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Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The mechanism for acute toxicity of lead (Pb) in rainbow trout (*Oncorhynchus mykiss*) was investigated at Pb concentrations close to the 96 h LC50 of 1.0 mg dissolved Pb l^{-1} (0.8–1.4, 95% C.I.) determined in dechlorinated Hamilton city tap water (from Lake Ontario, hardness = 140 mg l^{-1} $CaCO_3$). Tissue Pb accumulation associated with death was highest in the gill, followed by kidney and liver. Significant ionoregulatory impacts were observed in adult rainbow trout (200–300 g) fitted with indwelling dorsal aortic catheters and exposed to 1.1 ± 0.04 mg dissolved Pb l^{-1}. Decreased plasma [Ca^{2+}], [Na^+] and [Cl^-] occurred after 48 h of exposure through to 120 h, with increases in plasma [Mg^{2+}], ammonia, and cortisol. No marked changes in PaO_2, $PaCO_2$, pH, glucose, or hematological parameters were evident. Branchial Na^+/K^+ ATPase activity in juvenile trout exposed to concentrations close to the 96 h LC50 was inhibited by approximately 40% after 48 h of Pb exposure. Calcium ion flux measurements using ^{45}Ca as a radiotracer showed 65% inhibition of Ca^{2+} influx after 0, 12, 24 or 48 h exposure to the 96 h LC50 concentration of Pb. There was also significant inhibition (40–50%) of both Na^+ and Cl^- uptake, measured with ^{22}Na and ^{36}Cl simultaneously. We conclude that the mechanism of acute toxicity for Pb in rainbow trout occurs by ionoregulatory disruption rather than respiratory or acid/base distress at Pb concentrations close to the 96 h LC50 in moderately hard water.

Keywords: Lead; Rainbow trout; Waterborne; Acute toxicity; Ionoregulation

1. Introduction

Lead, a group IVA element in the periodic table, is a naturally occurring metal present in the earth's crust, rock, soil, and water. Lead enters aquatic environments by a number of pathways. The earth's crust, geologic weathering phenomena, and volcanic activity account for natural sources, but most waterborne lead derives from human activities such as mining and smelting, coal burning, cement manufacturing, and use in gasoline, batteries, and paint (World Health Organization, 1995). Contamination of water by such practices is the primary cause of lead poisoning in fish (Sorensen, 1991).

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Under normal conditions, waterborne lead falls within the range of 0.0006–0.12 mg l^{-1} (Demayo et al., 1982) though concentrations as high as 0.89 mg l^{-1} have been reported (Research Triangle Institute, 1999). In hard water, lead readily complexes to form $Pb(CO_3)$. Such complexes are less available for uptake and, therefore, less toxic to fish (Davies et al., 1976; Holcombe et al., 1976). Calcium, a prominent component of hard water, is believed to compete with lead for uptake, contributing to the protective effects of water hardness. Lead species such as Pb^{2+} and $Pb(OH)^{+}$ that commonly occur in soft, low pH waters are more available and toxic (Davies et al., 1976; Hodson et al., 1978).

Although a number of physiological effects of lead have been well characterized in various fish species (Davies et al., 1976; Hodson, 1976; Hodson et al., 1977; Weber et al., 1991; Weber, 1993), relative to other metals, little is known about the acute toxic mechanism of lead in fish. Circumstantial evidence suggests that a lead/calcium antagonism exists (Hodson et al., 1978; Varanasi and Gmur, 1978; Settle and Patterson, 1980) similar to that discovered for cadmium (Verbost et al., 1987, 1989) and zinc (Spry and Wood, 1985), by which these metals may directly compete with Ca^{2+} for uptake at calcium binding sites. This would result in hypocalcemia and death of the fish. Such a relationship, however, has not been proven directly. In addition to this possible mechanism, lead may also disrupt the balance of ions such as Na^{+} and Cl^{-} as exhibited by metals like copper (Lauren and McDonald, 1985) and silver (Wood et al., 1996; Morgan et al., 1997). Ahern and Morris (1998) observed disruption of Na^{+} balance and a 40% inhibition of Na^{+}/K^{+} ATPase activity in crayfish chronically exposed to 0.5 mg Pb l^{-1}. Another possibility is that respiratory effects may occur similar to those caused by aluminum at moderately acidic pH (Playle et al., 1989) or by nickel at circumneutral pH (Pane et al., 2003). It is unknown whether any of these mechanisms explain the toxic action of lead in fish.

Detailed knowledge of the acute toxic mechanisms for a number of water-borne metals (e.g. copper, silver) has been established, allowing development of models used to predict toxicity in many different water chemistries. The biotic ligand model (BLM) is one example (McGeer et al., 2000; DiToro et al., 2001). Currently, an accepted model for lead does not exist, a situation that is at least partially due to the lack of understanding of its acute toxic mechanism. Therefore, the objective of this study was to determine the mechanism of acute toxicity of lead in the rainbow trout (*Oncorhynchus mykiss*). The experimental approach used was similar to that earlier employed for silver (Wood et al., 1996) and nickel (Pane et al., 2003). It was designed to provide an appropriate 'diagnosis' of the nature of acute physiological disturbances within the fish when they were faced with a lead challenge at the acute level (i.e. close to the 96 h LC50).

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (1.5–3.5 g), for use in the LC50 tests, flux experiments, Na^{+}/K^{+} ATPase measurements, and adult rainbow trout (200–300 g), for use in cannulated fish studies, were obtained from Humber Springs Trout Farm in Orangeville, Ontario. Fish were held in dechlorinated City of Hamilton tap water (from Lake Ontario) at a temperature of 7–12 °C, and fed commercial trout pellets at a ration of 1% total body weight per day. Water composition was $Ca^{2+} = 1.0$, $Mg^{2+} = 0.2$, $Na^{+} = 0.6$, $Cl^{-} = 0.8$, $K^{+} = 0.05$ (mM), total Pb = 0.68 μg l^{-1}, dissolved organic carbon (DOC) = 3 mg l^{-1}, hardness (as $CaCO_3$) of approximately 140 mg l^{-1}, and pH 7.9–8.0. Experimental animals were starved 72 h prior to and throughout all experiments.

2.2. 96 h LC50 and bioaccumulation

The 96 h LC50 determination for rainbow trout was carried out under flow-through conditions following closely methods outlined in Sprague (1969). Juvenile trout were transferred to one of twelve 20 l exposure tanks (15 fish per tank) fed at a rate of 600 ml min^{-1} from a head reservoir and a series of mixing tanks with aerated dechlorinated

Hamilton city tap water. Trout were allowed to acclimate to these conditions for a period of 48 h before exposure to nominal total Pb concentrations of 0.25, 0.5, 1, 3, 5 mg l^{-1} plus one control treatment (two tanks per treatment). Pb exposure was carried out by dripping a stock solution of $Pb(NO_3)_2$ (Sigma-Aldrich) dissolved in double-distilled water (NANOpure II; Sybron/Barnstead, Boston, MA) at a rate of 1 ml min^{-1} into mixing tanks. At t = 0 h, the tanks were spiked using the stock solution to bring exposure water to the appropriate concentration.

Water samples were obtained daily, filtered (Acrodisc 0.45 μm filter; Pall Corporation, MI) and unfiltered. Filtered water samples were taken to quantify the concentration of dissolved Pb in solution. Water drawn was placed in clean plastic scintillation vials and acidified to 1% HNO_3 for storage until samples were analyzed for total and dissolved Pb concentrations. No glassware was employed. Mortality was monitored every 6 h. At each 6 h sampling point, dead fish were removed and gill, kidney, and liver samples were excised for tissue lead analysis. Gills were blotted dry and tissues were weighed, digested at 55 °C for 48 h in 1 N HNO_3 (Fisher Scientific; trace metal grade), centrifuged, and the supernatant drawn off for total Pb measurement. Both water samples and tissue digests were analyzed for Pb using graphite furnace atomic absorption spectrophotometry (GFAAS) (220 SpectrAA; Varian, Australia) against a certified multi-element standard (Inorganic Ventures Inc.). QA/QC procedures include GFAAS and flame atomic absorption spectrophotometry (FAAS) performance tests twice yearly, multiple determinations on the same samples, multiple blanks, addition-recovery tests, and the sample-addition method for quantification of low-level samples.

2.3. Cannulated fish studies

2.3.1. Experimental protocol

The nominal total Pb exposure concentration chosen for this experiment was 1 mg l^{-1}, based on the measured Pb 96 h LC50 in City of Hamilton tap water (see Section 3). Adult rainbow trout were anaesthetized using MS222 (0.1 g l^{-1} neutralized with NaOH) and surgically fitted with indwelling dorsal aortic catheters (PE50 tubing) (Soivio et al., 1972) filled with Cortland saline (Wolf, 1963). Immediately following surgery, fish were transferred to individual, darkened plexiglass chambers (volume = 8 l) served with individual aeration. Control chambers were fed from a vigorously aerated head tank at a rate of 100 ml min^{-1}. Pb exposure chambers received an equal flow rate but from an aerated mixing tank receiving a 1 ml min^{-1} drip of $Pb(NO_3)_2$ stock solution. At t = 0 h, Pb-exposed chambers were spiked with an appropriate volume of stock solution to achieve desired Pb concentrations.

Control and Pb treatments were run simultaneously using eight control fish and seven Pb-exposed fish. In both groups, blood samples (1 ml, with saline and red blood cell replacement) were taken prior to Pb exposure (t = 0 h) and at 24, 48, 72, 96 and 120 h. Blood was drawn anaerobically into an ice-cold, gas-tight Hamilton syringe for analysis of arterial pH (pH_a), O_2 tension (P_aO_2), plasma total CO_2 (C_aCO_2), hematocrit (Ht), hemoglobin (Hb), and plasma levels of Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, total Pb, glucose, lactate, cortisol, total protein, and total ammonia. Plasma was separated by centrifugation at 13 000 × *g* and frozen at −80 °C for these analyses. Erythrocytes were gently re-suspended in Cortland saline (Wolf, 1963) and re-injected into the fish in a total volume of 1.0 ml. Water samples, filtered (0.45 μm) and unfiltered, were drawn simultaneously from in front of the mouth and processed as in Section 2.2. Unfiltered samples were also taken for measurement of inspired oxygen tension (P_IO_2) and pH_I.

2.3.2. Analytical methods

For most blood parameters, the analytical procedures used followed those outlined in Wood et al. (1988). Radiometer electrodes and meters were used in the analysis of pH_a, PaO_2, inspired water pH (pH_I), and O_2 tension (P_IO_2). Hb measurements employed the colorimetric cyanmethemoglobin method (Sigma-Aldrich). Ht was measured directly from micro-hematocrit tubes, spun at 5000 × *g* for 4 min and the plasma removed anaerobically for measurement of total

CO_2 (C_aCO_2) with a Corning 965 CO_2 analyzer. Lactate was measured enzymatically (L-lactate dehydrogenase/NADH; Sigma-Aldrich) on samples deproteinized with ice cold 8% perchloric acid. Glucose was measured enzymatically from the same acid extracts neutralized with 1 M K_2CO_3 using the hexokinase/glucose-6-phosphate dehydrogenase method (Sigma-Aldrich). Plasma cortisol was determined using a ^{125}I radioimmunoassay (ICN Biomedicals; Montreal, Quebec). Total plasma ammonia concentration was measured enzymatically (glutamate dehydrogenase/NADP; Sigma-Aldrich). Plasma Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations were determined by FAAS (220FS SpectrAA; Varian), and plasma Pb by GFAAS. Plasma was diluted with 0.2% La for plasma Ca^{2+} determination. Plasma Cl^- was measured by coulometric titration (Radiometer CMT10).

2.4. Na^+/K^+ ATPase activity

Pb exposure was carried out using methods similar to those used during the 96 h LC50 experiment (20 l tanks, ten fish per tank, flow-through exposure at 600 ml min^{-1}, one control and five exposure tanks, each tank being used for a different exposure time to a lead level close to the 96 h LC50 (see Section 3)). Sampling for gill tissue took place at 0, 24, 48, 72, 96 and 120 h. Fish were removed from exposure water, killed immediately with a blow to the head, and dissected on ice. Excised gills were then placed into a 1.5 ml bullet tube, transferred to liquid nitrogen, and stored at −80 °C. Na^+/K^+ ATPase activity was determined using the microplate method outlined by McCormick (1993). For replicate measurements on the same samples, the coefficient of variation was 0.19. To ensure accurate interpretation of the Na^+/K^+ ATPase data, the effect of Pb on the coupling enzymes used in this assay (PK and LDH) was assessed in vitro via the addition of $Pb(NO_3)_2$ to the homogenate at the concentrations found in experimental samples; and was found to be insignificant. Protein was measured by the Bradford protein assay using bovine serum albumin protein standards (Sigma-Aldrich).

2.5. Ion flux experiments

2.5.1. Ca^{2+} influx measurements

Ca^{2+} influx determinations were carried out on control and experimental fish (juvenile rainbow trout) simultaneously. Experimental fish were pre-exposed to Pb prior to the 2 h flux periods for 0, 12, 24 or 48 h at concentrations close to the resulting 96 h LC50 (see Section 3). The exposure method and conditions were identical to those used in the 96 h LC50 tests. Fluxes were measured at the time intervals 0–2, 12–14, 24–26 and 48–50 h, using 250-ml Pyrex glass beakers fitted with a steady air supply and filled with 60 ml of control or Pb-containing dechlorinated city of Hamilton tap water ($n = 8$ for each treatment). The beakers were partially submerged on a light-shielded wet table receiving a constant water flow (for temperature control) for the entire flux period. Juvenile trout were transferred from exposure chambers to the flux beakers and were allowed a settling period of 0.5 h. After 0.5 h elapsed, ^{45}Ca radiotracer (as $CaCl_2$, specific activity = 15.538 mCi mg^{-1}, Perkin–Elmer, USA) was added to each beaker (6 μCi per chamber) and allowed to equilibrate for 0.25 h. Following equilibration, water samples were taken for the determination of initial ion concentration (800 μl) and for radioisotope counting (200 μl) (CPM). Equal volumes were taken at the end of the 2 h radiotracer exposure period for final ion and radioactivity measurements. Trout were removed from flux chambers at this time and killed with a blow to the head. Immediately thereafter, the dead fish were rinsed for 1 min in 1 mM ethylenediamine-tetraacetic acid (EDTA; Sigma-Aldrich) followed by a 1 min rinse in a 5 mM cold Ca^{2+} solution ($Ca(NO_3)_2$) (Sigma-Aldrich) to remove all surface-bound ^{45}Ca. Fish were then blotted dry, weighed, wrapped in foil, frozen in liquid nitrogen, and stored at −80 °C.

^{45}Ca radioactivity was measured by adding 200 μl water samples to 2 ml of aqueous counting scintillant (ACS™; Amersham) followed by scintillation counting (Rackbeta 1217; LKB Wallac, Turka, Finland). Water samples taken for the determination of total [Ca^{2+}] were diluted with 0.2% La^{3+} and analyzed by FAAS using the Varian 220FS Spectra AA.

Frozen whole fish were homogenized by grinding in liquid nitrogen, using methods similar to those of Hogstrand et al. (1994). Tissue aliquots (ca. 100 mg, exact weight recorded), in duplicate, were transferred to 20 ml glass scintillation vials and solubilized at 50 °C for 24 h in 1 ml of NCS-II™ tissue solubilizer (Amersham). The resulting digest was neutralized using glacial acetic acid (~ 30 μl) and diluted with 10 ml of organic counting scintillant (OCS™; Amersham). Samples were stored in darkness overnight to reduce chemoluminescence. ^{45}Ca radioactivity was measured by scintillation counting and quench-corrected to the same counting efficiency as water samples by the method of external standard ratios, using a ^{45}Ca quench curve generated from the tissue of interest in the same counting cocktail.

2.5.2. Na^+/Cl^- influx measurements

Methods used for Pb-exposure and flux measurements of Na^+ and Cl^- were similar to those of Ca^{2+} with a few differences. Radiotracers used were ^{22}Na (as NaCl, specific activity = 690.19 mCi mg^{-1}, NEN Life Science Products Inc., Boston, MA) and ^{36}Cl (as NaCl, specific activity = 0.012 mCi mg^{-1}, ICN Biomaterials Inc., Irvine, CA). These isotopes were added together for each round of fluxes at 0.2 μCi per flux beaker. Water samples were taken at 0.25 h and at the end of the 2 h flux period for radioisotope counting (1 ml) and for measurement of ion concentration (1 ml). At the end of the flux period, the fish were removed from flux chambers and killed with a blow to the head. Fish were then blotted dry, weighed, placed in plastic scintillation vials, and stored at −20 °C for radioisotope analysis. This method assumes that flux measurements reflect exchanges at the gills, consistent with the lack of absorption of monovalent ions such as Na^+ and Cl^- to plastic or fish surfaces (Wood, 1992).

^{22}Na is a dual γ and β emitter while ^{36}Cl is a pure β emitter. Exposure water samples were first measured for ^{22}Na by γ counting (Minaxi γ; Canberra-Packard, Meridan, CT), followed by addition of 5 ml of scintillation fluid (ACS; Amersham) to the same water samples for ^{36}Cl and ^{22}Na scintillation counting (Rackbeta 1217; LKB Wallac). Differences in counting efficiencies for ^{22}Na between the two instruments were corrected using common standards, and ^{36}Cl β radioactivity was measured by subtracting the corrected ^{22}Na activity from the total activity measured by scintillation counting. $[Na^+]$ was measured by FAAS, while $[Cl^-]$ was analyzed by the mercuric thiocyanate spectrophotometric method (Zall et al., 1956). The concentrations of the two radioisotopes were also measured in the fish. Whole-body ^{22}Na was measured by γ counting. For ^{36}Cl determination, whole fish were digested in 1 N HNO_3 (Fisher Scientific; trace metal grade) at 60 °C for 48 h, homogenized by vortexing, an aliquot removed (1.5 ml), centrifuged at $13\,000 \times g$ for 10 min, and the supernatant (1 ml) added to 5 ml of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT). ^{22}Na plus ^{36}Cl radioactivity was measured by scintillation counting and corrected as above to quantify only ^{36}Cl β emissions. Quenching observed for ^{36}Cl was negligible.

2.6. Calculations and statistical analysis

The 96 h LC50 for Pb with upper and lower 95% confidence intervals was calculated using probit analysis on SPSS statistical software.

Calculations of P_aCO_2 and plasma HCO_3^- in cannulated adult rainbow trout employed equations outlined in Playle et al. (1989) using the Henderson–Hasselbach equation and values for CO_2 solubility and apparent pK (pK′) in trout plasma at the corresponding temperature from Boutilier et al. (1984). Mean cell hemoglobin concentration (g Hb ml^{-1} of red blood cells) was calculated as the ratio of simultaneous Hb to Ht measurements.

Unidirectional calcium, sodium, and chloride influx rates in juvenile rainbow trout were calculated using the following formula:

$$J_{in}^{Ion} = \frac{\text{cpm g fish}^{-1}}{1/2\left[\frac{cpm_i}{[Ion]_i} + \frac{cpm_f}{[Ion]_f}\right]t}$$

where cpm_i, cpm_f, and t are as described in flux procedures, and $[Ion]_i$ and $[Ion]_f$ are initial and

final ion concentrations in the flux water (Wood, 1992).

Calculated data are expressed as mean ± 1 S.E.M. Experimental means were compared with corresponding control mean values by an unpaired two-tailed Student's *t*-test. Time-dependent responses in both control and experimental groups were tested against initial 0 h measurements using a one-way ANOVA with a two-sided Dunnett's post hoc multiple comparison. In addition, two-way ANOVA tests were run on data obtained from adult cannulated trout to investigate the possible significance of time on measured parameters. Where time was a factor, a Tukey honest significant difference (HSD) post-hoc comparison was run. All statistical significance is calculated at $P < 0.05$.

3. Results

3.1. 96 h LC50 and tissue lead distribution

The acute lead 96 h LC50 for juvenile rainbow trout in dechlorinated Hamilton city tap water was calculated to be 1.0 mg l^{-1} as dissolved Pb (total Pb = 1.04 mg l^{-1}), with lower and upper 95% confidence intervals of 0.8 and 1.4 mg Pb $^{-1}$, respectively.

The tissue lead distribution associated with acute mortality at waterborne Pb concentrations close to the 96 h LC50 is given in Fig. 1. Of the tissues sampled, Pb burden was greatest in the gill, reaching concentrations close to 200 μg g^{-1} wet weight in Pb-exposed fish. This was 343-times greater than Pb measured in the gills of control fish. The kidney also showed significant accumulation with a burden of approximately 35 μg g^{-1} wet weight that was about five-times greater than control burdens. Liver Pb was approximately 16 μg g^{-1} wet weight versus control concentrations of about 3 μg g^{-1} wet weight.

3.2. Cannulated fish studies

3.2.1. Physiological responses to lead exposure

The mean measured dissolved Pb concentration during the exposure period for these adult fish was 1.1 ± 0.04 mg l^{-1} (total Pb = 1.4 ± 0.15 mg l^{-1}), which was close to the 96 h LC50 of 1.0 mg l^{-1} for juvenile rainbow trout. The concentration of total Pb in control water was 0.68 ± 0.15 μg l^{-1}.

Rainbow trout exposed to acute levels of $Pb(NO_3)_2$ did not appear to be under any degree of respiratory distress. Measured PaO_2 (Fig. 2a) in Pb-exposed trout did not show any significant changes when compared with control fish, and did not deviate from levels observed at t = 0 h. Similarly, $PaCO_2$ did not change significantly in Pb-exposed or control individuals, though there was a small, non-significant increase at t = 120 h in control individuals (Fig. 2b). Ventilation rate remained stable (Fig. 2c) and plasma lactate unchanged (Fig. 2d) throughout the exposure period.

Acid–base measurements showed that pH_a also remained very stable (Fig. 3a). While no apparent respiratory or metabolic acidosis was evident, a progressive non-significant increase in plasma HCO_3^- occurred in Pb-exposed fish after 48 h of exposure (Fig. 3b).

Glucose, a classic indicator of stress, did not change significantly in control or Pb-exposed fish (Fig. 4a). However, significant increases in plasma cortisol levels were observed in Pb-exposed fish at 96 and 120 h while control values remained relatively stable (Fig. 4b). In addition to an observed cortisol elevation, Pb-exposed fish appeared to be retaining ammonia as plasma concentrations showed a progressive increase from 48 to 120 h of exposure that resulted in levels significantly greater than in control fish (Fig. 4c).

3.2.2. Ionoregulatory impacts

Plasma Pb increased dramatically from background concentrations of 24 to approximately 170 μg l^{-1} by 24 h in Pb-exposed fish (Fig. 5a). However, Pb concentrations did not continue to increase but stabilized from 24 to 120 h. Control plasma Pb levels remained low and unchanged (ca. 20 μg l^{-1}).

Significant hypocalcemia was observed in the Pb treatment (Fig. 5b). Plasma Ca^{2+} concentrations were impacted after 48 h of exposure, showing a 31% reduction by 72 h and significantly lower concentrations than in the control treatment at 72

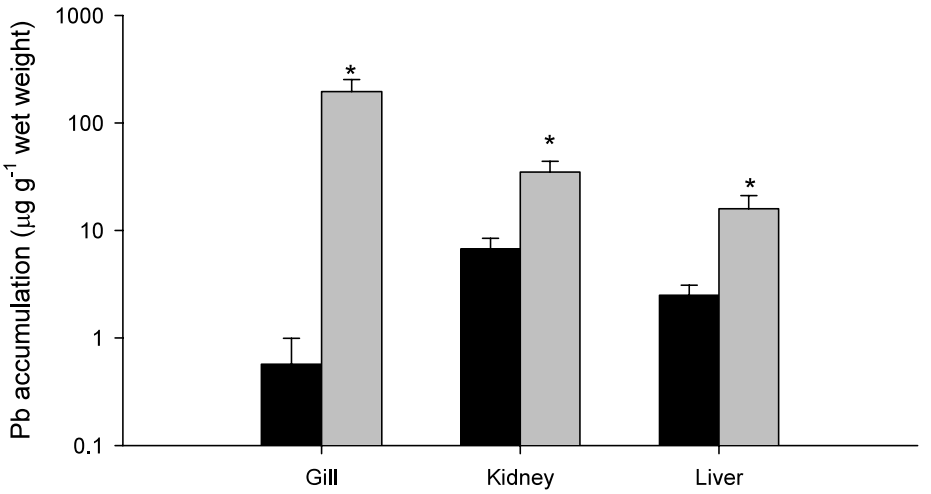

Fig. 1. Pb accumulation in tissues of juvenile rainbow trout exposed to control conditions (black bars) or elevated waterborne Pb (gray bars). The data represent the concentrations occurring at death in rainbow trout exposed to the 96 h LC50 concentration during the 96 h LC50 test. Pb concentrations are expressed as μg g^{-1} $tissue^{-1}$ wet weight. Data are expressed as mean ± 1 S.E.M. Asterisk "*" indicate significant difference from control values in analysis performed on log-transformed data ($P < 0.05$; two-tailed Student's t-test).

and 96 h. Control values remained stable. While Pb appeared to affect Ca^{2+} concentrations in exposed fish, it also caused a small increase in plasma Mg^{2+} (ca. 36%) that was significantly greater than control values at 96 and 120 h (Fig. 5c).

Plasma K^+ did not show significant changes due to treatment, however, there was an overall significant time effect on concentrations after repeated measuring (Fig. 6b). Pb-exposed fish exhibited significant decreases in plasma Na^+ and Cl^- concentrations. Plasma Na^+ (Fig. 6b) fell rapidly after 48 h in Pb-exposed fish from about 150 to 124 mM (18% decrease). Conversely, control values remained normal and relatively stable. Plasma Cl^- showed a similar trend with a 15% decrease, falling to 105 mM by 120 h (Fig. 6c). Again, control fish maintained stable plasma Cl^- levels (ca. 130 mM).

3.2.3. Hematology

Continuous daily blood sampling caused hemoglobin (Hb) and hematocrit (Ht) to decrease over time in both control and Pb treatments (Fig. 7a and b), despite the fact that the majority of the red blood cells were replaced after each sampling. This trend was significant only in control fish which exhibited a significant decrease in Ht at 96 h relative to t = 0 h and significantly lower [Hb] and Ht at 72 and 96 h compared with experimental fish. Mean cell hemoglobin concentration did not change, however (Fig. 7c) showing that erythrocyte swelling did not occur. Plasma protein remained unchanged in both control and Pb-exposed fish (Fig. 7d).

3.3. Na^+/K^+ ATPase activity

Rainbow trout were exposed to a dissolved Pb concentration of 1.5 ± 0.04 mg l^{-1} (total Pb = 1.6 ± 0.11 mg l^{-1}). When compared with values for unexposed fish at t = 0 h (control sampling), a significant 40% reduction in activity occurred by 48 h, and the inhibition remained stable and significant through to 120 h of exposure (Fig. 8).

3.4. Ion flux measurements

3.4.1. Ca^{2+} influx measurements

Calcium influx rates were significantly inhibited in juvenile trout through to 48 h compared with control values during exposure to 1.2 ± 0.04 mg

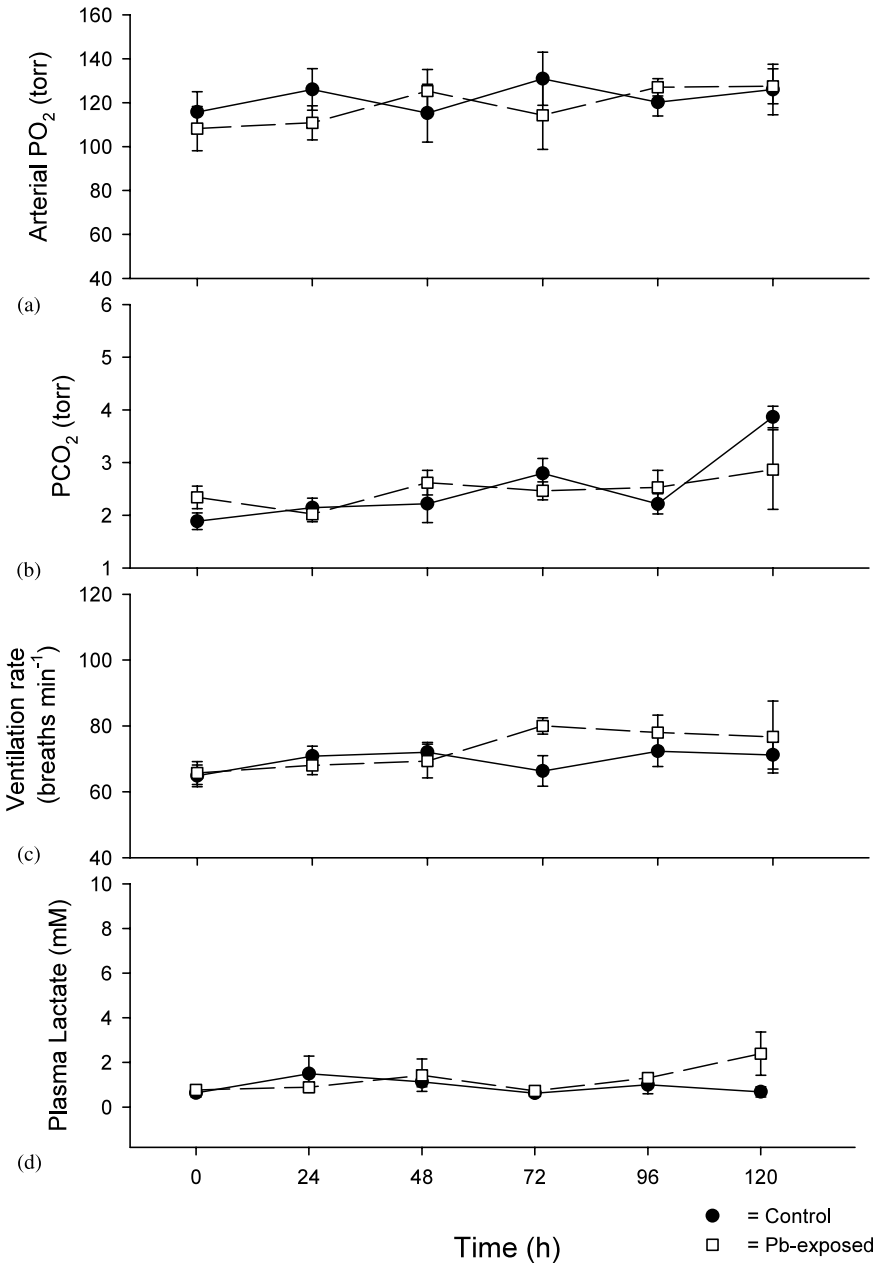

Fig. 2. Respiratory parameters obtained from cannulated adult rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb l^{-1}. (a) Arterial oxygen tension (PaO_2); (b) arterial carbon dioxide tension ($PaCO_2$); (c) ventilation rate; (d) plasma lactate. Data are expressed as mean ± 1 S.E.M. There were no significant differences ($P > 0.05$).

l^{-1} of dissolved Pb (total Pb $= 1.7 \pm 0.25$ mg l^{-1}), a concentration close to the 96 h LC50 for rainbow trout in Hamilton city dechlorinated tap water. The amount of total Pb in control exposure water was 0.85 ± 0.05 μg l^{-1}. Inhibition was immediate with influx rates being reduced in the first 2 h (initial flux period), and remaining stable at about 65% inhibition through to 48 h. No apparent time-

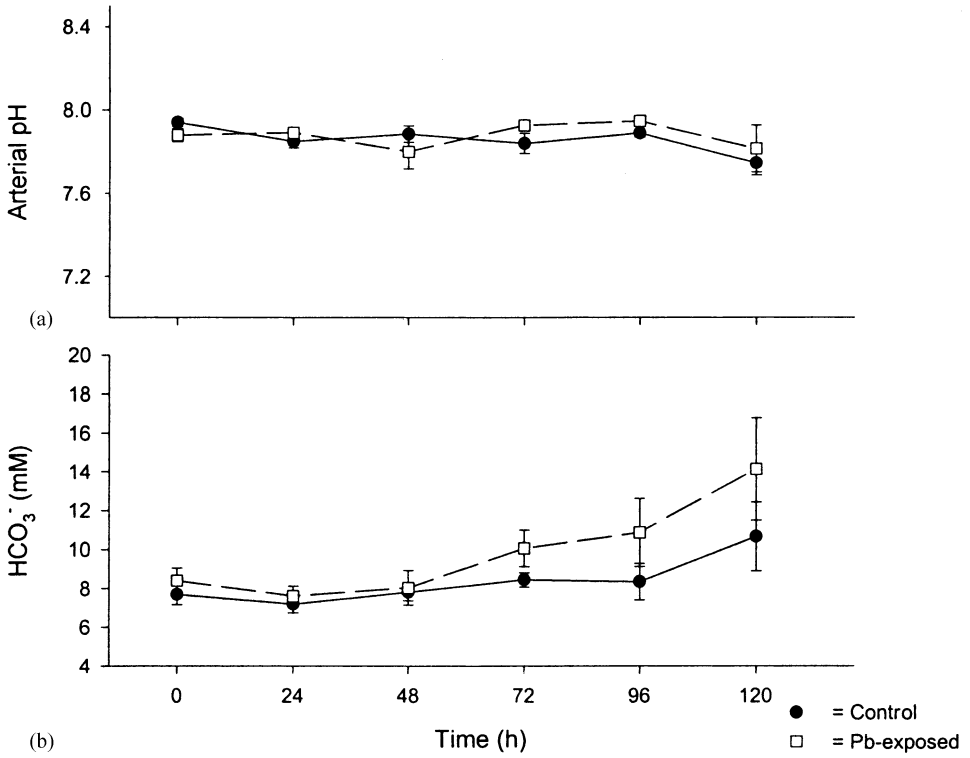

Fig. 3. Acid/base parameters obtained from cannulated rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb l^{-1}. (a) Arterial pH; (b) arterial plasma [HCO_3^-]. Data are expressed as mean ± 1 S.E.M. There were no significant differences ($P > 0.05$).

dependent changes in uptake rates that would indicate recovery or up-regulation of calcium influx occurred.

3.4.2. Na^+ and Cl^- flux measurements

Fig. 9b and c illustrate influx measurements for sodium and chloride, respectively, in control fish or fish exposed to 0.89 ± 0.05 mg Pb l^{-1} dissolved Pb (total Pb = 0.98 ± 0.07 mg l^{-1}). The amount of total Pb in control exposure water was 1.1 ± 0.17 μg l^{-1}. Both sodium and chloride uptake were significantly inhibited in Pb-exposed fish compared with rates measured in control fish. Inhibition took place immediately during the first 2 h flux period and continued at a stable value of about 50% at 12–14, 24–26 and 48–50 h. Chloride uptake showed similar reductions of about 40% at 0–2, 12–14, 24–26 and 48–50 h.

4. Discussion

4.1. 96 h LC50 and tissue accumulation

Considering the water hardness (140 mg l^{-1} as $CaCO_3$) and exposure method (flow-through) used, the 96 h LC50 of 1.0 mg dissolved Pb l^{-1} (1.04 mg l^{-1} total Pb) determined in this study is surprisingly close to previously reported LC50 values (expressed as dissolved Pb) which used very different exposure conditions. For example, Davies et al. (1976) reported an LC50 obtained from a static-renewal method in water with hardness = 353 mg l^{-1} (as $CaCO_3$) of 542 mg l^{-1} total Pb, of which only 1.47 mg Pb l^{-1} was dissolved. Davies et al. (1976) also determined an LC50 in relatively soft water (28 mg l^{-1} as $CaCO_3$) of 1.32 mg l^{-1} dissolved Pb. Hodson et al. (1978) reported

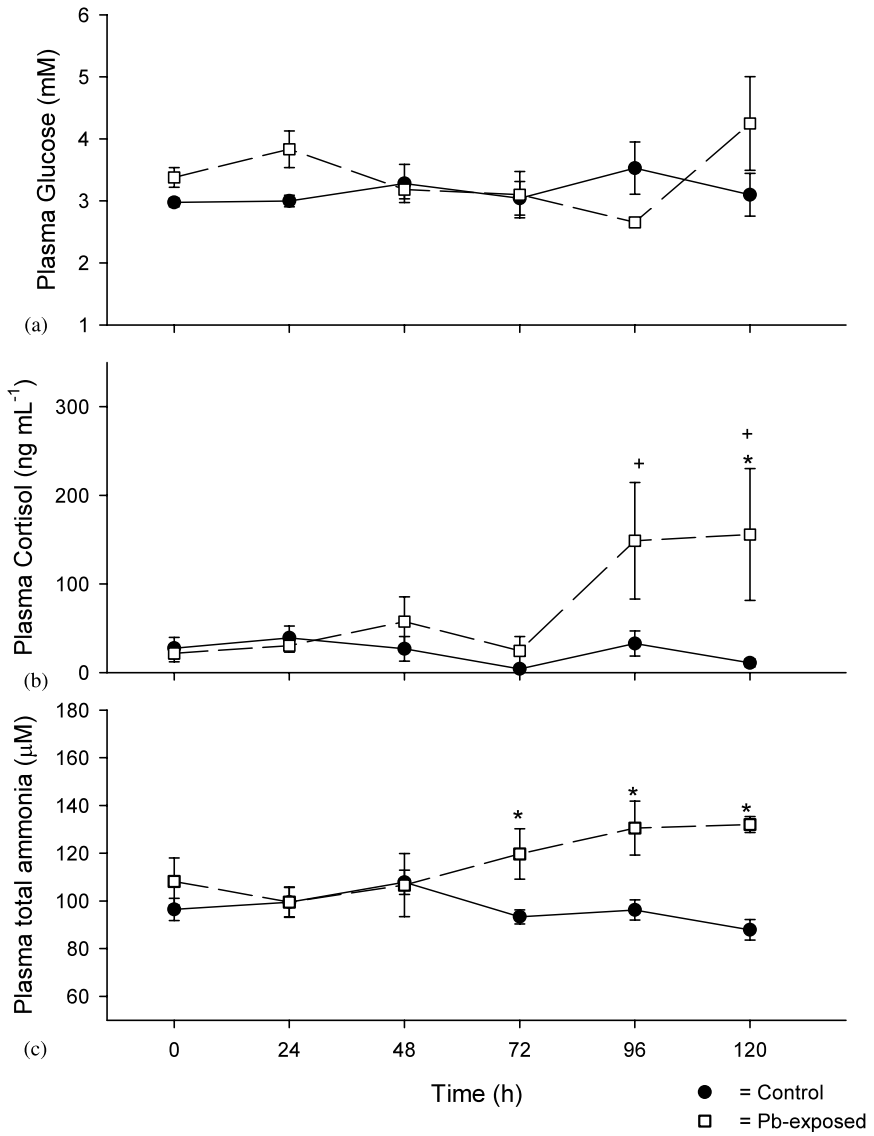

Fig. 4. Stress indices in arterial blood plasma obtained from cannulated adult rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb l^{-1}. (a) Arterial plasma [glucose]; (b) arterial plasma [cortisol]; (c) arterial plasma total ammonia. Data are expressed as mean ± 1 S.E.M. Asterisk "*" indicates significant difference ($P < 0.05$; two-tailed Student's t-test) from simultaneous control mean. "+" indicates significant difference ($P < 0.05$; Dunnett's one-way ANOVA) from 0 h control mean.

a value of 2.4 mg Pb l^{-1} in a 21-day LC50 done in Lake Ontario water (hardness = 135 mg l^{-1} as $CaCO_3$). Holcombe et al. (1976), using a flow-through method, reported an LC50 of 3.36 mg l^{-1} dissolved Pb (total Pb = 4.10 mg l^{-1}) using adult brook trout in relatively soft water (44 mg l^{-1} as $CaCO_3$). This slightly higher value may reflect a species or age difference. With the apparent uniformity of dissolved Pb LC50 values, despite a wide range of water qualities and total Pb LC50s, it appears that future research into the effects of water quality on Pb toxicity should concentrate on the dissolved component only. In the present study, LC50 measurements were useful

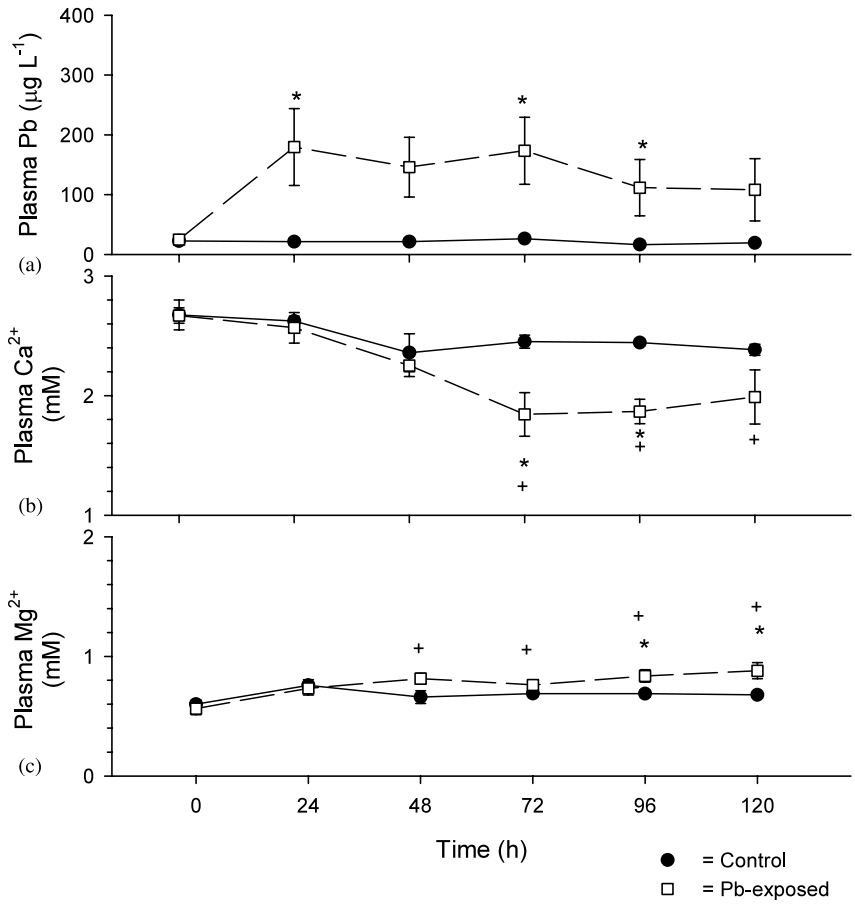

Fig. 5. Plasma ion concentrations obtained from cannulated adult rainbow trout exposed to control conditions or 1.1±0.04 mg dissolved Pb l^{-1}. (a) Arterial plasma [Pb^{2+}]; (b) arterial plasma [Ca^{2+}]; (c) arterial plasma [Mg^{2+}]. Data are expressed as mean±1 S.E.M. Other details as in legend of Fig. 4.

in establishing an acute toxic level of Pb under flow-through conditions in water of our hardness that could be used in further acute studies.

Tissue accumulation data (Fig. 1) point to the gill as the probable primary site for the acute toxic action of Pb based on the high Pb-burden measured here compared with other soft tissues sampled. This is consistent with the fact that the gill is the primary site of Pb uptake in fish (Varanasi and Gmur, 1978) and that a strong relationship may exist between gill metal burden and toxicity as has previously been demonstrated for other metals (Playle et al., 1993; Playle, 1998; DiToro et al., 2001). Chronically exposed rainbow trout also show elevated gill-Pb concentrations relative to other soft tissues such as the kidney and liver, accumulating approximately 15 μg g^{-1} wet weight after prolonged exposure to 0.1 mg Pb l^{-1} (Hodson et al., 1978). This accumulation is less than that reported in the present study, which could reflect a difference in rates of uptake in acute versus chronic Pb exposures. It is clear that Pb also crossed the gill and entered the fish as significant accumulation occurred in the kidney and liver, and Pb was substantially elevated in the blood plasma (Fig. 5a). In addition to the gill, the intestine may have been a route of uptake as demonstrated in Pb-exposed goldfish (Tao et al., 1999). Kidney Pb could contribute to acute toxicity, though studies investigating the nephrotoxic effects of Pb have

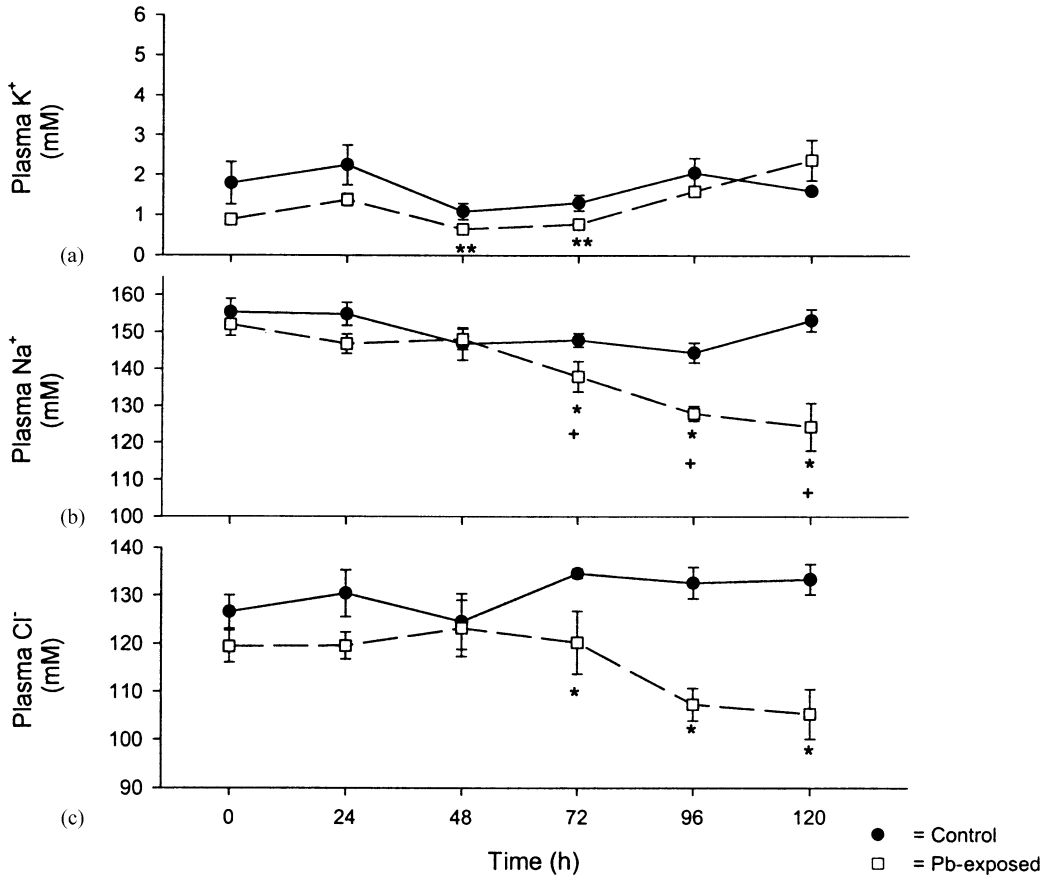

Fig. 6. Plasma ion concentrations obtained from cannulated rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb l^{-1}. (a) Arterial plasma [K^+]; (b) arterial plasma [Na^+]; (c) arterial plasma [Cl^-]. Data are expressed as mean ± 1 S.E.M. Other details as in legend of Fig. 4. "**" Indicates significant difference from 24 h control mean due to time-effect ($P < 0.05$; two-way ANOVA with Tukey HSD test.

not yet been performed in fish. Studies on the nephrotoxicity of Cd, initiated because selective accumulation was observed in the kidney tissue during chronic exposure (Sangalang and Freeman, 1979), found that disturbances in electrolyte balance do not result from impairment of renal function (Giles, 1984). Whether or not this is the case for Pb is still unclear. Similarly, the possible contribution of the liver Pb accumulation to acute toxicity is unknown.

4.2. Acute ionoregulatory disturbance

Cannulated fish exposed to the Pb 96 h LC50 showed significant ionoregulatory impairment after 48 h of exposure while experiencing no apparent respiratory or acid/base disturbance. The observed stability of PaO_2, $PaCO_2$, pH_a, plasma lactate, and ventilation rate in control and experimental trout over the course of the 120 h experiment contrasts with the clear respiratory toxicity induced by Al at moderately acidic pH (Playle et al., 1989) or by Ni at circumneutral pH (Pane et al., 2003). Exposure to Al and Ni results in substantial decreases in PaO_2 and pH_a, and increases in $PaCO_2$, blood lactate, and ventilation rate.

The time course of accumulation of Pb in the plasma of experimental fish saturated quickly, following a rapid 7-fold increase over the first 24

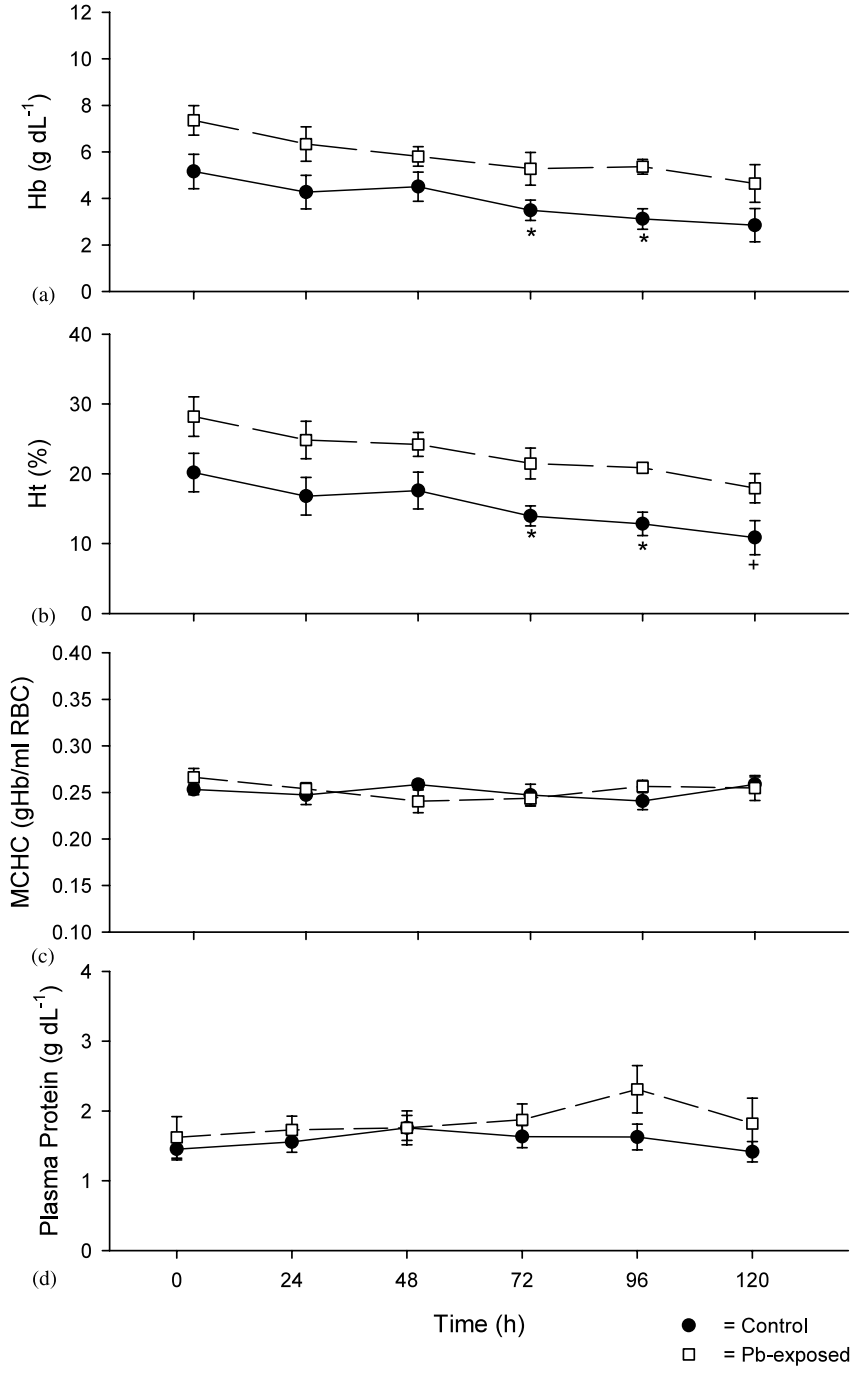

Fig. 7. Hematological parameters obtained from cannulated rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb l^{-1}. (a) Blood [Hb]; (b) hematocrit (Ht); (c) mean cellular hemoglobin concentration (MCHC); (d) arterial plasma [protein]. Data are expressed as mean ± 1 S.E.M. Other details as in legend of Fig. 4.

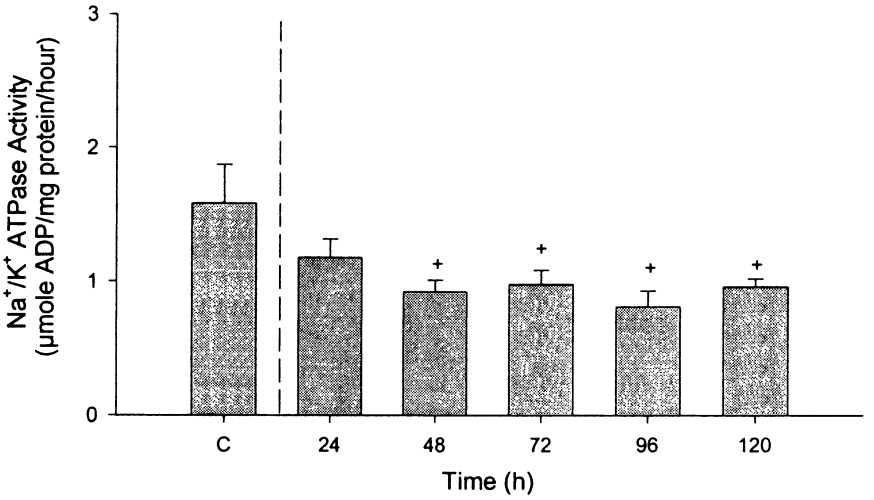

Fig. 8. Time course analysis of branchial Na^+/K^+ ATPase activity in juvenile rainbow trout at control sampling (c) (t = 0 h) and after exposure to 1.5 ± 0.04 mg dissolved Pb l^{-1} for up to 120 h. Data are expressed as mean ± 1 S.E.M ($N = 7–8$). "+" indicates significant difference ($P < 0.05$; Dunnett's one-way ANOVA) from control sampling mean.

h. This is similar to metals such as Ag, Cu, or Cd, which show saturation over time (Wood et al., 1996; Richards and Playle, 1999; Wood et al., 1999). Part of the explanation may be the ability of the erythrocyte to sequester Pb from the plasma (Holcombe et al., 1976; Hodson et al., 1977; Johansson-Sjobeck and Larsson, 1979). In addition, the saturation phenomenon may reflect clearance of Pb to the tissues and reduced uptake at the gill over time, possibilities which could be tested in future studies. The effect of Pb on hematological parameters was minimal in the present study (Fig. 7).

While the plasma Pb elevation saturated by 24 h, the fall in plasma Ca^{2+} in experimental fish was not significant until 48 h (Fig. 5d). This agrees with existing circumstantial evidence in favor of a Pb/Ca^{2+} antagonism (Hodson et al., 1978; Varanasi and Gmur, 1978; Settle and Patterson, 1980), and with similar antagonism and hypocalcemic responses induced by exposure to Cd (Giles, 1984; Verbost et al., 1987; Reid and McDonald, 1988; Wicklund-Glynn et al., 1994) or Zn (Spry and Wood, 1985; Hogstrand et al., 1994). The observed hypocalcemia is also consistent with the observed inhibition of Ca^{2+} influx by Pb in juvenile rainbow trout (Fig. 9a), but contrast to Sayer et al. (1989) who did not observe an effect on Ca^{2+} uptake in brown trout fry exposed to Pb in soft, acidic water. It is possible that direct competition for uptake between these two divalent ions at the same apical sites on the gill ionocytes is the basis of inhibition. This competition would eventually lead to hypocalcemia similar to that observed in cannulated fish in this study. This phenomenon has been seen for Cd, where evidence suggests that both Ca^{2+} and Cd^{2+} are transported through the gill epithelium by chloride cells (Perry and Wood, 1985; Verbost et al., 1987; Perry and Flik, 1988; Verbost et al., 1989; Perry et al., 1992; Flik et al., 1993) passing through the apical membrane passively via voltage-independent Ca^{2+} channels driven by electrochemical gradients (Perry and Flik, 1988; Verbost et al., 1989). Pb may also inhibit the basolateral transport mechanism, which is thought to involve a high affinity Ca^{2+} ATPase and/or a Na^+/Ca^{2+} exchanger (Flik et al., 1993). Competitive interaction between Pb^{2+} and Ca^{2+} could also explain the protective effects of increased water hardness as demonstrated in fish exposed to Pb (Sorensen, 1991). Recently, we have also found Pb inhibition of Ca^{2+} uptake at much lower Pb concentrations (J. Rogers and C.M. Wood, unpublished data), the severity of the inhibition increasing as Pb concentrations approached the LC50. It is also possible Ca^{2+} efflux

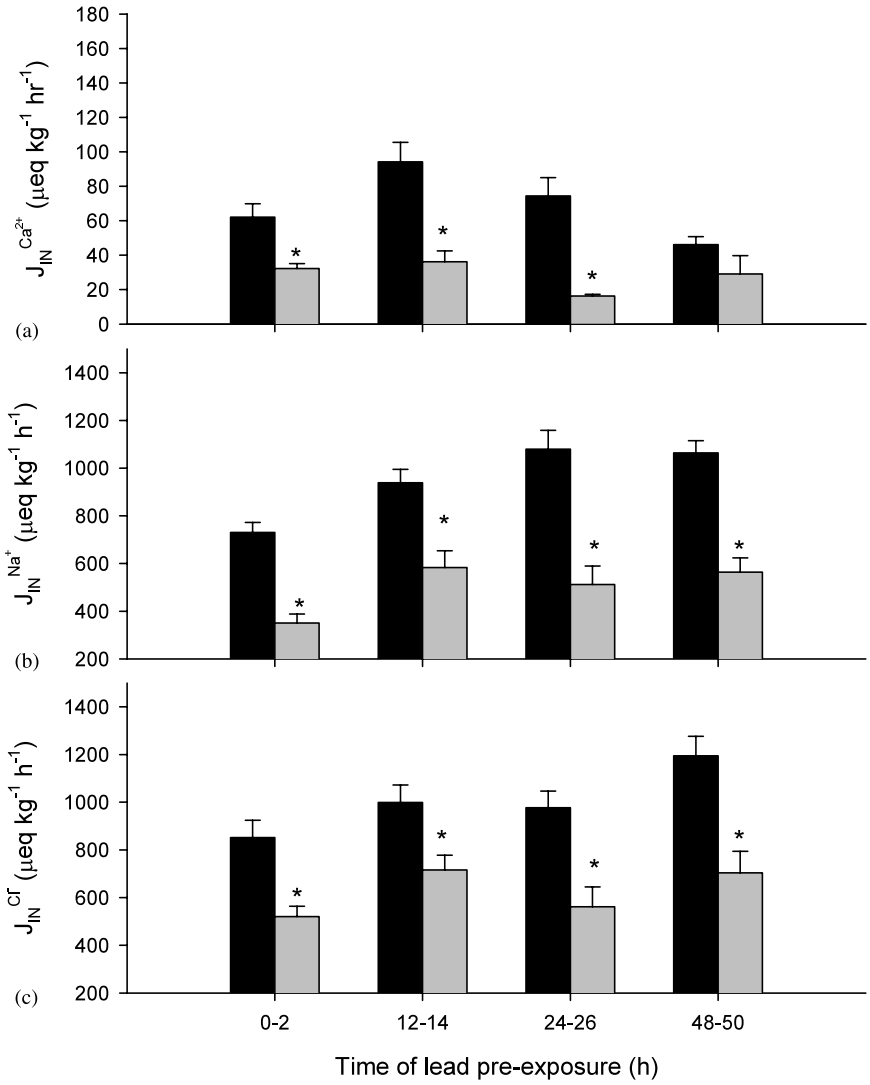

Fig. 9. Ion flux measurements in juvenile rainbow trout for (a) calcium influx in control water (black bars) or water with 1.2 ± 0.04 mg dissolved Pb l^{-1} (grey bars) after exposure to control conditions or 1.2 ± 0.04 mg dissolved Pb l^{-1} for 0, 12, 24, or 48 h; (b) sodium uptake in control water (black bars) or water with 0.89 ± 0.05 mg dissolved Pb l^{-1} after exposure for 0, 12, 24, or 48 h, and (c) chloride uptake in control water (black bars) or water with 0.89 ± 0.05 mg dissolved Pb l^{-1} after exposure for 0, 12, 24, or 48 h. Asterisk "*" indicates significant difference ($P < 0.05$; two-tailed Student's t-test) from corresponding control means ($N = 7–8$).

is stimulated in the presence of Pb. This was observed in soft-water flux measurements by Sayer et al. (1991) in brown trout and may be a component of the hypocalcemia and disturbed Ca^{2+} homeostasis observed in this study.

Magnesium is another tightly regulated ion in the rainbow trout, normally maintained at concentrations below 1 mM to maintain stable cellular and enzymatic functions (Bijvelds et al., 1998). The increase in Mg concentration observed in our experimental fish did not surmount this threshold, however, relative to control values, a significant increase was evident. Increased plasma concentrations of K were not observed suggesting that the Mg elevation did not result from red cell hemolysis. Possibly, it was in some way associated with

the hypocalcemic response. Although it is widely accepted that these cations enter fish via separate routes (Hardwick et al., 1990; Kayne and Lee, 1993; Marshall, 2002), circumstantial evidence exists for a Ca^{2+}/Mg^{2+} relationship. For example, low Mg^{2+} intake induces high body calcium concentrations in rainbow trout (Cowey et al., 1977). Mg^{2+} is also thought to move passively over apical membranes (gill and intestinal) down an electrochemical gradient (Flik et al., 1993). Ca^{2+} likely plays a role in this movement, so the observed hypocalcemia in Pb-exposed fish may cause disruption of the electrochemical gradient established at the apical surface.

Although no significant changes were observed with respect to K^+ regulation, (Fig. 6a), Na^+ and Cl^- balance was severely disrupted in experimental fish. Typically, divalent metals such as Pb, Cd or Zn are viewed as calcium antagonists; however, the ionoregulatory impacts of Pb appear to include these monovalent ions. Similar to the response to acute Ag exposure (Wood et al., 1996), net loss of Na^+ and Cl^- at the gill through the observed inhibition of influx (Fig. 9b and c), would result in the observed gradual decreases in plasma Na^+ and Cl^- (Fig. 6b and c). Inhibition of influx could occur at a number of levels; competition for uptake at the apical surface, inhibition of uptake at Na^+ channels or Na^+ and Cl^- exchangers, inhibition of the apical H^+ ATPase which is thought to polarize the Na^+ channel, inhibition of carbonic anhydrase that would limit the supply of H^+, NH_4^+ and HCO_3^- for these exchangers, or inhibition of the Na^+/K^+ ATPase at the basolateral membrane, which indirectly energizes the uptake mechanisms. Clearly the latter phenomenon occurs (Fig. 8), which is consistent with enzyme inhibition previously observed in Pb-exposed tilapia (Ay et al., 1999), but contrast to Sola et al. (1994) who did not observe an adverse effect of Pb on Na^+/K^+ ATPase activity in rainbow trout exposed to 1 mg Pb l^{-1}. From a time–course perspective, significant inhibition of Na^+/K^+ ATPase was observed during the time period where plasma Na^+ and Cl^- were found to decrease in cannulated trout. However, it is unclear whether the decrease in plasma Cl^- is electrochemically linked to inhibition of Na^+ transport or whether Pb acts to directly inhibit Cl^- transport by HCO_3^-/Cl^- exchange (Wood and Goss, 1990; Sullivan et al., 1995; Wilson et al., 2000) or active transport by a HCO_3^- dependent anion ATPase (Kerstetter and Kirschner, 1994). Sola et al. (1994) did not observe significant impairment of HCO_3^- ATPase activity in rainbow trout exposed to 1 mg Pb l^{-1}. While ion disruption resulting from inhibition of uptake was observed in this study, future research investigating the possible role of the efflux component in Na^+, Cl^-, and Ca^{2+} losses is necessary.

Na^+/K^+ ATPase inhibition upon Pb-exposure was not as severe as that observed in rainbow trout exposed to a 96 h LC50 level of Ag (10 μg l^{-1}) for 48 h in the same water quality (85% inhibition) (Morgan et al., 1997). The mechanisms of inhibition may be different for Ag and Pb. While Ag has a high-affinity for Na^+/K^+ ATPase based on strong binding to sulfhydryl groups (Morgan et al., 1997) resulting in potent inhibition of the enzyme, the less potent inhibition caused by Pb may result from Pb binding to carboxyl groups at the active site of Na^+/K^+ ATPase, causing a reduction in phosphorylation activity, a phenomenon demonstrated in vitro in human erythrocytes by Ong and Lee (1980a). This reduction in activity would result in a reduced rate of ion uptake, which is consistent with the ion disruption observed in this study. Generally, about a 40 mM reduction in plasma Na^+ results in death of the fish, as shown for Ag after 6 days of exposure (Wood et al., 1996). Prolonged exposure to Pb would undoubtedly result in similar mortality as plasma Na^+ dropped by 26 mM after 5 days of exposure in cannulated adult rainbow trout.

Low concentrations of plasma Na^+ and Cl^- observed after 48 h of Pb exposure (Fig. 6b and c) correspond with significant increases in plasma total ammonia at this time (Fig. 4c). Ammonia excretion, the mechanism of which is still controversial, occurs at the fish gill by passive diffusion, by indirect coupling to H^+ excretion/Na^+ uptake, or by Na^+/NH_4^+ exchange (Wilkie, 1997). Experiments using Na^+ transport blockers show that ammonia excretion is simultaneously inhibited (Kerstetter and Keeler, 1976; Payan, 1978; Wright and Wood, 1985; Wilson et al., 1994),

supporting the existence of a direct or indirect exchange mechanism. Thus, there may be an ionoregulatory basis for the observed increases in plasma total ammonia that accompany decreases in plasma Na^+ after 48 h of Pb exposure. Pb inhibition of Na^+ transport, therefore, would be a fitting explanation for observed rises in plasma total ammonia and would support an ionoregulatory rather than a respiratory or stress-related disturbance that could cause similar plasma ammonia elevations. This phenomenon has also been observed in fish exposed to Ag (Webb and Wood, 1998) and Cu (Beaumont et al., 1995).

Stress indices measured showed no change in plasma glucose concentrations, however, under ionoregulatory distress, slow activation of the pituitary interrenal axis likely resulted in the cortisol mobilization observed in cannulated adult trout at 96 and 120 h (Fig. 4b). Perry and Wood (1985) showed that cortisol treatment in rainbow trout increased influx rates for Ca^{2+} ($J_{in}^{Ca^{2+}}$) compared with untreated controls by cortisol-stimulated proliferation of lamellar chloride cells. Therefore, an ionoregulatory disruption such as hypocalcemia may have triggered the compensatory release of cortisol in experimental trout.

5. Conclusions

This study presents strong evidence that the mechanism of waterborne Pb toxicity in fish during acute exposure is ionoregulatory distress. Using the rainbow trout in moderately hard water as a model system, the toxic action of Pb appears to be midway between other known acute ionoregulatory toxicants like Ag and Cu which affect Na^+ and Cl^- balance, and Zn and Cd which disrupt Ca^{2+} homeostasis (see Section 1). This is in direct contrast with the toxic mechanisms of Al and Ni, which are primarily respiratory in nature. Data presented also open the possibility of predictive modelling approaches such as the BLM, which are 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; McGeer et al., 2000; DiToro et al., 2001). Binding models for Pb are presently being developed (MacDonald et al., 2002), and based on their predictions and the physiological evidence presented in this study, it appears that Pb is highly capable of out-competing Na^+ and Ca^{2+} for ion transport sites at the freshwater fish gill. In seawater, the physiology of Pb toxicity and the acute toxic mechanism of action may change. However, ionoregulation in marine fish follows different principles, with active transport processes dedicated to net ion excretion at the gills, rather than net ion uptake. Therefore, the effects of Pb in seawater cannot be extrapolated from freshwater data. Future research on the physiological effects of Pb in marine fish is needed.

This study has opened up promising avenues for further research. Kinetic analysis of Pb^{2+}/Ca^{2+} interaction similar to that done for Zn (Hogstrand et al., 1995), investigation into the effect of Pb^{2+} on the function of Ca^{2+} ATPase in the gills, and research into mechanisms of Na^+/Cl^- disruption are key aspects of Pb-toxicity that require further investigation. In addition, the possibility of Pb-induced nephrotoxicity as a component of the observed ionoregulatory distress is an important topic for future study.

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