Aquatic Toxicology 89 (2008) 222–231

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Modes of metal toxicity and impaired branchial ionoregulation in rainbow trout exposed to mixtures of Pb and Cd in soft water

Oana Birceanu[a], M. Jasim Chowdhury[b], Patricia L. Gillis[c], James C. McGeer[a], Chris M. Wood[b], Michael P. Wilkie[a,*]

[a] Department of Biology, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada
[b] Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada
[c] National Water Research Institute, Environment Canada, Burlington, Ontario L7R 4A6, Canada

ARTICLE INFO

Article history:
Received 20 February 2008
Received in revised form 4 July 2008
Accepted 4 July 2008

Keywords:
Biotic Ligand Model (BLM)
Gills
Metals
Toxic Unit Concept
Na^+ influx
Ca^{2+} influx

ABSTRACT

Models such as the Biotic Ligand Model (BLM) predict how natural organic matter (NOM) and competing ions (e.g., Ca^{2+}, H^+ and Na^+) affect metal bioavailability and toxicity in aquatic organisms. However, such models focus upon individual metals, not metal mixtures. This study determined whether Pb and Cd interact at the gill of rainbow trout (*Oncorhynchus mykiss*) when trout were exposed to environmentally relevant concentrations of these metals (Cd < 100 nmol L^{-1}; Pb < 500 nmol L^{-1}) in soft (<100 μmol Ca^{2+} L^{-1}), moderately acidic (pH 6.0) water. The 96-h LC50 for Pb was 482 nmol L^{-1}, indicating that Pb was one-order of magnitude more toxic in soft, acidic water than in harder, circumneutral pH waters. The LC50 for Cd alone was also low, 6.7 nmol L^{-1}. Surprisingly, fish acclimated to soft water had multiple populations of Pb-gill and Cd-gill binding sites. A low capacity, high affinity population of Pb-gill binding sites had a B_{max} of 18.2 nmol g^{-1} wet weight (ww) and apparent log $K_{Pb\text{-}gill}$ = 7.05, but a second low affinity population could not be saturated up to free Pb concentrations approaching 4000 nmol L^{-1}. Two populations of Cd-gill binding sites were characterized: a high affinity, low capacity population with an apparent log $K_{Cd\text{-}gill}$ = 7.33 and B_{max} = 1.73 nmol g^{-1} ww, and a low affinity, high capacity population with an apparent log $K_{Cd\text{-}gill}$ = 5.86, and B_{max} = 13.7 nmol g^{-1} ww. At low concentrations, Cd plus Pb accumulation was less than additive because Cd out-competed Pb for gill binding sites, which were likely apical Ca^{2+}-channels. While disturbances to Ca^{2+} influx were caused by Cd alone, Pb alone had no effect. However, Pb exacerbated Cd-induced disturbances to Ca^{2+} influx demonstrating that, although Pb- plus Cd-gill binding was less than additive due to competition, the effects (ionic disturbances) were more than additive (synergistic). Pb was also likely binding to intracellular targets, such as branchial carbonic anhydrase, which led to inhibited Na^+ influx. This ionic disturbance was exacerbated by Cd. We conclude that exposure to environmentally relevant concentrations of Pb plus Cd results in less than additive metal-gill binding in soft, moderately acidic waters. However, ionic disturbances caused by Cd plus Pb are greater than additive, and this may ultimately increase the toxicity of Cd–Pb mixtures to fishes. Our findings suggest that it may be necessary to re-evaluate water quality criteria and assumptions of the BLM for fish exposed to mixtures of Pb and Cd in the acidic, soft waters found in the Canadian Shield, Scandinavia and other sensitive regions.

1. Introduction

The Free Ion Activity Model (Morel, 1983; also see Campbell, 1995), Gill Surface Interaction Model (Pagenkopf, 1983), and more recently the Biotic Ligand Model (BLM; Playle et al., 1993; Di Toro et al., 2001) are used to predict how natural organic matter (NOM), other complexing moieties, and competing ions (e.g., Na^+, Ca^{2+} and H^+) affect metal bioavailability and toxicity in aquatic organisms (see Paquin et al., 2002; Playle, 1998; Niyogi and Wood, 2004a for reviews). A number of jurisdictions and regulatory bodies, including the United States Environmental Protection Agency (USEPA), are now considering or implementing BLM-based models in the establishment of water quality and risk assessment guidelines that are based on free metal rather than total metal concentrations. While the benefits of the BLM for predicting the toxicity of individual metals are well founded, a recognized

* Corresponding author at: Department of Biology, Wilfrid Laurier University, 75 University Avenue West, Waterloo, Ontario N2L 3C5, Canada.
Tel.: +1 519 884 0710x3313; fax: +1 519 746 0677.
E-mail address: mwilkie@wlu.ca (M.P. Wilkie).

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doi:10.1016/j.aquatox.2008.07.007

limitation is that it does not yet deal with metal mixtures, which are common in contaminated waters (e.g., Norwood et al., 2003, 2007; Playle, 2004). Moreover, assumptions of the BLM may not be valid in fishes that are acclimated to very soft waters of low ionic strength (e.g., Niyogi et al., 2004). Our overall goal was to determine how Pb and Cd interact at the gill of rainbow trout (*Oncorhynchus mykiss*) exposed to environmentally relevant concentrations of these metals (Cd < 100 nmol L^{-1}; Pb < 500 nmol L^{-1}) in soft waters that are found in the Canadian Shield and Scandinavia (see McDonald et al., 1989; Yan et al., 1996 for reviews). Accordingly, we separately determined the 96-h LC50s for both Pb and Cd in soft (Ca < 100 μmol L^{-1}), moderately acidic (pH 6) waters and then related these data to short-term (3-h and 24-h LA50) metal-gill binding using approaches pioneered by Playle et al. (1993).

Another objective of our study was to test the hypothesis that metal binding was additive when fish were exposed to mixtures of Pb and Cd. To achieve this objective, we first constructed Langmuir plots (e.g., Playle et al., 1993; Niyogi et al., 2004) generated from metal-gill binding saturation curves when fish were exposed to a range of Pb and Cd concentrations individually. The regression expressions developed were then used to predict Pb and Cd binding to the gill at given free concentrations of each metal, and we compared these estimates to actual metal accumulation when fish were exposed to mixtures of Cd and Pb.

A final objective was to determine if the ionoregulatory disturbances caused by Pb and Cd were exacerbated when fish were exposed to mixtures of these metals, at environmentally relevant concentrations. This was achieved by exposing fish to a matrix of Pb and Cd concentrations, and using the radiotracers $^{45}Ca^{2+}$ and $^{24}Na^{+}$ to determine the rates of whole body Ca^{2+} and Na^{+} influx. We predicted that Pb and Cd would act in an additive manner to inhibit Ca^{2+} influx because of competitive interactions at external Ca^{2+} channels on the gill cells (e.g., Verbost et al., 1989; Rogers and Wood, 2004). Based on this premise, we also predicted that Pb and Cd would have little effect on Na^{+} influx because such disturbances have been demonstrated to arise from effects within the intracellular space of the gill such as those caused by interference with carbonic anhydrase and/or with Na^{+}/K^{+} ATPases located on the basolateral membrane (Rogers et al., 2005).

2. Material and methods

2.1. Experimental animals and holding

Juvenile rainbow trout (*O. mykiss*) were purchased from Rainbow Springs Trout Hatchery, Thamesford, Ont., and held in aerated 100-L flow-through tanks receiving a 1:1 mixture of well water and ion-poor water produced by reverse osmosis (Culligan MP1000, Series E Reverse Osmosis (RO) System; Culligan of Canada Ltd., Mississauga, Ont.). The final water composition was $[Ca^{2+}]$ ~1.5 mmol L^{-1}, $[Na^{+}]$ ~500 μmol L^{-1}, Cl^{-} ~500 μmol L^{-1}, pH ~8 and temperature = 12 °C. After 2 days, fish were transferred to reverse osmosis water only, supplemented by a Ca^{2+} drip ($CaCl_2$) to produce soft, ion-poor holding water of the following composition: $[Ca^{2+}] = 72 \pm 2$ μmol L^{-1}, $[Na^{+}] = 346 \pm 8$ μmol L^{-1}, $[Cl^{-}] = 54 \pm 3$ μmol L^{-1}, [dissolved organic carbon (DOC)] = 1.1 ± 0.1 mg L^{-1}, pH 7.4 and temperature = 12 °C. Because Mg^{2+} and K^{+} were not added to the deionized water, their concentrations were considered negligible (<10 μmol L^{-1}). Fish were acclimated to these conditions for at least 2 weeks. Fish were fed ground HiPro Alevin-Parr fish food (Corey Feed Mills Ltd., Fredericton, New Brunswick) three times per week, but food was withheld 72 h prior to experiments. All the experiments were conducted in the same ion-poor, soft water, but in a static system maintained at pH 6.0–6.5 by the drop-wise addition of 8N HNO_3 or 2N KOH at regular intervals. Before gill metal binding experiments, fish were removed from their holding tanks and acclimated to the experimental containers (polyethylene buckets) for approximately 12 h prior to testing. The buckets had been acid-washed using 1% trace metal grade HNO_3 (Fisher Scientific Ltd., Unionville, Ont.), then thoroughly rinsed with reverse osmosis water. For measurements of Na^{+} and Ca^{2+} influx in the presence of Pb or Cd, fish were transferred directly from their holding tanks into the experimental chambers 1 h prior to experiments.

2.2. Experimental protocol

2.2.1. Acute toxicity of Cd and Pb

Trout (1–3 g) were randomly distributed in groups of eight to experimental buckets (in duplicate) containing exactly 10 L of ion-poor water to which sufficient $CdCl_2$ was added to yield nominal concentrations of 0 (controls), 1.0, 2.5, 5.0, 10.0, and 25 nmol L^{-1}. The acute toxicity of Cd was then determined over 96 h (96-h LC50). The 96-h LC50 of Pb was determined in a similar manner, but 10 fish were distributed to each container to which sufficient $PbCl_2$ was added to yield water Pb concentrations of 0 (controls), 0.25, 0.5, 1.0, 2.5, and 5.0 μmol L^{-1} (in duplicate). Water pH was measured using a handheld pH meter (Advanced Instruments, model 840035 pH Meter with 840016 epoxy body sealed combination electrode). Adjustments to pH were made using dilute 8N HNO_3 or 2N KOH (Sigma–Aldrich, St. Louis, MO, USA). Mortality was regularly monitored (every 3 h in the first 24 h, every 4–8 h thereafter) throughout the experiment.

For both Cd and Pb toxicity tests, 20 mL unfiltered and filtered water samples were collected at 0, 24, 48, 72, and 96 h. Sub-samples of this water (10 mL) were filtered using 0.45-μm Millex-HV syringe filters (Millipore, Bedford, MA). Additional sub-samples of filtered and unfiltered water (7 mL) were also acidified to 1% using 16N trace metal grade HNO_3, and stored at 4 °C until analyzed for Cd or Pb, or ions (Na^{+} and Ca^{2+}). The remaining unacidified water samples were saved in polyethylene vials for Cl^{-} ion determination, or in 5 mL borosilicate vials with no headspace for dissolved organic carbon (DOC) determination. DOC remained in the 0.9–1.7 mg L^{-1} range in all tests, and Ca^{2+} concentrations did not vary by more than 10% from levels in the holding water.

After acute toxicity experiments were completed, we then determined the Pb and Cd accumulation on the gills that would be predictive of the 96-h LC50 (the 3-h LA50 and 24-h LA50, see Niyogi and Wood 2004) by exposing the fish separately to the 96-h LC50 of each metal, individually for 3 or 24 h. In these experiments, juvenile rainbow trout (1–3 g) were added to polyethylene containers ($N = 5$ fish per container), and exposed to the pre-determined 96-h LC50 for Cd (6.7 nmol L^{-1}) and Pb (482 nmol L^{-1}), respectively (in triplicate), in acidic (nominal pH 6.0), ion-poor water. Water samples were collected at 0 and 3 h for the 3-h LA50 experiments, and at 0, 6, 12 and 24 h for the 24-h exposures, for later determination of water metal content. After the prescribed period (3 or 24 h), the fish were removed from their containers one at a time and killed by a blow to the head. The entire gill basket was then removed and the gills rinsed in deionized water, and then stored at −20 °C until gill metal concentration was quantified.

2.2.2. Determination of gill binding characteristics of Pb and Cd

Experiments to determine the binding characteristics of each metal were also conducted by completing metal-gill titrations. In these experiments, fish (1–3 g; $N = 6$–15) were added to 10 L of ion-poor water and then exposed for 3 h to nominal Cd concentrations of 0, 6, 25, 100, 250, 500, 1000, 2500, and 5000 nmol L^{-1}, or Pb

concentrations of 0, 300, 600, 1200, 2500, and 6000 nmol L^{-1}. Following experiments, fish were removed from the water, killed by a blow to the head, and the gills sampled as described above.

2.2.3. Effects of metal mixtures on branchial Cd and Pb accumulation

To determine if the presence of Cd interfered with branchial Pb accumulation, and *vice versa,* two experiments were performed. In the first, trout (1–3 g; $N = 10$) were exposed to nominal Pb concentrations of 0, 25, 50, 100, 250, 500 nmol L^{-1}, in the presence of 100 nmol L^{-1} of Cd in 10 L of acidic, ion-poor water for 3 h. A similar experiment was then repeated in which trout (1–3 g; $N = 10$) were exposed to nominal Cd concentrations of 0, 10, 25, 50, and 100 nmol L^{-1} in the presence of 100 nmol L^{-1} Pb. In both experiments, water samples were collected at 0, 1.5 and 3 h, and gill baskets were collected and processed for Pb and Cd analyses immediately following the 3-h exposure. Observed gill metal binding was then compared to predicted gill metal binding calculated using the regression formulae of the pre-determined Langmuir plots (see Section 2.2.2 and below for further details).

2.2.4. Effects of Cd and Pb mixtures on Ca^{2+} and Na^+ influx

To illuminate how Cd and Pb mixtures may exert their toxic effects, unidirectional ion flux rates were measured using radiotracers to assess whether these metals altered patterns of Ca-influx and Na-influx across the gills using the protocol of Hogstrand et al. (1994). These experiments were performed at McMaster University, where rainbow trout ($N = 144$, 5–13 g) were acclimated 1–2 weeks in ion-poor, acidic water similar to that used at Wilfrid Laurier (McMaster water composition: $[Ca^{2+}] = 80 \pm 1$ μmol L^{-1}, $[Na^+] = 141 \pm 3$ μmol L^{-1}, $Cl^- = 221 \pm 10$ μmol L^{-1}, pH 6.1 ± 0.01, DOC ~1.0 mg L^{-1} (not measured) and temperature = 10–12 °C].

One hour prior to experiments, the fish were added to well-aerated experimental chambers (four fish per chamber) containing 1 L of ion-poor water. Water pH was adjusted to pH 6.0, followed by the addition of the Pb plus Cd metal mixtures. The nominal concentrations of Cd (10 and 50 nmol L^{-1}) and Pb (0, 10, 50 and 100 nmol L^{-1}) used in these experiments were chosen to encompass metal concentrations likely to be found in both uncontaminated and contaminated waters (e.g., 62 nmol Cd L^{-1} (USEPA, 2000) and less than 580 nmol Pb L^{-1} (Demayo et al., 1982)). After addition of the Pb and/or Cd, the radiotracers ^{45}Ca (5 μCi as $CaCl_2$, specific activity 15.5 mCi mg^{-1}, PerkinElmer, USA) and ^{24}Na (5 μCi as Na_2CO_3, specific activity 20.0 μCi mg^{-1}) were added to each container, and allowed to equilibrate 15 min before water samples (20 mL) were collected at 0, 1 and 3 h. Exactly 10 mL water was saved for determination of gamma (γ) and beta (β) radioactivity. The remaining water (10 mL) was acidified by adding 0.1 mL 16N HNO_3 (trace metal grade) to each vial, and saved for later determination of Pb and Cd, and total ion (Ca^{2+}, Na^+ and Cl^-) concentrations. Immediately following the 3-h flux determination period, all four fish were removed from the flux chambers, killed by a blow to the head, and rinsed with 250 mL of ion-poor water (3 min), followed by 3 min in 250 mL of 20 mmol L^{-1} NaCl solution, and three more minutes in 250 mL of 5 mmol L^{-1} $Ca(NO_3)_2$ to remove all surface bound ^{24}Na and ^{45}Ca, respectively ("cold displacement"). The fish were then blotted dry, weighed, inserted into a 20-mL plastic scintillation vial, and immediately analyzed for ^{24}Na gamma radioactivity along with the water samples (see below).

2.3. Analytical techniques and calculations

2.3.1. Metal and ion analysis

Water and gill Cd and Pb concentrations were quantified in all experiments using graphite furnace atomic absorption spectrophotometry (GTA100 atomizer, SpectrAA 880, N_2 gas; Varian, Mississauga, Ont.). The sample injection volume was 10 μL for both metals. Both Pb and Cd concentrations were confirmed using precision standards. Total Ca^{2+} and Na^+ concentrations in the water were measured by flame atomic spectrophotometry (SpectrAA 880 or SpectraAA 220 FS Atomic Absorption; Varian, Mississauga, Ont.). Water Cl^- concentration was determined spectrophotometrically (Spectronic 301, Milton Roy, Rochester, NY) using the mercuric thiocyanate assay (Zall et al., 1956). Dissolved organic carbon was determined with a Shimadzu TOC 5050A Analyzer (Shimadzu Corporation, Kyoto, Japan), and precision standards.

2.3.2. Determination of water and tissue radioactivity

Determinations of Na^+ and Cl^- influx rates were based on respective increases in whole body gamma and beta radioactivity during exposure to the Pb and Cd mixtures (Section 2.2.4). Gamma counts of ^{24}Na in whole fish and water were determined using a 1480 Automatic Wallac Wizard Gamma Counter (PerkinElmer Life Sciences, Woodbridge, Ont.). Following gamma count determination the samples were then stored at −20 °C for 2 weeks, which allowed all radiation arising from the decay of ^{24}Na to become completely exhausted (^{24}Na half-life ~15 h) before beta radiation due to ^{45}Ca was quantified.

Tissue samples were prepared for ^{45}Ca beta counting by transferring the single, whole fish carcass into a 50-mL polyethylene tube, to which 25 mL of 1N HNO_3 (trace metal grade) was added, and digesting the contents at 60 °C for 48 h. Unlike previous studies, this procedure did not require the use of tissue solubilizer to sufficiently digest the tissue for beta count determination (Baldisserotto et al., 2006). Following digestion, the solution was vortexed, and 2 mL of the resulting mixture ("slurry") transferred to a borosilicate glass scintillation vial (20 mL). Exactly 10 mL of Ultima Gold AB scintillation cocktail (PerkinElmer Life and Analytical Sciences, Boston, MA) was then added to the vial, the slurry vortexed, and then left overnight in the dark to minimize chemiluminescence. Water samples (5 mL) were transferred to 20 mL glass scintillation vials, to which 10 mL of ACS scintillation cocktail (Amersham Biosciences UK Ltd., Little Chalfont Buckinghamshire, UK) was added, and also left overnight in the dark to minimize chemiluminescence. Beta counts per minute (cpm) were then measured using a LKB Wallac 1217 Rackbeta Liquid Scintillation Counter and corrected for quench using external standard ratio (ESR) quench correction.

2.4. Calculations and statistical analysis

The 96-h LC50 with upper and lower 95% confidence intervals was calculated using a log probit analysis program (USEPA Probit Analysis Program Used for Calculating LC/EC Value, Version 5.1).

Maximal Pb-gill and Cd-gill binding capacities (B_{max}) and the gill-binding strength (affinity) of each metal ($\log K_{Pb\text{-}gill}$ or $\log K_{Cd\text{-}gill}$) were determined using Langmuir plots in which the free metal/bound metal ratio was plotted against the free metal concentration (described in Richards and Playle, 1998). Briefly, free metal concentrations in the water were calculated using the web-based chemical equilibrium free-ware program Visual MINTEQ (Version 2.52) and the known water chemistry (DOC, Na, Cl, and Ca concentrations, and pH). The Gaussian DOM complexation model was selected when values for DOC were input into the Visual MINTEQ program. As described above, Mg^{2+} and K^+ were considered to be negligible and not included in calculations. Because the water containing the fish was open to the atmosphere, a CO_2 partial pressure of 0.00038 atm (default) was used for all calculations, instead of direct measurements of alkalinity.

The gill binding capacity (B_{max}) of each metal was calculated from the inverse slope of the Langmuir plot for Pb or Cd. The appar-

ent K_D (in nmol L^{-1}) for each metal was then determined by dividing the B_{max} by the inverse of the *y*-intercept of the Langmuir plot. The corresponding log $K_{metal\text{-}gill}$ for Pb or Cd was the negative logarithm of the molar equivalent of the apparent K_D (see Richards and Playle (1998) for further details).

Whole body Na and Ca influx rates in juvenile rainbow trout were determined based on the respective accumulation of gamma or beta radioactivity in the fish during the 3-h flux period (Hogstrand et al., 1994) using the following formula:

$$Na^+\text{- or } Ca^{2+}\text{-influx rate} = \frac{CPM_{fish}}{(MSA)T}$$

where CPM_{fish} is the gamma or beta radioactivity in the fish in cpm g^{-1}, MSA is the mean of the initial and final specific activity of the water in cpm $nmol^{-1}$ of Na or Ca, and *T* is the duration of the flux period in hours.

All data were expressed as the mean ± 1 standard error of the mean (S.E.M.). Significant differences in gill metal burden and ion influx rates were determined using one-way analysis of variance (ANOVA). Where significant variation was observed, statistical differences between the means were determined using the Tukey–Kramer post-test at the $P \leq 0.05$ level. In instances where the mean data compared had unequal variances, statistical analysis was performed using the Kruskal–Wallis test followed by a Dunn's Multiple Comparison's post-test at the $P < 0.05$ level. All statistical analysis was performed with GraphPad InStat, Version 3.02 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. 96-h LC50s and gill metal accumulation

The acute Cd and Pb 96-h LC50s for juvenile rainbow trout in ion-poor water were 6.7 nmol L^{-1} (95% CI = 3.9 to 9.2 nmol L^{-1}) and 482 nmol L^{-1} (95% CI = 233 to 868 nmol L^{-1}), respectively (Table 1). The 3-h and 24-h LA50 for Cd were 0.2 nmol g^{-1} ww and 1.1 nmol g^{-1} ww, respectively, at a measured dissolved Cd concentration of 4.5 nmol L^{-1} (intended nominal Cd concentration = 6.7 nmol L^{-1}; Table 1). The 3-h LA50 for Pb in the same water was 9.7 nmol g^{-1} ww at a measured dissolved Pb concentration of 605 nmol L^{-1}, while the 24 LA50 was 32.8 nmol g^{-1} ww at a measured dissolved Pb concentration of 648 nmol L^{-1} (intended nominal Pb concentration = 482 nmol L^{-1}; Table 1). In all cases the measured water Cd and Pb concentrations lay within the 95% confidence interval of the actual LC50, and a sensitivity analysis demonstrated that the error associated with the discrepancy between the measured and intended nominal concentrations was minor—i.e. it would have caused only a plus 0.07 nmol g^{-1} ww change in 3-h gill LA50 for Cd, and a minus 0.5 nmol g^{-1} ww change in 3-h LA50 for Pb.

Table 1
Acute toxicity data and gill metal accumulation by rainbow exposed to waterborne Pb and Cd

	Cd	Pb
96-h LC50 (nmol L^{-1})	6.7 (95% CI = 3.9–9.2)	482 (95% CI = 233–868)
3-h LA50 (nmol g^{-1} ww)	0.2 ± 0.03 (*N* = 15)	9.7 ± 0.8 (*N* = 15)
24-h LA50 (nmol g^{-1} ww)	1.1 ± 0.04 (*N* = 14)	32.8 ± 3.0 (*N* = 15)

LA50 data presented as the mean ± 1 S.E.M. when fish were exposed to nominal concentrations of Pb and Cd that were equivalent to their respective LC50s. The actual dissolved Cd concentration was 4.5 ± 0.2 nmol L^{-1} at both 3 and 24 h. The respective dissolved Pb concentrations were 605.1 ± 11.5 and 648.1 ± 20.4 nmol L^{-1}. These values were within the confidence intervals of the 96-h LC50 values for both Cd and Pb.

3.2. Binding characteristics of Pb and Cd

Pb accumulation on the gills increased steadily up to dissolved Pb concentrations of approximately 700 nmol L^{-1}, and then markedly increased when water Pb exceeded this concentration (Fig. 1A). Kinetic analysis, using the regression formulae of the Langmuir plot (Fig. 1A, inset), of the branchial Pb accumulation subsequently indicated that there were likely two populations of Pb-gill bindings sites; a high affinity, saturable population of Pb-gill binding sites with a log $K_{Pb\text{-}gill}$ of 7.05 (i.e. apparent K_D = 90 nmol L^{-1}) and a binding capacity (B_{max}) of 18.2 nmol g^{-1} ww, and a low affinity population which could not be saturated, at least up to free Pb concentrations close to 4000 nmol L^{-1} (Fig. 1A).

There were also two populations of Cd-gill binding sites, each saturable. When trout were exposed to relatively low concentrations of Cd, branchial Cd accumulation rose in a progressive manner between nominal water Cd concentrations of 0 and 100 nmol L^{-1} (Fig. 1B). The corresponding kinetics analysis (Fig. 1B, inset) revealed a high affinity, low capacity population of Cd-gill binding sites with a log $K_{Cd\text{-}gill}$ of 7.33 (i.e. apparent K_D = 46.8 nmol L^{-1}) and a B_{max} of 1.73 nmol g^{-1} ww (Fig. 1B). When trout were exposed to a greater range of water Cd concentrations, ranging from 200 to approaching 5000 nmol L^{-1}, a second, lower affinity, but higher capacity population of Cd-gill binding sites were revealed with a log $K_{Cd\text{-}gill}$ of 5.84 (i.e. apparent K_D = 1448 nmol L^{-1}) and a B_{max} of 13.7 nmol g^{-1} ww (Fig. 1C).

3.3. Effects of metal mixtures on gill Cd and Pb burdens

To determine if the presence of a second metal altered respective Cd- or Pb-gill binding kinetics, metal-gill binding was measured in different fish exposed to progressively greater water Cd or Pb concentrations, in the presence of nominal concentrations of 100 nmol L^{-1} of the second metal (either Pb at an actual measured concentration of 88.6 ± 5.0 nmol L^{-1}, or Cd at an actual measured Cd concentration of 78.6 nmol L^{-1}). The regression formulae of the Langmuir plots describing the kinetics for Pb or Cd binding (insets of Fig. 1A and B) were used to predict gill binding by the metal of interest (e.g., Cd) as its water concentration was increased in the absence of the second metal (e.g., Pb). This predicted Cd or Pb accumulation in the absence of the second metal was then compared to the actual measured Cd- or Pb-gill burden in the presence of the second metal.

The presence of Pb at a nominal concentration of 100 nmol L^{-1} did not appear to influence gill Cd accumulation, in which observed Cd accumulation closely matched the predicted Cd accumulation in the absence of Pb (Fig. 2A). Although the step-wise increases in water Cd concentration appeared to reduce Pb-gill binding by approximately 1 nmol g^{-1} ww (as Cd approached 70 nmol L^{-1} in the water), this decline was not significant (Fig. 2A).

The effects of water Cd on Pb-gill binding were clearer in fish exposed to a nominal water Cd concentration of 100 nmol L^{-1} and step-wise increases in water Pb concentration. Under these conditions, water Cd inhibited water Pb-gill binding at Pb concentrations between 20 and 230 nmol L^{-1}, but not at 470 nmol L^{-1} Pb, compared to predicted values for Pb in the absence of Cd (Fig. 2B). However, Cd-gill accumulation was not significantly affected by the gradual increases in water Pb concentration from approximately 3 to 400 nmol L^{-1} (Fig. 2B). It was notable, however, that the gill-Pb burden always exceeded the gill-Cd burden, in fish subjected to Pb–Cd mixtures (Fig. 2A and B). This trend persisted even when water Cd concentration matched or exceeded the water Pb concentration (Fig. 2B).

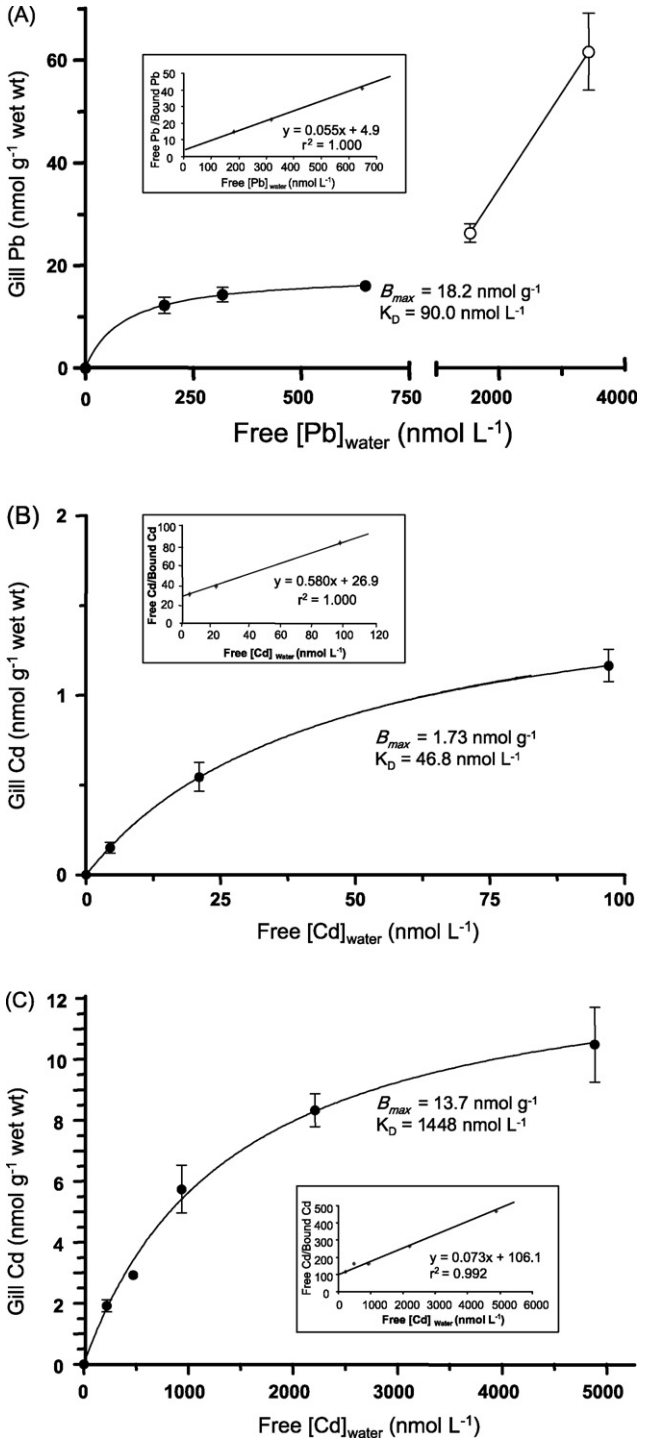

Fig. 1. Metal-gill accumulation by juvenile rainbow trout during exposure to increasing concentrations of (A) Pb, (B) low concentrations of Cd (5–97 nmol L^{-1}), and (C) high concentrations of Cd (219–4885 nmol L^{-1}) in ion-poor water after a 3-h exposure. Means ± 1 S.E.M. (N = 6–15 fish per concentration). The free metal/bound metal vs. the free metal concentration relationships (Langmuir plots; inset of each figure) were used to calculate the gill binding capacity (B_{max}), and the inverse of the gill binding strength (K_D) for each population of gill metal binding site identified. See text for further details.

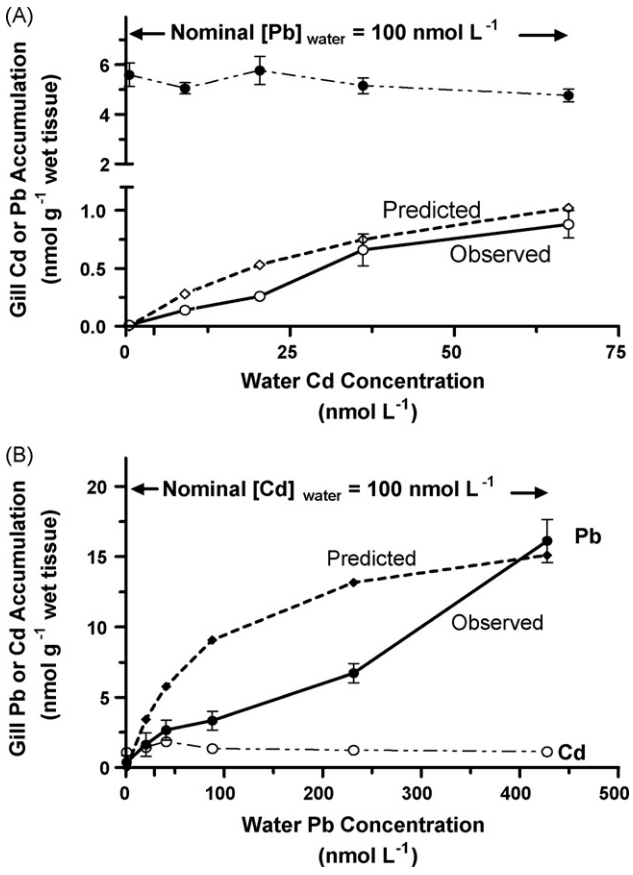

Fig. 2. Accumulation of Cd (open circles) or Pb (solid circles) in the gill tissue of juvenile rainbow trout during exposure to (A) increasing concentrations of Cd at constant Pb concentration (nominal concentration = 100 nmol L^{-1}), or (B) increasing concentrations of Pb at constant Cd concentration (nominal concentration = 100 nmol L^{-1}) in ion-poor water after a 3-h exposure. Diamonds represent predicted Pb (solid) and Cd (open) accumulation determined from the regressions of Langmuir plots (insets of Fig. 1A and B). Observed metal accumulation presented as the mean ± 1 S.E.M. (N = 10 fish per treatment).

3.4. Effects of Cd and Pb mixtures on Ca^{2+} and Na^{+} influx

Calcium influx was disrupted in a more than additive manner by the presence of Cd plus Pb in the exposure water. At nominal Pb and Cd concentrations of zero, the Ca^{2+} influx rate was approximately 27 nmol g^{-1} h^{-1}. In the absence of Cd, Pb had no significant effect on Ca^{2+} influx at Pb concentrations up to about 110 nmol L^{-1} (Fig. 3A). Similarly, Ca^{2+} influx was unaffected during exposure to a nominal Cd concentration of 10 nmol L^{-1} (actual [Cd] = 8.4 nmol L^{-1}) and no Pb (Fig. 3B). As Pb was added to the water at a nominal Cd equal to 10 nmol L^{-1}, however, it led to a progressive reduction in Ca^{2+} influx compared to the Cd-only exposure that approached 50% when the water Pb concentration exceeded 100 nmol L^{-1} (Fig. 3B). At a higher Cd concentration of 50 nmol L^{-1} (actual [Cd] = 45.2 nmol L^{-1}), and no Pb added, there was a significant 40% decrease in Ca^{2+} influx compared to the metal-free control (Fig. 3C). However, the addition of Pb did not exacerbate the Cd-induced inhibition of Ca^{2+} influx until the dissolved Pb concentration exceeded 100 nmol L^{-1}. At this Pb concentration, inhibition was greater: Ca^{2+} influx was now approximately 60% lower compared to the rate recorded in metal-free control fish (Fig. 3C).

Sodium influx was also affected by the presence of Pb plus Cd in the water. In the absence of Cd and Pb, the rate of Na^{+} influx was approximately 265 nmol g^{-1} h^{-1} (Fig. 4A). The Na^{+} influx was not significantly affected by the presence of Pb alone in the

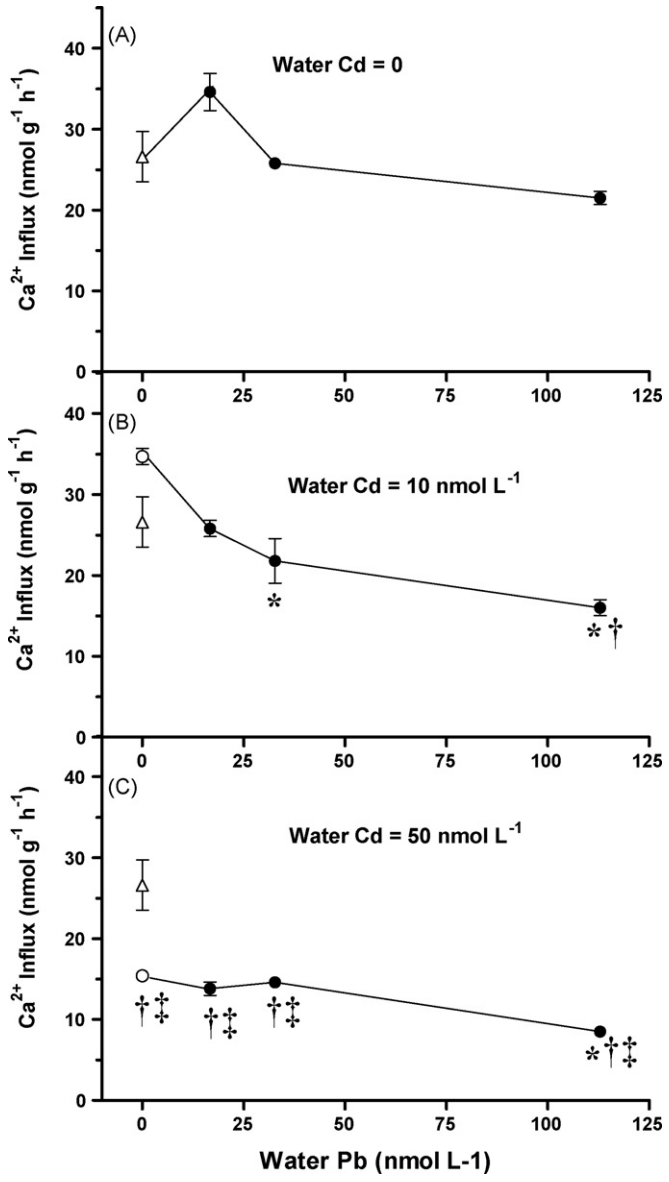

Fig. 3. Effects of metal exposure on rates of Ca^{2+} influx in juvenile rainbow trout ($N = 10–12$ fish per treatment) at different concentrations of Pb in the (A) absence of Cd, at (B) nominal [Cd] ~10 nmol L^{-1} (actual [Cd] = 8.7 nmol L^{-1}), and (C) nominal Cd ~50 nmol L^{-1} (actual Cd = 45.2 nmol L^{-1}), in ion-poor water over a 3 h measurement period. Data shown as mean ± 1 S.E.M. Significant differences ($p < 0.05$) between Pb exposure treatments (solid circles) and Pb = 0 exposures (open circles) at each Cd concentration are denoted by an asterisk (*). Significant differences from nominally metal-free controls (open triangle) are denoted by a dagger (†); double daggers (‡) represent significantly different rates of Ca^{2+} influx at given concentrations of Pb but different concentrations of Cd.

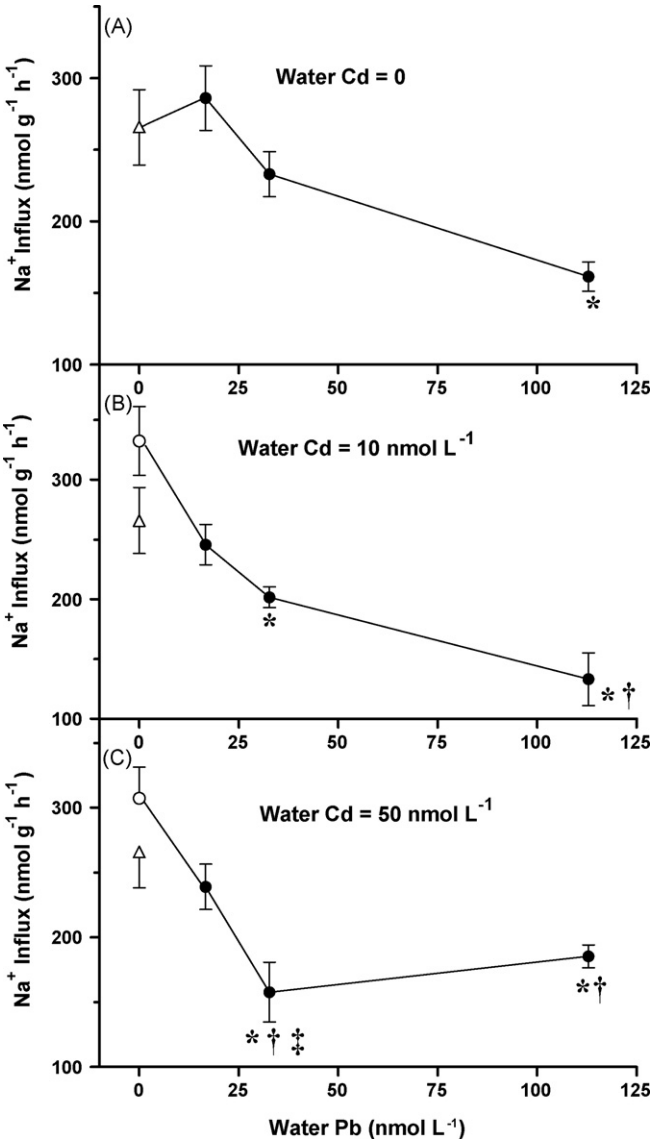

Fig. 4. Effects of metal exposure on rates of Na^+ influx in rainbow trout ($N = 10–12$ fish per treatment) exposed to increasing concentrations of Pb in (A) the absence of Cd, or (B) at [Cd] = 10 nmol L^{-1} and (c) at Cd = 50 nmol L^{-1}, in ion-poor water over a 3-h flux measurement period. Data shown as mean ± 1 S.E.M.. Significant differences ($p < 0.05$) between Pb exposure treatments (solid circles) and Pb = 0 exposures (open circles) at each Cd concentration are denoted by an asterisk (*). Significant differences from nominally metal-free controls (open triangle) are denoted by a dagger (†); double daggers (‡) represent significantly different rates of Na^+ influx at given concentrations of Pb but different concentrations of Cd.

water until dissolved Pb concentrations approached 110 nmol L^{-1}, at which time Na^+ influx was reduced by approximately 40% (Fig. 4A). When Cd was added to the water at a nominal concentration of 10 nmol L^{-1}, the threshold Pb concentration was reduced to approximately 25 nmol L^{-1}, where Na^+ influx was reduced by 25% compared to rates measured in fish held in nominally metal-free water, and 40% compared to the fish exposed to Cd only (Fig. 4B). These more than additive effects were even more pronounced at nominal Cd concentrations of 50 nmol L^{-1}, in which the combined effect with 25 nmol L^{-1} Pb resulted in a greater than 50% reduction in Na^+ influx compared to fish exposed to Cd alone, and a 40% reduction compared to fish held in nominally metal-free water (Fig. 4C).

4. Discussion

4.1. Acute toxicity of Pb alone in acidic, soft water

The trout exposed to Pb in the low ionic strength, acidic (~pH 6.0–6.5) water of the present study had a 96-h LC50 of 482 nmol L^{-1} (100 μg L^{-1}), which is approximately one-order of magnitude lower than previously reported values for trout, which range from 4.8 μmol L^{-1} (Rogers et al., 2003) to 6.4 μmol L^{-1} (Davies et al., 1976). Two factors, both associated with water chemistry, explain the greater Pb sensitivity of the trout in the present study. First, a greater proportion of the total Pb concentration was present in its ionized Pb^{2+} form in the moderately acidic waters of the present study (pH 6.0–6.5) compared to the more circumneutral waters

Table 2
Predicted Pb and Cd speciation (% of total metal concentration) in low ionic strength water, and within the intracellular fluid (ICF) of the trout gill

Species	Water			Gill ICF
	pH 6.0	pH 7.0	pH 8.0	pH 7.4
Pb				
Pb^{2+}	97.9	79.0	9.9	27.0
$PbOH^{+}$	1.5	12.1	14.8	5.3
$Pb(OH)_2$	–	0.1	0.7	0.05
$PbCl^{+}$	0.2	0.1	0.02	22.2
$PbCl_2$	–	–	–	2.0
$PbCO_3$	0.1	5.8	70.4	34.2
$PbHCO_3^{+}$	0.4	3.0	3.6	9.0
Cd				
Cd^{2+}	99.5	99.3	93.4	25.7
$CdOH^{+}$	–	0.03	0.2	–
$CdCl^{+}$	0.5	0.5	0.4	63.0
$CdCl_2$	–	–	–	10.7
$CdHCO_3^{+}$	0.02	0.1	1.4	0.3
$CdCO_3$	–	0.05	4.6	0.2

Parameters used to calculate Pb and Cd speciation using Visual MINTEQ (Version 2.52)—Water: $P_{CO_2} = 0.00038$ atm; $[Ca^{2+}] = 72\ \mu mol\ L^{-1}$; $[Na^{+}] = 346\ \mu mol\ L^{-1}$; $[Cl^{-}] = 54\ \mu mol\ L^{-1}$; [Mg2+] < 10 μmol L^{-1}; [K^{+}] < μmol L^{-1}; NOM = 0. Gill ICF: $P_{CO_2} = 0.00263$ atm; $[Ca^{2+}] = 200\ \mu mol\ L^{-1}$; $[Na^{+}] = 55\ mmol\ L^{-1}$; $[Cl^{-}] = 75\ mmol\ L^{-1}$; $[K^{+}] = 90\ mmol\ L^{-1}$. Intracellular fluid composition taken from Wood and LeMoigne (1991). Gill intracellular P_{CO_2} set to arterial P_{CO_2} (~2 Torr = 0.00263 atm; Wood and LeMoigne (1991)).

(pH 6.7–8.25 (Davies et al., 1976); pH 7.9–8.2 (Rogers et al., 2003)) examined previously. Calculations using the chemical speciation program, Visual MINTEQ, revealed that more than 90% of the total Pb concentration was in its Pb^{2+} form at pH 6.0–6.5, the pH range of the present study (Table 2). In contrast, approximately 80% of the total Pb is Pb^{2+} at pH 7.0, but less than 10% of Pb is present as the free ion at pH 8.0, with the balance being mainly $PbCO_3$ (70%) and $PbOH^{+}$ (15%; Table 2). Greater Pb toxicity and/or Pb uptake in moderately acidic waters has been demonstrated in a variety of fishes including trout (Hodson et al., 1978; MacDonald et al., 2002), fathead minnow larvae (*Pimephales promelas*: Grosell et al., 2006), and carp (*Cyprinius carpio*: Stouthart et al., 1994).

The soft nature of the ion-poor water (Ca = approximately 100 μmol L^{-1}) was the other factor contributing to the greater Pb toxicity in the present study. Water hardness is a key determinant of the sensitivity of fishes to metals (e.g., Pascoe et al., 1986; McDonald et al., 1989; Playle et al., 1993; Taylor et al., 2003) including Pb (Davies et al., 1976; MacDonald et al., 2002). Because Pb^{2+} has a similar hydrated radius to Ca^{2+} (R_{H-Pb} = 2.74 Å; R_{H-Ca} = 2.53 Å; Trivedi and Axe, 2001), it is thought to compete with Ca^{2+} for binding sites (Ca^{2+}-channels) on the gill (Stouthart et al., 1994; MacDonald et al., 2002; Rogers et al., 2003; Rogers and Wood, 2004), apparently leading to hypocalcemia and death (Rogers et al., 2003). Thus, the soft water used here likely resulted in less competitive interactions between Pb^{2+} and Ca^{2+} for Ca^{2+}-channel access at the apical membrane of the gill (Playle, 2004), a critical site for Ca^{2+} uptake and Ca^{2+} homeostasis in fishes (Flick et al., 1985; Perry and Wood, 1985; Stouthart et al., 1994). Although there was no significant reduction in Ca^{2+} influx in response to Pb alone (Fig. 4A), Pb was likely accumulating within the gill tissue because pronounced disturbances to Na^{+} influx were observed (also see below), and the Pb concentration range used was close to the apparent K_D (90 nmol L^{-1}) for Pb binding to the gills.

The LA50, in which the trout accumulated close to 10 nmol Pb g^{-1} gill tissue (Table 1) during a 3-h exposure to the 96-h LC50, was approximately 50% of the B_{max} (Fig. 1A). In other words, half of the available gill-binding sites were occupied by Pb in 3 h. This number of gill-Pb binding sites (2.1 nmol per fish at an average gill mass of 0.118 g/fish; N = 149) is similar to earlier estimates made by MacDonald et al., 2002 (1.4 nmol per fish) for comparably sized trout in water of similar hardness.

The rapid accumulation of Pb and associated disturbances to Na^{+} influx might also explain the greater Pb sensitivity of the trout in acidic, soft water and suggest that the mechanisms of Pb toxicity could be different in hard vs. soft water. In hard water, the mode of Pb toxicity is characterized by pronounced reductions in Ca^{2+}-influx leading to hypocalcaemia (Rogers et al., 2003; Rogers and Wood, 2004) with less marked reductions in Na^{+} transport (Rogers et al., 2005). However, the marked Pb-induced reduction in Na^{+}-influx we observed suggests that this might be the proximate toxic mechanism in soft water, such that toxicity might eventually result from reduced internal Na^{+}, or hyponatraemia. There is clearly a need to test this hypothesis during longer term Pb exposure in soft, acidic water.

4.2. Acute toxicity of Cd Alone in acidic, soft water

As Cd^{2+}, Cd is also a well-known Ca^{2+} analog, with a high affinity for Ca^{2+}-channels (Verbost et al., 1989; Pratap and Wendelaar-Bonga, 1993; Playle et al., 1993; Niyogi and Wood, 2004b), and it should have been highly toxic in the very soft water used. As predicted, the 96-h LC50 for Cd (6.7 nmol L^{-1}) was low, and comparable to that determined by Hollis et al., 2000 (~18 nmol L^{-1}) in slightly harder water (Ca = 130 μmol L^{-1}). In harder waters, Cd toxicity is lower due to greater competition by Ca^{2+} for Ca^{2+} channel access (Playle et al., 1993; Hollis et al., 2000). Indeed, 96-h LC50s may be 10- to 1000-fold greater in hard water (Pascoe et al., 1986; Hollis et al., 1999, 2000; Niyogi et al., 2004). Because Cd speciation is not strongly affected by a switch from acidic to circumneutral pH, it is less likely that the low pH of our test waters influenced Cd-gill binding (Table 2; also see McDonald et al., 1989 for review). However, the presence of two populations of Cd-gill binding sites complicated interpretation.

Titration experiments over a range of Cd concentrations (approaching 5000 nmol L^{-1} at the high end), yielded two populations of Cd-gill binding sites: a high affinity, low capacity population (Fig. 1B), and a previously uncharacterized low affinity, high capacity population (Fig. 1C). With a B_{max} of 1.73 nmol g^{-1} gill, the high affinity, low capacity Cd-gill binding sites were likely occupied when fish were exposed to the 96-h LC50 for Cd over 3 h (3-h LA50) and 24 h (24-h LA50). The LA50 at 3 h was about 10% of the measured B_{max}, and approximately 60% of the available Cd-gill binding sites were occupied after 24 h.

The B_{max} of the high affinity, low capacity population equates to 0.2 nmol Cd-gill binding sites per fish (average gill mass = 0.118 g per fish). This quantity is comparable to calculations by Playle et al. (1993) for fathead minnow, and B_{max} values (B_{max} ~0.7–2.6 nmol g^{-1} gill) reported for rainbow trout acclimated to soft water (Hollis et al., 2000; Niyogi et al., 2004). Compared to the high affinity, low capacity population, there were eightfold more low affinity, high capacity Cd binding sites on the gills, which equated to 1.6 nmol Cd-gill binding sites per fish. At first glance, it is debatable whether these low affinity, high capacity Cd-gill binding sites would need to be incorporated into BLMs because the range over which this population was defined, 200 to approaching 5000 nmol L^{-1}, is outside the range most fishes would encounter in uncontaminated or contaminated surface waters (CCME, 1995; USEPA, 2000). However, in fishes subjected to chronic Cd exposure in contaminated surface waters, such data may have important toxicity implications due to changes in gill metal binding properties that might affect Cd-gill binding site abundance and Cd-gill binding strength (Niyogi et al., 2004), and sub-lethal chronic toxicity.

4.3. Kinetics of Pb and Cd interactions with the gill

With a Pb 96-h LC50 that is approximately 100-fold greater than that of Cd, it is clear that the acute toxicity of Pb is substantially less than that of Cd. Yet, there was greater Pb binding than Cd binding to the gill when the trout were exposed to these metals, individually and in two-metal mixtures. The equilibrium dissociation constants commonly used in BLM predictions are $\log K_{Pb\text{-}gill} = 6.0$ for Pb, and $\log K_{Cd\text{-}gill} = 8.6$ for Cd (Playle et al., 1993; MacDonald et al., 2002), meaning that Cd should have a gill binding strength that is approximately 400 times greater than Pb (affinity is proportional to the $\log K_{metal\text{-}gill}$ value; Playle, 2004). There should have therefore been more Cd- than Pb-gill accumulation (i.e. new metal binding) when fish were exposed to Cd–Pb mixtures, not the opposite. We suggest this apparent contradiction was due in part to lower than expected differences in binding strength between Cd and Pb. In fact, the difference in gill binding affinity between these metals was less than twofold because the calculated $\log K_{Me\text{-}gill}$ for Cd ($\log K_{Cd\text{-}gill} = 7.33$; Fig. 1B) was only slightly greater than that of Pb ($\log K_{Pb\text{-}gill} = 7.05$; Fig. 1A).

Although Pb has a slightly lower metal-gill binding affinity than Cd, why was there greater Pb-gill binding than Cd-gill binding when fish were exposed to Pb–Cd mixtures? The most likely explanation was that there was a 10-fold higher number of Pb vs. Cd-binding sites (i.e. greater B_{max} for Pb; Fig. 1A and B). Theoretically, the B_{max} should have been identical if Pb and Cd were binding to the same population of external sites (i.e. Ca^{2+} channels) on the gill (Playle, 2004). One possibility for this apparent discrepancy is that Pb may be binding to an alternate or additional population(s) of gill binding sites. As the gill analysis used in this and in previous studies cannot distinguish between externally bound and internally bound metal in the gills (e.g., Playle et al., 1993; MacDonald et al., 2002), we suspected that the higher B_{max} for Pb was due to the trapping of Pb^{2+} as $PbCO_3$ within the more alkaline intracellular space (ICF) of the gill (intracellular pH ~7.4; Wood and LeMoigne, 1991).

To test this Pb-trapping hypothesis, simulations using Visual MINTEQ were conducted to determine how Pb and Cd speciation might be altered in the gill ICF (Table 2). As predicted, the simulation indicated that Pb speciation shifts would favor $PbCO_3$, but also $PbCl^+$ formation, in the more alkaline gill ICF, where the resulting proportion of Pb^{2+} would be less than 30% (Table 2). However, the high Cl^- within the gill ICF (~75 mmol L^{-1}) also favored $PbCl^+$ formation and a shift in Cd speciation from predominately Cd^{2+} in water, to mainly $CdCl^+$ and $CdCl_2$ within the gill ICF (Table 2). Thus, there is a possibility that both Pb and Cd could be trapped within the gill ICF. However, a much better knowledge of the gill's intracellular *milieu*, and the potential for metal precipitation and metal–protein binding, is needed to confirm this possibility.

Once in the gill cytosol, Pb and Cd could have bound to a variety of targets including sequestration by metallothionein and cytosolic vesicles (lysosomes/microsomes), but these metals also likely targeted physiologically sensitive sites including organelles and enzymes (Campbell et al., 2005). For instance, Pb-binding to carbonic anhydrase plus Pb- and Cd-binding to basolateral Na^+/K^+ ATPases (Rogers et al., 2005), may explain the rapid reductions in Na^+ influx that were observed in the presence of Pb alone, and Pb plus Cd (discussed further below).

At higher concentrations of Pb, it is clear that there is also a second, non-saturable (at least in the concentration range tested), population of Pb-gill binding sites (Fig. 1A), as first suggested by Rogers et al. (2005). We also characterized a second, saturable population of low affinity Cd-gill binding sites. Externally, these low affinity, high capacity binding sites for Pb and Cd could include sialic acid residues of mucus. It seems unlikely that Pb binding to intracellular ligands such as carbonic anhydrase and/or basolateral Na^+/K^+ ATPases represented this second population of Pb binding sites because a major effect attributed to these sites, reduced Na^+ influx, was observed at the relatively low Pb concentrations that fell within the saturable range for Pb binding. Regardless, it should be noted that the water Pb concentrations needed to occupy such secondary binding sites would be in the micromolar range and seldom, if ever, would be encountered even under the most contaminated conditions (Demayo et al., 1982). As pointed out previously, the Cd concentrations (greater than 200 nmol L^{-1}) required to occupy secondary Cd binding sites would seldom, if ever, approach such environmentally unrealistic values.

4.4. Interactions between Pb and Cd mixtures and the gill

At environmentally realistic concentrations of Pb (up to 580 nmol L^{-1}) and Cd (up to 70 nmol L^{-1}), metal mixture exposure had mixed effects on metal-gill binding. The presence of Pb (nominal concentration = 100 nmol L^{-1}) had little, if any, effect on Cd-gill binding in fish subjected to step-wise increases in the water Cd concentration. Indeed, the predicted Cd-gill binding curve, calculated from the corresponding Langmuir plot in the absence of Pb, virtually overlapped with the observed Cd-gill binding curve under these conditions (Fig. 2A). In contrast, Pb-gill binding appeared to be reduced by up to 50%, at a constant nominal water Cd concentration of 100 nmol L^{-1}, but only up to a Pb concentration of approximately 350 nmol L^{-1} (Fig. 2B). These findings therefore suggest that at low to moderate (and environmentally relevant) concentrations of Pb, Cd was out-competing Pb for metal-gill binding sites—i.e. the interaction for gill binding was less than additive due to competition.

We had originally predicted that Cd would out-compete Pb for Ca^{2+}-channel binding sites because it is known to bind to the external gill with greater strength than Pb, as illustrated by its greater binding constant ($\log K_{Cd\text{-}gill} = 7.2$–$8.6$ vs. $\log K_{Pb\text{-}gill} = 6.00$; Playle et al., 1993; MacDonald et al., 2002; Niyogi et al., 2004). Further, Rogers and Wood (2004) also demonstrated that Cd decreased Pb-gill binding in trout, but at substantially higher concentrations of both Cd (1 μmol L^{-1}) and Pb (4.8 μmol L^{-1}) than used here. Although our findings suggest that the difference in binding affinity between Cd and Pb is probably only about twofold (Cd higher than Pb) in the soft, acidic water used here, it is still sufficient to allow Cd to out-compete Pb for Ca^{2+}-channel binding sites. Thus, at environmentally realistic concentrations of each, Pb plus Cd bind to the gill in a less than additive manner. Our observation that exposure to Cd alone, but not Pb alone, caused reductions in Ca^{2+} influx also suggests that Cd binds to the Ca^{2+}-channel proper with greater affinity than Pb when both metals are present at comparable concentrations.

4.5. Effects of Cd and Pb mixtures on Ca^{2+} and Na^+ influx

As Pb^{2+} and Cd^{2+} both act as Ca^{2+} analogs, we predicted that they would act in an additive manner to inhibit Ca^{2+} influx as metal mixture concentrations increased. As expected, Cd inhibited Ca^{2+}-influx by rainbow trout, as described in earlier studies (e.g., Reid and McDonald, 1988; Niyogi and Wood, 2004b). There was, however, no reduction in Ca^{2+} influx in the presence of Pb alone (Fig. 2A), but it should be pointed out that the experiments only lasted 3 h and the Pb concentrations used were relatively low compared to previous studies in which Ca^{2+}-influx was inhibited. For instance, acute reductions in Ca^{2+} influx were reported in rainbow trout fingerlings exposed to Pb in hard water, but the exposure concentrations were more than 10-fold greater (2.3–5.8 μmol L^{-1}) than those used in this study (Rogers et al., 2003). Inhibition was seen at lower Pb levels in soft water by Rogers and Wood (2004), but their trout had been acclimated to hard water, not soft water. In our study, the trout

had been acclimated to soft water, which likely led to an increase in maximal Ca^{2+}-transport capacity. Three to fivefold increases in the maximal Ca^{2+} transport capacity were reported in trout acclimated to very soft water ($[Ca^{2+}]$ ~25 μmol L^{-1}; Perry and Wood, 1985). A similar increase in maximal Ca^{2+} transport capacity might have made the present fish more resistant to Pb-induced disturbances to Ca^{2+}-influx, especially during relatively short exposure periods and low Pb concentrations.

Although Pb alone had no effect, the presence of Pb in combination with Cd exacerbated disturbances to Ca influx caused by Cd alone, but only slightly. According to the classification of Norwood et al. (2003), this interaction would be classified as a more than additive effect of the synergistic type. At Cd concentrations of 10 and 50 nmol L^{-1}, the presence of Pb led to additional reductions in Ca^{2+} influx of approximately 10% relative to the nominally metal-free control fish. Thus, Pb's acute effects on Ca^{2+} influx are likely relatively minor compared to Cd. These conclusions are further re-inforced by the similar absence of an inhibition of Ca^{2+}-influx reported in brown trout (*Salmo trutta*) exposed to comparable concentrations of Pb in similar soft ($[Ca^{2+}]$ ~20–90 μmol L^{-1}), acidic water (pH 5.4–6.6; Sayer et al., 1991).

Pb did have a pronounced effect on Na^+ influx, however, and these effects were exacerbated by Cd, which by itself had no inhibitory effect on Na^+ influx (Fig. 4). Again this indicates more than additive effects. One likely mechanism of Pb action was inhibition of intracellular carbonic anhydrase within the gill leading to less CO_2 hydration to HCO_3^- and H^+ generation, which would have depleted internal substrate availability for the Na^+–H^+ transport system (Rogers et al., 2005). Rogers et al. (2005) also reported that Pb inhibited branchial Na^+/K^+ ATPase activity, which would gradually lead to increased Na^+ in the gill ICF, and a reduced water-gill ICF electrochemical gradient for Na^+ entry via Na^+-channels. These effects of Pb on Na^+ influx were exacerbated by Cd (~100 nmol L^{-1}) in a more than additive manner, possibly due to Cd-induced inhibition of the Na^+/K^+ ATPase. Exposure to Cd inhibits both gill and intestinal Na^+/K^+ ATPase activity in a variety of fishes (Schoenmakers et al., 1992; Pratap and Wendelaar-Bonga, 1993; Lemaire-Gony and Mayer-Gostan, 1994; Lionetto et al., 2000; Atli and Canli, 2006) and aquatic invertebrates (e.g., Postel et al., 1998). Thus, disturbances to both Ca^{2+} and Na^+ homeostasis will be more pronounced due to more than additive interactions between Cd and Pb in contaminated, moderately acidic, soft waters. Longer term studies (e.g., several days) are needed to determine whether actual toxicity at environmentally relevant concentrations of Pb and Cd under such conditions is due to altered Ca^{2+} homeostasis, Na^+ homeostasis or both.

4.6. The toxic unit concept and implications for environmental risk assessment

Playle (2004) used the toxic unit (TU) concept, where 1 TU is the 96-h LC50 for a particular toxicant (metal), to determine if it were possible to extend metal-gill binding models such as the BLM to multi-metal mixtures. His modeling of Pb-Cd and other multi-metal interactions (Zn, Co, Cu and Ag) assumed a single population of common binding sites. Based on this, he predicted that metal-gill binding would be greater than strictly additive or strictly additive at low to moderate concentrations of metals (exposure to less than 1 TU of each metal). A practical problem in testing Playle (2004) predictions experimentally is that at environmentally relevant concentrations, in which fish are exposed to a TU or less of metal, actual metal-gill binding can be very low (e.g., in the low to sub-nmol g^{-1} range; Table 1), making it difficult to infer whether Pb- and Cd-gill binding is strictly additive, greater than additive, or less than additive.

Playle's predictions of additive interactions were based on the presumption that metals such as Cd and Pb had a single common target on the gill, the Ca^{2+}-channel. Our measurements of Ca^{2+} influx in the presence of these metals therefore provided us with an alternate, yet sensitive, means to test Playle's predictions. Indeed, our observation that Pb exacerbated Cd-induced disturbances to Ca^{2+}-influx supports Playle's predictions of additive interactions between Pb and Cd at the gill. However, Pb also interfered with Na^+ influx, likely through rapid inhibition of intracellular carbonic anhydrase. Although Cd did not inhibit Na^+ influx, its presence at lower concentrations substantially exacerbated such Pb-induced ionic disturbances. These combined disturbances to Ca^{2+}-influx plus Na^+-influx suggest that a more than additive interaction between these two metals could cause toxicity due to combined disturbances to Ca^{2+} and Na^+ homeostasis. In other words, Cd- plus Pb-induced toxicity could be much greater than predicted if only less than or strictly additive interactions were taking place at the Ca^{2+}-channel of the gill. The possibility of "synergistic" interactions between Pb and Cd, and amongst other metals, should therefore be considered in future studies addressing the effects of multi-metal mixtures on fishes.

In conclusion, Cd and Pb bind to the gill in a less than additive manner, with Cd inhibiting Pb-gill binding. However, in combination they exacerbate disturbances to gill-mediated Ca^{2+} and Na^+ uptake in a more than additive fashion. Thus, fish exposed to such metal mixtures could be similarly more vulnerable to toxicity in a greater than additive manner. It may therefore be necessary to re-evaluate water quality criteria and assumptions of the BLM for both Pb and Cd under such conditions at environmentally relevant metal concentrations, so that accurate predictions can ultimately be made about Pb plus Cd toxicity in the acidic, soft waters that characterize the Canadian Shield, Scandinavia and many other vulnerable regions.

Acknowledgements

This paper is dedicated to the memory of the late Dr. Richard C. Playle, who originally conceived many of the ideas explored in this paper, and whose influence on the field of metal toxicology and development of the BLM was profound. We thank Anna Winter, Richard Smith, Linda Diao, Sunita Nadella, Yvonne Kara, and Joel Klinck for their excellent technical assistance, and the Animal Care Staff at Wilfrid Laurier University. The discussions and suggestions of Dr. Scott Smith, Dr. Uwe Borgmann, and Dr. Peter Campbell were also greatly appreciated. The Wilfrid Laurier University Science and Technology Endowment Program (STEP) provided financial assistance to OB. Finally, the authors gratefully acknowledge the funding provided to CMW and MPW by the NSERC Metals in the Human Environment Strategic Network (MITHE-SN). CMW is supported by the Canada Research Chair Program.

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