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Acute waterborne cadmium uptake in rainbow trout is reduced by dietary calcium carbonate

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Abstract

The effects of elevated dietary calcium (as $CaCO_3$) and acute waterborne Cd exposure (50 μg/l) on whole body uptake, tissue uptake, and internal distribution of newly accumulated Cd, Ca^{2+}, and Na^+ in juvenile rainbow trout were examined. Fish were fed with three diets (mg Ca^{2+}/g food): 20 (control), 30 and 60 for 7 days before fluxes were measured with radiotracers. The highest dietary Ca^{2+} elevation reduced waterborne whole body Ca^{2+} uptake, but did not protect against inhibition of waterborne Ca^{2+} uptake by waterborne Cd. Both Ca^{2+}-supplemented diets reduced newly accumulated Ca^{2+} in the gills in relation to the control treatment, but did not prevent the Cd-inhibiting effect against accumulation of new Ca^{2+} in most compartments. Fish fed with Ca^{2+}-supplemented diets showed markedly lower rates of whole body uptake and internalization (in some tissues) of waterborne Cd, illustrating that, while dietary Ca^{2+} supplementation did not protect against the impact of waterborne Cd on waterborne Ca^{2+} uptake, it did protect against the uptake of Cd. Waterborne Cd had no effect on Na^+ fluxes, total Cl^-, and in most body compartments, newly accumulated Na^+ and total Na^+ were also not affected. Dietary supplementation with $CaCO_3$ had the same protective effect as demonstrated by dietary supplementation with $CaCl_2$ in an earlier study. Thus, the reduction of waterborne Cd uptake and internalization by dietary Ca^{2+} was specifically due to Ca^{2+} and not to the anion.

Keywords: Cadmium uptake; Calcium uptake; Dietary calcium; Sodium uptake; Fish

1. Introduction

Cadmium is a metal without biological function in vertebrates, and can be found in waters that receive residues from industrial processes associated with the refining of other metals (Pratap et al., 1989). A chronic sublethal exposure to waterborne Cd leads to accumulation of this metal mainly in the kidney, liver, and gills (Giles, 1988; Hollis et al., 1999, 2001; McGeer et al., 2000; Szebedinszsky et al., 2001). Waterborne Cd provokes iono-regulatory disturbances in rainbow trout, but in most studies, the main changes observed are restricted to Ca^{2+} balance, with Na^+ and Cl^- levels not being altered or presenting only minor alterations (Larsson et al., 1981; Pratap et al., 1989; Hollis et al., 1999; McGeer et al., 2000; Baldisserotto et al., 2004). Chloride cells are the primary targets of waterborne Cd, because this metal decreases the activity of gill Ca^{2+}-ATPases, which leads to fish hypocalcemia (Wong and Wong, 2000).

It is well known that water hardness has a direct ameliorative effect against metal toxicity, with Ca^{2+} having a greater protective effect than Mg^{2+} (see review of Wood, 2001). Mortality due to waterborne Cd is reduced with the increase of waterborne Ca^{2+} (Hollis et al., 2000a; Meinelt et al., 2001; Hansen et al., 2002). Waterborne Ca^{2+} competes with Cd for binding sites (Playle et al., 1993), reducing Cd uptake in the gills (Hollis et al., 2000a).

Recently, a different approach to reduce metal toxicity in fish has been investigated: instead of changing water chemistry (for example, increase of waterborne Ca^{2+} to decrease waterborne Cd toxicity), the protective role of the diet against metal uptake (and consequently, toxicity) has been studied.

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This approach is based on the fact that freshwater fish have two main uptake pathways for ions: the gills (waterborne ions) and the gastrointestinal tract (dietary ions), and can control the total uptake by changing the proportion of each kind of uptake according to the situation. For example, goldfish (*Carassius auratus*) fed a Ca^{2+}-deficient diet increase their branchial uptake and tilapia (*Oreochromis mossambicus*) increase intestinal uptake when living in waters low in Ca^{2+} (Flik et al., 1995). Therefore, if the fish are acquiring more ions via the gastrointestinal route, they may reduce ion uptake rates at the gills, thereby simultaneously reducing metal uptake via the same branchial transport pathway. For example, Na^+ and Cu (Grosell and Wood, 2002) share the same transport pathway, and experiments showed that high dietary Na^+ (as NaCl) decreased gill uptake rates of both Na^+ and Cu and reduced internal accumulation of Cu in rainbow trout (Pyle et al., 2003; Kamunde et al., 2003). As Ca^{2+} and Cd compete for the same transport pathway (Verbost et al., 1989; Playle et al., 1993), this explains why elevation of dietary Ca^{2+} (as $CaCl_2$) protected against Cd accumulation in several fish tissues (Zohouri et al., 2001) . In addition, dietary Ca^{2+} supplementation reduced waterborne Ca^{2+} and Cd uptake in rainbow trout (Baldisserotto et al., 2004). However, when fed in the diet of some mammals, $CaCl_2$ may induce severe metabolic acidosis (reviewed by Zohouri et al., 2001), and rainbow trout fed a high concentration of this salt presented rapid mortality (Zohouri et al., 2001; Baldisserotto et al., 2004). This is likely because when fish ingest $CaCl_2$, these ions become independent in solution in the extracellular fluid and Ca^{2+} is incorporated into bone, together with carbonate, bicarbonate or phosphate, leaving behind H^+ and Cl^-, i.e., effectively hydrochloric acid, thereby producing metabolic acidosis.

Since the previous studies did not control for possible effects of the anion (Cl^-) in $CaCl_2$ supplementation of the diet, the objective of the present study was to see if the same protective effects against waterborne Cd exposure would occur with an entirely different calcium salt, $CaCO_3$. In contrast to $CaCl_2$, Ca^{2+} incorporation into bone from $CaCO_3$ should be matched by carbonate or bicarbonate incorporation as an acid–base neutral process, or if Ca^{2+} is incorporated with another anion, as an alkalinizing process. Specifically, our objective was to examine if experimentally elevating dietary Ca^{2+} by supplementation with $CaCO_3$ instead of $CaCl_2$ would affect whole body Ca^{2+} and Cd influx rates, and internal distributions of newly accumulated Ca^{2+} and Cd in juvenile rainbow trout in a similar pattern to that seen in an previous study with $CaCl_2$ supplementation of the diet (Baldisserotto et al., 2004). Fish were exposed to the experimental diets for 7 days to compare the results with a previous experiment with dietary $CaCl_2$ for the same period (Baldisserotto et al., 2004). Whole body Na^+ uptake rates and internal distributions of newly accumulated Na^+ were also measured to investigate possible nonspecific effects. In addition, we examined the effect of dietary Ca^{2+} on growth, mortality, and the accumulation of Na^+ , Cl^-, and Ca^{2+} in several internal body compartments.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (10–25 g) were purchased from Humber Springs Fish Hatchery (Orangeville, ON). Fish were maintained for at least 1 week in an aerated 200-l polypropylene tank supplied with approximately 0.9–1.5 l/min dechlorinated Hamilton tap water (mM): [Na]—0.60 ± 0.05, [Ca]–1.0 ± 0.1, [Cl]—0.7 ± 0.02, hardness = 140 mg/l as $CaCO_3$, alkalinity = 95 mg/l as $CaCO_3$, pH 7.8–8.0, Cd = 0.67 μg/l, temperature = 12–14 °C. Fish were fed twice a day with commercial trout food (Corey Hatchery Feed, Corey Feed Mills, Fredericton, NB; ionic composition given below) at a ration of 3% body mass/day. Photoperiod was maintained at 12-h light and 12-h dark.

After the acclimation period fish were randomly separated to three 200-l tanks in the same conditions as described above and fish of each tank (24 fish each) received a different diet (see *Diet Preparation* below). As the treatments were made in duplicate, this procedure was repeated. Uneaten food and feces were siphoned daily. Dead fish were removed daily and mortality was recorded. This cleaning regimen, in addition to the flow-through experimental design, ensured that excess Ca^{2+} from Ca^{2+}-supplemented diets did not accumulate in the water.

2.2. Diet preparation

All diets were prepared with Corey Hatchery Feed (manufacturer's specifications: [P] = 11 mg/g; crude protein = 55%; crude fat = 17%; crude fibre = 2%). This fish food was ground in a blender, followed by hydration with approximately 40% v/w deionized water. To prepare the treatment diets, the control diet (20 mg Ca^{2+}/g food) was supplemented with $CaCO_3$ to yield experimental diets with 30 and 60 mg Ca^{2+}/g food. $CaCO_3$ was dissolved in the deionized water and added to the food paste. The resulting paste was mixed and extruded through a pasta maker, air-dried, and broken into small pellets by hand. The control diet was prepared by the same method but with the addition of deionized water only. Actual measured Ca^{2+} concentrations ($\pm$ S.E.M., $N = 3$) in the three diets were 19.87 ± 0.40 (control), 34.00 ± 0.67, and 64.71 ± 1.64 mg Ca^{2+}/g food and Na^+ concentrations were 7.31 ± 0.09, 7.69 ± 0.23, and 6.98 ± 0.21 mg Na^+/g food, respectively. Cd content in control food was 0.26 μg/g food (single measurement).

2.3. Feeding and experimental regime

Fish were fed twice a day (8:00 am and 5:00 pm) with the control or treatment diets at a ration of 3% body mass/

day, with half of the ration being delivered at each feeding. Exposure lasted 14 days for growth experiments, but radioisotopic fluxes were measured at the end of 7 days. At the 7th day, the remaining fish were fed only after withdrawal of the specimens that were used in the flux experiments, so the latter were fasted overnight prior to the flux measurements.

2.4. Ion fluxes

After 7 days of exposure to experimental diets, 10 fish from each group were collected, weighed, and transferred to individual 450 ml flux chambers containing dechlorinated Hamilton tap water for each measurement of metal uptake rate (Cd, Ca, Na) for the whole body and of the incorporation of this newly accumulated metal uptake into individual tissue compartments (plasma, red blood cells, gills, kidney, liver, and the remaining carcass). For each diet, five flux (accompanied by five internal distribution) measurements were made: Ca^{2+} influx rate, Ca^{2+} influx rate in the presence of acute exposure to 50 μg/l Cd (as $CdCl_2$), Na^+ influx rate, Na^+ influx rate in the presence of acute exposure to 50 μg/l Cd, and Cd influx rate during acute exposure to 50 μg/l Cd. After a 1-h settling period, the appropriate radio-isotope (0.05 μCi/l ^{22}Na or 10 μCi/l $^{45}Ca^{2+}$ or 2 μCi/l ^{109}Cd, from New England Nuclear, Boston, MA) and $CdCl_2$ (to Cd-exposed fish) was added to each chamber and allowed 10 min of mixing. Water samples (10 ml) were taken then and 3 h later, and acidified with 100 μl 1 N HNO_3. This 3-h period for the experiments was chosen because it was previously demonstrated that Ca^{2+} and Cd uptakes and tissue-specific incorporation could be determined accurately within this time (Hollis et al., 1999, 2000b; Baldisserotto et al., 2004). Controls for each diet treatment were therefore submitted to the same treatment but without the addition of Cd to the water. After 3 h, fish were removed from the chambers, anesthetized with MS-222, rinsed with clean water, and blood was collected from the caudal vein with heparinized 1-ml syringes. Blood samples were centrifuged at $10{,}000 \times g$ for 5 min to separate plasma, leaving a red blood cell pellet. Fish were then sacrificed with a blow to the head, and gills, kidney, liver, and the remaining carcass were collected and weighed separately. Tissues were then partitioned for either radioactivity analysis (for newly accumulated metal concentrations) or frozen at −20 °C for later ionic analysis (for total Na^+, total Ca^{2+}, and total Cl^- concentrations).

Radioactivity in tissue and water samples containing ^{22}Na or ^{109}Cd was measured on a Canberra-Packard Minaxi Auto-Gamma 5000 series gamma counter (Canberra-Packard Instruments, Meriden, CT). There was no quenching for ^{22}Na or ^{109}Cd. Tissue (gill, carcass, plasma, liver, red blood cells, and kidney) and water samples were processed as described by Hogstrand et al. (1994) for counting ^{45}Ca. Samples were counted on a liquid scintillation counter (LKB Wallac 1217 Rackbeta, Pharmacia-LKB, Helsinki). Counting efficiencies for ^{45}Ca were determined by internal standardization, i.e., by addition/recovery of known amounts of ^{45}Ca.

Newly accumulated Na^+, Ca^{2+}, or Cd were calculated by the following equation (Grosell et al., 1997):

$$M_{\mathrm{New}} = \frac{a}{\left(\frac{b}{c}\right)},$$

where M_{New} is the newly accumulated Na^+, Ca^{2+}, or Cd concentration (nmol/g tissue), a is the number of counts per minute (cpm) per gram of tissue or ml of plasma as appropriate, b is the number of cpm per liter of water, and c is the total Na^+, Ca^{2+}, or Cd concentration per liter of water. For b/c (specific activity), we used the average of values at the start and end measurements. Unidirectional Na^+, Ca^{2+}, or Cd whole body uptake rates were determined by summing the newly accumulated uptake into all the individual tissues and dividing the result by fish weight and the length of the exposure period (3 h) to convert to a rate (μM/kg h or nM/kg h). For Na^+, it was also possible to detect small changes in the total Na^+ concentration of the water over the 3-h period, from which whole body net flux rates could be calculated. Sodium efflux rate was calculated from the difference between net Na^+ flux rate and influx rate (see Wood, 1992).

2.5. Water and tissue ion content analysis

Gills, kidney, carcass, liver, and red blood cells were digested in 3–5 volumes of 1 N HNO_3 for 24–48 h at 60 °C. These tissues and water samples were analyzed using flame (AAS; Na^+ and Ca^{2+}) or graphite furnace (GFAAS; Cd) atomic absorption spectrophotometry (Varian AA-1275 fitted with a GTA-95 graphite tube atomizer, Mississauga, ON). The method of Zall et al. (1956) was used for determining Cl^- concentration of these samples. Certified standards (Fisher Scientific and Radiometer, Copenhagen) were used throughout.

2.6. Statistical analysis

Data have been reported as means ± 1 S.E.M. (N). Homogeneity of variances among groups was tested with the Bartlett test. Data for some parameters were submitted to a log transformation to obtain homogeneity among groups. Comparisons among different diets were made by one-way analysis of variance and Tukey test or Kruskall Wallis–Anova (non-parametric) and Dunn's multiple comparison test. Comparisons between Cd-exposed and non-exposed fish were made by Student's t-test or Kolmogorov–Smirnov test. Analysis was performed using the software Statistica (version 5.1), and the minimum significance level was set at $P < 0.05$.

3. Results

3.1. Survival and growth

Mortality (2.54%) and biomass gain (2.34%/day) of both Ca^{2+}-supplemented diets did not show any significant difference from the control group over 14 days (4.6% and 2.83%/day, respectively).

3.2. Ca^{2+} uptake

Whole body uptake of waterborne Ca^{2+} was unaffected relative to the control group in fish that received a diet with 30 mg/g Ca^{2+}, but decreased (65%) in fish fed with 60 mg/g Ca^{2+}. Exposure to 50 μg/l Cd for 3 h greatly reduced Ca^{2+} uptake in all treatments in comparison to the control, and also in comparison to unexposed fish submitted to the same diet

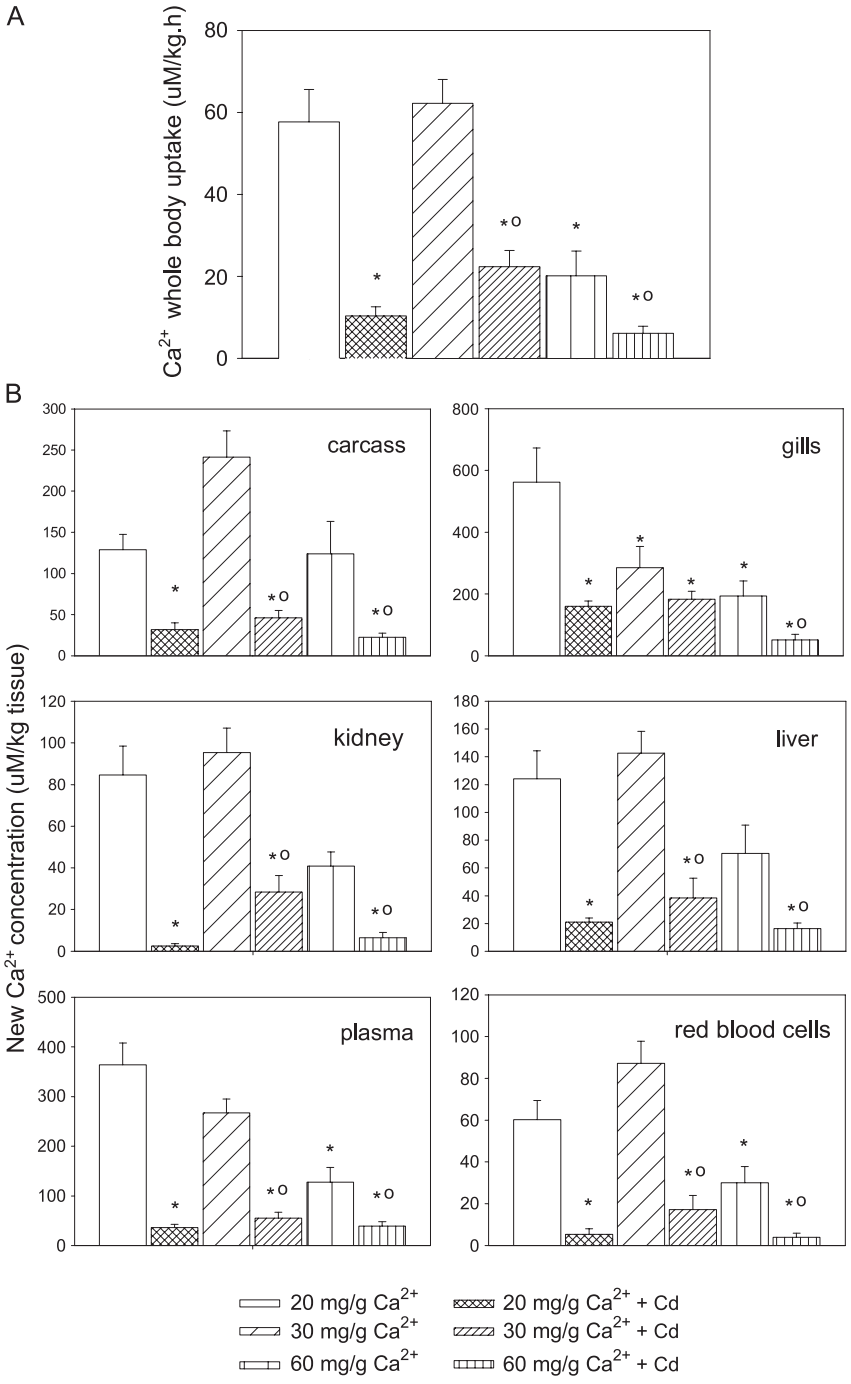

Fig. 1. Ca^{2+} whole body influx rates (A) and newly accumulated Ca^{2+} concentrations (3 h) (B) of rainbow trout exposed to diets with different Ca^{2+} concentrations for 7 days. +Cd—fish exposed to 50 μg/l Cd for 3 h. Means ± 1 S.E.M. ($N = 8–9$). *Significantly different from 20 mg/g Ca^{2+} ($P < 0.05$). ○Significantly different from group exposed to the same diet and not exposed to 50 μg/l Cd for 3 h ($P < 0.05$).

(Fig. 1A). Within each dietary treatment, the inhibition of Ca^{2+} uptake by Cd was 64–82%. Consequently, only the highest dietary Ca^{2+} elevation reduced waterborne Ca^{2+} uptake, but even this diet was unable to protect against inhibition of waterborne Ca^{2+} uptake by waterborne Cd.

The greatest accumulation of newly Ca^{2+} at the end of the 3-h measurement period occurred in the gills, followed by plasma, carcass, liver, kidney, and red blood cells. Both Ca^{2+}-supplemented diets reduced newly accumulated Ca^{2+} concentrations in the gills in relation to the control treatment. In addition, newly accumulated Ca^{2+} in the plasma and red blood cells was significantly reduced only in the fish fed with 60 mg Ca^{2+}/g food. Newly accumulated Ca^{2+} in the carcass, kidney, and liver were not significantly affected by diet, but their trends were similar to the other organs (Fig. 1B). Just as with whole body Ca^{2+} uptake rate (Fig. 1A), exposure to 50

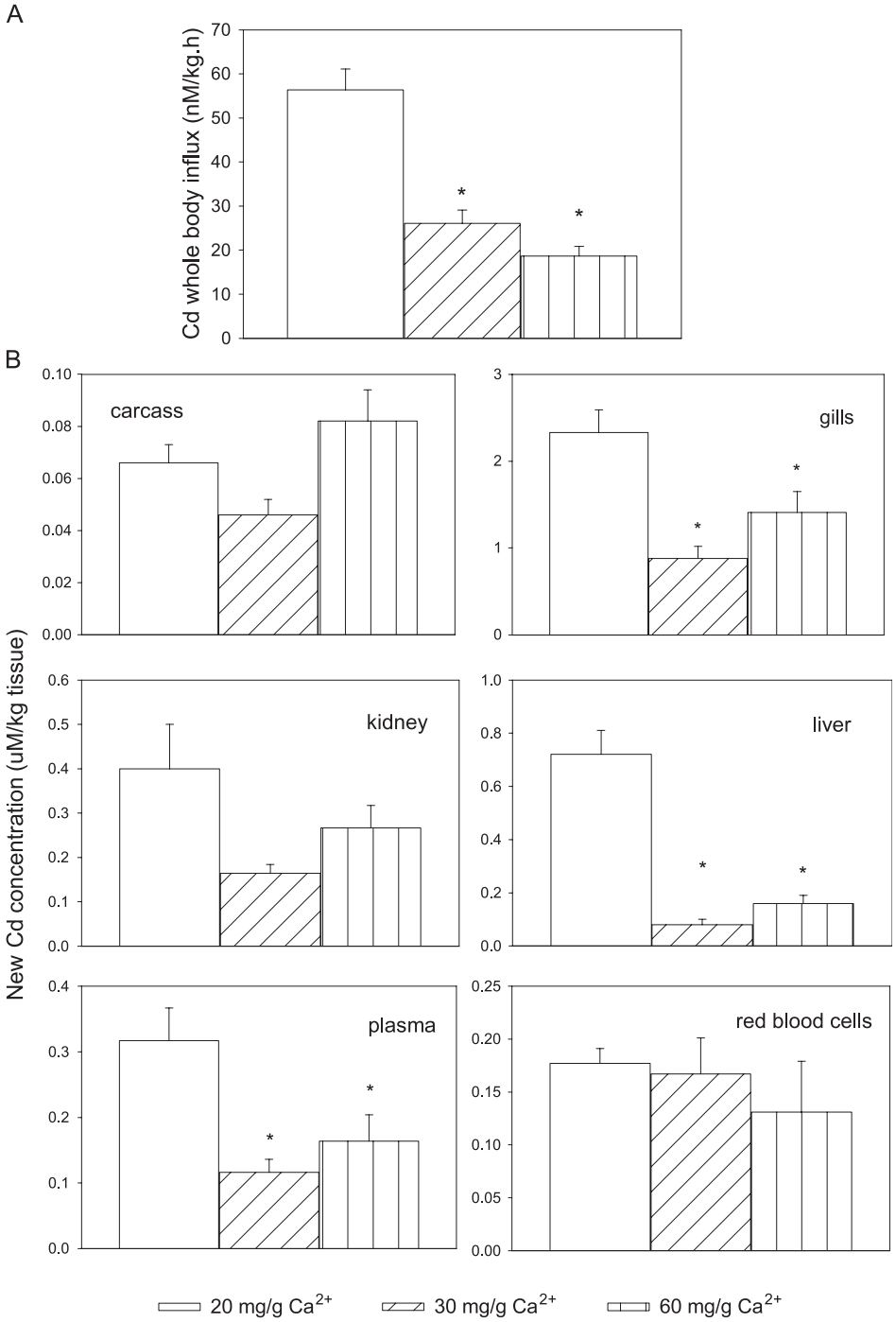

Fig. 2. Cd whole body influx rates (A) and newly accumulated Cd concentrations (3 h) (B) of rainbow trout exposed to diets with different Ca^{2+} concentrations for 7 days and to 50 μg/l waterborne Cd for 3 h. Means ± 1 S.E.M. ($N = 7–10$). *Significantly different from 20 mg/g Ca^{2+} ($P < 0.05$).

μg/l Cd for 3 h reduced newly accumulated Ca^{2+} in all studied compartments compared to unexposed fish (Fig. 1B). The Cd-inhibiting effect against newly accumulated Ca^{2+} was similar in all studied compartments irrespective of the diet, except for the gills (Cd inhibition was 36% for fish fed with 30 mg Ca^{2+}/g food, while the reduction of newly accumulated Ca^{2+} in fish fed with the other diets was around 70%). The greatest relative reduction occurred in the kidney of control fish, where inhibition was about 97% relative to unexposed fish.

3.3. Cd uptake

Fish fed with Ca^{2+}-supplemented diets showed markedly lower rates of whole body uptake of waterborne Cd relative to the control group (Fig. 2A). On a relative basis, the inhibition (67%) by the diet with 60 mg Ca^{2+}/g food was quantitatively comparable to the inhibition of whole body Ca^{2+} uptake rate (65%; Fig. 1A). The diet with 30 mg Ca^{2+}/g food did not change whole body Ca^{2+} uptake (Fig. 1A), but reduced whole body Cd uptake by 54%. The absolute magnitudes of Cd uptake rates were approximately 3 orders of magnitude lower than Ca^{2+} uptake rates, but as the Ca^{2+}/Cd molar ratios is around 2200, the relative uptake rates are similar.

As for whole body uptake rates, newly accumulated Cd concentrations at the end of 3 h (Fig. 2B) were lower (by 2 orders of magnitude) than newly accumulated Ca^{2+} concentrations (Fig. 1B) over the same time period. However, the tissue pattern accumulation was different than for Ca^{2+}. Newly accumulated Cd concentrations were greatest in the gills, followed by kidney and liver (about equal), then plasma, red blood cells, and finally carcass, where values were extremely low (Fig. 2B).

Both Ca^{2+}-supplemented diets strongly reduced the concentrations of newly accumulated Cd in the gills, liver, and plasma in comparison to the control diet. Newly accumulated Cd in the kidney, carcass, and red blood cells was not affected by diet (Fig. 2B). On a relative basis, inhibition was greatest (by 78–89%) in the liver.

When organ-specific newly accumulated Ca^{2+} was expressed relative to total body load, in all groups carcass represented the greatest percentage, followed by the gills, and with very small percentages for liver and kidney. Rainbow trout fed Ca^{2+}-supplemented diets showed a relative increase of Ca^{2+} internalization in the carcass (Fig. 3A). However, for newly accumulated Cd, the greatest percentage was found in the gills, followed by carcass, liver, and kidney in fish fed with the control diet. The increase of Ca^{2+} in the diet decreased the percentage of newly accumulated Cd in the gills, liver, and kidney and increased it in the carcass (Fig. 3B).

3.4. Na^+ uptake

The Ca^{2+}-supplemented diets did not change whole body Na^+ influx, efflux, and net flux in relation to control diet

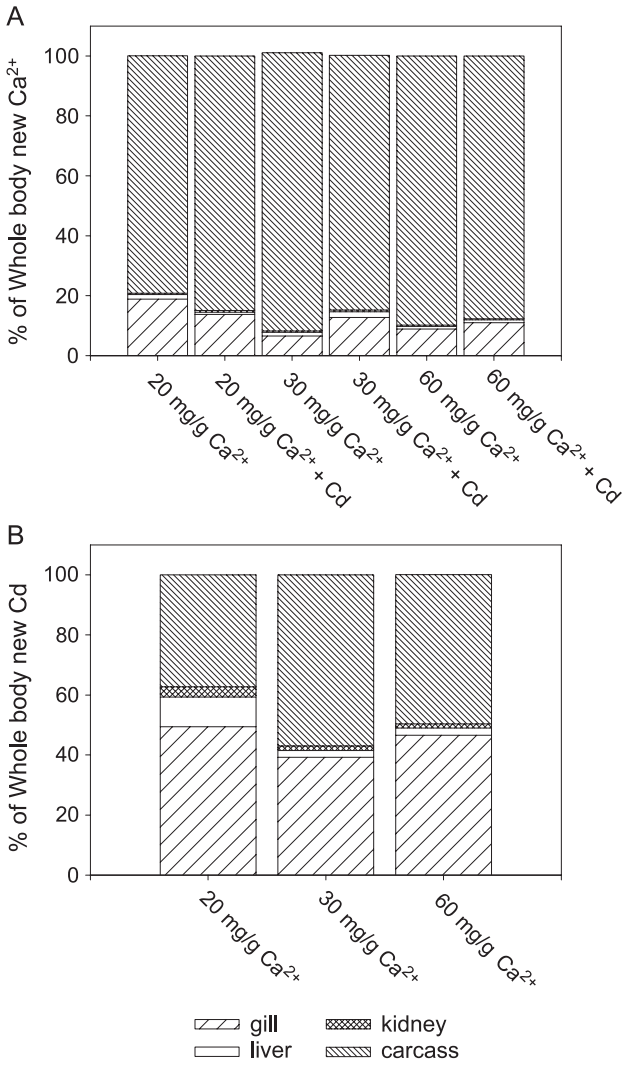

Fig. 3. Partitioning of whole-body newly accumulated Ca^{2+} (A) and Cd (B) of rainbow trout exposed to diets with different Ca^{2+} concentrations for 7 days, expressed as the relative contribution (i.e., mass-weighted contribution) of each organ (+Cd indicates fish exposed to 50 μg/l Cd for 3 h).

(Fig. 4). Both diets also did not influence newly accumulated Na^+ over the 3-h period in the studied compartments (Table 1). In contrast to newly accumulated Ca^{2+}, newly accumulated Na^+ concentrations were very similar among the various tissues, except for the plasma values which were three- to five-fold higher than for other compartments (Table 1). Irrespective of the diet, exposure to 50 μg/l of waterborne Cd did not change newly accumulated Na^+ in the gills, liver, carcass, and kidney, but in fish fed with Ca^{2+}-supplemented diets, newly accumulated Na^+ in the red blood cells was reduced and increased in plasma (only 60 mg/g Ca^{2+} food) by waterborne Cd (Table 1).

3.5. Ion levels in the tissues and plasma

Total Ca^{2+}, Na^+, and Cl^- levels in all studied internal compartments were not altered relative to the control group

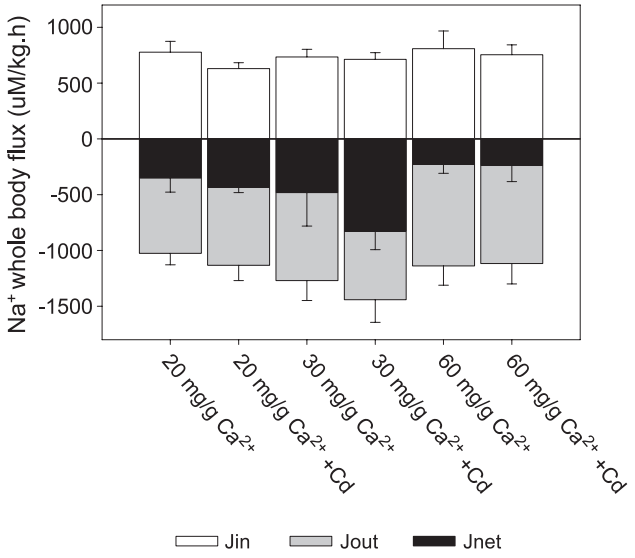

Fig. 4. Whole body Na^+ flux rates of rainbow trout exposed to diets with different Ca^{2+} concentrations for 7 days. +Cd indicates fish exposed to 50 μg/l Cd for 3 h. Upward white bars indicate influx (uptake), downward grey bars indicate efflux, and the black bars indicate net flux rates. Means ± 1 S.E.M. (N=7–10).

by Ca^{2+}-supplemented diets (Tables 1 and 2). Exposure to waterborne Cd for 3 h decreased plasma Ca^{2+} in fish fed with control diet, and total Na^+ in the gills, red blood cells, and plasma in fish fed with 60 mg/g Ca^{2+} food (Tables 1 and 2). Total Cl^- in all studied compartments was unaffected by exposure to waterborne Cd (Table 1).

4. Discussion

A combination of low waterborne (0.125 mM) and dietary Ca^{2+} reduced growth rate in brook trout (*Salvelinus fontinalis*) (Rodgers, 1984), demonstrating that a minimum Ca^{2+} uptake by gills and/or intestine is needed for normal fish growth. The commercial food used in this experiment contained enough Ca^{2+} for fish growth because the minimum dietary Ca^{2+} is in the 3–7 mg Ca^{2+}/g food range (O'Connell and Gatlin, 1994). In addition, waterborne Ca^{2+} in this experiment was around 1.0 mM, which is above the minimum level for rainbow trout without dietary Ca^{2+} (0.575 mM) (Ogino and Takeda, 1978). Consequently, it is not surprising that the Ca^{2+}-supplemented diets used in this experiment did not alter rainbow trout growth. Zohouri et al. (2001) reported that a diet supplemented with $CaCl_2$ to yield 30 mg Ca^{2+}/g food also did not change rainbow trout growth, but a higher concentration of $CaCl_2$ (60 mg Ca^{2+}/g food) led to 21.6% mortality and decreased weight gain. The deaths observed in the treatment with a high amount of $CaCl_2$ probably were due to metabolic acidosis (as verified in some mammals) and/or to a sharp increase on Ca^{2+} plasma levels seen after the first feeding with this diet (Baldisserotto et al., 2004). In our present study, we did not measure ion plasma levels shortly after feeding, but apparently Ca^{2+}-supplemented diets with $CaCO_3$ did not induce these dramatic ionic changes, because no mortality, decrease on fish growth, or symptoms described by Baldisserotto et al. (2004) were observed.

Table 1
Newly accumulated Na^+ (μM/g tissue) (A), total Na^+ (μM/g tissue) (B) and total Cl^- (μM/g tissue) (C) in several compartments of rainbow trout fed a range of dietary Ca^{2+} and exposed (or not) to waterborne Cd for 3 h

	Gill	Carcass	Liver	Kidney	RBC	Plasma
(A) Diet						
20 mg/g	3.57 ± 0.58	2.24 ± 0.28	1.74 ± 0.21	3.10 ± 0.44	3.22 ± 0.49	9.17 ± 1.75
20 mg/g +Cd	2.75 ± 0.27	2.11 ± 0.32	1.73 ± 0.20	2.30 ± 0.25	2.56 ± 0.24	6.39 ± 0.97
30 mg/g	3.12 ± 0.36	2.09 ± 0.19	2.21 ± 0.16	3.60 ± 0.32	3.36 ± 0.49	8.54 ± 0.62
30 mg/g +Cd	2.81 ± 0.25	2.04 ± 0.17	2.05 ± 0.12	3.40 ± 0.32	1.90 ± 0.31*	7.26 ± 0.36
60 mg/g	2.87 ± 0.55	2.36 ± 0.46	1.91 ± 0.38	3.79 ± 0.91	2.05 ± 0.32	5.51 ± 0.29
60 mg/g +Cd	3.02 ± 0.43	2.19 ± 0.26	1.54 ± 0.17	2.69 ± 0.36	1.30 ± 0.15*	7.54 ± 0.48*
(B) Diet						
20 mg/g	54.9 ± 2.0	39.1 ± 1.5	28.2 ± 1.3	61.0 ± 1.8	57.4 ± 4.1	136.2 ± 5.1
20 mg/g +Cd	55.4 ± 2.3	38.8 ± 0.9	31.6 ± 1.2	58.2 ± 1.5	55.2 ± 5.1	129.5 ± 3.1
30 mg/g	52.6 ± 2.7	36.7 ± 2.4	35.6 ± 1.5	51.6 ± 2.6	65.1 ± 4.4	139.2 ± 8.0
30 mg/g +Cd	51.7 ± 2.7	39.8 ± 1.8	37.3 ± 1.8	56.1 ± 3.0	55.0 ± 3.3	123.2 ± 8.2
60 mg/g	51.3 ± 1.3	40.9 ± 0.6	34.5 ± 1.1	57.9 ± 1.6	53.3 ± 2.6	138.4 ± 1.6
60 mg/g +Cd	45.9 ± 3.4*	41.0 ± 1.0	34.1 ± 1.6	60.2 ± 0.7	41.3 ± 3.1*	127.5 ± 4.4*
(C) Diet						
20 mg/g	50.2 ± 5.5	35.1 ± 3.9	47.8 ± 4.2	60.5 ± 5.3	82.3 ± 7.3	111.3 ± 5.4
20 mg/g +Cd	52.7 ± 7.1	39.4 ± 5.9	51.2 ± 4.9	58.7 ± 7.4	93.6 ± 7.2	107.3 ± 7.9
30 mg/g	56.4 ± 4.9	44.8 ± 6.8	49.0 ± 6.0	56.2 ± 9.1	96.9 ± 8.5	130.4 ± 7.5
30 mg/g +Cd	48.6 ± 5.8	46.3 ± 6.6	51.7 ± 3.6	54.8 ± 7.3	88.4 ± 9.3	126.8 ± 8.7
60 mg/g	44.0 ± 3.3	29.7 ± 4.3	51.6 ± 4.2	56.0 ± 5.5	90.1 ± 5.0	108.3 ± 7.2
60 mg/g +Cd	51.4 ± 2.5	29.6 ± 3.1	54.1 ± 5.3	59.0 ± 5.7	71.7 ± 6.4	109.7 ± 4.2

RBC—red blood cells. Means ± 1 S.E.M. N=8–10 fish.
* Significantly different from group exposed to the same diet and not exposed to 50 μg/L Cd for 3 h (P<0.05) by Student t-test.

Therefore, supplementation with $CaCO_3$ seems to be safer than with $CaCl_2$. There is no measurement (to our knowledge) of dietary Ca^{2+} levels of fish in the wild, but $CaCO_3$ is a common natural form of calcium (e.g. in crustacean exoskeleton, mollusc shells), and Sherwood et al. (2000) reported that yellow perch (*Perca flavescens*) in metal-contaminated (Cd, Zn, Cu) lakes tended to eat more invertebrates and less fish than perch from reference lakes, a strategy which would likely increase the Ca^{2+} content of their diet.

In the present study, both Ca^{2+}-supplemented diets reduced newly accumulated Ca^{2+} in the gills (by more than 50%) (Fig. 1B). However, fish fed with 30 mg Ca^{2+}/g food did not show any significant decrease of Ca^{2+} internalization in the other tissues (Fig. 1B), which suggests that apical entry is reduced, but basolateral transfer from gills to blood is not significantly affected. At 60 mg Ca^{2+}/g food, both transfers seem to be affected, as internalization of newly accumulated Ca^{2+} was significantly reduced in several internal tissues. We speculate that dietary Ca^{2+} supplementation would induce a rise on intracellular Ca^{2+} in the gill ionocytes, which would cause a down-regulation of branchial apical Ca^{2+} channels and lower Ca^{2+} internalization in the gills. However, activity of the basolateral Ca^{2+} pump would not be significantly reduced, at least at the lower dietary Ca^{2+} dose, allowing transfer of Ca^{2+} from gills to other tissues. In addition, as there was a significant decrease of Ca^{2+} internalization in all compartments of fish exposed to waterborne Cd (compared to fish fed with the same diet but not exposed to waterborne Cd) (Fig 1A), it is probable that the branchial basolateral Ca^{2+} pump was significantly inhibited by Cd and not by dietary Ca^{2+} supplementation.

The lower Ca^{2+} internalization in the gills in fish fed Ca^{2+}-supplemented diets results in lower internalization of Cd in the gills, since both metals share the same depressed pathway, and consequently lower liver, plasma (Fig. 2B), and whole body Cd uptake (Fig. 2A), but only the highest elevation of dietary Ca^{2+} led to a decrease of whole body Ca^{2+} influx (Fig. 1A). These results are in agreement with the previous findings of Zohouri et al. (2001), who also demonstrated that Cd accumulation in the gill, liver, and kidney of rainbow trout exposed to sublethal waterborne Cd was lower if they were fed with twice the amount of dietary Ca^{2+} (as $CaCl_2$) of control group. Our findings (with $CaCO_3$) and those (with $CaCl_2$) of Baldisserotto et al. (2004) demonstrate that diets with triple Ca^{2+} content relative to usual commercial diets (60 mg Ca^{2+}/g food with $CaCl_2$ or $CaCO_3$ addition) lead to similar results regarding whole body waterborne Ca^{2+} and Cd uptake and internalization. However, a 50% increase of dietary Ca^{2+} (30 mg Ca^{2+}/g food) with addition of $CaCl_2$ reduced whole body waterborne Ca^{2+} uptake and internalization (Baldisserotto et al., 2004), but the same increase in dietary Ca^{2+} with $CaCO_3$ did not change these parameters (except branchial Ca^{2+} internalization). As metabolic acidosis associated with low water pH reduces Ca^{2+} influx (Wood, 2001), probably the acidosis caused by $CaCl_2$ could have been a contributory factor to in the reduced whole body Ca^{2+} uptake observed by Baldisserotto et al. (2004). Therefore, a 50% increase of dietary Ca^{2+} with $CaCO_3$ is enough to reduce waterborne Cd uptake while at the same time maintaining waterborne whole body Ca^{2+} uptake. The increase of dietary Ca^{2+} would increase Ca^{2+} uptake via the gastrointestinal route, and consequently would reduce branchial Ca^{2+} uptake.

The internal distribution of newly accumulated Ca^{2+} and Cd after 3 h (Fig. 3) showed that Cd movement from the gills to the carcass is somewhat slower than Ca^{2+}, as was also observed by Baldisserotto et al. (2004). However, exposure to waterborne Cd for a longer period (30 days) allows enough time for Cd to accumulate in a higher percentage in the carcass (Szebedinszsky et al., 2001), indicating that the pattern observed of Cd internalization after 3 h of exposure is not yet equilibrated. An increase of dietary Ca^{2+} reduced the relative internalization of both Ca^{2+} and Cd in the gills and increased their relative internalization in the carcass (Fig. 3). The same was observed to rainbow trout fed with $CaCl_2$-supplemented diets (Baldisserotto et al., 2004). Therefore, in fish fed Ca^{2+}-supplemented diets, there would be a reduced Cd uptake by branchial apical channels, but Cd movement from the gills to the plasma across by the basolateral membrane would continue, leading to an increase of relative Cd internalization in the carcass in spite of lower Cd uptake by the gills.

Waterborne Cd at 50 μg/l inhibited whole body Ca^{2+} uptake as well as internalization of newly accumulated

Table 2
Effect of dietary Ca^{2+} and acute waterborne Cd exposure (+Cd) on total Ca^{2+} in several compartments of rainbow trout fed a range of dietary Ca^{2+} and exposed (or not) to waterborne Cd for 3 h

Diet	Gill	Liver	Kidney	RBC	Plasma
20 mg/g	121.7 ± 4.2	0.44 ± 0.05	1.74 ± 0.23	0.53 ± 0.14	2.06 ± 0.09
20 mg/g +Cd	127.9 ± 4.0	0.63 ± 0.08	1.77 ± 0.22	0.47 ± 0.08	1.77 ± 0.08*
30 mg/g	119.9 ± 8.1	0.49 ± 0.09	1.87 ± 0.23	0.40 ± 0.05	1.68 ± 0.16
30 mg/g +Cd	109.8 ± 3.1	0.63 ± 0.16	1.51 ± 0.16	0.35 ± 0.04	1.45 ± 0.14
60 mg/g	114.9 ± 3.7	0.41 ± 0.07	1.24 ± 0.22	0.27 ± 0.03	1.67 ± 0.22
60 mg/g +Cd	117.3 ± 6.9	0.52 ± 0.06	1.76 ± 0.27	0.24 ± 0.03	1.83 ± 0.15

Values in μM/g tissue or μM/ml (plasma). RBC—red blood cells. Means ± 1 S.E.M. $N = 8–10$ fish.
* Significantly different from group exposed to the same diet and not exposed to 50 μg/l Cd for 3 h ($P < 0.05$) by Student *t*-test.

Ca^{2+}, and Ca^{2+}-supplemented diets did not protect against this effect. This lack of effect of elevation of dietary Ca^{2+} against inhibition of waterborne Ca^{2+} uptake by waterborne Cd was also found by Zohouri et al. (2001) (chronic effect) and Baldisserotto et al. (2004) (acute effect). Therefore, elevation of dietary Ca^{2+} with either $CaCl_2$ or $CaCO_3$ reduced waterborne Ca^{2+} uptake but did not change the effect of waterborne Cd on Ca^{2+} uptake, demonstrating that both the effect and lack of effect, respectively, are related to Ca^{2+} itself and not to the anions used. Waterborne Cd also led to hypocalcemia on fish fed with control diet, but those which received high dietary Ca^{2+} were not significantly affected by Cd (Table 2). Therefore, dietary Ca^{2+} supplementation with $CaCO_3$ avoided hypocalcemia due to acute exposure to waterborne Cd. Dietary Ca^{2+} supplementation with $CaCl_2$ only protected against plasma hypocalcemia when the amount of Ca^{2+} was tripled relative to the control load, while lower concentrations were ineffective (Zohouri et al., 2001; Baldisserotto et al., 2004).

Recent studies showed that dietary ion supplementation reduced both its own branchial uptake, and consequently also the uptake of its toxic mimic, as seen with Na^+ versus Cu (Kamunde et al., 2003; Pyle et al., 2003), and Ca^{2+} versus Cd (Zohouri et al., 2001; Baldisserotto et al., 2004; present study). Therefore, the bioaccumulation of a given metal and potentially its toxicity, both can be altered by the water quality and the chemistry of the diet.

Waterborne Cd had no effect on Na^+ fluxes, total Cl^-, and in most body compartments, newly accumulated Na^+ and total Na^+ were also not affected. These results are in agreement with most other studies where Na^+ fluxes, and plasma Na^+ and Cl^- measurements have been made, and no change was detected after acute (3 h) (Baldisserotto et al., 2004) or chronic exposure to waterborne Cd (Larsson et al., 1981; Pratap et al., 1989; Wood, 2001). However, on rainbow trout fed with Ca^{2+}-supplemented diets, Na^+ internalization in the red blood cells was reduced by waterborne Cd (Table 1). Waterborne Cd also decreased Na^+ internalization in the plasma and total Na^+ in the red blood cells and plasma of those fed with the highest amount of $CaCO_3$ (Table 1). Since this effect of waterborne Cd was not observed when rainbow trout were fed with $CaCl_2$ supplementation (Baldisserotto et al., 2004), it is possible that this decrease of newly accumulated Na^+ in the red blood cells was due to a synergistic effect of waterborne Cd and dietary CO_3^{2-}. The membrane of the red blood cells has a Na^+/H^+ transporter (Randall and Brauner, 1998), which could have been inhibited by waterborne Cd and dietary CO_3^{2-}.

Our present results lead to the conclusion that dietary Ca^{2+} supplementation with $CaCO_3$ has the same protective effect as supplementation with $CaCl_2$ to reduce the uptake and internalization of waterborne Cd in rainbow trout, demonstrating that this effect is due to Ca^{2+} and not to the anion. Moreover, due to the lack of mortality, $CaCO_3$ seems to be safer for elevating Ca^{2+} in commercial diets than $CaCl_2$.

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References

Baldisserotto, B., Kamunde, C., Matsuo, A., Wood, C.M., 2004. A protective effect of dietary calcium against acute waterborne cadmium uptake in rainbow trout. Aquat. Toxicol. 67, 57–73.

Flik, G., Verbost, P.M., Wendelaar Bonga, S.E., 1995. Calcium transport processes in fishes. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Fish Physiology. Cellular and Molecular Approaches to Fish Ionic Regulation, vol. 14. Academic Press, San Diego, pp. 317–341.

Giles, M.A., 1988. Accumulation of cadmium by rainbow trout, *Salmo gairdneri*, during extended exposure. Can. J. Fish. Aquat. Sci. 45, 1045–1053.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Grosell, M.H., Hogstrand, C., Wood, C.M., 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 38, 257–276.

Hansen, J.A., Welsh, P.G., Lipton, J., Cacela, D., Dailey, A.D., 2002. Relative sensitivity of bull trout (*Salvelinus confluentus*) and rainbow trout (*Oncorhynchus mykiss*) to acute exposures of cadium and zinc. Environ. Toxicol. Chem. 21, 67–75.

Hogstrand, C., Wilson, R.W., Polgar, D., Wood, C.M., 1994. Effects of zinc on the kinetics of branchial uptake in freshwater rainbow trout during adaptation to waterborne zinc. J. Exp. Biol. 186, 55–73.

Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 1999. Cadmium accumulation, gill Cd binding, acclimation, and physiological effects during long term sublethal Cd exposure in rainbow trout. Aquat. Toxicol. 46, 101–109.

Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 2000a. Protective effects of calcium against chronic waterborne cadmium exposure to juvenile rainbow trout. Environ. Toxicol. Chem. 19, 2725–2734.

Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 2000b. Effects of sublethal Cd exposure in rainbow trout during soft water exposure: implications for biotic ligand modelling. Aquat. Toxicol. 51, 93–105.

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, 468–474.

Kamunde, C.N., Pyle, G.G., McDonald, D.G., Wood, C.M., 2003. Influence of dietary sodium and waterborne copper exposure on copper and sodium homeostasis, sublethal copper toxicity, and gill copper binding in rainbow trout, *Oncorhynchus mykiss*. Environ. Toxicol. Chem. 22, 342–350.

Larsson, Å., Bengtsson, B.E., Haux, C., 1981. Disturbed ion balance in flounder, *Platichthys flesus* L. exposed to sublethal levels of cadmium. Aquat. Toxicol. 1, 19–35.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout: 1. Iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231–243.

Meinelt, T., Playle, R.C., Pietrock, M., Burnison, B.K., Wienke, A., Steinberg, C.E.W., 2001. Interaction of cadmium toxicity in embryos and larvae of zebrafish (*Danio rerio*) with calcium and humic substances. Aquatic Toxicol. 54, 205–215.

O'Connell, J.P., Gatlin, D.M., 1994. Effects of dietary calcium and vitamin D3 on weight gain and mineral composition of the blue tilapia (*Oreochromis aureus*) in low-calcium water. Aquaculture 125, 107–117.

Ogino, C., Takeda, H., 1978. Requirements of rainbow trout for dietary calcium and phosphorus. Bull. Jpn. Soc. Sci. Fish. 42, 793–799.

Playle, R.C., Dixon, D.G., Burnison, K., 1993. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modeling of metal accumulation. Can. J. Fish Aquat. Sci. 50, 2678–2687.

Pratap, H.B., Fu, H., Lock, R.A.C., Wendelaar Bonga, S.E., 1989. Effects of waterborne and dietary cadmium on plasma ions of the teleost *Oreochromis mossambicus* in relation to water calcium levels. Arch. Environ. Contam. Toxicol. 18, 568–575.

Pyle, G.G., Kamunde, C.N., McDonald, D.G., Wood, C.M., 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 206, 609–618.

Randall, D.J., Brauner, C., 1998. Interactions between ion and gas transfer in freshwater teleost fish. Comp. Biochem. Physiol. 119A, 3–8.

Rodgers, D.W., 1984. Ambient pH and calcium concentration as modifiers of growth and calcium dynamics of brook trout, *Salvelinus fontinalis*. Can. J. Fish. Aquat. Sci. 41, 1774–1780.

Sherwood, G.D., Rasmussen, J.B., Rowan, D.J., Brodeur, J., Montela, A., 2000. Bioenergetic cost of heavy metal exposure in yellow perch (*Perca flavescens*): in situ estimates with a radiotracer technique. Can. J. Fish. Aquat. Sci. 57, 441–450.

Szebedinszsky, 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. Environ. Toxicol. Chem. 20, 597–607.

Verbost, P.M., Van Rooij, J., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. J. Exp. Biol. 145, 185–197.

Wong, C.K.C., Wong, M.H., 2000. Morphological and biochemical changes in the gills of tilapia (*Oreochromis mossambicus*) to ambient cadmium exposure. Aquat. Toxicol. 48, 517–527.

Wood, C.M., 1992. Flux measurements as indices of H^+ and metal effects on freshwater fish. Aquat. Toxicol. 22, 239–264.

Wood, C.M., 2001. Toxic responses of the gill. In: Schlenk, D.W., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts. Organs, vol. 1. Taylor and Francis, Washington, DC, pp. 1–89.

Zall, D.M., Fisher, M.D., Garner, Q.M., 1956. Photometric determination of chlorides in water. Anal. Chem. 28, 1665–1678.

Zohouri, M.A., Pyle, G.G., Wood, C.M., 2001. Dietary Ca inhibits waterborne Cd uptake in Cd-exposed rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol., C Pharmacol. Toxicol. 130, 347–356.