ratios $<$ 0.6; Figure 3B), and probably not in the kidney (ratios around 1.0; Figure 3C), when Cd accumulation is considered. Thus although there was a clear induction of MT in all tissues, it was much less than the Cd buildup on a molar binding site basis, yet the theoretical MT-Cd binding capacity remained adequate in kidney, more than adequate in liver, but obviously insufficient in gills. With regard to the latter, recent radioisotopic labeling experiments suggest that the majority of the Cd built up in the gills during chronic sublethal exposure is compartmentalized into a very slowly exchanging pool (Hollis *et al.* 1999). Clearly, this is not an MT pool.

When the ratio of actual metal to theoretical maximum metal-MT was calculated for (Cd + Zn + Cu) accumulation in the tissues, the ratio was now much greater than 1.0 for gills, liver, and kidney, even under control conditions. Obviously, this reflects the fact that Cu and Zn are essential micronutrients, associated with a vast number of enzymes and other proteins. Only a relatively small fraction could have been bound by endogenous MT levels under control conditions. Although there was no indication of displacement of Zn by Cd during the 30-day exposure to 3 μg/L Cd (Table 1), given that Cd has a higher affinity than Zn for most MTs, it is likely that that Zn would be the "displaced" metal from MT-binding sites (even though the absolute concentrations are little affected). Potentially, this could cause cellular effects even in tissues where all the Cd is immobilized by metallothionein.

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