

## Characterization of branchial lead–calcium interaction in the freshwater rainbow trout *Oncorhynchus mykiss*

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### Summary

The mechanism of branchial lead uptake and interplay with  $\text{Ca}^{2+}$  transport was investigated in the freshwater rainbow trout *Oncorhynchus mykiss*. Lead significantly reduced  $\text{Ca}^{2+}$  influx by approximately 40% and 30% after exposure to  $2.3 \pm 0.1$  and  $1.4 \pm 0.2 \mu\text{mol l}^{-1}$  dissolved lead, respectively, for 0–48 h. Acute inhibition of  $\text{Ca}^{2+}$  influx by lead exhibited typical Michaelis–Menten kinetics with an approximate 16-fold increase in  $K_m$ , whereas  $J_{\text{max}}$  values did not significantly change, yielding an inhibitor constant ( $K_{i,\text{Pb}}$ ) of  $0.48 \mu\text{mol l}^{-1}$ . Alternative analyses suggest the possibility of a mixed competitive/non-competitive interaction at the highest lead concentration tested ( $4.8 \mu\text{mol l}^{-1}$ ). Branchial lead accumulation was reduced with increasing waterborne  $\text{Ca}^{2+}$  concentrations, suggesting a protective effect of  $\text{Ca}^{2+}$  against lead uptake at the gill. The apical entries of  $\text{Ca}^{2+}$  and lead were both inhibited (55% and 77%, respectively) by the addition of lanthanum ( $1 \mu\text{mol l}^{-1}$ ) to the exposure water. The use of cadmium ( $1 \mu\text{mol l}^{-1}$ ) and zinc ( $100 \mu\text{mol l}^{-1}$ ) as voltage-independent calcium channel competitors also reduced

branchial lead uptake by approximately 56% and 47%, respectively. Nifedipine and verapamil (up to  $100 \mu\text{mol l}^{-1}$ ), both voltage-dependent calcium channel blockers, had no effect on gill lead accumulation.  $\text{CaCl}_2$  injection reduced both  $\text{Ca}^{2+}$  and lead uptake by the gills. This suggests transport of lead through apical voltage-independent calcium channels, similar to the entry of  $\text{Ca}^{2+}$ . High-affinity  $\text{Ca}^{2+}$ -ATPase activity was not acutely affected by lead, but a significant 80% reduction in activity occurred during exposure for 96 h to  $5.5 \pm 0.4 \mu\text{mol l}^{-1}$  dissolved lead, indicating a possible non-competitive component to lead-induced  $\text{Ca}^{2+}$  disruption. The effect of lead on  $\text{Ca}^{2+}$  efflux was investigated and found to be insignificant. We conclude that uptake of lead occurs, at least in part, by the same mechanism as  $\text{Ca}^{2+}$ , which results in disruption of  $\text{Ca}^{2+}$  influx and ultimately  $\text{Ca}^{2+}$  homeostasis.

Key words: waterborne lead, calcium, competition, rainbow trout, *Oncorhynchus mykiss*, branchial uptake.

### Introduction

Lead is a common contaminant in the natural environment that can enter the water column through geologic weathering and volcanic action, or by various anthropogenic practices including smelting, coal burning and use in gasoline, batteries and paint (World Health Organization, 1995). Though waterborne lead concentrations do not normally exceed  $0.6 \mu\text{mol l}^{-1}$  (Demayo et al., 1982), levels as high as  $4.3 \mu\text{mol l}^{-1}$  have previously been reported (US Environmental Protection Agency, 1986). Contamination of water through anthropogenic practices is the primary cause of lead poisoning in fish (Sorensen, 1991).

Recent evidence has shown that the acute toxic mechanism for waterborne lead in the rainbow trout *Oncorhynchus mykiss* is ionoregulatory disruption, with observed effects on  $\text{Na}^+$  and  $\text{Cl}^-$  balance and  $\text{Ca}^{2+}$  homeostasis (Rogers et al., 2003). These effects are manifested through an inhibition of ion influx and corresponding decreases in the plasma levels of these ions. This places lead midway between other known acute ionoregulatory toxicants like copper and silver, which affect

$\text{Na}^+$  and  $\text{Cl}^-$  balance (Laurén and McDonald, 1985; Wood et al., 1996; Morgan et al., 1997), and zinc and cadmium, which disrupt  $\text{Ca}^{2+}$  homeostasis (Spry and Wood, 1985; Verbost et al., 1987).

Branchial uptake of  $\text{Ca}^{2+}$  is thought to be primarily by passive movement through apical voltage-insensitive channels in the ‘chloride’ cells of the fish gill (Flik et al., 1993). Once entering the chloride cell,  $\text{Ca}^{2+}$  is transported *via*  $\text{Ca}^{2+}$ -binding proteins to the basolateral membrane where it is actively extruded into the circulation by way of a high-affinity  $\text{Ca}^{2+}$ -ATPase enzyme (Flik et al., 1985; Verbost et al., 1994; Marshall 2002) and/or a  $\text{Na}^+/\text{Ca}^{2+}$  exchange mechanism (Flik et al., 1994, 1997; Verbost et al., 1997). Lead could potentially have an impact at any one of these steps of calcium entry. Recently, MacDonald et al. (2002) speculated that lead disrupts  $\text{Ca}^{2+}$ -homeostasis by competitive inhibition at apical  $\text{Ca}^{2+}$  channels in the fish gill, thus entering the fish by the same mechanism as  $\text{Ca}^{2+}$ . There is an abundance of circumstantial evidence in support of this. For example, the toxicity of

waterborne lead is greatly reduced with increasing water hardness, since the  $\text{Ca}^{2+}$  component probably exerts protective effects by inhibiting entry of lead (Sorensen, 1991). Elevated dietary  $\text{Ca}^{2+}$  levels have also been shown to reduce lead uptake in fish (Varanasi and Gmur, 1978). Once crossing the gill and entering the systemic circulation, lead accumulates in bone, suggesting similarities between the handling of lead and  $\text{Ca}^{2+}$  within the organism (Davies et al., 1976; Holcombe et al., 1976; Settle and Patterson, 1980). These indirect relationships suggest that lead may share one or multiple points of entry with calcium; however, despite such circumstantial evidence, this relationship has not been proven directly.

Using both physiological and pharmacological techniques, metals like cadmium and zinc have been shown to be  $\text{Ca}^{2+}$  antagonists. Both metals have been found to reduce rates of  $\text{Ca}^{2+}$  influx (Verboost et al., 1987; Spry and Wood, 1985) resulting in hypocalcemia. Kinetic analyses of  $\text{Ca}^{2+}$  interaction with both zinc (Spry and Wood, 1989; Hogstrand et al., 1994, 1998) and cadmium (Niyogi and Wood, in press) have demonstrated typical Michaelis–Menten relationships, i.e. increased  $K_m$  values (decreased affinity of the apical calcium channel) with little or no change in the maximal rate of uptake ( $J_{\text{max}}$ ). This suggests direct competition. Additionally, apical calcium channel blockers such as lanthanum have been shown to reduce the rate of  $\text{Ca}^{2+}$ , cadmium and zinc uptake, suggesting that these metals share the same lanthanum-inhibitable route of uptake (Verboost et al., 1989; Hogstrand et al., 1995, 1996). Using the rainbow trout as a model species, the objective of the present study was to characterize the branchial interaction of lead and  $\text{Ca}^{2+}$  by incorporating kinetic analysis and the use of apical channel blockers (both voltage sensitive and voltage insensitive). The potential stimulation of  $\text{Ca}^{2+}$ -efflux, as reported in lead-exposed brown trout (Sayer et al., 1991), and possible inhibition of high-affinity  $\text{Ca}^{2+}$ -ATPase were also investigated as potential factors in lead-induced disruption of  $\text{Ca}^{2+}$ -homeostasis. Characterization of lead binding to the rainbow trout gill may aid in the development of water chemistry-based predictive models for lead, such as the Biotic Ligand Model (BLM; Paquin et al., 2002). This process requires further understanding and characterization of key binding sites involved in lead toxicity.

## Materials and methods

### *Experimental animals*

Juvenile rainbow trout *Oncorhynchus mykiss* Walbaum (3–10 g) for use in influx experiments and experiments using calcium channel blockers/competitors and  $\text{CaCl}_2$  injection, larger juvenile trout (40–60 g) for use in  $\text{Ca}^{2+}$ -efflux measurements, and adult rainbow trout (200–300 g) for use in the high-affinity  $\text{Ca}^{2+}$ -ATPase assay, were obtained from Humber Springs Trout Farm in Orangeville, Ontario, Canada. Fish were held in dechlorinated City of Hamilton tapwater (from Lake Ontario) at a temperature of 7–12°C, and fed commercial trout pellets at a ration of 1% total body mass per day. Water composition ( $\text{mmol l}^{-1}$ ) was  $\text{Ca}^{2+}$  1.0,  $\text{Mg}^{2+}$  0.2,

$\text{Na}^+$  0.6,  $\text{Cl}^-$  0.8,  $\text{K}^+$  0.05, total lead  $0.003 \mu\text{mol l}^{-1}$  ( $0.68 \mu\text{g l}^{-1}$ ), dissolved organic carbon (DOC)  $3 \text{ mg l}^{-1}$ , hardness (as  $\text{CaCO}_3$ ) of approximately  $140 \text{ mg l}^{-1}$ , and pH 7.9–8.0. Experiments were conducted at a temperature of 9–12°C and experimental animals were starved for 72 h prior to and throughout all experiments.

### *Ca<sup>2+</sup> influx measurements*

$\text{Ca}^{2+}$  influx determinations were carried out using methods almost identical to those outlined by Rogers et al. (2003), differing only in lead-exposure concentrations used and duration of the pre-exposure period. In brief,  $\text{Ca}^{2+}$  influx was measured by relating the specific activity of  $^{45}\text{Ca}$  in the exposure water to the accumulation of isotope in the fish following the flux period. In the present study, influx was measured under control conditions and at lead concentrations that were approximately 50% and 25% of the 96 h  $\text{LC}_{50}$  of  $4.8 \mu\text{mol l}^{-1}$  dissolved lead determined in City of Hamilton dechlorinated tapwater by Rogers et al. (2003). The nominal lead-exposure concentrations implemented were  $2.4 \mu\text{mol l}^{-1}$  and  $1.2 \mu\text{mol l}^{-1}$ . Juvenile rainbow trout were subject to pre-flux exposure periods of 0 h, 12 h and 24 h before undergoing a flux period of 2 h in control water or lead-containing water.

### *Kinetic analysis of the interaction between lead and Ca<sup>2+</sup>*

The differential effects of lead on unidirectional calcium influx were assessed *in vivo* using methods similar to those outlined in Zohouri et al. (2001). Sixteen polyethylene bags representing a series of four different calcium concentrations in control and three lead concentrations, were filled with 3 l calcium-free synthetic water ( $0.7 \text{ mmol l}^{-1}$   $\text{Na}^+$  and  $\text{Cl}^-$  added as  $\text{NaCl}$ ,  $1.9 \text{ mmol l}^{-1}$   $\text{KHCO}_3$ , pH 8.0). Each bag was fitted with an airline and placed on a water bath for temperature control (approximately 10°C). Three bags of each set were spiked with a  $\text{Pb}(\text{NO}_3)_2$  stock solution (Sigma; Aldrich, Oakville, ON, Canada) to obtain nominal lead concentrations of 0.48, 2.4 and  $4.8 \mu\text{mol l}^{-1}$  lead (control contained  $0 \mu\text{mol l}^{-1}$  lead). Each bag was then spiked with  $\text{Ca}(\text{NO}_3)_2$  (Fisher Scientific, Markham, ON, Canada) to achieve nominal calcium concentrations of 150, 300, 600 and  $1200 \mu\text{mol l}^{-1}$ . Finally, flux bags were injected with  $7 \mu\text{Ci l}^{-1}$   $^{45}\text{Ca}$  (as  $\text{CaCl}_2$ , specific activity  $0.14 \mu\text{Ci mol}^{-1}$ ; Perkin-Elmer, Boston, MA, USA).

Juvenile rainbow trout were transferred to each of the 16 flux bags (seven fish per bag) and an initial 15 min period was allowed for 'settling' and isotopic equilibration. The exposure period was 3 h; initial and final water samples (5 ml) were drawn in duplicate for determination of  $^{45}\text{Ca}$  activity and total calcium concentration. Water samples were also drawn for determination of total lead (unfiltered) and dissolved lead (filtered;  $0.45 \mu\text{m}$  filter) concentrations. These samples were stored in plastic scintillation vials in 1%  $\text{HNO}_3$  for analysis. Following the 3 h flux period, fish were removed and killed by a single blow to the head. The fish were then rinsed for 1 min in  $1 \text{ mmol l}^{-1}$  EDTA (Sigma-Aldrich) followed by a 1 min rinse in a  $5 \text{ mmol l}^{-1}$  cold  $\text{Ca}^{2+}$  solution [ $\text{Ca}(\text{NO}_3)_2$ ; Sigma-Aldrich] to remove all surface-bound  $^{45}\text{Ca}$ . Whole bodies were

blotted dry, placed in scintillation vials, and digested in 1 mol l<sup>-1</sup> HNO<sub>3</sub> (Fisher Scientific; trace metal grade) at 55°C for 48 h. Samples were then homogenized by vortexing, a sample (1.5 ml) was removed, centrifuged at 13 000 g for 10 min, and the supernatant (1 ml) added to 5 ml of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT, USA). <sup>45</sup>Ca radioactivity was measured by scintillation counting (Rackbeta 1217; LKB Wallac, Turku, Finland). Water samples taken for determination of <sup>45</sup>Ca radioactivity were added to 10 ml of aqueous counting scintillant (ACS™: Amersham, Piscataway, NJ, USA) and scintillation counted as above. <sup>45</sup>Ca radioactivity was quench-corrected to the same counting efficiency as water samples by the method of external standard ratios, using a <sup>45</sup>Ca quench curve generated from the tissue of interest in the same counting cocktail.

Water samples taken for the determination of total [Ca<sup>2+</sup>] were diluted with 0.2% lanthanum and analyzed by flame atomic absorption spectrophotometry (FAAS) using the Varian 220FS Spectr AA (Varian, Mulgrave, VC, Australia). Determination of total and dissolved waterborne lead concentrations was done using graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA) against a certified multi-element standard (Inorganic Ventures, Inc., Ventura, CA, USA).

#### *The effect of calcium on branchial lead accumulation*

The effect of waterborne calcium concentration on the branchial accumulation of lead in juvenile rainbow trout was assessed using methods similar to those implemented in the kinetic analysis of lead and Ca<sup>2+</sup>, though precision was lower due to the lack of a suitable lead radioisotope. Following a 3-h exposure period, experimental animals were removed from the flux bags, killed by a blow to the head, and the gills dissected. The gills were rinsed for 1 min in deionized water, blotted dry, weighed and digested in 1 mol l<sup>-1</sup> HNO<sub>3</sub> at 55°C for 48 h. Samples were then homogenized by vortexing, a sample (approximately 1.5 ml) was removed, centrifuged at 13 000 g for 10 min, and the supernatant analyzed for total lead concentration using GFAAS.

#### *Calcium-channel blocker experiments*

The role of apical calcium channels in mediating branchial lead uptake was investigated by the use of the voltage-independent blocker, lanthanum, antagonists of voltage-independent Ca<sup>2+</sup> uptake, cadmium and zinc, and the voltage-dependent channel blockers nifedipine and verapamil. In assessing the effects of lanthanum on branchial lead accumulation, a series of six polyethylene bags representing one control (0 μmol l<sup>-1</sup> lanthanum) and five lanthanum concentrations were filled with 3 l of synthetically modified water obtained by reverse osmosis (0.7 mmol l<sup>-1</sup> Na<sup>+</sup> and Cl<sup>-</sup> added as NaCl), made carbonate-free to reduce complexation of waterborne lanthanum (Verbost et al., 1987; Hogstrand et al., 1996) and calcium-free to maximize branchial lead accumulation. Flux bags were then spiked with a LaCl<sub>3</sub>

(Sigma-Aldrich) stock solution to achieve nominal waterborne lanthanum concentrations of 0.0001 to 1 μmol l<sup>-1</sup>. Juvenile rainbow trout were transferred to each of the six flux bags (seven fish per bag) and allowed a 10-min settling period for equilibration with waterborne lanthanum. The bags were subsequently spiked with a Pb(NO<sub>3</sub>)<sub>2</sub> stock solution to achieve a nominal lead concentration of 4.8 μmol l<sup>-1</sup>, followed by an additional settling period to allow for lead equilibration. Water samples were taken, filtered and unfiltered, for determination of total and dissolved lead concentrations. The fish were then exposed for 3 h. Following exposure, fish were removed from flux bags and the gills were dissected. Processing of the dissected gills for total lead concentrations used procedures identical to those outlined above. Using <sup>45</sup>Ca as a radiotracer, simultaneous measurements of Ca<sup>2+</sup> influx were made at control and 1 μmol l<sup>-1</sup> lanthanum to assess the effect of lanthanum on Ca<sup>2+</sup> uptake. Influx measurements were performed using a waterborne Ca<sup>2+</sup> concentration of 600 μmol l<sup>-1</sup>. Further procedures used were identical to those outlined above under 'calcium influx measurements'. The effects of cadmium and zinc on branchial lead accumulation and Ca<sup>2+</sup> influx were assessed using methods almost identical to those used for lanthanum. For cadmium, flux bags were spiked with a stock solution of Cd(NO<sub>3</sub>)<sub>2</sub> to achieve nominal concentrations of 0.0001, 0.001, 0.01, 0.1 and 1 μmol l<sup>-1</sup> cadmium. For zinc, bags were spiked with ZnSO<sub>4</sub> to achieve nominal concentrations of 0.01, 0.1, 1, 10 and 100 μmol l<sup>-1</sup> zinc. These experiments also included measurements of gill cadmium burden from the same gill digests analyzed for total lead, using GFAAS.

The use of nifedipine and verapamil as L-type calcium channel blockers was evaluated using methods almost identical to those used for lanthanum, cadmium and zinc with the exception of waterborne concentrations employed. For nifedipine, bags were spiked with a stock solution made using 75% ethanol (to solubilize the blocking agent; final ethanol concentration in the flux medium was 0.1%) to achieve concentrations of 0.1, 1, 10 and 100 μmol l<sup>-1</sup> nifedipine. A control was run by spiking nifedipine-free water with ethanol to control for the effects of ethanol on lead accumulation and to maintain consistency between blocker experiments. For verapamil, flux bags were spiked with a stock solution made using deionized water (NANOpure II; Sybron/Barnstead, Boston, MA, USA) to achieve final concentrations of 0.1, 1, 10 and 100 μmol l<sup>-1</sup> verapamil.

#### *Effect of CaCl<sub>2</sub> injection on Ca<sup>2+</sup> influx and branchial lead accumulation*

Methods used to test the effects of CaCl<sub>2</sub> injection on Ca<sup>2+</sup> influx and branchial lead accumulation followed closely those outlined in Hogstrand et al. (1996). Ionic calcium was injected into juvenile rainbow trout with the goal of reducing uptake of Ca<sup>2+</sup> and lead through the stanniocalcin-regulated pathway. In the present study, fish were injected intraperitoneally with 0.22 μmol l<sup>-1</sup> Ca<sup>2+</sup> g<sup>-1</sup> body mass using an injection solution made from CaCl<sub>2</sub>.H<sub>2</sub>O dissolved in 0.9% NaCl. Control fish

were sham-injected with 0.9% NaCl (vehicle only). Approximately 30 min following injection, measurements of  $\text{Ca}^{2+}$  influx were performed on experimental and control fish ( $N=8$  per treatment) using methods described in the previous section. Branchial lead accumulation was measured in a separate group of control and experimental fish ( $N=8$  per treatment), again using methods outlined above.

#### *Determination of $\text{Ca}^{2+}$ -efflux*

The possibility of lead-induced stimulation of  $\text{Ca}^{2+}$ -efflux was investigated by following closely the methods of Perry and Flik (1988). Fish were subject to either no pre-flux exposure (control), or a pre-exposure period of 24 or 48 h to nominal waterborne lead concentrations close to the 96 h  $\text{LC}_{50}$  of  $4.8 \mu\text{mol l}^{-1}$ . Lead exposure was carried out by dripping a stock solution of  $\text{Pb}(\text{NO}_3)_2$  (Sigma-Aldrich) dissolved in deionized water (NANOpure II; Sybron/Barnstead, Boston, MA, USA) at a rate of  $1 \text{ ml min}^{-1}$  into a mixing tank fed with  $500 \text{ ml min}^{-1}$  of City of Hamilton dechlorinated tapwater. The mixing tank then fed an exposure tank ( $\sim 200 \text{ l}$ ) holding 20 rainbow trout. At  $t=0 \text{ h}$ , the exposure tank was spiked with the lead stock solution to achieve the appropriate lead concentration.

After the appropriate pre-exposure period, rainbow trout were anaesthetized using MS-222 and given an intra-peritoneal injection of a  $^{45}\text{Ca}$  solution ( $30 \mu\text{Ci } ^{45}\text{Ca}$  in  $1 \text{ ml}$  saline) in preparation for efflux measurements. A period of 8–12 h was allowed for recovery of the fish, and for isotopic equilibration. Fish were then transferred to darkened, Plexiglass boxes (volume  $450 \text{ ml}$ ), each fitted with an air supply. The boxes were filled with either lead-free water or water spiked with  $\text{Pb}(\text{NO}_3)_2$  to achieve a nominal lead concentration of  $4.8 \mu\text{mol l}^{-1}$ , and placed in a water bath for temperature control. Following a 0.5 h settling period, a 3 h  $\text{Ca}^{2+}$  flux measurement was started. Initial water samples were taken in duplicate for  $^{45}\text{Ca}$  activity and total calcium concentration ( $5 \text{ ml}$ ), and for determination of dissolved lead concentrations ( $10 \text{ ml}$ ). At the end of the flux period, comparable final water samples were drawn, fish were removed from their respective exposure boxes and anaesthetized with MS-222. A terminal blood sample was taken from each fish by caudal puncture, centrifuged at  $13\,000 \text{ g}$ , and the plasma frozen for measurement of  $^{45}\text{Ca}$  activity and total calcium concentration.  $\text{Ca}^{2+}$ -efflux  $J$  was calculated using the following formula:

$$J_{\text{out}}^{\text{Ca}^{2+}} = \frac{(R_i - R_f)V}{\text{SAM}t}, \quad (1)$$

where  $R_i$  and  $R_f$  are initial and final water radioactivities in c.p.m.  $\text{ml}^{-1}$ ,  $V$  is the volume of the external flux medium in  $\text{ml}$ ,  $\text{SA}$  is the  $t=3 \text{ h}$  specific activity of the plasma in c.p.m.  $\mu\text{mole}$ ,  $t$  is the duration of the flux period in  $\text{h}$ , and  $M$  the fish mass in  $\text{kg}$ . Water samples were analysed using methods identical to those outlined in the lead/ $\text{Ca}^{2+}$  kinetic experiments.

#### *The effect of lead on high-affinity $\text{Ca}^{2+}$ -ATPase activity*

High-affinity  $\text{Ca}^{2+}$ -ATPase activity was assayed in adult rainbow trout exposed to control conditions or to a nominal

lead concentration of  $4.8 \mu\text{mol l}^{-1}$  for 3 h, 24 h or 96 h in City of Hamilton dechlorinated tapwater. Lead exposure was carried out using methods identical to those used in measurements of  $\text{Ca}^{2+}$ -efflux. Isolation of the basolateral membrane from gill epithelium was carried out to assay enzyme activity. Procedures followed closely those outlined in Perry and Flik (1988). Trout were anaesthetized with MS-222 and injected intravenously with  $2500 \text{ U}$  of sodium heparin dissolved in  $1 \text{ ml}$  of saline. After 20 min, the fish were decapitated just posterior to the pectoral fins. The head was placed onto an operating table where the gills, irrigated with freshwater, were perfused with ice-cold isotonic saline ( $0.6\%$  NaCl) containing  $20 \text{ U ml}^{-1}$  of sodium heparin. Perfusion was carried out at a pressure of  $60 \text{ cmH}_2\text{O}$  via a catheter (PE 60 tubing) inserted into the bulbus arteriosus until the gills appeared to be free of trapped red blood cells. Following perfusion, the gill basket was quickly excised. Further preparations were carried out on ice ( $0\text{--}4^\circ\text{C}$ ) from this point.

The gill epithelium was scraped from the gill arches onto a glass plate using a glass microscope slide. The scrapings were then placed into a douncer with a loose pestle and homogenized in  $15 \text{ ml}$  of a hypotonic buffer consisting of  $25 \text{ mmol l}^{-1}$  NaCl and  $20 \text{ mmol l}^{-1}$  Hepes-Tris (pH 7.4). The volume was brought to  $50 \text{ ml}$  and the homogenate centrifuged for  $15 \text{ min}$  at  $550 \text{ g}$  (Beckman Ti 70 Rotor, Palo Alto, USA) to remove nuclei and cellular debris. Membranes were then collected by ultracentrifugation of the remaining supernatant at  $30\,000 \text{ g}$  for  $30 \text{ min}$ . The resulting pellet, fixed to the centrifuge tube, was resuspended with 100 strokes of a douncer in  $15 \text{ ml}$  of an isotonic buffer containing  $250 \text{ mmol l}^{-1}$  sucrose,  $12.5 \text{ mmol l}^{-1}$  NaCl,  $0.5 \text{ mmol l}^{-1}$   $\text{H}_2\text{EDTA}$ , and  $5 \text{ mmol l}^{-1}$  Hepes-Tris (pH 7.5). The volume of the suspension was then brought to  $30 \text{ ml}$  with the same buffer. The resulting suspension was then centrifuged differentially in the following manner:  $1000 \text{ g}$  for  $10 \text{ min}$ ,  $10\,000 \text{ g}$  for  $10 \text{ min}$ ,  $30\,000 \text{ g}$  for  $30 \text{ min}$ . The resulting pellet was obtained by adding  $300 \mu\text{l}$  of a buffer containing  $20 \text{ mmol l}^{-1}$  Hepes-Tris (pH 7.4) and  $200 \text{ mmol l}^{-1}$  sucrose, and resuspended by passing the mixture through a 23-gauge syringe needle. The membrane preparations contained approximately  $3 \text{ mg ml}^{-1}$  of bovine serum albumin protein equivalents and were used on the same day of isolation. High-affinity  $\text{Ca}^{2+}$ -ATPase activity was assayed in the presence of oligomycin B ( $5 \mu\text{g ml}^{-1}$ ) and  $5 \text{ mmol l}^{-1}$  sodium azide, at  $1.0 \mu\text{mol l}^{-1}$  and  $0 \mu\text{mol l}^{-1}$  free  $\text{Ca}^{2+}$  in an assay medium containing  $100 \text{ mmol l}^{-1}$  NaCl,  $0.1 \text{ mmol l}^{-1}$  ouabain,  $0.5 \text{ mmol l}^{-1}$  EGTA,  $0.5 \text{ mmol l}^{-1}$   $N$ -hydroxyEDTA,  $5 \text{ mmol l}^{-1}$   $\text{MgCl}_2$ ,  $3 \text{ mmol l}^{-1}$   $\text{Na}_2\text{ATP}$  and  $20 \text{ mmol l}^{-1}$  Hepes-Tris (pH 7.4). Membrane vesicles were permeabilized with  $20 \mu\text{g}$  saponin  $\text{mg}^{-1}$  membrane protein. High-affinity  $\text{Ca}^{2+}$ -ATPase activity was measured as the difference in inorganic phosphate release ( $\text{P}_i$ ) in the presence and absence of  $\text{Ca}^{2+}$  in the assay medium.

The *in vitro* effect of lead on  $\text{Ca}^{2+}$ -ATPase activity was tested by adding lead [from a  $\text{Pb}(\text{NO}_3)_2$  stock solution] to the final membrane suspension, just prior to addition to the assay

reaction medium, at concentrations similar to those measured in the final membrane suspension from the *in vivo* exposure. Finally, branchial lead accumulation was measured in a separate group of fish, similar in size and exposed to control conditions or to  $4.8 \mu\text{mol l}^{-1}$  lead for 3 h, 24 h or 96 h. Procedures used for measurement of gill lead burden were identical to those outlined in the section ‘Calcium-channel blocker experiments’ above.

#### Statistical analysis

In kinetic experiments, Michaelis–Menten analysis of the relationship between the rate of  $\text{Ca}^{2+}$  influx ( $J_{\text{in}}^{\text{Ca}^{2+}}$ ) and waterborne calcium (substrate) were performed using Lineweaver–Burk linear regression (double reciprocal plots), where  $J_{\text{max}}$  (maximum rate of influx) is the inverse of the  $y$ -intercept of the regression line, and  $-K_m$  (Michaelis constant) is the inverse of the  $x$ -intercept of the regression line. For comparison, data were also analysed by Eadie–Hofstee plots and by non-linear regression (Sigmaplot 2000).

Calculated data are expressed as means  $\pm 1$  S.E.M. ( $N$ ). Experimental means were compared to corresponding control mean values by an unpaired two-tailed Student’s  $t$ -test. Both time-dependent and dose-dependent responses in both control and experimental groups were tested against initial 0 h or control measurements using a one-way analysis of variance (ANOVA) with a two-sided Dunnett’s *post hoc* multiple comparison. All statistical significance was calculated at  $P < 0.05$ .

## Results

### Water chemistry

As an integral component to the characterization of lead/ $\text{Ca}^{2+}$  interaction in the rainbow trout, speciation analysis was conducted for these waterborne ions using MINEQL+, a computer-based geochemical modeling program (Schecher and McAvoy, 1994). For each experiment, simulations were run taking into account all chemical species present in the medium. These analyses indicated that 98% of the calcium in exposure water was present in the free ionic form,  $\text{Ca}^{2+}$ . In determining the speciation of lead in the exposure water, it was found that  $>95\%$  of waterborne lead was present in the bioavailable forms  $\text{Pb}^{2+}$  ( $\sim 50\%$ ),  $\text{Pb}(\text{OH})^+$  ( $\sim 30\%$ ) and  $\text{Pb}(\text{OH})_2$  ( $\sim 15\%$ ), with a small component being bound to carbonate ( $\text{PbCO}_3$ ; 3–5%). These data are consistent with dissolved lead concentrations measured using filtration with a  $0.45 \mu\text{mol l}^{-1}$  filter.

### Effect of lead on $\text{Ca}^{2+}$ influx

Juvenile rainbow trout exposed to  $2.3 \pm 0.1 \mu\text{mol l}^{-1}$  dissolved lead (measured) showed significant reductions in  $\text{Ca}^{2+}$ -influx rate compared to control fish (Fig. 1A). Effects on influx were immediate, with an approximate 40% inhibition occurring at 0–2 h of lead exposure, this effect carrying through to 24–26 h, though non-significant at 12–14 h. Exposure to  $1.4 \pm 0.2 \mu\text{mol l}^{-1}$  dissolved lead resulted in similar inhibition of  $\text{Ca}^{2+}$  influx (Fig. 1B), though significant only at

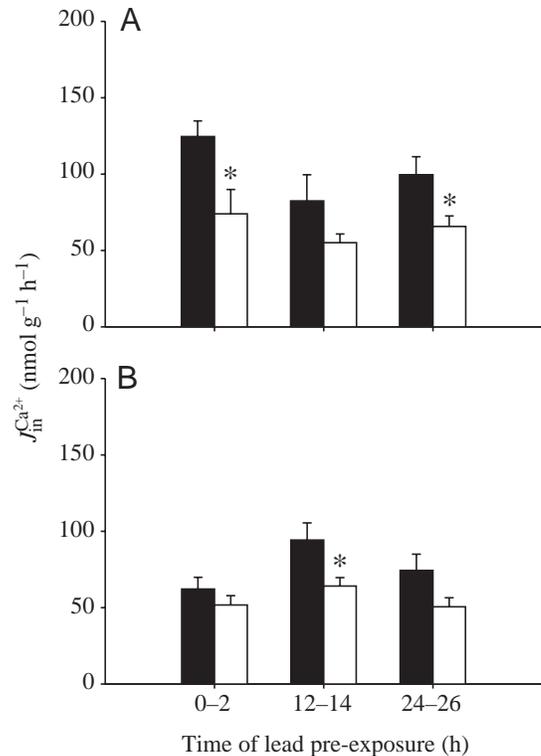


Fig. 1. (A) Calcium influx rates  $J_{\text{in}}^{\text{Ca}^{2+}}$  in juvenile rainbow trout in City of Hamilton dechlorinated tapwater in control conditions (black bars) or water containing  $2.3 \pm 0.1 \mu\text{mol l}^{-1}$  dissolved lead (white bars) after exposure for 0, 12 or 24 h. (B) Calcium influx rates in control water (black bars) or water containing  $1.4 \pm 0.2 \mu\text{mol l}^{-1}$  dissolved lead (white bars) after exposure for 0, 12 or 24 h. Values are means  $\pm 1$  S.E.M. ( $N=8$  throughout). \*Significant difference ( $P < 0.05$ ; two-tailed Student’s  $t$ -test) from corresponding control means.

12–14 h where the reduction was 32%. The concentration of total lead in control water was  $0.002 \pm 0.0001 \mu\text{mol l}^{-1}$ .

### Kinetic analysis of the interaction between lead and $\text{Ca}^{2+}$

The saturable nature of calcium influx with increasing waterborne calcium concentrations obeyed typical Michaelis–Menten kinetic analysis, in the presence and absence of lead (Fig. 2). Measured waterborne lead concentrations were  $0.46 \pm 0.03 \mu\text{mol l}^{-1}$ ,  $2.5 \pm 0.2 \mu\text{mol l}^{-1}$  and  $4.9 \pm 0.3 \mu\text{mol l}^{-1}$ . Consistent with the immediate inhibition of  $\text{Ca}^{2+}$ -influx documented in the previous experiment, waterborne lead exposure resulted in inhibition of calcium influx in juvenile rainbow trout over a 3 h exposure period, the inhibition increasing with increased lead concentrations. From analysis of a double reciprocal Lineweaver–Burk plot (Fig. 3A), it is apparent that  $K_m$  values increased significantly with increasing waterborne lead concentration (Table 1), for example,  $4.8 \mu\text{mol l}^{-1}$  lead exposure resulted in an approximate 16-fold increase in  $K_m$  value from control levels. By contrast,  $J_{\text{max}}$  was not significantly altered as a function of waterborne lead concentration (Table 1). Kinetic analysis

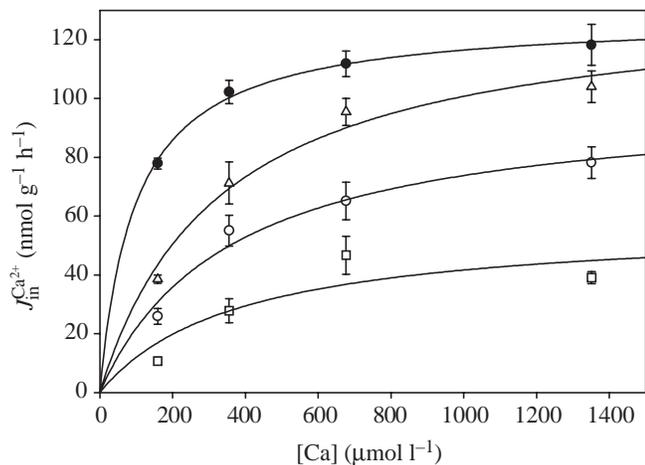


Fig. 2. Unidirectional branchial calcium influx rates  $J_m^{Ca^{2+}}$  in juvenile rainbow trout at various waterborne calcium concentrations in synthetic water for four different lead concentrations. Control fish, black circles; fish exposed to  $0.46 \pm 0.03 \mu\text{mol l}^{-1}$  dissolved lead, white triangles;  $2.5 \pm 0.2 \mu\text{mol l}^{-1}$  lead, white circles;  $4.9 \pm 0.3 \mu\text{mol l}^{-1}$  lead, white squares. Values are means  $\pm 1$  S.E.M. ( $N=7$  per treatment).

using Eadie–Hofstee and non-linear regression also indicated significant increases in  $K_m$ ; however, unlike the results obtained by Lineweaver–Burk regression, calculations revealed a significant decrease (approximately 57%) in  $J_{\text{max}}$ , but only at the highest concentration,  $4.8 \mu\text{mol l}^{-1}$  lead (Table 1).

Because of the competitive nature of  $\text{Ca}^{2+}$ -influx inhibition by lead, an inhibitor constant was determined from a regression plot of apparent  $K_m$  (measured  $K_m$  values from Table 1)/ $J_{\text{max}}$  vs. waterborne lead concentration (Fig. 3B; Segel, 1976). The  $K_{i,\text{Pb}}$  ( $-K_i$ ,  $x$ -intercept of the regression line) was calculated to be  $0.48 \mu\text{mol l}^{-1}$  lead.

#### The effect of calcium on branchial lead accumulation

The effect of waterborne calcium on branchial lead uptake is shown in Fig. 4. Overall a protective effect of calcium was

Table 1.  $J_{\text{max}}$  and apparent  $K_m$  for unidirectional branchial calcium influx rates in juvenile rainbow trout at various waterborne lead concentrations

Waterborne [Pb] ( $\mu\text{mol l}^{-1}$ )	$J_{\text{max}}$ ( $\text{nmol g}^{-1} \text{h}^{-1}$ )			Apparent $K_m$ ( $\mu\text{mol l}^{-1}$ )		
	LB	EH	NLR	LB	EH	NLR
Control	135.1	128.5	127.9	166.2	100.5	98.0
0.48	156.3	141.0	134.3	475.6*	385.4*	332.6*
2.4	147.1	102.2	100.4	798.7*	391.4*	360.2*
4.8	144.9	54.4*	56.5*	1911.7*	490.0*	395.0*

Values are means.

Kinetics were characterized by Lineweaver–Burk regression (LB), Eadie–Hofstee regression (EH) and non-linear regression (NLR).

\*Significant difference ( $P < 0.05$ ) from control ( $N=7$ ).

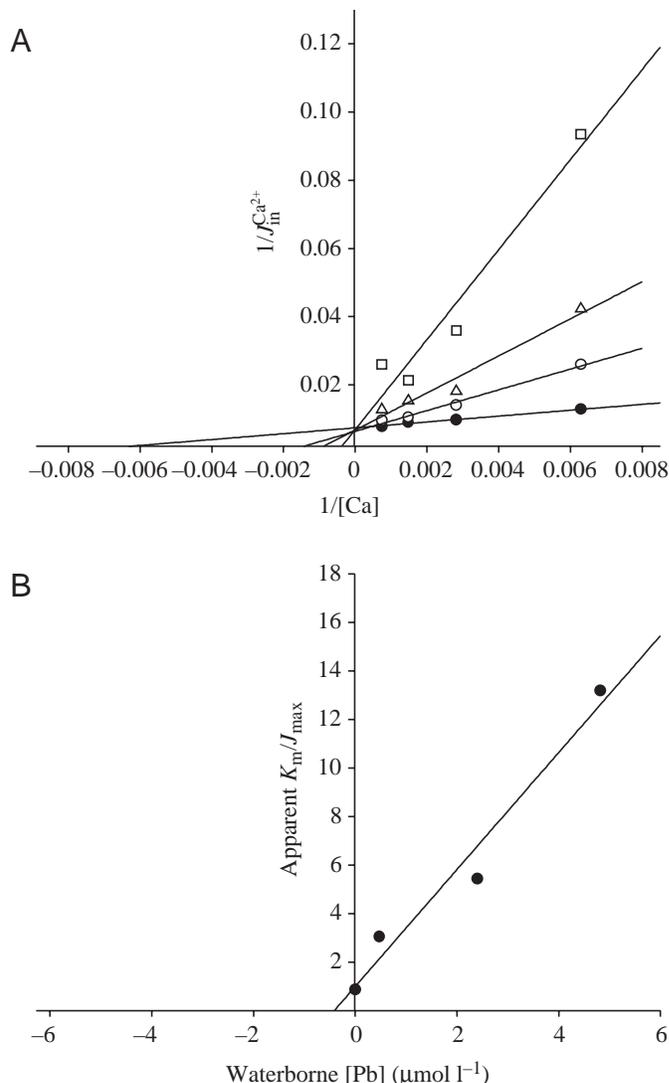


Fig. 3. Analytical plots for data presented in Fig. 2 using (A) Michaelis–Menten analyses by Lineweaver–Burk regression (double reciprocal plot) illustrating the competitive relationship between lead and calcium uptake in the rainbow trout, and (B) regression analyses of apparent  $K_m/J_{\text{max}}$  vs. waterborne lead concentration (using apparent  $K_m$  and  $J_{\text{max}}$  values presented in Table 1). The regression equation was  $y=2.38x+1.12$  ( $r^2=0.95$ ). The inhibitor constant ( $-K_{i,\text{Pb}}$ , the  $x$ -intercept of the regression line) was  $0.48 \mu\text{mol l}^{-1}$  lead.

observed, with the amount of branchial lead accumulation decreasing with increasing waterborne  $\text{Ca}^{2+}$  concentration. At a nominal waterborne lead concentration of  $0.48 \mu\text{mol l}^{-1}$ , accumulation was consistent over the range of  $\text{Ca}^{2+}$  concentrations ( $150\text{--}1300 \mu\text{mol l}^{-1}$ ). With increases in waterborne lead concentrations to  $2.4$  (dissolved  $2.5 \pm 0.2 \mu\text{mol l}^{-1}$ ) and  $4.8 \mu\text{mol l}^{-1}$  (dissolved  $4.9 \pm 0.3 \mu\text{mol l}^{-1}$ ), the concentration of waterborne  $\text{Ca}^{2+}$  required to yield the same protective effects increased. For example, upon exposure to  $4.8 \mu\text{mol l}^{-1}$  lead (dissolved  $4.9 \pm 0.3 \mu\text{mol l}^{-1}$ ), lead accumulation at  $150 \mu\text{mol l}^{-1}$   $\text{Ca}^{2+}$  was

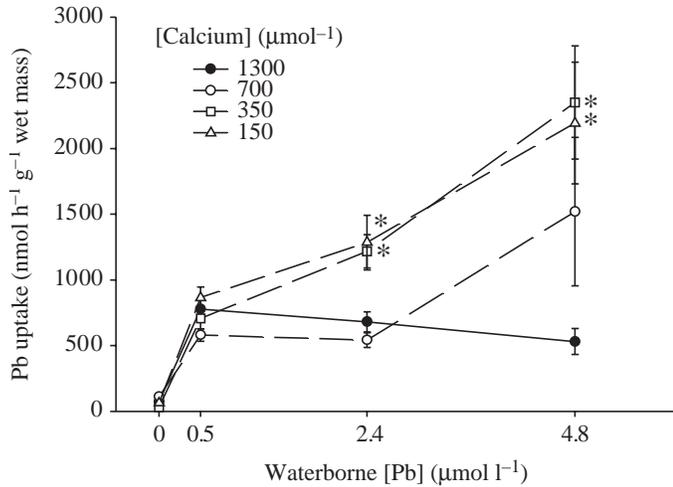


Fig. 4. Measurements of branchial lead accumulation in juvenile rainbow trout at various waterborne calcium concentrations in synthetic water for four different lead concentrations. Values are means  $\pm$  1 S.E.M. ( $N=7$  per treatment). \*Significant difference ( $P<0.05$ ; two-tailed Student's  $t$ -test) from corresponding lead uptake in the presence of 1300  $\mu\text{mol l}^{-1}$  calcium.

approximately 35 times higher than control ( $0 \mu\text{mol l}^{-1}$  lead) accumulation, while at 1300  $\mu\text{mol l}^{-1}$   $\text{Ca}^{2+}$ , accumulation was only 6 times greater than control gill lead accumulation. At a waterborne  $\text{Ca}^{2+}$  concentration of approximately 1300  $\mu\text{mol l}^{-1}$ , branchial lead accumulation measured after exposure to 0.48, 2.4 and 4.8  $\mu\text{mol l}^{-1}$  lead did not differ significantly.

#### The effect of $\text{Ca}^{2+}$ channel blockers on branchial lead accumulation

The effect of lanthanum, a voltage-independent  $\text{Ca}^{2+}$ -channel inhibitor, on  $\text{Ca}^{2+}$  uptake and branchial lead accumulation is shown in Fig. 5A,B. From  $\text{Ca}^{2+}$  influx measurements determined for control conditions at 600  $\mu\text{mol l}^{-1}$   $\text{Ca}^{2+}$  and 1  $\mu\text{mol l}^{-1}$  lanthanum (added as  $\text{LaCl}_3$ ), an approximate 55% reduction in  $\text{Ca}^{2+}$  uptake occurred in the presence of 1  $\mu\text{mol l}^{-1}$  lanthanum. Upon exposure to  $2.3 \pm 0.2 \mu\text{mol l}^{-1}$  dissolved lead, waterborne lanthanum significantly reduced gill lead accumulation in a dose-dependent fashion. In the presence of 0.0001  $\mu\text{mol l}^{-1}$  lanthanum, an approximate 39% reduction in lead accumulation occurred compared to control fish exposed to the same lead concentration in the absence of lanthanum. This

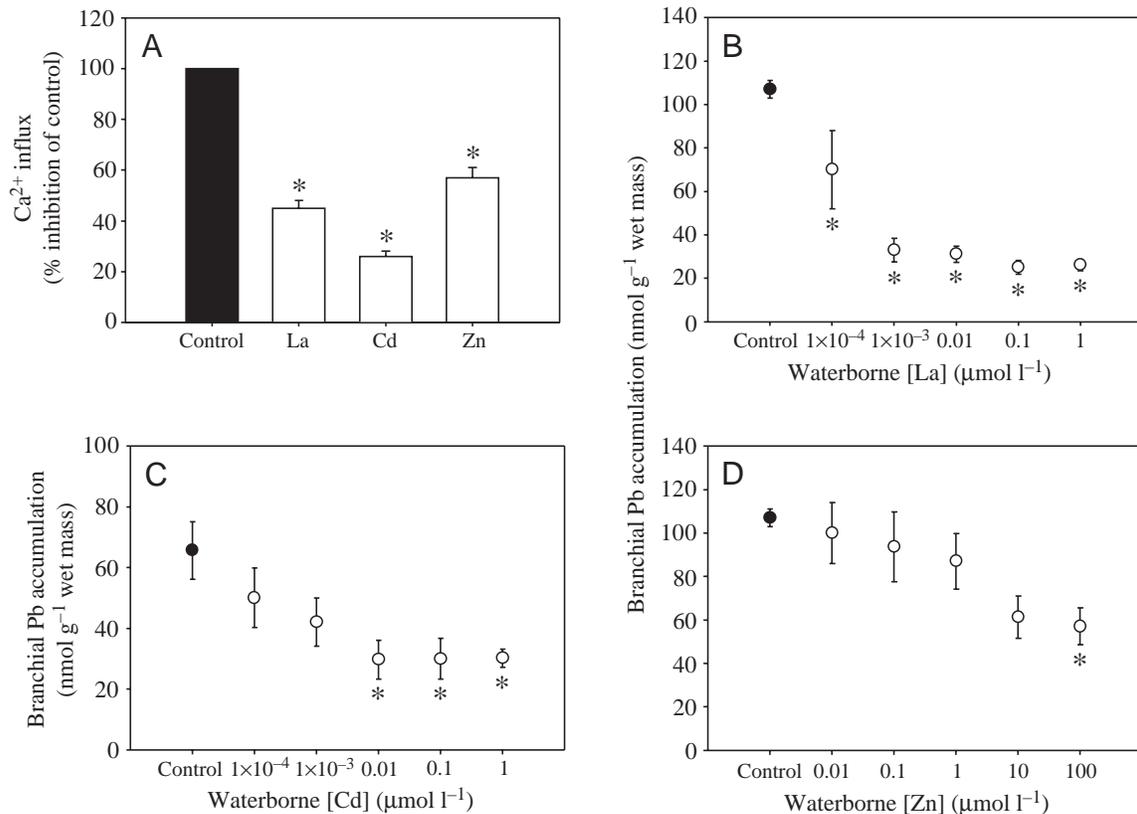


Fig. 5. (A) Percentage inhibition of calcium uptake in juvenile rainbow trout exposed to control conditions (black bar) or 1  $\mu\text{mol l}^{-1}$  lanthanum, 1  $\mu\text{mol l}^{-1}$  cadmium or 100  $\mu\text{mol l}^{-1}$  zinc (white bars) in synthetically modified water ( $600 \mu\text{mol l}^{-1}$   $\text{Ca}^{2+}$ ). (B–D) Branchial lead accumulation in juvenile rainbow trout exposed to control conditions (black circles) or (B) a series of waterborne lanthanum concentrations, (C) a series of waterborne cadmium concentrations, and (D) a series of waterborne zinc concentrations. Values are means  $\pm$  1 S.E.M. ( $N=7$  per treatment). \*Significant difference ( $P<0.05$ ; two-tailed Student's  $t$ -test) from corresponding control mean.

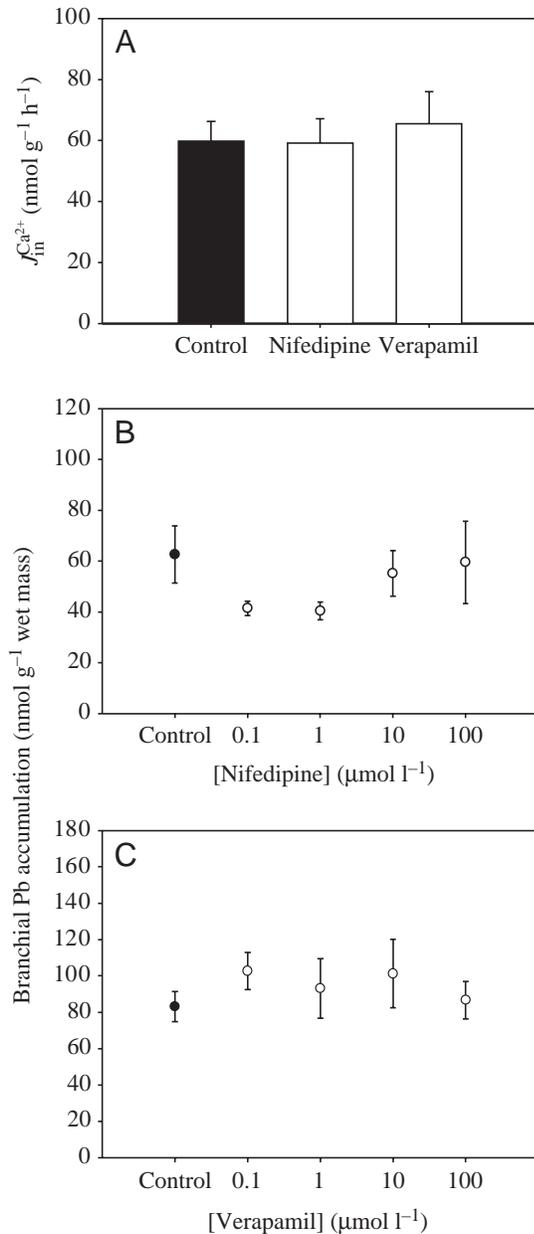


Fig. 6. (A) The effects of the voltage-dependent  $\text{Ca}^{2+}$  channel blockers nifedipine and verapamil on calcium uptake  $J_{\text{in}}^{\text{Ca}^{2+}}$  in juvenile rainbow trout exposed to control conditions (black bars) or 100  $\mu\text{mol l}^{-1}$  nifedipine and 100  $\mu\text{mol l}^{-1}$  verapamil (white bars) in synthetically modified water (600  $\mu\text{mol l}^{-1}$   $\text{Ca}^{2+}$ ). (B,C) The effect of nifedipine on branchial lead accumulation in juvenile trout exposed to control conditions (black circle) or (B) to 0.1, 1, 10 and 100  $\mu\text{mol l}^{-1}$  nifedipine (white circles), (C) to 0.1, 1, 10 and 100  $\mu\text{mol l}^{-1}$  verapamil (white circles). Values are means  $\pm 1$  S.E.M. ( $N=7$  per treatment).

inhibition of lead uptake was dramatic from 0.001 to 1  $\mu\text{mol l}^{-1}$  lanthanum, with a reduction of approximately 77% (compared to control) in lead accumulation in the presence of these waterborne lanthanum concentrations.

Waterborne cadmium had similar effects on  $\text{Ca}^{2+}$  uptake and

branchial lead accumulation. A waterborne concentration of 1  $\mu\text{mol l}^{-1}$  cadmium reduced the rate of  $\text{Ca}^{2+}$  influx by approximately 74% compared to controls (Fig. 5A). Branchial cadmium accumulation occurred in a dose-dependent fashion when waterborne cadmium concentration was increased from control levels to 1  $\mu\text{mol l}^{-1}$ , with the largest increase occurring at the highest concentration (data not shown). This dose-dependent uptake corresponded with reduced levels of branchial lead accumulation (Fig. 5C). While gill cadmium accumulation increased by approximately 20-fold, lead uptake was reduced by 56% upon exposure to  $2.3 \pm 0.2 \mu\text{mol l}^{-1}$  dissolved lead. Results using zinc as a calcium-channel blocker were similar.  $\text{Ca}^{2+}$  uptake was reduced by approximately 43% in the presence of 100  $\mu\text{mol l}^{-1}$  zinc (Fig. 5A). This inhibition corresponded with a reduction of approximately 47% in branchial lead accumulation in fish exposed to  $2.3 \pm 0.2 \mu\text{mol l}^{-1}$  dissolved lead in the presence of zinc (Fig. 5D). Similar to cadmium, zinc also accumulated dose-dependently at the gill, with the highest accumulation occurring at 100  $\mu\text{mol l}^{-1}$  zinc (data not shown).

Voltage-dependent, L-type calcium-channel blockers did not appear to affect  $\text{Ca}^{2+}$  influx or branchial lead accumulation. Exposure to 100  $\mu\text{mol l}^{-1}$  nifedipine or 100  $\mu\text{mol l}^{-1}$  verapamil did not significantly reduce the rate of  $\text{Ca}^{2+}$  influx at 600  $\mu\text{mol l}^{-1}$   $\text{Ca}^{2+}$  (Fig. 6A). The effects of nifedipine on lead accumulation were found to be not significant compared to controls (Fig. 6B). Exposure to verapamil yielded similar results when comparing lead accumulation in the presence and absence of the blocking agent (Fig. 6C).

#### *Effect of $\text{CaCl}_2$ injection on $\text{Ca}^{2+}$ influx and branchial lead accumulation*

Injection of  $\text{CaCl}_2$  significantly reduced  $\text{Ca}^{2+}$  influx by approximately 52% compared to fish sham-injected with vehicle only (Fig. 7A). Inhibition of  $\text{Ca}^{2+}$  influx corresponded with a significant decrease in branchial lead accumulation in  $\text{CaCl}_2$  injected fish (Fig. 7B). This reduction was approximately 37% compared to controls (NaCl injected).

#### *The role of efflux in lead-induced hypocalcemia*

The role of efflux in lead-induced disruption of  $\text{Ca}^{2+}$ -homeostasis was assessed during exposure to control conditions or 6.3  $\pm 0.1 \mu\text{mol l}^{-1}$  dissolved lead. The results are summarized in Table 2. Rainbow trout exposed to control conditions (0 h) had an average  $\text{Ca}^{2+}$ -efflux ( $J_{\text{out}}^{\text{Ca}^{2+}}$ ) of approximately 32.8  $\pm 4.6 \text{ nmol g}^{-1} \text{ h}^{-1}$ . Lead-exposed fish did not show significant deviations from this value after exposures of 3, 27 and 51 h to the experimental conditions.

#### *High-affinity $\text{Ca}^{2+}$ -ATPase activity*

Adult rainbow trout were exposed to a dissolved lead concentration of 5.5  $\pm 0.4 \mu\text{mol l}^{-1}$ . When compared to values for unexposed fish at  $t=0$  h (control sampling), activity after 3 h of lead exposure was not reduced. However, prolonged exposure resulted in a reduction in activity after 24 h, and a significant 80% inhibition by 96 h of lead exposure (Fig. 8A).

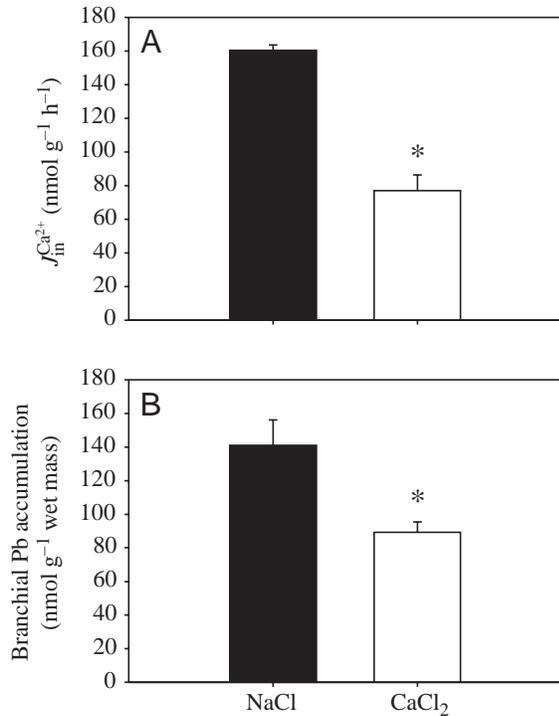


Fig. 7. The effect of CaCl<sub>2</sub> injection on (A) Ca<sup>2+</sup> influx  $J_{in}^{Ca^{2+}}$  in juvenile rainbow trout in synthetically modified water (600  $\mu\text{mol l}^{-1}$  Ca<sup>2+</sup>), and (B) branchial lead accumulation in juvenile rainbow trout. Values are means  $\pm$  1 S.E.M. ( $N=7$  per treatment). \*Significant difference ( $P<0.05$ ; two-tailed Student's  $t$ -test) from corresponding control mean.

Table 2. Unidirectional calcium efflux rates in juvenile rainbow trout in City of Hamilton dechlorinated tapwater in control water or after exposure to  $6.3\pm 0.14 \mu\text{mol l}^{-1}$  dissolved lead for 0, 3, 27 or 51 h

Time of Pb exposure (h)	$J_{out}^{Ca^{2+}}$ ( $\text{nmol g}^{-1} \text{h}^{-1}$ )
0 (Control)	$32.8\pm 4.6$
0–3	$39.6\pm 7.2$
24–27	$37.1\pm 4.0$
48–51	$48.6\pm 8.8$

Values are means  $\pm$  S.E.M. ( $N=7$ ). There were no significant differences.

Branchial lead accumulation increased significantly after 24 h and 96 h in fish exposed to an identical waterborne lead concentration (Fig. 8B). This accumulation appeared to correlate well with the inhibition of high-affinity Ca<sup>2+</sup>-ATPase activity (Fig. 8C). The *in vitro* effects of directly added Pb(NO<sub>3</sub>)<sub>2</sub> on the Ca<sup>2+</sup>-ATPase assay were insignificant.

### Discussion

Previous research has presented circumstantial evidence that lead may compete with Ca<sup>2+</sup> for the same binding sites on the

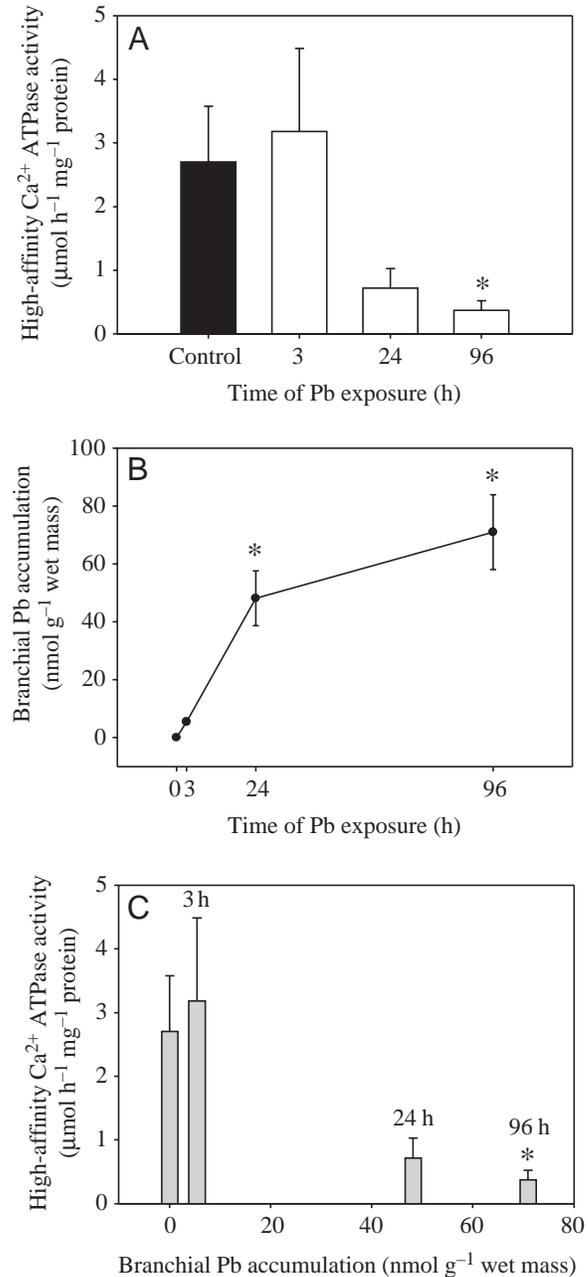


Fig. 8. Time course analysis of (A) branchial high affinity Ca<sup>2+</sup>-ATPase activity in adult rainbow trout, and (B) branchial lead accumulation, and (C) plot of high-affinity Ca<sup>2+</sup>-ATPase activity versus branchial lead accumulation in rainbow trout exposed to control conditions and to  $5.5\pm 0.4 \mu\text{mol l}^{-1}$  dissolved lead for 3 h, 24 h and 96 h. Values are means  $\pm$  1 S.E.M. ( $N=6$ ). \*Significant difference ( $P<0.05$ ; one-way ANOVA with Dunnett's *post-hoc* multiple comparison) from control sampling mean.

gills of freshwater fish (Davies et al., 1976; Varanasi and Gmur, 1978; Sorensen, 1991; MacDonald et al., 2002; Rogers et al., 2003). In the present study we provide direct evidence suggesting that lead does in fact compete with calcium for uptake and that interference with branchial Ca<sup>2+</sup> transport

includes sharing of at least two phases of this transport: an immediate interaction with apical entry and a more slowly developing interaction with high-affinity  $\text{Ca}^{2+}$ -ATPase.

The observed rapid inhibition of calcium influx (Fig. 1) after exposure to 1.4 and 2.3  $\mu\text{mol l}^{-1}$  lead is consistent with previous studies demonstrating reduced  $\text{Ca}^{2+}$  influx after exposure to lead concentrations close to 4.8  $\mu\text{mol l}^{-1}$  (Rogers et al., 2003). This is contrary to findings presented by Sayer et al. (1989, 1991) that lead exposure does not result in disruption of  $\text{Ca}^{2+}$  influx. From the present data (Figs 1 and 2), it is apparent that at exposure concentrations (0.48  $\mu\text{mol l}^{-1}$  upwards) that approach environmentally relevant or normal levels (0.003–0.58  $\mu\text{mol l}^{-1}$  lead; Demayo et al., 1982), the hypocalcemic effects of lead are still prominent. Additionally, influx inhibition occurs immediately, suggesting that the interaction may be competitive in nature, similar to metals like cadmium (Verboost et al., 1987, 1989) and zinc (Spry and Wood, 1985; Hogstrand et al., 1994, 1996). Therefore, measurements of  $\text{Ca}^{2+}$  influx were useful for validating a kinetic approach to the analysis of the relationship between lead and  $\text{Ca}^{2+}$ .

The construction of uptake curves at a number of waterborne substrate ( $\text{Ca}^{2+}$ ) concentrations using various waterborne lead levels allowed for characterization of the effect of lead on branchial  $\text{Ca}^{2+}$  influx kinetics. From these curves,  $K_m$  and  $J_{\text{max}}$  values were calculated for each lead concentration used (Table 1). These data indicated a typical Michaelis–Menten competitive relationship with a significant increase in  $K_m$  value with increasing waterborne lead concentrations. Increases in  $K_m$  suggest that as waterborne lead concentrations are elevated, the affinity of the calcium-binding site for the calcium ion decreases, due to the presence of a competitor. Consequently, the amount of  $\text{Ca}^{2+}$  required to achieve half maximum transport is elevated. The stability of  $J_{\text{max}}$  values obtained from this kinetic study confirmed the competitive nature of the lead/ $\text{Ca}^{2+}$  relationship, as the maximum rate of  $\text{Ca}^{2+}$  transport, or the integrity of  $\text{Ca}^{2+}$  binding sites, remained unchanged. These findings are consistent with those made for zinc (Hogstrand et al., 1994) and for cadmium (Niyogi and Wood, in press), metals that are known calcium antagonists.

It is also important to note, however, that exposure to the highest lead concentration of 4.8  $\mu\text{mol l}^{-1}$  may have caused a reduction in the maximum rate of  $\text{Ca}^{2+}$  influx. This was not detected by Lineweaver–Burk analysis, but was indicated by significant decreases in  $J_{\text{max}}$  value when using Eadie–Hofstee and non-linear regression to interpret kinetic data. This would suggest that the branchial interaction between lead and  $\text{Ca}^{2+}$  might be described as a mixed competitive and non-competitive interaction upon exposure to elevated waterborne lead concentrations. This seems to be consistent with data presented in Fig. 2, that suggest the possibility of a  $J_{\text{max}}$  decrease, Fig. 4, which suggests that exposure to 4.8  $\mu\text{mol l}^{-1}$  waterborne lead results in a linear or diffusive component to lead influx when the waterborne concentration of  $\text{Ca}^{2+}$  is reduced (350 and 150  $\mu\text{mol l}^{-1}$ ), and Fig. 8, which indicates that a high concentration of accumulated lead in the gill may

inhibit baso-lateral high-affinity  $\text{Ca}^{2+}$ -ATPase activity, albeit over a longer exposure period.

Kinetic analysis of lead and  $\text{Ca}^{2+}$  interaction allowed calculation of an inhibitor constant ( $K_{i,\text{Pb}}$ ) for calcium influx of 0.48  $\mu\text{mol l}^{-1}$ . Based on this value, it is evident that the affinity of lead for  $\text{Ca}^{2+}$  binding sites on the fish gill is less than that for cadmium, but greater than that for zinc. Niyogi and Wood (in press) reported an inhibitor constant of 0.12  $\mu\text{mol l}^{-1}$  for cadmium in rainbow trout, a value 4 times lower than that reported in the present study for lead. From the data of Hogstrand et al. (1994, 1998), the inhibition constant of zinc appears to be above 2  $\mu\text{mol l}^{-1}$ . This variation in affinity for  $\text{Ca}^{2+}$  binding sites could explain the difference in potency between lead, cadmium and zinc in terms of acute toxicity; cadmium is the most toxic with a 96 h  $\text{LC}_{50}$  of 0.2  $\mu\text{mol l}^{-1}$  (Hollis et al., 1999) compared to an  $\text{LC}_{50}$  of 4.8  $\mu\text{mol l}^{-1}$  for lead (Rogers et al., 2003) and 13.3  $\mu\text{mol l}^{-1}$  for zinc (Alsop et al., 1999). All values were determined in the same water quality.

Branchial lead accumulation was largely dependent upon waterborne  $\text{Ca}^{2+}$  concentrations, as shown in Fig. 4. At the lowest waterborne  $\text{Ca}^{2+}$  concentration of 150  $\mu\text{mol l}^{-1}$ , lead accumulation significantly increased as waterborne lead concentrations increased from control to 4.8  $\mu\text{mol l}^{-1}$ . This relationship changed as  $\text{Ca}^{2+}$  concentration increased. Accumulation at 0.48, 2.4 and 4.8  $\mu\text{mol l}^{-1}$  lead was not significantly different at higher levels of waterborne  $\text{Ca}^{2+}$ , demonstrating strong protective effects of waterborne  $\text{Ca}^{2+}$  against branchial lead uptake. This confirms other existing evidence highlighting the protective effects of water hardness in lead toxicity (Sorensen, 1991) and suggests that it is the  $\text{Ca}^{2+}$  component of hard water that dictates the toxicity of dissolved lead to fish. Richards and Playle (1999) reported similar protective effects of  $\text{Ca}^{2+}$  against cadmium accumulation on the gills in synthetically modified soft water. Alsop and Wood (1999) demonstrated reduced zinc uptake and toxicity with increasing waterborne  $\text{Ca}^{2+}$  concentration. Considering the differential branchial lead accumulation and kinetic lead/ $\text{Ca}^{2+}$  analysis from the present study, it is evident that the gill is probably the major site of a predominately competitive interaction between lead and  $\text{Ca}^{2+}$  that contributes to lead toxicity in the rainbow trout.

Further evidence supporting the existence of a competitive relationship between lead and  $\text{Ca}^{2+}$  was acquired through the use of apical calcium-channel blockers. While inhibiting  $\text{Ca}^{2+}$  uptake (Fig. 5A), waterborne lanthanum, a classic  $\text{Ca}^{2+}$  channel blocker (Weiss, 1974), significantly reduced the amount of lead accumulation on the gill (Fig. 5B). This suggests that apical entry of lead into the chloride cells probably occurs through a lanthanum-sensitive, voltage-independent apical calcium channel. These observations are similar to those reported for zinc by Hogstrand et al. (1996), with an approximate 53% decrease in zinc uptake occurring in the presence of 1  $\mu\text{mol l}^{-1}$  lanthanum compared to controls. Comhaire et al. (1998) reported a 48% inhibition of cobalt uptake at low-level lanthanum; however, 1  $\mu\text{mol l}^{-1}$

waterborne lanthanum exposure resulted in a stimulation of cobalt influx. A similar effect was observed in the present study at waterborne lanthanum concentrations exceeding  $1 \mu\text{mol l}^{-1}$  (data not shown). Stimulated lead accumulation may have been due to disruption of apical membrane integrity by high lanthanum concentrations, resulting in an increase in the diffusive component of lead uptake. Finally, the results of the present study are consistent with those of Verbost et al. (1987), demonstrating an inhibition of cadmium influx by lanthanum, and leading to the conclusion that cadmium and  $\text{Ca}^{2+}$  share the same apical entry sites. This has led to the use of cadmium for the purpose of calcium-channel antagonist experiments (Comhaire et al., 1998), as in the present study.

The effects of cadmium on  $\text{Ca}^{2+}$  influx were significant (Fig. 5A). As gill cadmium accumulation increased with increased waterborne cadmium concentrations, gill lead accumulation was reduced in a dose-dependent fashion compared to uptake observed in the absence of the blocker (Fig. 5C). This is consistent with the results of Comhaire et al. (1998), who reported significant inhibition of cobalt uptake upon exposure to similar waterborne cadmium concentrations. The inhibition of lead accumulation by cadmium, a metal that traverses the chloride cell apical membrane through voltage-independent, lanthanum-inhibitable channels (Verbost et al., 1989), is further evidence supporting a similar entry route for lead. In further support of this theory is the effect of zinc on lead accumulation (Fig. 5D). The ability of zinc, a metal believed to enter fish *via*  $\text{Ca}^{2+}$  uptake pathways (Spry and Wood 1985; Hogstrand et al., 1994, 1995, 1996, 1998), to reduce branchial lead accumulation suggests a similar route of entry for these metals.

The possibility of lead and  $\text{Ca}^{2+}$  transport through voltage-dependent, L-type calcium channels at the apical surface of the chloride cell was discounted by the lack of effect of the voltage-dependent  $\text{Ca}^{2+}$  channel blockers verapamil and nifedipine (Perry and Flik, 1988) (Fig. 6A–C). Perry and Flik (1988) reported similar results when assessing the effect of nifedipine on  $\text{Ca}^{2+}$  uptake, leading to the suggestion that  $\text{Ca}^{2+}$  flux across the apical membrane occurs through voltage-independent  $\text{Ca}^{2+}$  channels.

Injection of ionic  $\text{Ca}^{2+}$  was also employed to manipulate apical  $\text{Ca}^{2+}$  channels so as to inhibit  $\text{Ca}^{2+}$  influx *via* the stanniocalcin-controlled pathway. Previous studies have shown that activation of this pathway results in a decreased permeability of chloride cells to  $\text{Ca}^{2+}$  entry, thereby reducing influx (Perry et al., 1989; Verbost et al., 1993). In the present study, the hypocalcemic effects of stanniocalcin induction reduced both  $\text{Ca}^{2+}$  influx and lead accumulation (Fig. 7A,B). This provides further evidence that lead uptake is by the same mechanism as  $\text{Ca}^{2+}$  uptake. These results are similar to those reported by Hogstrand et al. (1996), where both  $\text{Ca}^{2+}$  and zinc influx rates were reduced by injection of  $\text{Ca}^{2+}$ .

The other significant process in the transepithelial uptake of  $\text{Ca}^{2+}$  is the movement across the baso-lateral membrane against an electrochemical gradient *via* a high affinity  $\text{Ca}^{2+}$ -ATPase mechanism (Perry and Flik, 1988). The importance of

this enzyme to calcium transport makes it a vulnerable target for  $\text{Ca}^{2+}$  antagonists such as cadmium, which, following sufficient accumulation within the chloride cell, inhibits  $\text{Ca}^{2+}$ -ATPase activity contributing to disturbances in  $\text{Ca}^{2+}$  homeostasis (Verbost et al., 1989). The present study revealed that prolonged lead exposure results in significant inhibition of high affinity  $\text{Ca}^{2+}$ -ATPase activity (Fig. 8A). This reduction in activity occurred after 24–96 h of lead exposure, suggesting that similar to cadmium, lead must accumulate in sufficient amounts at the gill to have a negative effect on  $\text{Ca}^{2+}$ -ATPase activity (Fig. 8C). Accumulation at the gill was significantly elevated after 24 h and 96 h of lead exposure (Fig. 8B), the same time points where substantial inhibition of enzyme activity was observed. This mechanism of  $\text{Ca}^{2+}$  homeostasis disruption is also similar to that proposed for zinc (Hogstrand et al., 1996). With increased amounts of branchial lead accumulation following prolonged exposure, a slower, non-competitive component to disruption of  $\text{Ca}^{2+}$  homeostasis would occur. This would ultimately have an effect on  $J_{\text{max}}$ , decreasing the overall efficiency of the  $\text{Ca}^{2+}$  transport system.

$\text{Ca}^{2+}$  efflux was much less sensitive to lead than  $\text{Ca}^{2+}$  influx (Table 2). This is evident by the absence of efflux stimulation at a waterborne lead concentration ( $6.3 \mu\text{mol l}^{-1}$ ) that significantly reduced  $\text{Ca}^{2+}$  influx (Rogers et al., 2003) and is at least 4 times higher than the minimum concentration required to elicit  $\text{Ca}^{2+}$  influx inhibition. These findings are consistent with those for cadmium (Verbost et al., 1987; Reid and McDonald, 1988). Verbost et al. (1987) found the effect of cadmium on  $\text{Ca}^{2+}$  efflux was insignificant, except for measurements made at the highest cadmium concentration. Reid and McDonald (1988) found no effect of cadmium on branchial  $\text{Ca}^{2+}$  efflux at circumneutral pH and at moderately acidic pH (4.8). While significant stimulation of efflux by lead was not observed, a slight, non-significant increase in efflux rate did occur after 48 h of lead exposure. This could reflect a secondary effect of lead on the integrity of paracellular routes, through which  $\text{Ca}^{2+}$  efflux is thought to occur (Perry and Flik, 1988).

### Conclusions

To our knowledge, this study is the first to present direct evidence that the uptake of waterborne lead by freshwater adapted rainbow trout is by the same mechanism as  $\text{Ca}^{2+}$ . The uptake involves competitive inhibition of apical entry at lanthanum-sensitive  $\text{Ca}^{2+}$  channels and interference with the function of the ATP-driven baso-lateral  $\text{Ca}^{2+}$  pump. Similarities are evident between lead and other known calcium antagonists such as cadmium, zinc and cobalt, suggesting avenues for further research that include characterization of the interaction between lead and  $\text{Ca}^{2+}$ -ATPase as well as the intracellular handling of lead by  $\text{Ca}^{2+}$  transport proteins such as calmodulin.

This study could have potential implications for a predictive modelling approach such as the Biotic Ligand Model (BLM) (Paquin et al., 2002), which is based on a toxic metal binding to the gills in competition with protective and

nutrient ions in the water column (Playle et al., 1993; Playle, 1998; Paquin et al., 2002). Currently, binding models for lead are being developed (MacDonald et al., 2002), and based on their predictions and the physiological evidence presented in this study, it appears that lead is highly capable of out-competing  $\text{Ca}^{2+}$  for specific transport sites at the freshwater fish gill.

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