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Aquatic Toxicology 78 (2006) 217–232

AQUATIC TOXICOLOGY

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# The chronic effects of dietary lead in freshwater juvenile rainbow trout (*Oncorhynchus mykiss*) fed elevated calcium diets

Lara C. Alves *, Chris M. Wood

*McMaster University, Department of Biology, Hamilton, Ont., Canada L8S 4K1*

Received 26 December 2005; received in revised form 11 March 2006; accepted 12 March 2006

**Abstract**

This study examined the impact of elevated dietary $Ca^{2+}$ on the responses to chronic dietary Pb exposure in juvenile rainbow trout. Trout were fed reference (0.3 μg Pb/g, ~20 mg $Ca^{2+}$/g) and Pb-enriched diets (~50 or 500 μg Pb/g) in the presence of background $Ca^{2+}$ (~20 mg $Ca^{2+}$/g) or (~60 mg $Ca^{2+}$/g) of added $Ca^{2+}$ (as $CaCO_3$) for 42 days. The quantitative order of Pb accumulation in tissues reflected the exposure pathway of Pb via the diet (per tissue wet weight): gut > bone > kidney > liver > spleen > gill > carcass > brain > white muscle. The anterior intestine accumulated the most Pb per tissue wet weight, while the bone accumulated the most Pb per fish weight. Pb concentrations were much higher in the posterior kidney than the anterior kidney. Simultaneous addition of $Ca^{2+}$ to the diet had an overall protective effect in all the tissues analysed in reducing Pb accumulation. The RBCs accumulated 100 times more Pb when compared to the plasma, while the whole blood δ-aminolevulinic acid dehydratase was inhibited in the high treatment group without added $Ca^{2+}$, by the end of the exposure. Neither plasma $Cl^-$, $K^+$, $Mg^{2+}$ nor $Na^+$, $K^+$-ATPase activities in the gills, mid- and posterior intestine were affected. However, there were mild disruptions in plasma $Na^+$ and $Ca^{2+}$ levels in the elevated Pb and $Ca^{2+}$ treatment groups, and a significant up-regulation in $Na^+$, $K^+$-ATPase activity at the anterior intestine in fish fed the high Pb diets with background or added $Ca^{2+}$. By day 42, Pb levels in most tissues had either stabilized or started to decrease, indicating some capacity for regulation of accumulated loads. We conclude that elevated dietary $Ca^{2+}$ levels will be protective in reducing Pb burdens in freshwater juvenile rainbow trout exposed to environments contaminated with waterborne Pb.


*Keywords:* Dietary; Lead (Pb); Calcium (Ca); Accumulation; Ionoregulation; δ-Aminolevulinic acid dehydratase (ALAD)

## 1. Introduction

In contaminated aquatic environments, fish can take up Pb through the water, diet and to a lesser extent the skin (e.g. Dallinger et al., 1987; Hodson et al., 1978; Köck et al., 1998; Rogers et al., 2003, 2005; Rogers and Wood, 2004). Waterborne Pb causes the disruption of $Na^+$, $Cl^-$ and $Ca^{2+}$ regulation during acute exposure, the induction of spinal deformities and black tails during chronic exposure, and disruption in hemoglobin synthesis during both types of exposure (Hodson et al., 1978; Rogers et al., 2003, 2005; Rogers and Wood, 2004). Dietary Pb is less well studied than waterborne Pb. However, physiological changes and morphological damage in the enterocytes at the mid- and posterior intestine have been observed in rainbow trout fed 10 μg Pb/g fish/day for 15–30 days (Crespo et al., 1986).

In contrast to the many acute waterborne Pb experiments (Hodson, 1976; Hodson et al., 1977, 1978; Varanasi and Gmur, 1978; Rogers et al., 2003, 2005; Rogers and Wood, 2004), only four studies have looked at the effects of dietary Pb exposure to rainbow trout. Hodson et al. (1978) found that Pb was not taken up via the diet, but in contrast, the studies of Alves et al. (2006), Mount et al. (1994), and Crespo et al. (1986) all found that dietary Pb accumulates in the whole body and in a number of internal tissues when present in the diet.

Alves et al. (2006) found mild physiological disturbances in juvenile rainbow trout fed diets containing up to 520 μg Pb/g for 21 days. These disturbances included transient decreases in plasma $Mg^{2+}$ and $Ca^{2+}$ levels, and increases in whole body waterborne $Na^+$ influx rates. Despite an absence of effects on growth and survival rates, rainbow trout accumulated significant Pb burdens in the intestine, kidney, gills, liver and carcass, with the RBCs accumulating 105 times more Pb when compared to

* Corresponding author at: Environment Canada, 867 Lakeshore Road, P.O. Box 5050, Burlington, Ont., Canada L7R 4A6. Tel.: +1 905 336 4405; fax: +1 905 336 4420.

*E-mail address:* Lara.Alves@ec.gc.ca (L.C. Alves).

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doi:10.1016/j.aquatox.2006.03.005

the plasma by the end of the experiment. Pb accumulated to the greatest extent in the intestine, suggesting that the intestine may be a site of dietary Pb toxicity. Notably, there was some indication of stabilization or even depuration of tissue Pb burdens by day 21, but the exposure was not long enough to establish clear trends. Similarly, Mount et al. (1994) found that despite an absence of effects on survival and growth, rainbow trout accumulated whole body dietary Pb burdens when fed for 60 days with brine shrimp contaminated with Pb concentrations as high as 170 μg Pb/g dw. In addition, Crespo et al. (1986) reported morphological changes, decreased $Na^+$, $K^+$-ATPase activity, and decreased $Na^+$ and $Cl^-$ absorption in the intestine of trout fed for 15–30 days with a comparable dietary load of Pb.

Generally, divalent metals such as Pb, Cd and $Zn^{2+}$ are considered $Ca^{2+}$ antagonists. In an early study (Varanasi and Gmur, 1978), coho salmon (*Oncorhynchus kisutch*) force-fed gelatin capsules containing 8.4 mg of calcium chloride ($CaCl_2$) and then exposed to 1300 μg/L of waterborne Pb for 168 h, had reduced Pb tissue burdens (Varanasi and Gmur, 1978). Similarly, several more recent studies (Zohouri et al., 2001; Baldisserotto et al., 2004a, 2004b, 2005; Franklin et al., 2005) have shown that dietary $Ca^{2+}$ (as $CaCl_2$ or $CaCO_3$) is protective against the uptake of both waterborne and dietary Cd, as well as against the uptake of waterborne $Zn^{2+}$ (Niyogi and Wood, 2006).

δ-Aminolevulininc acid dehydratase (ALAD), an enzyme that catalyses the formation of porphobilinogen (PGB) from the substrate aminolevulinic acid (ALA), has long been used as a biomarker for Pb toxicity in humans (Secchi et al., 1974) and fish (Hodson et al., 1977; Schmitt et al., 1984, 1993, 2002; Burden et al., 1998). Pb inhibits ALAD by binding to essential sulfhydryl (SH) groups and displacing the $Zn^{2+}$ cofactors on ALAD (World Health Organization, 1977; Sassa, 1982). Hodson (1976) and Hodson et al. (1977, 1978) found that ALAD activity in rainbow trout was inhibited after exposing fish to waterborne Pb concentrations as low as 13 μg/L for 4 weeks, but the effects of dietary Pb exposure on ALAD activity in freshwater fish are not known.

Therefore, the objectives of the present investigation were: (a) to investigate the accumulation of Pb in various tissues; (b) to assess any possible physiological and toxicological effects on growth and survival rates, plasma $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ regulation, the enzyme ALAD and $Na^+$, $K^+$-ATPase activity in gills or intestine, in juvenile rainbow trout fed with two different levels of dietary Pb, in the presence of either background or elevated $Ca^{2+}$ levels in the diet.

## 2. Materials and methods

### 2.1. Fish

Juvenile rainbow trout (*O. mykiss*) ($N \sim 400$) weighing 20–23 g were obtained from Humber Springs Trout Hatchery (Orangeville, Ont.). After arrival, fish were randomly selected and placed into eight, 200 L polypropylene flow-through, aerated tanks that were divided into $2 \times 100$ L sections (25 fish per section). The division was achieved using a 0.2 $mm^2$ mesh screen, which allowed free-mixing of water, but not the food or feces between the two sides. Each tank was supplied with 1 L/min of dechlorinated Hamilton water, pumped only once through the tanks and with the composition of $Na^+ = 0.65$ mM, $Cl^- = 0.8$ mM, $Ca^{2+} = 1.0$ mM, $Mg^{2+} = 0.4$ mM, $K^+ = 0.06$ mM and water hardness as $CaCO_3 = 140$ mg/L, total Pb = $1.3 \pm 0.1$ μg/L. The pH and temperature were kept at ambient conditions, 7.4–7.7 and 11–13 °C, respectively. Photoperiod was maintained at 12 h light and 12 h dark. Fish were acclimated three weeks prior to their use in the 42 day experiment. Prior to the start of the experiment juvenile rainbow trout had a mean weight of $25.8 \pm 0.5$ g.

Fish were fed commercial salmon fry pellets once daily (Silver Cup feed; Murray, UT, USA, see below for composition) at a ration of 1.5% body mass/day upon arrival and until the beginning of the experiment. At the start of the experiment, each tank section was assigned to one of six replicated nominal dietary Pb and/or $Ca^{2+}$ treatments: 0 μg Pb/g + 20 mg $Ca^{2+}$/g dry weight (A); 0 μg Pb/g + 60 mg $Ca^{2+}$/g (B); 50 μg Pb/g + 20 mg $Ca^{2+}$/g (C); 50 μg Pb/g + 60 mg $Ca^{2+}$/g (D); 500 μg Pb/g + 20 mg $Ca^{2+}$/g (E), and 500 μg Pb/g + 60 mg $Ca^{2+}$/g (F) (Fig. 1). Additional replicated control tank sections (0 μg Pb/g + 20 mg $Ca^{2+}$/g and 0 μg Pb/g + 60 mg $Ca^{2+}$/g, Fig. 1) in tanks 1 and 5, in comparison to tanks 3 and 7, and 4 and 8, where the same treatments were paired with high dietary treatments, were used to control for possible waterborne Pb contamination as a result of Pb leaching across from the feces and/or food in the neighbouring tank section.

### 2.2. Diet

Pb-enhanced diets were made by adding Pb, in the form of lead nitrate, $Pb(NO_3)_2$ (Sigma–Aldrich) into 0.5 pt. commercial salmon fry food (Silver Cup feed, Murray, UT, USA).

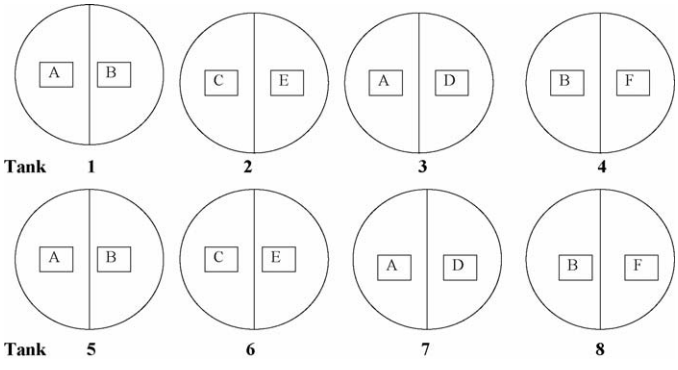


Fig. 1. Tank set-up. Tanks were set up by sectioning 200 L tanks into $2 \times 100$ L sections using a 0.2 $mm^2$ mesh screen. The letter in each section corresponds to the diet given in each section: (A) 0 μg Pb/g + 20 mg $Ca^{2+}$/g; (B) 0 μg Pb/g + 60 mg $Ca^{2+}$/g; (C) 50 μg Pb/g + 20 mg $Ca^{2+}$/g; (D) 500 μg Pb/g + 20 mg $Ca^{2+}$/g; (E) 50 μg Pb/g + 60 mg $Ca^{2+}$/g; (F) 500 μg Pb/g + 20 mg $Ca^{2+}$/g. Each of the above tanks were replicated. Tanks 1 and 5 were used as a control for possible waterborne Pb as a result of Pb leaching from the feces and food into neighbouring tank sections (i.e. treatment A was replicated in tanks 3 and 7, and treatment B was replicated in tanks 4 and 8, both of which had the highest dietary Pb levels on the opposite side). 20 mg $Ca^{2+}$/g represents background Ca concentrations in the salmon fry food used to prepare the diets.

Table 1
The nominal and achieved concentrations of Pb and $Ca^{2+}$, in custom made diets

| Treatment | [Pb][a] (μg/g) | [Ca][a] (mg/g) |
|---|---|---|
| 0 μg Pb/g + 20 mg $Ca^{2+}$/g | 0.3 ± 0.1 | 19.2 ± 0.3 |
| 0 μg Pb/g + 60 mg $Ca^{2+}$/g | 0.1 ± 0.0 | 61.0 ± 0.9 |
| 50 μg Pb/g + 20 mg $Ca^{2+}$/g | 45.2 ± 2.1 | 18.5 ± 0.2 |
| 50 μg Pb/g + 60 mg $Ca^{2+}$/g | 51.6 ± 3.6 | 60.4 ± 1.2 |
| 500 μg Pb/g + 20 mg $Ca^{2+}$/g | 480.2 ± 30 | 18.2 ± 0.4 |
| 500 μg Pb/g + 60 mg $Ca^{2+}$/g | 495.2 ± 12.9 | 59.3 ± 1.6 |

[a] $N = 15$. Data are expressed as mean ± 1 S.E.M. 20 mg $Ca^{2+}$/g represents background $Ca^{2+}$ concentration in the salmon fry food used prepare the diets.

The manufacturer's specifications for the food were of 52% crude protein (minimum); 14% crude fat (minimum); 3% crude fiber (maximum); 12% ash (maximum), and 1% sodium (actual). The background levels of Pb were 0.3 ± 0.1 μg Pb/g, and for $Ca^{2+}$ were 19.2 ± 0.3 mg $Ca^{2+}$/g. The commercial food was powdered, then hydrated with 40% (v/w) of double distilled water (NANOpure II; Sybron/Barnstead, Boston, MA) containing different proportions of dissolved $Pb(NO_3)_2$. For the $Ca^{2+}$-enhanced diets, the powdered food was mixed with powdered $CaCO_3$ before adding water that contained $Pb(NO_3)_2$. The control diets were not supplemented with equivalent amounts of nitrate or carbonate, to avoid confounding effects of another anion. The mixtures were blended in a pasta maker (Popeli, Ronco Inventions, Chastworth, CA, USA) for 1 h, to yield 0, 50 and 500 μg Pb/g diet (nominal concentrations), each with 20 (approximate background $Ca^{2+}$ levels found in salmon fry food, control) or 60 mg $Ca^{2+}$/g (nominal concentrations, added as $CaCO_3$). The mixed paste was then passed through a cutter where small strands of approximately 5 mm were broken into small pellets by hand. All food pellets were air-dried to a constant weight for approximately 4 days and frozen until further use. Moisture in the food was determined by placing 10 g of food from each treatment into a 60 °C oven and drying to a constant weight which took about 12 h. The moisture content was only 6%, so the reported concentrations of elements in the diet on a wet weight basis are very close to values on a dry weight basis.

Measured Pb concentrations in food (Table 1) were determined by heat digesting the food pellets in five volumes of 6N $HNO_3$ at 60 °C for 48 h. The supernatant was then diluted in 1% $HNO_3$ and measured against a certified multi-element Pb standard (Anachemia Inc., Quebec), blank samples, reference samples (Anachemia Inc., Quebec), and spiked samples on the graphite furnace atomic absorption spectrophotometer (AAS; 220 SpectrAA; Varian GTA-110; Varian, Australia) with a detection limit of 0.06 μg Pb/L. Mean spike recovery for the food spiked with a known amount of Pb was 84 ± 1.6%. The data were not corrected for total Pb recovery. $Ca^{2+}$ concentrations (Table 1) were measured as above, expect that the samples were diluted in 0.5% $LaCl_3$ and measured against known standards made with 0.5% $LaCl_3$ on a flame AAS (220FS Spectra AA; Varian, Australia).

### 2.3. Feeding, mean weight, specific growth rates and food conversion efficiency

Fish from each tank section were bulk-weighed on days 0, 8, 15, 22, 29, and 36, by removing all the fish and placing them into a 4.5 L bucket filled with water. The total biomass was determined from the difference between the weight of the bucket and water with and without fish. The mean fish weight was calculated by dividing the total biomass by the number of fish in each tank section. Each of the six replicated treatments and the replicated control tanks (i.e. A/B, Fig. 1) were fed a daily ration of 1.5% total body weight per tank section per day determined weekly by bulk weighing. The bottom of each tank was siphoned approximately 3 h post-feeding to control for any leaching of Pb and $Ca^{2+}$ from both the food and feces. Water samples were taken twice weekly, 1 h after siphoning (on the sampling day and 3 days post-sampling), to detect total and dissolved Pb contamination.

Specific growth rates (SGR) expressed as a % per day were determined using linear regression (SigmaStat, 3.0) of the natural logarithm of mean bulk weight versus time data. The food conversion efficiency (FCE; %) for each treatment was calculated using the formula:

$$\mathrm{FCE} = (\mathrm{SGR}_{\mathrm{ave}}/R) \times 100, \quad (1)$$

where $\mathrm{SGR}_{\mathrm{ave}}$ is the mean specific growth rate (%/day), and $R$ is the ration (%/day).

### 2.4. Tissue sampling and Pb analysis

On day 0, prior to the implementation of the different dietary treatments, two randomly selected fish per tank section were sacrificed with 1.0 g/L MS 222. On days 14, 28, and 42, four randomly selected fish per tank section were similarly sacrificed, 16 h post-fish feeding. Blood samples (see below) were taken immediately. Fish wet weights $W$, in (g) and fork length $L$, in (cm) were taken in order to measure individual condition factors ($W/L^3 \times 100\%$; Brett, 1979). Vertebral bone, brain, gill baskets, anterior, mid- and posterior intestine, anterior and posterior kidney, liver, muscle, spleen, stomach, and remaining carcass were dissected out in order to determine Pb tissue burden.

The gills, once dissected, were rinsed with 0.6% NaCl in order to remove any fine particulate matter. The stomach and the three sections of the intestine were longitudinally cut and rinsed with 0.6% NaCl to remove any undigested food and feces. A section of bone was removed from the posterior neural spine of the vertebrae, while white muscle was removed anterior to the dorsal fin. The bone, brain, gills, intestine sections, kidney sections, liver, muscle, spleen, stomach, and remaining carcass were blotted dry, placed into pre-weighed plastic tubes, re-weighed and stored in a 4 °C cold room until further analyses.

Each tissue was digested in five volumes of the weight of the tissue in 1N $HNO_3$. The acid digests were placed in an oven for 48 h at 60 °C. The tissues were then centrifuged at 14 000 × $g$ (Eppendof 5145C) for 20 min; the supernatant was diluted in

1% $HNO_3$ and measured by the graphite furnace AAS for Pb burden. The mean spike recovery for tissue (carcass) spiked with a known amount of Pb was 98 ± 2.4%. The data were not corrected for total Pb recovery.

Water samples were acidified to 1% $HNO_3$ (trace-metal grade acid, Fisher Scientific) and analysed on the furnace AAS. Dissolved Pb was measured similarly to total Pb, except that the water was first passed through a 0.45 μm Supor® low protein binding non-pyrogenic membrane acrodisc syringe filter (Pall Corporation, MI, USA) and then acidified with 1% $HNO_3$.

### 2.5. Determination of whole body Pb and percent Pb distribution

Whole body Pb distribution was calculated in order to determine Pb distribution in different tissues of the fish using the formula:

$$\mathrm{WB} = \left[\sum(T_n \mathrm{Twt}_n)\right] / \mathrm{Fwt}, \quad (2)$$

where WB is the whole body Pb accumulation (μg/g wet weight), $T$ the accumulation of Pb in each tissue (μg/g), Twt the weight of each individual tissue (g), and Fwt is the combined weight of all tissues (g) in the whole fish. The lower case $n$ represents an individual tissue (i.e. WB = [(liver Pb × liver weight) + ⋯ + (carcass Pb × carcass weight)]/(liver weight + ⋯ + carcass weight)).

Since only a small section of the bone and white muscle was removed from the fish, estimates of tissue weight (Twt, g/kg fish) in rainbow trout from Gingerich et al. (1990) were used to determine whole body bone and white muscle weight in fish using the formula:

$$\mathrm{Twt} = \mathrm{Tewt} \times \mathrm{aveFwt}, \quad (3)$$

where Tewt represents the estimated tissue weight (g/kg fish) from Gingerich et al. (1990), and aveFwt is the mean fish weight in the study (kg). Once total bone weight and muscle weight were determined, these values were substituted into Eq. (2), for WB Pb determination. The carcass remnants represents the leftover tissue, after the estimate of total bone and white muscle are considered, by the equation above.

Percent of total Pb distribution contributed by each tissue in reference to whole body Pb burden was determined using the formula:

$$\mathrm{PD} = [T\ \mathrm{Twt}/\mathrm{Fwt}] \times 100, \quad (4)$$

where PD represents percent of the total distribution (%) contributed by an individual tissue, while $T$, Twt and Fwt represent the same notation as above.

### 2.6. Blood analytical techniques

Blood was taken by caudal puncture using an ice-chilled 1-mL disposable syringe and 21-gauge needle pre-rinsed with lithium heparin (50 i.u./mL). In addition to the blood samples taken at the times of tissue sampling (days 0, 14, 28, and 42), blood samples were also taken on days 0, 7, 21 and 35 from fish sacrificed for the $Na^+$, $K^+$-ATPase assay (see below) in order to measure plasma $Ca^{2+}$, $Cl^-$, $K^+$, $Mg^{2+}$, $Na^+$ and total Pb. RBCs were analysed for total Pb. Whole blood taken on days 0, 14, 28 and 42 was used to measure δ-aminolevulinic acid dehydratase (ALAD), total protein (TP), hemoglobin (Hb), total Pb, and total Zn.

Plasma was obtained by centrifuging whole blood at 14 000 × $g$ for 2 min. The plasma and RBCs were decanted into separate pre-weighed tubes, then placed in liquid nitrogen and stored in a −70 °C freezer until further use. Plasma $Ca^{2+}$ and $Mg^{2+}$ concentrations were determined by diluting the sonicated plasma with 0.5% $LaCl_3$ and assaying it against known standards diluted in 0.5% $LaCl_3$ using the flame AAS. Plasma $Na^+$ and $K^+$ were handled similarly, except that the plasma $Na^+$ and standards were diluted in 1% $HNO_3$, and plasma $K^+$ and standards were diluted in 0.1% $CsCl_2$. Total plasma Pb was determined by diluting the plasma with 1% $HNO_3$ and measuring it against a certified multi-element Pb standard (Anachemia Inc., Quebec) on the graphite furnace AAS. Plasma $Cl^-$ was measured using the coulometric titration method (Radiometer CMT10). The RBCs were acid-digested and assayed for total Pb concentration following the protocol as above for the tissues.

Whole blood was directly transferred into liquid nitrogen and stored at −70 °C, until analysis for Hb, TP, ALAD, total Pb, and total Zn. Hb levels were determined in duplicate using the cyanmethemoglobin method (540 nm) on an LKB UltraSpec Plus spectrophotometer with Drabkin's reagent and known standards from Pointe Scientific Inc (Michigan). TP was assayed in duplicate using the Bradford (1976) method with bovine serum albumin standards (Sigma–Aldrich) and measured on a microplate reader (SpectraMax 340 PC, Molecular Devices, CA) at 540 nm. The whole blood was acid-digested and assayed for total Pb concentration following the same protocol as above for the tissues and RBCs. The mean recovery for whole blood spiked with a known amount of Pb was 68% ± 3.7%. Data were not corrected for total Pb recovery.

Total Zn concentrations in the whole blood were determined by diluting the acid-digested whole blood with 1% $HNO_3$ and assaying it against known Zn standards (Sigma–Aldrich) diluted in 1% $HNO_3$ using the flame AAS.

### 2.7. δ-Aminolevulinic acid dehydratase (ALAD) assay

On day 0, two fish, while on days 14, 28 and 42, four randomly selected fish per tank section were used to determine ALAD in the blood, using a procedure based on the Ehrlich colour reaction modified from Schmitt et al. (1993, 2005) and Whyte (2002). In short, frozen blood was thawed and homogenized in an equal volume of ice cold double-distilled water, and 15 μL of this homogenate was placed into four tubes, two tubes which contained 75 μL of 0.2% Triton X-100 in 0.1 M phosphate buffer, used as the blank, and two other tubes containing 75 μL of 0.2% Triton X-100 in 0.1 M phosphate buffer plus 670 μg/mL of δ-aminolevulinic acid hydrochloride (ALA–HCl), used for ALAD determination. Each tube was vortexed and incubated in a 37 °C

water bath for 1 h. The reaction was terminated with 600 μL of TCA/*n*-ethylmaleimide solution (4.0 g trichloroacetic acid and 2.7 g *N*-ethylmaleimide in 100 mL of double-distilled water). The tubes were then centrifuged at 1000 × *g* for 5 min. One hundred microliters of the supernatant plus 100 μL of Ehrlich reagent (3 mL of double distilled water, 42 mL glacial acetic acid and 10 mL 70% perchloric acid and 1.0 g *p*-dimethylamino benzaldehyde) solution were mixed and measured against porphobilinogen standards at 540 nm on the microplate reader.

### 2.8. $Na^+$, $K^+$-ATPase assay

On day 0, two fish were randomly selected per tank section, while on days 7, 21, and 35, three randomly selected fish per tank section were used to determine $Na^+$, $K^+$-ATPase activity in the gill and intestinal segments. Each gill and intestinal segment was placed into a pre-weighed plastic tube, frozen in liquid nitrogen, and stored at −70 °C, until further use. $Na^+$, $K^+$-ATPase activity was measured using the UV detection microplate method of McCormick (1993), and then normalized against total protein using the Bradford (1976) method.

### 2.9. Statistical analysis

All statistical tests were performed using SigmaStat version 3.0. Prior to analysis of variance, all data were tested for homogeneity of variances among groups using the Bartlett test. Those that failed were subjected to various transformations to obtain homogeneity among groups. Data that could not be normalized were subjected to the Kruskal–Wallis rank test, where all ranks were corrected for ties and a Dunn multiple comparison was used to determine differences between treatments and groups ($P < 0.05$). Comparisons in normalized data between treatments and groups were made by one-way, two-way or three-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for differences among treatments and groups ($P < 0.05$). All data have been reported as mean ± S.E.M., $N$ = sample size.

## 3. Results

### 3.1. Water samples

Pb concentrations in both filtered and unfiltered water samples were not significantly different between treatments (not shown). Waterborne Pb concentrations were not significantly greater than waterborne Pb background levels of 1.3 ± 0.1 μg Pb/L measured during the pre-exposure acclimation period. When the additional control tanks were compared to the other control treatment tanks (Fig. 1) that were sectioned with high dietary Pb levels, there were no significant differences in waterborne Pb concentrations, Pb accumulation in the various tissues, hematological, or ionoregulatory parameters, indicating that leaching of dietary Pb and transport of waterborne Pb between the tank sections was not a problem.

### 3.2. Growth and survival

Chronic dietary exposure in the range of 50–500 μg Pb/g resulted in no significant effects on the growth and survival of juvenile rainbow trout over 42 days (Table 2). Enhanced-$Ca^{2+}$ diets (60 mg $Ca^{2+}$/g) also had no effects on survival, despite a tendency for SGR to be lower in fish fed these diets. The mean fish weight (g) remained constant across all treatments during the course of the experiment. FCE was significantly lower in the 0 μg Pb/g + 60 mg $Ca^{2+}$/g, 500 μg Pb/g + 20 mg $Ca^{2+}$/g and 500 μg Pb/g + 60 mg $Ca^{2+}$/g, compared to the 0 μg Pb/g + 20 mg $Ca^{2+}$/g and 50 μg Pb/g + 20 mg $Ca^{2+}$/g treatments.

### 3.3. Pattern of Pb accumulation in tissues

In terms of accumulated whole body Pb burden (μg/kg fish; Fig. 2) throughout the duration of the experiment, the fish fed the 500 μg Pb/g + 20 mg $Ca^{2+}$/g diet accumulated 18–34 times more Pb when compared to the control (500 μg Pb/g + 20 mg $Ca^{2+}$/g), and 6–9 times more when compared to the 50 μg Pb/g + 20 mg $Ca^{2+}$/g treatment. The fish receiving the 50 μg Pb/g + 20 mg $Ca^{2+}$/g diet accumu-

Table 2
Mean fish weight, specific growth rates (SGR), rations, food conversion efficiency (FCE) and condition factors (CF) of juvenile rainbow trout fed different Pb and/or $Ca^{2+}$ diets for 42 days

| Treatment | Mean fish wt.[a] (g) | SGR[a,b] (%/day) | Ration (%/day) | FCE[c] (%) | CF[d] |
|---|---|---|---|---|---|
| 0 μg Pb/g + 20 mg $Ca^{2+}$/g | 48 ± 4 a | 1.46 ± 0.12 ab | 1.5 ± 0.1 | 97 ± 2 ac | 1.60 ± 0.06 a |
| 0 μg Pb/g + 60 mg $Ca^{2+}$/g | 40 ± 4 a | 1.03 ± 0.07 a | 1.5 ± 0.1 | 69 ± 1 b | 1.51 ± 0.08 a |
| 50 μg Pb/g + 20 mg $Ca^{2+}$/g | 48 ± 4 a | 1.66 ± 0.09 b | 1.5 ± 0.1 | 104 ± 1 c | 1.54 ± 0.05 a |
| 50 μg Pb/g + 60 mg $Ca^{2+}$/g | 40 ± 2 a | 1.30 ± 0.13 ab | 1.5 ± 0.1 | 87 ± 3 a | 1.53 ± 0.03 a |
| 500 μg Pb/g + 20 mg $Ca^{2+}$/g | 37 ± 4 a | 1.16 ± 0.14 a | 1.5 ± 0.1 | 77 ± 4 b | 1.45 ± 0.06 a |
| 500 μg Pb/g + 60 mg $Ca^{2+}$/g | 41 ± 2 a | 1.19 ± 0.12 a | 1.5 ± 0.1 | 79 ± 2 b | 1.59 ± 0.03 a |

Lower case letters indicate significant differences ($P < 0.05$) between treatments. Values sharing the same letter indicates no significant differences. Values not sharing the same letter indicate significant differences. No mortality was observed in any of the treatments.

[a] Values are in mean ± 1 S.E.M.

[b] SGR was calculated using the linear regression (SigmaPlot version 8.0) of the natural logarithm of mean bulk weight vs. time. Mean ± 1 S.E.M. is based on the regression, not on the tank divisions.

[c] FCE was calculated by SGR/ration multiplied by 100.

[d] CF was calculated using the formula $K$ = weight (g)/(fork length (cm))$^3$ × 100 (Brett, 1979).

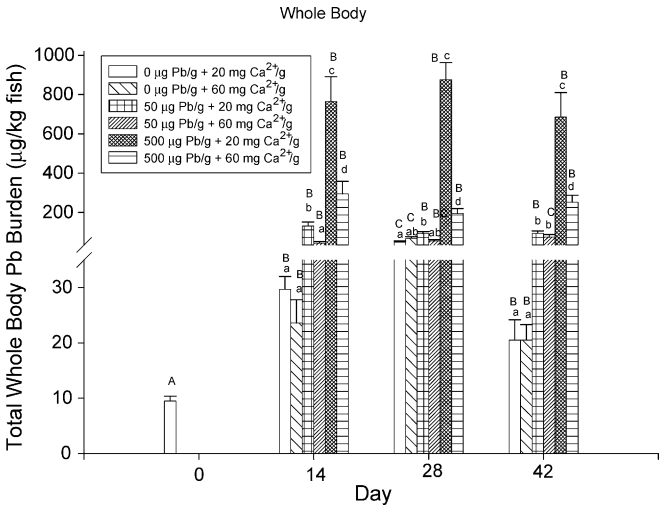


Fig. 2. Accumulated whole body Pb burden in juvenile rainbow trout fed contaminated Pb and/or $Ca^{2+}$ supplemented diets. Data reported as mean ± 1 S.E.M.; $N = 8$. Upper case letters represent significant differences ($P < 0.05$) between days within the same treatment. Lower case letters represent significant differences ($P < 0.05$) between treatments within the same day. Values sharing the same letters indicates no significant differences. Values not sharing the same letters indicates significant differences. An overall significant effect of $Ca^{2+}$ ($P < 0.05$) was determined using a three-way ANOVA with a Tukey's multiple comparison test.

lated two to five times more Pb when compared to the controls.

Fig. 3 shows the percent of total Pb distribution (per fish weight) in the different tissues of juvenile rainbow trout fed the 500 µg Pb/g + 20 mg $Ca^{2+}$/g diet for 42 days. In short, the bone accumulated the highest percentage of Pb (37.7%), while the brain accumulated the least burden of Pb (0.1%). In terms of Pb distribution in the gastrointestinal tract, the anterior intestine (18.6%) had the highest Pb burden followed by the stomach (9.9%), the mid-intestine (1.0%), and the posterior intestine (0.9%). The posterior kidney, however, had a much higher Pb load (2.4%) when compared to the anterior kidney (0.3%). Despite the spleen's relatively small weight when compared to the posterior kidney, posterior intestine, and mid-intestine, it accumulated a substantial percentage of Pb burden (2.7%).

Pb concentrations in the control treatment (0 µg Pb/g + 20 mg $Ca^{2+}$/g) of all tissues were low (<0.05 µg/g tissue wet weight), but were significantly elevated on days 14 and 28, and occasionally on day 42, when compared to day 0. In the high dietary Pb treatments without enhanced $Ca^{2+}$, Pb accumulated in all the tissues analysed when compared to the controls (Figs. 4–7; Table 3). The order of Pb accumulation (in terms of concentration) reflected the exposure pathway of fish fed the high Pb diet (500 µg Pb/g + 20 mg $Ca^{2+}$/g) after 42 days: gut > bone > kidney > liver > spleen > gills > carcass > brain > white muscle. In the gastrointestinal tract, Pb levels were the highest in anterior intestine (Fig. 5b) > mid-intestine (Fig. 5c) > stomach (Fig. 5a) > posterior intestine (Fig. 5d). Within the internal tissues the following order of Pb accumulation was observed: bone (Fig. 7) > posterior kidney (Fig. 6b) > anterior kidney (Fig. 6a) > liver (Fig. 4b) > spleen (Table 3) > gills (Fig. 4a) > carcass (Table 3) > brain (Table 3) > white muscle (Table 3) in fish exposed to the high Pb diet.

Similarly, for the low Pb diet (50 µg Pb/g + 20 mg $Ca^{2+}$/g), the order reflected the exposure pathway after 42 days: anterior intestine > mid-intestine > bone > posterior kidney >

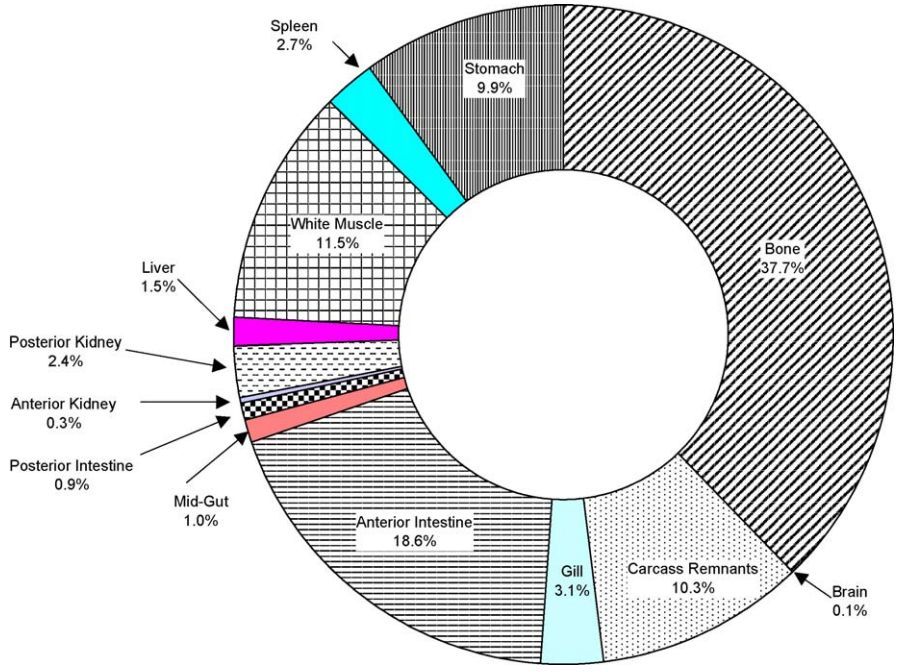


Fig. 3. Average percent distribution of total Pb burden in different tissues of the juvenile rainbow trout fed the 500 µg Pb/g + 20 mg $Ca^{2+}$/g diet for 42 days. The bone accumulated the highest Pb burden, while the brain accumulated the least Pb burden in fish fed the 500 µg Pb/g + 20 mg $Ca^{2+}$/g diet. Calculations to determine percent Pb burden in each tissue are explained in Section 2.5.

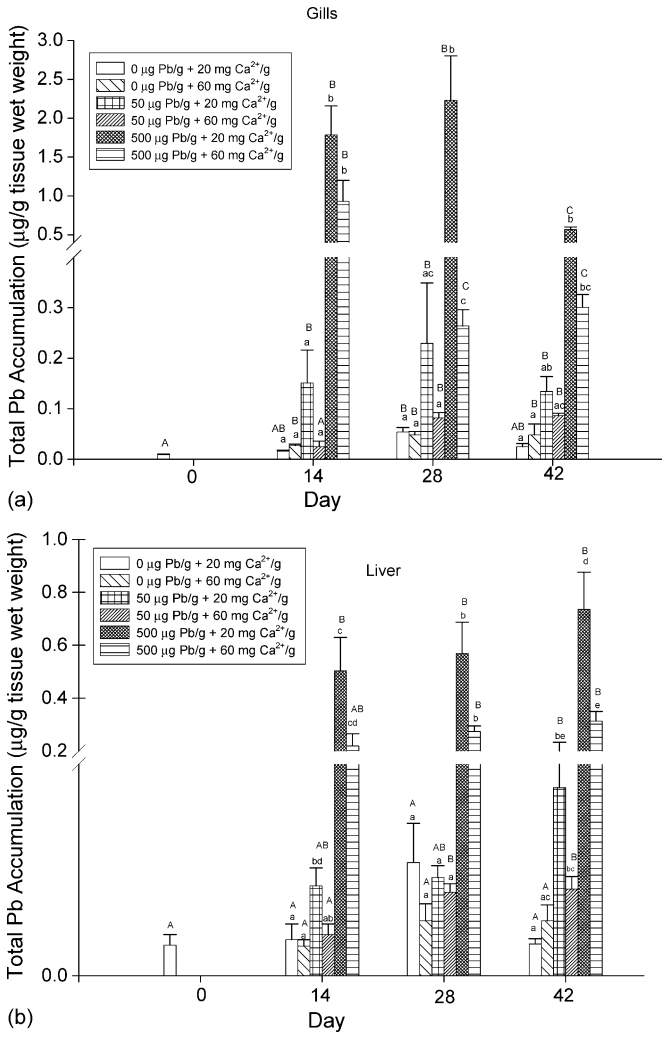


Fig. 4. Lead accumulation in the gills (a) and liver (b) of juvenile rainbow trout over 42 days. Other details as in legend of Fig. 2.

posterior intestine > stomach > anterior kidney > liver > spleen > gills > white muscle > brain > carcass. In the gastrointestinal tract in the low dietary Pb treatment (50 μg Pb/g + 20 mg $Ca^{2+}$/g), anterior intestine accumulated the most Pb > mid-intestine > posterior intestine > stomach. Within the internal tissues, the bone accumulated the highest Pb burden followed by > posterior kidney > anterior kidney > liver > spleen > gills > white muscle > brain > carcass.

The gills significantly accumulated Pb from days 0 to 28 (2.2 μg Pb/g tissue wet weight) in the 500 μg Pb/g + 20 mg $Ca^{2+}$/g exposed fish, followed by a significant depuration by day 42 (0.6 μg Pb/g tissue wet weight; Fig. 4a). A similar pattern was observed in the mid-intestine (Fig. 5c), and the anterior kidney (Fig. 6a), whereas Pb burdens in most other tissues were more or less stable from days 14 through 42. Taken together, these data suggest that Pb regulation may have taken place in the high dietary Pb treatments. Similarly, fish exposed to the 50 μg Pb/g + 20 mg $Ca^{2+}$/g exhibited approximately stable Pb burden from days 14 to 42 in many tissues, suggesting homeostatic regulation.

The bone (Fig. 7) had a distinct pattern of Pb accumulation in fish fed the 500 μg Pb/g + 20 mg $Ca^{2+}$/g diet. In short, Pb accumulation was significantly increased on day 14 with a significant reduction on day 28. No significant differences were observed in Pb accumulation between days 28 and 42, and days 14 and 42. In fish fed the low dietary Pb, bone Pb levels remained fairly close to control background levels.

The two tissues with the lowest Pb accumulation, the white muscle (Table 3) and brain (Table 3) had approximately three times greater Pb accumulations than background control levels in fish fed the high Pb diets over the duration of the experiment. The carcass (Table 3) had an initially high Pb accumulation by day 14 (0.55 μg Pb/g), with Pb levels remaining stable around 0.43 μg Pb/g over the duration of the experiment in fish fed a 500 μg Pb/g + 20 mg $Ca^{2+}$/g diet. In fish fed the low dietary Pb, Pb accumulations in these compartments were close to control background levels.

### 3.4. *Effects of elevated dietary $Ca^{2+}$ on patterns of Pb accumulation in tissue*

Elevated dietary $Ca^{2+}$ levels had a protective effect in reducing whole body Pb burden (Fig. 2) on all days in fish fed the high dietary Pb diet. Whole body Pb burden was significantly reduced by 61–78% on all days in fish fed the 500 μg Pb/g + 60 mg $Ca^{2+}$/g treatment when compared to fish fed the 500 μg Pb/g + 20 mg $Ca^{2+}$/g diet. However, elevated dietary $Ca^{2+}$ levels had a significant effect in reducing whole body Pb burden by 66% only on day 14 in fish fed the 50 μg Pb/g + 60 mg $Ca^{2+}$/g diet when compared to fish fed the 50 μg Pb/g + 20 mg $Ca^{2+}$/g diet.

A three-way ANOVA was done to factor in time, dietary $Ca^{2+}$ and Pb levels on the overall effect of $Ca^{2+}$ on each tissue. Overall, elevated $Ca^{2+}$ levels had a protective effect in reducing tissue Pb burden in all tissues, except the brain of trout fed the high dietary Pb levels. The same was true for fish fed the low dietary Pb levels with elevated $Ca^{2+}$ but only in the anterior intestine, anterior kidney, bone and liver. Despite, the overall effect of $Ca^{2+}$, the individual time points were not significantly different in many of the tissues between the 500 μg Pb/g + 20 mg $Ca^{2+}$/g and 500 μg Pb/g + 60 mg $Ca^{2+}$/g treatments and the 50 μg Pb/g + 20 mg $Ca^{2+}$/g and 50 μg Pb/g + 60 mg $Ca^{2+}$/g treatments.

The following effects of elevated dietary $Ca^{2+}$ are particularly notable.

Fish fed the elevated $Ca^{2+}$ diets (500 μg Pb/g + 60 mg $Ca^{2+}$/g) exhibited a 90% reduction in gill Pb concentrations when compared to fish fed elevated Pb alone (500 μg Pb/g + 20 mg $Ca^{2+}$/g) on day 28, with lesser reductions also present on other days (Fig. 4a).

The stomach had an equally high Pb accumulation on day 14 in fish fed control and elevated $Ca^{2+}$ diets contaminated with Pb, but thereafter, there was a continuous decrease in the latter (Fig. 5a) A significant six-fold lower Pb burden in fish fed the 500 μg Pb/g + 60 mg $Ca^{2+}$/g was observed on day 42 in the stomach relative to the 500 μg Pb/g + 20 mg $Ca^{2+}$/g treatment.

Pb accumulations in the anterior intestine (Fig. 5b) of fish fed the elevated dietary $Ca^{2+}$ levels were stable around 2.7 μg Pb/g

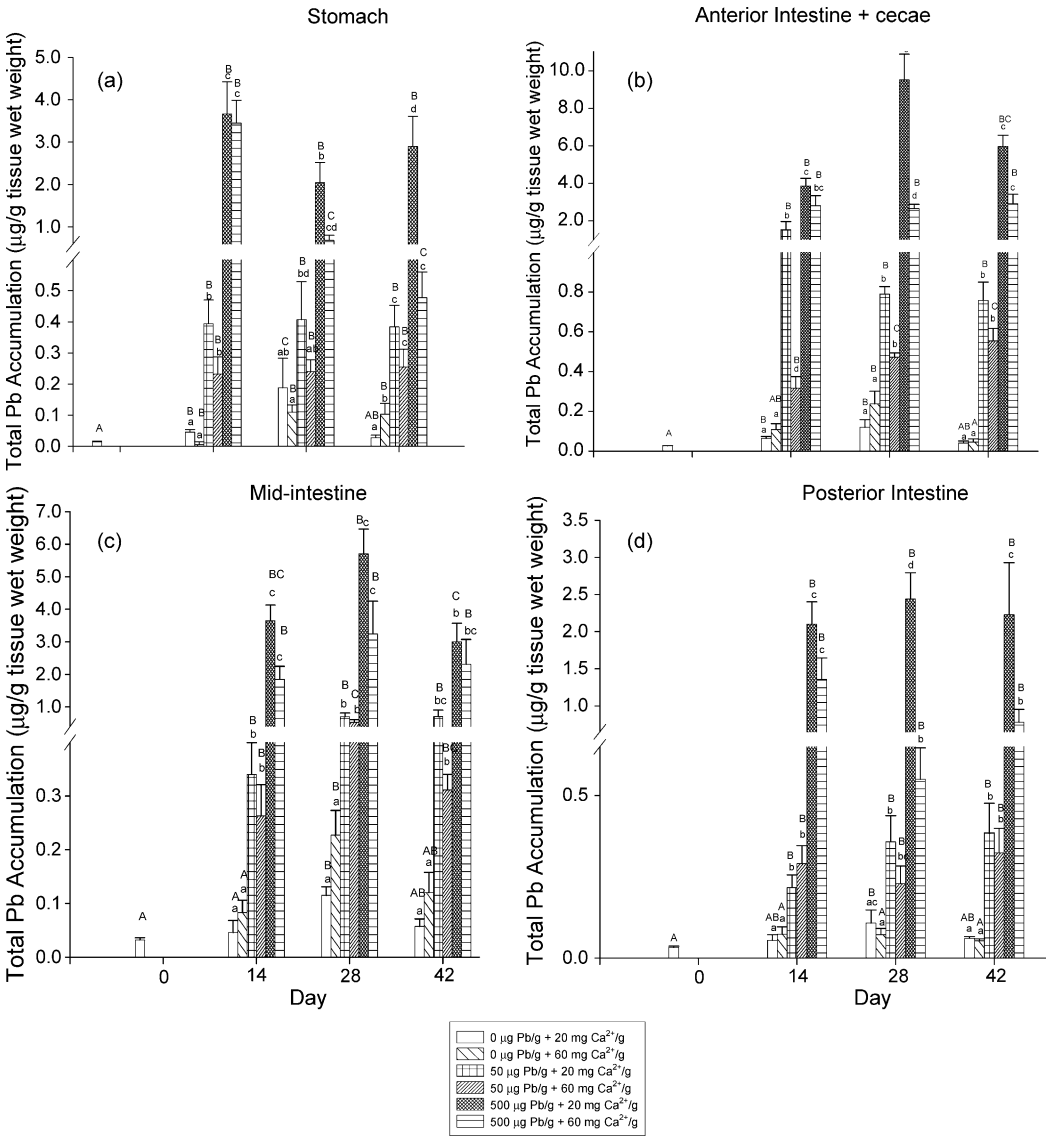


Fig. 5. Lead accumulation along the gastrointestinal tract of juvenile rainbow trout over 42 days. Note different scale used for different sections: (a) stomach; (b) anterior intestine + pyloric cecae; (c) mid-intestine; (d) posterior intestine. Other details as in legend of Fig. 2.

tissue wet weight over the duration of the experiment. Elevated dietary $Ca^{2+}$ significantly reduced Pb burden in the anterior intestine by 67% on day 28 between 500 µg Pb/g + 20 mg $Ca^{2+}$/g and 500 µg Pb/g + 60 mg $Ca^{2+}$/g treatments. In the mid-intestine (Fig. 5c), fish fed elevated dietary $Ca^{2+}$ had a similar pattern of Pb uptake as that of fish fed the high Pb diet alone. Despite elevated dietary $Ca^{2+}$ having an overall effect on Pb burden in the mid-intestine, elevated $Ca^{2+}$ did not have a significant effect in reducing Pb burden on individual sampling days in this tissue. Elevated $Ca^{2+}$ was effective in reducing Pb burden in the posterior intestine (Fig. 5d) by 65–80% on days 28 and 42 when compared to high dietary Pb alone.

Dietary $Ca^{2+}$ was significant in reducing Pb burden by 70–90% over the duration of the experiment in the anterior kidney (Fig. 6a). Similarly, dietary $Ca^{2+}$ was effective in reducing Pb burden in the posterior kidney (Fig. 6b) by 70% on day 28 when compared to the high dietary Pb alone.

Elevated dietary $Ca^{2+}$ significantly reduced Pb burden in the liver by 41% on day 42 (Fig. 4b). Dietary $Ca^{2+}$ (500 µg Pb/g + 60 mg $Ca^{2+}$/g) resulted in a significant three- and seven-fold decrease in Pb accumulation in the bone on days 28 and 42, respectively.

Notably, Pb burdens in the white muscle (Table 3) were significantly reduced by 66% on day 14 in fish fed the high Pb, high $Ca^{2+}$ diet, when compared to fish fed the high Pb, low $Ca^{2+}$ diet. Compared to the high Pb diet alone, the elevated $Ca^{2+}$ diet significantly reduced Pb burden in the carcass by approximately 70% on days 14 and 28 (Table 3).

### *3.5. Plasma and RBC Pb burden, whole blood burden and ALAD activity*

When the blood plasma (Table 3) and RBC (Fig. 8) were compared in terms of Pb burden on day 35 in all dietary treatments, it was found that virtually all the Pb (~98–99%) was present

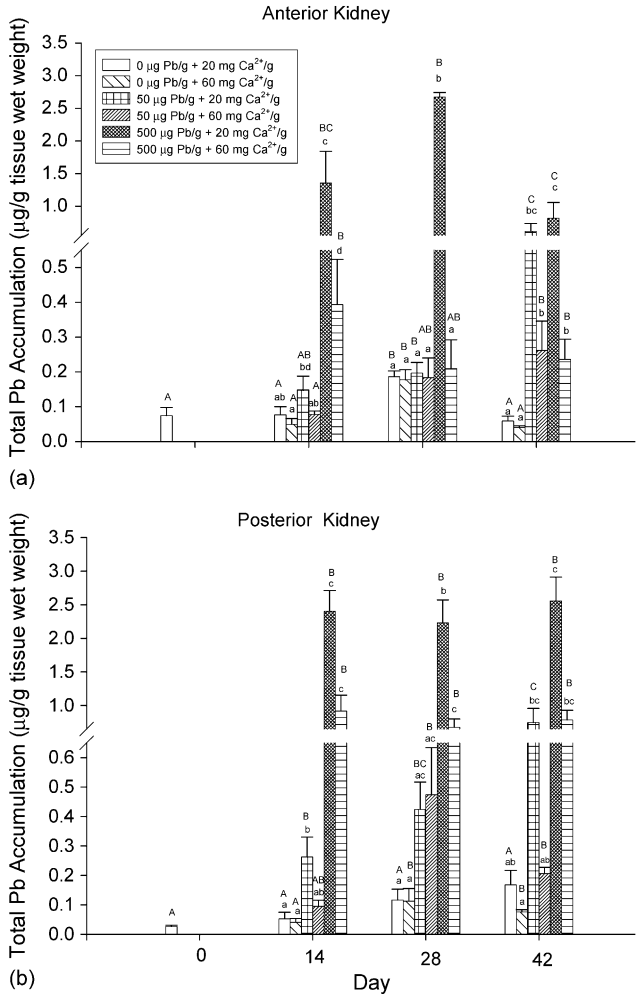


Fig. 6. Lead accumulation in the anterior and posterior kidney of juvenile rainbow trout over 42 days: (a) anterior kidney; (b) posterior kidney. Other details as in legend of Fig. 2.

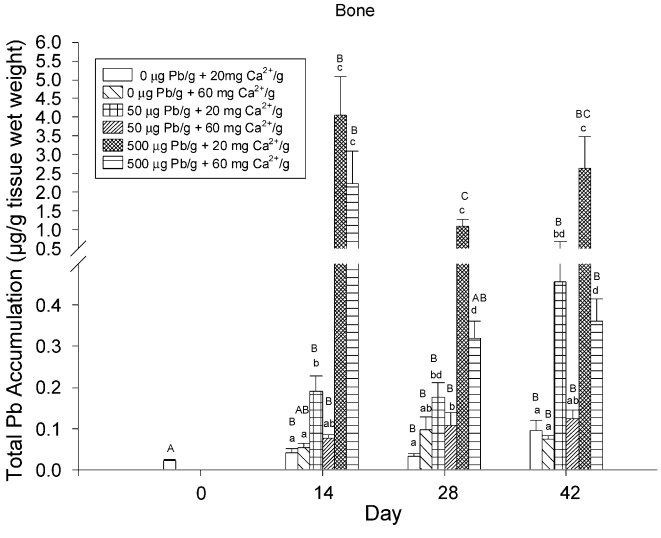


Fig. 7. Lead accumulation in the bone of juvenile rainbow trout over 42 days. Other details as in legend of Fig. 2.

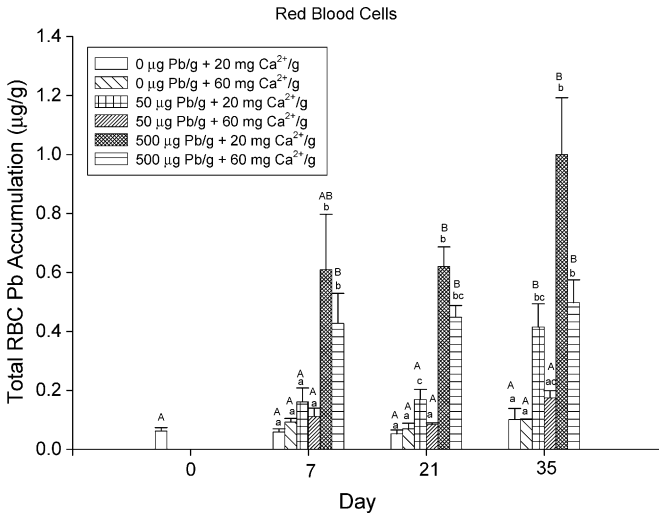


Fig. 8. Lead accumulation in the red blood cells of juvenile rainbow trout over 35 days. Data reported as mean ± 1 S.E.M.; $N = 6$. Other details as in legend of Fig. 2.

in the RBCs, with almost no Pb (1–2%) being present in the plasma. Effects of elevated dietary Pb and/or $Ca^{2+}$ on plasma Pb concentrations could not be detected, though there was a general tendency for plasma Pb levels to decrease with time (Table 3). Pb burden in the RBCs increased gradually over time from day 7 (0.6 µg Pb/g RBC wet weight) to day 35 (1.0 µg Pb/g RBC wet weight) in the 500 µg Pb/g + 20 mg $Ca^{2+}$/g treatment. RBC Pb burden in the 500 µg Pb/g + 60 mg $Ca^{2+}$/g treatment increased from 0.4 ug Pb/g RBC wet weight on day 7 to 0.6 µg Pb/g RBC wet weight on day 35. Despite there being no significant differences on individual days, elevated $Ca^{2+}$ did have a significant overall effect in reducing Pb burdens in the RBC (Fig. 8) and whole blood (Table 4).

Whole blood Pb burden was significantly elevated in the high Pb treatments groups with and without $Ca^{2+}$ when compared to the controls on days 14–42 (Table 4). Elevated dietary $Ca^{2+}$ levels were significant in reducing whole blood Pb burden between the 500 µg Pb/g + 20 mg $Ca^{2+}$/g (0.30 µg Pb/g blood wet weight) and the 500 µg Pb/g + 60 mg $Ca^{2+}$/g (0.15 µg Pb/g blood wet weight) treatments on day 28. Similarly, a significant reduction in Pb accumulation was also present between the 50 µg Pb/g + 20 mg $Ca^{2+}$/g diet (0.14 µg Pb/g blood wet weight) and the 50 µg Pb/g + 60 mg $Ca^{2+}$/g diet (0.07 µg Pb/g blood wet weight) on day 42.

When ALAD activity (nmol PBG/g/h) was plotted against the natural logarithm of whole blood Pb concentration (Fig. 9), only 9% of variability in ALAD activity was explained by dietary Pb in the blood in all treatments tested; this relationship was not significant ($P = 0.45$). However, a significant reduction in the ALAD activity between the 0 µg Pb/g + 20 mg $Ca^{2+}$/g treatment and 500 µg Pb/g + 20 mg $Ca^{2+}$/g treatment on day 42 was observed, with whole blood Pb concentrations of 0.07 µg Pb/g and 0.47 µg Pb/g, respectively (Table 4). Despite this significant decrease, there were no significant effects on Hb levels,

Table 3
Pb tissue burden in the brain, carcass, spleen and white muscle (μg Pb/g tissue wet wt.) over 42 days

| Tissue | Day | 0 μg Pb/g + 20 mg $Ca^{2+}$/g | 0 μg Pb/g + 60 mg $Ca^{2+}$/g | 50 μg Pb/g + 20 mg $Ca^{2+}$/g | 50 μg Pb/g + 60 mg $Ca^{2+}$/g | 500 μg Pb/g + 20 mg $Ca^{2+}$/g | 500 μg Pb/g + 60 mg $Ca^{2+}$/g |
|---|---|---|---|---|---|---|---|
| Brain (μg Pb/g tissue wet wt.) | 0 | 0.07 ± 0.02 A | 0.07 ± 0.02 A | 0.07 ± 0.02 A | 0.07 ± 0.02 AB | 0.07 ± 0.02 A | 0.07 ± 0.02 A |
| | 14 | 0.06 ± 0.01 Aa | 0.06 ± 0.04 Aa | 0.13 ± 0.02 Ab | 0.04 ± 0.01 Aa | 0.27 ± 0.08 Bb | 0.26 ± 0.1 Ab |
| | 28 | 0.12 ± 0.02 Aa | 0.10 ± 0.02 Aa | 0.12 ± 0.03 Aa | 0.20 ± 0.07 Ba | 0.31 ± 0.09 Ba | 0.26 ± 0.1 Aa |
| | 42 | 0.08 ± 0.03 Aac | 0.04 ± 0.01 Aa | 0.08 ± 0.02 Aac | 0.07 ± 0.02 ABa | 0.24 ± 0.09 ABbc | 0.18 ± 0.04 Abc |
| Carcass (μg Pb/g tissue wet wt.) | 0 | 0.01 ± 0.00 A | 0.01 ± 0.00 A | 0.01 ± 0.00 A | 0.01 ± 0.00 A | 0.01 ± 0.00 A | 0.01 ± 0.00 A |
| | 14 | 0.03 ± 0.00 ABab | 0.02 ± 0.00 Ba | 0.08 ± 0.03 Bbd | 0.04 ± 0.01 Bab | 0.55 ± 0.08 Bc | 0.17 ± 0.03 Bd |
| | 28 | 0.07 ± 0.02 Bac | 0.07 ± 0.01 Cac | 0.05 ± 0.01 ABa | 0.07 ± 0.02 Bac | 0.42 ± 0.08 Bb | 0.13 ± 0.02 Bc |
| | 42 | 0.02 ± 0.00 Aa | 0.02 ± 0.00 ABa | 0.06 ± 0.01 Bb | 0.06 ± 0.01 Bb | 0.44 ± 0.09 Bc | 0.23 ± 0.05 Bc |
| Spleen (μg Pb/g tissue wet wt.) | 0 | 0.04 ± 0.01 A | 0.04 ± 0.01 A | 0.04 ± 0.01 A | 0.04 ± 0.01 A | 0.04 ± 0.01 A | 0.04 ± 0.01 A |
| | 14 | 0.07 ± 0.01 Aa | 0.04 ± 0.01 Aa | 0.21 ± 0.05 Bcd | 0.09 ± 0.02 Bac | 0.69 ± 0.21 Bb | 0.41 ± 0.17 Bbd |
| | 28 | 0.07 ± 0.02 Aa | 0.08 ± 0.02 Aa | 0.09 ± 0.03 Ba | 0.10 ± 0.02 Ba | 0.29 ± 0.03 Bb | 0.42 ± 0.28 Bab |
| | 42 | 0.06 ± 0.01 Aa | 0.09 ± 0.02 Aac | 0.16 ± 0.03 Bbc | 0.15 ± 0.02 Bbc | 0.52 ± 0.11 Bd | 0.24 ± 0.04 Bbd |
| White muscle (μg Pb/g tissue wet wt.) | 0 | 0.02 ± 0.01 A | 0.02 ± 0.01 A | 0.02 ± 0.01 A | 0.02 ± 0.01 A | 0.02 ± 0.01 A | 0.02 ± 0.01 A |
| | 14 | 0.08 ± 0.02 Bac | 0.12 ± 0.01 Bbc | 0.07 ± 0.01 Bac | 0.05 ± 0.01 Ba | 0.29 ± 0.07 Bb | 0.10 ± 0.02 Bac |
| | 28 | 0.05 ± 0.02 ABa | 0.09 ± 0.04 ABab | 0.09 ± 0.01 Bab | 0.05 ± 0.01 Ba | 0.14 ± 0.02 Bb | 0.07 ± 0.01 Bab |
| | 42 | 0.07 ± 0.02 Bac | 0.04 ± 0.01 ABa | 0.04 ± 0.01 ABa | 0.09 ± 0.03 Bab | 0.17 ± 0.04 Bb | 0.11 ± 0.02 Bbc |
| Plasma Pb (μg/L) | 0 | 9.7 ± 1.8 A | 9.7 ± 1.8 A | 9.7 ± 1.8 A | 9.7 ± 1.8 A | 9.7 ± 1.8 A | 9.7 ± 1.8 A |
| | 7 | 7.5 ± 0.9 Aab | 9.9 ± 1.6 Aab | 8.9 ± 1.3 Aa | 6.1 ± 0.3 ABa | 11.4 ± 2.4 Aab | 13.4 ± 1.5 Aab |
| | 21 | 9.1 ± 1.5 Aa | 8.2 ± 0.5 Aa | 8.0 ± 0.3 Aa | 9.4 ± 1.7 Aa | 7.7 ± 1.0 ABa | 7.2 ± 0.9 Aa |
| | 35 | 7.1 ± 0.4 Aa | 5.1 ± 1.6 Aab | 2.8 ± 0.4 Bb | 3.7 ± 0.4 Bab | 3.7 ± 0.4 Bab | 4.5 ± 0.6 Bab |

Data are expressed as mean ± 1 S.E.M. Upper case letters indicate significant differences between days within the same treatment ($P < 0.05$). Lower case letters represent significant differences between treatments within the same day; values sharing the same letter are not significantly different ($P < 0.05$).

total blood protein, and whole blood Zn levels (Table 4) over the duration of the experiment in all treatments.

### 3.6. Ion regulation and $Na^+$, $K^+$-ATPase activity

There were no significant differences observed in plasma $Cl^-$ (overall mean = 129 ± 3 mmol $L^{-1}$) and $K^+$ (overall mean = 5.1 ± 0.4 mmol $L^{-1}$) in all treatments when compared to the controls over the duration of the experiment (data not shown). However, significant differences did exist in plasma $Na^+$ levels on day 21 and $Ca^{2+}$ levels on days 7 and 21 (Table 5). In short, plasma $Na^+$ levels were significantly increased in the 50 μg Pb/g + 20 mg $Ca^{2+}$/g and 500 μg Pb/g + 20 mg $Ca^{2+}$/g treatments when compared to both of the controls. Plasma $Na^+$ concentrations in the 500 μg Pb/g + 60 mg $Ca^{2+}$/g treatment were significantly elevated when compared to the 0 + 60 mg $Ca^{2+}$/g diet. Plasma $Ca^{2+}$ levels were significantly decreased on day 7 in the 500 μg Pb/g + 60 mg $Ca^{2+}$/g treatment when compared to the 0 μg Pb/g + 20 mg $Ca^{2+}$/g and 50 μg Pb/g + 20 mg $Ca^{2+}$/g treatments. However, $Ca^{2+}$ levels were significantly elevated on day 21 in the above treatments when compared to the controls. Furthermore, $Mg^{2+}$ and $Ca^{2+}$ levels were significantly lower on days 21 and 35 within the same treatment when compared to days 0 and 7 (Table 5).

There were no significant effects of dietary Pb on $Na^+$, $K^+$-ATPase activity in the gills, mid-intestine, and posterior intestine on day 35 (Fig. 10). However, there was a significant up-regulation in $Na^+$, $K^+$-ATPase activity in the 500 μg Pb/g + 20 mg $Ca^{2+}$/g and 500 μg Pb/g + 60 mg $Ca^{2+}$/g when compared to the 0 μg Pb/g + 20 mg $Ca^{2+}$/g treatment on day 35 in the anterior intestine.

Table 4
Mean hemoglobin, total blood protein, whole blood-Pb, ALAD concentrations and whole blood Zn concentrations following 42 days exposure to different dietary Pb diets

| Treatment | Hemoglobin (g/L) | Total blood protein (g/L) | Blood Pb (μg/L) | ALAD (nMol PBG/g RBC/h) | Blood–Zn (μg/L) |
|---|---|---|---|---|---|
| 0 μg Pb/g + 20 mg $Ca^{2+}$/g | 132 ± 6 a | 167 ± 6 a | 68 ± 7 a | 447 ± 33 a | 7500 ± 1500 a |
| 0 μg Pb/g + 60 mg $Ca^{2+}$/g | 120 ± 7 a | 159 ± 9 a | 67 ± 10 a | 336 ± 27 ab | 10700 ± 1000 a |
| 50 μg Pb/g + 20 mg $Ca^{2+}$/g | 130 ± 5 a | 153 ± 7 a | 137 ± 18 b | 337 ± 11 ab | 10300 ± 700 a |
| 50 μg Pb/g + 60 mg $Ca^{2+}$/g | 128 ± 4 a | 161 ± 4 a | 70 ± 7 a | 428 ± 54 ab | 10400 ± 1900 a |
| 500 μg Pb/g + 20 mg $Ca^{2+}$/g | 127 ± 5 a | 153 ± 8 a | 473 ± 127 c | 322 ± 31 b | 10100 ± 500 a |
| 500 μg Pb/g + 60 mg $Ca^{2+}$/g | 124 ± 6 a | 164 ± 8 a | 242 ± 27 c | 439 ± 68 ab | 11300 ± 800 a |

Other details as in legend of Table 3.

Table 5
Plasma $Na^-$, $Ca^{2+}$, and $Mg^{2+}$ concentrations (mM)

| Plasma ion | Day | 0 μg Pb/g + 20 mg $Ca^{2+}$/g | 0 μg Pb/g + 60 mg $Ca^{2+}$/g | 50 μg Pb/g + 20 mg $Ca^{2+}$/g | 50 μg Pb/g + 60 mg $Ca^{2+}$/g | 500 μg Pb/g + 20 mg $Ca^{2+}$/g | 500 μg Pb/g + 60 mg $Ca^{2+}$/g |
|---|---|---|---|---|---|---|---|
| $Na^+$ (mM) | 0 | 158 ± 5 A | 158 ± 5 A | 158 ± 5 AC | 158 ± 5 A | 158 ± 5 A | 158 ± 5 A |
| | 7 | 155 ± 5 Aab | 143 ± 6 Aa | 173 ± 7 ABb | 149 ± 3 Aab | 155 ± 5 Aab | 157 ± 10 Aab |
| | 21 | 145 ± 3 Aac | 143 ± 5 Aa | 184 ± 2 Bb | 158 ± 4 Aab | 179 ± 7 Ab | 168 ± 8 ABbc |
| | 35 | 155 ± 12 Aab | 158 ± 3 Aab | 151 ± 4 Cb | 149 ± 7 Ab | 158 ± 12 Aab | 189 ± 9 Ba |
| $Ca^{2+}$ (mM) | 0 | 2.9 ± 0.1 A | 2.9 ± 0.1 AB | 2.9 ± 0.1 A | 2.9 ± 0.1 AB | 2.9 ± 0.1 A | 2.9 ± 0.1 A |
| | 7 | 2.9 ± 0.1 Aa | 2.5 ± 0.1 Aab | 2.8 ± 0.1 Aa | 2.6 ± 0.1 Aab | 2.4 ± 0.2 Bab | 2.1 ± 0.2 Bb |
| | 21 | 2.6 ± 0.1 Aa | 2.5 ± 0.1 Aa | 2.7 ± 0.1 Aab | 2.5 ± 0.1 Aa | 2.7 ± 0.1 ABab | 3.0 ± 0.1 Ab |
| | 35 | 2.5 ± 0.3 Aa | 3.1 ± 0.3 Ba | 3.0 ± 0.2 Aa | 3.1 ± 0.1 Ba | 3.2 ± 0.2 Aa | 2.7 ± 0.2 Aa |
| $Mg^{2+}$ (mM) | 0 | 1.1 ± 0.1 A | 1.1 ± 0.1 A | 1.1 ± 0.1 A | 1.1 ± 0.1 A | 1.1 ± 0.1 A | 1.1 ± 0.1 AC |
| | 7 | 0.9 ± 0.1 ABa | 0.9 ± 0.1 ABa | 0.9 ± 0.1 ABa | 0.8 ± 0.1 ABa | 0.9 ± 0.1 ABa | 0.9 ± 0.1 Aa |
| | 21 | 0.9 ± 0.0 Ba | 0.8 ± 0.0 Ba | 0.8 ± 0.0 Ba | 0.7 ± 0.1 Ba | 0.8 ± 0.0 Ba | 0.7 ± 0.1 BCa |

Other details as in legend of Table 3.

## 4. Discussion

### 4.1. Diet

Dietary Pb concentrations (Table 1) were chosen to duplicate reported Pb concentrations found in contaminated benthic invertebrates in the environment (Woodward et al., 1994, 1995; Farag et al., 1994, 1999). Previous studies have found significant reductions in whole body Cd uptake from the water and diet when rainbow trout were fed a diet supplemented with 60 mg $CaCO_3$/g (Baldisserotto et al., 2004b, 2005; Franklin et al., 2005), or 60 mg $CaCl_2$ (Zohouri et al., 2001; Baldisserotto et al., 2004a), though some negative physiological effects associated with the $Cl^-$ anion were found in the latter studies. Since Cd is a Pb antagonist, a dietary $Ca^{2+}$ concentration of 60 mg $CaCO_3$/g was chosen for the present study.

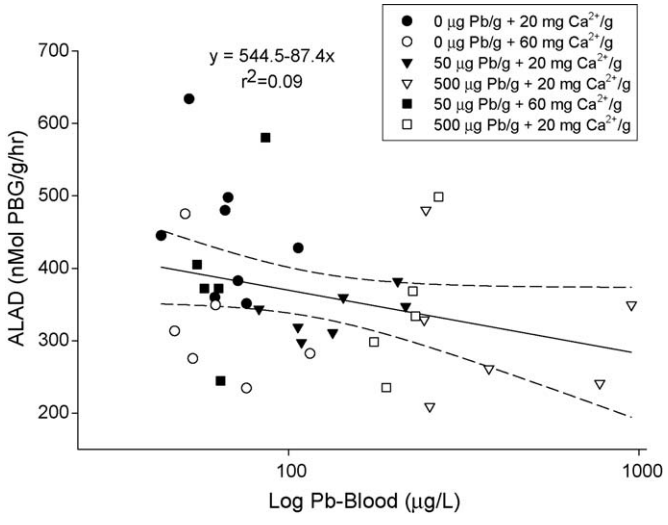


Fig. 9. Regression between linear ALAD activity (on a linear scale) and log blood Pb concentration on day 42. Each symbol represents a fish in an individual treatment ($N$ = 5–8 per treatment). Not enough blood was available for some of the ALAD and Pb burden assays, thus explaining the low N value in some treatments. Solid line represents the linear regression, while the dashed lines represents the upper and lower 95% confidence intervals.

### 4.2. Growth and survival

There were no mortalities (Table 2), no significant effects on fish weight or CF (Table 2) and only minor differences in FCE in this experiment. These results are consistent with Alves et al. (2006) who found no effects on survival and growth when juvenile rainbow trout were fed commercial trout pellets amended with various levels of Pb (7–520 μg Pb/g) for 21 days. They are also consistent with the results of Hodson et al. (1978) who found no effects on survival of rainbow trout fed beef liver diets contaminated with Pb levels up to 118 μg Pb/g dw for 32 weeks, and Mount et al. (1994) who found no effects on survival and growth of rainbow trout fed brine shrimp contaminated with 170 μg Pb/g dw for 60 days.

Fish fed the high $Ca^{2+}$ and Pb diets, nevertheless, did have non-significantly lower SGR, weight gains, and FCE than those fed the low dietary $Ca^{2+}$ and Pb diets. Andrews et al. (1973) noted that when channel catfish (*Ictalurus punctatus*) fingerling

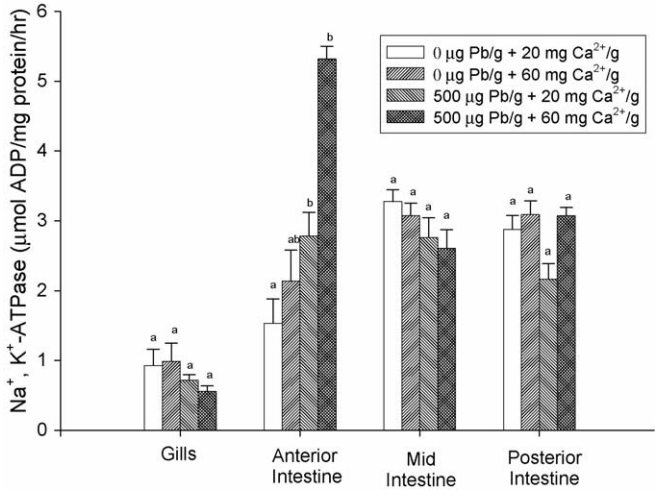


Fig. 10. $Na^+$, $K^+$-ATPase activity in the gills and intestine on day 35. Data represented as mean ± 1 S.E.M.; $N$ = 6. Other details as in legend of Fig. 2.

were fed diets containing 2% $Ca^{2+}$ in the form of $CaCO_3$ and reared at waterborne $Ca^{2+}$ levels of 56 mg $Ca^{2+}$/L (1.4 mM $Ca^{2+}$), there were decreases in growth and FCE in these fish compared to fish fed diets containing 0.5, 1, and 1.5% $Ca^{2+}$. In the present study the background and enhanced $Ca^{2+}$ diets contained 2% and 6% $Ca^{2+}$, respectively. The authors suggested that these reductions may be the result of $Ca^{2+}$ competing with the uptake of Zn and $Mg^{2+}$ from the diet, which are essential nutrients. In contrast to channel catfish, juvenile rainbow trout may need a higher dietary $Ca^{2+}$ diet threshold (>6%) in order for there to be a significant decrease in growth and FCE. Overall, growth rates do not appear to be sensitive indicators of dietary Pb toxicity.

### 4.3. Tissue specific accumulation

The present exposures were completed without significant elevations in waterborne Pb levels. It can be concluded unequivocally that Pb of dietary origin does accumulate in the internal tissues and whole body in agreement with Crespo et al. (1986) and Mount et al. (1994) respectively, but in contrast to Hodson et al. (1978) who found that dietary Pb (4–118 μg Pb/g dw; 32 weeks) did not accumulate in the internal tissues. However, the latter study used beef liver as the diet, and reported that Pb bound to beef liver was not leached from the food when placed in water and acid solutions, suggesting that Pb was not bioavailable for uptake by the fish in this diet.

Ten out of the 13 tissues (stomach, mid-intestine, liver, posterior kidney, gills, bone, carcass, brain, spleen, and white muscle) analysed in the low dietary Pb treatments with and without supplemented $Ca^{2+}$ were within a factor of 3 of background tissue levels (0.07 μg Pb/g). This suggests that concentrations greater than 50 μg Pb/g are necessary to result in clear Pb accumulation in these tissues.

This study points towards the intestine as a potential target tissue for dietary Pb toxicity. When the whole body tissue burdens are considered in μg of Pb in the total tissue (not shown), the anterior intestine + pyloric cecae (Fig. 5b), mid-intestine (Fig. 5c), and posterior intestine (Fig. 5d) accumulated 2.9 μg Pb (total tissue), 0.32 μg Pb (total tissue) and 0.18 μg Pb (total tissue), respectively in fish fed the high Pb, low $Ca^{2+}$ diets. This suggests that the anterior intestine + pyloric cecae may act as a sink for Pb, when compared to the mid-intestine and posterior intestine. The importance of the anterior intestine + pyloric cecae in Pb uptake is probably due to its large surface area, and ability to digest proteins and lipids (Kleinow and James, 2001). Pb's ability to inhibit protein/enzyme activity by either directly binding to the enzymes, or by reacting with sulfhydryl (SH), amine, phosphate and carboxyl groups (Goering, 1993) on other compounds may help explain the high concentration of Pb at this site.

Pb's ability to bind to the intestinal mucus may serve to sequester Pb that can be absorbed by the gut or excreted or sloughed off during the renewal of the epithelial cells in freshwater fish (Stroband and Debets, 1978), therefore, preventing Pb accumulation and toxicity, and explaining the high Pb concentrations along the gastrointestinal tract. For instance, Glover and Hogstrand (2002) found that mucus and epithelial cells accumulated up to 74% of the retained Zn, whereas Chowdhury et al. (2004) observed that <7% of the Cd infused into the gastrointestinal tract was absorbed by the intestine, whereas, 10–24% remained bound to the gut wall and 16–33% was present in the lumen.

The stomach (Fig. 5a) accumulated a significant concentration of Pb throughout the whole study (3.7–2.5 μg Pb/g tissue wet weight, days 14 and 42) when compared to the controls. This is consistent with Farag et al. (1994) who found that rainbow trout fed a contaminated mixed metal (Al, As, Cd, Cu, Cr, Pb, Hg, Zn) benthic invertebrate diet for 21 days exhibited higher Pb burden in the stomach and pyloric cecae when compared to the gills and kidney. Moreover, Köck et al. (1998) found that the Pb burden of the stomach and gut tissues of arctic char (*Salvelinus alpinus*) was positively correlated with Pb concentrations in their diets. The acidic environment of the stomach (pH 2–4) (Kleinow and James, 2001) may permit Pb to exist in the dissolved ionic form. However, mucus, which is secreted copiously in the stomach, and which acts as a pH buffer, may prevent Pb absorption (Whitehead et al., 1996). Nevertheless, this mucus may bind and sequester the Pb, as in the intestine, thus explaining the high Pb concentrations in the stomach.

The posterior kidney (Fig. 6b) accumulated more dietary Pb on average than the anterior kidney (Fig. 6a) in fish fed the 500 ug Pb/g + 20 mg $Ca^{2+}$/g treatment on days 14 and 42. These results are similar to Reichert et al. (1979) who found that coho salmon exposed to 150 μg/L of waterborne Pb for 15 days accumulated Pb primarily in the posterior kidney when compared to the anterior kidney. The posterior kidney mainly functions in renal ion transport, such as $Ca^{2+}$ re-absorption (Larsen and Perkins, 2001). Since Pb is a $Ca^{2+}$ antagonist, Pb may compete with $Ca^{2+}$ for uptake at the posterior kidney explaining the high Pb burden present in this tissue, and the possible disturbances in plasma $Ca^{2+}$ regulation seen in fish on high Pb diets (Table 5).

In light of the significant amount of Pb accumulated by the whole blood (0.47 μg Pb/g on day 42 in the 500 μg Pb/g + 20 mg $Ca^{2+}$/g treatment; Table 4), the percentage of accumulated Pb that could be explained purely by trapped whole blood within each tissue was calculated using the estimated mean blood volumes in different tissues of freshwater rainbow trout from Olson (1992) (not shown). In short, the high blood content of the spleen was sufficient to explain the full extent (i.e. 100%) of its accumulated Pb burden. Vascularization also helps to explain the high Pb burden in the renal tissue, accounting for about 20% of the Pb burden. Other tissues where the contribution of the trapped blood was significant were the brain (∼12%), gills (∼11%), and liver (∼10%). The liver (Fig. 4b) continuously accumulated Pb in fish fed the high Pb, low $Ca^{2+}$ diet throughout this study. Pb levels in the liver were lower than those of the intestine, kidney and bone. Studies with Pb (Reichert et al., 1979; Campana et al., 2003) have found fish livers to lack Pb-binding proteins, such as metallothioneins, that help in the detoxification of other metals. This may explain why the liver on average had a relatively low Pb burden. In all

other tissues, trapped blood could explain less than 1% of the observed Pb burdens.

The bone (Fig. 7) accumulated a substantial concentration of Pb (2.6 μg Pb/g tissue wet weight) when compared to many other tissues. Hodson et al. (1978) found that the opercular bone had the greatest accumulation of Pb in rainbow trout exposed to waterborne Pb when compared to the gills and kidney. These authors suggested that the bone provides a site for Pb storage and detoxification. Since Pb is a $Ca^{2+}$ antagonist, the high concentrations of Pb in the bone may be explained by Pb using similar uptake pathways to those of $Ca^{2+}$ (see discussion below). In the long term, this detoxification mechanism may have negative consequences. For instance, when dietary and/or waterborne $Ca^{2+}$ levels are low, $Ca^{2+}$ may be relocated from the bone, and this could simultaneously release Pb into circulation, and cause disturbances in $Ca^{2+}$ ion regulation.

The white muscle (Table 3) had significant levels of Pb accumulation. When Pb distribution (Fig. 2) in terms of whole body is considered, the white muscle accounted for 12% of the Pb accumulation. The same was true for the carcass remnants, which made up 10% of whole body Pb accumulation. These results are not surprising since the carcass and white muscle when combined make up 80–85% of the fish mass.

### 4.4. Is dietary Pb accumulation regulated?

When accumulated whole body Pb in μg/kg fish was considered (Fig. 2), Pb burden in all the Pb treatments with and without elevated dietary $Ca^{2+}$ levels remained more or less stable from days 14 through 42. Taken together, these data suggest that fish may be able to regulate Pb.

Gill burden (Fig. 4a) in this study was maintained around 2 μg Pb/g on days 14 and 28, with a significant reduction to 0.6 μg Pb/g by day 42 when rainbow trout were exposed to the 500 μg Pb/g + 20 mg $Ca^{2+}$/g treatment. This suggests that the gill may be able to adjust, regulate and redistribute Pb to other tissues for detoxification or excretion of Pb.

Pb concentrations in both the posterior intestine (2.3 μg Pb/g tissue wet weight; Fig. 5d) and posterior kidney (2.5 μg Pb/g tissue wet weight, Fig. 6b) remained stable on all days analysed in fish exposed to 500 μg Pb/g + 20 mg $Ca^{2+}$/g treatment. This suggests that the posterior intestine and posterior kidney are both able to regulate and excrete Pb, perhaps via the feces and urine, respectively. In addition, in most of the other tissues Pb burden was stable from days 14 to 42, reinforcing the idea that Pb may be regulated.

Evidence in favour of some sort of homeostatic regulation of Pb was the relationship between Pb accumulation and Pb dose in the diet. Despite a 10-fold difference between the two elevated Pb levels (500 versus 50 μg Pb/g), there was much less than a 10-fold difference in Pb accumulation in the whole body and most tissues. For instance, whole body Pb burden (Fig. 2) was only six to nine times higher in fish fed the high Pb diets when compared to the low diets. Similarly, a two- to eight-fold difference between the two elevated dietary Pb levels (500 μg Pb/g versus 50 μg Pb/g) was also evident in most individual tissues (Fig. 11a and b).

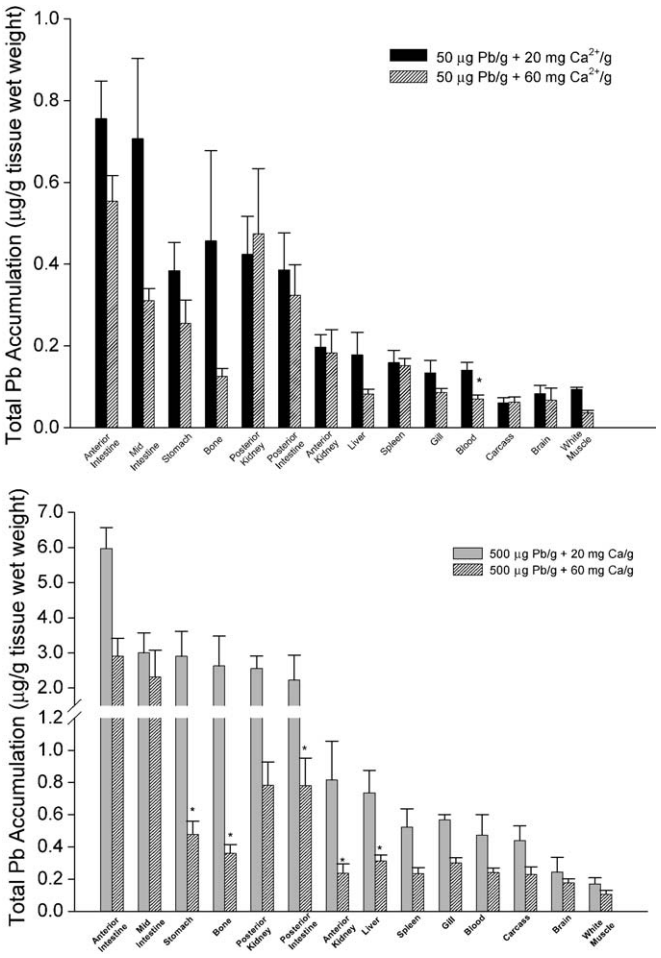


Fig. 11. Pb concentrations in different tissues of juvenile rainbow trout fed 50 μg Pb/g diet with and without supplemented dietary $Ca^{2+}$ and 500 μg Pb/g diet with and without supplemented dietary $Ca^{2+}$ over 42 days. (a) 50 μg Pb/g diet with and without supplemented dietary $Ca^{2+}$. (b) 500 μg Pb/g diet with and without supplemented dietary $Ca^{2+}$. Other Data represented as mean ± 1 S.E.M.; $N = 8$. Asterisk (*) indicates a significant difference ($P < 0.05$) between treatments within the same tissue.

### 4.5. Is elevated dietary $Ca^{2+}$ protective?

Elevated dietary $Ca^{2+}$ was clearly protective against the bioaccumulation of Pb and exerted its greatest effect in the stomach, bone, posterior intestine, anterior kidney, and liver, and the least effect at the anterior intestine, mid-intestine, posterior kidney, spleen, gill, carcass, brain and white muscle. This conclusion is based on data taken at the end of the experiment in fish exposed to the 500 μg Pb/g + 60 mg $Ca^{2+}$/g diet when compared to 500 μg Pb/g + 20 mg $Ca^{2+}$/g diet (Fig. 11b). This protective effect of elevated dietary $Ca^{2+}$ against Pb bioaccumulation may result in part from direct $Ca^{2+}$ versus Pb competition for uptake mechanisms at the gastrointestinal tract, in part from similar competition at the plasma–tissue interface, and in part from physiological mechanisms as discussed below.

Despite no effects of elevated dietary $Ca^{2+}$ on different days in the mid-intestine, and spleen in fish fed the high Pb, high $Ca^{2+}$ diet, elevated dietary $Ca^{2+}$ had an overall significant effect in reducing Pb burden in all tissues, except the brain. The low

Pb, elevated $Ca^{2+}$ diet had an overall effect in reducing Pb burdens only in the anterior intestine, anterior kidney, bone, and liver when compared to the low Pb diet alone. There was not a significant effect of elevated dietary $Ca^{2+}$ on individual tissues at the end of the experiment in fish fed the low Pb, high $Ca^{2+}$ diet when compared to the low Pb, low $Ca^{2+}$ diet (Fig. 11a).

Elevated dietary $Ca^{2+}$ levels resulted in significant reductions in whole body Pb burdens (Fig. 2) compared to fish fed the high dietary Pb alone, on all days. This is consistent with Franklin et al. (2005) who found that rainbow trout fed elevated dietary $Ca^{2+}$ levels exhibited a reduced whole body dietary Cd burden. In addition, Varanasi and Gmur (1978) found that the accumulation of waterborne Pb was greatly reduced in the gills, blood, liver, brain, bone and kidney of the coho salmon force-fed 8.4 mg $Ca^{2+}$ gelatin capsules.

The protective effect of elevated dietary $Ca^{2+}$ was observed on day 28 at the gills in fish fed the high Pb, high $Ca^{2+}$ diet when compared to the high Pb, low $Ca^{2+}$ diet. Since plasma $Ca^{2+}$ levels were significantly elevated on day 21 in fish fed the 500 μg Pb/g + 60 mg $Ca^{2+}$/g diet, it is suggested that elevated dietary $Ca^{2+}$ and increased $Ca^{2+}$ absorption at the intestine may trigger regulatory mechanisms such as a decrease in $Ca^{2+}$ influx rates and an increase in $Ca^{2+}$ efflux rates at the gills in order to maintain $Ca^{2+}$ balance. Since Pb is a $Ca^{2+}$ antagonist and mimic, an increase in $Ca^{2+}$ efflux rates at the gills may also increase Pb efflux rates, thus explaining the protective effect of elevated dietary $Ca^{2+}$ against dietary Pb burden at the gills.

Franklin et al. (2005) found that dietary $Ca^{2+}$ reduced dietary Cd burden in the stomach by at least two-fold. Similarly in this study, $Ca^{2+}$ significantly, reduced stomach burden by six-fold on day 42. Despite Pb having a higher affinity for gastrointestinal mucin than $Ca^{2+}$ in rats (Powell et al., 1999), elevated dietary $Ca^{2+}$ concentrations may be sufficient to out-compete dietary Pb for mucin binding sites in fish, thus explaining the low Pb burdens in the high Pb, high $Ca^{2+}$ diets when compared to the high Pb, low $Ca^{2+}$ diet, along the gastrointestinal tract (Fig. 5a–d).

The protective effects of elevated $Ca^{2+}$ at the posterior kidney and anterior kidney when compared to the 500 μg Pb/g + 20 mg $Ca^{2+}$/g treatment may be explained by kidney's role in the re-absorption of $Ca^{2+}$ (Larsen and Perkins, 2001). Elevated $Ca^{2+}$ levels in the glomerular filtration may compete with Pb at the kidney during $Ca^{2+}$ re-absorption, explaining the protective effect of $Ca^{2+}$ against Pb burden in the renal tissues. Since elevated dietary $Ca^{2+}$ was significant in reducing Pb burdens by 70–90% over the duration of the experiment in the anterior kidney (Fig. 6a) in trout fed the high Pb, high $Ca^{2+}$ diet when compared to the high Pb, low $Ca^{2+}$ diet, it is suggested that $Ca^{2+}$ and Pb may share a similar transport mechanism at the kidney.

Pb burden in the bone was 72–86% lower in fish fed elevated $Ca^{2+}$ diets. This is consistent with Varanasi and Gmur (1978) who found the coho salmon had reduced waterborne Pb burdens in the bone when exposed to increased levels of dietary $Ca^{2+}$. These authors suggested that Pb competes with $Ca^{2+}$ for a common pathway and/or transport mechanism at the bone.

Overall, dietary $Ca^{2+}$ was protective in reducing dietary Pb burdens in the whole body and individual tissues in this study. The protective effects of elevated dietary $Ca^{2+}$ may become important in new environmental regulations and approaches to Pb toxicity (e.g. Biotic Ligand Model; Paquin et al., 2002) that already take into account the water chemistry of exposure. Internal $Ca^{2+}$ status becomes very important to fish that live in Pb-contaminated soft water environments which tend to have lower pH's, since Pb in this environment is more likely to exist in the free ionic form, which is the most toxic to fish (Davies et al., 1976). Fish in these environments may choose diets that are enriched with $Ca^{2+}$, such as mollusks and crustaceans, not only to maintain $Ca^{2+}$ ion regulation, but also to protect against sublethal Pb toxicity. For example, increases in dietary $Ca^{2+}$ may reduce Pb uptake at the intestine, may down-regulate branchial $Ca^{2+}$ uptake pathway (and therefore Pb uptake pathways), and may increase $Ca^{2+}$ efflux rates (and therefore Pb efflux rates) so as to maintain $Ca^{2+}$ ion-regulation. For instance, Varanasi and Gmur (1978) found that elevated $Ca^{2+}$ levels in the diet were more protective against waterborne Pb burden than elevated $Ca^{2+}$ levels in the water, stressing the importance of dietary $Ca^{2+}$ in protecting against Pb accumulation and toxicity in aquatic environments.

### 4.6. Plasma ions and ionoregulatory effects

In freshwater fish the absorption of $Ca^{2+}$ via the intestine to the blood plasma is primarily by way of a $Na^+/Ca^{2+}$ exchanger and to a lesser degree by $Ca^{2+}$-ATPase. Therefore, $Ca^{2+}$ uptake at the intestine is dependent on $Na^+$ concentration at the epithelium and $Na^+$, $K^+$-ATPase activity (Flik et al., 1993).

Plasma $Ca^{2+}$ levels were found to be significantly decreased on day 7 in the 500 μg Pb/g + 60 mg $Ca^{2+}$/g treatment when compared to the controls (Table 5). Similarly, Alves et al. (2006) found plasma $Ca^{2+}$ levels to be decreased on day 7 in fish fed a 520 μg Pb/g diet for 21 days, suggesting that dietary Pb interferes with $Ca^{2+}$ ion regulation.

However, plasma $Ca^{2+}$ and $Na^+$ levels were significantly increased by day 21 in the 500 μg Pb/g + 60 mg $Ca^{2+}$/g treatment, and the 500 μg Pb/g + 60 mg $Ca^{2+}$/g and 500 μg Pb/g + 20 mg $Ca^{2+}$/g, respectively, when compared to the controls (Table 5). $Na^+$, $K^+$-ATPase activity was also up-regulated at the anterior intestine on day 35 in the 500 μg Pb/g + 20 mg $Ca^{2+}$/g and 500 μg Pb/g + 60 mg $Ca^{2+}$/g treatments (Fig. 10). Taken together, these results suggest that dietary Pb indirectly disrupts plasma $Na^+$ levels. Since, $Ca^{2+}$ absorption at the intestine is dependent on $Na^+$ status at the intestine, declines in plasma $Ca^{2+}$ levels, as a result of Pb competing with $Ca^{2+}$ at the intestine, may trigger regulatory mechanisms (such as the up-regulation of $Na^+$, $K^+$-ATPase) to increase $Na^+$ concentrations at the intestine to aid in $Ca^{2+}$ absorption at the intestine and thus maintain $Ca^{2+}$ ion balance.

$Mg^{2+}$ and $Ca^{2+}$ are considered to be complementary ions (Bijvelds et al., 1998). Alves et al. (2006) found that $Mg^{2+}$ levels were decreased on day 14 when compared to the control. In the present study no significant effects on $Mg^{2+}$ levels (Table 5) when compared to the controls were observed. These differences may perhaps be explained by the fact that waterborne $Mg^{2+}$

levels were two times higher (0.4 mM) in this study than those of Alves et al. (2006, 0.2 mM).

$Na^+$, $K^+$-ATPase activity (Fig. 10) in the gills was not affected in this study. This is not surprising since gill burden was 100 times less than that of the Rogers et al. (2003) study that found a 40% inhibition of $Na^+$, $K^+$-ATPase at waterborne Pb concentrations of about 1.0 mg/L, associated with gill tissue burdens of 200 μg Pb/g.

Crespo et al. (1986) found that $Na^+$, $K^+$-ATPase activity was inhibited at the mid-intestine in adult fresh water rainbow trout fed 10 μg Pb/g fish/day for 15–30 days. In the present study, there were no significant effects on $Na^+$, $K^+$-ATPase activity at the mid- and posterior intestine. Crespo et al. (1986) did not measure Pb burden in the mid-intestine, but they did measure Pb concentrations in the kidney (21.82 μg Pb/g tissue dw, day 15). Assuming that wet weight weighs five times more than dry weight, the kidney in the Crespo et al. (1986) accumulated approximately two-fold higher Pb concentrations (∼4 μg/g tissue wet weight) than in the present study (∼2.4 μg/g wet weight, Fig. 6a and b) on day 42. Taken together this suggests that a higher Pb burden threshold may be needed to inhibit $Na^+$, $K^+$-ATPase activity at the intestine.

### 4.7. RBC and ALAD activity

When plasma Pb (Table 3) was compared to RBC Pb, it was found that 99% of the Pb was bound on or in the RBCs. This is consistent with Alves et al. (2006) who found that 98–99% of dietary Pb was present in the RBCs. The constant Pb accumulation in the whole blood and RBCs (Table 4 and Fig. 8, respectively) suggests that besides transporting Pb to various tissues, blood may act as a reservoir of excess Pb in the fish system.

Overall, there were no significant effects of dietary exposure of Pb on Hb levels and ALAD activity in the present study (Table 4). However, a significant 28% inhibition of ALAD activity was observed between the 0 μg Pb/g + 20 mg $Ca^{2+}$/g treatment and the 500 μg + 20 mg $Ca^{2+}$/g treatment on day 42. ALAD activity has been found to be negatively correlated with blood Pb concentrations (over the range 30–5400 μg/L) and waterborne Pb concentrations up to 100 μg Pb/L in fish (Hodson et al., 1977, 1978; Schmitt et al., 1984, 1993). In the present study, a non-significant linear response of log blood Pb levels versus ALAD activity was evident, with 9% of the variation of ALAD activity being explained by Pb in the blood after 42 days (Fig. 9). This suggests that a longer exposure time may be necessary in order to see a clear dietary Pb effect.

ALAD requires $Zn^{2+}$ cofactors and SH groups in order to function (Finelli, 1977; Sassa, 1982). It has been suggested that Zn alleviates the effects of Pb inhibition on ALAD activity by protecting SH groups from oxidation and the displacement of Zn by Pb on ALAD (Schmitt et al., 1984, 1993, 2002; Dwyer et al., 1988). Blood Zn levels were about 100 times greater than blood Pb levels (0.47 μgPb/g in the 500 μg Pb/g + 20 mg $Ca^{2+}$/g, Table 4), and remained constant on all days in all treatments (not shown) and did not differ significantly between treatments. Taking all these observations together, ALAD is not a sensitive biomarker of Pb toxicity in fish exposed to dietary Pb.

## Acknowledgements

We like to thank Dr. Chris N. Glover for general helpful input and Dr. Jeffrey J. Whyte of U.S. Geological Survey, Biochemistry and Physiology Branch, Columbia Environmental Research Center for helpful advice on the microplate assay for ALAD analysis in blood. This work was supported by the Natural Sciences and Engineering Research Council of Canada CRD Program, the International Lead Zinc Research Organization, the Nickel Producers Environmental Research Association, the International Copper Association, the Copper Development Association, Teck-Cominco, Noranda-Falconbridge, and Inco. CMW is supported by the Canada Research Chair Program.

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