Comparative Biochemistry and Physiology, Part C 158 (2013) 10–21

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Acute toxicity, critical body residues, Michaelis–Menten analysis of bioaccumulation, and ionoregulatory disturbance in response to waterborne nickel in four invertebrates: *Chironomus riparius*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Daphnia pulex*

Erin M. Leonard *, Chris M. Wood

Department of Biology, McMaster University, Hamilton, ON, L8S 4K1, Canada

ARTICLE INFO

Article history:
Received 27 February 2013
Received in revised form 27 March 2013
Accepted 30 March 2013
Available online 6 April 2013

Keywords:
Nickel
Acute
Invertebrates
Bioaccumulation
Ionoregulation

ABSTRACT

We investigated the bioaccumulation and acute toxicity (48 h or 96 h) of Ni in four freshwater invertebrate species in two waters with hardness of 40 (soft water) and 140 mg L^{-1} as $CaCO_3$ (hard water). Sensitivity order (most to least) was *Lymnaea stagnalis* > *Daphnia pulex* > *Lumbriculus variegatus* > *Chironomus riparius*. In all cases water hardness was protective against acute Ni toxicity with LC50 values 3–3.5× higher in the hard water vs. soft water. In addition, higher water hardness significantly reduced Ni bioaccumulation in these organisms suggesting that competition by Ca and Mg for uptake at the biotic ligand may contribute to higher metal resistance. CBR50 values (Critical Body Residues) were less dependent on water chemistry (i.e. more consistent) than LC50 values within and across species by ~2 fold. These data support one of the main advantages of the Tissue Residue Approach (TRA) where tissue concentrations are generally less variable than exposure concentrations with respect to toxicity. Whole body Ni bioaccumulation followed Michaelis–Menten kinetics in all organisms, with greater hardness tending to decrease B_{max} with no consistent effect on K_d. Across species, acute Ni LC50 values tended to increase with both K_d and B_{max} values — i.e. more sensitive species exhibited higher binding affinity and lower binding capacity for Ni, but there was no correlation with body size. With respect to biotic ligand modeling, log K_{NiBL} values derived from Ni bioaccumulation correlated well with log K_{NiBL} values derived from toxicity testing. Both whole body Na and Mg levels were disturbed, suggesting that disruption of ionoregulatory homeostasis is a mechanism of acute Ni toxicity. In *L. stagnalis*, Na depletion was a more sensitive endpoint than mortality, however, the opposite was true for the other organisms. This is the first study to show the relationship between Na and Ni.

1. Introduction

It is well established that water hardness is protective against metal toxicity and bioaccumulation due to competition by Ca and/or Mg with the metal for binding sites at the biotic ligand, as well as their actions in stabilizing membrane permeability (Miller and MacKay, 1980; Pagenkopf, 1983; Playle, 1998; Paquin et al., 2000; Wood, 2001). Protection against nickel (Ni) toxicity at higher water hardness has been shown in vertebrates (Meyer et al., 1999; Pyle et al., 2002), and invertebrates (Deleebeeck et al., 2007b; Kozlova et al., 2009). However, for the latter, information is sparse on the effects of water hardness on the physiology of the organisms, metal toxicity and bioaccumulation. In addition, invertebrates are the most sensitive group in the ecotoxicity database for Ni (ECB, 2008), emphasizing the need for more information on these species.

Current models deriving either water quality guidelines or criteria in Canada and the US, respectively, use models based on water hardness. In both jurisdictions, formulae are given which incorporate hardness to determine maximum allowable concentrations of metal within aquatic environments (CCREM, 1987; US EPA, 1986). However, within the past decade there has been further research into more complex models which predict the amount of biologically available metal based on a suite of water chemistry parameters, such as dissolved organic carbon (DOC), alkalinity, pH, and other cations as well as hardness. The European Union has adopted a version of these more complex models for deriving Environmental Quality Standards (EQS; ECB, 2008). Such models include the Biotic Ligand Model (BLM; Paquin et al., 2000; Di Toro et al., 2001; Niyogi and Wood, 2004), which utilize site-specific water chemistry parameters

* Corresponding author at: McMaster University, Life Science Building, 1280 Main St. W., Hamilton, ON, L8S 4K1, Canada. Tel.: +1 905 525 9140x23237; fax: +1 905 522 6066.
E-mail address: leonarem@mcmaster.ca (E.M. Leonard).

1532-0456/$ – see front matter
http://dx.doi.org/10.1016/j.cbpc.2013.03.008

to predict the bioavailability of the metal in conjunction with the binding constants of the biotic ligand of the organism. This allows prediction of whether the amount of metal theoretically bound to the organism is sufficient to cause toxicity. On a physiological basis, if metal binding is saturable, the binding constants of the biotic ligand can be characterized by their binding affinity (K_d) and binding site density (B_{max}) using Michaelis–Menten analysis. Currently, there are several validated acute BLMs for nickel (Ni) in freshwater organisms (Meyer et al., 1999; Hoang et al., 2004; Deleebeeck et al., 2007a; Kozlova et al., 2009).

A complementary, related approach is the Tissue Residue Approach (TRA: Luoma et al., 2009; Adams et al., 2011; Schmidt et al., 2011), which predicts toxicity as a function of metal levels within the organism, independent of water chemistry. The TRA uses bioaccumulation as an endpoint, which may prove to vary less with external factors such as water chemistry (and specifically in the context of this study, water hardness). This approach has been suggested as a possible tool for risk assessment where Critical Body Residues (CBR values) may be used to predict the toxicity across species (Luoma et al., 2009; Adams et al., 2011; Schmidt et al., 2011).

With this in mind, four invertebrates were selected for studies on Ni bioaccumulation and acute toxicity, based on their relatively high sensitivity (the gastropod: *Lymnaea stagnalis* and the cladoceran: *Daphnia pulex*) and low sensitivity (the dipteran: *Chironomus riparius* and the oligochaete: *Lumbriculus variegatus*) to metal toxicity, respectively.

Gastropods in general have been neglected in terms of toxicological studies (Grosell and Brix, 2009), as they were originally regarded as relatively insensitive to metals in terms of acute toxicity (Nebeker et al., 1986). However, more recent evidence suggests that they are in fact one of the most sensitive groups (Brix et al., 2011, 2012; Ng et al., 2011). Most notably, *L. stagnalis* has replaced *Ceriodaphnia dubia* in the species sensitivity distribution (SSD) as the most sensitive species to chronic Ni exposure (Schlekat et al., 2010). Second to gastropods, cladocerans are regarded as very sensitive species to Ni (Kozlova et al., 2009), as well as to cadmium (Cd) and zinc (Zn) (Shaw et al., 2006; Clifford and McGeer, 2009). Chironomids, along with the similarly tolerant oligochaetes, represent the other end of the sensitivity spectrum. Benthic surveys have found these organisms to be the predominant species in polluted aquatic environments (Winner et al., 1980). In addition, chironomids are the most tolerant aquatic organism in the SSD for Cd (U.S. EPA, 2000).

Pane et al. (2003b) suggested that the mechanisms of Ni toxicity were different for vertebrates and invertebrates. In the teleost rainbow trout, *Oncorhynchus mykiss*, Ni acts as a respiratory toxicant (Pane et al., 2003a; Pane and Wood, 2004), whereas in the cladoceran *Daphnia magna*, Ni was shown to be an ionoregulatory toxicant which disrupts Mg homeostasis (Pane et al., 2003b). In addition, water Mg and Ca concentration has a large protective effect against acute Ni toxicity in both *D. magna* (Deleebeeck et al., 2007a,b) and *D. pulex* (Kozlova et al., 2009). Therefore, one aim of the present study was to determine whether this phenomenon of Mg and/or Ca antagonism is characteristic of aquatic invertebrates in general.

With this background in mind, our aims were: (1) to determine acute (48- or 96-h) LC50 values for Ni in both soft water (operationally defined as 40 mg L^{-1} as $CaCO_3$) and hard water (operationally defined as 140 mg L^{-1} as $CaCO_3$) in the four invertebrate species; (2) to assess whether Ni bioaccumulation is linked to mortality within and across species and in this manner define CBR50 values to compare with LC50 values; (3) to determine if Ni bioaccumulation is saturable in nature, and if there are relationships between Michaelis–Menten uptake parameters (B_{max} and K_d values) with toxicity that can be related to BLM constants; and finally (4) to elucidate if disruption of the homeostasis of Mg (or of two other essential ions, Na and Ca) is an indicator of the acute toxic mechanism of waterborne Ni in these four invertebrates.

2. Methods

2.1. Experimental organisms

C. riparius and *D. pulex* cultures are currently maintained at McMaster University and were initiated from cultures from J. Webber (Environment Canada, Burlington, Ontario, Canada) and J. McGeer and E.-J. Costa (Wilfred Laurier University, Waterloo, Ontario, Canada), respectively. *L. stagnalis* cultures are also currently maintained at McMaster University and were originally obtained from M. Grosell and S. Ebanks (University of Miami, Florida, USA), Z.-P. Feng (University of Toronto, Toronto, Ontario, Canada), N. Syed (University of Calgary, Calgary, Alberta, Canada), G. Spencer (Brock University, St. Catharine's, Ontario, Canada) and D. Spafford (University of Waterloo, Waterloo, Ontario, Canada). *L. variegatus* were purchased from Aquatic Foods Inc. (Fresno, California, USA). All organisms were kept in dechlorinated Hamilton tap water with an ionic composition of (in mmol L^{-1}) Na^+ (0.6), Cl^- (0.8), Ca^{2+} (1.0), K^+ (0.4), Mg^{2+} (0.4), and Ni ($<0.4 \times 10^{-5}$). Water pH was 7.8–8.0, while hardness and alkalinity were 120–140 mg L^{-1} and 95 mg L^{-1} as $CaCO_3$ equivalents, respectively, and dissolved organic carbon (DOC) was 2.3 mg L^{-1}. The cultures were maintained under a 16 h:8 h light:dark photoperiod, with the exception of *D. pulex* which were maintained under a 12 h:12 h light:dark photoperiod.

2.1.1. Chironomus riparius

Culture chambers for *C. riparius* consisted of 20-L aquaria with one part silica sand and three parts dechlorinated tap water, and were continuously aerated. Culture media was changed every life cycle (~28 days). *C. riparius* were fed ad libitum every other day with ground Big Al's Staple Flake Food (45% protein, 5% crude fat, 2% crude fiber and 8% moisture) (Big Al's Aquarium Supercentres, Woodbridge, ON, Canada). 3rd and 4th instar larvae were used for Ni exposures.

2.1.2. Lumbriculus variegatus

L. variegatus were kept in 80-L aquaria with a flow-through of continuously aerated dechlorinated tap water at turnover rate of 20 L day^{-1}. *L. variegatus* were fed the same commercial ground flake food as the described above, once every two weeks.

2.1.3. Daphnia pulex

D. pulex were kept in non-aerated 500-ml beakers. Water was changed bi-weekly and organisms were fed three times per week with unicellular green algae (*Selenastrum capricornutum*) plus YCT [Yeast (Fleischmann's Active Dry Yeast, Burns Philp Food Ltd., LaSalle, Quebec, Canada), CEROPHYL® (Cerophyl Laboratories Inc., Kansas City, MO, USA) and Martin's commercial dried pellet feed (Martin Mills Inc., Elmira, ON, Canada)]. Daphnids used in Ni exposures were 6–8 days old.

2.1.4. Lymnaea stagnalis

L. stagnalis were kept in 5-L aquaria on a flow-through system of dechlorinated tap water with a turnover rate of 2 L day^{-1}. Snails were fed fresh romaine lettuce three times weekly and carrots once per week. Juvenile snails (25–40 days post-hatch, ~2.0–2.5 cm in length) were used for all experiments. Ni toxicity for *L. stagnalis* was only assessed at a water hardness of 140 mg L^{-1} as $CaCO_3$ equivalents as this organism is a calciphile and exhibits reduced growth and increased mortality at environmental hardness below 50 mg L^{-1} as $CaCO_3$ (Dalesman and Lukowiak, 2010).

2.1.5. Soft water acclimation

For all organisms tested in 40 mg L^{-1} as $CaCO_3$ (soft water), water hardness was gradually decreased by ~10 mg L^{-1} per day (as $CaCO_3$ equivalents) using reverse osmosis (RO) water. All organisms

were left in the final soft water (approximately 40 mg L^{-1} as $CaCO_3$ equivalents) under static conditions with 75% water renewal every other day for 13 (*C. riparius*), 15 (*D. pulex*) and 18 (*L. variegatus*) days prior to metal exposure.

2.2. Toxicity and bioaccumulation tests

2.2.1. Acute (96- or 48-h) LC50 tests

L. stagnalis, *L. variegatus* and *C. riparius* acute LC50 tests were 96 h, whereas *D. pulex* was 48 h as per the U.S. EPA guidelines for deriving WQC (Stephan et al., 1985). All organisms were acclimated to testing temperatures (*L. stagnalis* = 22 ± 1 °C, *D. pulex* = 22 ± 1 °C, *L. variegatus* = 21 ± 1 °C and *C. riparius* = 22 ± 1 °C) and starved for 24 h (48 h for *L. stagnalis*) prior to exposure to allow sufficient time for gut clearance and to standardize metabolic rate. All experiments were conducted in 250-ml glass beakers (500-ml glass beakers for *L. stagnalis*) with static renewal every 24 h. Mortality was checked every 24 h prior to water renewal. Fifteen organisms in 200 ml of aerated exposure water (10 for *L. stagnalis* in 500 ml) were used for each exposure concentration. Each concentration was tested in triplicate to assess acute toxicity. Water was sampled every 24 h, before and after water change. Mean measured water chemistry parameters for all experiments are shown in Table 1 and measured Ni concentrations in the exposure waters are shown in Supplementary Table 1. Measured total and dissolved Ni concentration were generally close to the nominal values and dissolved values were used to determine LC50 values.

2.2.2. Ni bioaccumulation and whole body ion measurements

At 48 or 96 h, surviving organisms were transferred to hard water or soft water as appropriate containing no added Ni for 5 min to remove adsorbed Ni, followed by a brief (5 s) rinse in nanopure water (18.2 MΩ cm, Millipore Corporation, Billerica, MA, USA). Organisms were then transferred to filter paper and patted dry. Whole-body wet weights were recorded. *L. stagnalis* was placed in a −20 °C freezer for 24 h in order to detach the shell from the soft tissue. All weight data for *L. stagnalis* refer to soft tissue only. Whole organisms (or soft tissue for *Lymnaea*) were digested at room temperature with 65% HNO_3 (trace metal grade, Fisher Scientific, Ottawa, ON, Canada; 10 μL of HNO_3 per mg of tissue wet wt) for one week and then hydrogen peroxide (4 μL of H_2O_2 per mg of tissue wet wt) was added for 24 h to complete the digestion process. The digest was then diluted with a 1% HNO_3 solution for later measurements of tissue Ni, Na, Mg and Ca concentrations.

2.3. Analytical techniques

Ni in water samples (non-filtered and filtered through 0.45 μm Acrodisk filters, Pall Corporation, Ann Arbor, Michigan, USA) and tissue samples were measured using Graphite Furnace Atomic Absorption Spectroscopy (GFAAS; Varian SpectrAA — 220 with graphite tube atomizer (GTA — 110), Mulgrave, Australia) against certified atomic absorption standards (Aldrich Chemical Company, Oakville, ON, Canada). Measurements were conducted at a wavelength and slit width of 232.0 nm and 0.2 nm, respectively, to obtain a lower working limit of 0.2 μg L^{-1} or 0.003 μmol L^{-1}. Ni recovery was 94 ± 1.1% as determined by Environment Canada certified reference materials, TM-24.3 (lot # 0310) and TM-25.3 (lot # 0809). Ni concentration measurements were not corrected for recovery.

Ions (Na, Mg, and Ca) in water samples and tissues were analyzed by Flame Atomic Absorption Spectroscopy (FAAS; Varian SpectrAA — FS-220, Mulgrave, Australia). Na, Mg and Ca reference standard solutions (Fisher Scientific, Ottawa, ON, Canada) were used to obtain standard curves. Water pH and DOC were measured using a Accumet® Basic AB15 pH meter (Fisher Scientific, Ottawa, ON, Canada) and a total organic carbon analyzer (Mandel Scientific Company Inc.; TOC- V_{CPN} series; Shimadzu, Kyoto, Japan), respectively.

Table 1
Water chemistry for all Ni exposures in hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$) and soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$). All ion concentrations are represented in μmol L^{-1} with the exception of DOC (mg L^{-1}), hardness and alkalinity (mg L^{-1} as $CaCO_3$) and pH. Values are means ± S.E.M., n = 20–30 per value.

	SW	HW
Na	395 ± 4.9	824 ± 5.1
K	29 ± 3.1	38 ± 2.4
Cl	410 ± 5.4	970 ± 6.1
Ca	336 ± 2.7	1051 ± 7.4
Mg	174 ± 1.3	357 ± 3.0
Hardness	50.9 ± 4.2	140.7 ± 3.1
DOC	1.2 ± 0.3	2.3 ± 0.4
Alkalinity	84 ± 6	95 ± 4
pH	7.2 ± 0.04	7.8 ± 0.05

2.4. Calculations and statistical analyses

Acute LC50 values with 95% confidence intervals (C.I.) were calculated using measured dissolved Ni concentrations and ToxCalc-Toxicity Data Analysis Software v5.0.32 (Tidepool Scientific Software, McKinleyville, CA, USA). When the 95% C.I. of two LC50 values overlapped, a simplified manual method (Litchfield and Wilcoxon, 1949) was applied to determine if they were significantly different. Water Ni concentrations have been expressed as nominal, total, dissolved, ionic and active Ni concentrations in the supplementary information section (Supplementary Table 1). Acute LC10 values, which are often used for regulatory purposes, are also summarized in Supplementary Table 2. Non-filtered and filtered (0.45 μm, Acrodisk filter, Pall Corporation, Ann Arbor, Michigan, USA) water samples comprise the total and dissolved fractions, respectively. Ionic (Ni^{2+}) and active fractions (determined by the Ni concentration and by attractive and/or repulsive interactions of other molecules in solution) of Ni were calculated using measured water chemistry data reported in Table 1 using Visual MINTEQ software (ver. 3.0, beta, KTH, Department of Land and Water Resources Engineering, Stockholm, Sweden). The NICA–Donnan Model (Benedetti et al., 1995) was used in the model to estimate the effect of DOC on Ni speciation. The critical whole-body residue (CBR50) was the Ni bioaccumulation in whole body that corresponded to 50% mortality. Regression analyses were performed on relationships between Ni bioaccumulation and survival. When the regression was significant at $p < 0.05$ or the coefficient of determination (r^2) was greater than 0.6, a goodness-of-fit curve was plotted. CBR50 were calculated from the regressions of logit mortality against log Ni bioaccumulation. Ni bioaccumulation and survival were corrected for control levels prior to analysis.

Non-linear regression analyses of Ni bioaccumulation kinetics were performed with a hyperbolic curve fit (single rectangular two parameters $y = ax/(x + b)$; SigmaPlot for Windows version 10.0; Systat Inc., Chicago, IL, USA) in order to fit the parameters of the Michaelis–Menten equation:

$$\text{Specific binding} = B_{max} x\,[L]/[L] + K_d$$

where [L] is the concentration of the ligand (in this case, Ni), B_{max} is the binding site density for the ligand (μmol kg^{-1} wet wt), and K_d is the binding affinity (expressed in μmol Ni L^{-1}).

Ni bioaccumulation and essential ion data have been presented as means ± SEM (n), where n is the number of organisms. All data passed normality and homogeneity tests, or were transformed as necessary before statistical analyses were performed. Statistically significant differences between two groups were evaluated by unpaired Student's t tests (two-tailed). Comparisons amongst multiple treatment groups were assessed using a one-way analysis of variance (ANOVA) followed by the Fisher LSD Method (Sigma Plot 10.0, Chicago, IL, USA).

For all tests, statistical significance was allotted to differences with $p < 0.05$.

3. Results

3.1. Water chemistry

Water chemistry data for both soft and hard water are reported in Table 1. Ni water concentrations expressed as nominal, total, dissolved, ionic and active fractions of the metal, taking into account this measured water chemistry, are reported in the Supplementary Information section (Supplementary Table 1). All Ni water concentrations presented in this study are reported as the dissolved fraction of the metal, which averaged to be 93% of nominal values and 96% of total values (Supplementary Table 1).

3.2. Acute Ni LC50 values in soft and hard water

The comparative sensitivity order for acute Ni toxicity, from most sensitive to least sensitive, was: *L. stagnalis* > *D. pulex* > *L. variegatus* > *C. riparius* (Table 2). Acute 48- or 96-h LC50 values for Ni ranged from 7.5 to 246.8 μmol Ni L^{-1} (33 fold difference; or above the solubility of Ni, >11,000 μmol Ni L^{-1}). LC10 values are provided in Supplementary Table 2. In all cases where LC50 values were derived in both hard and soft water, organisms were ~3–3.5× more sensitive in soft water than in hard water (Table 2). No correlation was observed between the comparative sensitivity order for Ni and the mass of the organisms tested (Fig. 1). Note that for the snail (*L. stagnalis*), the mass plotted in Fig. 1 refers to soft tissue mass only, and does not include the shell.

3.3. Correlation between survival and whole body Ni bioaccumulation

As Ni bioaccumulation increased in the whole-body of HW acclimated *L. stagnalis*, survival decreased in a linear manner (Fig. 2A). This linear relationship between survival and whole body Ni was also observed in SW acclimated *D. pulex* and *L. variegatus*. However, in HW acclimated *D. pulex* and *L. variegatus*, a sigmoidal relationship occurred where survival remained high at lower whole-body Ni levels before reaching threshold concentrations at about 700 and 85 μmol kg^{-1} wet wt. in the two species, respectively (Fig. 2B, C). Beyond the threshold concentrations, mortality steadily increased (Fig. 2B, C). As no mortality was observed in *C. riparius* below the solubility point of Ni (~11,000 μmol Ni L^{-1}), there was no relationship between Ni bioaccumulation and survival (Fig. 2D).

CBR50 values ranged from 117.5 to 1259.0 μmol kg^{-1} wet wt. (Table 3), an 11-fold difference in comparison to the LC50 value range of 33-fold (Table 2). The CBR50 value for *L. stagnalis* was not determined as regression analysis between Ni bioaccumulation and survival in HW was not significant at $p > 0.05$. However, extrapolation of the regression line would yield an approximate value of 260 μmol kg^{-1} wet wt., within the range of the other CBR50 values. In *D. pulex*, there was a significant 3.5 fold difference between LC50 values in SW vs. HW (Table 2), but there was no significant difference between CBR50 values (Table 3). A similar trend was observed in *L. variegatus*, where there was a 3.0 fold difference between LC50 values (Table 2) in the two water hardness; however, no significant difference between CBR50 values (Table 3).

Table 2
Acute (48- or 96-h) LC50 values for waterborne Ni in μmol L^{-1} in soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$) and hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$) with lower and upper 95% confidence intervals in brackets. *Indicates a significant difference in LC50 values between SW and HW.

	Lymnaea stagnalis (96 h)	*Daphnia pulex* (48 h)	*Lumbriculus variegatus* (96 h)	*Chironomus riparius* (96 h)
LC50				
SW	NT	7.5* (3.5–15.1)	81.7* (51.6–109.5)	>11,000
HW	8.2 (6.6–11.1)	26.0 (19.5–37.7)	246.8 (209.8–281.8)	>11,000

NT indicates "not tested".

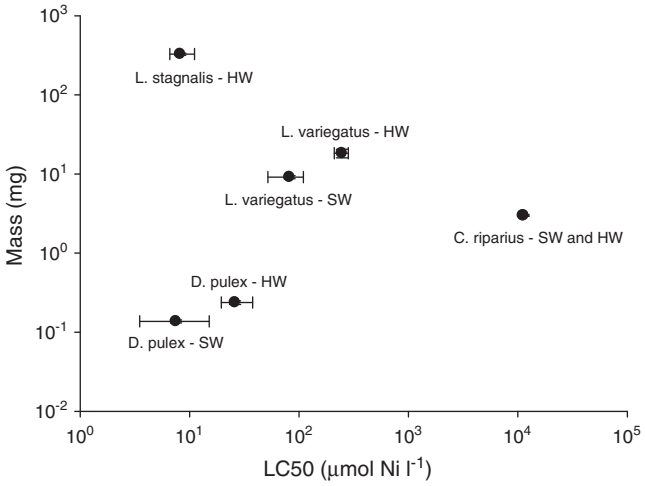

Fig. 1. Comparative sensitivity order for Ni LC50 values ($n = 3$, with 95% confidence intervals) in relation to mean body mass in mg ($n > 30$, with S.E.M.), in *Daphnia pulex*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Chironomus riparius* in hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$ – open symbols) and soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$ — shaded symbols).

3.4. Ni bioaccumulation parameters

Ni bioaccumulation increased with increasing exposure concentration, and there were clear hyperbolic, saturable relationships in all four organisms (Figs. 3A, 4A, 5A, and 6A), allowing for Michaelis–Menten constants to be calculated (Table 4). However, note that in Fig. 5A, the B_{max} for SW acclimated animals occurred at concentrations well beyond the highest Ni concentration tested, so this value should be interpreted with caution.

In comparison to the other organisms, *L. stagnalis* had the highest affinity (lowest K_d value) and a relatively low capacity (low B_{max}) for Ni (Table 4). K_d values were next lowest for *D. pulex*, followed by *L. variegatus* and finally *C. riparius* with exceptionally high values. After *L. stagnalis*, B_{max} values were slightly greater in *L. variegatus*, much greater in *D. pulex*, and again extremely high in *C. riparius*. In general B_{max} values tended to be higher in SW than in HW, while there was no clear effect of hardness on K_d values; the only significant hardness-related difference was the 2.8 fold higher B_{max} value in SW vs. HW chironomids. In comparison to the other organisms, *C. riparius* had by far the highest K_d or B_{max} values, indicating the lowest affinity and highest capacity (Table 4).

Across species, there were significant correlations between acute sensitivity to Ni and both kinetic parameters. Acute Ni LC50 values tended to increase with both B_{max} (Fig. 7A) and K_d values (Fig. 7B) — i.e. more sensitive species exhibited lower binding capacity and higher binding affinity for Ni. Notably, however, for B_{max} the relationship was driven solely by the extreme *C. riparius* values (Fig. 7A), whereas this was less true for the K_d vs. toxicity relationship (Fig. 7B).

3.5. Essential ion homeostasis

In general, exposure to Ni disrupted Na (Figs. 3B–5B) and Mg (Figs. 3C–5C) homeostasis at exposure concentrations below the LC50 values (cf. Table 2) with the exception of *C. riparius* (Fig. 6). In

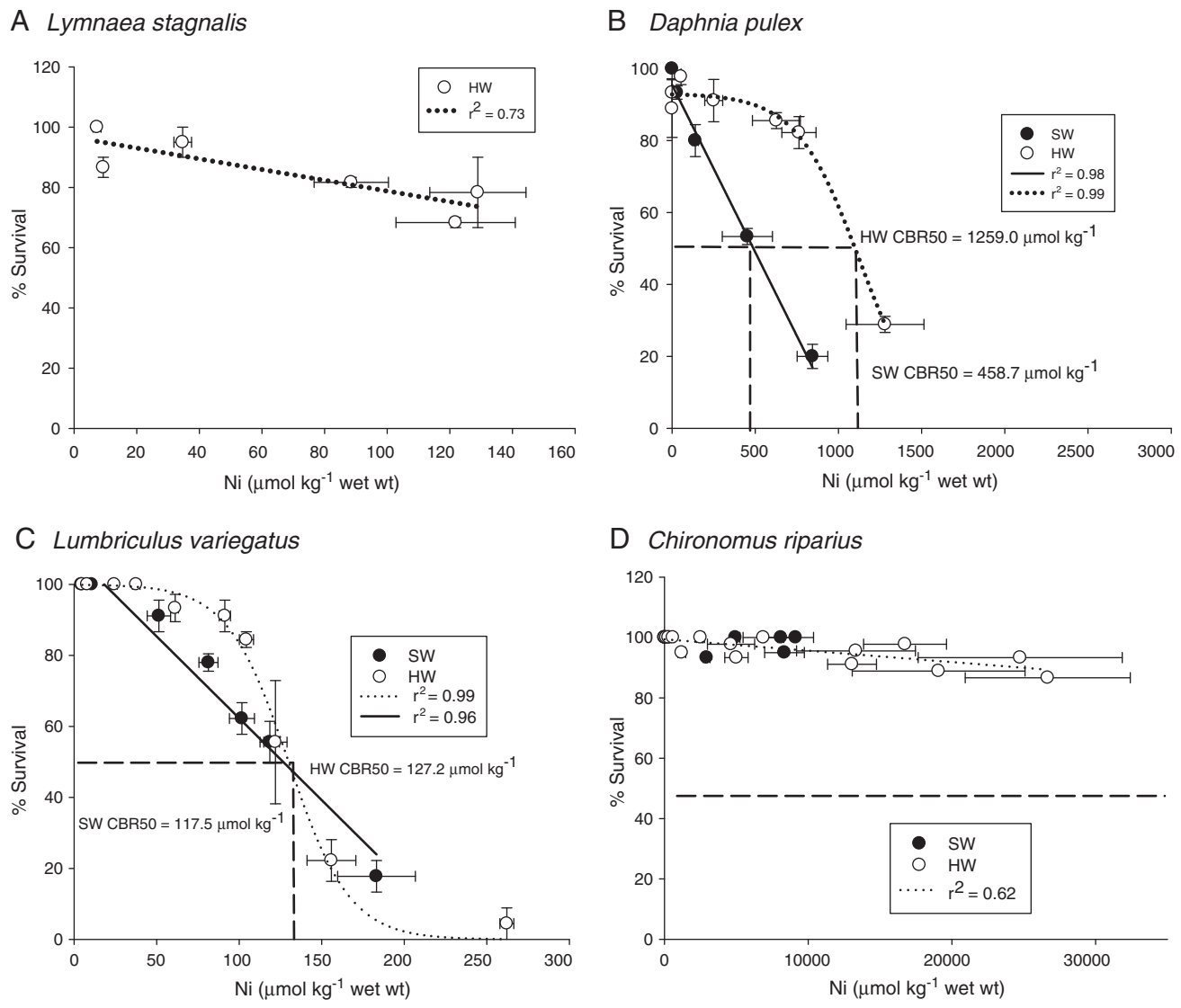

Fig. 2. Correlation between survival and whole body Ni bioaccumulation in *Daphnia pulex* (A), *Lymnaea stagnalis* (B), *Lumbriculus variegatus* (C) and *Chironomus riparius* (D) in hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$) and soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$). Values are means ± S.E.M.; $n = 3$ for % survival and $n = 10–15$ for Ni bioaccumulation. * denotes significant regression ($p < 0.05$). The dashed lines at 50% survival intersect the bioaccumulation vs. mortality relationships at the CBR50 values, which are indicated on the figure panels.

L. stagnalis, soft tissue Mg dropped by 25%, while Na declined by 72% in HW (Fig. 3). In *D. pulex*, there were 34% and 25% drops in whole body Mg in SW and HW respectively, as well as 35 and 22% decreases in whole body Na (Fig. 4).

Table 3
48- or 96-h CBR50 values for Ni in µmol kg^{-1} wet wt. with lower and upper 95% confidence intervals for *Daphnia pulex*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Chironomus riparius* in hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$) and soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$) water.

	Lymnaea stagnalis	*Daphnia pulex*	*Lumbriculus variegatus*	*Chironomus riparius*
CBR50	(96 h)	(48 h)	(96 h)	(96 h)
SW	NT	458.7 (479.3–1309.4)	117.5 (138.6–213.5)	N/A
HW	ND+	1259.0 (1153.6–1420.3)	127.2 (133.8–173.4)	N/A

There were no significant differences ($p > 0.05$) between HW and SW CBR50 values for the same organism. When the regression analysis between Ni bioaccumulation and survival was not significant at $p > 0.05$ or the coefficient of determination (r^2) was less than 0.6, no CBR50 values were calculated and are indicated by ND+. NT indicates "not tested".

However, different trends were observed in *L. variegatus*, depending on hardness. Although there was a 30% decrease in whole body Na with Ni exposure in SW, there was a significant increase of Na by 10% in HW. In addition, whole body Mg increased by 20% and 24% in SW and HW, respectively (Fig. 5).

There were no significant changes in whole body Ca with Ni exposure in any of the organisms, though a tendency for increase in SW *L. variegatus* (Figs. 3D–6D). No disruption of essential ion homeostasis was observed in *C. riparius* (Fig. 6).

4. Discussion

4.1. Acute (48- or 96-h) LC50 values for Ni in SW and HW

Organisms were 3–3.5 times more tolerant to waterborne Ni in HW (nominally 140 mg/L as $CaCO_3$) in comparison to SW (nominally 40 mg/L as $CaCO_3$). The protection against Ni toxicity was also seen with respect to Ni bioaccumulation, and was most likely due to the increased competition of the water hardness cations (Ca and Mg) with the metal, as well as their actions in stabilizing membrane

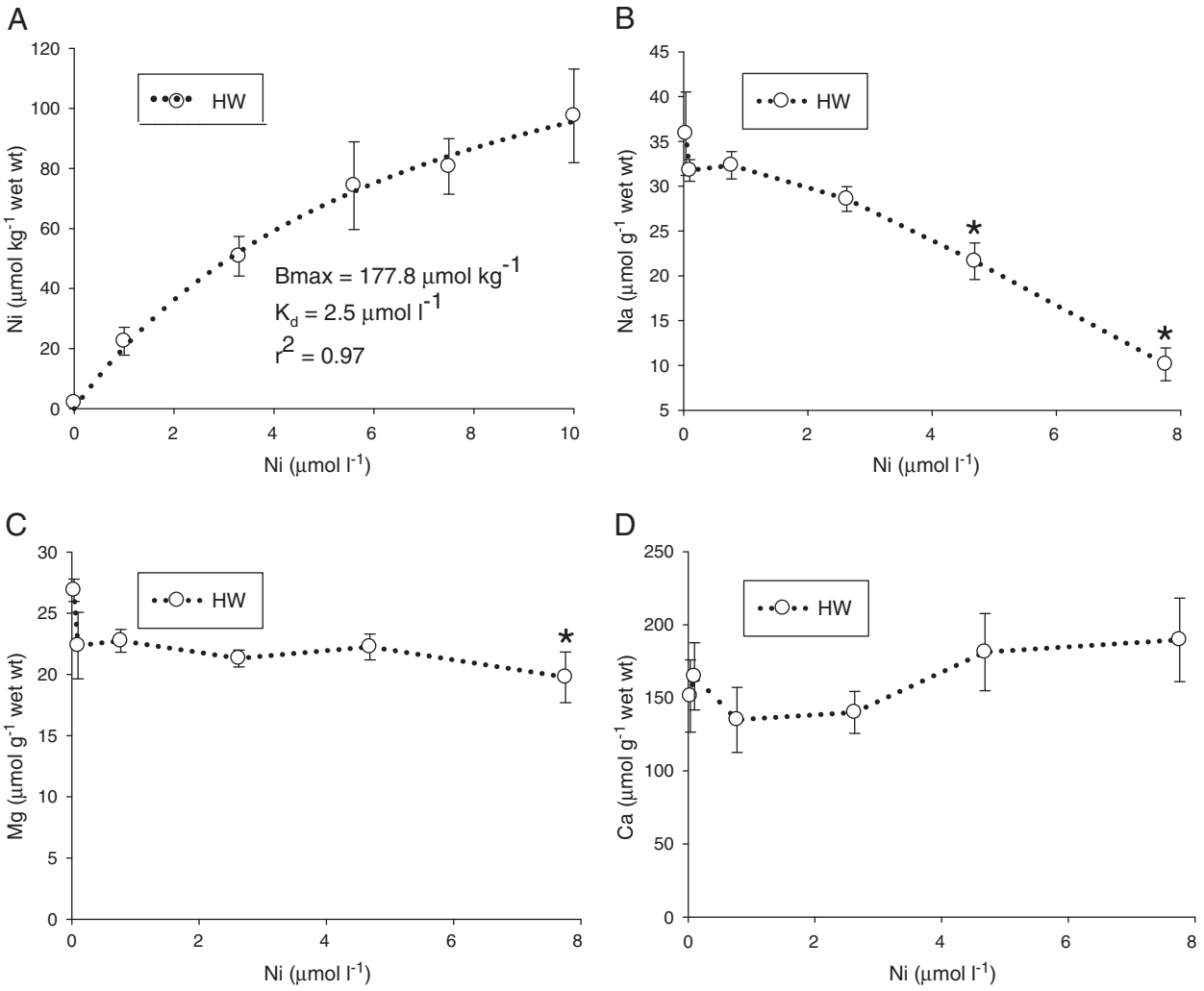

Fig. 3. Whole body (soft tissue) Ni (A), Na (B), Mg (C) and Ca (D) levels over a range of exposure concentrations following a 96-h exposure to *Lymnaea stagnalis* in hard water (nominally 140 mg/L as $CaCO_3$). * Denotes a significant difference ($p < 0.05$) in whole body ion concentration (Na, Mg, Ca) relative to the respective control value. Values are means ± S.E.M.; $n = 8–10$ per treatment.

permeability (Miller and MacKay, 1980; Pagenkopf, 1983; Playle, 1998; Paquin et al., 2000; Wood, 2001). This protection has previously been shown in several invertebrate species: *D. magna* (Deleebeeck et al., 2007b), *D. pulex* (Kozlova et al., 2009) and *C. dubia* (Keithly et al., 2004), as well as some vertebrate species: *Pimephales promelas* (Meyer et al., 1999; Pyle et al., 2002; Hoang et al., 2004) and *O. mykiss* (Deleebeeck et al., 2007a). However, the protection against Ni toxicity at higher water hardness may not be solely attributed to the increased presence of competitive cations; Boisen et al. (2003) demonstrated that uptake mechanisms for Na were different in HW and SW acclimated zebrafish. Therefore, differences in the osmoregulatory physiology of the organisms at the different water hardness may also influence LC50 values. Ni speciation analysis using Visual MINTEQ showed no marked differences in Ni complexation between HW and SW (see Supplementary Table 1).

Acute LC50 values recorded in the present study ranged from 7.5 to 246.8 μmol Ni L^{-1}, and LC10 values from 0.60 to 146.3 μmol Ni L^{-1} (Supplementary Table 2) depending on the hardness and species. Currently, Canadian Water Quality Guidelines (WQG) for Ni are presented for either a range of water hardness (e.g. for the two water hardness values of this study; 0.43 μmol Ni L^{-1} at a water hardness between 0 and 60 mg L^{-1} as $CaCO_3$, and 1.87 μmol Ni L^{-1} at water hardness between 120 and 180 mg L^{-1} as $CaCO_3$; CCME, 2007) or as a water hardness based equation ($e^{0.76*\ln[\text{hardness}] + 1.06}$ (expressed in μg L^{-1}); with guidelines of 0.8 μmol Ni L^{-1} at a water hardness of 40 mg L^{-1} as $CaCO_3$, and 2.1 μmol Ni L^{-1} at water hardness of 140 mg L^{-1} as $CaCO_3$ (CCREM, 1987)). It should be noted that these Canadian WQG are chronic values being compared against acute 96- or 48- LC50 values from the current study. In the United States, the Criterion Maximum Concentration (CMC — acute) for Ni is also based on water hardness by an equation ($CMC = e^{0.846*(\ln \text{ hardness}) + 2.255}$; U.S. EPA, 1995) and is 3.7 μmol Ni L^{-1} at 40 mg L^{-1} as $CaCO_3$ and 10.6 μmol Ni L^{-1} at 140 mg L^{-1} as $CaCO_3$. For the European Union Water Framework Directive, Environmental Quality Standards (EQS) are based on a "user friendly" BLM which incorporates Ca, DOC and pH (ECB, 2008). The EQS values for the European Union are chronic values and are based primarily on DOC and pH and not dependent on water hardness for Ni. The EQS derived using the water chemistry parameters in Table 1 are 0.058 μmol Ni L^{-1} in SW (nominally 40 mg L^{-1} as $CaCO_3$) and 0.043 μmol Ni L^{-1} in HW (nominally 140 mg L^{-1} as $CaCO_3$; ECB, 2008). Therefore, all species are protected by current North American Water Quality Guidelines/Criteria as well as the European Union Environmental Quality Standards.

To our knowledge, no acute Ni LC50 values for *L. stagnalis* have been reported previously. Nebeker et al. (1986) found the acute LC50 value for another snail species, *Physa gyrina*, to be 4. 1 μmol Ni L^{-1} at a water hardness of 26 mg L^{-1} as $CaCO_3$. Normalization to a water

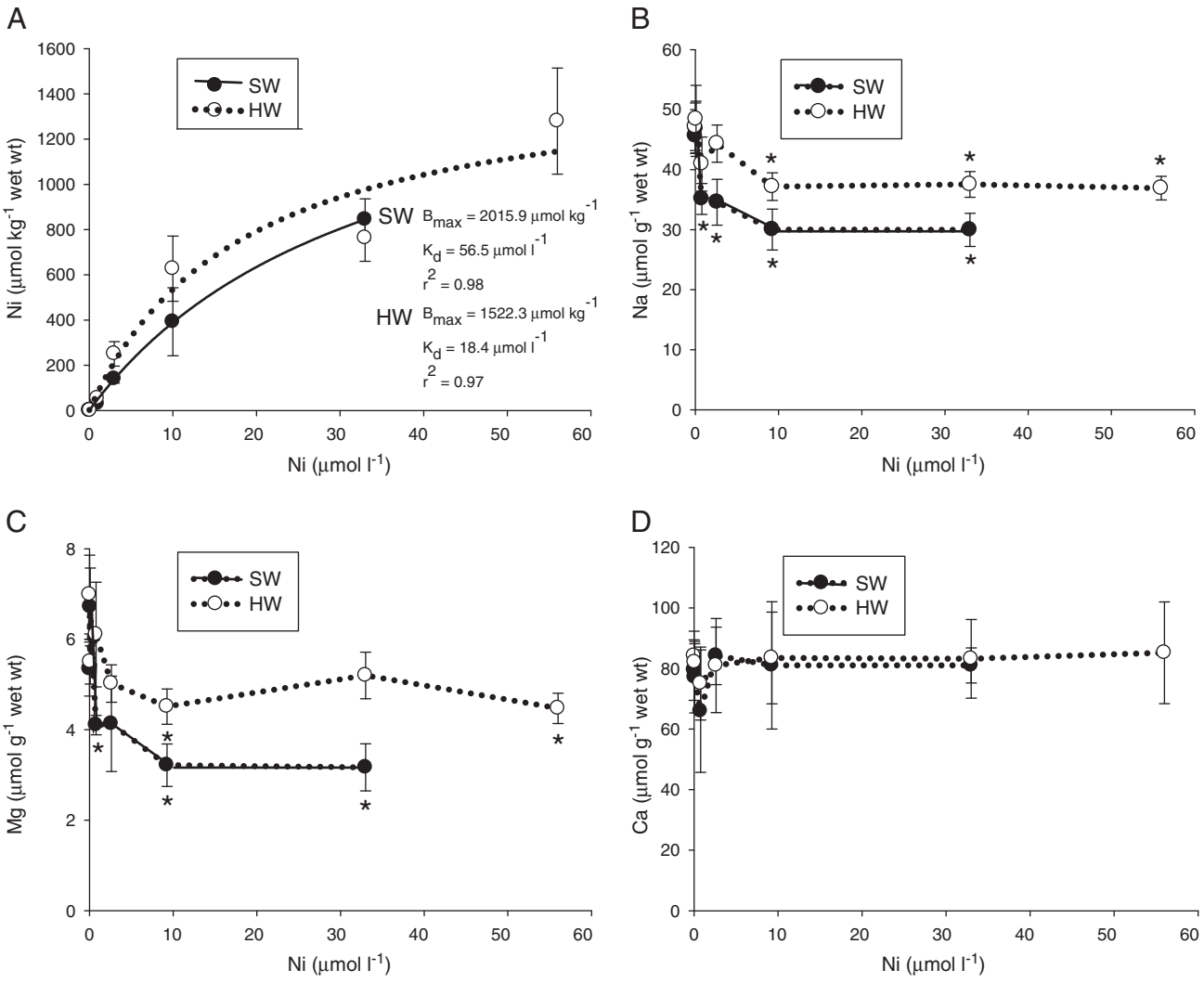

Fig. 4. Whole body Ni (A), Na (B), Mg (C) and Ca (D) levels over a range of exposure concentrations following a 48-h exposure to *Daphnia pulex* in hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$) and soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$). * Denotes a significant increase or decrease ($p < 0.05$) in whole body ion concentration (Na, Mg, Ca) relative to the respective control value. Values are means ± S.E.M.; $n = 8$–10 per treatment.

hardness of 85 mg L^{-1} as $CaCO_3$ using the U.S. EPA hardness correction (U.S. EPA, 1986), gives LC50 value estimates of 5.4 and 11.1 μmol Ni L^{-1} for the current study and Nebeker et al. (1986), respectively. In terms of chronic endpoints, *L. stagnalis* are known to be one of the most sensitive species to Co, Cu, Ni and Pb (De Schamphelaere et al., 2008; Schlekat et al., 2010; Brix et al., 2011; Ng et al., 2011, 2012; Brix et al., 2012).

In general, cladocerans are known to be fairly sensitive to acute Ni exposure. Keithly et al. (2004) reported 48-h LC50 values of 1.4 μmol Ni L^{-1} (at water hardness = 50 mg L^{-1} as $CaCO_3$) and 4.4 μmol Ni L^{-1} (at water hardness = 161 mg L^{-1} as $CaCO_3$) for *C. dubia*. As well, Pane et al. (2003b) found a 48-h LC50 of 18.2 μmol Ni L^{-1} (at water hardness = 45 mg L^{-1} as $CaCO_3$) for *D. magna*. Therefore, it is not surprising that in the current study, *D. pulex* is one of the most sensitive species with LC50 values of 7.5 μmol Ni L^{-1} (at water hardness = 50 mg L^{-1} as $CaCO_3$) and 26.0 μmol Ni L^{-1} (at water hardness = 140 mg L^{-1} as $CaCO_3$). In soft water comparable to that of the present study, Kozlova et al. (2009) determined the EC50 value for *D. pulex* to be 46 μmol Ni L^{-1}, ~6× higher than our current SW LC50.

L. variegatus are known to be relatively tolerant to Ni with an acute LC50 value of 250 μmol Ni L^{-1} (U.S. EPA, 1995). *Chironomus* species are known to be extremely tolerant to Ni toxicity (Béchard et al., 2008), where the 1st instar *C. riparius* larvae 24-h LC50 values in soft water (8 mg L^{-1} as $CaCO_3$) were at least 25 times higher than the current Canadian Council of the Ministers of the Environment (CCME) or U.S. EPA Water Quality Guidelines/Criteria. In addition, Powlesland and George (1986) determined the 96-h LC50 value for the same species (2nd instar larvae) to be 4,531 μmol Ni L^{-1} at a water hardness of 55 mg L^{-1} as $CaCO_3$. In the current study, we used the 3–4th instar larvae, which is a more resistant life stage (U.S. EPA, 1995), and found even higher LC50s (>11,000 μmol Ni L^{-1}).

With respect to the comparative sensitivity order, smaller organisms are thought to be more sensitive to metal toxicity (Grosell et al., 2007), which is logical: smaller organisms have a larger surface area to volume ratio, allowing for more metal uptake and/or more ion loss, leading to the potential for greater toxicity. However, for the four invertebrates species assessed in the current study, the Ni comparative sensitivity order was not a function of mass (Fig. 1) as it is for other metals such as copper (Grosell et al., 2007).

4.2. Correlation between survival and whole body Ni bioaccumulation

A sigmoidal relationship was observed between % survival and Ni bioaccumulation in hard water for *D. pulex* and *L. variegatus* (Fig. 2B, C). At low Ni bioaccumulation levels, survival is high, until the bioaccumulation in the organisms reaches apparent thresholds at approximately 700 and 85 μmol kg^{-1} wet wt. in *D. pulex* and

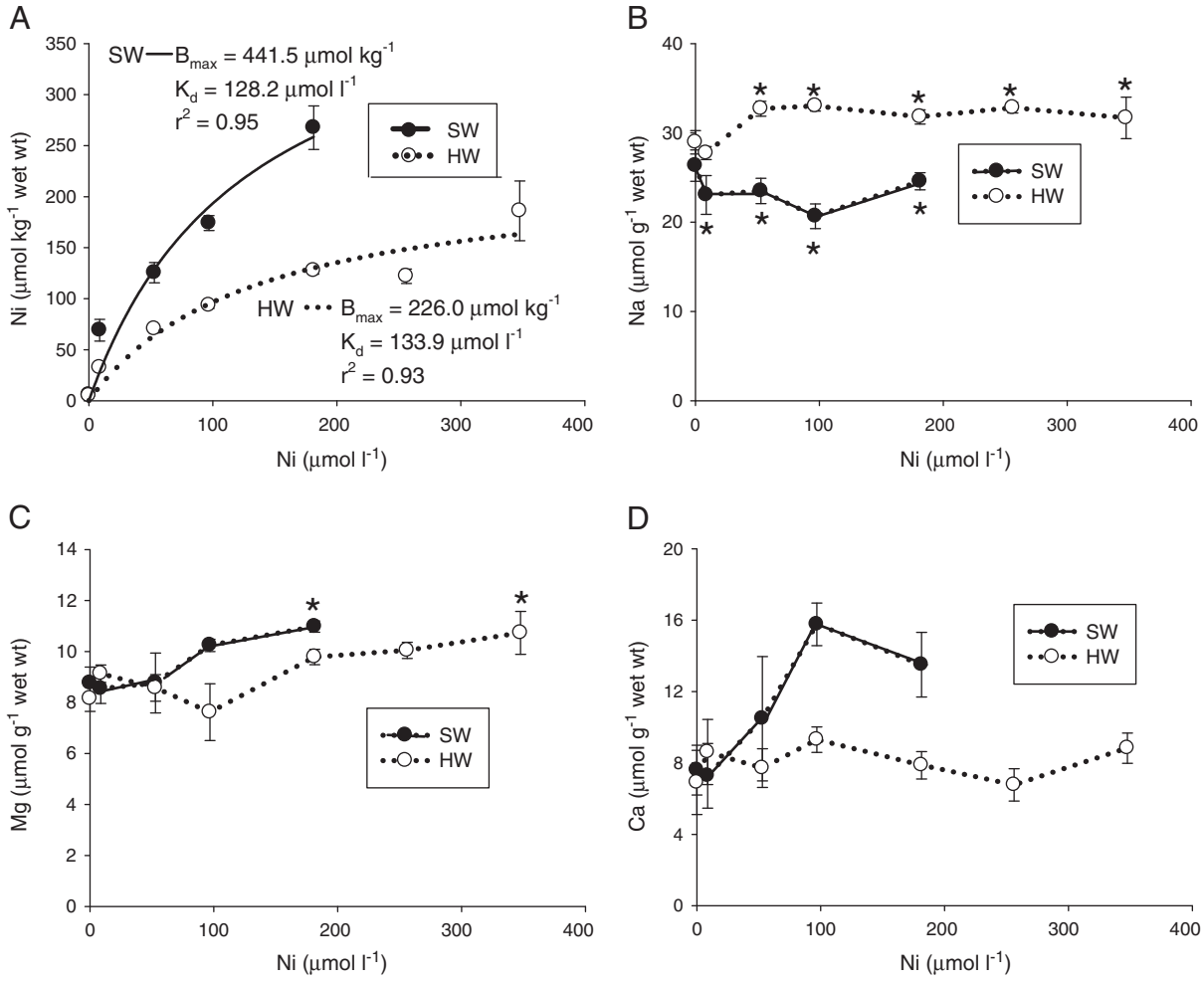

Fig. 5. Whole body Ni (A), Na (B), Mg (C) and Ca (D) levels over a range of exposure concentrations following a 96-h exposure to *Lumbriculus variegatus* in soft (40 mg/L as $CaCO_3$) and hard (140 mg/L as $CaCO_3$) water. * Denotes a significant increase or decrease ($p < 0.05$) in whole body ion concentration (Na, Mg, Ca) relative to the respective control value. Values are means ± S.E.M.; $n = 8$–10 per treatment.

L. variegatus, respectively. This suggests that at lower concentrations, either Ni excretion is maintained at or above Ni uptake and that the Ni which does accumulate is detoxified into non-biologically active pools, such as metallothionein-like proteins (MTLP) or metal rich granules (MRG: Ng et al., 2012). However, above this threshold, Ni excretion falls below Ni uptake and/or Ni levels in non-biologically active pools reach a saturation point and Ni spills over into biologically active pools such as organelles and heat denaturable proteins (HDP), causing mortality (Wallace et al., 2003). This sigmoidal relationship, which is observed for *D. magna* and *L. variegatus* in HW acclimated organisms, is in agreement with other freshwater studies on invertebrates with Cu in *L. variegatus* (Ng et al., 2012) and Cd in *Tubifex tubifex* (Redeker and Blust, 2004). This pattern has also been seen in some marine invertebrates: Ni in *Litopenaeus vannamei* and *Excirolana armata* (Leonard et al., 2011) and Zn and Cu in *Palaemon elegans* (White and Rainbow, 1982).

Linear relationships between % survival and Ni bioaccumulation appear to be more characteristic of SW Ni exposures. There are two possible explanations for this type of relationship — 1) at the lowest exposure concentrations, bioaccumulation in the organisms has already exceeded the threshold level before which bioaccumulation does not cause mortality, or 2) there is no real threshold level, such that as the organisms bioaccumulate Ni, toxicity starts to occur. The former explanation is opposed by the observation that the LC10 values (Supplementary Table 2), which can be used to estimate thresholds, are higher than the lowest exposure concentrations. Regardless, in the SW acclimated *D. pulex* and *L. variegatus*, the toxicity thresholds had already been surpassed by exposure concentrations (nominal) of 0.1 µmol Ni L^{-1} for *D. pulex* and 1 µmol Ni L^{-1} for *L. variegatus*, further reflecting the increased sensitivity of organisms in SW environments.

CBR50 values for significant relationships between % survival and Ni bioaccumulation were calculated. In *D. pulex*, there is a 3.5 fold difference between LC50 values in the two water hardness (Table 2), whereas, there was no significant difference between CBR50 values (Table 3). Similarly, in *L. variegatus*, there is a 3.0 fold difference between LC50 values in the two water hardness, however, again no significant difference between CBR50 values. This suggests that CBR50 values are less dependent on water chemistry than LC50 values within a species. In addition, when comparing across species, the variability in LC50 values across three phyla is 33-fold, but only 11-fold when comparing CBR50 values (Tables 2, 3). These data support one of the main advantages of the TRA where tissue concentrations are generally less variable than exposure concentrations with respect to a toxicity response (Luoma et al., 2009; Adams et al., 2011; Schmidt et al., 2011). There is a small but growing body of evidence supporting the concept of the TRA for metals (e.g. Redeker and Blust, 2004; Leonard et al., 2011; Ng et al., 2012). In addition, the TRA is related to more widely studied models such as the BLM — which has gained acceptance as a regulatory tool (e.g. BLM is used for calculating the Ni Environmental Quality Standard under the European Union's Water Framework Directive). Models such as the BLM utilize free

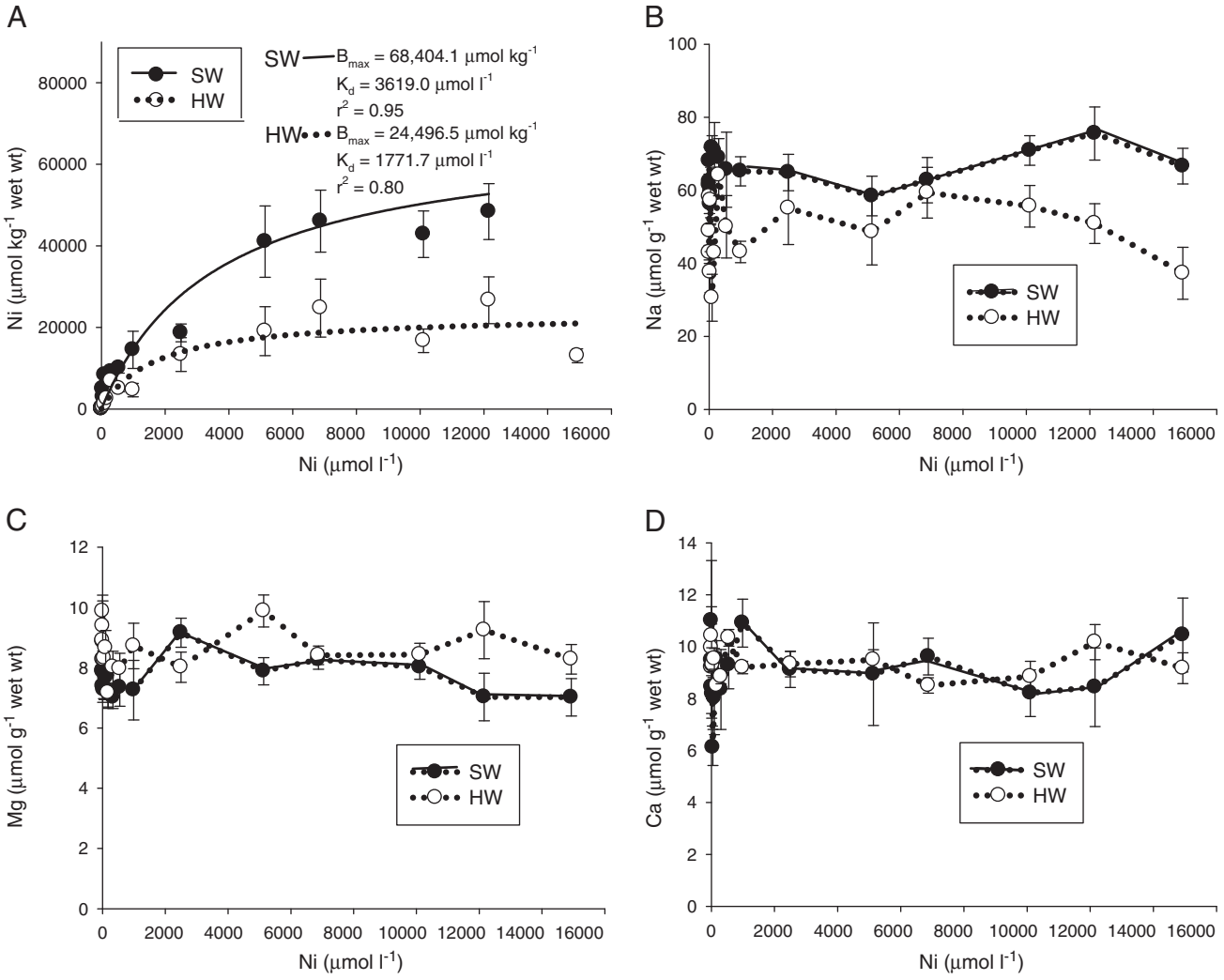

Fig. 6. Whole body Ni (A), Na (B), Mg (C) and Ca (D) levels over a range of exposure concentrations following a 96-h exposure to *Chironomus riparius* in soft (40 mg/L as $CaCO_3$) and hard (140 mg/L as $CaCO_3$) water. * There were no significant differences ($p < 0.05$) in whole body ion concentrations (Na, Mg, Ca) relative to the respective control values. Values are means ± S.E.M.; n = 8–10 per treatment.

Table 4
Michaelis–Menten kinetic constants (B_{max} and K_d) for saturable Ni bioaccumulation in *Daphnia pulex*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Chironomus riparius* in hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$) and soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$).

	B_{max} (µmol kg^{-1} wet wt)	K_d (µmol Ni L^{-1})	r^2
Lymnaea stagnalis			
SW	NT	NT	NT
HW	177.8 ± 30.1	2.5 ± 1.1	0.97
Daphnia pulex			
SW	2015.9 ± 552.2	56.5 ± 26.9	0.98
HW	1522.3 ± 300.0	18.4 ± 9.7	0.97
Lumbriculus variegatus			
SW	441.5 ± 152.8	128.2 ± 85.8	0.95
HW	226.0 ± 47.6	133.9 ± 69.4	0.93
Chironomus riparius			
SW	68,404.1 ± 9839.8*	3619.0 ± 1425.2	0.95
HW	24,496.5 ± 4167.3	1771.7 ± 1183.9	0.80

NT indicates "not tested".
* Indicates a significant difference in kinetic constants between SW and HW. Values are means ± S.E.M.

metal ion activity in conjunction with binding constants to predict toxicity with the understanding that the same bound metal concentrations will exert the same toxicological effect within a species. In this manner, Keithly et al. (2004) demonstrated the congruence of two separately determined lethal accumulation values for Ni in *Hyalella azteca* — LA20 following a 14-d study (Borgmann et al., 2001) and LA25 following a 28-d study (Keithly et al., 2004). Hence, the same critical body burden is estimated for the same species regardless of the external exposure concentration, water chemistry parameters and length of exposure.

4.3. Ni bioaccumulation parameters and their relation to BLM constants

As the exposure concentration of Ni increased, Ni bioaccumulation increased in a hyperbolic fashion until reaching a threshold where it leveled off in all four invertebrate species (Figs. 3A–6A). In general, more sensitive organisms (lower LC50 values) exhibited a lower K_d (higher affinity of the organism for Ni) and lower B_{max} values than more tolerant organisms (Fig. 7A, B). While this makes sense and is in general accord with BLM theory (Pagenkopf, 1983; Playle, 1998; Paquin et al., 2000; Niyogi and Wood, 2004), in future it would be of interest to expand this analysis to a wider range of species.

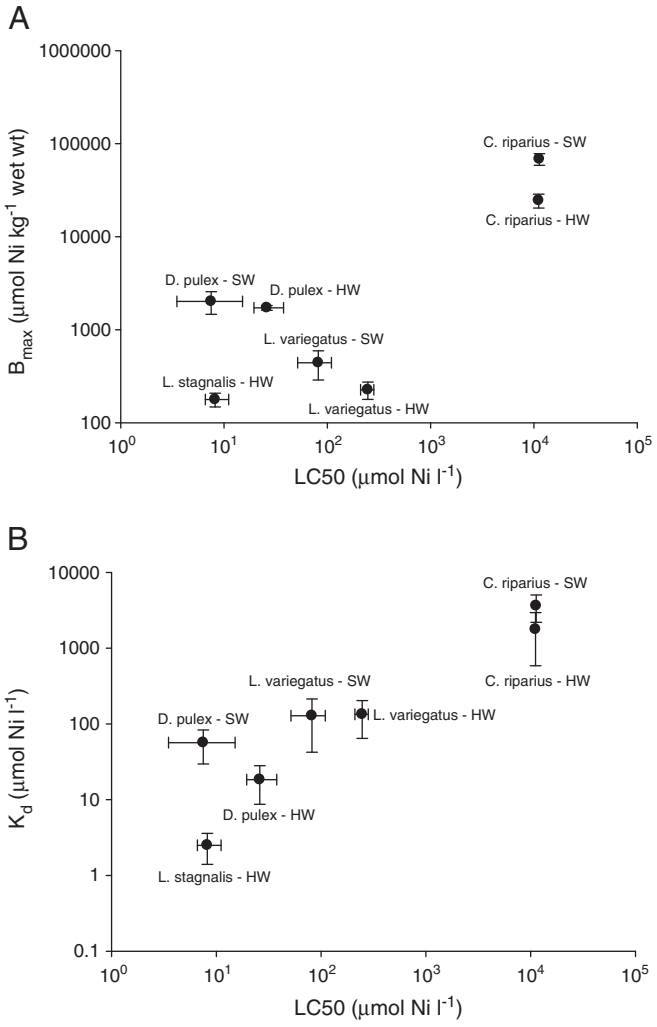

Fig. 7. Correlation between Michaelis–Menten uptake parameters ((A) B_{max} and (B) K_d) and LC50 values for Ni. Michaelis–Menten uptake parameters are means ± S.E.M.; $n = 8$–10 per treatment. $n = 3$ for LC50 values with 95% confidence intervals. For panel A, $r^2 = 0.76$, $p = 0.01$; for panel B, $r^2 = 0.86$, $p = 0.003$.

Models such as the Biotic Ligand Model (BLM) (Paquin et al., 2000; Di Toro et al., 2001; Niyogi and Wood, 2004), utilize water geochemistry and fitted binding constants to predict the amount of metal theoretically bound to the 'biotic ligand' of the organism which is sufficient to cause acute toxicity. On a physiological basis, if metal binding is saturable, the binding constants of the biotic ligand can be characterized by their binding affinity (K_d) using Michaelis–Menten analysis. In the present study, we evaluated whether this concept could be extended to Ni bioaccumulation in the whole body rather than bioaccumulation on a theoretical 'biotic ligand' (target site for toxicity). Specifically, in Table 5, we have compared the log K_{NiBL} values derived from the ionic component of the LC50 value (toxicity) with those derived from the ionic component of the K_d (ionic Ni concentration causing half saturation of Ni bioaccumulation in the whole organism). In general, there was relatively good agreement between the two sets of values (Table 5), suggesting that whole body bioaccumulation can serve as a surrogate for Ni binding to the theoretical 'biotic ligand' which causes toxicity. This further validates the modeling approach of the BLM because estimating the concentration of Ni theoretically bound to the biotic ligand using the ionic component of the LC50 value (the BLM approach) correlates with the observed Ni bound to the biotic ligand in the current study. In the cases where a comparison can be made between log K_{NiBL} values for bioaccumulation and toxicity within a species in different water chemistries, we observe two different trends. For *L. variegatus*, the log K_{NiBL} values for bioaccumulation differed by only 0.02 log units in comparison to those of toxicity which differed by 0.44. However in *D. pulex*, the log K_{NiBL} values for bioaccumulation varied by approximately the same in both bioaccumulation and toxicity (0.52 and 0.51 log units, respectively). To the best of our knowledge there are no other published comparisons of this nature.

Our values of log K_{NiBL} for toxicity ranged from 3.72 to 5.21, which are comparable to previously published log K_{NiBL} values based on toxicity. Specifically, the published log K_{NiBL} values for *D. magna* (4.0; Wu et al., 2003) and *D. pulex* (4.87; Kozlova et al., 2009) are similar to those of the current study for *D. pulex* of 5.21 and 4.70 in SW and HW, respectively (Table 5). The very low log K_{NiBL} values (2.56-2.87) derived from the bioaccumulation data of *C. riparius* are in accord with the very high tolerance of chironomids in which the LC50 lay beyond the range of testing, such that the log K_{NiBL} values for toxicity could not be determined (Table 5).

4.4. Is the disruption of Mg, Na and/or Ca homeostasis an indicator of acute toxic mechanism of waterborne Ni?

Pane et al. (2003b) suggested that the mechanisms of Ni toxicity were different for vertebrates and invertebrates. Specifically, in rainbow trout, Ni acts as a respiratory toxicant (Pane et al., 2003a; Pane and Wood, 2004), whereas in *D. magna*, Ni acts as an ionoregulatory toxicant, disrupting Mg homeostasis (Pane et al., 2003b). However,

Table 5
Log K_{NiBL} values based on bioaccumulation (K_d) and toxicity (LC50) values, in *Lymnaea stagnalis*, *Daphnia pulex*, *Lumbriculus variegatus* and *Chironomus riparius* in hard water (HW, nominally 140 mg L^{-1} as $CaCO_3$) and soft water (SW, nominally 40 mg L^{-1} as $CaCO_3$).

	Bioaccumulation			Toxicity		
	K_d value (μmol Ni L^{-1})	Ionic Ni component of K_d value (μmol Ni L^{-1})	logK_{NiBL} values	LC50 value (μmol Ni L^{-1})	Ionic Ni component of LC50 value (μmol Ni L^{-1})	logK_{NiBL} values
L. stagnalis						
HW	2.5 ± 1.1	1.9 ± 0.8	5.72	8.2	6.2	5.21
D. pulex						
SW	56.5 ± 26.9	47.2 ± 22.5	4.33	7.5	6.2	5.21
HW	18.4 ± 9.7	14.0 ± 7.3	4.85	26.0	19.9	4.70
L. variegatus						
SW	128.2 ± 85.8	99.6 ± 66.6	4.00	81.7	68.4	4.16
HW	133.9 ± 69.4	104.0 ± 53.7	3.98	246.8	192.2	3.72
C. riparius						
SW	3619.0 ± 1425.2	2750.4 ± 1097.6	2.56	N/A		
HW	1771.7 ± 1183.9	1346.5 ± 923.4	2.87			

to the best of our knowledge, the extent of the mechanistic data in freshwater invertebrates is on this species alone, and no other freshwater invertebrates have been studied in a mechanistic context. The 25–35% and 26% decreases in whole body Mg in *D. pulex* and *L. stagnalis*, respectively, provide strong evidence that this antagonistic relationship is not limited to *D. magna*. This is not to say that disruption of Mg homeostasis is the only mechanism of acute Ni toxicity in freshwater invertebrates, but rather to emphasize the inter-dependent relationship of Ni and Mg (Pyle and Couture, 2012). In addition, there is evidence of this relationship in the marine invertebrate, *L. vannamei*, where acute (96 h) exposures to waterborne Ni caused 60–70% decreases in whole body Mg in both brackish water (5 ppt) and sea water (25 ppt; Leonard et al., 2011). This relationship is well supported in the mammalian literature where increased Mg has the largest protective effect against Ni binding to DNA when compared with other divalents such as manganese, calcium, zinc and copper (Kasprzak et al., 1986). In addition, Mg has been shown to antagonize the genotoxicity, cell transformation and animal tumor induction actions of Ni compounds (Kasprzak et al., 1986, 1987; Conway et al., 1987).

A novel finding was the disruption of Na homeostasis by exposure to Ni in some of the species (Figs. 3B, 4B, 5B). Pane et al. (2003b) observed a slight but non-significant decrease in whole body Na in *D. magna* when assessing Ni toxicity at 65% of the acute LC50 value (12 μmol Ni L^{-1}). Ni and Na are generally not thought of as antagonistic ions, as they do not share the same chemical characteristics or even valence as seen with Ni and Mg (see above). The decline of whole body Na in *L. stagnalis* was substantial, with a 72% decrease in whole body Na at the highest exposure concentration of 7.8 μmol dissolved Ni L^{-1} (Fig. 3B). The calculated $EC50_{Na}$ for *L. stagnalis* was 6.1 μmol Ni L^{-1} which is 25% lower than the lethality endpoint (LC50) of 8.2 μmol Ni L^{-1}. This suggests that for *L. stagnalis*, physiological endpoints are more sensitive than lethality. A similar trend was observed for Cu in *L. stagnalis* by Ng et al. (2011), where the $EC50_{Na}$ for *L. stagnalis* was 0.17 μmol Cu L^{-1}, which was 58% lower than the 96-h LC50 (0.40 μmol Cu L^{-1}; Ng et al., 2011).

However, for the other organisms, lethality was a more sensitive endpoint than ion depletion. Nonetheless, the 10–30% decreases in whole body Na in SW and HW for *D. pulex* and *L. variegatus* (Figs. 4B, 5B) suggest that disruption of Na homeostasis may also contribute to Ni toxicity. Recently, a common mechanism of toxicity across all metals was suggested in the zebrafish, *Danio rerio*, where metal exposure led to the increased permeability and subsequent leakage of ions from the fish to the dilute external medium (Alsop and Wood, 2011). As Na has the largest ionic gradient from plasma to the surrounding water, there was the largest leakage of Na. Therefore, this unified mechanism of metal toxicity may span more taxa than just cyprinids as is suggested by the loss of whole body Na in three of the four freshwater invertebrates.

In contrast to the decreases in Mg and Na, there was no significant change in whole body (or soft tissue for *L. stagnalis*) Ca in any of the organisms. The absence of an effect of Ni on Ca is surprising as many have found Ca to be protective against Ni toxicity (Pane et al., 2005; Deleebeeck et al., 2007b; Kozlova et al., 2009). In addition, mollusks, such as *L. stagnalis*, have extremely high Ca requirements for proper shell formation (Greenaway, 1971). There are three possible explanations for this discrepancy (1) the acute nature of this study has not captured the ionoregulatory disruption of Ca or (2) although external Ca is protective against Ni toxicity, this does not correlate to internal Ca disruption as a mechanism of Ni toxicity or (3) in the case of the organisms tested in soft water, the short acclimation time of at least 13 days to soft water prior to metal exposure may not have allowed for the establishment of a calcium equilibrium and therefore confounded the results.

There was no depletion of whole body Na, Mg or Ca in *C. riparius* in either soft or hard water (Fig. 6B, C). This is most likely due to the extreme tolerance of this organism to metal exposure in general (Béchard et al., 2008; Gillis and Wood, 2008). *Chironomus* species are well known for their tolerance to metal toxicity and this tolerance may relate to their ability to maintain internal ion homeostasis upon exposure to metals (Gillis and Wood, 2008). In fact, disruption of Na and Ca homeostasis by cadmium was not seen until the mg Cd L^{-1} range (Gillis and Wood, 2008).

5. Overall conclusions

In summary, water hardness was protective against acute Ni toxicity. LC50 values in SW vs. HW were significantly different in *D. pulex* and *L. variegatus*; however, CBR50 values were less dependent on water chemistry. These data support one of the main advantages of the TRA where tissue concentrations are generally less variable than exposure concentrations with respect to toxicity. We suggest that with further study, TRA may be used as a tool for risk assessment in conjunction with the BLM. Whole body Ni bioaccumulation followed Michaelis–Menten kinetics in all organisms, with greater hardness tending to decrease B_{max} with no consistent effect on K_d. Across species, acute Ni LC50 values tended to increase with both K_d and B_{max} values. With respect to biotic ligand modeling, log K_{NiBL} values derived from Ni bioaccumulation correlated well with log K_{NiBL} values derived from toxicity testing. Both whole body Na and Mg levels were disturbed by acute Ni exposure, suggesting that disruption of ionoregulatory homeostasis is a mechanism of acute Ni toxicity.

Acknowledgements

We wish to thank Josias Grobler and Dr. Tania Ng (McMaster University, Hamilton, ON, Canada) for their technical assistance with the Daphnia and snails, and Dr. Kevin Brix for his advice. Dr. Chris Schlekat of NiPERA also provided very useful comments on the MS. This research was supported by a NSERC Strategic Grant (C.M. Wood and J.C. McGeer, P.I.s), Environment Canada, and Rio Tinto Alcan. Special thanks to Bill Adams of Rio Tinto for facilitating this research. CMW is supported by the Canada Research Chair Program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2013.03.008.

References

Adams, W.J., Blust, R., Borgmann, U., Brix, K.V., DeForest, D.K., Green, A.S., Meyer, J., McGeer, J.C., Paquin, P., Rainbow, P.S., Wood, C.M., 2011. Utility of tissue residue approach for predicting effects of metals on aquatic organisms. Integr. Environ. Assess. Manag. 7, 75–98.

Alsop, D., Wood, C.M., 2011. Metal uptake and acute toxicity in zebrafish: common mechanisms across multiple metals. Aquat. Toxicol. 105, 385–393.

Béchard, K.M., Gillis, P.L., Wood, C.M., 2008. Acute toxicity of waterborne Cd, Cu, Pb, Ni and Zn to first instar *Chironomus riparius* larvae. Arch. Environ. Contam. Toxicol. 54, 454–459.

Benedetti, M.F., Milne, C.J., Kinniburgh, D.G., Van Riemsdijk, W.H., Koopal, L.K., 1995. Metal ion binding to humic substances: application of the nonideal competitive absorption model. Environ. Sci. Technol. 29, 446–457.

Boisen, A.M.Z., Amstrup, J., Novak, I., Grosell, M., 2003. Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). Biochim. Biophys. Acta 1618, 207–218.

Borgmann, U., Neron, R., Norwood, W.P., 2001. Quantification of bioavailable nickel in sediments and toxic thresholds to *Hyalella azteca*. Environ. Pollut. 111, 189–198.

Brix, K.V., Esbaugh, A.J., Grosell, M., 2011. The toxicity and physiological effects of copper on the freshwater pulmonate snail, *Lymnaea stagnalis*. Comp. Biochem. Physiol. 154C, 261–267.

Brix, K.V., Esbaugh, A.J., Munley, K.M., Grosell, M., 2012. Investigations into the mechanisms of lead toxicity to the freshwater pulmonate snail, *Lymnaea stagnalis*. Aquat. Toxicol. 106-107, 147–156.

CCME (Canadian Council of Ministers of the Environment), 2007. Canadian water quality guidelines for the protection of aquatic life: summary table. Updated December, 2007 Canadian Environmental Quality Guidelines, 1999. Canadian Council of Ministers of the Environment, Winnipeg.

CCREM (Canadian Council of Resource and Environment Ministers), 1987. Canadian water quality guidelines. Prepared by the Task Force on Water Quality Guidelines.
Clifford, M., McGeer, J.M., 2009. Development of a biotic ligand model to predict the acute toxicity of cadmium to *Daphnia pulex*. Aquat. Toxicol. 98, 1–7.
Conway, K., Wang, X.W., Xu, L.S., Costa, M., 1987. Effects of magnesium on nickel-induced genotoxicity and cell transformations. Carcinogenesis 8, 1115–1121.
Dalesman, S., Lukowiak, K., 2010. Effect of acute exposure to low environmental calcium on respiration and locomotion in *Lymnaea stagnalis*. J. Exp. Biol. 213, 1471–1476.
De Schamphelaere, K.A.C., Koene, J.M., Heijerick, D.G., Janssen, C.R., 2008. Reduction of growth and haemolymph Ca levels in the freshwater snail *Lymnaea stagnalis* chronically exposed to cobalt. Ecotoxicol. Environ. Saf. 71, 65–70.
Deleebeeck, N.M.E., DeSchamphelaere, K.A.C., Janssen, C.R., 2007a. A bioavailability model predicting the toxicity of nickel to rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*) in synthetic and natural waters. Ecotoxicol. Environ. Saf. 67, 1–13.
Deleebeeck, N.M.E., Muyssen, B.T.A., DeLaender, F., Janssen, C.R., DeSchamphelaere, K.A.C., 2007b. Comparison of nickel toxicity to cladocerans in soft versus hard surface waters. Aquat. Toxicol. 84, 223–235.
Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical Basis. Environ. Toxicol. Chem. 20, 2383–2396.
ECB (European Chemicals Bureau), 2008. European Union Risk Assessment Report: Nickel, Ispra, Italy: European Commission, Joint Research Centre, European Chemicals Bureau. http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/nickelreport311.pdf.
Gillis, P.L., Wood, C.M., 2008. The effect of extreme waterborne cadmium exposure on the internal concentrations of cadmium, calcium and sodium in *Chironomus riparius* larvae. Ecotoxicol. Environ. Saf. 71, 56–64.
Greenaway, P., 1971. Calcium regulation in the freshwater mollusk, *Limnaea stagnalis* (L.) (Gastropoda: Pulmonata). I. The effects of internal and external calcium concentration. J. Exp. Biol. 54, 199–214.
Grosell, M., Brix, K.V., 2009. High net calcium uptake explains the hypersensitivity of the freshwater pulmonate snail, *Lymnaea stagnalis*, to chronic lead exposure. Aquat. Toxicol. 91, 302–311.
Grosell, M., Blanchard, J., Brix, K.V., Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquat. Toxicol. 84, 162–172.
Hoang, T.C., Tomasso, J.R., Klaine, S.J., 2004. Influence of water quality and age on nickel toxicity to fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 23, 86–92.
Kasprzak, K.S., Waalkes, M.P., Poirier, L.A., 1986. Effects of magnesium acetate on the toxicity of nickelous acetate in rats. Toxicology 42, 57–68.
Kasprzak, K.S., Waalkes, M.P., Poirier, L.A., 1987. Effects of essential divalent metals on carcinogenicity and metabolism of nickel and cadmium. Biol. Trace Elem. Res. 13, 253–273.
Keithly, J., Brooker, J.A., Deforest, D.K., Wu, B.K., Brix, K.V., 2004. Acute and chronic toxicity of nickel to a cladoceran (*Ceriodaphnia dubia*) and an amphipod (*Hyalella azteca*). Environ. Toxicol. Chem. 23, 691–696.
Kozlova, T., Wood, C.M., McGeer, J.C., 2009. The effect of water chemistry on the acute toxicity of nickel to the cladocerans *Daphnia pulex* and the development of a biotic ligand model. Aquat. Toxicol. 91, 221–228.
Leonard, E.M., Barcarolli, I., Silva, K.R., Wasielesky, W., Wood, C.M., Bianchini, A., 2011. The effects of salinity on acute and chronic Ni toxicity and bioaccumulation in two euryhaline crustaceans: *Litopenaeus vannamei* and *Excirolana armata*. Comp. Biochem. Physiol. C 154, 409–415.
Litchfield, J.T., Wilcoxon, F., 1949. A simplified method of evaluating dose–effect experiments. J. Pharmacol. Exp. Ther. 96, 99–113.
Luoma, S.N., Cain, D.J., Rainbow, P.S., 2009. Calibrating biomonitors to ecological disturbance: a new technique for explaining metal effects in natural waters. Integr. Environ. Assess. Manag. 6, 199–209.
Meyer, J.S., Santore, R.C., Bobitt, J.P., DeBrey, L.D., Boese, C.J., Paquin, P.R., Allen, H.E., Bergman, H.L., DiToro, D.M., 1999. Binding of nickel and copper to fish gills predicts toxicity when water hardness varies, not free-ion activity does not. Environ. Sci. Technol. 33, 913–916.
Miller, T.G., Mackay, W.C., 1980. The effects of hardness, alkalinity and pH of test water on the toxicity of copper to rainbow trout (*Salmo gairdneri*). Water Res. 14, 129–133.
Nebeker, A.V., Stinchfield, A., Savonen, C., Chapman, G.A., 1986. Effects of copper, nickel and zinc on three species of Oregon freshwater snails. Environ. Toxicol. Chem. 5, 807–811.
Ng, T.Y.T., Pais, N.M., Wood, C.M., 2011. Mechanisms of waterborne Cu toxicity to the pond snail *Lymnaea stagnalis*: physiology and Cu bioavailability. Ecotoxicol. Environ. Saf. 74, 1471–1479.
Ng, T.Y.T., Pais, N.M., Dhaliwal, T., Wood, C.M., 2012. Use of whole-body and subcellular residues of *Lumbriculus variegatus* to predict waterborne Cu toxicity in fresh water. Chemosphere 87, 1208–1214.
Niyogi, S., Wood, C.M., 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.
Pagenkopf, G.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH and water hardness. Environ. Sci. Technol. 17, 347–352.
Pane, E.F., Wood, C.M., 2004. Mechanistic analysis of acute, Ni-induced respiratory toxicity in the rainbow trout (*Oncorhynchus mykiss*): an exclusively branchial phenomenon. Aquat. Toxicol. 69, 11–24.
Pane, E.F., Richards, J.G., Wood, C.M., 2003a. Acute waterborne nickel toxicity in rainbow trout (*Oncorhynchus mykiss*) occurs by a respiratory rather than ionoregulatory mechanism. Aquat. Toxicol. 63, 65–82.
Pane, E.F., Smith, C., McGeer, J.C., Wood, C.M., 2003b. Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. Environ. Sci. Technol. 37, 4382–4389.
Pane, E.F., Bucking, C., Patel, M., Wood, C.M., 2005. Renal function in the freshwater rainbow trout (*Oncorhynchus mykiss*) following acute and prolonged exposure to waterborne nickel. Aquat. Toxicol. 72, 119–133.
Paquin, P.R., Santore, R.C., Wu, K.B., Kavvadas, C.D., Di Toro, D.M., 2000. The biotic ligand model: a model of the acute toxicity of metals to aquatic life. Environ. Sci. Pol. 3, 175–182.
Playle, R.C., 1998. Modeling metal interactions at fish gills. Sci. Total Environ. 219, 147–163.
Powlesland, C., George, J., 1986. Acute and chronic toxicity of nickel to larvae *Chironomus riparius* (Meigen). Environ. Pollut. 42, 47–64.
Pyle, G.G., Couture, P., 2012. Homeostasis and toxicology of essential metals. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology, Vol. 31A. Elsevier Inc., pp. 253–289.
Pyle, G.G., Swanson, S.M., Lehmkuhl, D.M., 2002. The influence of water hardness, pH, and suspended solids on nickel toxicity to larval fathead minnows (*Pimephales promelas*). Water Air Soil Pollut. 133, 215–226.
Redeker, E.S., Blust, R., 2004. Accumulation and toxicity of cadmium in aquatic oligochaetes *Tubifex tubifex*: a kinetic modeling approach. Environ. Sci. Technol. 38, 537–543.
Schlekat, C.E., Van Genderen, E.J., De Schamphelaere, K.A.C., Antunes, P.M.C., Rogevich, E.C., Stubblefield, W.A., 2010. Cross-species extrapolation of chronic nickel biotic ligand models. Sci. Total. Environ. 408, 6148–6157.
Schmidt, T.S., Clements, W.H., Zuellig, R.E., Mitchell, K.A., Church, S.E., Wanty, R.B., San Juan, C.A., Adams, M., Lamothe, P.J., 2011. Critical tissue residue approach linking accumulated metals in aquatic insects to population and community-level effects. Environ. Sci. Technol. 45, 7004–7010.
Shaw, J.R., Dempsey, T.D., Chen, C.Y., Hamilton, J.W., Folt, C.I., 2006. Comparative toxicity of cadmium, zinc and mixtures of cadmium and zinc to daphnids. Environ. Toxicol. Chem. 25, 182–189.
Stephan, C.E., Mount, D.I., Hansen, D.J., Gentile, J.H., Chapman, G.A., Brungs, W.A., 1985. Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses. U.S. EPA Office of Research Laboratories, Duluth, Minnesota.
U.S. Environmental Protection Agency, 1986. Water Quality Criteria for Nickel — 1986. EPA/440/5-86/004 Office of Water, Washington, DC.
U.S. Environmental Protection Agency, 1995. Updates: Water Quality Criteria Documents for the Protection of Aquatic Life in Ambient Water. EPA-820-B-96-001 Office of Water, Washington, DC.
U.S. Environmental Protection Agency, 2000. Update of Ambient Water Quality Criteria for Cadmium. U.S. Environmental Protection Agency Office of Water Office of Science and Technology, Washington, DC (EPA Contract No. 68-C-98-134).
Wallace, W.G., Lee, B.-G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). Mar. Ecol. Prog. Ser. 249, 183–197.
White, S.L., Rainbow, P.S., 1982. Regulation and accumulation of copper, zinc and cadmium by the shrimp, *Palaemon elegans*. Mar. Ecol. Prog. Ser. 8, 95–101.
Winner, R.W., Bossel, M.W., Farrel, M.P., 1980. Insect community structure as an index of heavy-metal pollution in lotic ecosystems. Can. J. Fish. Aquat. Sci. 37, 647–655.
Wood, C.M., 2001. Toxic responses of the gill. In: Schlenk, D.W., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, Volume 1 — Organs, pp. 1–89.
Wu, K.B., Paquin, P.R., Navab, V., Mathew, R., Santore, R.C., Di Toro, D.M., 2003. Development of a biotic ligand model for nickel: phase I. Water Environment Research Foundation (report 01-ECO-10T).