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Metal–metal interactions of dietary cadmium, copper and zinc in rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

The influence of metal–metal interactions on uptake, accumulation, plasma transport and chronic toxicity of dietary Cu, Cd and Zn in rainbow trout (*Oncorhynchus mykiss*) was explored. Juvenile rainbow trout were fed diets supplemented with (μg/g) 500 Cu, 1000 Zn and 500 Cd singly and as a ternary mixture at 2.5% body weight daily ration for 28 days. Complex interactions among the metals dependent on the tissue/organ, metals ratios and duration of exposure were observed. While Zn did not accumulate, whole-body Cd and Cu concentrations increased following linear and saturation patterns, respectively. Early enhanced whole-body Cu accumulation in fish exposed to the metals mixture was correlated with reduced Cd concentration whereas late enhancement of Cd accumulation corresponded with elevated Cd concentration. This suggests early mutual antagonism and late cooperation between Cd and Cu probably due to interactions at temporally variable metal accumulation sites. At the level of uptake, Cd and Cu were either antagonistic or mutually increased the concentrations of each other depending on the duration of exposure and section of the gut. At the level of transport, enhanced Cd accumulation in plasma was closely correlated with reduced concentrations of both Zn and Cu indicating competitive binding to plasma proteins and/or antagonism at uptake sites. Compared to the Cu alone exposure, Cu concentrations were either lower (gills and carcasses) or higher (liver and kidney) in fish exposed to the metals mixture. On the other hand, Cd accumulation was enhanced in livers and carcasses of fish exposed to the mixture compared to those exposed to Cd alone, while Zn stimulated Cu accumulation in gills. Chronic toxicity was demonstrated by elevated malondialdehyde levels in livers and reduced concentrations of Zn and Cu in plasma. Overall, interactions of Cd, Cu and Zn are not always consistent with the isomorphous competitive binding theory.

1. Introduction

Due to common natural and anthropogenic release processes, copper (Cu), cadmium (Cd) and zinc (Zn) often occur together in contaminated aquatic environments (ASTDR, 2004) and potentially can be taken up by fish from both the water and the food. Concurrent exposure of fish to multiple metals may precipitate metal–metal interactions at uptake sites, binding sites on transport proteins or at cellular target and storage sites. These interactions, thought to result from physical and chemical similarities among these metals, occur via the mechanism of ionic and molecular mimicry (Bridges and Zalups, 2005) and can influence metals accumulation and toxicity in aquatic organisms (Rainbow et al., 2000). Because Cd, Cu and Zn belong to the borderline group of metals and have comparable ionic radii and affinity for similar binding sites (sulphur-, oxygen- and nitrogen-containing ligands) in organic macromolecules (Nieboer and Richardson, 1980; Brzóska and Moniuszko-Jakoniuk, 2001; Thévenod, 2010), it is logical to infer that their interactions are consistent with the isomorphous competitive binding theory (Hill and Matrone, 1970; Bremner and Campbell, 1978; Bremner and Beattie, 1995). Surprisingly, the actual mechanisms of the metal–metal interactions at biological sites in animals remain controversial, with both competitive and non-competitive effects having been observed (Brzóska and Moniuszko-Jakoniuk, 2001). In fish, while some strides have been made toward understanding the metal–metal interactions following waterborne exposures (Pelgrom et al., 1995; Dethloff et al., 1999; Amiard-Triquet and Amiard, 1998; Komjarova and Blust, 2009), knowledge of dietary metals interactions remains very scant.

The contribution of dietary exposure to metals toxicity remains a topic of interest and uncertainty in aquatic toxicology. Previous studies have shown that dietary metals accumulate in a variety fish tissues (Szebedinszky et al., 2001; Clearwater et al., 2002; Kamunde et al., 2002b; Chowdhury et al., 2005) and modify uptake kinetics and toxicity (e.g., increased tolerance) of waterborne metals (Szebedinszky et al., 2001; Niyogi and Wood, 2004). Moreover metals accumulation from the food may be dominant (Dallinger et al., 1987; Clearwater et al.,

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2002; Kamunde et al., 2002a). This accumulation has been linked with adverse effects in fish including behavioural impairment such as reduced swimming activity and histological changes in gill and liver (Handy et al., 1999; Farag et al., 1999), membrane lipid peroxidation (Berntssen et al., 2000; Khan et al., 2010) and reduced growth and survival (Farag et al., 1999, 2003; Meyer et al., 2005; Ng and Wood, 2008). However, the majority of dietary metals studies to date utilised single metals exposures leaving an unambiguous necessity to explore how metal–metal interactions influence chronic uptake, accumulation and toxicity of metals mixtures.

The objective of the present study therefore was to characterize the interactions between Cu, Cd and Zn at the whole-body and organ/tissue levels during chronic dietary co-exposure to the three metals in rainbow trout. It was predicted that metals accumulation during concurrent dietary exposure would be governed by competitive interactions on the basis of ionic and molecular mimicry hypothesis. Specific attention was paid to interactions at the levels of uptake, plasma binding and transport and internal accumulation, with a view to understanding how they may impact chronic toxicity, bioelements (Cu and Zn) homoeostasis and oxidative stress response.

2. Materials and methods

All experimental procedures that fish were subjected to were approved by the University of Prince Edward Island Animal Care Committee in accordance with the Canadian Council on Animal Care.

2.1. Fish

Juvenile rainbow trout were obtained from Ocean Trout Farm, Brookvale, PE, and acclimated to laboratory conditions for 1 month at the Atlantic Veterinary College (AVC) Aquatic Research Facility. Laboratory conditions consisted of a single 250 l tank supplied with aerated flow-through well water containing: Na 47.1, Cl 137.3, Ca 58.8, Mg 27.6, hardness 260 (as $CaCO_3$), all in mg/l. The water pH and temperature were 7.6 and 12.5 °C, respectively, while the background Cu, Cd and Zn concentrations were (μg/l): 1.2, below limit of detection (0.03), and 32.8, respectively. During the acclimation period, fish were fed 2% bw daily ration of commercial trout chow (Corey Feed Mills Ltd., Fredericton, NB) containing crude protein 46% (minimum), crude fat 26% (minimum), crude fibre 1.7% (maximum), Ca 1.3% (actual), phosphorous 1.0% (actual), Na 0.6% (actual), vitamin A 4400 IU/kg (minimum), vitamin D_3 3200 IU/kg (minimum) and vitamin E 2000 IU/kg (minimum).

2.2. Experimental diets

Experimental diets were made by supplementing un-pelleted trout chow (Corey Feed Mills Ltd.) with levels of Cu, Zn and Cd to give nominal concentrations of 500, 1000 and 500 μg/g in diet singly and as a tri-metal mixture, respectively. Briefly, the metals were dissolved in 40% volume to diet weight of double-distilled water and mixed for 30 min in a pasta maker. An additional 20% volume to diet weight of double-distilled water was added and mixed for a further 15 min. The food paste was then extruded via a 3 mm die, air-dried and broken into small pellets (approximately 3 mm^3) by hand. Control diet was processed in a similar manner without added metals. The experimental diets were kept at −20 °C till use. The actual concentrations of the metals in the diets were measured by atomic absorption spectrometry (AAnalyst800, Perkin-Elmer, Foster City, California, USA) in graphite furnace (GFAAS; Cu and Cd) and flame (FAAS; Zn) modes after digestion with a 15:1 mixture of 70% HNO_3 (trace metal grade) and 30% H_2O_2. Table 1 shows the metal concentrations in the experimental diets.

2.3. Experimental protocol

At the start of the experiment the fish weighed approximately 20 g. The experimental design consisted of 5 groups: control (no supplemental metals in the diet), Cu-exposed (500 μg/g Cu with background Cd and Zn), Cd-exposed (500 μg/g Cd with background Cu and Zn), Zn-exposed (1000 μg/g Zn with background Cd and Cu) and metals mixture-exposed (μg/g: 500 Cu+500 Cd+1000 Zn). These concentrations provided non-toxic metal doses (Clearwater et al., 2002) that were high enough to result in accumulation essential to reveal potential metal–metal interactions. A total of 225 fish were randomly distributed among 15 spatially randomized 10 l tanks (15 fish/tank) comprising 3 replicates of each treatment group and control. The fish were maintained on 2.5% bw daily ration of designated experimental diet, a 12 h light:12 h dark photoperiod and water flow rate of 600 ml/min. The daily metals doses (μg/g bw/day) provided to the fish at this feeding rate are presented in Table 2. To check for potential leaching of metals from food to water, in-tank water samples were collected 2 times a week before and 1 h after feeding and analysed for metals. Cadmium remained undetectable while the before (b) and after (a) concentrations of Cu and Zn were (n=24): Cu 1.22 ± 0.06 (b); 1.34 ± 0.04 (a) and Zn 32.79 ± 2.66 (b); 31.47 ± 4.93 (a). Additionally water dissolved oxygen ranged from 9.9 to 10.8 mg/l over the experimental period. The exposure was carried out for 28 days with sampling of fish for tissue harvesting at the start of the exposure (day 0) and on days 7, 14 and 28. The tissue sampling was preceded by withholding food for 24 h and involved sacrificing 2 fish per replicate by cephalic blows and weighing them. Blood was immediately collected in heparinised 1 ml syringes by caudal venipuncture and centrifuged at 10000 × g for 4 min to separate plasma which was stored at −80 for measurement of the oxidative stress biomarker, malondialdehyde (MDA) and metals concentrations. Upon dissection, gills, kidneys, stomach, pyloric caeca, intestine and carcasses were wet-weighed and dried at 80 °C to constant (dry) weight and subsequently processed for Cd, Cu and Zn analyses. Note that all gut tissues were thoroughly washed with physiological saline to remove un-evacuated ingesta and blotted dry before weighing. Additionally, livers were immediately frozen in liquid nitrogen and subsequently stored at −80 °C for MDA and metals measurements.

2.4. Metal analysis

Dried gills, kidneys, stomach, pyloric caeca and intestines were digested with 10 × volumes of a 15:1 mixture of 70% HNO_3 and 30% H_2O_2 while dried carcasses were digested in 6 × volumes of the same digestion mixture, all for 48 h. Analytical quality control (QC) was maintained using bovine liver (SRM 1577b, National Institute of Standards and Technology, Gaithersburg, MD, USA) and lobster hepatopancreas (Tort-1, National Research Council, Ottawa, Ontario, Canada). These QC samples and blanks were digested in a similar manner as the samples. Metals concentrations were then measured by AAS on furnace (Cu and Cd) and flame (Zn) modes after appropriate dilution with double-distilled water. The bovine and Tort-1 QC samples, blanks and a certified reference material (TMDA 54.4; National Water Research Institute, Burlington, ON) were analysed along with the experimental samples. The recovery rates (means ± S.D.) for the bovine liver, Tort-1 and TMDA were 96 ± 3%, 94 ± 4% and 98 ± 4%, respectively, while metals were not detected in blanks.

Table 2
Daily dietary doses (μg/g bw/day) of Cu, Cd and Zn provided to rainbow trout. Nominal doses are in brackets MM=metals mixture.

Group	Daily dietary metal doses		
	Copper	Cadmium	Zinc
Control	0.57 (0)	0.03 (0)	6.08 (0)
Cu-exposed	11.8 (12.5)	0.03 (0)	6.46 (0)
Cd-exposed	0.58 (0)	12.4 (12.5)	6.29 (0)
Zn-exposed	0.58 (0)	0.03 (0)	29.0 (25.0)
MM-exposed	12.0 (12.5)	12.5 (12.5)	29.2 (25.0)

Table 1
Measured concentrations of Cu, Cd and Zn in experimental diets. Values are means ± S.E.M., n=6; MM=metals mixture. Nominal concentrations are in brackets.

Group	Dietary metal concentration		
	Copper	Cadmium	Zinc
Control	22.76 ± 0.18 (0)	1.05 ± 0.04 (0)	243.31 ± 2.07 (0)
Cu-exposed	472.15 ± 10.97 (5 0 0)	1.13 ± 0.05 (0)	258.41 ± 4.28 (0)
Cd-exposed	23.26 ± 0.18 (0)	495.32 ± 8.34 (5 0 0)	251.53 ± 4.26 (0)
Zn-exposed	23.18 ± 0.28 (0)	1.06 ± 0.04 (0)	1160.45 ± 18.04 (1000)
MM-exposed	481.66 ± 4.33 (5 0 0)	498.63 ± 7.64 (5 0 0)	1166.97 ± 25.98 (1000)

2.5. MDA concentrations

The concentration of MDA was measured in plasma and liver using a commercial kit (Northwest Life Sciences Specialties LLC, Vancouver, Washington) as per the manufacturer's instructions. Livers were initially homogenised and centrifuged at 10000 × *g* for 2 min at 4 °C, with the supernatant being used for the measurements, while plasma was analysed without additional processing.

2.6. Statistical analysis

The data are expressed as means ± S.E.M. Assumptions of normality of distribution and homogeneity of variances were tested using Kolmogorov–Smirnov and Levene's tests, respectively. All the data met these assumptions and were subsequently submitted to a 2-way analysis of variance (ANOVA, Statistica version 5.1, Statsoft, Inc., Tulsa, OK) with time and treatment as independent variables. Differences between means were delineated using Tukey's honest significant difference (HSD) test, $p < 0.05$.

3. Results

3.1. Growth and toxicity

The fish increased in weight from approximately 20 g to a final average weight of 30 g and exposure to dietary Cu, Cd and Zn, singly or as a mixture, did not impair weight gain (Fig. 1). Of the 225 fish, 3 treatment-unrelated mortalities occurred over the experimental period.

3.2. Metals accumulation

There was substantial Cd accumulation in whole-bodies of Cd- and metals mixture-exposed fish (Fig. 2A), attaining peak concentrations about 1500 and 1800 times the day 0 controls levels, respectively. Furthermore, an early (day 7) diminution and late (day 28) enhancement of Cd accumulation in whole-bodies of fish exposed to the metals mixture was observed. Copper exposure alone also enhanced accumulation of background Cd on day 28. On the other hand, whole-body Cu accumulation in the Cu- and metals mixture-exposed groups peaked on day 7 (metals mixture-exposed) and 14 (Cu-exposed) and saturated thereafter, with enhanced accumulation in the metals mixture group on day 7 (Fig. 2B). Unlike Cu and Cd, Zn exposure did not increase whole-body Zn concentrations, rather, Zn concentrations decreased with time in all of the groups (Fig. 2C).

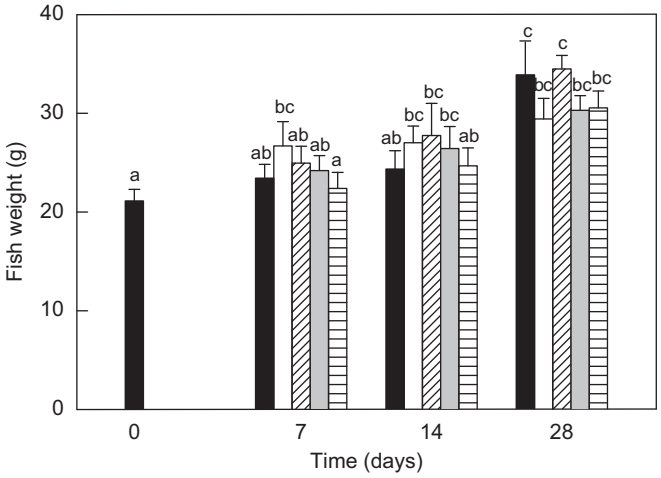

Fig. 1. Growth of rainbow trout maintained on control or metals contaminated diets for 28 days. Black bars, controls; open bars, Cu-diet; diagonally-hatched bars, Zn-diet; grey bars, Cd-diet and cross-hatched bars, metals mixture diet. Values are means ± S.E.M. Different letters denote significant difference, 2-way ANOVA, $p < 0.05$.

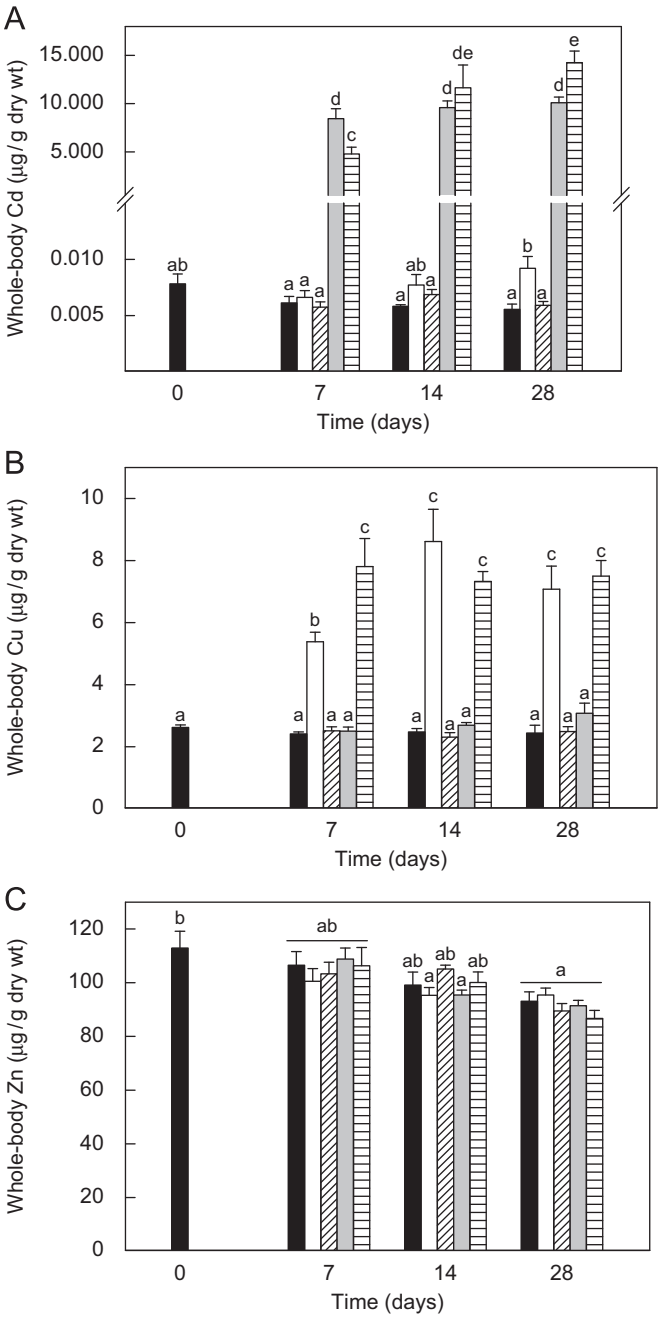

Fig. 2. Concentrations of Cd, Cu and Zn in whole-bodies of rainbow trout fed control or metals contaminated diets for 28 days. Black bars, control diet; open bars, Cu-diet; diagonally-hatched bars, Zn-diet; grey bars, Cd-diet and cross-hatched bars, metals mixture diet. Values are means ± S.E.M. Different letters denote significant difference, 2-way ANOVA, $p < 0.05$.

Metals accumulation in gut tissues is shown in Fig. 3. In the stomach, Cd concentrations increased in the Cd- and metals mixture-exposed groups to attain, respectively, final concentrations 500 and 420 times above the day 0 controls (Fig. 3A). The metals mixture-exposed group appeared to saturate by day 14, while the Cd group maintained linear accumulation throughout the exposure. Interestingly, background Cd concentrations declined in the Zn-exposed fish. Copper (Fig. 3B) accumulated significantly in the Cu- and metals mixture-exposed groups to reach terminal (day 28) concentrations 21 and 15 times above the day 0 control levels. Less Cu was accumulated in the stomach when metals were presented as a mixture relative to Cu alone.

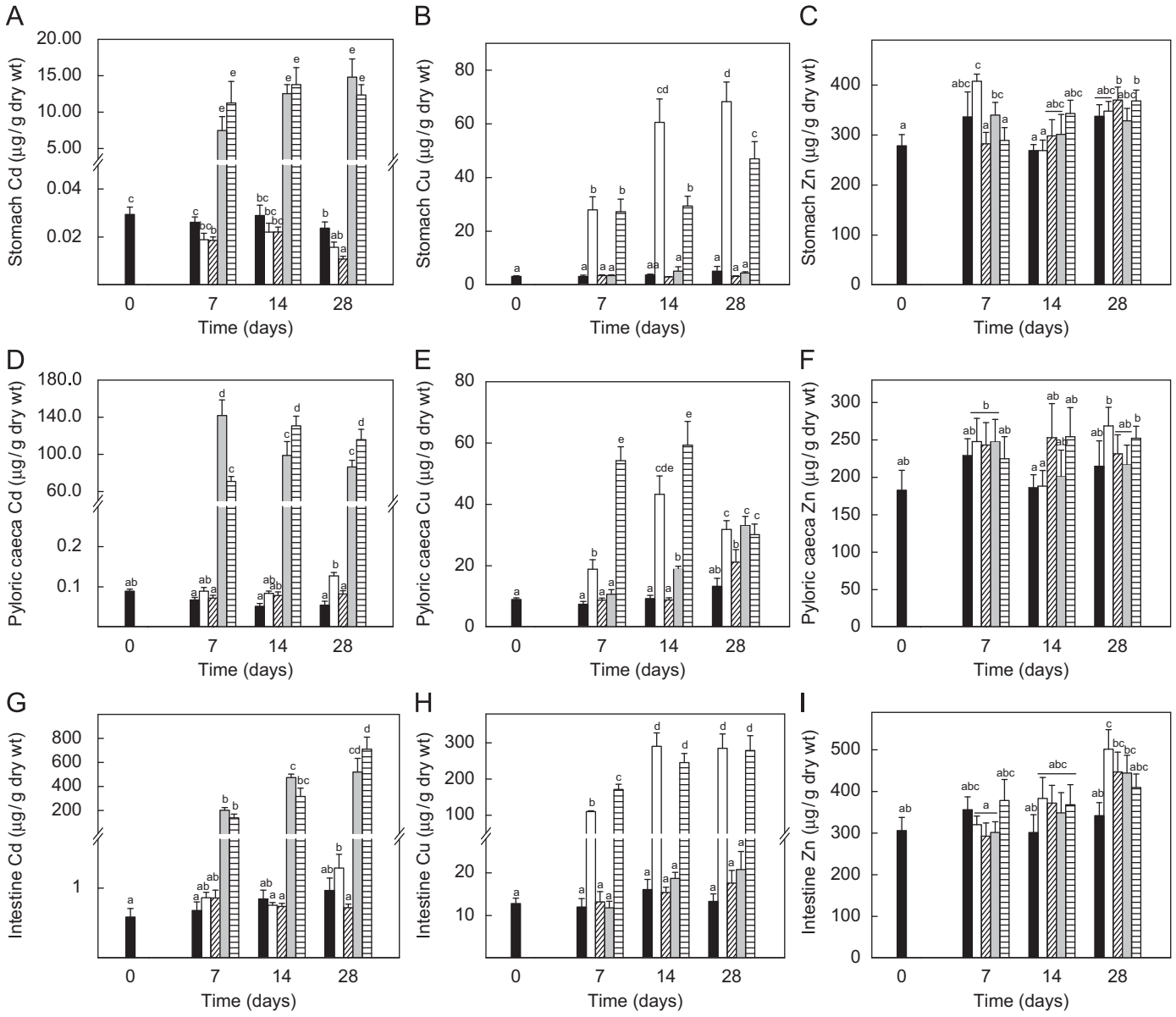

Fig. 3. Concentrations of Cd, Cu and Zn in gut tissues [stomach (A–C), pyloric caeca (D–F) and intestines (G–I)] of rainbow trout fed control or metals contaminated diets for 28 days. Black bars, control diet; open bars, Cu-diet; diagonally-hatched bars, Zn-diet; grey bars, Cd-diet and cross-hatched bars, metals mixture diet. Values are means ± S.E.M. Different letters denote significant difference, 2-way ANOVA, $p < 0.05$.

Unlike Cu and Cd, there was no time-matched accumulation of Zn in the stomach with the concentration remaining within the range of 280–320 µg/g dry wt. (Fig. 3C).

In the pyloric caeca (Fig. 3D) marked accumulation of Cd was observed in the Cd and metals mixture exposure groups. Fish exposed to the metals mixture accumulated significantly less Cd than those exposed to Cd alone on day 7. However, by day 28 fish exposed the metals mixture accumulated more Cd than those exposed to Cd alone. Interesting Cu exposure alone resulted in significantly increased concentrations of Cd relative to the controls on day 28. Copper accumulation, on the other hand, was enhanced early (day 7) in the metals mixture-exposed fish compared to those exposed to Cu alone. These concentrations peaked on day 14 at approximately 5 and 7 times the day 0 control levels in the Cu- and metals mixture-exposed groups, respectively, and declined thereafter (Fig. 3E). Additionally, fish exposed to Cd alone accumulated more Cu than the respective controls on days 14 and 28. Compared to Cu and Cd, Zn accumulation in the pyloric caeca was modest with inconsistent trends of increased accumulation in the Zn-, Cu- and metals mixture-exposed groups (Fig. 3F).

Cadmium concentrations in the intestine increased linearly to attain day 28 concentrations about 885 and 1210 times above the day 0 values in the Cd- and metals mixture-exposed fish, respectively (Fig. 3G). Concentrations of Cu also increased in the Cu- and metals mixture-exposed groups with saturation occurring after 14 days at about 20 times the day 0 control concentrations in both groups (Fig. 3H). There was a significant enhancement of Cu accumulation in the metals mixture-exposed group compared to the Cu-exposed group on day 7. For Zn (Fig. 3I), the intestinal concentrations were variable with the notable observations being enhanced accumulation in the Cu-exposed fish on day 28 and, surprisingly, lack of accumulation in the Zn-exposed fish.

In plasma, Cd concentrations increased in the Cd-exposed and metals mixture-exposed fish attaining maximal accumulation of 25–43 ng/ml by day 7 (Fig. 4A). More importantly, there was enhanced accumulation of Cd in the metals mixture-exposed group

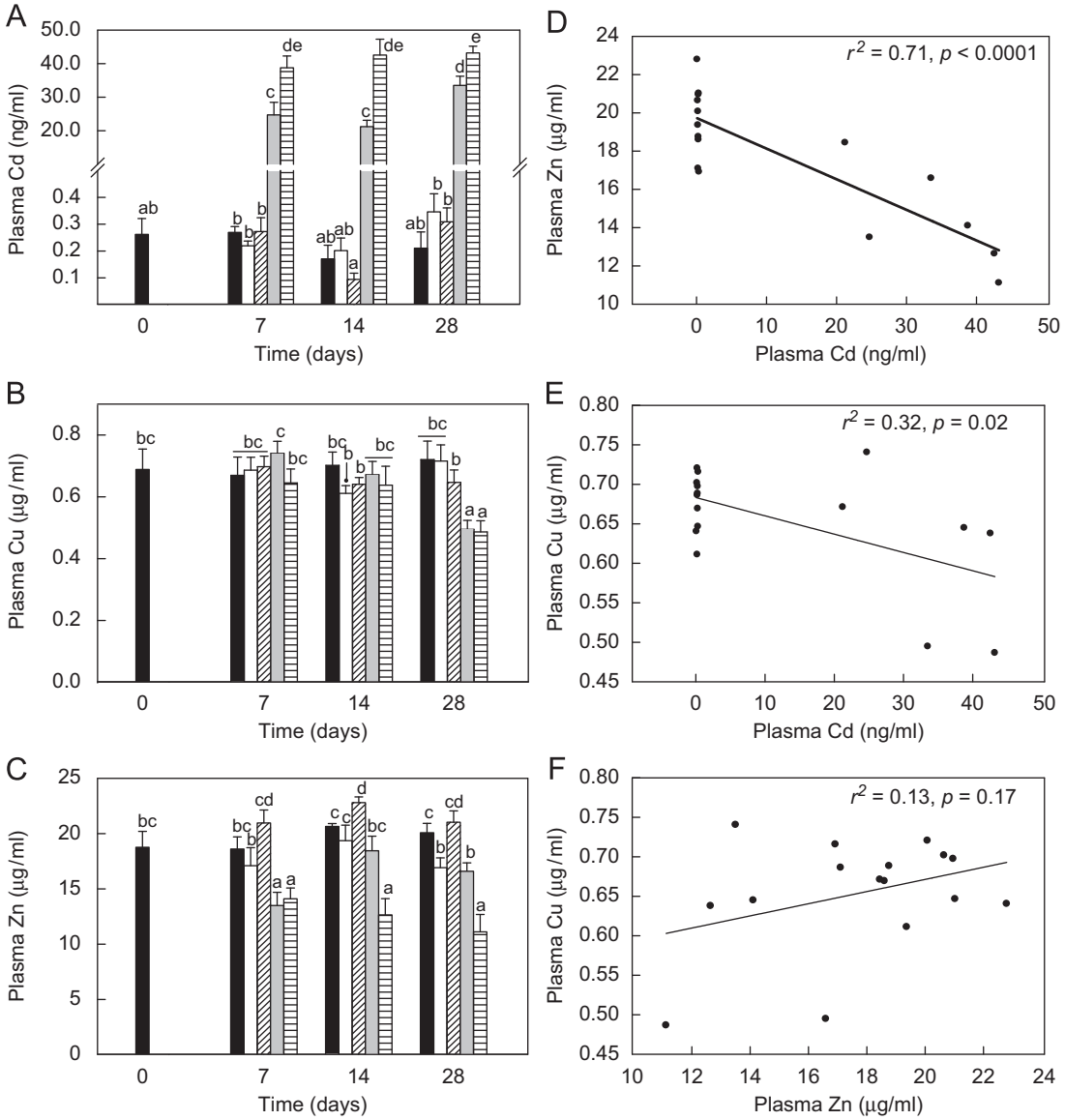

Fig. 4. Concentrations of Cd, Cu and Zn in plasma of rainbow trout fed control or metals contaminated diets for 28 days. Black bars, control diet; open bars, Cu-diet; diagonally-hatched bars, Zn-diet; grey bars, Cd-diet and cross-hatched bars, metals mixture diet. Values are means ± S.E.M. Different letters denote significant difference, 2-way ANOVA, $p < 0.05$.

(relative to the Cd-exposed group) which was closely associated with reduced accumulation of Zn (days 7, 14, 28) and Cu (day 28 only). Other than for the afore-mentioned decline on day 28 in metals mixture-exposure, plasma Cu concentrations remained relatively constant between 0.6 and 0.7 µg/ml (Fig. 4B). Plasma Zn concentrations on the other hand (Fig. 4C) ranged between 13 and 23 µg/ml and were increased following dietary Zn exposure and reduced consistently by exposure to dietary Cd, Cu and metals mixture. Overall correlations between the metals concentrations in plasma revealed significant negative relationships between Cd and both Zn (Fig. 4D) and Cu (Fig. 4E). The relationship between plasma Cu and Zn (Fig. 4F) was not significant.

The patterns of metals accumulation in the liver and kidney are shown in Fig. 5. Both Cd (Fig. 5A) and Cu (Fig. 5B) accumulated time-dependently in livers of fish exposed to the respective metal and mixture. Compared to the single metals exposures, accumulations of both Cd and Cu were enhanced in the metals mixture-exposed group on day 28. Enhancements of Cu accumulation in fish exposed to Cd (days 14 and 28) and Zn (day 14) were also observed. For Zn, modest hepatic accumulation was observed with significant accumulation relative to the time-matched controls occurring in the Zn-exposed group on day 14 and in metals mixture-exposed group on day 28 (Fig. 5C).

Renal Cd accumulation was linear over time in the Cd- and metals mixture-exposed groups, both attaining comparable day 28 concentrations about 100 times above the day 0 control levels (Fig. 5D). Similarly, renal Cu concentrations increased with time, however, relative to Cu alone exposure, there was a distinct enhancement of Cu accumulation in the metals mixture-exposed group (Fig. 5E). Worth noting is the increased accumulation of Cu in the Cd-exposed fish on day 28 (Fig. 5E) and the lack of significant Zn accumulation in all the groups (Fig. 5F).

The gill and carcass metals concentration data (Fig. 6) show that Cd accumulated time-dependently to the same extent in gills of the Cd- and metals mixture-exposed groups, with the final Cd concentrations being about 90 times the day 0 concentrations (Fig. 6A). Gill Cu concentrations, however, did not change in the Cu- or metals mixture-exposed groups but there was enhanced accumulation of background Cu in the Zn-exposed group resulting in Cu concentration significantly higher than those of the controls

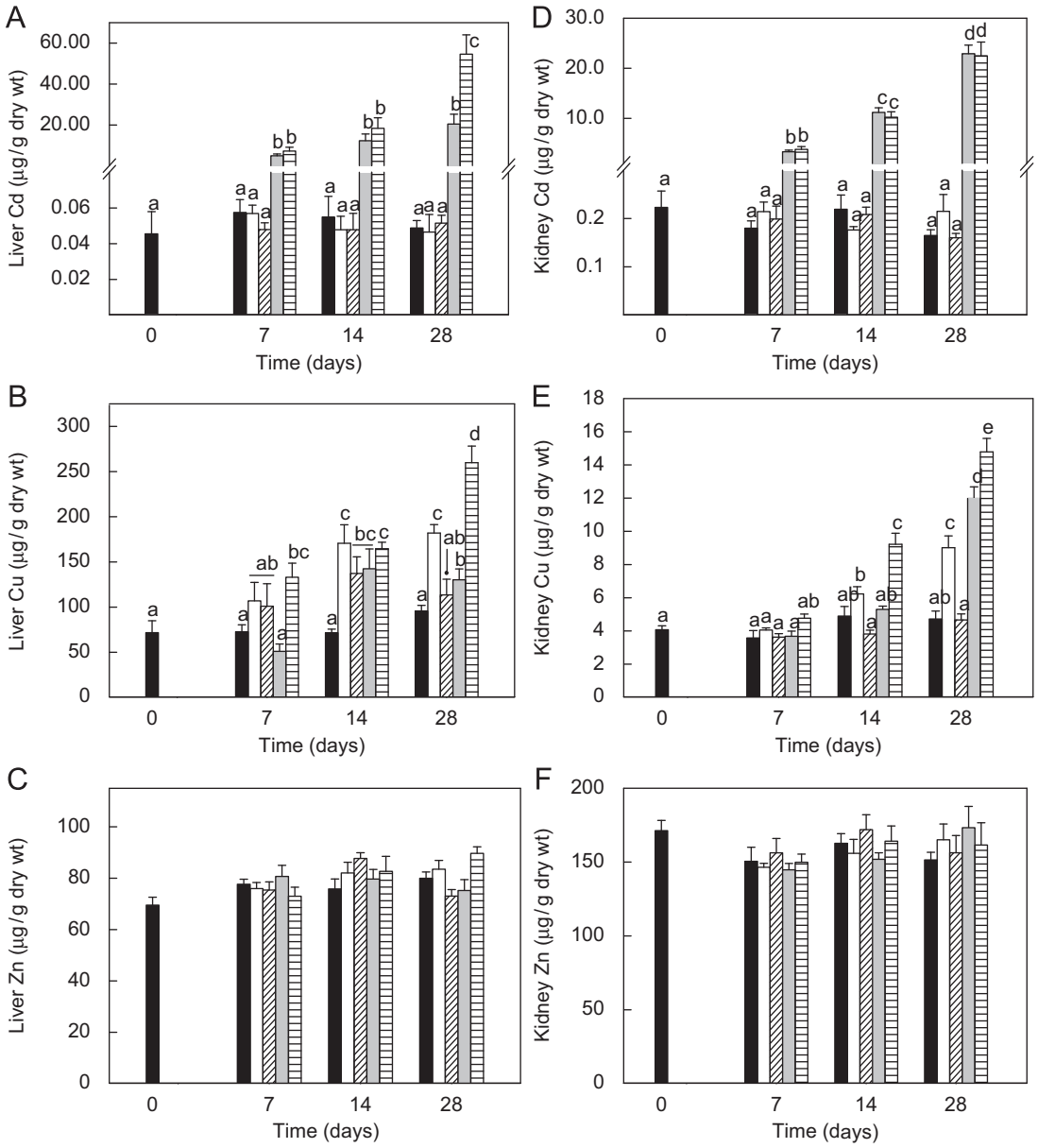

Fig. 5. Concentrations of Cd, Cu and Zn in livers (A–C) and kidneys (D–F) of rainbow trout fed control or metals contaminated diets for 28 days. Black bars, control diet; open bars, Cu-diet; diagonally-hatched bars, Zn-diet; grey bars, Cd-diet and cross-hatched bars, metals mixture diet. Values are means ± S.E.M. Different letters denote significant difference, 2-way ANOVA, $p < 0.05$.

on days 14 and 28 (Fig. 6B). As well, exposure to the metals mixture or Cd decreased gill Cu concentrations on day 28. Lastly, similar to most of the other tissues examined, Zn did not accumulate in gills; instead, a general reduction in its concentration with time was observed (Fig. 6C).

In the carcass, Cd accumulated linearly in the Cd- and metals mixture-exposed fish with enhanced accumulation in fish exposed to the mixture compared to Cd only exposure on day 14 (Fig. 6D). Copper exposed fish also accumulated more Cd than the controls. Overall the Cd- and metals mixture-exposed groups achieved comparable terminal Cd concentrations. Regarding Cu, significant accumulation occurred in fish exposed to Cu and the metals mixture, with peaks on day 7 followed by gradual decline (Fig. 6E). Exposure to Zn, Cd and the metals mixture resulted in carcass Cu concentrations lower than those of the controls on days 14 and/or 28. In contrast with Cd and Cu, there was no accumulation of Zn in the carcass, rather, the concentrations tended to decline with time regardless of the dietary Zn exposure concentration (Fig. 6F).

3.3. MDA concentrations

Concentrations of MDA in liver (Fig. 7A) and plasma (Fig. 7B) measured on days 7 and 28 were increased only in livers of fish exposed to Zn and the metals mixture on day 7 and returned to control levels on day 28. Exposure to Cd and Cu had no effect on plasma and liver MDA concentrations.

4. Discussion

4.1. Metals accumulation, interactions and toxicity

Whole-body metals concentrations reflect the net accumulation and integrate all metals uptake and loss processes including cross-organ homoeostatic redistribution. The non-essential metal, Cd, exhibited clear time-dependent accumulation in fish exposed to Cd alone or as a component of a tri-metal dietary mixture. Evidence of

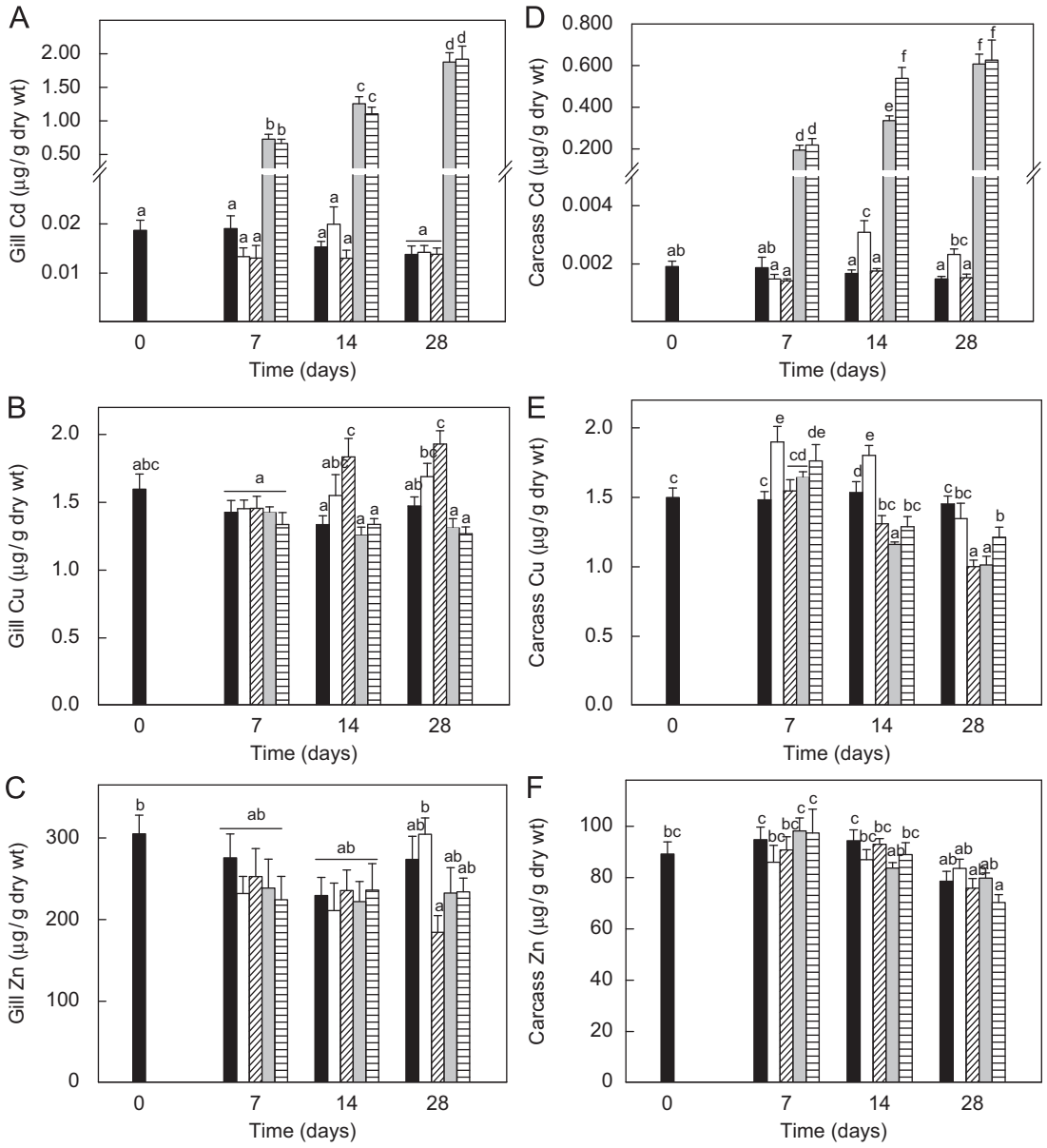

Fig. 6. Concentrations of Cd, Cu and Zn in gills (A–C) and carcasses (D–F) of rainbow trout fed control or metals contaminated diets for 28 days. Black bars, control diet; open bars, Cu-diet; diagonally-hatched bars, Zn-diet; grey bars, Cd-diet and cross-hatched bars, metals mixture diet. Values are means ± S.E.M. Different letters denote significant difference, 2-way ANOVA, $p < 0.05$.

Cd–metal interactions included the early diminution and late enhancement of accumulation in fish exposed to the metals mixtures relative to those expose to Cd. The late enhancement of Cd accumulation in the metals mixture group was likely a Cu effect, e.g., induction of proteins that bind Cd, because there was a corresponding enhancement of Cd accumulation in the Cu exposure alone (Fig. 2A). Previous waterborne studies reported that the co-exposure of Cu reduced whole-body Cd concentration in tilapia (Pelgrom et al., 1994) and zebrafish (Komjarova and Blust, 2009). These reports are similar to our day 7, but not day 28, findings (Fig. 2A). Thus the duration of exposure appears to influence the Cd–metal interactions. For Cu, there was an early enhancement of accumulation when the metals were presented as a mixture compared to Cu alone exposure, with apparent saturation after 14 days, suggesting faster occupation of a limited number of Cu binding sites and/or reduced uptake and increased excretion. That this enhancement of Cu accumulation corresponded with reduced whole-body Cd concentration suggests antagonistic interactions between Cu and Cd. Compared to Cd and Cu, whole-body Zn concentrations did not increase at the dietary Zn exposure concentration used in the present study, nor were they altered by exposure to Cu and Cd. This underscores the existence of efficient mechanisms to regulate body levels of Zn in rainbow trout (Hogstrand and Wood, 1996; Sappal et al., 2009). Indeed we observed a decline in body Zn concentration with time, likely due to dilution of a relatively constant pool of Zn with increase in body mass. Overall the observed changes in whole-body metals concentrations did not have overt toxic effects. However, Cu and Zn concentrations in plasma were reduced suggesting disturbed homoeostasis of these bioelements.

The pattern of metals accumulation in gut tissues (intestine > pyloric caeca > stomach) was similar to those reported previously for single metals exposures (Clearwater et al., 2002; Kamunde et al., 2002a; Chowdhury et al., 2005). Due to chemical and physical similarity of the 3 metals used in present study, we predicted competitive interactions at binding sites on metal influx/efflux

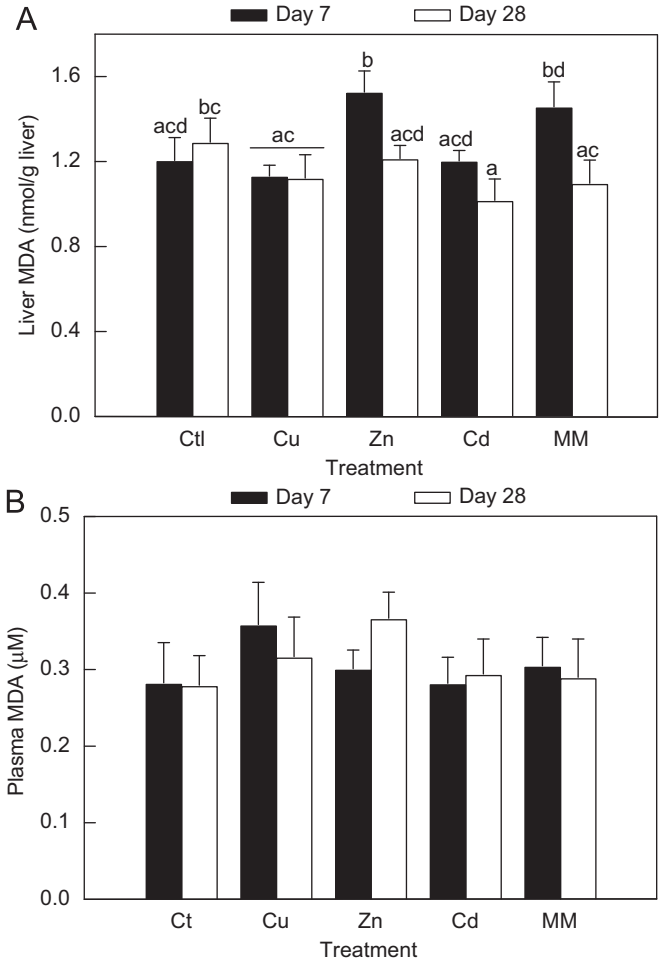

Fig. 7. Concentration of malondialdehyde (MDA) in liver (A) and plasma (B) of rainbow trout fed control or metals contaminated diets for 28 days. Values are means ± S.E.M. Different letters denote significant difference, 2-way ANOVA, $p < 0.05$.

transporters in the gut. Surprisingly the interactions observed in the present study were not always competitive or mutual and varied with the part of the gut, metals concentrations and duration of exposure. Thus in the stomach (Fig. 3A–C), Cu accumulation was inhibited by Cd and Zn co-exposure without corresponding increase in the accumulation of either Cd or Zn. Additionally, high dietary Zn exposure alone caused a reduction in the background concentrations of Cd but high dietary Cd had no effect on background concentrations of either Cu or Zn. In contrast, Cu accumulation in the pyloric caeca (Fig. 3D–F) was enhanced in fish exposed to the metals mixture, an effect attributable to displacement of Cd because there was a corresponding reduction in the accumulation of Cd in as much as elevated Cd alone increased the retention of background dietary Cu. There was, however, no evidence of interaction between Zn with Cd or Cu in the stomach or pyloric caeca under the experimental conditions used in the present study. Finally in the intestine (Fig. 3G–I), Cd accumulation was not affected by either Cu or Zn, contrasting the results obtained using a gut *in vitro* model (Ojo and Wood, 2008) in which Cd uptake was reduced by Zn. Our data also are contrary to Chowdhury et al. (2005) who observed decreased concentration of Zn in dietary Cd-exposed rainbow trout and Pelgrom et al. (1995) who reported enhanced accumulation of Cd in the intestine after waterborne Cd–Cu mixture exposure. We did, however, observe enhanced accumulation of Cu in the intestine early in the exposure in fish exposed to the metals mixture which was not associated with changes in concentrations of either Cd or Zn. This is partly similar to the enhanced accumulation of Cu in the intestine observed after exposure to a waterborne Cd–Cu mixture in tilapia (Pelgrom et al., 1995). In the present study, enhancement of intestinal Zn accumulation by Cu without an effect of Cu on Zn concentration was observed contrasting findings in mammals that Zn and Cu are mutually antagonistic (Bremner and Beattie, 1995).

Overall the mechanisms of gastrointestinal interactions of metals in animals are not clearly understood. Our data suggest that high dietary Cd intake could have induced synthesis of metal binding proteins such as metallothionein as previously reported (Chowdhury et al., 2005; Sappal et al., 2009) with subsequent increased sequestration and retention of Cu within the stomach and pyloric caeca. This retention would limit the transfer of Cu to blood for distribution to other tissues and possibly promote Cu excretion via epithelial cell denudation. However, the notion of sequestration of metals by induced metallothionein (MT) in the gastrointestinal tract with subsequent inhibition of its absorption does not entirely explain metal–metal interactions because high dietary Zn was found to reduce Cu concentrations in mice devoid of the MT gene (Reeves, 1998). This spawned the alternative hypothesis that Zn inhibits Cu efflux proteins and/or non-competitively stimulates Cu influx proteins (Reeves et al., 1998). In the present study, the key conclusions regarding gastrointestinal uptake/accumulation are that, first, dependent on exposure duration and metals concentration ratios, Cd and Cu were either antagonistic or mutually increased each other's uptake/accumulation in the pyloric caeca without the effect on Zn. Because Cd is not a substrate for the Cu influx carriers, copper transporters (Ctr) 1 and 2 (Kaplan and Lutsenko, 2009), this interaction likely occur via other metal transporters, e.g., the divalent metal transporter 1 (DMT1), ZIP and ZnT families of transporters (Garrick et al., 2006; Thévenod, 2010) and MT binding. Second, the stomach appears to be an important site for metals absorption in which Zn exposure reduced Cd uptake/accumulation. Third, Cu–Cd–Zn interactions were less evident in the intestine, with the only significant effect being that Cu exposure enhanced Zn accumulation. It is possible that in the intestine, competitive interactions at common uptake and accumulation sites were more or less balanced by uptake/accumulation at metal-specific/non-interacting sites.

We investigated the Cu–Cd–Zn interactions at the level of transport by monitoring changes in their plasma concentrations. Although metal binding molecules in fish plasma have not been fully characterised, available data suggest they are similar to those found in mammalian plasma (De Smet et al., 2001). In mammals, transcuprein and ceruloplasmin are specific Cu transporters with the later binding up to 90% of circulating Cu in humans, while α2-microglobulin, transferrin and immunoglobulin G transport Zn. Serum albumin transports Cu, Cd and Zn (and other metals) albeit upon binding at different sites. Specifically, Cu preferentially binds to the amino-terminal copper and nickel (ATCUN) site, Zn to site A which also binds other divalent metals, whereas Cd binds to site B (Lu et al., 2008). Another avenue of metals transport in plasma is the amino acids histidine and cysteine. Although the present study did not specifically investigate the types of metal binding molecules in rainbow trout plasma, the metals accumulation pattern clearly shows antagonistic interactions in which enhanced accumulation of Cd in the metals mixture was closely correlated with reduced accumulation of Zn (Fig. 4D; significant negative slope, $r^2 = 0.71$) and Cu (Fig. 4E; significant negative slope, $r^2 = 0.32$). Previous studies similarly reported negative relationships between serum Zn/Cu and Cd in rats fed Cd-supplemented diet (Revees and Rossow, 1996; Brzóska and Moniuszko-Jakoniuk, 2001). Generally under physiological conditions, concentrations of plasma proteins, hence metal binding

sites, exceed the concentrations of the biometals Cu and Zn. In the present study antagonism between the metals was evident after 14 days of metals exposure/accumulation, suggesting that the metal binding capacity become limiting at this time point. The ensuing competition for binding sites culminated in Cd displacing Cu and Zn likely due to its higher affinity for these sites. In addition, release of Cd-metallothionein from the liver (Nomiyama et al., 1998) likely contributed to the increase in plasma Cd concentration. Competitive metal–metal interactions at gastrointestinal uptake sites also could have influenced plasma metals loading.

The liver (see Fig. 5A–C) displayed enhanced accumulation of Cd and Cu but not Zn in fish exposed to the metals mixture compared to the respective single metals exposures suggestive of an excess of binding sites relative to the metals concentrations. Similarly, enhanced Cu accumulation occurred in largemouth bass liver after dietary Cd exposure (Weber et al., 1992) and a positive correlation between Cd and Zn concentrations was observed in rat livers after Cd–Zn exposure (Oishi et al., 2000). Mutual enhancement of accumulation can be explained by induction of metal binding proteins such as MT and its stability state upon binding of various metal ions. At physiological state MT first saturates with Zn or/and Cu but during exposure to elevated Cd, replacement of Zn by Cd occurs because Cd has higher affinity for MT than Zn (Brzóska and Moniuszko-Jakoniuk, 2001). The displaced Zn then induces MT synthesis, thus increasing the overall metal binding capacity of the liver. Because the α-domain of MT is thermodynamically stable when saturated by Cd, while the β-domain is not stable when it contains Cd (Funk et al., 1987), feeding fish diets elevated in Cd–Cu–Zn results in increased metals accumulation by binding to induced MT.

We observed marked renal Cd accumulation without Cd–metal interactions upon exposure of fish to the Cu–Cd–Zn mixture (Fig. 5D). In contrast, Cu accumulation (Fig. 5E) was clearly enhanced in fish exposed to the mixture and Cd, a result akin to that reported in tilapia co-exposed to waterborne Cu and Cd (Pelgrom et al., 1995). Since renal Zn concentrations did not change (Fig. 5F), the enhanced Cu accumulation was likely due to interactions between Cu and Cd, which, similar to the liver, can be explained by metal binding to induced metal-binding proteins.

Branchial accumulation of dietary Cd, representing both epithelial- and blood-bound Cd, was unambiguously consistent with earlier findings in rainbow trout (Szebedinszky et al., 2001; Chowdhury et al., 2005). Although accumulation of dietary Cd in gills is deemed to have less severe direct toxicological impact than that accumulated from the water, it modifies gill Cd binding with implications on biotic ligand modelling for prediction of acute toxicity (Szebedinszky et al., 2001; Niyogi and Wood, 2004). In the present study, branchial Cd accumulation (Fig. 6A) was not affected by the co-exposure to Cu and/or Zn, contrasting increased accumulation of Cd observed after waterborne Cd and Cu co-exposure in tilapia (Pelgrom et al., 1995) and reduced Cd uptake in zebrafish after elevated waterborne Zn exposure (Glynn, 2001; Komjarova and Blust, 2009). Because Cd exposure in water or food had no effect on background branchial Zn and Cu concentrations (Hollis et al., 2001; Chowdhury et al., 2005), the inconsistencies of the interactions of these metals at the gill among various studies likely result from experimental variables such the experimental duration and ratios of metals concentrations in exposure media.

Unlike Cd, Cu and Zn concentrations (Fig. 6B and C) did not increase in gills on exposure to elevated dietary levels of the respective metal, singly or as a mixture. However, Zn exposure enhanced background Cu accumulation while Cd exposure alone did not influence Cu accumulation (Fig. 6B). These results contrast with the enhanced accumulation of Cu in gills of eels (Gill et al., 1992) and tilapia (Pelgrom et al., 1995) after waterborne Cd–Cu co-exposures. Moreover, although Zn exposure enhanced accumulation of background Cu, fish exposed to elevated concentrations of the metals in the dietary mixture did not have increased Cu concentration, indicating that metals ratio and route of exposure influence metal–metal interactions.

The carcass, comprising mainly muscle and bone, exhibited clear accumulation of Cd in fish exposed to Cd or the mixture. This accumulation likely occurred in bone by displacement of Ca because Cd accumulation in muscle is generally minimal (Pelgrom et al., 1995). Moreover, the enhanced accumulation of Cd (day 14) was likely an interaction with Cu because there was a corresponding decline in Cu concentration, and elevated dietary Cu alone increased accumulation of background Cd (Fig. 6D and E). The accumulation pattern of Cu in the carcass (Fig. 6 E) in which an early (day 7) peak accumulation was followed by a gradual decline in Cu concentration highlights the effect of exposure duration and growth dilution. Moreover, antagonism between Cu and Cd/Zn was also revealed because the two metals reduced accumulation of Cu in the carcass. In rats and mice no Cd–Zn interactions were found in the muscle while in bone, Cd either decreased or did not affect the Zn concentration depending on the exposure concentration (see Brzóska and Moniuszko-Jakoniuk, 2001 for review). In the present study both Cd and Cu did not affect carcass Zn concentrations.

4.2. Oxidative stress response

Among the metals used in the present study, Cu and Cd are known to promote oxidative stress via redox cycling (Cu) and impairing the antioxidant system (Cd), while Zn has been shown to counteract oxidation (Bray and Bettger, 1990; Stohs and Bagchi, 1994; Zago and Oteiza, 2001). Our results are inconsistent with this theme because exposure to Zn increased MDA concentrations in the liver, while Cu and Cd had no effect. It is possible that Zn binds to –SH groups and/or occupies binding sites of redox active metals, e.g., Cu/Fe, impairing the antioxidant defence system and enhancing redox cycling, thus promoting oxidation. Additionally, elevated cellular Zn was recently shown to induce oxidative stress by impairing mitochondrial enzyme systems in hepatocytes (Lemire et al., 2008). Thus whether or not Zn is protective against cellular oxidation may in part depend on the exposure concentration. Overall the fact that a consistent trend of metals-induced oxidative stress response in fish has not yet emerged highlights the importance of the exposure conditions in determining the outcome. More studies are clearly necessary to clarify the oxidative stress inducing role of dietary Zn exposure in fish.

5. Conclusions

The present study demonstrates that accumulation and distribution patterns of Cu, Cd and Zn may differ when these metals are presented to fish in food singly or as a tri-metal mixture. Metal–metal interactions were not always consistent with the isomorphous competitive binding theory, indicating that prediction of accumulation and chronic toxicity of dietary metals mixtures would be difficult to accomplish. Metal–metal interactions appear to depend on the organ/tissue, ratio of concentrations of the metals involved and duration of exposure. The oxidative stress response observed on Zn exposure suggests that the antioxidant properties of Zn also may depend on the exposure duration and relative concentrations of metals in the diet. Overall metal–metal interactions significantly influence accumulation and toxicological responses and should be considered in tools for predicting the metals' impact in aquatic systems.

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References

Amiard-Triquet, C., Amiard, J.C., 1998. Influence of ecological factors on accumulation of metal mixtures. In: Langston, J.W., Bebianno, M. (Eds.), Metal Metabolism in Aquatic Environments. Chapman and Hall, London, UK, pp. 351–386.

ATSDR (Agency for Toxic Substance and Disease Registry), Draft, 2004. Evaluation of the Toxicology of Chemical Mixtures Commonly Found at Hazardous Waste Sites. US Public Health Service, US Department of Human Services, Atlanta, GA.

Berntssen, M.H.G., Lundebye, A.K., Hamre, K., 2000. Tissue lipid peroxidative responses in Atlantic salmon (*Salmo salar* L.) parr fed high levels of dietary copper and cadmium. Fish Physiol. Biochem. 23, 35–48.

Bray, T.M., Bettger, W.J., 1990. The physiological role of zinc as an antioxidant. Free Radic. Biol. Med. 8, 281–291.

Bremner, I., Beattie, J.H., 1995. Copper and zinc metabolism in health and disease: speciation and interactions. Proc. Nutr. Soc., 489–499.

Bremner, I., Campbell, J.K., 1978. Effect of copper and zinc status on susceptibility to cadmium intoxication. Environ. Health Perspect. 25, 125–128.

Bridges, C.C., Zalups, R.K., 2005. Molecular and ionic mimicry and the transport of toxic metals. Toxicol. Appl. Pharmacol. 204, 274–308.

Brzóska, M.M., Moniuszko-Jakoniuk, J., 2001. Interactions between cadmium and zinc in the organism. Food Chem. Toxicol. 39, 967–980.

Chowdhury, M.J., Baldisserotto, B., Wood, C.M., 2005. Tissue-specific cadmium and metallothionein levels in rainbow trout chronically acclimated to waterborne or dietary cadmium. Arch. Environ. Contam. Toxicol. 48, 381–390.

Clearwater, S.J., Farag, A.M., Meyer, J.S., 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp. Biochem. Physiol. C 132, 269–313.

Dallinger, R., Prosi, F., Segner, H., Back, H., 1987. Contaminated food and uptake of heavy-metals by fish–a review and a proposal for further research. Oecologia 73, 91–98.

De Smet, H., Blust, R., Moens, L., 2001. Cadmium-binding to transferrin in the plasma of the common carp *Cyprinus carpio*. Comp. Biochem. Physiol. C 128, 45–53.

Dethloff, G.M., Schlenk, D., Hamm, J.T., Bailey, H.C., 1999. Alterations in physiological parameters of rainbow trout (*Oncorhynchus mykiss*) with exposure to copper and copper/zinc mixtures. Ecotoxicol. Environ. Saf. 42, 253–264.

Farag, A.M., Skaar, D., Nimick, D.A., MacConnell, E., Hogstrand, C., 2003. Characterizing aquatic health using salmonid mortality, physiology, and biomass estimates in streams with elevated concentrations of arsenic, cadmium, copper, lead, and zinc in the Boulder River watershed, Montana. Trans. Am. Fish. Soc. 132, 450–467.

Farag, A.M., Woodward, D.E., Brumbaugh, W., Goldstein, J.N., MacConnell, E., Hogstrand, C., Barrows, F.T., 1999. Dietary effects of metals-contaminated invertebrates from the Coeur d'Alene River, Idaho, on cutthroat trout. Trans. Am. Fish. Soc. 128, 578–592.

Funk, A.E., Day, F.A., Brady, F.O., 1987. Displacement of zinc and copper from copper-induced metallothionein by cadmium and by mercury: in vivo and ex vivo studies. Comp. Biochem. Physiol. C, 1–6.

Garrick, M.D., Singleton, S.T., Vargas, F., Kuo, H.C., Zhau, L., Knopfel, M., Davidson, T., Costa, M., Paradkar, P., Roth, J.A., Garrick, L., 2006. M. DMT1: Which metals does it transport? Biol. Res. 39 79–85.

Gill, T.S., Bianchi, C.P., Epple, A., 1992. Trace metal (Cu and Zn) adaptation of organ systems of the American eel, *Anguilla rostrata*, to external concentrations of cadmium. Comp. Biochem. Physiol. C 102, 361–371.

Glynn, A.W., 2001. The influence of zinc on apical uptake of cadmium in the gills and cadmium influx to the circulatory system in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. C 128, 165–172.

Handy, R.D., Sims, D.W., Giles, A., Campbell, H.A., Musonda, M.M., 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. Aquat. Toxicol. 47, 23–41.

Hill, C.H., Matrone, G., 1970. Chemical parameters in the study of *in vivo* and *in vitro* interactions of transition elements. Fed. Proc. 29, 1474–1481.

Hogstrand, C., Wood, C.M., 1996. The physiology and toxicology of zinc in fish. In: Taylor, E.W. (Ed.), Toxicology of Aquatic Pollution. Cambridge University Press, Cambridge, pp. 61–84.

Hollis, L., Hogstrand, C., Wood, C.M., 2001. Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. Arch. Environ. Contam. Toxicol. 41, 468–471.

Kamunde, C., Clayton, C., Wood, C.M., 2002a. Waterborne vs. dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R69–R78.

Kamunde, C., Grosell, M., Higgs, D., Wood, C.M., 2002b. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. J. Exp. Biol. 205, 279–290.

Kaplan, J.H., Lutsenko, S., 2009. Copper transport in mammalian cells: special care for a metal with special needs. J. Biol. Chem. 284, 25461–25465.

Khan, F.R., Bury, N.R., Hogstrand, C., 2010. Cadmium bound to metal rich granules and exoskeleton from *Gammarus pulex* causes increased gut lipid peroxidation in zebrafish following single dietary exposure. Aquat. Toxicol 96, 124–129.

Komjarova, I., Blust, R., 2009. Multimetal interactions between Cd, Cu, Ni, Pb, and Zn uptake from water in the Zebrafish. *Danio rerio.* Environ. Sci. Technol. 43, 7225–7229.

Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M. (Eds.), 2005. Toxicity of Dietborne Metals to Aquatic Organisms. SETAC Press, Pensacola.

Lemire, J., Mailloux, R., Vasu, D., Appanna, V.D., 2008. Zinc toxicity alters mitochondrial metabolism and leads to decreased ATP production in hepatocytes. J. Appl. Toxicol. 28, 175–182.

Lu, J., Stewart, A.J., Sadler, P.J., Pinheiro, T.J.T., Blindauer, C.A., 2008. Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. Biochem. Soc. Trans. 36, 1317–1321.

Nieboer, E., Richardson, D.H.S., 1980. The replacement of the non-descript term 'Heavy Metals' by a biologically and chemically significant of metal ions. Environ. Pollut. Ser. B 1, 3–26.

Niyogi, S., Wood, C.M., 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.

Ng, T.Y.T., Wood, C.M., 2008. Trophic transfer and dietary toxicity of Cd from the oligochaete to the rainbow trout. Aquat. Toxicol. 87, 47–59.

Nomiyama, K., Nomiyama, H., Kamed, K., 1998. Plasma cadmium-metallothionein, a biological exposure index for cadmium-induced renal dysfunction, based on the mechanism of its action. Toxicology 129, 157–168.

Oishi, S., Nakagawa, J.-I., Ando, M., 2000. Effects of cadmium administration on the endogenous metal balance in rats. Biol. Trace Elem. Res. 76, 257–278.

Ojo, A.A., Wood, C.M., 2008. In vitro characterization of cadmium and zinc uptake via the gastrointestinal tract of the rainbow trout (*Oncorhynchus mykiss*): interactive effects and the influence of calcium. Aquat. Toxicol. 89, 55–64.

Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M., Wendelaar Bonga, S.E., 1995. Effects of combined waterborne Cd and Cu exposures on ionic composition and plasma cortisol in tilapia, *Oreochromis mossambicus*. Comp. Biochem. Physiol. C 111, 227–235.

Pelgrom, S.M.G.J., Lamers, L.P.M., Garritsen, J.A.M., Pels, B.M., Lock, R.A.C., Balm, P.H.M., Wendelaar Bonga, S.E., 1994. Interactions between copper and cadmium during single or combined metal exposure in juvenile tilapia, *Oreochromis mossambicus*–influence of feeding condition on whole body metal accumulation and the effect of metals on tissue water and ion content. Aquat. Toxicol. 30, 117–135.

Rainbow, P.S., Amiard-Triquet, C., Amiard, J.C., Smith, B.D., Langston, W.J., 2000. Observations on the interaction of zinc and cadmium uptake rates in crustaceans (amphipods and crabs) from coastal sites in U.K. and France differentially enriched with trace metals. Aquat. Toxicol 50, 189–204.

Reeves, P.G., 1998. Copper metabolism in metallothionein-null mice fed a high-zinc diet. J. Nutr. Biochem. 9, 598–601.

Reeves, P.G., Briske-Anderson, M., Johnson, L., 1998. Physiologic concentrations of zinc affected the kinetics of copper uptake and transport in the human intestinal cell model, Caco-2. J. Nutr 128, 1794–1801.

Reeves, P.G., Rossow, K.L., 1996. Zinc-and/or cadmium induced intestinal metallothionein and copper metabolism in adult rats. Nutr. Biochem 7, 128–134.

Sappal, R., Burka, J., Dawson, S., Kamunde, C., 2009. Bioaccumulation and subcellular partitioning of zinc in rainbow trout (*Oncorhynchus mykiss*): cross-talk between waterborne and dietary uptake. Aquat. Toxicol. 91, 281–290.

Stohs, S.J., Bagchi, D., 1994. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. 18, 321–336.

Szebedinszky, C., McGeer, J.C., McDonald, D.G., Wood, C.M., 2001. Effects of chronic Cd exposure via the diet or water on internal organ-specific distribution and subsequent gill Cd uptake kinetics in juvenile rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem 20, 597–607.

Thévenod, F., 2010. Catch me if you can! Novel aspects of cadmium transport in mammalian cells. Biometals. doi:10.1007/s10534-010-9309-1.

Weber, D.N., Eisch, S., Spieler, R.E., Petering, D.H., 1992. Metal redistribution in largemouth bass (*Micropterus salmoides*) in response to restrainment stress and dietary cadmium: role of metallothionein and other metal-binding proteins. Comp. Biochem. Physiol. C 101, 255–262.

Zago, M.P., Oteiza, P.I., 2001. The antioxidant properties of zinc: interactions with iron and antioxidants. Free Radic. Biol. Med. 31, 266–274.