NICKEL BIOACCUMULATION AS A PREDICTOR OF TOXICITY

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By

ERIN M. LEONARD, B.SC., M.SC.

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AUTHOR: Erin Michelle Leonard,
                   B.Sc., M.Sc. (McMaster University, Canada)

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ABSTRACT

Recently, the focus of metal toxicity has shifted from concentrations within the aqueous environment to bioaccumulation within the organism. In this regard, the Biotic Ligand Model (BLM) relates the binding of metal at specific toxic sites ("ligands") to eventual toxicity, whereas the Tissue Residue Approach (TRA) relates metal burdens at whole body, tissue, or subcellular levels to eventual toxicity. However, much less is currently known regarding Ni in comparison to other metals. This thesis addresses this knowledge gap by evaluating the use of Ni bioaccumulation as a predictor of toxicity in a number of fish and invertebrate species; bioaccumulation endpoints examined included Michaelis-Menten uptake parameters (K_d, B_{max}), a BLM parameter (log K_{NIBL} values) and critical body residues (CBR50 values)

More sensitive species exhibited higher binding affinities and lower binding capacities for Ni. In invertebrates, a strong overall correlation was observed between log K_{NiBL} values for whole organism binding and acute toxicity to the extent that measurement of toxicity was an acceptable alternative to measurement of binding affinity, and vice versa. However, in two teleosts, the same comparison showed that a Ni BLM built on bioaccumulation would be more protective than one built on toxicity. The results further validated a central concept of the BLM - that short term metal bioaccumulation is predictive of longer term toxicity. Acute (96-h) Ni bioaccumulation predicted chronic (15 or 30-day) mortality in both salt and fresh water. In the latter, acute (96-h) sub-cellular bioaccumulations of Ni in either biologically active (BAM) or biologically inactive metal pools (BIM) of one fish species (round goby) were also predictive of 30-d mortality. However, goby were more sensitive to Ni bioaccumulation in the BAM versus BIM fraction.

This thesis advances the use of bioaccumulation as a predictor of Ni toxicity and may have implications for metal toxicity frameworks such as the BLM and TRA.

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TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 2

THE EFFECTS OF SALINITY ON ACUTE AND CHRONIC NI TOXICITY AND BIOACCUMULATION IN TWO EURYHALINE CRUSTACEANS: *LITOPENAEUS VANNAMEI AND EXCIROLANA ARMATA*

CHAPTER 3

ACUTE TOXICITY, CRITICAL BODY RESIDUES, MICHAELIS-MENTEN ANALYSIS OF BIOACCUMULATION, AND IONOREGULATORY DISTURBANCE IN RESPONSE TO WATERBORNE NICKEL IN FOUR INVERTEBRATE SPECIES: *CHIRONOMUS RIPARIUS*, *LYMNAEA STAGNALIS*, *LUMBRICULUS VARIEGATUS* AND *DAPHNIA PULEX*

CHAPTER 4

CRITICAL BODY RESIDUES, MICHAELIS-MENTEN ANALYSIS OF BIOACCUMULATION, LETHALITY, AND BEHAVIOUR AS ENDPOINTS OF WATERBORNE TOXICITY IN TWO TELEOSTS

CHAPTER 5

CHRONIC NICKEL BIOACCUMULATION AND SUB-CELLUAR FRACTIONATION IN TWO FRESHWATER TELEOSTS, THE ROUND GOBY AND THE RAINBOW TROUT: EXPOSED SIMULTANEOUSLY TO WATERBORNE AND DIETARY NICKEL

CHAPTER 6

GENERAL SUMMARY AND CONCLUSIONS

FIGURE LEGEND

TABLE LEGENDS

LIST OF ABBREVIATIONS

℃	Degree Celsius
μm	Micrometer
μmol/L	Micromoles per liter
ANOVA	Analysis of variance
BAM	Biologically Active Metal
BLM	Biotic Ligand Model
BIM	Biologically Inactive Metal
B_{max}	Binding capacity
Ca	Calcium
$CaCO_3$	Calcium carbonate
CBR50	Critical body residue at 50 % survival
CD	Cellular Debris
Cd	Cadmium
Cl^-	Chloride
Cm	Centimeter
Co	Cobalt
Cu	Copper
d	Day
DOC	Dissolved organic carbon
FIAM	Free ion activity model
g	Gram
GFAAS	Graphite furnace atomic absorption spectroscopy
GSIM	Gill surface interaction model

h	Hour
H_2O	Water
HCO_3^-	Bicarbonate
HDP	Heat denaturable proteins
HNO_3	Nitric acid
HW	Hard water
K	Potassium
K_d	Binding affinity
L	Liter
LA50	Lethal accumulation at 50 % survival
LC50	Lethal concentration at 50 % survival
Mg	Magnesium
min	Minute
ml	Milliliter
mM	Millimolar
MRG	Metal rich granules
MS-222	Tricaine methane sulphonate
MT	Metallothionein
n	Sample size
Na	Sodium
Ni	Nickel
ORG	Organelles
Pb	Lead
pH	Measure of acidity or basicity/$-\log[H^+]$
ppt	Parts per thousand
r^2	Coefficient of determination

Rho	Correlation coefficient
SEM	Standard error of the mean
SO_4^{2-}	Sulphate
SSD	Species sensitivity distribution
SW	Soft water
TRA	Tissue Residue Approach
WQC	Water Quality Criteria
wt	Weight
Zn	Zinc

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in the "sandwich thesis" format, as decided with my supervisory committee and approved by McMaster University. This thesis comprises six chapters. Chapter 1 provides a general introduction of the thesis research. Chapters 2 through 5 are manuscripts that have been published, accepted or ready to be submitted for publication in peer reviewed scientific journals. Chapter 6 discusses the findings of the experimental chapters, the results of which may inform strategies for the regulation of nickel in the aquatic environment.

CHAPTER 1:   **GENERAL INTRODUCTION**

CHAPTER 2:   **THE EFFECTS OF SALINITY ON ACUTE AND CHRONIC NICKEL TOXICITY AND BIOACCUMULATION IN TWO EURYHALINE CRUSTACEANS: *Litopenaeus vannamei* and *Excirolana armata*.**

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CHAPTER 3:   **ACUTE TOXICITY, CRITICAL BODY RESIUDES, MICHAELIS-MENTEN ANALYSIS OF BIOACCUMULATION, AND IONOREGULATORY DISTURBANCE IN RESPONSE TO WATERBORNE NICKEL IN FOUR INVERTEBRATE SPECIES:** *Chironomus riparius*, *Lymnaea stagnalis*, *Lumbriculus variegatus* **and** *Daphnia pulex*

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CHAPTER 5:   **CHRONIC NICKEL BIOACCUMULATION AND SUB-CELLULAR FRACTIONATION IN TWO FRESHWATER TELEOSTS, THE ROUND GOBY AND THE RAINBOW TROUT, EXPOSED SIMULTANEOUSLY TO WATERBORNE AND DIETBORNE NICKEL**

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CHAPTER 6:   **GENERAL SUMMARY AND CONCLUSIONS**

CHAPTER 1

GENERAL INTRODUCTION

Introduction to Nickel (Ni)

Ni is ubiquitous in the aquatic environment (Eisler, 1998). Ni (atomic number = 28, atomic weight = 58.71) is a transition metal which has 5 stable isotopes with valence states between 0 and +2, however, the dominant species of Ni found in the aqueous environment exists in the Ni^{2+} form as $(Ni(H_2O)_6)^{2+}$ (WHO, 1991, Chau and Kulikovsky-Cordeiro, 1995; Eisler, 1998). Ni enters aquatic environments via both natural and anthropogenic sources. In un-impacted freshwater, Ni levels usually range from 0.02-0.17 µmol Ni/L, but in some areas of Canada in the North West Territories, Ni levels are several orders of magnitude higher from natural volcanic processes (Chau and Kulikovsky-Cordeiro, 1995; Eisler, 1998). In impacted industrial areas, Ni levels reach as high as 34 µmol Ni/L because of mining, refining, smelting, alloy processing, scrap metal reprocessing, fossil fuel combustion and waste incineration (NAS, 1975; WHO, 1991; Chau and Kulikovsky-Cordeiro, 1995; Eisler, 1998).

Ni is generally regarded as an essential micronutrient (Phipps et al., 2002) where both deficiency and excess of Ni cause reduced survival consistent with other essential micronutrients such as copper (Cu) and zinc (Zn; Simkiss and Taylor, 1989). Ni essentiality has been proven in many terrestrial organisms including: rats, chickens, pigs, cows, sheep and goats (Phipps et al., 2002) as well as in many bacterial and plant species (Ragsdale, 1998). In aquatic organisms, Ni essentiality is suspected but not yet proven in fish as well as invertebrates (Eisler, 1998; Muyssen et al., 2004); nevertheless, Chowdhury et al. (2008) showed that Ni is homeostatically regulated in rainbow trout.

Physiology and toxicity of Ni

It is well documented that hardness cations, magnesium (Mg) and calcium (Ca) together or Ca alone have protective effects against Ni toxicity (Hoang et al., 2004; Meyer et al., 1999; Pyle et al., 2002; Deleebeeck et al., 2007a, 2008a; 2009 Kozlova et al., 2009). The reasons are two-fold: 1) both Ca and Mg are involved with the regulation of membrane permeability (Ebel and Günther, 1980; McWilliams, 1983; Hunn, 1985) and 2) Ca and Mg compete with Ni for negative binding sites on the surface of the gill (Schwartz and Playle, 2001).

The association between Mg and Ni is well supported in mammalian literature where these cations interact in some of the same enzyme, endocrine and

transport systems (Kenney and McCoy, 1992). Competitive studies have shown the ability for Ni to replace Mg as a cofactor for DNA polymerase (Kasprzak et al., 1986a). In the prokaryote, *Salmonella typhimonium*, Ni competitively inhibits three different types of Mg transporters (Snavely et al., 1991). These interactions are most likely due to the similar dehydrated ionic radii these two cations share (Ni^{2+} - 0.066 nm, Mg^{2+} - 0.069 nm; Weast, 1973). In more recent years, the correlation between Ni and Mg has been proven in aquatic vertebrates and invertebrates. In invertebrates, Ni inhibits unidirectional Mg uptake in the cladoceran, *Daphnia magna* (Pane et al., 2003b) and Mg protects against Ni toxicity in a closely related cladoceran, *D. pulex* (Kozlova et al., 2009). In vertebrates, Mg inhibits Ni uptake into the brush border membrane vesicles isolated from the kidneys of *Oncorhynchus mykiss* (Pane et al., 2006a, b). In the same species, elevated Mg reduced the unidirectional uptake of Ni across the gastrointestinal tract (Leonard et al., 2009). Again, in *O. mykiss*, Mg was more protective against Ni toxicity than Ca (Deleebeeck et al., 2007a).

Despite the correlation between Ni and the cations Mg and Ca, current knowledge suggests that the mechanisms of Ni toxicity are different for vertebrates and invertebrates (Pane et al., 2003b). In the teleost, specifically *O. mykiss*, Ni acts as a respiratory toxicant significantly increasing ventilation rate, ventilatory stroke volume and oxygen consumption, as well as causing the swelling of the gill lamellae (Pane et al. 2004), whereas in invertebrates, Ni appears to be an ionoregulatory toxicant by disrupting Mg homeostasis (Pane et al., 2003b).

Background on Water Quality Criteria (WQC)

Since the mid-1980s, WQC for freshwater shifted from being based on total or dissolved fractions of the metal (regardless of water chemistry) to being based on water hardness (in $CaCO_3$ equivalents) in a given body of water. It has become 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 and uptake 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). Current WQC in both Canada and the U.S. are derived from these same hardness-based equations which use logarithmic regressions based on the results of experimental tests, namely LC50 tests which determine the concentration of a metal in the water column which causes 50% mortality; U.S. EPA, 1986; CCREM, 1987, U.S. EPA, 1995; CCME, 2007).

However, in recent decades, much of the developing research has focused in greater detail on the influence of water chemistry on the bioavailability and incipient toxicity of metals. Studies in the early 1980s by Pagenkopf (1983) and

Morel (1983) proposed the Gill Surface Interaction Model (GSIM) and the Free Ion Activity Model (FIAM), respectively, which incorporated the theory that the ionic component of the metal is responsible for toxicity and that water chemistry parameters affect not only the speciation of the metal in the aquatic environment but also the binding sites on the organism (Pagenkopf, 1983; Morel, 1983). In these models, overall metal toxicity related to the degree of metal binding to physiologically important sites on the surface of the organism (Pagenkopf, 1983; Morel, 1983).

Additionally, a series of studies in the 1980s and 1990s demonstrated the inhibitory effect of metals on ion transport at the gills at concentrations close to the 96-h LC50 values (the lethal concentration of the metal causing 50% mortality; see Wood et al., 2001 for review). Specifically, copper (Cu; Laurén et al., 1987) and silver (Ag; Wood et al., 1996) blocked sodium (Na) channels or transporters while cadmium (Cd; Verbost et al., 1988), zinc (Zn; Hogstrand et al., 1994) and cobalt (Co; Comhaire et al., 1994) blocked calcium (Ca) channels or transporters; supporting the theory that metals act as ionoregulatory toxicants. Shortly following this, Playle et al. (1998) devised gill metal binding models which allowed for prediction of metal binding to physiologically important sites on the gills of fish during short term exposures.

This research set the foundation for the development of the biotic ligand model (BLM; Di Toro et al. 2001; Paquin et al., 2000; Niyogi and Wood, 2004). The BLM is a regulatory approach which incorporates the physiological impact of a metal on an organism with a clear understanding of the interactions between the metal and the organism (Niyogi and Wood, 2004). More specifically, the BLM is a computer based model which uses site-specific water chemistry parameters (e.g. cations, anions, dissolved organic carbon (DOC), alkalinity, pH, and hardness) in conjunction with geochemical constants and the binding characteristics of the biotic ligands to predict the toxicity of a metal. Affinity constants (log K) and binding site densities (B_{max}) of the biotic ligands on the gills are calculated using Langmuir adsorption or Scatchard analysis. These values are derived from the Michaelis-Menten equation: specific binding = B_{max} x [L]/ [L] + K_d; where [L] is the concentration of the ligand, B_{max} is the binding site density for the ligand, and K_d represents the inverse relationship to the binding affinity (Michaelis and Menten, 1913; Johnson and Goody, 2011). At a binding site on the biotic ligand, log K values for gill binding correlate to toxicity (i.e. higher log K values corresponded to higher toxic action of the metal and vice versa).

Most of the original work which went into the development of the BLM was conducted on fish, mainly juvenile rainbow trout and fathead minnows, based on their size and ease of use. This is somewhat ironic considering that invertebrates such as *Daphnia magna* are 5-10 x more sensitive to metal toxicity in comparison to fish and therefore regulatory application of these models has required adjustment of BLM sensitivity parameters to suit the most sensitive

species in the Species Sensitivity Distributions (SSD; Niyogi and Wood, 2004). This has been done by changing the LA50 parameter in the model, which is the amount of metal on the biotic ligand which is required to cause 50% mortality. The lower the LA50 in the model, the more sensitive is the target organism. Currently, there is still great need to expand the knowledge base on invertebrates. In fact, many of the current BLMs use the measurement of toxicity as an alternative to directly determining metal binding affinity at the biotic ligand (MacRae et al., 1999), which for invertebrates is the whole organism.

Currently, the BLM is the best modeling tool for predicting acute metal toxicity to aquatic organisms and versions of the BLM have been incorporated in both the European Union's Water Quality Standards for Cu, Zn and Ni (ECB, 2008) and the U.S. EPA's Ambient Water Quality Criteria for Cu (U.S. EPA, 2003). The BLM is also now applied in metal regulatory applications in Australia, New Zealand, and China, and is under consideration for use in Canada.

Historically, most WQC were based on knowledge of acute metal toxicity, 96-h or less, however, in the natural environment, chronic exposure is more characteristic. There have been several validated chronic BLMs for Cu (De Schamphelaere and Janssen, 2004; De Schamphelaere et al., 2006a), Zn (Heijerick et al., 2005), Pb (Mager et al., 2011) as well as Ni (discussed in the next section). Some jurisdictions, such as Canada and the European Union, only set WQC to protect species against chronic metal exposure (CCME, 2007; ECB, 2008). However, the U.S. EPA has both acute (Criteria Maximum Concentration; CMC) and chronic (Criteria Continuous Concentration – CCC) ambient WQC for protection of aquatic life.

The development of the BLM and the theories which supported its growth mark another shift in our understanding of metal toxicity and as a result, the frameworks setting WQC, where bioaccumulation either at the gills in fish or whole organism for invertebrates, is a key factor in predicting metal toxicity. The BLM can be considered a highly sophisticated bioaccumulation model that relies on only one source, waterborne exposure, and one site, the theoretical biotic ligand. However, expressing toxicological effect as a function of the total bioaccumulation of a metal by a whole organism or tissue has many advantages: it integrates all exposure routes (e.g. water column and food), incorporates changes in water chemistry over time, and assimilates the toxicokinetics of different species (U.S. EPA, 2007). Indeed, bioaccumulation of metals has been found to be better predictor of toxicity than exposure water concentrations (Borgmann et al., 1991; Borgmann and Norwood, 1997; Borgmann et al., 1998; Borgmann et al., 2004).

The Tissue Residue Approach (TRA) correlates tissue bioaccumulation with adverse biological effects (e.g. mortality) and in this manner bioaccumulation can be used to predict the toxicity within and across species

(Connolly, 1985; McCarty and McKay, 1993; Luoma et al., 2009; Borgmann et al., 2001; Adams et al., 2011; Schmidt et al., 2011). The TRA makes use of Critical Body Residues (CBR values), a similar concept to the LA50 values used in the BLM, calculated from the concentration of bioaccumulated Ni which correlates with mortality. The TRA has been used successfully with organic pollutants (McCarty and Mackay, 1993; Connell, 1995; Hickie et al., 1995), however, it is only in the early development phase for metals (Borgmann et al., 2001; Luoma et al., 2009; Adams et al., 2011; Schmidt et al., 2011). The TRA and BLM can be used independently or together as a tool for assessing the toxicity to aquatic organisms.

The concept of correlating Ni bioaccumulation with toxicity endpoints can be more complex. Organisms can cope with low levels of intracellular bioaccumulation by regulation of rate of uptake and loss (Rainbow, 2002) or via sequestration by metal-binding proteins, incorporation into inorganic crystalline concretions (metal-rich granules), and/or incorporation into lysosomal compartments, all of which render the metal less capable or incapable of exerting its toxic effects (Naimo, 1995; Mason and Jenkins, 1995; Wallace et al., 2003). However, toxicity can occur when the concentration exceeds a threshold value where metal detoxification strategies of the cell are at capacity and the excess metal binds to physiologically important sites causing disruption of function (Rainbow, 2002). These latter sites include small peptides and enzymes – classified as heat-denaturable proteins (HDP) or nuclei, mitochondria, lysosomes and endoplasmic reticulum – classified as organelles (ORG; Wallace et al., 2003).

Wallace et al. (2003) devised a protocol for separating intracellular compartments/fractions which can be further classified into biologically inactive metal (BIM: metal-rich granules (MRG) and metallothionein protein or metallothionein-like proteins (MT)) and biological active metal (BAM: organelles (ORG) and heat-denaturable proteins (HDP); Rainbow, 2002). Therefore, when metal levels exceed a threshold value in the BIM, there is metal spill-over into the BAM fractions which lead to deleterious effects. These effects can then be correlated to adverse biological effects such as failed recruitment or mortality. In this manner, the use of subcellular metal residues for the TRA may improve the effectiveness of the model for toxicity assessment (Adams et al., 2011).

Ni WQC in freshwater

In comparison to other metals such as Cu, Ag, and Zn, toxicity data for Ni are far less extensive. Meyer et al. (1999) was the first to demonstrate that the BLM concept could be extended to Ni by showing that the short-term (24-h) gill Ni binding was a constant predictor of acute Ni toxicity (96-h LC50 values) in fathead minnows across a wide range of water hardness levels. In general, log

K_{NiBL} values are relatively low, while B_{max} and LA50 values are relatively high in comparison to other metals (Meyer et al., 1999; Water Environment Research Foundation, 2002) which reflects the low toxicity of the metal. The BLM for Ni was re-calibrated with relatively good success to invertebrates by lowering the LA50 to account for the greater sensitivity of *D. magna* and *C. dubia* for Ni (Schubauer-Berigan, 1993; Chapman et al., 1980).

Currently, there are several validated acute BLMs for Ni in freshwater organisms (Meyer et al., 1999; Hoang et al., 2004; Deleebeeck et al., 2007b; Deleebeeck et al., 2008a; Kozlova et al., 2009), as well as several chronic freshwater BLMs for invertebrates (*C. dubia* – De Schamphelaere et al., 2006b and *D. magna* – Deleebeeck et al., 2008b) and one vertebrate (*O. mykiss* – Deleebeeck et al., 2007a).

As discussed above, the TRA is only in the early development phase for metals. The information available to date for Ni bioaccumulation is not as robust as other metals and therefore more data are required.

Ni WQC in salt water

Knowledge on the mechanisms of Ni toxicity in salt water is very limited in comparison to fresh water. Canada and the European Union do not currently have criteria established for salt water. Australia and New Zealand have saltwater WQC for Ni which are similar to their values for very hard freshwaters. The U.S. EPA criteria for Ni in salt water are lower than the respective fresh water values in low hardness. This is based on some marine invertebrates demonstrating adverse biological effects at low exposure concentrations (Eisler, 1998). Overall, much more information is required on the effects of Ni on marine organisms. This would help determine whether a marine Ni BLM or TRA could be implemented for the development of more reliable saltwater WQC.

Test organisms

The gastropod, *Lymnaea stagnalis*, is a common air-breathing freshwater pond snail which inhabits Europe, the Northern United States and parts of Asia. It is currently known as one of the most sensitive species to both acute and chronic metal toxicity (Schlekat et al., 2010; Brix et al., 2011, 2012) and has previously been neglected in terms of toxicological studies (Grosell and Brix, 2009). Recently, *L. stagnalis* has replaced *C. dubia* in the species sensitivity distribution (SSD) as the most sensitive species to chronic Ni toxicity. Recently, Schlekat et al. (2010) demonstrated that the chronic Ni BLM, originally developed for *C. dubia*, can be extrapolated to the more sensitive *L. stagnalis* in the context of the

European Nickel Aquatic Effects Database. Little is currently known regarding the effects of acute Ni toxicity on this organism and to the best of our knowledge, there is no published acute LC50 value for this species.

The cladoceran, *Daphnia pulex*, is also very sensitive to metal toxicity (Shaw et al., 2006; Kozlova et al., 2009; Clifford and McGeer, 2009). *D. pulex* are small translucent crustaceans commonly found in freshwater lakes and streams of North America, Europe and Australia. An acute Ni BLM was validated in *D. pulex* by Kozlova et al. (2009), where in soft water, the acute 48-h EC50 value was 46 µmol Ni/L (Kozlova et al., 2009).

Due to their metal sensitivity, *L. stagnalis* and *D. pulex* are currently driving most metal SSDs which in turn set WQC in many countries. In Canada, *Daphnia* species are used in the Environment Canada Biological Test Methods for invertebrates and in this manner, WQC are driven by this genus (Environment Canada, 1996).

Chironomus riparius, along with the similarly tolerant oligochaete, *Lumbriculus variegatus*, represent the other end of the sensitivity spectrum. Benthic surveys have found these organisms to be the predominant species in highly polluted aquatic environments (Winner et al., 1980).

C. riparius are dipterans found in ecologically diverse habitats of North America and Europe, and spend the majority of their life cycle as benthic larvae (Pinder, 1995). The larval stage is extremely resistant to Cd, Cu, Ni, Pb and Zn with LC50 values orders of magnitude above environmental guidelines (Béchard et al., 2008; Gillis and Wood, 2008). For Cd, chironomids represent the most tolerant aquatic organism in the SSD (U.S. EPA, 2000). Published 48-h LC50 values for *C. riparius* are 1350 µmol Ni/L and 2880 µmol Ni/L for 1st and 2nd instar larvae, respectively, further demonstrating this species tolerance to metals (Powlesland and George, 1986).

L. variegatus are benthic oligochaete worms distributed widely over North America. In comparison to other organisms, *Lumbriculus* are more resistant to Cu than *Tubifex tubifex*, *Hyalella azteca*, and *Gammarus pulex*, but more sensitive than *C. riparius* (Roman et al., 2007). Published Ni LC50 values for *L. variegatus* are 1277 µmol Ni/L (Schubauer-Berigan, 1993) and 250 µmol Ni/L (U.S. EPA, 1995).

The rainbow trout, *Oncorhynchus mykiss*, is a recreational sport fish that inhabits the pelagic zones of many lakes and rivers. Rainbow trout are one of the most sensitive fish species tested to date for Ni (Nebeker et al. 1985; U.S. EPA 1995; Brix et al., 2004), as well as one of the most well studied freshwater fish used in the diagnosis of metal toxicity (Pane et al., 2004). The 96-h LC50 in moderately hard water (140 mg/L as $CaCO_3$) determined by Pane et al. (2003b) was 260 µmol Ni/L. Similarly, Nebeker et al. (1985) estimated the 96-h LC50 in

soft water (33 mg/L) to be 170 μmol Ni/L, while Brix et al. (2004) reported the 96-h LC50 (water hardness of 91 mg/L) to be 354 μmol Ni/L.

The round goby, *Neogobius melanostomus*, is a benthic and philopatric species which relies on shelters and burial in loose substrate to avoid predators (Belanger and Corkum, 2003). This species is native to Eurasia, and was first discovered in the North American Great Lakes in the early 1990s. Since their establishment, the round goby has become a predominant species in many Great Lake regions, heavily disrupting the native ecosystem, food web structure and nutrient cycling (Walsh et al., 2007; Ng et al., 2008). They are of great concern due to their ability to transfer contaminants up the food chain (Morrison et al., 2000). To date, most research has focused on the transfer and biomagnification of PCBs from invertebrates to gobies to smallmouth bass and yellow perch (Belanger and Corkum, 2003; Kwon et al., 2006; Ng et al., 2008). In general, *N. melanostomus* is known as a pollution-tolerant species (Pinchuk et al. 2003); however, little is known of the gobies sensitivity to metals.

In response to the limited knowledge of Ni toxicity in marine environments, two euryhaline crustaceans, the white shrimp, *Litopenaeus vannamei,* and the cirolanid isopod, *Excirolana armata*, were used to assess the effects of salinity on Ni toxicity. These species live in tropical and subtropical areas of the world, in estuarine environments where salinity is not maintained at full seawater (~35 ppt) but can range from 0 to 35 ppt. *L. vannamei*, formerly *Penaeus vannamei*, inhabits estuaries, salt marshes and open oceans in the eastern Pacific, from Sonora in Mexico to northern Peru (Holthuis, 1980) and is currently heavily exploited for aquaculture in the U.S. *L. vannamei* is an osmoconformer at salinities between 20 and 30 ppt, but hyperosmoregulates at salinities <20 ppt (Castille and Lawrence, 1981; Lin et al., 2000; Sowers et al., 2006).

E. armata lives in intertidal zones of sandy beaches from Rio de Janeiro to Chubut Province, Argentina (Thompson and Sánchez de Bock, 2007), however cirolanid isopods of the genus *Excirolana* span the world and dominate the supra-littoral and intertidal zones in terms of biomass and abundance (Yannicelli et al., 2001).These crustaceans are known to tolerate a salinity range of 1.5-70 ppt for up to a month (Santos and Bianchini, 1998), however, little else is known regarding their osmoregulatory strategy. To the best of our knowledge, there is no published information on the acute or chronic toxicity of Ni to either of these species.

Goals of this thesis

Overall, as more information is gained regarding metal toxicity to aquatic species, more emphasis is shifting to the use of metal bioaccumulation to predict metal toxicity. However, in comparison to other metals, far less is known

regarding Ni bioaccumulation with respect to BLM and TRA approaches for both aquatic invertebrates and vertebrates. Therefore, with this background in mind, we sought to demonstrate the use of bioaccumulation as a predictor of various Ni toxicity endpoints. These include lethality, essential ion disruption, critical-body residues, BLM parameters, Michaelis-Menten parameters, behaviour and sub-cellular analysis.

Chapter summary

Chapter 2 examined the acute and chronic impact of Ni on two marine invertebrates: *Litopenaeus vannamei* and *Excirolana armata*, which demonstrated that salinity protects against acute Ni toxicity, where closer to the isosmotic point of the organism, less toxicity occurs. This salinity-dependent difference in acute Ni toxicity could not be explained by differences in Ni bioaccumulation. However, salinity does not affect chronic Ni toxicity, suggesting that water chemistry as well as the osmoregulatory strategy no longer influence toxicity. In *L. vannamei*, a critical tissue residue threshold value for Ni was determined suggesting the ability to develop a marine Ni TRA. In accord with the general BLM theory, chronic (15-day) mortality in both species could be predicted by acute (96-h) Ni bioaccumulation patterns.

Chapter 3 investigates the bioaccumulation and acute toxicity (48h or 96 h) of Ni in four freshwater invertebrate species: *Chironomus riparius*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Daphnia pulex*, in two water chemistries with hardness values of 40 (soft water) and 140 mg/L as $CaCO_3$ (hard water). These two hardness conditions are representative of the Canadian Shield and adjoining areas (e.g. the Great Lakes) and of the species which inhabit these waters. Principal conclusions were that water hardness is protective against acute Ni toxicity and Ni bioaccumulation, and that Critical Body Residues (CBR50 values) are less dependent on water chemistry (i.e. more consistent) than LC50 values both within and across species. In addition, the results further validated the modeling approach of the BLM by demonstrating that estimating the concentration of Ni theoretically bound to the biotic ligand using the ionic component of the LC50 value (the BLM approach) correlates well with the observed Ni bound to the biotic ligand.

Chapter 4 compared BLM, LC, and CBR parameters for various organs, as well as behavioural responses (spontaneous activity) in two freshwater teleosts: *Oncorhynchus mykiss* and *Neogobius melanostomus* (round goby), following an acute (96-h) Ni exposure. The data suggested that a BLM built on bioaccumulation would be more protective than one built on toxicity. Behavioural endpoints were more sensitive than LC50 values in round goby and in these two

species behavioural responses to waterborne Ni were more closely related to Ni bioaccumulation in the tissues than to waterborne Ni concentrations.

Chapter 5 assessed chronic Ni toxicity, bioaccumulation and sub-cellular distribution of Ni in both the gills and the gut of these same two freshwater teleosts at various sampling times. One of the overall advantages of this study was the integration of exposure routes (both waterborne and dietary) when assessing the TRA as a method for predicting toxicity to aquatic organisms. The use of acute subcellular bioaccumulation as the residue indicator of chronic Ni toxicity shows promise. Gill CBR50 values were determined for round goby as well as for BIM and BAM fractions.

Chapter 6 summarizes the key findings of the experimental chapters of this thesis and how these data may help provide more empirical evidence for the use of the BLM and TRA for Ni. Additionally, chapter 6 summarizes several bioaccumulation parameters ability to predict Ni toxicity in a number of fish and invertebrate species.

CHAPTER 2

THE EFFECTS OF SALINITY ON ACUTE AND CHRONIC NI TOXICITY AND BIOACCUMULATION IN TWO EURYHALINE CRUSTACEANS: *Litopenaeus vannamei and Excirolana armata*

ABSTRACT

We investigated the influence of salinity (5 ppt versus 25 ppt) on acute (96-h LC50) and chronic toxicity (15-30 day LC50) of Ni in two euryhaline crustaceans, the shrimp (*Litopenaeus vannamei*) and the isopod (*Excirolana armata*). 96-h LC50 values were 41 µmol/L and 362 µmol/L for *L. vannamei* and 278 µmol l^{-1} and >1000 µmol/L for *E. armata* at 5 ppt and 25 ppt, respectively. Speciation analysis demonstrated that complexation with anions such as SO_4^{2-}, HCO_3^- and Cl^- at 25 ppt had a negligible effect on reducing the free Ni^{2+} ion component in comparison to 5 ppt. The salinity-dependent differences in acute Ni toxicity could not be explained by differences in Ni bioaccumulation. Therefore, differences in physiology of the organisms at the two salinities may be the most likely factor contributing to differences in acute Ni toxicity. Chronic LC50 values (2.7 - 23.2 µmol/L) were similar in the two species, but salinity had no significant effect, indicating that water chemistry and osmoregulatory strategy do not influence chronic toxicity. However chronic (15-day) mortality in both species could be predicted by acute (96-h) Ni bioaccumulation patterns.

INTRODUCTION

Water chemistry can greatly alter the bioavailability of a metal and its consequent toxicity (Di Toro et al., 2001). In general, free ionic metal concentrations are reduced in high salinity environments in comparison to low salinity or freshwater because of the increased presence of complexing anions. For nickel (Ni) in sea water, the two of importance are SO_4^{2-} and Cl^- (Sadiq, 1989). In addition, at higher salinities, there should be increased competition with metal ions by protective cations such as Na^+, Mg^{2+} and Ca^{2+} for binding to sites at the biotic ligand (Paquin et al., 2000; Janssen et al., 2003). Therefore, salinity is thought to act protectively against the toxicity of many metals, including Ni (Eisler, 1998).

Currently, Ni toxicity data tend to be less extensive than for other divalent cationic metals (Eisler, 1998), especially in marine environments. Anthropogenic Ni entry into marine and estuarine waters can occur via several means, including coastal mining effluents, sewage disposal, atmospheric deposition and marine mining and drilling (Bryan, 1984). Ni levels can reach 0.04 µmol/L in the open ocean and as high as 1.4 µmol/L in coastal and estuarine environments where the confined space causes greater Ni accumulation (Boyden, 1975).

Euryhaline species are good model organisms to assess the influence of salinity on Ni toxicity, as the effects of salinity are two-fold: it affects the bioavailability of the metal, as well as the physiology of the organisms. Euryhaline crustaceans such as the white shrimp, *Litopenaeus vannamei,* and the cirolanid isopod, *Excirolana armata*, live in tropical and subtropical areas of the world, where in the estuarine environment salinity is not maintained at full seawater (~35 ppt) but can range from 0 to 35 ppt. Therefore, both species may be required to either osmoconform or to hyperosmoregulate at different salinities. In general, aquatic organisms, including crustaceans (Bianchini et al., 2003; Bianchini et al., 2004; Pedroso et al., 2007a,b; Pinho et al., 2007; Pinho and Bianchini, 2010), are more sensitive to metal stress when they are hyperosmoregulating, rather than when they are closer to their isosmotic point (Grosell et al., 2007).

L. vannamei, formerly *Penaeus vannamei*, inhabits estuaries, salt marshes and open oceans in the eastern Pacific, from Sonora in Mexico to northern Peru (Holthuis, 1980) and is currently heavily farmed in the U.S. *L. vannamei* is an osmoconformer at salinities between 20 and 30 ppt, but hyperosmoregulates at salinities <20 ppt (Castille and Lawrence, 1981; Lin et al., 2000; Sowers et al.,

2006). *E. armata* lives in intertidal zones of sandy beaches from Rio de Janeiro to Chubut Province, Argentina (Thompson and Sánchez de Bock, 2007), however cirolanid isopods of the genus *Excirolana* span the world and dominate the supra-littoral and intertidal zones in terms of biomass and abundance (Yannicelli et al., 2001). Little is known regarding the osmoregulatory strategy of *E. armata*; however these crustaceans are known to tolerate a salinity range of 1.5-70 ppt for up to a month (Santos and Bianchini, 1998) and a close relative, *Idotea chelipes* osmoconforms between 14 and 25 ppt and hyper-osmoregulates between 3 and 11 ppt (Lapucki and Normant, 2008).

Most marine invertebrates, including crustaceans maintain the ionic composition of their haemolymph isotonic to the surrounding water, with the exception of Mg which is often maintained at a concentration much lower than the surrounding water, as narrow changes in the extracellular concentration of Mg may cause very large increases in intracellular Mg (Morritt and Spicer, 1993). Excess intracellular Mg shuts off enzymes involved in the transfer of phosphate groups (e.g. ATPases, phosphatases and kinases). As well, at high levels, Mg has a narcotizing effect on crustaceans (Pantin, 1946), significantly decreasing heart rate and movement. It is well known that Ni commonly acts as an antagonist of Mg. This has been observed in many species including mammals, birds, bacteria, fungi (Eisler, 1998), and most recently trout, where Ni transport was facilitated by Mg transporters (Pane et al., 2003a).

Current models, such as the Biotic Ligand Model (BLM) (DiToro et al. 2001; Paquin et al., 2002; Niyogi and Wood, 2004), utilize site-specific water chemistry parameters to predict the bioavailability of the metal to an organism and its subsequent toxicity. The basis of the BLM is to understand the speciation of metals in different water chemistries, and using this knowledge, in conjunction with knowledge of the binding constants on target surfaces of organisms, to determine whether sufficient metal will bind on or in the organism to cause acute toxicity. Short term metal burdens are used to predict longer term toxicity. At present, there are several successful BLM's for Ni in freshwater organisms (Deleebeeck et al., 2007a; Kozlova et al., 2009), but none to our knowledge for Ni in organisms that live in estuarine and marine waters.

A complementary approach is the Tissue Residue Approach (TRA), which originated in organic contaminant toxicology (McElroy et al., 2010), and has worked well for predicting chronic Ni toxicity to at least one freshwater organism, the amphipod *Hyallela azteca* (Borgmann et al., 2001). The TRA predicts

toxicity as a function of metal levels within the organism, i.e. independent of water chemistries and potentially, length of exposure. In addition, the TRA has been proposed recently as a risk assessment tool by which metal burden in a resistant, metal-accumulating organism can be used to predict the fate of more sensitive organisms in a field situation (Adams et al., 2010). The TRA uses bioaccumulation as an endpoint, and from this, bioconcentration factors (BCF) can be calculated, as the ratio of the concentration of the metal in the organism to that in the water. Application of the BCF approach to metals, especially putative essential metals such as Ni, has proven controversial (Radenac et al., 2001; McGeer et al., 2003; DeForest et al., 2007).

With this background in mind, the present study aimed to identify the relationship between salinity and Ni toxicity (both acute and chronic) in two euryhaline crustaceans. More specifically, we asked the following questions: (i) What are the acute (96-h) and chronic LC50 (15 or 30-d) values for Ni at two different salinities (5 and 25 ppt) for the shrimp *L. vannamei* and the isopod *E. armata*? Both species would be expected to hyperosmoregulate at 5 ppt but osmoconform at 25 ppt. (ii) If differences in LC50 values occur at the two salinities, can they be correlated to Ni speciation within the water column, as would be expected by BLM theory? (iii) Can we relate Ni bioaccumulation patterns to acute and/or chronic toxicity? By BLM theory, early Ni bioaccumulation should be predictive of chronic mortality, whereas the TRA would identify a Critical Tissue Residue value indicative of chronic toxicity. (iv) What can bioconcentration factors (BCFs) tell us about Ni regulation? (v) How does acute and chronic Ni exposure affect essential ion homeostasis, with a particular focus on Mg, in these two very different organisms?

Our approach was to conduct simultaneous acute and chronic exposures, as tests performed in conjunction will help to explain toxic effects (Watts and Pascoe, 2000). This information can be used to inform current attempts to improve water quality guidelines for Ni in the marine and estuarine environment.

METHODS

Experimental organisms

Litopenaeus vannamei and Excirolana armata

Post-larvae *L. vannamei* (common name: white shrimp; individual weight of 0.2-2 g) were obtained from Universidade Federal do Rio Grande – FURG, Estação Marinha de Aquacultura, Instituto de Oceanografia (Rio Grande, RS, Brazil) and kept in static, aerated aquaria. *E. armata* (individual weight of 0.002-0.010 g) were collected in sand beaches at São José do Norte (RS, Brazil; water Ni concentration on day of collection = 0.09 µmol Ni/L) and acclimated to laboratory conditions (i.e. water chemistry, photoperiod and temperature) for one week prior to salinity change.

Salinity acclimation and experimental conditions

Organisms were held in filtered (0.45 µm mesh filter, Durapore PVDF Membrane, Millipore, São Paulo, SP, Brazil) water from Cassino Beach (Rio Grande, RS, Brazil; salinity $\cong$35 ppt) and acclimated to the appropriate salinity (either 5 or 25 ppt) by addition of distilled water to achieve a salinity decrease of 2 ppt per day above 10 ppt and by 1 ppt per day below 10 ppt. Organisms were kept at the appropriate salinity for a minimum of one week prior to experimentation. The measured ionic compositions of the exposure media at the two salinities were (in mmol/L) for 5 ppt: Na^+ (71), Cl^- (85), Ca^{2+} (1.8), K^+ (1.4), Mg^{2+} (5.8), SO_4^{2-} (4.2), Ni (<0.05 x 10^{-5}) with an alkalinity of 38 mg/L as $CaCO_3$, Dissolved Organic Carbon (DOC) of 0.13 mg/L and pH of 6.5. And at 25 ppt: Na^+ (350), Cl^- (392), Ca^{2+} (8.6), K^+ (6.8), Mg^{2+} (25), SO_4^{2-} (15), Ni (<0.04 x 10^{-5}) with an alkalinity of 240 mg/L as $CaCO_3$, DOC of 1.1 mg C/L and pH of 7.0. Temperature and photoperiod were fixed at 20ºC and 12L:12D, respectively. Prior to experiment, organisms were fed daily to satiation with a pellet diet composed of 10% crude protein, 38% carbohydrates, 7.5% fiber, 5% ash, 13% calcium and 3% phosphorus, containing a Ni concentration of 58 µmol/kg dry wt.

Experimental set-up

All glass aquaria were acid-washed in 10% HNO_3 and rinsed with distilled water before use. Exposure media were made from filtered Cassino Beach water

and diluted with distilled water as described above. Desired Ni concentrations were prepared from a primary Ni stock solution (25 mmol/L NiCl$_2$.6H$_2$O (Merck, Haar, Germany)) and allowed to reach equilibrium for 24 h in the exposure aquaria before addition of organisms. Aquaria were lightly aerated and water was changed daily with a similarly aged Ni solution and acid-washed glassware. Non-filtered and filtered (0.45 µm) samples (1.5 ml) from the different experimental media were collected daily for measurement of Ni, Na$^+$, Mg^{2+} and Ca^{2+} levels, and 50-ml water samples were collected every five days for measurement of pH and DOC. All samples were acidified to a final concentration of 1% HNO$_3$ (Suprapur, Merck, Haar, Germany) for sample preservation.

L. vannamei exposures were conducted in 2-L aquaria; water was aerated and changed daily. L. vannamei were fed daily with a pellet diet (composition as above) one hour prior to water change to avoid Ni complexation with the food. E. armata exposures were conducted in 250-ml beakers, with no aeration and daily water changes. E. armata were fed (same diet as above) twice per week, left to feed for 2 hours and then water was changed.

Acute (96-h) and chronic (15- or 30-d) LC50s

Acute (96-h) LC50 values for L. vannamei were determined using nominal Ni concentrations of 0, 1, 10, 33, 100, 333, 1000 µmol Ni/L. Chronic (30-d) LC50 values for L. vannamei were determined using nominal Ni concentrations of 0, 1, 3.3, 5.6, 10, 33 and 56 µmol Ni/L. For E. armata, 0, 1, 10, 33, 56, 100, 333, 1000 µmol Ni/L were the nominal Ni concentrations employed for acute and chronic LC50 values. Measured total and dissolved Ni concentration were within 10% of the nominal values and were used in all calculations. Each concentration was tested in triplicate to assess both acute and chronic toxicity. L. vannamei were fed daily and E. armata were fed twice per week during the exposure. At the end of the 30-d (L. vannamei) or 15-d (E. armata) chronic exposures, organisms were patted dry and whole-body wet weights were obtained. Organisms were digested at room temperature with 65% HNO$_3$ (Suprapur, Merck, Haar, Germany; 10 µl of HNO$_3$ per mg of tissue wet wt). Digestion acid was then diluted with 1% HNO$_3$ solution made with Suprapur HNO$_3$ and Milli-Q water for later Ni, Na, Mg and Ca analyses.

Time-dependent Ni bioaccumulation and essential ion homeostasis

With a different subset of organisms, time-dependent sampling of organisms occurred on days 0, 2, 4, 15, and 30 (only 15-d chronic exposure for *E. armata*). For acute *L. vannamei* and acute and chronic *E. armata* tests, the nominal Ni concentrations used were 0, 1, 10, 33, 56 (*E. armata* only), 100, 333 µmol Ni/L. Chronic *L. vannamei* bioaccumulation was determined using nominal Ni concentrations of 0, 1, 3.3, 5.6 and 10 µmol Ni/L. Five organisms were sampled to assess time-dependent Ni bioaccumulation and essential ion homeostasis at each point in time. Wet weights were documented and the digestion protocol was followed as outlined above at each of these sampling days.

Analytical techniques and calculations

Ni, Na, Mg and Ca concentrations in water samples and tissues (whole-body) were analyzed by Flame Atomic Absorption Spectroscopy (AAS, Avanta, 932 Plus – GBC, Hampshire, IL, U.S.A) against certified standards (Tritisol-Merck, Haar, Germany). The detection limit for measurement of Ni was 0.09 µg Ni/ml (1.5 µmol/L). Water pH and DOC were measured using a Digimed DM 20 pH meter and a total organic carbon analyzer (VCPN series; Shimadzu, Japan), respectively.

Calculations and statistical analyses

Data have been presented as means $\pm$ SEM (*n*), where *n* is the number of organisms. Measured total and dissolved Ni concentrations along with specific water chemistries at 5 and 25 ppt were used to estimate the free ionic nickel (Ni^{2+}) concentrations and Ni^{2+} activity using Visual MINTEQ software (ver. 3.0, beta, KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden). The active fraction is a measure of the effective activity of Ni in these water chemistries, which is determined by concentration and by interactions (attraction or repulsion) of other molecules in solution. The NICA-Donnan model (Benedetti et al., 1995) was used in the model to estimate the effect of DOC on Ni speciation. Acute and chronic LC50 values with 95% confidence intervals were calculated using ToxCalc – Toxicity Data Analysis Software v5.0.32 (Tidepool Scientific Software, McKinleyville, U.S.A.). When the 95% CI of two LC50 values overlapped, a simplified method (Litchfield and Wilcoxon, 1949) was

applied to determine if they were significantly different. Bioconcentration factors (BCFs) were routinely calculated as the control concentration (in non-exposed animals) subtracted from the concentration in Ni-exposed animals divided by the dissolved Ni concentration in the exposure medium. This calculation method was used instead of the McGeer et al. (2003) calculations due to high background levels in the organisms as well as the inability to measure low water Ni concentrations in the control exposures. 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 Fisher LSD Method. For all tests, statistical significance was allotted to differences with $p < 0.05$.

RESULTS

Acute and chronic LC50 values

Acute (96-h) LC50 values for *L. vannamei* were approximately 9-fold lower in 5 ppt versus 25 ppt (41 µmol/L and 362 µmol/L as dissolved Ni, respectively; Table 2.1). This trend remained consistent when considering the nominal, total, dissolved, free ion and active fractions of the metal (Table 2.1). In contrast, chronic (30-d) LC50 values were not significantly different between 5 ppt (2.7 µmol/L) and 25 ppt (7.6 µmol/L), and this consistency was again independent of the Ni fraction being examined (Table 2.1). Note that acute LC50 values were approximately 15- and 50-fold higher than chronic LC50 values at 5 and 25 ppt, respectively (Table 2.1).

A similar trend was observed in *E. armata* where acute (96-h) LC50 values were more than 3-fold lower in 5 ppt versus 25 ppt (278 µmol/L and >1000 µmol/L as dissolved Ni respectively (Table 2.2). Note that organisms were not exposed to Ni concentrations above 1000 µmol/L so as to retain environmental relevance. There was a significant difference between the acute (96-h) LC50 values at the two salinities when assessing the nominal, total, dissolved, free ion and active fractions of the metal. Chronic (15-d) LC50 values were not significantly different between 5 ppt (7.9 µmol/L) and 25 ppt (23 µmol/L), and this trend was independent of the Ni fraction being examined (Table 2.2). Acute LC50 values were 35- and >45-fold higher than chronic LC50 values at 5 and 25 ppt, respectively (Table 2.2).

Concentration-dependent Ni bioaccumulation

In *L. vannamei*, time-dependent Ni accumulation at days 0, 2, 4, 15 and 30 demonstrated that by day 2 bioaccumulation values were representative of chronic values in the surviving shrimp (Fig. 2.1A, B). In contrast, Ni bioaccumulation continued to increase over time in *E. armata* (Fig. 2.1C, D).

Acute Ni bioaccumulation (96-h whole body burdens in surviving animals) in both *L. vannamei* and *E. armata* demonstrated that as the concentration of Ni increased in the exposure medium, more Ni accumulated within the organism; however, there were no significant differences in Ni body burden within an exposure concentration between the two salinities (Fig. 2.2A-D). In contrast, for chronic exposures, in surviving *L. vannamei* at day 30, Ni bioaccumulation was independent of exposure concentration and salinity, averaging approximately 0.1 µmol/g wet wt, not significantly different from the mean value in non-exposed control animals (Fig. 2.2B). For *E. armata* the chronic bioaccumulation pattern was different than in *L. vannamei*, inasmuch as chronic Ni burden was not constant but rather followed a similar pattern as acute, where an increase in the exposure medium concentration resulted in an increase in Ni bioaccumulation (Fig. 2.2D). However, again, Ni burden did not differ within an exposure concentration at the two salinities (Fig. 2.2D).

Early Ni body burdens (at 96-h) were predictive of chronic (15-d) mortality in both species, as Ni bioaccumulation gradually increased with increasing mortality (Fig. 2.3A, B). It should be noted that 15-d mortality was used for *L. vannamei* in Fig. 2.3A, as a more appropriate comparison between the two organisms.

However, in *L. vannamei*, when 96-h body burdens were plotted against 30-d mortality, whole body Ni had not exceeded background Ni levels (~0.1 µmol Ni/g wet wt) in the organisms which survived until day 30. Above this background level, the organisms could not regulate or tolerate Ni on a chronic basis, resulting in high chronic (30–d) mortality (Fig. 2.3C).

Bioconcentration factors for Ni

Acute bioconcentration factors (BCFs) for *L. vannamei* were 40 – 60 l/kg wet wt. at 1 µmol/L, but at 10 µmol/L significantly decreased by 85% and 80% in 5 ppt and 25 ppt, respectively (Fig. 2.4A). Between 10 and 333 µmol/L, acute

BCFs did not change significantly, averaging approximately 5 l/kg wet wt., and were independent of salinity (Fig. 2.4A). Chronic BCFs in surviving animals at 30-d exhibited similar patterns to acute BCFs, and were again independent of salinity (Fig. 2.4B).

A similar trend was observed for *E. armata* where acute and chronic BCFs decreased with increasing exposure concentrations (Fig. 2.4, D). At 1 µmol/L acute BCFs were 153 and 336 l/kg wet wt. for 5 and 25 ppt, respectively and decreased by 75 and 94% by 10 µmol/L. In 5 ppt, the acute BCF continued to significantly decrease in the 33 µmol/L exposure. Above this concentration, only slight variations in BCFs were observed (Fig. 2.4C). Chronic BCFs showed a similar, less marked pattern across exposure concentrations (Fig. 2.4D). At 5 ppt, the BCF significantly decreased from 70 l/kg wet wt. at an exposure concentration of 1 µmol/L to 35 l/kg wet wt. at 33 µmol/L. At 25 ppt, the BCF significantly decreased by 75% from concentrations of 1 µmol/L to 10 µmol/L, and remained constant over higher exposure concentrations (Fig. 2.4D).

Essential ions homeostasis

Average Na values were 180 and 243 µmol/g wet wt for *L. vannamei* in 5 and 25 ppt, respectively (Fig. 2.5A, B). At 25 ppt, whole body Na levels were maintained across all exposure days and Ni concentrations. However, at the lower salinity (5 ppt), *L. vannamei* Na levels significantly decreased by 70% from 170 µmol/g wet wt in the controls to 50 µmol/g wet wt in the 333 µmol Ni/L treatment on day 4 (Fig. 2.5A). Mg whole body concentrations averaged 7.1 and 12 µmol/g wet wt at 5 and 25 ppt respectively (Fig. 2.5C, D). However, at 5 ppt, whole body Mg significantly decreased by 60% in the 333 µmol Ni l^{-1} treatment on day 4 (Fig. 2.5C), and significantly decreased by 65% in the 10 µmol Ni/L treatment on day 15 (Fig. 2.5C). At 25 ppt on day 4, there was a significant 70% decrease in whole body Mg in the 10 µmol Ni/L exposure (Fig. 2.5D). All significant decreases in Mg could be directly correlated with significant increases in whole body Ni (Fig. 2.6A, B). There were no significant differences in whole body Ca concentrations with increased Ni concentrations at either 5 or 25 ppt for acute or chronic exposures in *L. vannamei* (Fig. 2.5E, F). Whole body Ca concentrations averaged 91 and 138 µmol/g wet wt for *L. vannamei* in 5 and 25 ppt, respectively (Fig. 2.5E, F).

There were no significant differences in whole body Na (558 µmol/g wet wt at 5 ppt and 1260 µmol/g wet wt at 25 ppt), Mg (28 µmol/g wet wt at 5 ppt and 30 µmol g^{-1} wet wt at 25 ppt) or Ca concentrations (282 µmol g^{-1} wet wt at 5 ppt and 865 µmol/g wet wt at 25 ppt) with increased Ni concentrations for *E. armata* (data not shown).

DISCUSSION

Acute and chronic LC50 values for L. vannamei and E. armata

Currently, little is known regarding the impacts of Ni on marine organisms. Therefore, acute and chronic toxicity of Ni in two euryhaline crustaceans: the white shrimp, *Litopenaeus vannamei*, and the cirolanid isopod, *Excirolana armata*, at two salinities (5 and 25 ppt) were assessed. Salinity acts protectively against acute Ni toxicity with 96-h LC50 values of 41 µmol/L and 362 µmol/L for *L. vannamei* and 278 µmol/L and >1000 µmol/L for *E. armata* at 5 ppt and 25 ppt, respectively. The present results add to the growing body of evidence that euryhaline marine invertebrates are more sensitive to acute metal toxicity at low salinity than at high salinity (Jones, 1975; McLusky et al., 1986; Verslycke et al., 2003; Table 2.1 and 2.2). However, to the best of our knowledge this is the first study to illustrate this for Ni as well as to examine both acute and chronic endpoints for Ni.

Speciation analysis demonstrated that the greater presence of anions such as SO_4^{2-}, HCO_3^- and Cl^- (as predicted by Visual MINTEQ software, ver. 3.0) at 25 ppt vs. 5 ppt only slightly reduced the free Ni^{2+} ion component (free Ni^{2+} = 78 % in 25 ppt and 89 % in 5 ppt) (Tables 2.1 and 2.2), therefore complexation had only a minor influence on the difference between acute LC50 values at the two salinities. The more pertinent influence can be attributed to greater cation competition (by higher concentrations of Na^+, Ca^{2+}, and particularly Mg^{2+} - see below) and/or differences in the physiology of the organisms at the two salinities. However, recent evidence for the marine teleost, *Fundulus heteroclitus*, exposed to varying salinity, confirmed that speciation and competition analysis fail to account for the pattern of Cu sensitivity in seawater, whereas there was a significant correlation between Na gradients and acute toxicity (Grosell et al., 2007). Similar findings were also reported for the euryhaline copepod *Acartia tonsa* exposed to Ag (Pedroso et al., 2007a) and Cu (Pinho and Bianchini, 2010). This supports the concept that disruption of osmoregulation (i.e. interference with

Na homeostasis) is the key contributor in acute Cu and Ag toxicity. Therefore in the current study, differences in physiology of the organisms at the two salinities may be the most likely factor contributing to differences in acute Ni toxicity, as both organisms are at their isosmotic point at 25 ppt and are consequently osmoconforming, leading to more metal tolerance (Jones, 1975; Henry and Cameron, 1982; Sprague, 1985; Grosell et al., 2007; Pedroso et al., 2007b; Pinho et al., 2007). At lower salinities such as 5 ppt, these organisms must hyper-osmoregulate by active transport processes (Lin et al., 2000; Grosell et al., 2007; Pedroso et al., 2007b; Pinho et al., 2007), and are in turn more sensitive (in terms of LC50 values) to metal toxicity.

However, after 30-d exposure, salinity no longer significantly affected Ni toxicity (Tables 2.1 and 2.2) with 30-d LC50 values of 2.7 μmol/L and 7.6 μmol/L (n.s.) for *L. vannamei* (Table 2.1) and 15-d LC50 values of 7.9 μmol/L and 23 μmol/L (n.s.) for *E. armata* at 5 ppt and 25 ppt, respectively (Table 2.2). This suggests that water chemistry as well as the osmoregulatory strategy of *L. vannamei* and *E. armata* at the two different salinities does not influence chronic toxicity, which contradicts what would be expected by the BLM theory. However, it should be noted that if there had been more intermediate Ni exposure concentrations, yielding LC50 values with more precise confidence intervals, there might have been a significant difference in chronic LC50 values between the two salinities. Nevertheless, the much lower chronic LC50 values together with the difference in acute and chronic ratios between the two salinities suggest that acute and chronic toxicity mechanisms may be different. This has been shown in the freshwater crustacean, *Daphnia magna*, where the acute mechanism of Ni toxicity is Mg antagonism, however, chronically Ni impaired respiratory function (Pane et al., 2003b). In addition, both Heijerick et al., (2005) and De Schamphelaere and Janssen (2004) showed, through the development of chronic BLMs for Zn and Cu, respectively, in the same species, *D. magna*, that competitive ions are much less important than in acute BLMs. Therefore, the role that competitive ions play in acute toxicity is greatly reduced in chronic toxicity (Schwartz and Vigneault, 2007).

Currently there is a growing body of work related to metal toxicity in euryhaline crustaceans (Bianchini et al., 2003; Bianchini et al., 2004; Pedroso et al., 2007a,b; Pinho et al., 2007; Pinho and Bianchini, 2010; Martins et al., 2011). Acute LC50 data for other shrimp species include *Metapenaeus ensis* at 150 μmol/L (48-h, 30-34 ppt; Wong et al., 1993), which are similar to the marine copepod, *Tisbe holothuriae* (Verriopoulous and Dimas, 1988) and the larval crab,

Cancer magister (34 ppt; Martin et al., 1981). These values correlate well with acute LC50 values of both *L. vannamei* and *E. armata* in the current study.

In contrast, much less information is available for metal toxicity in *E. armata*. Bianchini et al. (2003, 2004) have reported a significant protective effect of salinity against acute waterborne Cu toxicity in *E. armata* in the range of 1.5-30 ppt, in either the absence or the presence of food in the water. Also, they showed that the mechanism of acute Cu toxicity in isopods exposed to the metal in low salinity (1.5 ppt) is associated with a whole body disturbance in essential ions (Na^+ and Cl^-) homeostasis. In addition, previous studies on other isopods have also shown that external stressors such as lower salinity led to higher sensitivity to Zn, Cd and Cu, phenomena which appeared to be related to osmotic disturbance in the haemolymph of these organisms due to changes in gill structure (Jones, 1975). However, to our knowledge, there have been no acute or chronic Ni exposures to isopods at varying salinity. In general, there is a requirement for more chronic metal analysis on marine and estuarine crustaceans.

The Criterion Maximum Concentration (CMC – i.e. acute criterion) and Criterion Continuous Concentration (CCC – i.e. chronic criterion) in seawater outlined by the U.S. EPA (2004) for dissolved Ni are 1.3 µmo/L and 0.14 µmol/L, respectively. The Canadian Water Quality Guideline (chronic) by the CCME (2007) is 0.4 µmol Ni/L. Therefore, *L. vannamei* and *E. armata* would be protected in both the U.S. and Canada against acute and chronic Ni toxicity (Table 2.1), though the margin of protection is considerably lower at 5 ppt than at 25 ppt.

Concentration-dependent Ni bioaccumulation and essential ion homeostasis

The significant difference between acute LC50 values at 5 and 25 ppt for *L. vannamei* (Table 2.1) cannot be explained by a difference in tissue Ni burden (Fig. 2.2A). However, one possible mechanism of acute toxicity may be the perturbation of the homeostasis of the essential ion, Mg (Figs. 2.5C, D and 2.6). All significant decreases in whole body Mg can be correlated with significant increases in whole body Ni concentration at both salinities (Fig. 2.6). Historically, Mg is recognized as a specific Ni antagonist in physiological as well as toxicological studies (Pane et al., 2003a,b). More specifically, Ni has been implicated as a competitive inhibitor of Mg uptake via three different types of Mg transporters in the prokaryote, *Salmonella typhimurium* (Snavely et al., 1991). Additionally, in *D. magna*, long term Ni exposure led to a reduction in whole

body Mg and unidirectional Mg uptake (Pane et al., 2003a,b). In trout, *Oncorhynchus mykiss*, elevated Mg reduced the unidirectional uptake of Ni across the gastrointestinal tract (Leonard et al., 2009). Therefore, this interplay between Ni and Mg may be a phenomenon not only in freshwater organisms, but may also hold true in marine animals.

In penaeid shrimps, Mg is maintained within narrow limits (Geddes, 1975), where slight increases in extracellular Mg can drastically affect intracellular Mg causing a narcotizing effect (Pantin, 1946), while slight decreases can interfere with the stability of RNA and DNA, as well as interfere with enzymes involved in transferring phosphate groups (Morritt and Spicer, 1993). Therefore Ni may be replacing Mg at binding sites, destabilizing RNA and DNA and disrupting enzyme function causing acute Ni toxicity.

In 5 ppt, the acute 70 % depletion in whole body Mg correlated with chronic mortality in the 333 µmol/L treatment for *L. vannamei* (Fig. 2.5C). However, in 25 ppt, Mg homeostasis was restored in *L. vannamei*, where this same 70 % reduction (10 µmol/L treatment) in whole body Mg returned to control values by the day 15 sampling time (Fig. 2.5D). Similar trends have been observed in rainbow trout with metals such as Cu, Zn, and Cd, where over a chronic exposure there is an initial temporary loss of Na and Ca followed by a recovery to control values, however, there was a longer lag of recovery for Ca in comparison to Na (McGeer et al., 2000). The authors suggested that this recovery was aided by dietary uptake of the ions and/or linked to changes in gill morphology (McGeer et al., 2000). The same ion recovery is observed in freshwater rainbow trout, where Ni interferes acutely with Mg reabsorption by the kidney, thereby increasing Mg loss through the urine. This apparent antagonism disappears by chronic endpoints (Pane et al., 2005). As well, Brix et al. (2004) showed that over a chronic (85-d) exposure that Ni was not acting as an ionoregulatory toxicant, however, there was some initial loss of Na, Ca and Mg.

In addition to acute Mg loss, there was significant Na depletion observed acutely at the lower salinity. This interplay between Na and Ni is not as readily observed as the interactions between Mg and Ca with Ni, however, Brix et al. (2004) observed a 15% reduction of plasma Na of rainbow trout at 24-h in freshwater, suggesting a trend for acute Na loss in freshwater or low salinity environments.

There was no significant decline in whole-body ions observed in *E. armata* in response to Ni exposure. In light of the higher tolerance of *E. armata*,

there may be an alternative mechanism of toxicity at the higher metal concentrations.

As metal accumulation was measured in the whole body of both crustaceans, it cannot be determined what fraction is associated with soft tissue vs. the exoskeleton. It is well known that a fraction (anywhere from 19-97 %) of the accumulated metal can be associated with the chitinous exoskeleton (Munger and Hare, 1997; Keteles and Fleeger, 2001). The fate of the metal bound to the exoskeleton is uncertain, however, it is hypothesized that the metal is either adsorbed to the surface or is bound to the inner exoskeletal matrix after being incorporated into the organism (White and Rainbow, 1982). In the latter, shedding of the exoskeleton during ecdysis may contribute to elimination of the metal (Reinfelder and Fisher, 1994) or if the metal is bound to the procuticle, some fraction may be mobilized and released into the tissues along with Ca prior to ecdysis (Keteles and Flegger, 2001). In a different shrimp species, *Palaemonetes pugio*, 36-52 % of the whole body Cu, Zn and Cd was associated with the exoskeleton, however, the fate of metals following ecdysis varied depending on the metal (Keteles and Fleeger, 2001). Future studies would benefit from determining the contribution of binding to the exoskeleton on Ni toxicity and bioaccumulation.

Contrary to current evidence that more metal-sensitive organisms tend to be smaller (see Bianchini et al., 2002 and Grosell et al., 2002 for reviews of species sensitivity distribution to Ag and Cu); the present study shows the opposite, where the larger organism, *L. vannamei*, is more sensitive to acute Ni toxicity. However, chronically, there is no significant difference between the LC50 values for the two species.

Freshwater studies have shown no correlation between Ni exposure and disruption of whole-body Na homeostasis (Pane et al., 2003a,b), however, variations in whole-body Ca are characteristic of Ni exposures. Classically, Ni interacts antagonistically with Ca and is an effective blocker of several different types of Ca channels (McFarlane and Gilly, 1998; Todorovic and Lingle, 1998; Lee et al., 1999). In addition, Ca has been shown to protect against waterborne Ni toxicity in rainbow trout, *Oncorhynchus mykiss*, fathead minnows, *Pimephales promelas* (Meyer et al., 1999; Deleebeeck et al., 2007a) and the water flea, *Daphnia pulex* (Kozlova et al., 2009). However, this interaction between Ni and Ca was not observed in these two marine invertebrates. This may be explained by the active calcium metabolism required in all crustaceans to undergo continuous

moulting of the exoskeleton to allow for growth (Greenaway, 1985). Therefore, regeneration of Ca throughout the moulting cycle of these crustaceans may allow for rapid Ca replenishment (Huner et al., 1979), allowing internal Ca levels to be maintained.

Chronically Ni bioaccumulation appears to be well regulated in the shrimp at relatively low Ni exposure levels in that there are only slight variations in Ni whole body burden among the surviving shrimp chronically exposed to different concentrations of Ni (control, 1, 3 and 5.6 µmol/L for 5 ppt and control, 1, 3 and 10 µmol/L for 25 ppt; Fig. 2.2B as well as Fig. 2.1A, B). Total Ni concentrations were in good agreement with previous studies on decapod crustaceans (Mwangi, and Alikhan, 1993; Khan and Nugegoda, 2003), including *L. vannamei* (Nunez-Nogueira and Botello, 2007), which shows the capacity to regulate Ni. This Ni body burden of ~0.1 µmol g^{-1} wet wt. for chronic exposures (Fig. 2.2C) may define a Critical Tissue Residue Threshold for Ni in this species (Adams et al., 2010). Elevations above this level resulted in high mortality over 30 days.

For *L. vannamei* and *E. armata*, chronic (15-d) mortality was well predicted by early (96-h) bioaccumulation patterns (Fig. 2.3A, B). This suggests that there is a relationship between bioaccumulation and toxicity which could be used in the development of a BLM approach to predict chronic Ni toxicity in marine and estuarine environments.

Bioconcentration factors for Ni

Acute and chronic bioconcentration factors for *L. vannamei* and *E. armata* followed a similar pattern where there was an inverse relationship between BCFs and water concentrations, which suggests that at lower environmentally relevant exposures, Ni is actively being taken up by the organism to meet metabolic needs (Philips and Rainbow, 1989), but at higher toxic concentrations, internal Ni concentrations are being regulated and therefore do not increase in proportion to waterborne Ni levels (Figs. 2.3A-D). Meta-analyses have shown that an inverse relationship is characteristic of all metals (McGeer et al., 2003; DeForest et al., 2007). To the best of our knowledge this is the first study to examine BCF values in a marine environment for Ni. Ni BCF patterns in these two euryhaline crustaceans are following the general pattern for all metals.

Conclusions

Salinity acts protectively against acute Ni toxicity, where closer to the isosmotic point of the organism, less toxicity occurs. This is in contrast to organisms at lower salinities which are hyperosmoregulating and are more sensitive to Ni toxicity. Salinity-dependent differences in acute Ni toxicity cannot be explained by Ni bioaccumulation; however, an acute mechanism of toxicity appears to be interference with Mg and Na regulation in *L. vannamei*. Salinity does not affect chronic Ni toxicity, suggesting that water chemistry as well as the osmoregulatory strategy of *L. vannamei* no longer influence toxicity. All fractions of the metal appear to equally predict metal bioavailability. Chronic LC50's are much lower than acute LC50's and Ni bioaccumulation patterns are very different, indicating that acute and chronic toxicity mechanisms may differ. Assessing chronic (30-d) Ni bioaccumulation patterns, *L. vannamei* regulates Ni at ~ 0.1 µmol/g wet wt. Body burdens above this concentration eventually lead to death, therefore defining a Critical Tissue Residue threshold value for Ni in this species for marine and estuarine environments. Chronic mortality in *E. armata* can be predicted by early (96-h) bioaccumulation patterns. BCF values for Ni in these two euryhaline species acclimated to 5 and 25 ppt follow the general inverse pattern with concentration as described for virtually all metals by McGeer et al. (2003). Overall, in these two euryhaline species, salinity is protective against acute Ni toxicity; however chronic Ni toxicity is independent of salinity. These species are protected by the current U.S. EPA and Canadian Water Quality Criteria/Guidelines.

ACKNOWLEDGEMENTS

Table 2.1
Acute (96-h) and chronic (30-d) LC50 values (µmol/L) for waterborne Ni toxicity
in *Litopenaeus vannamei* (*n*=7 per treatment, with 3 replicates) acclimated to 5
and 25 ppt. The values are expressed as different fractions of Ni (nominal =
desired exposure concentration, total and dissolved fractions = measured by
FAAS, total fraction passed through 0.45 µm filter to obtain dissolved fraction,
and ionic and active fractions = speciated by Visual MINTEQ). The active
fraction is a measure of the effective activity of Ni in these water chemistries,
which is determined by concentration and by interactions (attraction or repulsion)
of other molecules in solution. 95% confidence intervals are presented in brackets
in µmol/L. * Indicates a significant difference in LC50 values between the two
salinities.

Salinity	Nominal	Total	Dissolved	Ionic	Active
Acute (96-h)					
5 ppt	42	41	41	37	14
	(25-69)	(25-67)	(25-66)	(23-61)	(8.6.1-23)
25 ppt	385*	363*	362*	295*	85*
	(270-540)	(255-510)	(254-508)	(207-415)	(59-119)
Chronic (30-d)					
5 ppt	2.9	2.9	2.7	2.5	0.9
	(1.8-4.1)	(1.9-4.2)	(1.7-4.0)	(1.6-3.7)	(0.6-1.4)
25 ppt	8.3	8.0	7.6	6.1	1.8
	(2.3-24)	(1.9-25)	(1.7-25)	(1.4-19)	(0.5-4.9)

Table 2.2
Acute (96-h) and chronic (15-d) LC50 values (µmol/L) for waterborne Ni toxicity
in *Excirolana armata* (*n*=5 per treatment, with 3 replicates) acclimated to 5 and
25 ppt. The values are expressed as different fractions of Ni. The values are
expressed as different fractions of Ni (nominal = desired exposure concentration,
total and dissolved fractions = measured by FAAS, total fraction passed through
0.45 µm filter to obtain dissolved fraction, and ionic and active fractions =
speciated by Visual MINTEQ). The active fraction is a measure of the effective
activity of Ni in these water chemistries, which is determined by concentration
and by interactions (attraction or repulsion) of other molecules in solution. 95%
confidence intervals are presented in brackets in µmol/L. * Indicates a significant
difference in LC50 values between the two salinities. Note organisms were not
exposed to Ni concentrations above 1000 µmol/L due to lack of environmental
relevance.

Salinity	Nominal	Total	Dissolved	Ionic	Active
Acute (96-h)					
5 ppt	290	279	278	257	95
	(214-357)	(206-342)	(205-342)	(190-316)	(69-118)
25 ppt	>1000*	>1000*	>1000*	>1000*	>1000*
Chronic (30-d)					
5 ppt	8.4	8.0	7.9	7.4	2.8
	(2.1-17)	(2.0-17)	(2.0-16)	(1.8-15)	(0.7-5.8)
25 ppt	24	24	23	19	5.4
	(12-45)	(12-44)	(11-42)	(9.0-34)	(2.6-9.9)

Fig 2.1
Time dependent Ni bioaccumulation over a range of exposure concentrations from 1 to 333 µmol Ni/L on days 0, 2, 4, 15 and 30 at (A, C) 5 ppt and (B, D) 25 ppt for *Litopenaeus vannamei* (A, B) and *Excirolana armata* (C, D). Values are means ± S.E.M.; n = 5-16 per treatment.

A *L. vannamei* - 5 ppt

B *L. vannamei* - 25 ppt

C *E. armata* - 5 ppt

D *E. armata* - 25 ppt
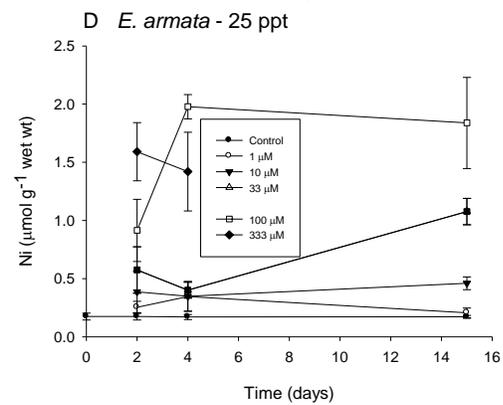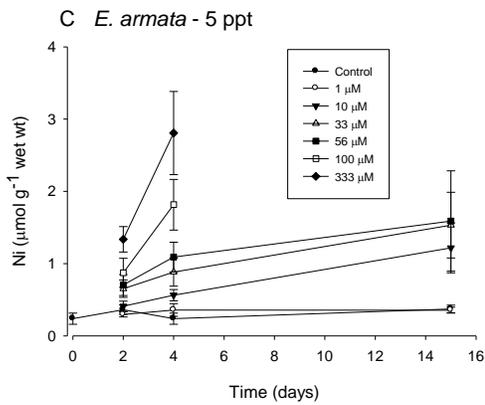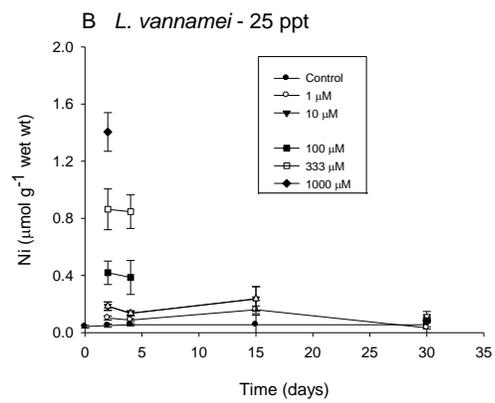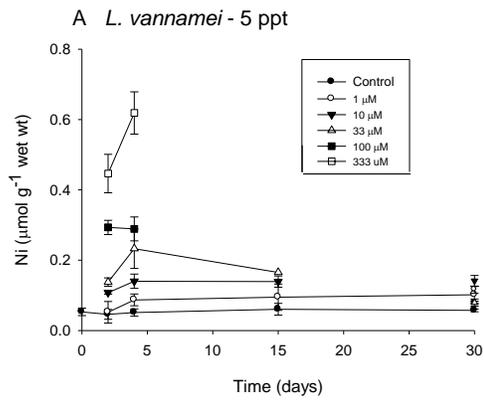
Fig 2.2
Concentration-dependent Ni uptake over a range of exposure concentrations from 1 to 333 µmol Ni/L at 96-h (A) and day 30 (B) for *Litopenaeus vannamei* and at 96-h (C) and day 15 (D) for *Excirolana armata*. Values are means ± S.E.M.; $n = 5$ per treatment (acute) and $n = 7$-16 per treatment (chronic). There was no significant difference in Ni bioaccumulation within the same exposure concentration between the two salinities.

A *L. vannamei* - 96-h

B *L. vannamei* - day 30

C *E. armata* - 96-h

D *E. armata* - day 15
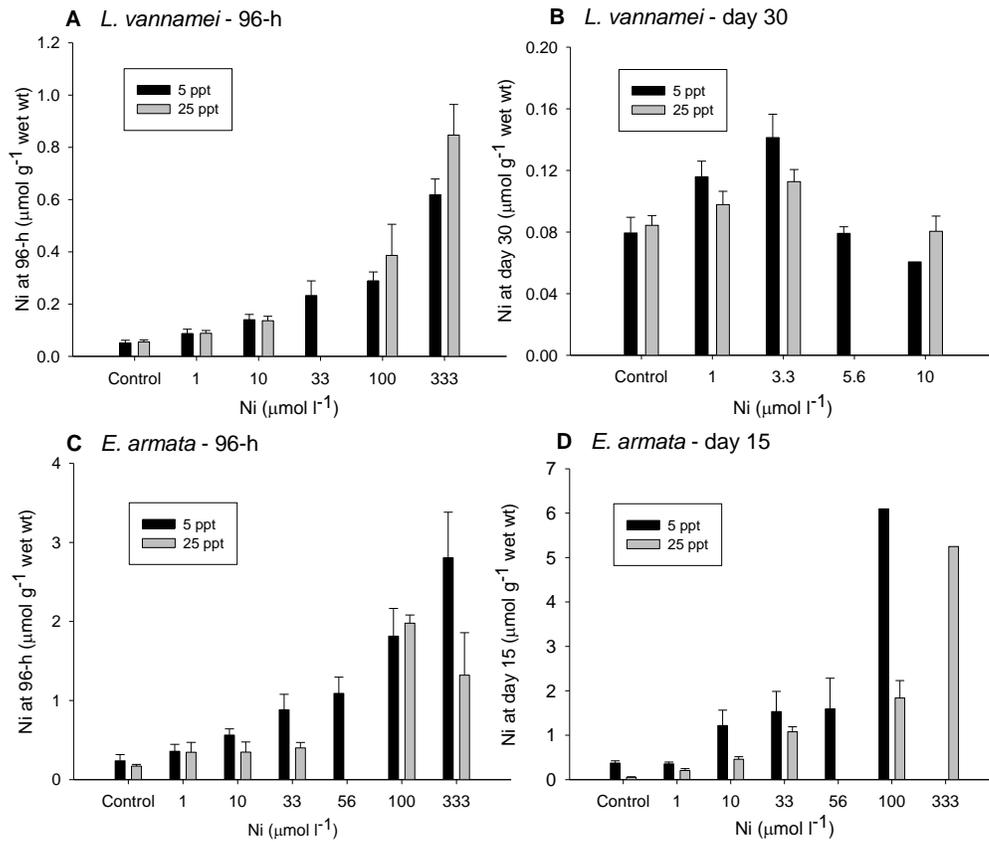
Fig 2.3
Early (96-h) Ni bioaccumulation plotted against chronic (15 or 30-d) mortality at both salinities. (A) *Litopenaeus vannamei* – 15-d mortality (B) *Excirolana armata* – 15-d mortality and (C) *L. vannamei* – 30-d mortality. Values are means ± S.E.M.; n = 5-16 per treatment.
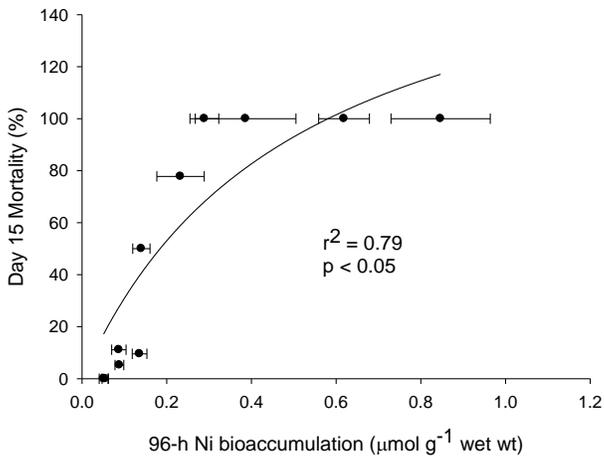
A *L. vannamei*

B *E. armata*
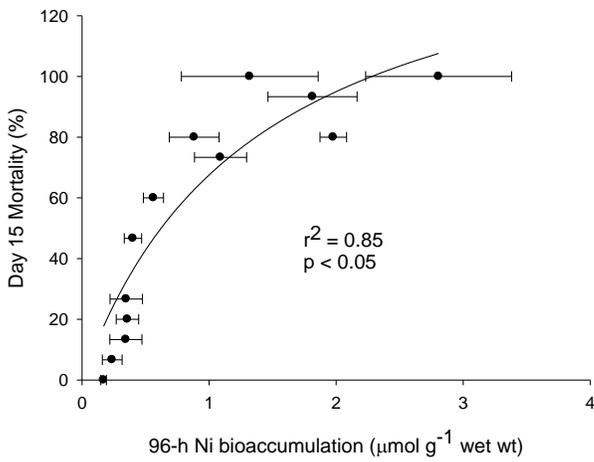
C *L. vannamei*
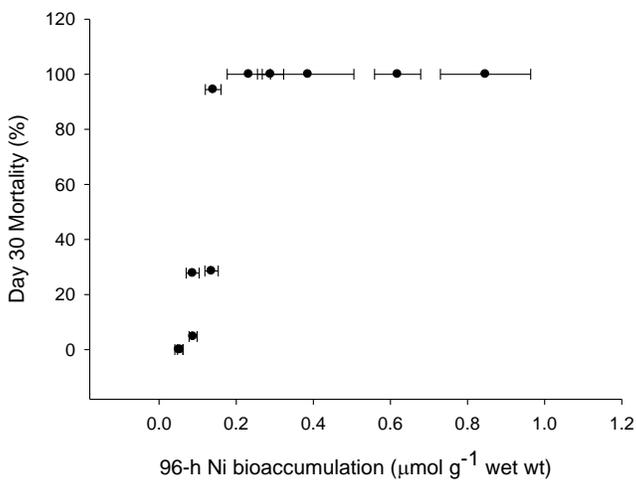
Fig 2.4
Bioconcentration factor (BCF = concentration of Ni in organism expressed in μmol/kg wet wt divided by the Ni concentration in exposure medium expressed in μmol/L) in *Litopenaeus vannamei* at 96-h (A) and day 30 (B) and in *Excirolana armata* at 96-h (C) and day 15 (D). Values are means ± S.E.M.; n = 5-16 per treatment. Different letters denote significant differences in bioconcentration factors.
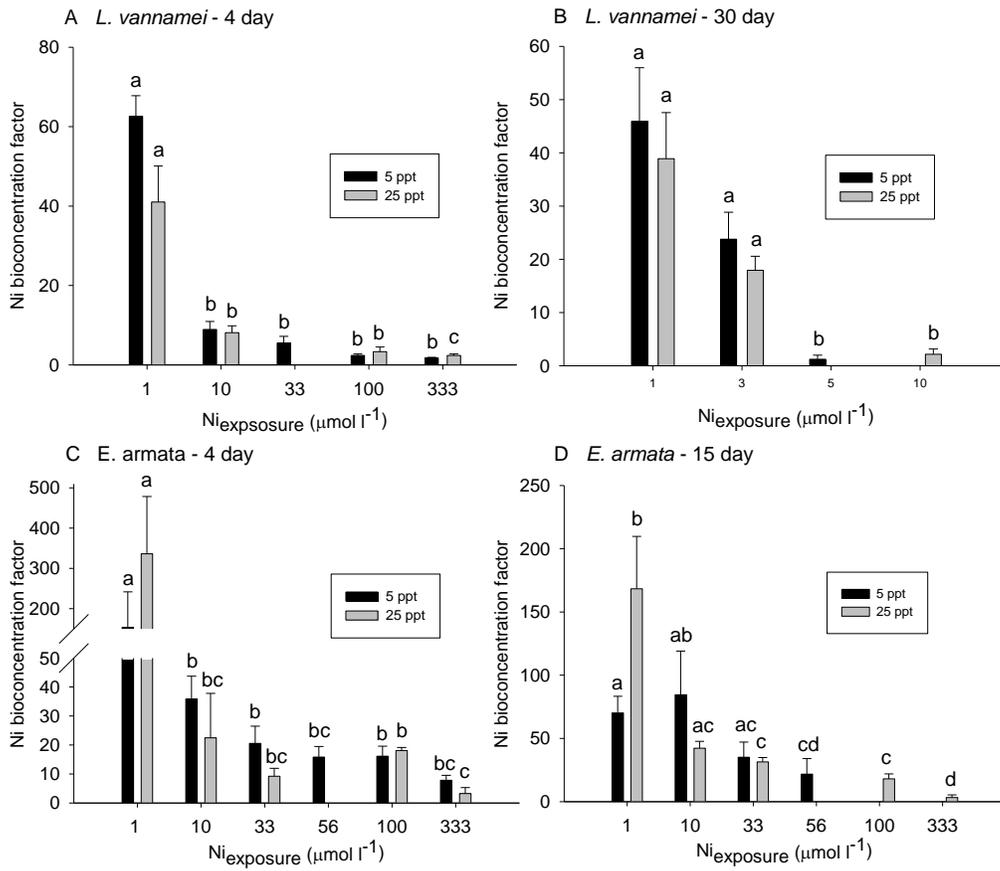
Fig 2.5
Whole body ion concentrations in 5 ppt (A, C, E) and 25 ppt (B, D, F) for
Litopenaeus vannamei over chronic (30-d) Ni exposure. Organisms were sampled
on days 0, 2, 4, 15 and 30. Values are means ± S.E.M.; $n = 5$ per treatment.
Different letters denote significant differences in whole body ions within a
sampling day. No letters denote no significant differences in whole body ions
within a sampling day (B, E and F).
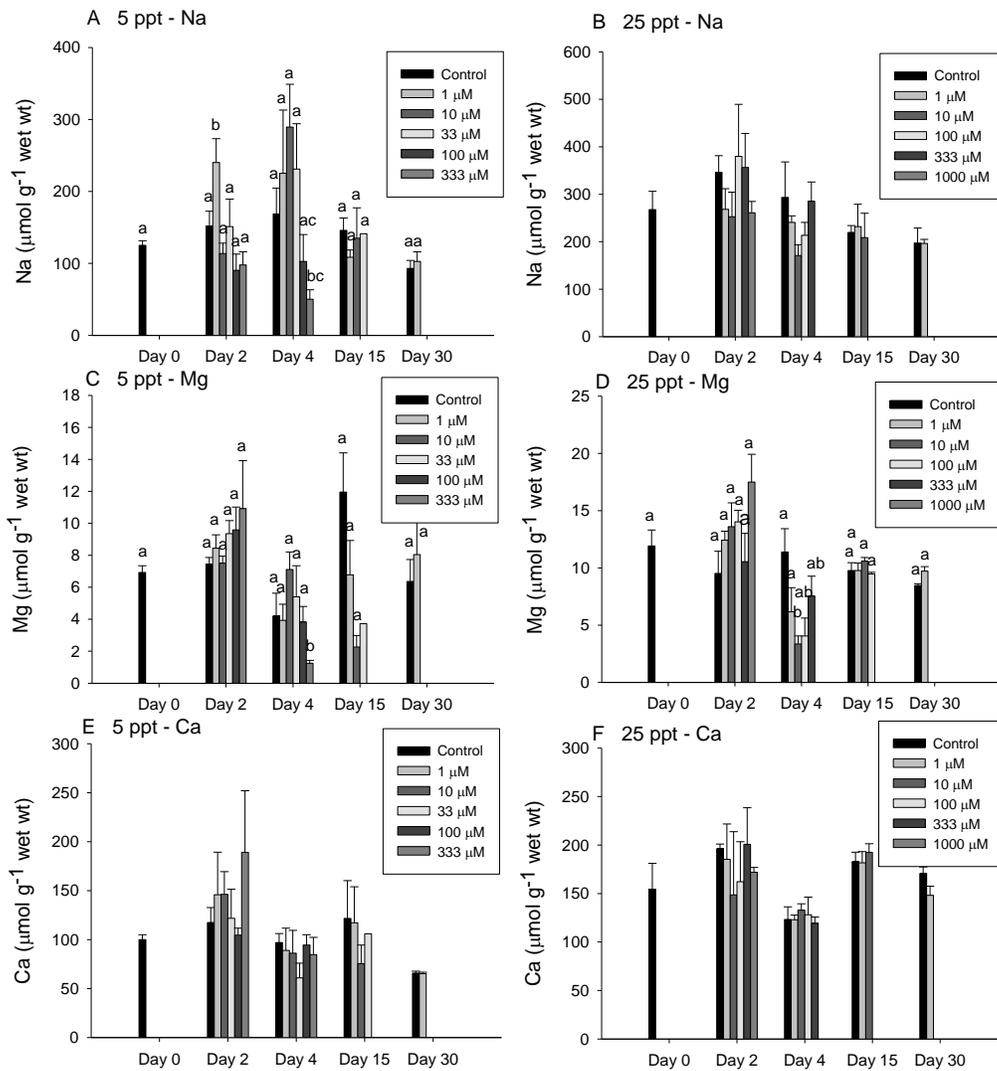
Fig 2.6
Whole body Ni and Mg plotted against Ni exposure concentrations on day 4 at 5 ppt (A) and 25 ppt (B) of Ni exposure in *Litopenaeus vannamei*. No significant difference in whole body Mg was found in *Excirolana armata*. Values are means ± S.E.M.; $n = 5$ per treatment. Different letters denote significant differences in whole body ion concentration. Capital letters denote Ni and lower case letters denote Mg.

A *L. vannamei* - 96-h - 5 ppt
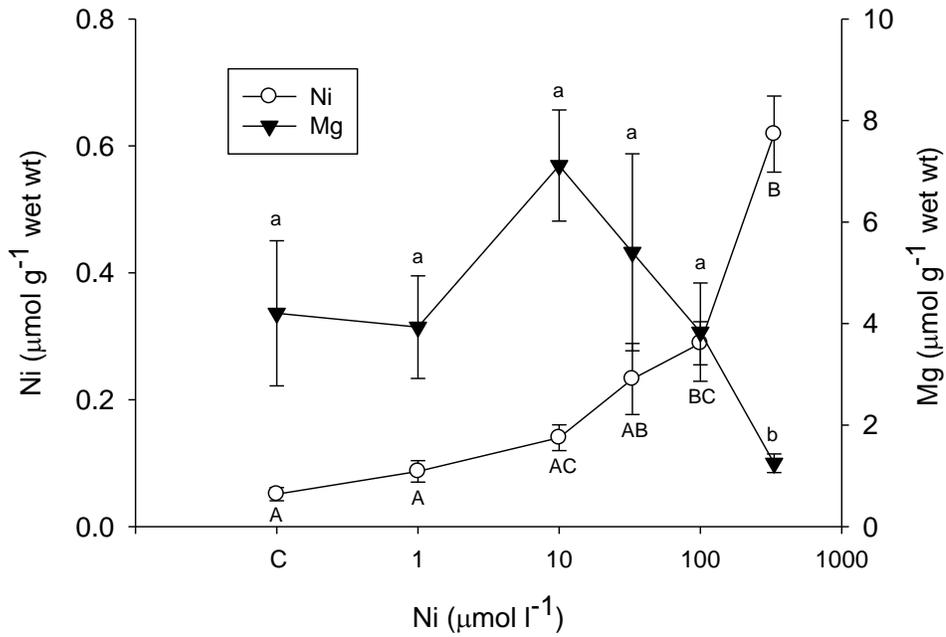
B *L. vannamei* - 96-h - 25 ppt
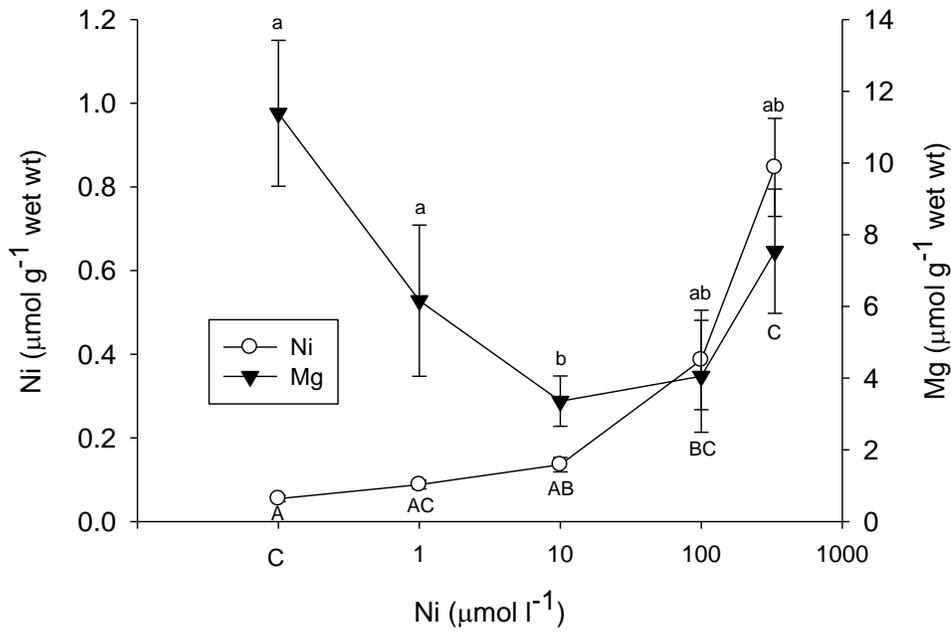
CHAPTER 3

ACUTE TOXICITY, CRITICAL BODY RESIUDES, MICHAELIS-MENTEN ANALYSIS OF BIOACCUMULATION, AND IONOREGULATORY DISTURBANCE IN RESPONSE TO WATERBORNE NICKEL IN FOUR INVERTEBRATE SPECIES:
Chironomus riparius*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Daphnia pulex

ABSTRACT

We investigated the bioaccumulation and acute toxicity (48h or 96 h) of Ni in four freshwater invertebrate species in two waters with hardness of 40 (soft water) and 140 mg/L 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 x 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 *Lymnaea 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.

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, 1995). 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; DiToro et al. 2001; Paquin et al., 2000; Niyogi and Wood, 2004), which utilize site-specific water chemistry parameters 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 as CaCO$_3$) and hard water (operationally defined as 140 mg/L 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.

METHODS

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 tapwater with an ionic composition of (in mmol/L) 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 and 95 mg/L as $CaCO_3$ equivalents, respectively, and dissolved organic carbon (DOC) was 2.3 mg/L. 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.

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.

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. *L. variegatus* were fed the same commercial ground flake food as the described above, once every two weeks.

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.

Lymnaea stagnalis

 L. stagnalis were kept in 5-L aquaria on a flow-through system of dechlorinated tapwater with a turnover rate of 2 L/day. 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 as $CaCO_3$ equivalents as this organism is a calciphile and exhibits reduced growth and increased mortality at environmental hardness below 50 mg/L as $CaCO_3$ (Dalesman and Lukowiak, 2010).

Soft Water Acclimation

 For all organisms tested in 40 mg/L as $CaCO_3$ (soft water), water hardness was gradually decreased by ~10 mg/L per day (as $CaCO_3$ equivalents) using reverse osmosis (RO) water. All organisms were left in the final soft water (approximately 40 mg/L 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.

Toxicity and Bioaccumulation Tests

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℃, *D. pulex* = 22 ± 1 ℃, *L. variegatus* = 21 ± 1 ℃ and *C. riparius* = 22 ± 1 ℃) 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.

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 ºC 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.

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 or 0.003 µmol/L. 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.

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:

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

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

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.

RESULTS

Water chemistry

Water chemistry data for both soft and hard water are reported in Table 3.1. 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 3.1). 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 3.1).

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 3.2). Acute 48- or 96-h LC50 values for Ni ranged from 7.5 – 246.8 µmol Ni/L (33 fold difference; or above the solubility of Ni, >11,000 µmol Ni/L). LC10 values are provided in Supplementary Table 3.2. In all cases where LC50 values were derived in both hard and soft water, organisms were ~3-3.5 x more sensitive in soft water than in hard water (Table 3.2). No correlation was observed between the comparative sensitivity order for Ni and the mass of the organisms tested (Fig. 3.1). Note that for the snail (*L. stagnalis*), the mass plotted in Fig. 3.1 refers to soft tissue mass only, and does not include the shell.

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. 3.2A). 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 wet wt. in the two species, respectively (Fig. 3.2B, C). Beyond the threshold concentrations, mortality steadily increased (Fig. 3.2B, C). As no mortality was observed in *C. riparius* below the solubility point of Ni

(~11,000 µmol Ni/L), there was no relationship between Ni bioaccumulation and survival (Fig. 3.2D).

CBR50 values ranged from 117.5 to 1259.0 µmol/kg wet wt. (Table 3.3), an 11-fold difference in comparison to the LC50 value range of 33-fold (Table 3.2). 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 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 3.2), but there was no significant difference between CBR50 values (Table 3.3). A similar trend was observed in *L. variegatus*, where there was a 3.0 fold difference between LC50 values (Table 3.2) in the two water hardness, however, no significant difference between CBR50 values (Table 3.3).

Ni bioaccumulation parameters

Ni bioaccumulation increased with increasing exposure concentration, and there were clear hyperbolic, saturable relationships in all four organisms (Figs. 3.3A, 3.4A, 3.5A, and 3.6A), allowing for Michaelis-Menten constants to be calculated (Table 3.4). However, note that in Fig. 3.5A, 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 3.4). 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 3.4).

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. 3.7A) and K_d values (Fig. 3.7B) - 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. 3.7A), whereas this was less true for the K_d vs. toxicity relationship (Fig. 3.7B).

Essential ion homeostasis

In general, exposure to Ni disrupted Na (Fig. 3.3B-3.5B) and Mg (Fig. 3.3C-3.5C) homeostasis at exposure concentrations below the LC50 values (cf. Table 3.2) with the exception of *C. riparius* (Fig. 3.6). In *L. stagnalis*, soft tissue Mg dropped by 25%, while Na declined by 72% in HW (Fig. 3.3). 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. 3.4).

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. 3.5).

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 3.3D-3.6D). No disruption of essential ion homeostasis was observed in *C. riparius* (Fig. 3.6).

DISCUSSION

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 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; Hoang et al., 2004; Pyle et al., 2002) 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 3.1).

Acute LC50 values recorded in the present study ranged from 7.5 to 246.8 µmol Ni/L, and LC10 values from 0.60 to 146.3 µmol Ni/L (Supplementary

Table 3.2) 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 at a water hardness between 0 and 60 mg/L as $CaCO_3$, and 1.87 μmol Ni/L at water hardness between 120 and 180 mg/L as $CaCO_3$; CCME, 2007) or as a water hardness based equation ($e^{0.76*ln\,[hardness]+1.06}$ (expressed in μg/L); with guidelines of 0.8 μmol Ni/L at a water hardness of 40 mg/L as $CaCO_3$, and 2.1 μmol Ni/L at water hardness of 140 mg/L 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 ($CMC = e^{0.846*(ln\,hardness) + 2.255}$; U.S. EPA, 1995) and is 3.7 μmol Ni/L at 40 mg/L as $CaCO_3$ and 10.6 μmol Ni/L at 140 mg/L 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 in SW (nominally 40 mg/L as $CaCO_3$) and 0.043 μmol Ni/L in HW (nominally 140 mg/L 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 at a water hardness of 26 mg/L as $CaCO_3$. Normalization to a water hardness of 85 mg/L 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 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 (DeSchamphelaere et al., 2008; Schlekat et al., 2010; Brix et al., 2011; 2012, Ng et al., 2011, 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 (at water hardness = 50 mg/L as $CaCO_3$) and 4.4 μmol Ni/L (at water hardness = 161 mg/L as $CaCO_3$) for *C. dubia*. As well, Pane et al. (2003b) found a 48-h LC50 of 18.2 μmol Ni/L (at water hardness = 45 mg/L 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 (at water hardness = 50 mg/L as $CaCO_3$) and 26.0 μmol Ni/L (at water hardness = 140 mg/L 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, ~6 x 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 (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 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 at a water hardness of 55 mg/L 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).

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. 3.1) as it is for other metals such as copper (Grosell et al., 2007).

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. 3.2B, 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 *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 3.2), 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 for *D. pulex* and 1 µmol Ni/L 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 3.2), whereas, there was no significant difference between CBR50 values (Table 3.3). 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 3.2, 3.3). 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 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.

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. 3.3A-3.6A). 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. 3.7A,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.

Models such as the Biotic Ligand Model (BLM) (DiToro et al. 2001; Paquin et al., 2000; 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 3.5, 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 3.5), 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 3.5). 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 3.5).

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, 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., 1986a). In addition, Mg has been shown to antagonize the genotoxicity, cell transformation and animal tumor induction actions of Ni compounds (Conway et al., 1987; Kasprzak et al., 1986b; Kasprzak et al., 1987).

A novel finding was the disruption of Na homeostasis by exposure to Ni in some of the species (Figs 3.3B, 3.4B, 3.5B). 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). 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. 3.3B). The calculated EC50$_{Na}$ for *L. stagnalis* was 6.1 µmol Ni/L which is 25% lower than the lethality endpoint (LC50) of 8.2 µmol Ni/L. 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, which was 58% lower than the 96-h LC50 (0.40 µmol Cu/L; 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. 3.4B, 3.5B*)* 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 (Deleebeeck et al., 2007b; Kozlova et al., 2009; Pane et al., 2005). 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 3.6B,C). This is most likely due to the extreme tolerance of this organism to metal exposure in general (Gillis and Wood, 2008; Béchard et al., 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 range (Gillis and Wood, 2008).

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

Table 3.1
Water chemistry for all Ni exposures in hard water (HW, nominally 140 mg/L as
$CaCO_3$) and soft water (SW, nominally 40 mg/L as $CaCO_3$). All ion
concentrations are represented in µmol/L with the exception of DOC (mg/L),
hardness and alkalinity (mg/L 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

Table 3.2

Acute (48- or 96-h) LC50 values for waterborne Ni in μmol/L in soft water (SW, nominally 40 mg/L as $CaCO_3$) and hard water (HW, nominally 140 mg/L 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)	> 11000
HW	8.2 (6.6-11.1)	26.0 (19.5-37.7)	246.8 (209.8-281.8)	> 11000

NT indicates "not tested".

Table 3.3

48- or 96-h CBR50 values for Ni in μmol/kg 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 as $CaCO_3$) and soft water (SW, nominally 40 mg/L as $CaCO_3$) water. 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".

	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

Table 3.4
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 as $CaCO_3$) and soft water (SW, nominally 40 mg/L as $CaCO_3$). *Indicates a significant difference in kinetic constants between SW and HW. Values are means ± S.E.M.

	B_{max}	K_d	r^2
	(µmol/kg wet wt)	(µmol Ni/L)	
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".

Table 3.5
Log KNiBL 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 as $CaCO_3$) and soft water (SW, nominally 40 mg/L as $CaCO_3$).

	Bioaccumulation			Toxicity		
	K_d value (μmol Ni/L)	Ionic Ni component of K_d value (μmol Ni/L)	logK$_{NiBL}$ values	LC50 value (μmol Ni/L)	Ionic Ni component of LC50 value (μmol Ni/L)	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**			
HW	1771.7 ± 1183.9	1346.5 ± 923.4	**2.87**		**N/A**	

Fig. 3.1
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 as $CaCO_3$ – open symbols) and soft water
(SW, nominally 40 mg/L as $CaCO_3$ – shaded symbols).
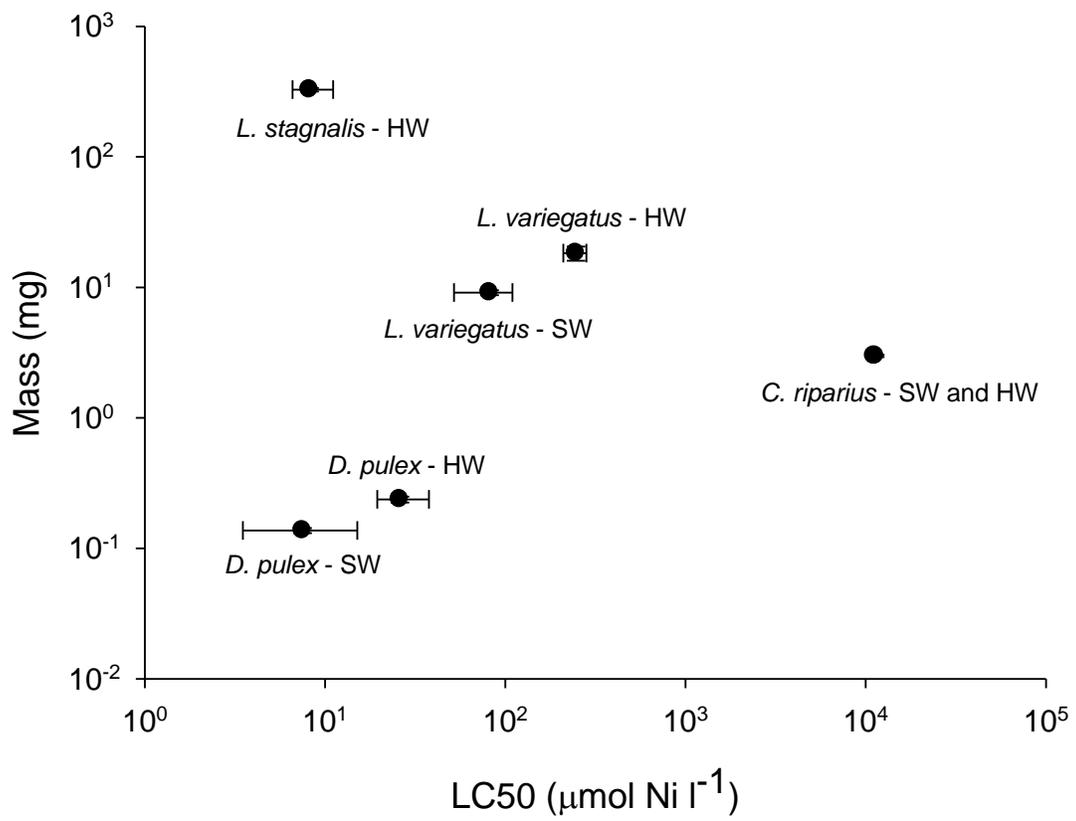
Fig. 3.2
Correlation between survival and whole body Ni bioaccumulation in *Daphnia pulex* (A), *Lymnaea stagnalis* (B), *Lumbriculus variegatus* (C) and *Chironomus riparius* (D) in hard water (HW, nominally 140 mg/L as $CaCO_3$) and soft water (SW, nominally 40 mg/L as $CaCO_3$). Values are means ± 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.

A   *Lymnaea stagnalis*
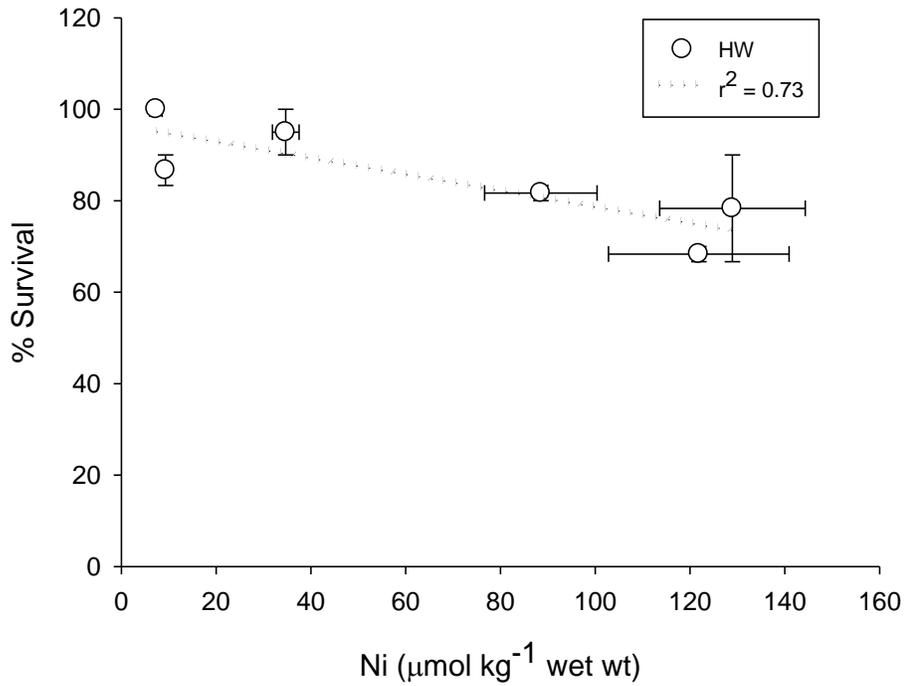
B   *Daphnia pulex*
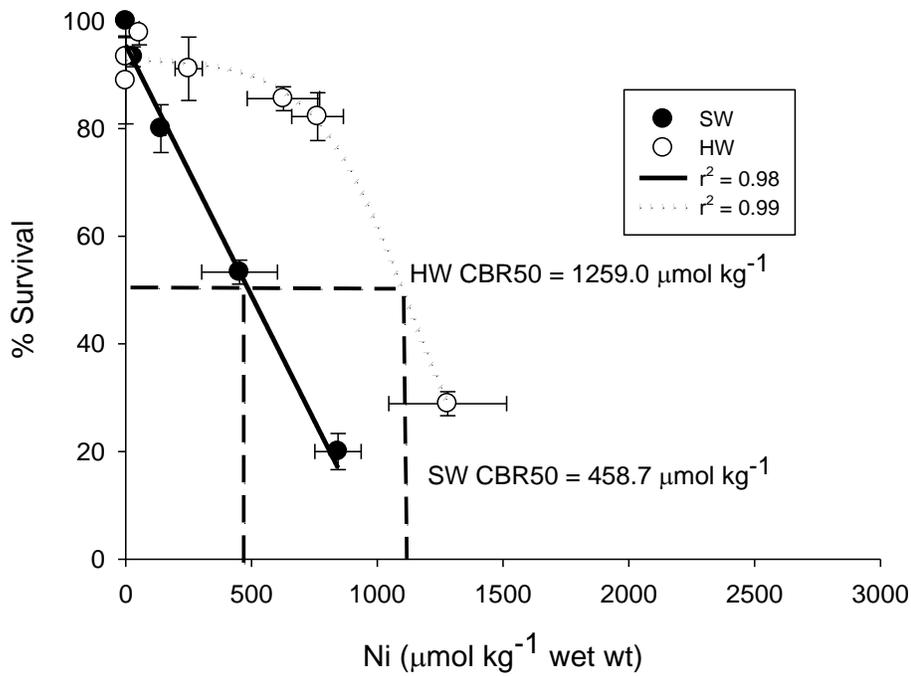
C *Lumbriculus variegatus*
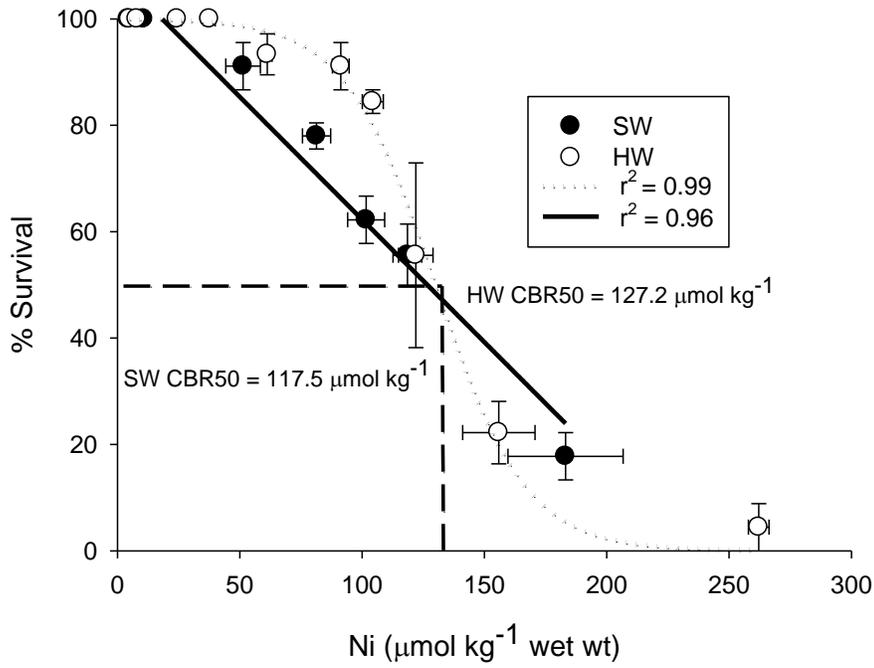
D *Chironomus riparius*
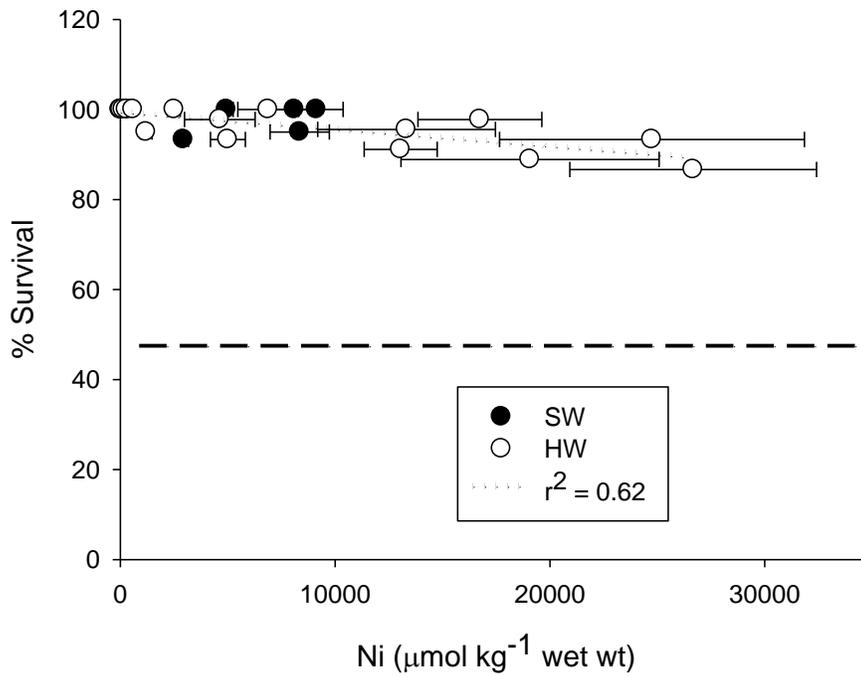
Fig. 3.3
Whole body (soft tissue) Ni (A), Na (B), Mg (C) and Ca (D) levels over a range of exposure concentrations following a 96-h exposure to *Lymnaea stagnalis* in hard water (nominally 140 mg/L as $CaCO_3$) . * Denotes a significant difference ($p < 0.05$) in whole body ion concentration (Na, Mg, Ca) relative to the respective control value. Values are means ± S.E.M.; $n = 8\text{-}10$ per treatment.

A

$B_{max} = 177.8$ μmol kg^{-1}
$K_d = 2.5$ μmol l^{-1}
$r^2 = 0.97$

B

C

D
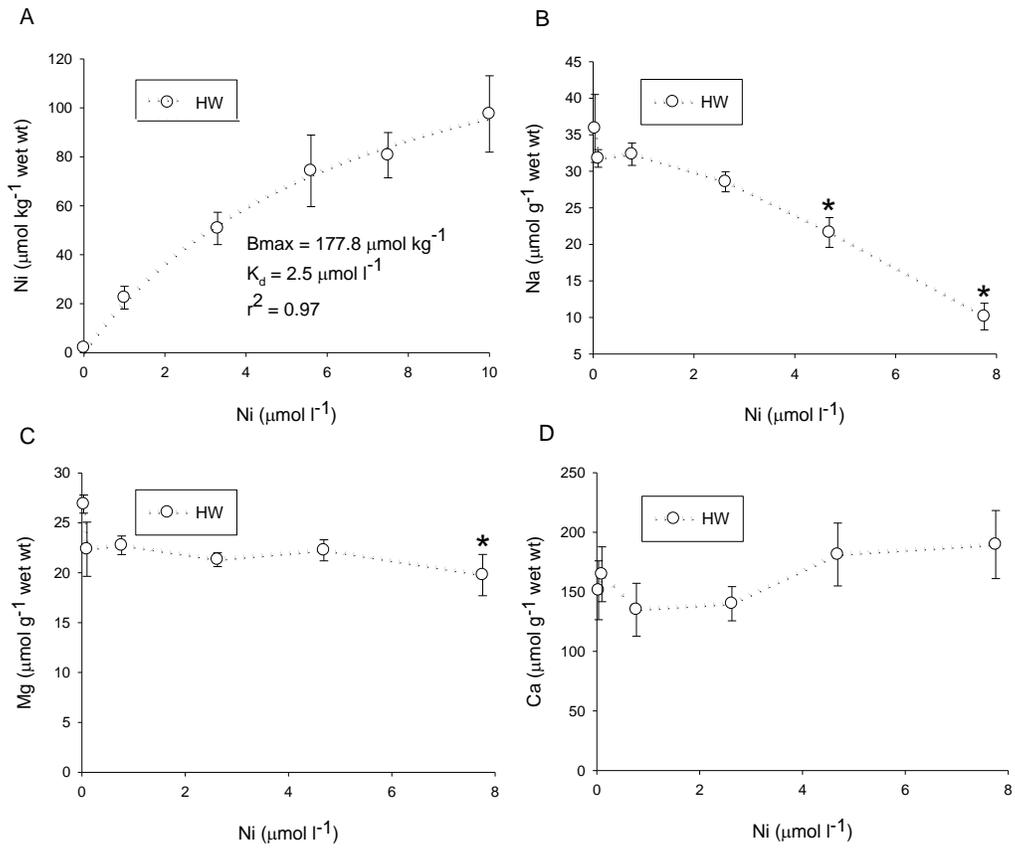
Fig. 3.4

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 as $CaCO_3$) and soft water (SW, nominally 40 mg/L 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.

A

B

C

D
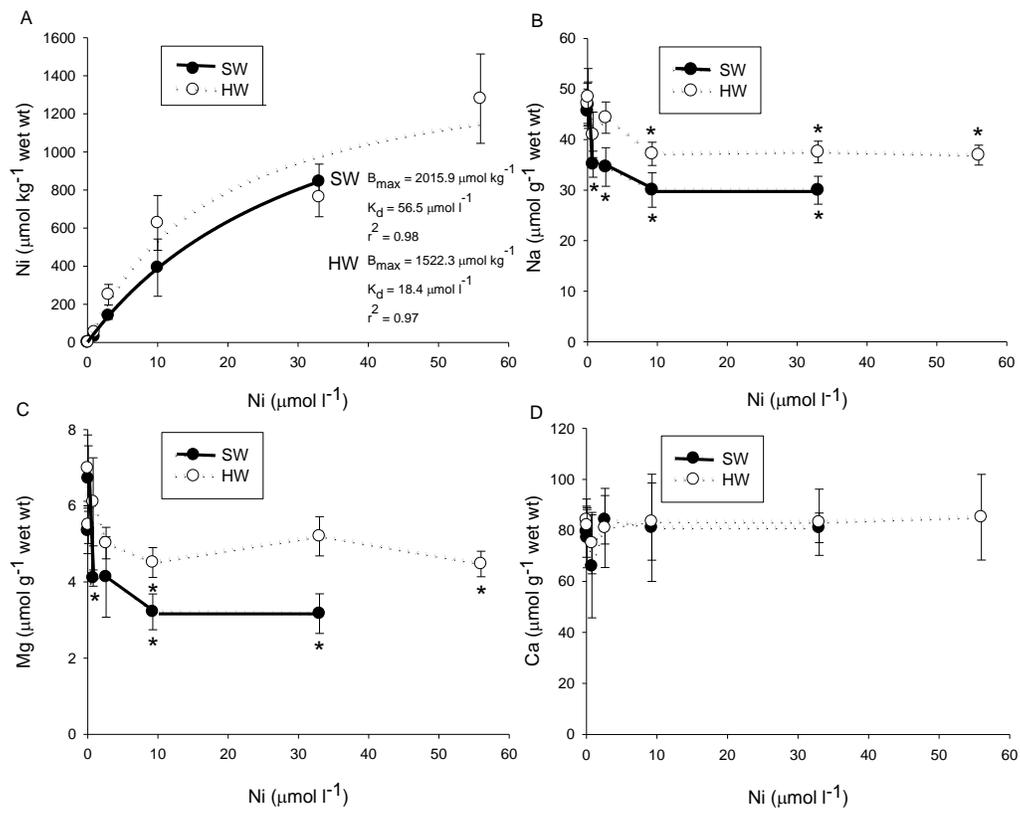
Fig. 3.5

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 $\pm$ S.E.M.; n = 8-10 per treatment.

Fig. 3.6
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.

Fig. 3.7

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.
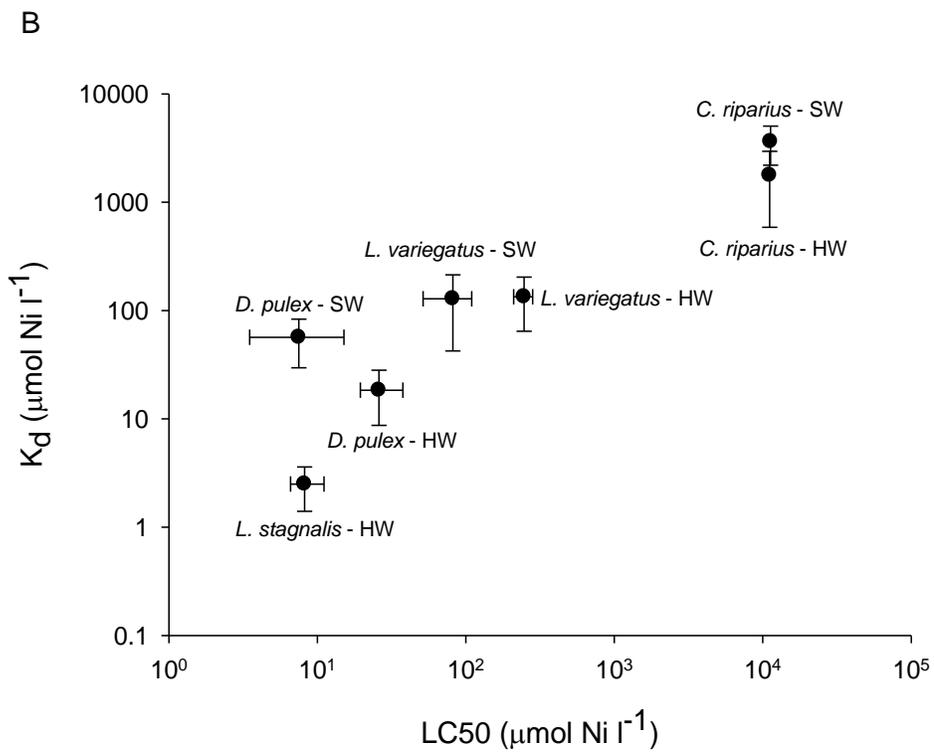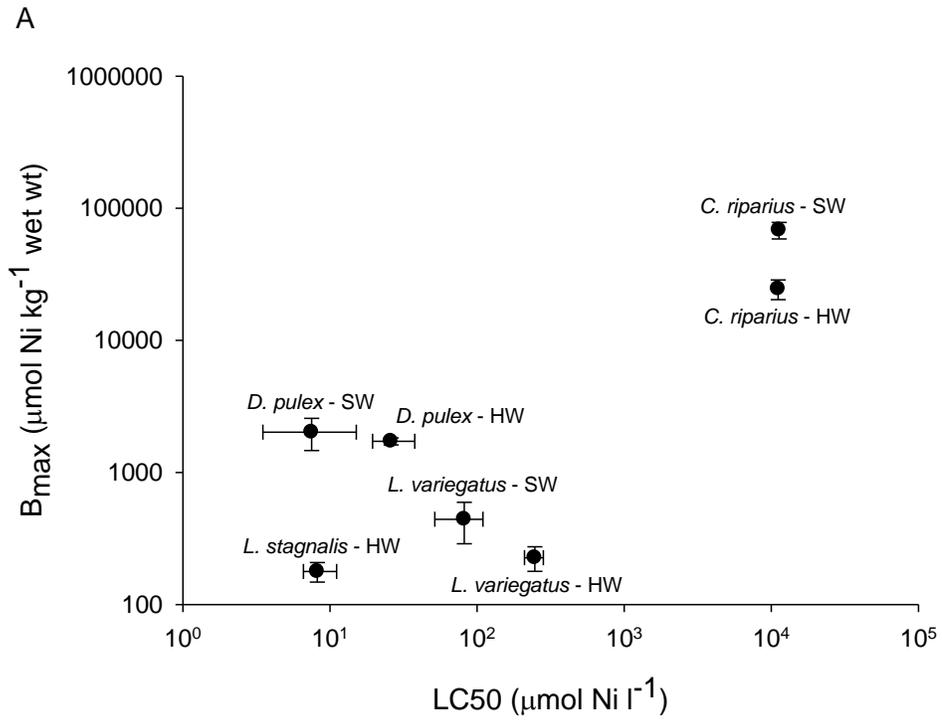
Supplementary Table 3.1
Average Ni exposure concentrations expressed as different fractions over the 48-h or 96-h test periods for each species in hard water (HW) and soft water (SW).

	Nominal (µmol Ni/L)	Total (µmol Ni/L)	Dissolved (µmol Ni/L)	Ionic (µmol Ni/L)	Active (µmol Ni/L)
Lymnaea stagnalis - HW					
	Control	0.02	0.02	0.01	0.01
	1	0.8	0.8	0.6	0.5
	3.3	2.9	2.6	2.0	1.5
	5.6	4.8	4.7	3.6	2.7
	10	8.0	7.8	5.9	4.5
	33.3	30.4	30.1	22.9	17.3
	56	52.5	51.8	39.4	29.8
Daphnia pulex - SW					
	Control	0.02	0.01	0.01	0.01
	0.1	0.1	0.1	0.08	0.07
	1	1.0	0.9	0.7	0.6
	3.3	2.6	2.6	2.2	1.8
	10	9.3	8.9	7.4	6.0
	33.3	31.3	29.9	24.8	20.2
	56	55.4	54.6	43.4	36.9
Daphnia pulex - HW					

	Control	0.03	0.02	0.01	0.01
	0.1	0.2	0.1	0.08	0.06
	1	0.9	0.8	0.6	0.6
	3.3	3.1	2.6	2.0	1.5
	10	11.0	9.3	7.1	5.3
	33.3	33.1	32.8	24.9	18.8
	56	52.9	52.3	39.8	30.0
Lumbriculus variegatus - SW					
	Control	0.02	0.02	0.02	0.01
	1	0.9	0.8	0.7	0.5
	10	9.1	8.8	7.3	5.9
	33.3	32.8	30.1	25.0	20.3
	56	50.0	48.2	40.0	32.6
	100	96.6	95.7	79.5	64.7
	178	179.1	173.4	144.1	117.2
	333.3	322.2	316.7	263.1	214.0
Lumbriculus variegatus - HW					
	Control	0.04	0.03	0.02	0.02
	1	1.0	0.8	0.6	0.5
	10	9.5	8.7	6.6	5.0
	33.3	33.8	30.2	23.0	17.4
	56	49.4	48.9	37.2	28.1

	100	97.0	95.5	72.6	54.9
	178	178.3	172.9	131.5	99.4
	333.3	345.5	317.1	241.2	182.2
Chironomus riparius - SW					
	Control	0.04	0.03	0.02	0.02
	1	1.1	0.9	0.7	0.6
	10	8.7	8.7	7.2	5.9
	33.3	32.6	31.7	26.3	21.4
	56	51.0	50.9	42.3	34.4
	100	97.3	96.4	80.1	65.2
	180	173.2	170.3	141.5	115.1
	333.3	304.9	304.2	252.7	205.6
	500	549.1	541.3	422.2	320.9
	1,000	992.6	967.9	861.5	657.0
	3,000	2,498.8	2,510.0	2,391.1	1,817.2
	5,000	5,133.3	5,131.1	4,982.8	3,836.7
	7,000	6,875.1	6,662.4	6,440.2	4,350.6
	10,000	10,104.8	10,098.0	9,330.0	7,330.9
	12,000	11,488.2	10,905.2	10,880.9	8,369.1
	15,000	13,633.1	10,821.3	10,945.2	8,297.7
Chironomus riparius - HW					
	Control	0.04	0.04	0.03	0.02

	1	0.9	0.8	0.6	0.5
	10	8.7	8.3	6.3	4.8
	33.3	32.6	31.7	24.1	18.2
	56	53.8	50.9	38.7	29.2
	100	96.8	96.4	73.3	55.4
	180	173.3	170.3	129.5	97.9
	333.3	326.1	313.3	238.3	180.0
	500	441.5	429.8	394.8	289.3
	1,000	925.4	913.5	805.6	578.8
	3,000	3,137.7	3,121.7	2,553.1	1,907.7
	5,000	4,463.1	4,315.3	4,396.6	3,769.1
	7,000	7,129.8	6,943.9	6,289.2	4,762.4
	10,000	10,167.2	9,561.1	9,177.3	6,142.7
	12,000	11,543.3	11,429.4	11,122.5	8,020.9
	15,000	13,686.4	11,128.1	10,056.1	7,968.2

Supplementary Table 3.2

Acute (48- or 96-h) LC10 values for waterborne Ni in µmol/L in soft water (SW, nominally 40 mg/L as CaCO3) and hard water (HW, nominally 140 mg/L as CaCO$_3$) with lower and upper 95% confidence intervals in brackets. *Indicates a significant difference in LC10 values between SW and HW.

LC10 (µmol Ni/L)	*Lymnaea stagnalis* 96-h	*Daphnia pulex* (48-h)	*Lumbriculus variegatus* (96-h)	*Chironomus riparius* (96-h)
SW	NT	0.6* (0.1-1.4)	33.4* (11.0-52.5)	> 11000
HW	3.9 (2.6-4.9)	8.8 (5.1-12.3)	146.3 (103.8-178.1)	> 11000

NT indicates "not tested".

CHAPTER 3 ADDENDUM

Note that discrepancies between the data reflect the fact that multiple test per organism were performed.

Correction to Table 3.3
48- or 96-h CBR50 values for Ni in µmol/kg 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 as $CaCO_3$) and soft water (SW, nominally 40 mg/L as $CaCO_3$) water. 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". Note changes to 95% confidence intervals.

	Lymnaea stagnalis	*Daphnia pulex*	*Lumbriculus variegatus*	*Chironomus riparius*
CBR50	(96-h)	(48-h)	(96-h)	(96-h)
SW	NT	458.7 (119.4-1452.1)	117.5 (97.3-139.3)	N/A
HW	ND[+]	1259.0 (875.6-1745.3)	127.2 (110.7-142.9)	N/A

Correction toTable 3.4
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 as $CaCO_3$) and
soft water (SW, nominally 40 mg/L as $CaCO_3$). *Indicates a significant difference
in kinetic constants between SW and HW.Values are means ± S.E.M. Note
change to K_d value for *L. stagnalis*.

	B_{max}	K_d	r^2
	(µmol/kg wet wt)	(µmol Ni/L)	
Lymnaea stagnalis			
SW	NT	NT	NT
HW	177.8 ± 30.1	6.9 ± 1.2	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".

Correction to Fig. 3.3
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. Note change to K_d value in (A).
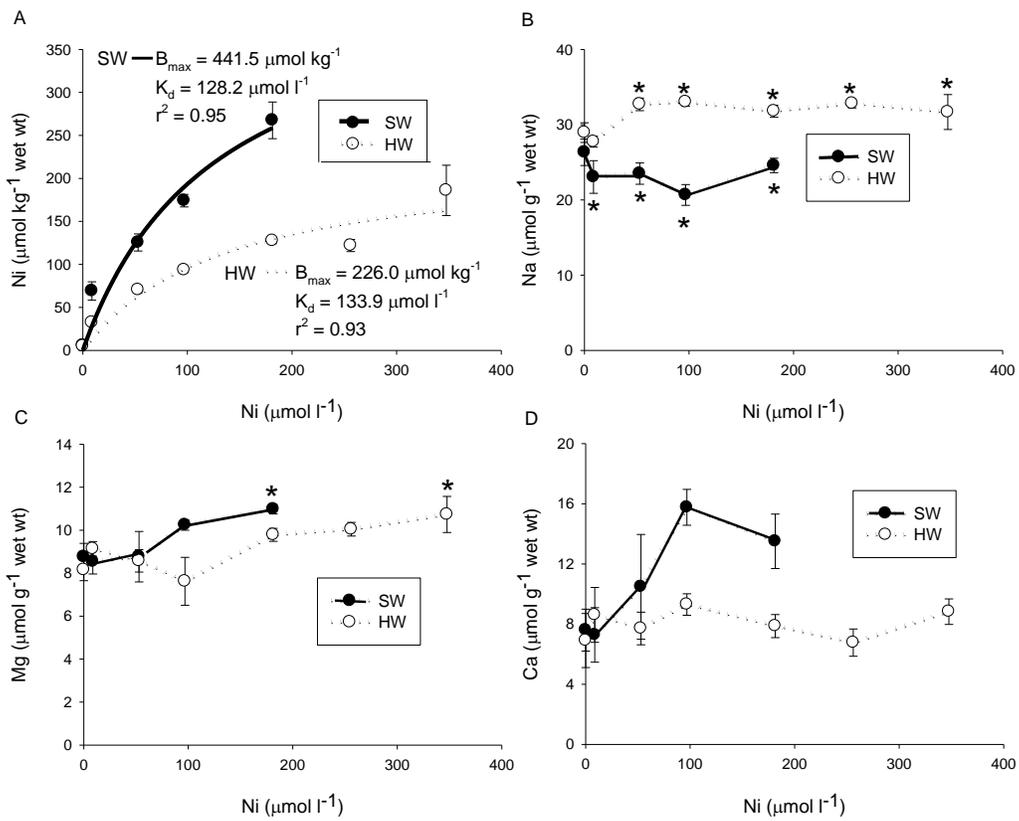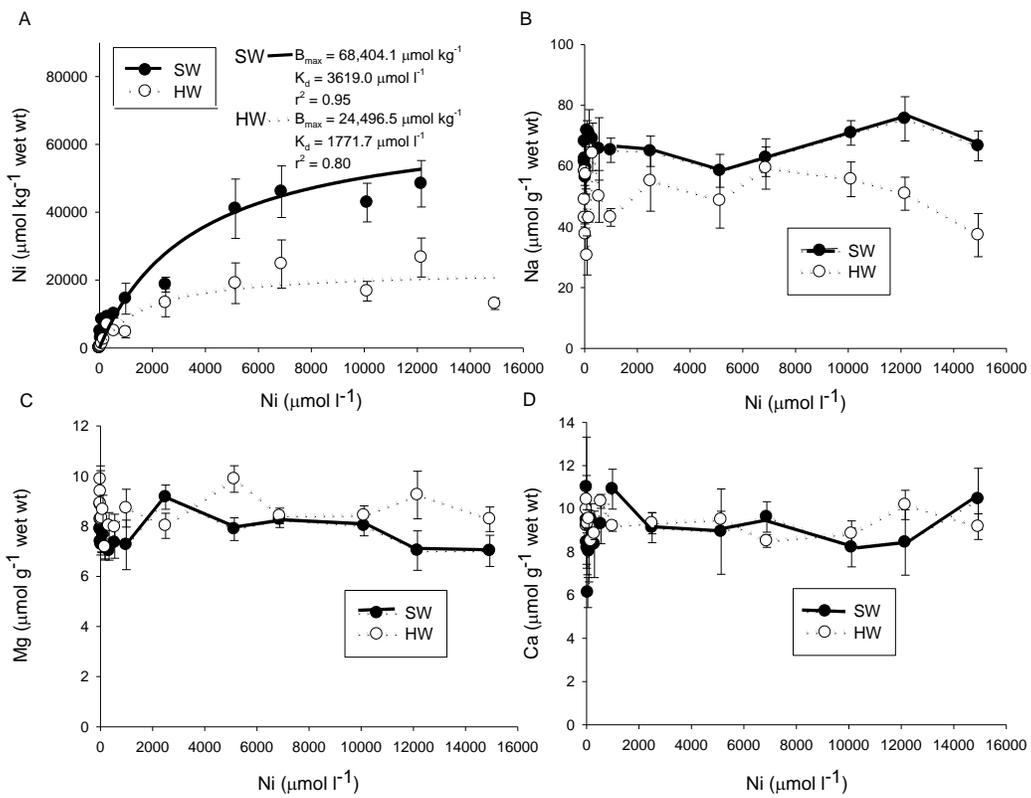
A

B

C

D
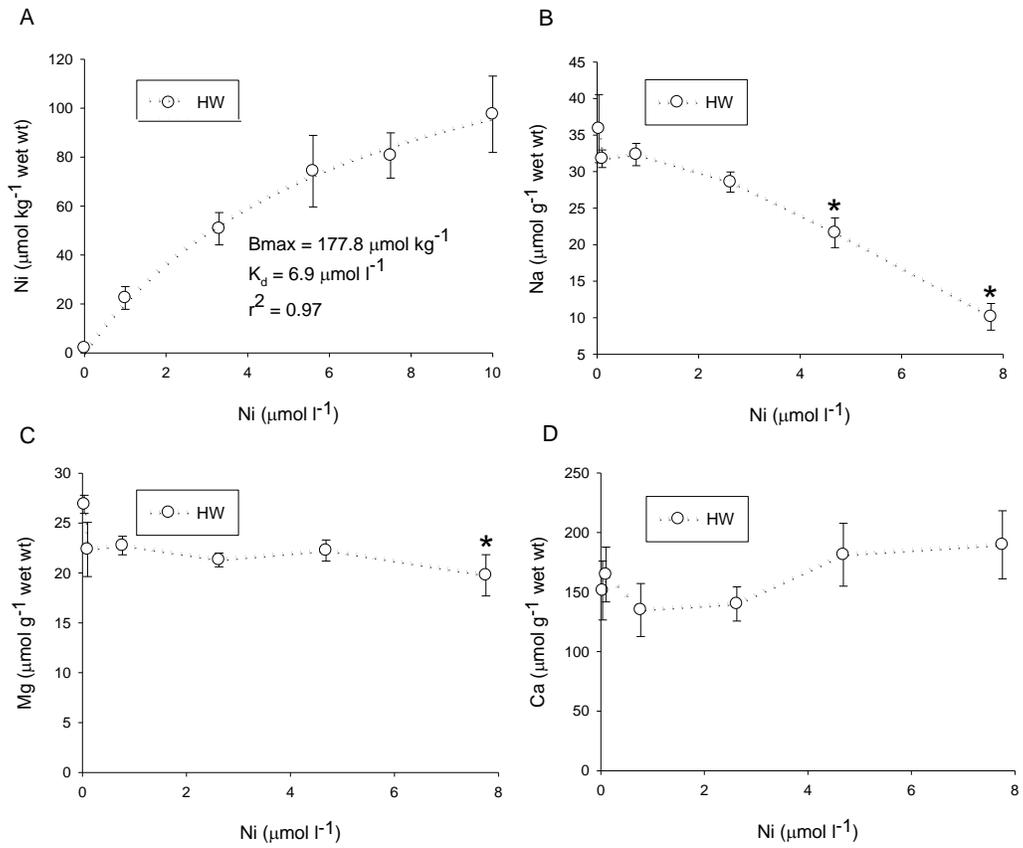
Correction to Fig. 3.7
Correlation between Michaelis-Menten uptake parameters ((A) B_{max} and (B) K_d) and LC50 values for Ni. Michaelis-Menten uptake parameters are means ± S.E.M.; n = 8-10 per treatment. n = 3 for LC50 values with 95% confidence intervals. For panel A, r^2 = 0.76, p = 0.01; for panel B, r^2 = 0.86, p = 0.003. Note change to K_d value in panel B.
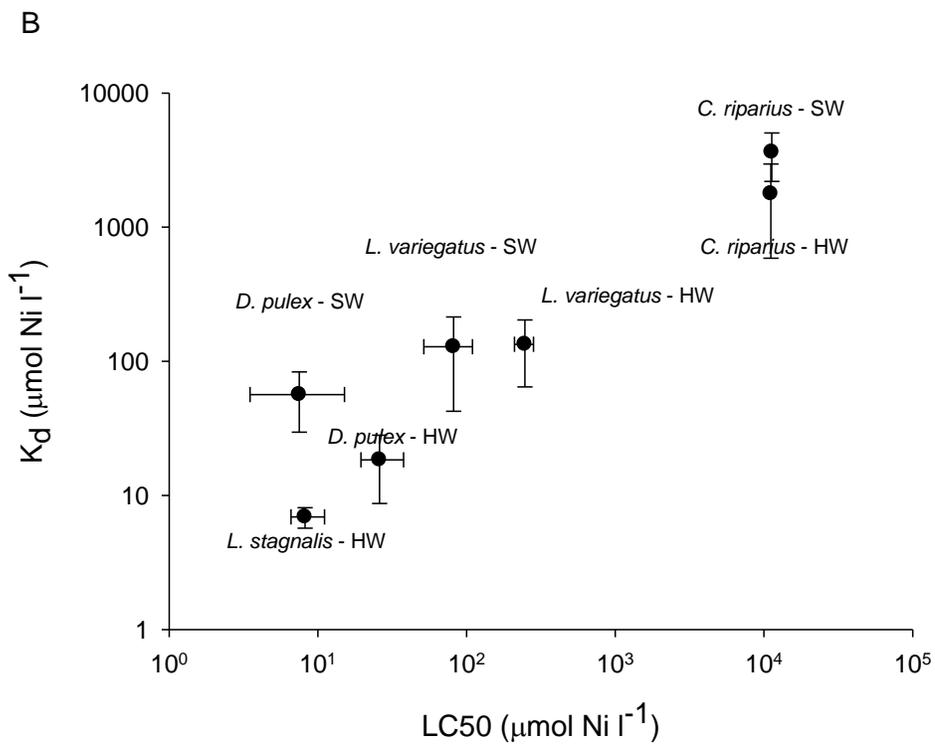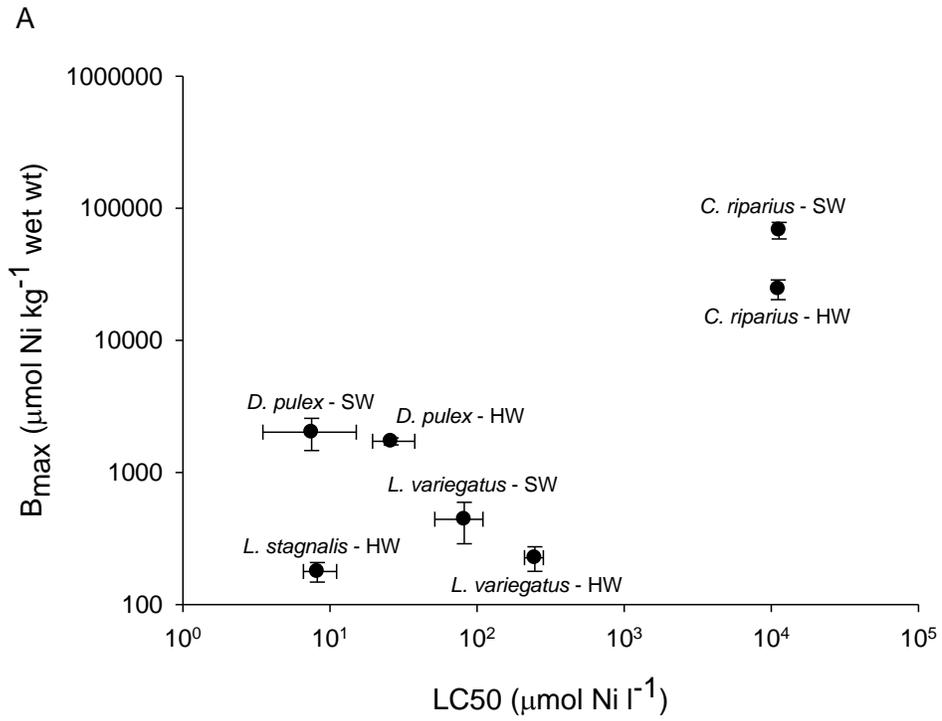
A

B

CHAPTER 4

CRITICAL BODY RESIDUES, MICHAELIS-MENTEN ANALYSIS OF BIOACCUMULATION, LETHALITY, AND BEHAVIOUR AS ENDPOINTS OF WATERBORNE NI TOXICITY IN TWO TELEOSTS

ABSTRACT

Traditionally, water quality guidelines/criteria are based on lethality tests where results are expressed as a function of waterborne concentrations (e.g. LC50). However, there is growing interest in the use of uptake and binding relationships, such as Biotic Ligand Models (BLM), and in bioaccumulation parameters, such as Critical Body Residue Values (e.g. CBR50), to predict metal toxicity in aquatic organisms. Nevertheless, all these approaches only protect species against physiological death (e.g. mortality, failed recruitment), and do not consider ecological death which can occur at much lower concentrations when the animal cannot perform normal behaviours essential for survival. Therefore, we investigated acute (96-h) Ni toxicity in two freshwater fish species, the round goby (*Neogobius melanostomus*) and rainbow trout (*Oncorhynchus mykiss*) and compared LC, BLM, and CBR parameters for various organs, as well as behavioural responses (spontaneous activity). In general, round goby were more sensitive. Ni bioaccumulation displayed Michaelis-Menten kinetics in most tissues, and round goby gills had lower K_d (higher binding affinity) but similar B_{max} (binding site density) values relative to rainbow trout gills. Round goby also accumulated more Ni than did trout in most tissues at a given exposure concentration. Organ-specific 96-h acute CBR values tended to be higher in round goby but 96-h acute CBR50 and CBR10 values in the gills were very similar in the two species. In contrast, LC50 and LC10 values were significantly higher in rainbow trout. With respect to BLM parameters, gill log K_{NiBL} values for bioaccumulation were higher by 0.4-0.8 log units than the log K_{NiBL} values for toxicity in both species, and both values were higher in goby (more sensitive). Round goby were also more sensitive with respect to the behavioural response, exhibiting a significant decline of 63-75% in movements per minute at Ni concentrations at and above only 8% of the LC50 value; trout exhibited no clear behavioural response. Across species, diverse behavioral responses may be more closely related to tissue Ni burdens than to waterborne Ni concentrations. To our knowledge, this is the first study to link Ni bioaccumulation with behavioural

endpoints. In future it would be beneficial to expand these analyses to a wider range of species to determine whether Ni bioaccumulation, specifically in the gills, gut and whole fish, may be a good predictor of behavioural changes from metal exposure; which in the wild can lead to ecological death.

INTRODUCTION

In most jurisdictions, current water quality guidelines/criteria for Ni rely on approaches such as hardness-correction which were developed more than two decades ago (CCREM, 1987; USEPA, 1986, 1995), and do not reflect the effects of other water chemistry factors that are known to affect metal toxicity. Several promising newer approaches are now available and these take into account the receptor characteristics of target organisms and the bioavailability of the metal as a function of water chemistry. For example, the European Union (ECB, 2008) has recently adopted Biotic Ligand Modeling (BLM) approaches (DiToro et al., 2001; Paquin et al., 2000; Niyogi and Wood, 2004) which utilize water chemistry parameters to predict the bioavailability of Ni in conjunction with the binding constants of the biotic ligand (log K values) to predict toxicity. Another developing approach, currently not being used to derive Ni water quality guidelines, is the Tissue Residue Approach (TRA; Luoma et al., 2009; Adams et al., 2011; Schmidt et al., 2011) which utilizes Ni bioaccumulation within the whole organism or within an organ to predict toxicity. The TRA makes use of Critical Body Residues (CBR values) calculated from the concentration of Ni bioaccumulated which correlates with mortality, and therefore, in this manner, bioaccumulation can be used to predict the toxicity within and across species. The BLM and TRA methods may be used independently or in combination as a tool for protection of aquatic organisms. Expressing toxicological effect as a function of the bioaccumulation of a metal such as Ni has many potential benefits: integration of all exposure routes (e.g. water column, food and sediments), independence from differences and/or changes in water chemistry that may affect uptake, and incorporation of toxicokinetics of different species (USEPA, 2007). The available information to date for CBR for metals, specifically Ni, is not robust and therefore more data are required.

Other ways in which Ni bioaccumulation can be related to Ni toxicity are by characterization of binding constants from saturable metal binding tests using Michalis-Menten analysis, where binding affinity (K_d) and binding site density (B_{max}) can correlate with Ni toxicity. Previous analysis on invertebrates demonstrated that, in general, more sensitive organisms (lower LC50 values) exhibit a higher binding affinity (lower K_d) and lower binding site density B_{max} values for Ni than more tolerant organisms (Leonard and Wood, 2013).

Both the traditional and more modern approaches (BLM, TRA) protect a species only against physiological death (e.g. mortality, failed recruitment), while the ecological and behavioural impacts can also greatly affect survival in nature (Scott and Sloman, 2004). Such metal-induced "ecological death" can occur at much lower concentrations, as the organism itself may not be overtly harmed but also is unable to perform normal behaviours such as foraging, predator evasion, or searching for mates (Kania and O'Hara, 1974, Little and Finger, 1990, Barron, 2002). To date, behavioural disturbances from exposure to metals have not been

employed as an endpoint to derive water quality guidelines (Wood, 2012). However, behaviour may be a very sensitive and valuable endpoint. For example, behavioural dysfunction has been shown extensively for the olfactory toxicity of Cu (Pyle and Mirza, 2007; McIntyre et al., 2008; Green et al., 2010) and is of concern as well for Ni (Pyle and Couture, 2012). Other behavioural endpoints include disruptions of predator avoidance, schooling behaviour, reproductive behavior, and formation and maintenance of social hierarchies (Atchison et al., 1987; Beitinger, 1990; Scott and Sloman, 2004; Sloman and Wilson, 2006; Sloman, 2007; Sopinka et al., 2010). The most frequently studied behavioural responses to contaminant-related toxicity in fish involve swimming activity (Little and Finger, 1990). Swimming activity includes frequency and duration of movements, endpoints which are appropriate for a variety of species and relevant to survival (Rand, 1984; Little and Finger, 1990).

The information available on the impact of Ni on behaviour is scarce in comparison to other metals such as copper, cadmium and mercury (Scott and Sloman, 2004). To address this deficit, we explored the effects of waterborne Ni exposure on two species of freshwater fish: the round goby and rainbow trout. The round goby (Gobiidae: *Neogobius melanostomus*) is an invasive species to the North American Great Lakes basin since the early 1990's (Jude et al., 1995). It is known as a pollution-tolerant species (Pinchuk et al., 2003), and is benthic and philopatric, relying on shelters and burial into loose substrate to avoid predators (Belanger and Corkum, 2003). The second species, rainbow trout (Salmonidae: *Oncorhynchus mykiss*) is a recreational sport fish that inhabits the pelagic zones of many lakes and rivers. Rainbow trout are one of the most sensitive fish species tested to date for Ni (Nebeker et al., 1985; U.S. EPA, 1995). These two species not only have different habitats and lifestyles but they may also be at different ends of the sensitivity spectrum for fish which makes them a good comparison to assess trends in bioaccumulation vs. toxicity and behaviour.

Therefore, with this background in mind, our aims were: (1) to measure and compare organ and whole body Ni concentrations over a wide range of waterborne Ni concentrations in the two species as well as to calculate their K_d, B_{max}, 96-h acute CBR10 and CBR50 values; (2) to determine acute (96-h) LC10 and LC50 values for Ni in these two species and compare the resulting toxicity-derived log K values with those (log K_d) derived from the bioaccumulation tests; (3) to determine the impact of Ni on behavioural endpoints following an acute waterborne Ni exposure; (4) to assess the possible correlations between changes in behaviour (which may lead to ecological death) and either the Ni bioaccumulation or the Ni exposure concentration in these two species.

We hypothesized that round goby would be more resistant than rainbow trout, and that 96-h acute CBR50 values would vary less across species than LC50 values. As well, we expected that K_d and B_{max} values would be lower in the more sensitive species and that behaviour of round goby and rainbow trout would be

affected by exposure to acute waterborne Ni at concentrations well below the LC50 value, and closer to LC10 values. Finally, we postulated that both Ni exposure concentration and Ni bioaccumulation would correlate to changes in behaviour (spontaneous movement).

METHODS

Experimental Organisms

Round goby, *N. melanostomus*, were collected from Hamilton Harbour at LaSalle Park (43º18'1" N, 79º 50'47"W; weeks of June 29 – July 10th, 2009, background Ni concentration = 0.31 ± 0.03 µmol/L). Forty-eight round goby (mean body mass 10 ± 3 g), were caught in commercial minnow traps baited with frozen corn, set at a depth of 1 m or less, for 24 h. Fish were then transported back to the laboratory and acclimated to laboratory conditions in 5-L containers, with a flow-through of aerated, dechlorinated Hamilton (Ontario, Canada) tap water. PVC tubes, approximately 5-10 cm in length, were used for shelter and the round goby were fed *ad libitum* every other day with Big Al's Staple Flake Food (45 % protein, 5 % crude fat, 2 % crude fibre and 8 % moisture; Big Al's Aquarium Supercentres, Woodbridge, ON, Canada).

All round goby were treated with oxytetracycline (2.5 g/L for 2 hours), a formalin dip (1:6000 for 1 h) and chloramine T (2 mg/L for 4 h) to prevent and minimize infections. All treatments were completed two weeks prior to nickel exposure.

Forty-two rainbow trout, *O. mykiss*, 13 ± 1 g were purchased from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada. They were initially held in 500-L tanks receiving flow-through dechlorinated Hamilton tap water. Trout were fed 2 % body weight, every other day, with Martin's commercial dried pellet feed (Martin Mills Inc., Ontario, Canada).

All fish were kept in Hamilton dechlorinated tap water with an ionic composition of (in mmol/L) Na (0.9), Cl (1.0), Ca (1.0), K (0.4), Mg (0.4), and Ni ($<4 \times 10^{-5}$). Water hardness was ~140 mg/L as $CaCO_3$ equivalents; pH was 7.8, alkalinity 96 mg/L, water temperature was 13 ± 2ºC, dissolved organic carbon (DOC) was 2.3 mg/L and the photoperiod was 16:8 h light:dark. Fish were fasted for 48 h prior to exposure to allow sufficient time for gut clearance and to standardize metabolic rate.

Flow-through exposure system

Ni stock solutions, made with NiCl$_2$·6 H$_2$O (Sigma Aldrich, St. Louis, Missouri, USA), were held in Mariotte bottles above the exposure tanks. A flow of 0.5 ml/min of Ni stock solutions from the Mariotte bottles was mixed with a flow of 750 ml/min dechlorinated Hamilton tap water in a mixing bucket before being administered to the exposure tanks containing the fish.

Mariotte bottle drip rates and flow rates of dechlorinated water were monitored daily. Water samples were taken every 24 h from each exposure to determine total and dissolved (0.45 µm filtration, see below) concentrations of Ni.

Acute (96-h) LC50 tests

Mean water chemistry parameters for all experiments are shown in Table 4.1. Measured total and dissolved Ni concentration were generally close to the nominal values and dissolved values were used to determine LC50 values. Measured Ni concentrations in the exposure waters are shown in Supplementary Table 4.1. Eight round goby and six rainbow trout were transferred to each exposure tank (20-L) for 24 h prior to Ni exposure to allow time for acclimation to the new environment. Each concentration was tested in duplicate to assess acute toxicity. Impacted fish (defined as fish which had lost equilibrium and had turned upside down) were removed from the exposure tanks and euthanized.

Behavioural assay

Round goby

At the end of the 96-h tests, all surviving round goby in each exposure were isolated in a clear plastic bin (75 x 30 cm) filled with 11 L of control water at 13 ± 2°C. Fish were allowed to acclimate for 30 min before spontaneous activity was filmed from above with a video camera for 5 min. Each fish in each group was later scored from the video tapes, for the mean number of movements made per minute. Round goby are discrete movers, making single distinct hops or swims interspersed with long periods of inactivity that are easily observed and scored during video analysis (Marentette et al., 2011, Marentette et al., 2012, Marentette and Balshine, 2012).

Rainbow trout

Similarly at the end of the 96 h tests, all surviving rainbow trout in each exposure group were placed in the same clear plastic bin (75 x 30 cm) filled with

11 L of control water at 13 ± 2°C. Fish were allowed to acclimate for 30 min before spontaneous activity was filmed from above for 5 min. A different video scoring system was used here because rainbow trout tend to move more smoothly and continuously than the round goby. Videotapes were converted to .jpg images at 2 frames per second, and velocity in cm/sec determined from 1-min sequences using ImageJ's Manual Tracker plug-in (NIH). Each fish was assigned a mean velocity in cm/sec.

Tissue sampling

Following behavioural measurements, both rainbow trout and round goby were rinsed briefly (5 s) in nanopure water (18.2 MΩ cm, Millipore Corporation, Billerica, MA, USA) and then euthanized with 0.80 mg/L of tricaine methanesulfonate (*MS-222*) (Syndel Laboratories Ltd. Vancouver, BC, Canada; adjusted to pH 7.8 with NaOH). The body mass of each fish was measured and recorded. Gills, gut, kidney, and liver were excised, weighed and then preserved for further analysis. Gills and gut were rinsed with 0.9% NaCl solution and blotted dry for 5 min before storing them in 15-ml Falcon^TM tubes. The kidney, liver and brain were stored in 2-ml bullet tubes and the remaining carcass (consisting of the remainder of the fish which was largely muscle, bones, skin and scales) was stored in aluminum foil. All organs were kept at -4°C for later tissue analysis.

Analytical techniques

The various organs and the carcass of each fish were digested in sealed vials with 2 N HNO_3 (trace metal grade, Fisher Scientific, Ottawa, ON, Canada) with a volume of 3-5 times the weight of the tissue. These were incubated in a Precision Oven (Jouan Inc., Virginia, USA) at 60 °C for 48 h, with vortexing at 24 h. Tissue digests were then stored at 4 °C for later analysis. Ni concentrations in water samples 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 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 or 0.003 μmol/L. Ni recovery was 91 ± 2.3 % as determined by Environment Canada certified reference materials, TM-24.3 (lot # 0310) and TM-25.3 (lot # 0809). Ni concentrations were not corrected for recovery.

Concentrations of major cations (Na, Mg, Ca and K) in water samples were analyzed by flame atomic absorption spectroscopy (FAAS; Varian

SpectrAA – FS-220, Mulgrave, Australia). Samples were diluted using 1 % HNO_3 for Na analysis and 1 % HNO_3 with 1 % $LaCl_3$ for Ca and Mg analysis. Na, K, Mg and Ca reference standard solutions (Fisher Scientific, Ottawa, ON, Canada) were used to obtain standard curves. Water pH and DOC were measured using an 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.

Mean water chemistry parameters for all acute Ni exposures are shown in Table 4.1. Ni water concentrations in each exposure level tested were expressed as five fractions: nominal, total, dissolved, ionic and active fractions of the metal, were calculated taking into account the measured water chemistry from Table 4.1, and are reported in the Supplementary Information Section (Supplementary Table 4.1). Note that the active fraction represents the concentration of the ion in its fully dissociated, freely diffusible form, and is less than the total ionic concentration because of the tendency of ions to interact with other atoms and molecules in solution (Hill et al., 2012). All water Ni concentrations presented in this study are reported as the dissolved fractions of the metal, which averaged 95% of nominal values and 94% of the total values (Supplementary Table 4.1).

Statistical analyses

Data have been presented as means ± SEM (*n*), where *n* is the sample size. Measured total and dissolved Ni concentrations along with specific water chemistries were used to estimate the free ionic nickel (Ni^{2+}) concentrations and Ni^{2+} activity using Visual MINTEQ software (ver. 3.0, beta, KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden). The active fraction is a measure of the effective activity of Ni in these water chemistries, which is determined by concentration and by interactions (attraction or repulsion) of other molecules in solution. The NICA-Donnan model (Benedetti et al., 1995) was used in the calculations to estimate the effect of DOC on Ni speciation. Acute LC50 and LC10 values with 95% confidence intervals (C.I.) were calculated using ToxCalc – Toxicity Data Analysis Software ver.5.0.32 (Tidepool Scientific Software, McKinleyville, CA, U.S.A.). The 96-h acute critical whole-body residue (CBR50) was the Ni bioaccumulation in an organ or whole fish (combined organs) that corresponded to 50% mortality. We used two different methods for determining the 96-h acute CBR50 values with 95% C.I.: 1) ToxCalc software (as above, substituting Ni bioaccumulation for waterborne Ni concentration) and 2) linear regressions of logit mortality vs. log Ni bioaccumulation. For the latter method, non-linear regressions of log bioaccumulation vs. logit mortality were used to determine the 96-h acute CBR10 and CBR50 values, and 95% C.I. were derived for these CBR values in Sigma Plot for Windows version 10.0 (Systat Inc., Chicago, IL, USA). 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 also was fitted to the original data (see Leonard and Wood, 2013). Ni bioaccumulation and survival were corrected for control levels prior to analysis.

Non-linear regression analyses were performed to determine the concentration-dependent kinetics of Ni bioaccumulation, again as in Leonard and Wood (2013), with a hyperbolic curve fit (single rectangular two parameters $y = ax/(x + b)$; Sigma Plot for Windows version 10.0; Systat Inc., Chicago, IL, USA) in order to determine the parameters of the Michaelis–Menten equation:

Specific binding = $(B_{max}$ x [L]) / ([L] + K_d);

where [L] is the concentration of the ligand (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).

Significant differences between two groups were evaluated using 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) or by Dunnett's test for the behavioural analysis, in order to compare treatment groups to the control group. Spearman non-parametric rank correlation was performed to compare water Ni exposure concentration or Ni bioaccumulation in organs or whole fish against behavioural data. For all tests, statistical significance was allotted to differences with $p < 0.05$.

RESULTS

Ni bioaccumulation and Michaelis-Menten parameters

Prior to Ni exposure in the laboratory, the gills, gut, carcass and whole fish of round goby had 1.2-2.6 x more Ni in comparison to trout, however, the liver and brain of rainbow trout had 2.1 and 3.8 x more Ni, respectively, than the round goby. There was no significant difference in the kidney Ni level. In general, as the Ni exposure concentration increased so did Ni bioaccumulation (this was true for all organs and for both species apart from trout carcass/muscle, Fig. 4.1). Ni bioaccumulation in organs and whole fish (all organs combined) were comparable between the two species on a per weight basis (Fig. 4.1). Primarily, Ni bioaccumulation occurred in the gills, kidney and gut, with less Ni in the guts of rainbow trout than round goby. Interestingly, little Ni was detected in the brain, suggesting that the blood-brain barrier is fairly efficient for this important behavioural control center. In round goby brains, elevated Ni were observed only at the highest exposure concentration tested (221. 3 μmol Ni/L), while in rainbow

trout brains, Ni bioaccumulation never exceeded the levels observed in the control fish.

However, when accounting for the weight of the organs, the order of Ni bioaccumulation on a percent basis in organs from highest to lowest for both species was: carcass > gill > gut > kidney > liver > brain (Fig. 4.2). In comparison to control fish, the round goby from the highest exposure concentration had 2 x more Ni bioaccumulated in their gills, gut and kidney. The pattern was even more pronounced in the trout with fish from the highest exposure concentration having 8, 2.5, and 6.4 x more Ni in their gills, gut and kidney, respectively compared to control fish (Fig. 4.2).

Ni bioaccumulation displayed a hyperbolic, saturable relationship with respect to exposure concentration in the gills (Fig. 4.3A), gut (Fig. 4.3B), kidney (Fig. 4.3C) and whole fish (organs combined; Fig. 4.3G). Table 4.2 shows the calculated Michaelis-Menten constants from these relationships. Note that in Fig. 4.3G, the B_{max} for whole fish (organs combined) in the round goby occurred at concentrations well beyond the highest Ni concentration tested, so this value should be interpreted with caution. Between species, the binding site density for Ni, or B_{max} value, of the gut was 7.5 × higher in round goby in comparison to rainbow trout. In round goby, the various organs had similar B_{max} values, but in rainbow trout, B_{max} values (on a per weight basis) decreased with the increasing size of the organ (kidney > gill > gut > whole fish (all organs combined; Table 4.2)).

In both species, the binding affinity for Ni did not differ significantly across the various organs: gills, gut, kidney and whole fish. However, the K_d values (inverse relationship to binding affinity) were 4.9 × lower in the gills and 2.0 × higher in the guts of round goby in comparison to rainbow trout (Table 4.2).

In the liver of both species and in the carcass of round goby, there was a linear relationship between Ni bioaccumulation and exposure concentration and therefore no Michaelis-Menten parameters were calculated (Fig. 4.3D, F).

96-h acute critical body residues

Critical Body Residues (CBR values) are the concentrations of Ni in tissues which correlate to a percent mortality (in the case of the present study: 96-h acute CBR50 and CBR10; Fig. 4.4). However, methods for calculating these values are not standardized in the literature; therefore we have included a comparison of two commonly published methods: logit mortality vs. log bioaccumulation (Ng et al. 2012; Leonard and Wood 2013) and Toxcalc for determining CBR values (Table 4.3). In general, both methods calculate similar CBR values, however, CBR values were more variable (95% confidence intervals

were larger) when calculated by the logit mortality vs. log bioaccumulation method. In addition, the Toxcalc method calculates CBR values which correspond slightly better to CBR values interpolated from the goodness-of-fit lines (Table 4.3, Fig 4.4).

Using the Toxcalc method, 96-h acute CBR50 values in organs of round goby ranged from 23.5 μmol Ni/kg wet wt. in the liver to 247.5 μmol Ni/kg wet wt. in the gills, whereas, in rainbow trout, 96-h acute CBR50 values ranged from 16.9 μmol Ni/kg wet wt. in the whole fish to 264.9 μmol Ni/kg wet wt. in the kidney. In general 96-h acute CBR values were higher in round goby (i.e. a greater Ni bioaccumulation was associated with a given level of mortality), but only a few of the differences were statistically significant. An organ comparison between the two species showed similar 96-h acute CBR50 values for the gills, in fact there was no significant difference by the logit mortality vs. log bioaccumulation method, and only a small difference by the Toxcalc method (Table 4.3). There was also little variation between the 96-h acute CBR50 values in the kidneys and livers of the two species. Although the 96-h acute CBR50 values of the gut and whole fish were ~ 2-3 fold different between the two species, these differences were not significant by the logit mortality vs. log bioaccumulation method (Table 4.3).

The 96-h acute CBR10 values (similar to threshold values) for organs and whole body ranged from 7.5 to 193 μmol/kg wet wt in the two species (Table 4.3). An organ comparison between the two species revealed no significant difference between gill, gut or whole fish 96-h acute CBR10 values whereas there were 4.0 and 2.8 x differences for the kidney and liver, respectively (Table 4.3).

Acute Ni LC10 and LC50 values for round goby and rainbow trout and comparison between log K values derived from toxicity vs. bioaccumulation

The LC values in the two species were significantly different with the round goby being more sensitive (lower LC50 and LC10 values) than the rainbow trout (Table 4.4). The difference in LC50 values was 2.19 fold, and the difference in LC10 values was 3.80 fold.

Table 4.5 compares 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).

The log K_{NiBL} values for toxicity were 4.09 for round goby and 3.76 for rainbow trout. Round goby log K_{NiBL} values for bioaccumulation ranged from 3.38 to 4.86, whereas these values ranged from 3.78 to 4.44 in rainbow trout (Table

4.5). Notably, for both species, the gill log K_{NiBL} values for bioaccumulation are higher by 0.4-0.8 log units than the log K_{NiBL} values for toxicity.

Impact of Ni on behavioural endpoints following an acute waterborne Ni

The behavioral effect of acute Ni exposure on round goby was much more pronounced than in rainbow trout. In round goby, the selected behavioural endpoint of movements per minute significantly decreased by 63-75 % at all exposure concentrations of 8.1 µmol Ni/L or higher (up to 221.3 µmol Ni/L), indicating a clear locomotion threshold between 0.7 and 8.1 µmol Ni/L (Fig. 4.5A). The lowest observed effect concentration (LOEC) represents 8% of the LC50 or 28% of the LC10 (cf. Table 4.4). In contrast, in the rainbow trout, there was no significant difference in the mean velocity or swimming speed between the fish in control vs. exposure treatments, although the mean velocity significantly declined in the 32.6 µmol Ni/L exposure concentration in comparison to the 0.8 µmol Ni/L (Fig. 4.5B).

Linking physiological endpoints (bioaccumulation) to ecological endpoints (behaviour)

To determine if the very different patterns in behavioral responses in the two species could be integrated by either the Ni exposure concentrations or the Ni bioaccumulation data, Spearman rank correlation analyses were conducted. As Ni bioaccumulation increased in the gills and whole fish, behaviour/movement decreased with Spearman rank correlations of rho = -0.6044, p = 0.03. A slightly weaker correlation was observed with Ni bioaccumulation in the gut (rho = -0.5604, p = 0.05) (Table 4.6). There was also a trend suggesting that increased exposure Ni concentrations correlated with decreased movement (rho = -0.544, p = 0.05). There were no significant correlations between Ni bioaccumulation in the other organs (kidney, liver, brain or carcass) and changes in behaviour (Table 4.6).

DISCUSSION

Overview

Contrary to our predictions, round goby were more sensitive to acute waterborne Ni exposure than rainbow trout; however, as expected the 96-h acute CBR50 values varied less between the two species than 96-h acute LC50 values. As well, in agreement with published data, the K_d values calculated from

bioaccumulation and toxicity for the gills were lower (higher affinity) in the more sensitive round goby in comparison to rainbow trout. The Ni concentration that reached a behavioural threshold of reduced movement was well below the LC10, and the inhibition of movement was already maximal at this concentration, indicating that this is a very sensitive endpoint in the round goby. In marked contrast, no consistent behavioural effects were observed in rainbow trout.

Overall, one of the fundamental concepts of the BLM is that gill metal bioaccumulation is a good predictor of acute toxicity (Meyer et al., 1999; Pane et al. 2004). This study assessed whether this concept could be expanded to other organs for the purposes of the BLM and TRA and also if this framework could be extended to another endpoint such as behaviour. In general, gill, gut and whole fish Ni bioaccumulation were the most consistent predictors of toxicity and there was promising evidence that these organs may be used to predict behavioural endpoints. More work on a wider range of taxa are now needed.

Organ Ni bioaccumulation and Ni bioaccumulation parameters

On a per weight basis, Ni primarily bioaccumulated in the gills, kidney and gut (Fig. 4.1). A similar trend was previously observed in adult rainbow trout exposed for 117 h to 198 µmol/L of waterborne Ni (Pane et al., 2003a). In previous studies, gill Ni bioaccumulation was found to be cellularly incorporated or loosely bound versus being blood-bound (Pane et al., 2004). Also a high Ni bioaccumulation in the gut contradicts the general belief that freshwater fish do not drink. Pane et al. (2003a) suggested stress-induced drinking as a potential mechanism for gastric Ni bioaccumulation, and ruled out hepatic clearance and subsequent biliary excretion as the cause due to the low level of bioaccumulation in the liver – as was also observed in the current study (Figs. 4.1, 4.2D). Both the brain and carcass (which is primarily comprised of muscle, bones, skin and scales) are not organs of large Ni bioaccumulation on a per weight basis, which is consistent with studies by Pane et al. (2003a). Therefore, on a per weight basis, the gills, gut and kidney are likely the prime target organs.

However, when the relative weights of each of the organs are considered on a percent basis the carcass is the main sink of Ni bioaccumulation in the whole animal as would be expected from an "organ" comprising 90% of the total weight of the fish (Fig. 4.2). Ni bioaccumulation in gills and gut correspond to ~ 8-12% of the total Ni in the control fish and 20-60% of the total Ni in the highest exposure. The contributions of the other organs to Ni bioaccumulation are relatively minor.

Saturable Ni binding in the gills, gut, kidney and whole fish (all organs combined) and subsequent calculation of Michaelis-Menten parameters allows for

the characterization of Ni binding (Fig. 4.3, Table 4.2). To the best of our knowledge this is the first study to show a comparison of binding constants (affinity and capacity) in organs other than the gills and to compare between two fish species of different habitat and lifestyle., though we have recently made such measurements in invertebrates (Leonard and Wood, 2013). Neither this study nor Leonard and Wood (2013) found a strong correlation between capacity values (B_{max}) and Ni toxicity in different species, but this was not the case with affinity values (K_d). The more sensitive species (lower LC50 value; round goby in this case) exhibited a higher affinity for Ni (lower K_d) in the gills (and although not significant, also in the kidney) than the more tolerant organism, rainbow trout (Table 4.2). This same trend of a lower K_d correlating with higher sensitivity was previously observed in a range of invertebrates acutely exposed to Ni (Leonard and Wood, 2013).

The opposite was true for the gut, where the affinity of the rainbow trout gut for Ni was over 3 x higher (i.e. lower K_d) than that of the round goby. The gut of round goby sampled from Hamilton Harbour contained mainly dipterans, oligochaetes, cladocerans, copepods, ostracods and dreissenids, some of which, namely dipterans and oligochaetes (Taraborelli et al., 2010) are predominant species in polluted aquatic environments (Winner et al., 1980), and are known to bioaccumulate toxins to a high degree (Seidman et al., 1986). Therefore, the gut of the round goby may have been previously exposed to contaminants via the diet. It is well established that when fish are chronically acclimated to sublethal metal concentrations there is an increase in the low-affinity, high capacity binding sites (Niyogi and Wood, 2003). Therefore, this may be reflective of the "pre-exposure" in Hamilton Harbour to Ni and other contaminants (see below).

It does not appear that much can be drawn from the Michaelis-Menten parameters in the whole fish; combining the organs may have obscured the results, and this is reflected in the high 95% confidence intervals on Kd and B_{max} estimates (Table 4.2). Nevertheless, the much greater accumulation of Ni in the carcass and therefore in the whole fish in the round goby (Fig. 4.3G) may relate to its greater sensitivity to Ni.

If a sigmoidal relationship is observed between % survival and Ni bioaccumulation, CBR values can be calculated, specifically a 96-h acute CBR50 value which corresponds to the tissue concentration at 50% mortality (Fig. 4.4) and a 96-h acute CBR10 value which represents a threshold value after which survival decreases as Ni bioaccumulation increases (Fig. 4.4). Both these parameters facilitate a standardized comparison of different tissues and different organisms. This sigmoidal relationship has also been observed in two invertebrate species (*Daphnia pulex* and *Lumbriculus variegatus*) acutely exposed to Ni (Leonard and Wood, 2013), as well as for Cu exposed *Lymnaea stagnalis* (Ng et al., 2012).

When comparing the two different methods for determining the 96-h acute CBR50 values with 95% C.I. (ToxCalc software and linear regressions of logit mortality vs. log Ni bioaccumulation), 96-h acute CBR50 values were less variable when calculated using the Toxcalc method in comparison to the logit mortality vs. log bioaccumulation method. This is most likely because the Toxcalc method is less influenced by 0 and 100% mortalities. However, both methods derive similar 96-h acute CBR50 values (Table 4.3). In addition, the Toxcalc software allows for calculation of more sensitive endpoints such as CBR10 values.

External effect concentrations (LC values) can vary due to water chemistry's influence on Ni bioavailability, however, internal effect concentrations (CBR50 values) should in theory vary much less. For Ni, Meyer et al. (1999) demonstrated that 24 h gill Ni bioaccumulation (LA50) in the fathead minnow (*Pimephales promelas*) was constant across wide range of water hardness even though the 96 h acute LC50s varied by 10 fold. In the current study, we made a comparison between two different species rather than varying the external water chemistry. There was a 2.19 fold difference between 96-h acute LC50 values whereas there was only a 1.09-1.25 fold difference (depending on the method used) between 96-h acute CBR50 values for the gills between these two species (Table 4.3). This suggests that 96-h acute CBR50 values for the gills are less species-dependent than LC50 values, which supports one of the main objectives of the TRA (and fundamental concept of the BLM) where tissue concentrations of the target organ (the gills) are generally less variable than exposure concentrations with respect to a toxicity response (Meyer et al., 1999; Luoma et al., 2009; Adams et al., 2011; Schmidt et al., 2011). Evidence similar to that of the current study which supports the TRA for metals is expanding (e.g. Redeker and Blust, 2004; Leonard et al., 2011; Ng et al., 2012; Leonard and Wood, 2013). This suggests that despite the large differences between these two fish (as well as the round goby's possible pre-exposure to Ni and other contaminants in Hamilton Harbour – see below), the threshold concentrations of Ni within a tissue which causes an effect are quite similar.

96-h acute CBR50 values are similar to the gill LA50 parameter (lethal accumulation which causes 50% mortality) which is incorporated into the calculation of the BLM. In the present study, 96-h acute CBR50 values were 263 µmol/kg wet wt. in goby and 239 µmol/kg wet wt. in trout. Reported 96-h gill LA50 values were 2079 µmol/kg wet wt. (Brix et al., 2004) for rainbow trout and 250 µmol/kg wet wt. for *P. promelas* (Meyer et al., 1999). The Brix et al. (2004) study used rainbow trout 18-d postswim-up which averaged 1.6 g wet wt, 6-8 x smaller than the fish in the current study, possibly explaining the much larger 96-h acute CBR50 value. The value for *P. promelas* correlates relatively well with the 96-h acute CBR50 values of the gill in the current study.

The fold differences for kidney (1.38-1.73), liver (1.00-1.90), and kidney (1.38 – 1.73) 96-h acute CBR50 values (Table 4.3) were also less variable than the differences in 96-h acute LC50 values. More information is required to determine whether these organs would be good predictors of Ni toxicity.

In the same manner, we can also derive 96-h acute CBR10 values using the Toxcalc method which are representative of a threshold value of the fish. There were no significant differences in 96-h acute CBR10 values of the gills and gut between the two species (Table 4.3), suggesting that in both species a similar bioaccumulation of Ni in these organs correlates to the onset of mortality. There was a significant difference between kidney, liver and whole fish 96-h acute CBR10 values, perhaps due to the larger concentration of Ni in the trout vs. the goby prior to laboratory exposure to Ni.

It should be noted that using the logit mortality vs. log bioaccumulation method, there is no significant difference between 96-h acute CBR50 values in the gills, gut, liver and whole fish however, this is due to the larger 95% confidence intervals, as we see this trend does not exist when using the Toxcalc method which derives less variable 95% confidence intervals (Table 4.3).

Acute Ni toxicity values and comparison between log K values derived from toxicity vs. bioaccumulation

To the best of our knowledge, there are no previous acute Ni toxicity studies on round goby. However, other studies on rainbow trout have reported acute (96-h) LC50 values for Ni of 138 and 255 μmol Ni/L with water hardness of 22 and 120 mg/L as $CaCO_3$, respectively (Atchison et al., 1987; Pane et al., 2003a) and are in good agreement with the current LC50 of 228 μmol Ni/L at a water hardness of 140 mg/L as $CaCO_3$.

Contrary to our predictions, round goby were 2.19 times more sensitive to waterborne Ni than rainbow trout with LC50 values of 104.1 μmol Ni/L and 228.1 μmol Ni/L, respectively. We had expected round goby to be more resistant to Ni due to the prevalence of this species in highly contaminated areas (Pinchuk et al., 2003). Potentially, while resistant to many toxicants, the round goby might be quite sensitive to Ni. However, a possible alternate explanation is that the goby used in this study had been collected from the wild. The collection site (LaSalle Park on Hamilton Harbour) is considered to be a "clean site" (Marentette et al., 2010), but Hamilton Harbour itself is a Canadian Area of Concern designated by the International Joint Commission (International Joint Commission, 1999). Many contaminants are known to be at problematic levels in other areas of Hamilton Harbour, namely: polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and metals including cadmium,

arsenic, lead, iron, mercury, zinc, and nickel (Hamilton Harbour RAP, 1992, 2003). Indeed, the goby had 2x more Ni in the gills and the gut than the rainbow trout purchased from a hatchery. It is possible that some residual influence of the Ni and other contaminants in either the diet or the water reduced the overall tolerance of the gobies leading to a higher sensitivity to Ni.

One of the main concepts of the BLM is that there is a strong overall correlation between log K values for gill binding and acute toxicity (Niyogi and Wood, 2004) to the extent that measurement of binding affinity based on gill metal binding as an acceptable alternative to measurement of toxicity and vice versa (MacRae et al., 1999; Niyogi and Wood, 2004). In the present study, we evaluated whether this concept could be extended beyond the biotic ligand of the gills (the known target side for acute toxicity of most metals in fish) to other organs. Specifically, in Table 4.5, 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). The log K_{NiBL} values calculated from the ionic component of the K_d value were considerably higher than log K_{NiBL} values for the ionic component of the LC50 value (toxicity) for the various organs or whole fish (Table 4.5). Specifically for the gills (considered the toxic site of action), there is a 0.8 log unit difference between the log K_{NiBL} for bioaccumulation vs. toxicity in round goby and 0.4 log unit difference in rainbow trout. This suggests that a BLM built on bioaccumulation would be more protective than one built on toxicity. A similar trend was observed for invertebrates (namely *Lymnaea stagnalis* and *Lumbriculus variegatus*) where the log K_{NiBL} for bioaccumulation was higher (~0.3-0.5 log units) than for toxicity (Leonard and Wood, 2013). However, the kidney log K_{NiBL} values are the same for round goby and very similar for rainbow trout (Table 4.5), indicating the kidney as an organ of interest with regards to Ni as was previously shown by Pane et al. (2005, 2006a,b). The correlation between log K_{NiBL} values for binding and acute toxicity does not appear to extend to other organs (gut or whole fish) which are not considered the toxic site of action.

Impact of Ni on fish behaviour

In the current study, we have shown that swimming activity, specifically movement, is a sensitive endpoint in comparison to lethality in round goby where the significant decline in movements per minute occurred at only 28% of the LC10. This is consistent with other studies that showed that the average toxicant exposure concentration that caused significant alterations in swimming behaviour was less than 16% of the concentration that caused 50% mortality (Little and Finger, 1990). To the best of our knowledge little is known regarding the effects of Ni on behavioural endpoints, however, acute sub-lethal concentrations of Ni

(25-85 µmol/L) have been shown to impact respiratory and aggressive behaviour as well as cause stress-related discomfort movements in the tilapia, *Oreochromis niloticus* (Alkahem, 1994).

The acute behavioural effect concentration in round goby was at or below 8.1 µmol Ni/L. Currently, the Canadian Water Quality Guideline (WQG) for Ni at a water hardness of 140 mg/L as $CaCO_3$ is 2.1 µmol Ni/L (calculated using the water hardness based equation $e^{0.76*\ln [hardness]+1.06}$ (expressed in µg/L); CCREM, 1987). In the United States, the Criterion Maximum Concentration (CMC – acute) for Ni is also based on water hardness (CMC $= e^{0.846*(\ln hardness) + 2.255}$; U.S. EPA 1995) and is 10.6 µmol Ni/L at 140 mg/L as $CaCO_3$. For the European Union, the Water Framework Directive, Environmental Quality Standard (EQS; based on a "user friendly" BLM which incorporates Ca, DOC and pH (ECB, 2008)) is 0.043 µmol Ni/L (based on water chemistry from Table 4.1). It should be noted that the Canadian WQG and European Union's EQS values are chronic values. Therefore, round goby may be at risk of ecological death in the United States based on the current CMC for Ni; however, this species is protected by Ni water quality guidelines in Canada and the European Union. The behavioural effects of chronic exposures to Ni in round goby, however, remain uncertain.

The behavioral effect of acute Ni exposure on rainbow trout was much less pronounced than in round goby. Metals often, but not always, affect swimming activity at levels lower than the LC50, in a range of fish taxa (Little and Finger, 1990; Scott and Sloman, 2004). The 96-h LC50 for rainbow trout exposed to copper was approximately 0.5 µmol/L (hardness as $CaCO_3$ of 30-102 mg/L, Howarth and Sprague, 1978), while LOECs for homing behaviour after 37-40 weeks (Saucier et al., 1991, Saucier and Astic, 1995) were 0.3 µmol/L, at 61 mg/L hardness. The 96-h LC50 for rainbow trout exposed to cadmium was 0.2 µmol/L at 140 mg/L hardness (Szebedinsky et al., 2001, Hollis et al., 1999), while LOECs for alarm substance responses after 7 days (Scott et al., 2003) and agonistic behaviours after 24 hours (Sloman et al., 2003a,b) were 0.02 µmol/L at 120 mg/L hardness, respectively. It may be the case that Ni, like Cu, only affects trout behaviours at levels comparable to the 96-h LC50; however it is also possible that if we had measured activity levels after different exposure times to Ni, or different behaviours altogether (such as olfaction or aggression), different patterns would have been revealed.

Other metals have been shown to cause effects on behaviour either through the olfactory system, the brain (see Sloman, 2007 for review), or through alterations to metabolic load (Allin and Wilson, 1999). The results of the current study, suggest there is an efficient blood-brain barrier against Ni in both species. However, we cannot exclude indirect effects on the brain as a copper-exposed carp showed no accumulation of Cu in the brain, but exhibited an indirect effect of decreased 5-HT in the brain (De Boeck et al., 1995).

Link between changes in behaviour and Ni exposure concentration or Ni bioaccumulation

Traditionally, most toxicity endpoints are linked to the concentration of the metal, in this case Ni, in the environment (Meyer et al., 1999; DiToro et al., 2001; Niyogi and Wood, 2004); however, there is increasing evidence that Ni bioaccumulation may also be a good predictor of toxicity endpoints (see above; Luoma et al., 2009; Adams et al., 2011; Schmidt et al., 2011; Leonard and Wood, 2013), and perhaps even behavioural endpoints. Here we have compared the correlation between changes in behaviour with both Ni exposure concentrations and Ni bioaccumulation in organs or whole fish (Table 4.6). We observe a stronger correlation between Ni bioaccumulation in the gills or whole fish and behaviour than is found between Ni exposure concentration and behaviour. To our knowledge, this is the first study to link Ni bioaccumulation with behavioural endpoints such as swimming/spontaneous movement. Our integrative approach explored the response of two very different species, one of which showed a clear behavioural effect of exposure to Ni. In future it would be of interest and benefit to expand these analyses to a wider range of species to determine whether Ni bioaccumulation, specifically in the gills, gut and whole fish, may be a good predictor of behavioural changes from metal exposure; which in the wild can lead to ecological death.

ACKNOWLEDGEMENTS

Table 4.1
Water chemistry for Ni exposures. All ion concentrations are represented in μmol/L with the exception of DOC (mg/L), hardness and alkalinity (mg/L as $CaCO_3$) and pH. Values are means ± S.E.M., n = 20-30 per value.

	Round goby	Rainbow trout
Na	881 ± 4	856 ± 2
K	42 ± 3	40 ± 2
Cl	971 ± 6	980 ± 6
Ca	1078 ± 3	1069 ± 5
Mg	401 ± 2	370 ± 6
Hardness	148 ± 2	144 ± 5
DOC	2.4 ± 0.2	2.1 ± 0.4
Alkalinity	95 ± 5	97 ± 3
pH	7.8 ± 0.1	7.8 ± 0.1

Table 4.2

Michaelis-Menten kinetic constants (B_{max} and K_d) for saturable Ni bioaccumulation in the gill, gut, kidney and whole fish of round goby and rainbow trout. Values are means ± S.E.M. Different letters denote a significant difference between B_{max} or K_d values within a species. An asterisk * denotes a significant difference between a B_{max} or K_d values of an organ or whole fish between the two species. The B_{max} and K_d values were not calculated for the liver in either species and the carcass for round goby as the relationship between Ni exposure concentration and organ Ni bioaccumulation was linear. As well, in the brain and carcass of trout, no relationship was found between Ni exposure and organ Ni concentration.

	B_{max}	K_d	r^2
	(µmol/kg wet wt)	(µmol Ni/L)	
Goby			
Gill	303.9 ± 54.1^a	17.8 ± 11.7^a	0.87
Gut	509.4 ± 71.8^a	242.6 ± 58.1^a	0.99
Kidney	396.2 ± 84.6^a	107.8 ± 52.0^a	0.97
Whole fish	275.5 ± 422.4^a	539.6 ± 1116.8^a	0.85
Trout			
Gill	277.6 ± 20.1^a	$86.4 \pm 16.8^{a*}$	0.99
Gut	$67.7 \pm 9.6^{b*}$	$75.3 \pm 29.5^{a*}$	0.94
Kidney	512.1 ± 101.8^c	211.2 ± 86.4^a	0.97
Whole fish	19.5 ± 1.9^b	46.5 ± 15.6^a	0.91

Table 4.3

96-h acute CBR values for Ni in μmol/kg wet wt. with lower and upper 95% confidence intervals in brackets. 96-h acute CBR50 values were calculated either by logit vs. log method or by Toxcalc.* denotes a significant difference in 96-h acute CBR50 or CBR10 values between the two species within an organ or whole fish. The CBR50 values were not calculated for brain and carcass as the regression analysis between organ Ni bioaccumulation and survival was not significant at $p > 0.05$ or the coefficient of determination (r^2) was less than 0.6.

	96-h acute CBR50 values calculated from logit vs. log (µmol/kg wet wt)	Fold difference	96-h acute CBR50 values calculated from Toxcalc (µmol/kg wet wt)	Fold difference	96-h acute CBR10 values calculated from Toxcal (µmol/kg wet wt)	Fold difference
Gills						
Goby	263.0 (210.2-305.3)	1.09	247.5 (234.-262.5)	1.25	167.0 (152.9-178.5)	1.02
Trout	239.3 (190.7-267.7)		197.4* (191.4-203.8)		164.3 (155.0-171.3)	
Gut						
Goby	141.8 (63.0-254.7)	3.02	148.9 (91.4-297.6)	2.89	49.7 (10.9-82.9)	1.21
Trout	130.9 (8.57-512.9)		51.4* (49.5-53.5)		41.0 (38.2-43.2)	
Kidney						
Goby	173.4 (125.2-230.1)	1.73	190.6 (70.1-3335.2)	1.38	48.6 (0.1-113.7)	3.98
Trout	299.8* (279.2-358.8)		264.9 (251.2-279.8)		193.3* (147.9-207.7)	
Liver						
Goby	9.0 (ND)	1.90	23.5 (14.9-49.8)	1	7.5 (3.8-11.7)	2.79
Trout	17.5 (8.4-26.7)		23.5 (23.1-24.0)		20.9* 20.2-21.5	
Whole fish						
Goby	60.9 (16.1-357.3)	2.40	46.4 (36.3-66.4)	2.74	22.0 (14.9-28.1)	1.53
Trout	25.4 (8.1-37.2)		16.9* (16.5-17.4)		14.4* (13.8-14.8)	

Table 4.4
96-h acute LC10 and LC50 values for Ni in µmol/L with lower and upper 95% confidence intervals in brackets for round goby and rainbow trout.* denotes a significant difference in toxicity values between the two species.

	LC10 (µmol/L)	Fold difference	LC50 (µmol/L)	Fold difference
Goby	29.0 (12.2-46.8)	3.80	104.1 (68.8-160.4)	2.19
Trout	110.3* (89.2-129.7)		228.1* (201.5-257.5)	

Table 4.5
Log K_{NiBL} values based on bioaccumulation (K_d values, various organs) and log K_{NiBL} values based on toxicity (LC50) values in round goby and rainbow trout. There was no significant difference between K_d values for various organs within a species. An asterisk * denotes a significant difference between K_d values of an organ or whole fish between the two species. Different letters denotes significant difference between log K_{NiBL} values within a species. [+] denotes a significant difference between log K_{NiBL} values for bioaccumulation and toxicity

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	Bioaccumulation			Toxicity		
	K_d value (µmol Ni/L)	Ionic Ni component of K_d value (µmol Ni/L)	$\log K_{NiBL}$ values	LC50 value (µmol Ni/L)	Ionic Ni component of LC50 value (µmol Ni/L)	$\log K_{NiBL}$ values
Goby						
Gill	17.8 ± 11.7	13.9 ± 9.1	4.86[a+] (4.64-5.32)			
Gut	242.6 ± 58.1	189.2 ± 45.3	3.72[b+] (3.63-3.84)	104.1 (68.8-160.4)	80.2 (53.0-123.5)	4.09 (3.91-4.28)
Kidney	107.8 ± 52.0	81.9 ± 39.5	4.09[c] (3.92-4.37)			
Whole fish	539.6 ± 1131.8	415.5 ± 871.5	3.38[abc] (ND)			
Trout						
Gill	86.4 ± 16.8*	67.4 ± 13.1	4.17[a+] (4.09-4.26)			
Gut	75.3 ± 29.5*	58.7 ± 23.0	4.23[a+] (4.09-4.45)	228.1 (201.6-257.5)	175.6 (155.2-198.3)	3.76 (3.70-3.81)
Kidney	211.2 ± 86.4	164.7 ± 67.4	3.78[b+] (3.63-4.01)			
Whole fish	46.5 ± 15.6	36.0 ± 12.0	4.44[a+] (4.32-4.62)			

Table 4.6
Spearman rank correlation between fish behavioral inhibition and Ni
concentration in the exposure water or Ni bioaccumulation within an organ or
whole fish. A negative Spearman correlation coefficient corresponds to a decrease
in behaviour as the Ni exposure concentration or Ni bioaccumulation within an
organ or whole fish increases. $P < 0.05$ is considered significant.

1st variable	2nd variable	Spearman rank correlation coefficient (rho value)	P value
Behaviour	Water exposure concentration	-0.544	0.05
Behaviour	Gill	-0.6044	0.03
Behaviour	Gut	-0.5604	0.05
Behaviour	Kidney	-0.4835	0.09
Behaviour	Liver	-0.0769	0.80
Behaviour	Brain	-0.0055	0.98
Behaviour	Carcass	-0.3846	0.19
Behaviour	Whole fish	-0.6044	0.03

Fig. 4.1

Ni bioaccumulation, represented in μmol/kg wet wt, in organs of round goby and rainbow trout over a range of exposure concentrations following a 96-h waterborne exposure. Values are means ± S.E.M.; n = 8 per treatment for round goby and n = 6 per treatment for rainbow trout.

A   Round goby
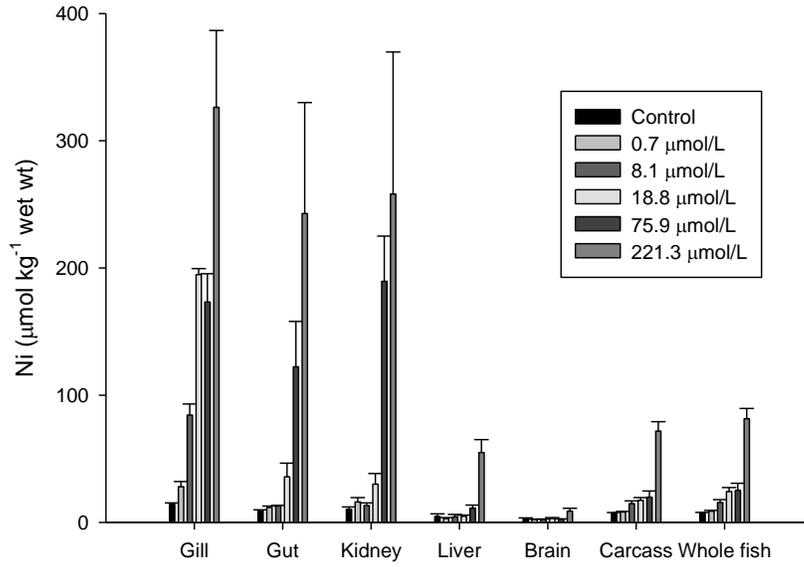
B   Rainbow trout
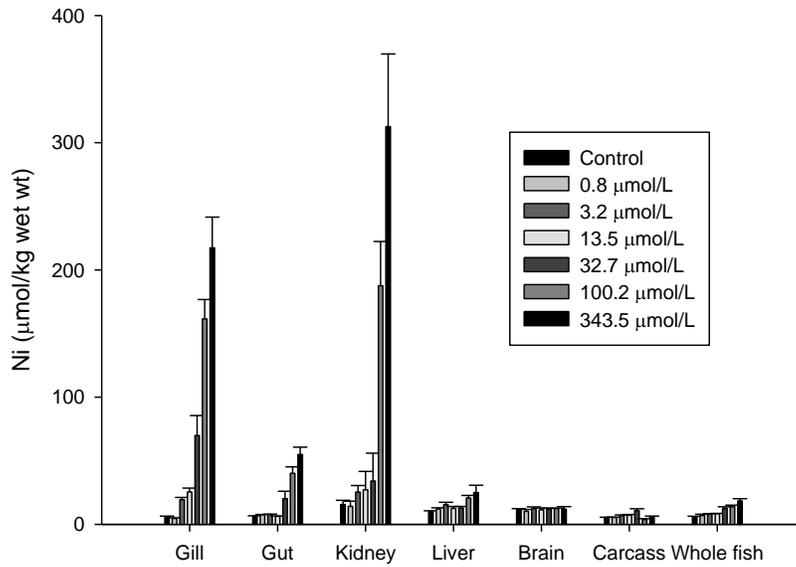
Fig. 4.2
Pie charts reflecting the average percentage Ni content (i.e. as a percentage of the whole body burden) in each organ in the (A,C) control fishes and (B,D) highest exposure fishes following an acute (96-h) waterborne exposure to Ni. N = 8 per treatment for round goby and n = 6 per treatment for rainbow trout.

A Control - Round goby
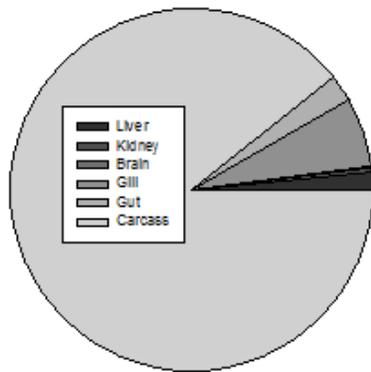
B 221.3 µmol Ni/L - Round goby
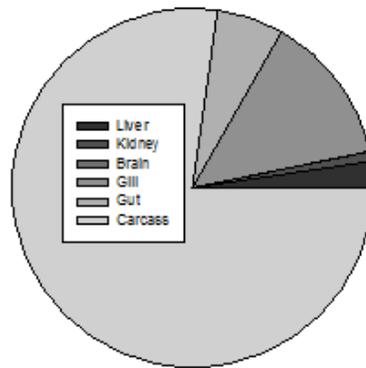
C Control - Rainbow trout
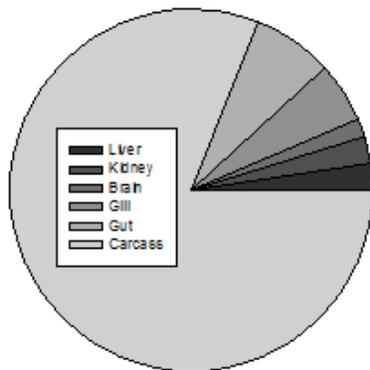
D 343.5 µmol Ni/L - Rainbow trout
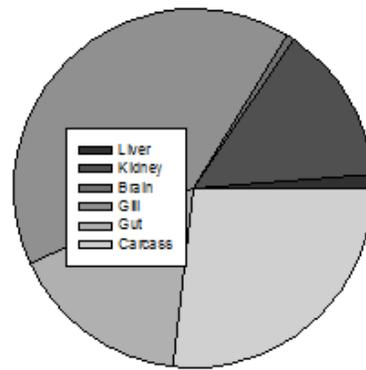
Fig. 4.3
Ni bioaccumulation in (A) gills, (B) gut, (C) kidney, (D) liver, (E) brain, (F) carcass and (G) whole fish (organs combined) over a range of exposure concentrations following a 96-h exposure to round goby and rainbow trout. Values are means ± S.E.M.; n = 8 per treatment for round goby and n = 6 per treatment for rainbow trout. Non-linear regression analyses were performed to determine the concentration-dependent kinetics of Ni bioaccumulation with a hyperbolic curve fit. Curves were not fitted if the relationship between exposure Ni concentration and Ni bioaccumulation was not saturable.

A

Gill Ni (μmol/kg wet wt)

- Trout
- Goby

K_d = 17.8 μmol/L
Bmax = 303.9 μmol/kg
r^2 = 0.87

K_d = 86.4 μmol/L
Bmax = 277.6 μmol/kg
r^2 = 0.99

Ni (μmol/L)

B

Gut Ni (μmol/kg wet wt)

- Trout
- Goby

K_d = 242.6 μmol/L
Bmax = 509.4 μmol/kg
r^2 = 0.99

K_d = 75.3 μmol/L
Bmax = 67.7 μmol/kg
r^2 = 0.94

Ni (μmol/L)

C

Kidney Ni (μmol/kg wet wt)

- Trout
- Goby

K_d = 107.8 μmol/L
Bmax = 396.2 μmol/kg wet wt
r^2 = 0.97

K_d = 211.2 μmol/L
Bmax = 512.1 μmo/kg wet wt
r^2 = 0.97

Ni (μmol/L)

D

Liver Ni (μmol/kg wet wt)

- Trout
- Goby

$y = 0.23x + 1.43$
r^2 = 0.96

$y = 0.04x + 12.80$
r^2 = 0.80

Ni (μmol/L)

E

Brain Ni (μmol/kg wet wt)

- Trout
- Goby

Ni (μmol/L)

F

Carcass Ni (μmol/kg wet wt)

- Trout
- Goby
- $y = 0.27x + 8.40$
  r^2 = 0.96

Ni (μmol/L)

G

Whole fish Ni (μmol/kg wet wt)

K_d = 539.6 μmol/L
Bmax = 275.5 μmol/kg wet wt
r^2 = 0.85

- Trout
- Goby

K_d = 46.5 μmol/L
Bmax = 19.5 μmol/kg wet wt
r^2 = 0.96

Ni (μmol/L)

Fig. 4.4

Correlation between survival and (A) gill, (B) gut, (C) kidney, (D) liver Ni bioaccumulation in round goby and rainbow trout. Values are means ± S.E.M.; n = 2 replicate toxicity tests with 6 (rainbow trout) and 8 (round goby) per exposure concentration for % survival and n = 6-8 for Ni bioaccumulation. The lines at 50% survival intersect the bioaccumulation vs. mortality relationships at the 96-h acute CBR50 values, which are indicated on the Figure panel and were calculated using Toxcalc software.
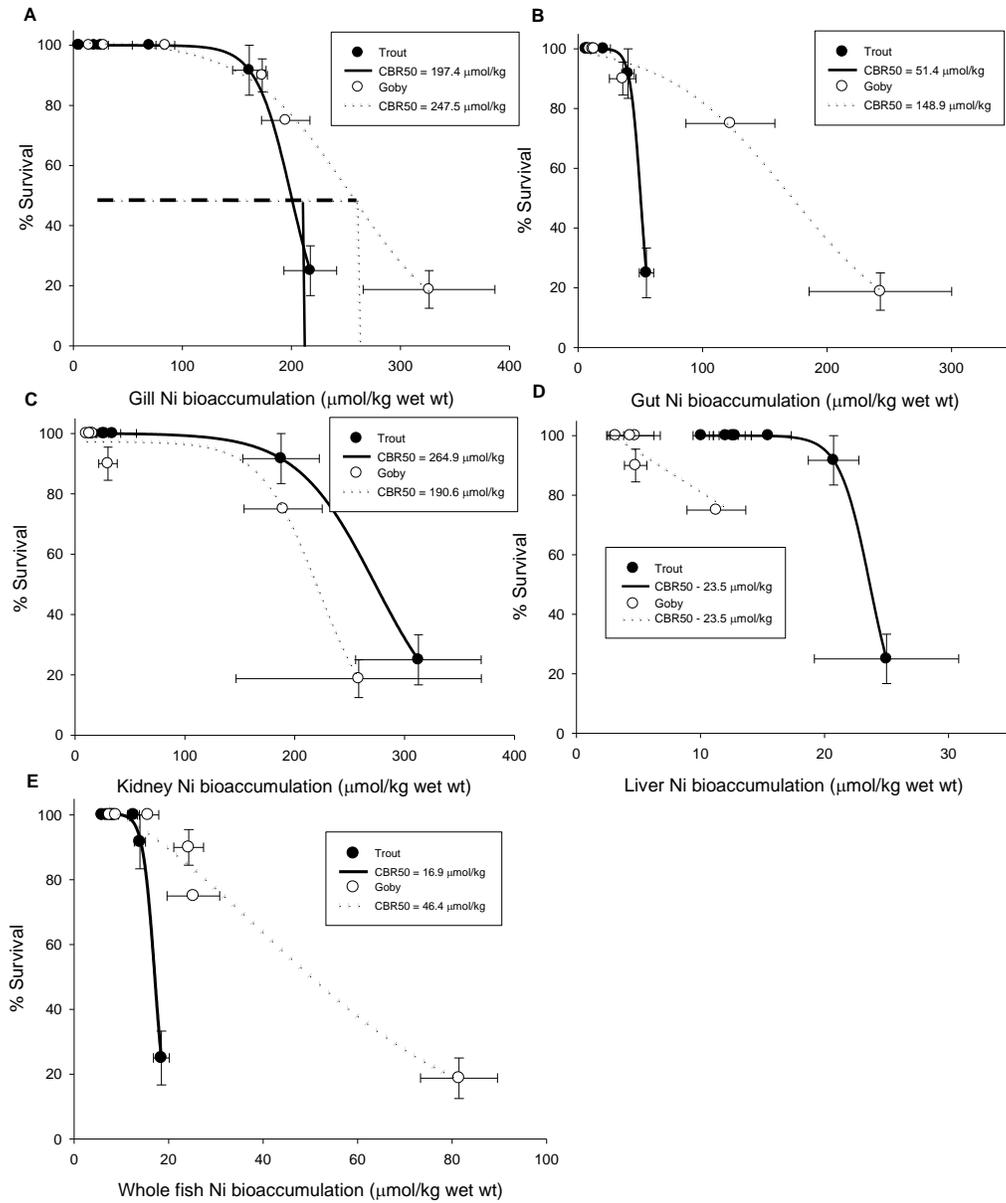
Fig. 4.5

Impact of Ni on behavioural endpoints in round goby (A; movements/min) and rainbow trout (B; mean velocity/min) exposed to 96-h of waterborne Ni. Values are means ± S.E.M.; n = 8 per treatment for round goby and n = 6 per treatment for rainbow trout. Statistical significance was determined using Dunnett's test to compare treatment groups to the control group and * denotes a significant difference from control value. There was no significant difference in mean velocity/min relative to the control value in rainbow trout at any exposure concentration.

A   Round goby
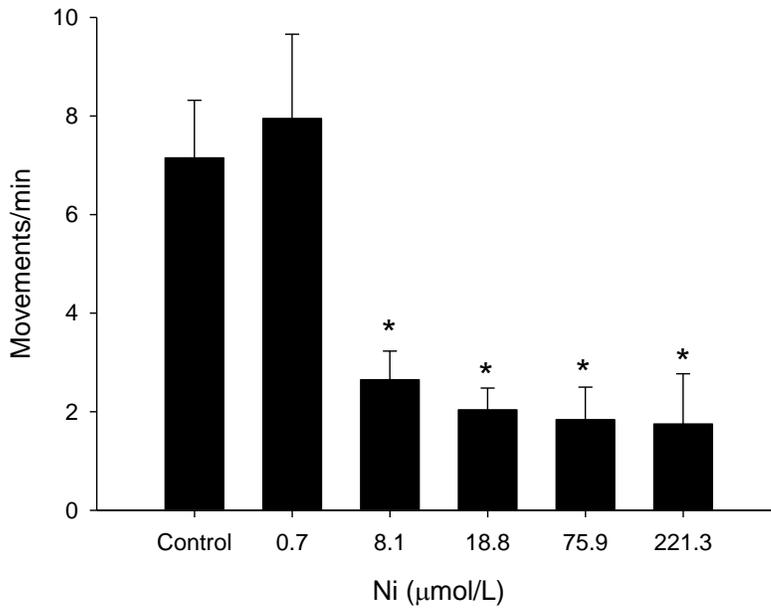
B   Rainbow trout
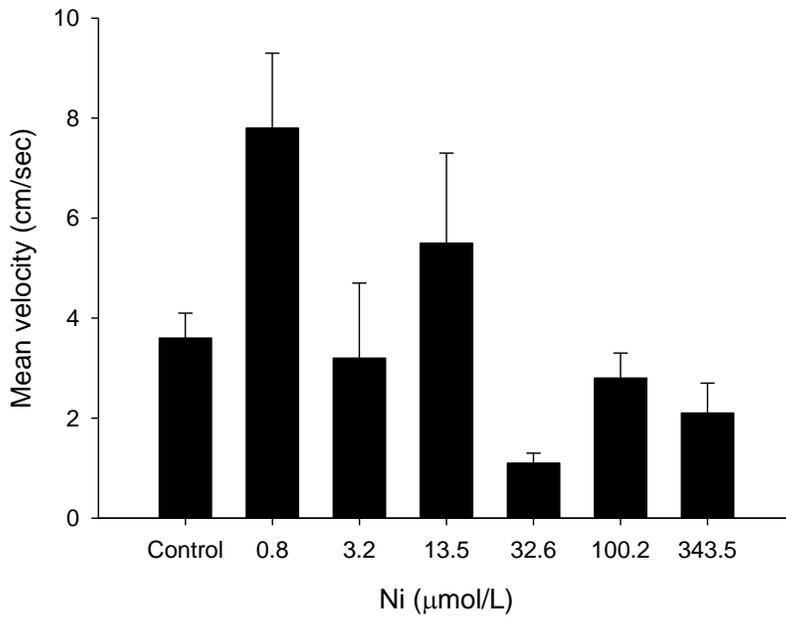
Supplementary Table 4.1
Average Ni exposure concentrations expressed as different fractions over the 96-h test period for each species.

	Nominal (μmol Ni/L)	Total (μmol Ni/L)	Dissolved (μmol Ni/L)	Ionic (μmol Ni/L)	Active (μmol Ni/L)
Round goby					
	Control	0.03	0.03	0.02	0.01
	1	0.9	0.7	0.6	0.5
	10	8.4	8.1	6.1	4.6
	33.2	21.9	18.8	14.4	10.7
	100	77.3	75.9	58.6	43.5
	250	234.6	221.3	173.0	127.2
Rainbow trout					
	Control	0.02	0.03	0.02	0.01
	1	0.9	0.8	0.6	0.4
	3.3	3.2	3.2	2.4	1.8
	10	14.1	13.5	10.3	7.7
	33.3	33.0	32.7	25.0	18.7
	100	100.2	100.2	77.3	57.4
	333.3	351.3	343.5	268.1	197.5

CHAPTER 5

CHRONIC NICKEL BIOACCUMULATION AND SUB-CELLULAR FRACTIONATION IN TWO FRESHWATER TELEOSTS, THE ROUND GOBY AND THE RAINBOW TROUT: EXPOSED SIMULTANEOUSLY TO WATERBORNE AND DIETARY NICKEL

ABSTRACT

Rainbow trout and round goby were exposed for 30 days to waterborne and dietary Ni in combination at two waterborne concentration ranges (5.8 – 11.2 µmol/L, 62.0 – 79.0 µmol/L), the lower of which is typical of contaminated environments. The prey (black worms; *Lumbriculus variegatus*) were exposed for 48 h in the effluent of the fish exposure tanks before being fed to the fish (ration = 2% body weight/day). Ni in gills, gut, and prey was fractionated into biologically inactive metal [BIM =metal-rich granules (MRG) and metallothioneins (MT)] and biological active metal [BAM = organelles (ORG) and heat-denaturable proteins (HDP)]. Goby were more sensitive than trout to chronic Ni exposure. Possibly, this greater sensitivity may have been due to the goby's pre-exposure to pollutants at their collection site, as evidenced by ~2-fold greater initial Ni bioaccumulation in both gills and gut relative to trout. However, this was followed by ~2-16 x larger bioaccumulation in both the gills and the gut during the experimental exposure. On a subcellular level, ~ 3-40 x more Ni was associated with the BAM fraction of goby in comparison to trout. Comparison of the fractional distribution of Ni in the prey versus the gut tissue of the predators suggested that round goby were more efficient than rainbow trout in detoxifying Ni taken up from the diet. Assessing sub-cellular distribution of Ni in the gills and gut of two fish of different habitat and lifestyles revealed two different strategies of Ni bioaccumulation and sub-cellular distribution. On the one hand, trout exhibited an ability to regulate gill Ni bioaccumulation and maintain the majority of the Ni in the MT fraction of the BIM. In contrast goby exhibited large Ni spillovers to both the HDP and ORG fractions of the BAM in the gill. However, the same trend was not observed in the gut, where the potential acclimation of goby to pollutants from their collection site may have aided their ability to regulate Ni spillover to the BAM more so than in trout. Overall, chronic mortality observed in goby may be associated more with Ni bioaccumulation in gills than in gut; the former at either 4-d or 30-d was predictive of chronic Ni toxicity. BIM and BAM fractions of the

goby gills were equally predictive of chronic (30-d) mortality. However, critical body residue (CBR50) values of the BIM fraction were ~ 2-4 x greater than CBR50 values of the BAM fraction, suggesting that goby are more sensitive to Ni bioaccumulation in the BAM fraction. There was insufficient mortality in trout to assess whether Ni bioaccumulation was predictive of chronic mortality.

INTRODUCTION

Total and dissolved metal concentrations in the water alone can be poor predictors of environmental threats to aquatic organisms (Borgmann, 1983; Borgmann et al., 2004). Therefore, much emphasis has been placed on the role that chemical speciation of metals in the environment plays in metal toxicity (Pagenkopf, 1983; Morel, 1983; Campbell, 1995). However, in recent decades, the emphasis has shifted to the role of metal bioaccumulation in metal toxicity. For example, the Biotic Ligand Model (BLM) uses site-specific water chemistry parameters in conjunction with the binding constants of the biotic ligand to predict whether sufficient metal will bind to the organism to cause acute toxicity (Paquin et al., 2000; Di Toro et al., 2001; Niyogi and Wood, 2004). Short term metal bioaccumulation is used to predict longer term toxicity (e.g. Meyer et al., 1999). In addition, the Tissue Residue Approach (TRA) correlates tissue bioaccumulation with adverse biological effects (e.g. mortality) and in this manner bioaccumulation can be used to predict the toxicity within and across species (Connolly, 1985; McCarty and McKay, 1993; Luoma et al., 2009; Borgmann et al., 2001; Adams et al., 2011; Schmidt et al., 2011). These methods may be used independently or together as a tool for assessing the toxicity to aquatic organisms.

Expressing toxicological effect as a function of the bioaccumulation of a metal such as Ni has many advantages: it integrates all exposure routes (e.g. water column and food), it incorporates changes in water chemistry over time, and it assimilates the toxicokinetics of different species (U.S. EPA, 2007). Indeed, bioaccumulation of metals has been found to be a better predictor of toxicity than exposure water concentrations (Borgmann et al., 1991; Borgmann and Norwood, 1997; Borgmann et al., 2001). However, metal bioaccumulation is not constant over time; there can be regulation of uptake and/or elimination, or sub-cellular compartmentalization, both of which can change, rendering the metal more or less toxic (Adams et al., 2011).

In order to better understand the latter, Wallace et al. (2003) devised a protocol for separating intracellular fractions which can be classified into biologically inactive metal (BIM; comprising metal-rich granules (MRG) and metallothionein protein or metallothionein-like proteins (MT)) and biological active metal (BAM; comprising organelles (ORG) and heat-denaturable proteins (HDP); Rainbow, 2002). When metal levels exceed a threshold value in the BIM, there is metal spill-over into the BAM fractions which leads to deleterious chemical effects. This may be correlated to adverse biological effects such as failed recruitment or mortality. In this manner, the use of subcellular metal residues for the TRA may improve the effectiveness for toxicity assessment (Adams et al., 2011). Therefore assessing changes to bioaccumulation patterns

both on a whole organ level and a sub-cellular level over a chronic time scale may further explain mechanisms of Ni toxicity.

Traditionally, the majority of information gained on metal toxicity has correlated gill metal bioaccumulation with mortality in models such as the BLM. Overall, gill metal (e.g. Ni) bioaccumulation from waterborne metal exposure is a good predictor of acute toxicity (Meyer et al., 1999); however, aquatic organisms such as fish use not only their gills, but also their gut to exchange the necessary nutrients and minerals, and to eliminate waste (Randall et al., 2002). In fact, in terms of metal exposure, many studies have shown that dietary exposure via prey can be as important as the waterborne route for metal bioaccumulation and associated toxicity (Spry et al., 1988; Munger and Hare, 1997; Zhang and Wang, 2005; Pyle et al., 2005; Farag et al., 2007). Therefore, in the present study, both the gills and the gut were assessed as potential biotic ligands for nickel following a joint waterborne and dietary exposure so as to best simulate their natural exposure conditions. The overall advantage of this approach is the integration of exposure routes when using the TRA as a method for predicting toxicity to aquatic organisms.

We chose to compare two fish species with different habitats and lifestyles: the round goby (*Neogobius melanostomus*) and the rainbow trout (*Oncorhynchus mykiss*). The round goby were collected from a "clean site" at LaSalle Park on Hamilton Harbour (Marentette et al., 2010), but the Harbour itself was designated by the International Joint Commission as a Canadian Area of Concern due to the many contaminants known to be problematic namely: polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and metals including cadmium, arsenic, lead, iron, mercury, zinc, and nickel (Hamilton Harbour RAP 1992, 2003). In contrast, the rainbow trout were purchased from a hatchery served with pristine water. The prey species, *Lumbriculus variegatus*, was chosen based on its ability to meet nutritional requirements, to be accepted by fish and its prevalence as a food source for these fish (Taraborelli et al., 2010; Mount et al., 2006). In our experimental design, the prey was pre-exposed to the same waterborne concentration of Ni before being fed to the two fish species, so as to simulate natural exposure conditions. Round goby are known to be a pollution-tolerant species (Pinchuk et al., 2003). However, a recent study conducted in our laboratory showed a greater sensitivity of this species to acute Ni toxicity (Leonard et al., submitted = chapter 4) in comparison to rainbow trout - one of the most sensitive teleosts to metals (Nebeker et al., 1985; U.S. EPA, 1995).

Therefore, our overall goal was to compare the Ni bioaccumulation patterns of these two species to joint waterborne and dietary exposures at an environmentally relevant waterborne Ni concentration (nominally 10 µmol Ni/L) and a more toxic concentration (nominally 60 µmol Ni/L). By way of reference, Ni levels as high as 17 µmol Ni/L have been reported in contaminated natural waters (Eisler, 1998), while levels of regulatory significance in Canada are 2.1

μmol Ni/L (a chronic value; CCREM, 1987) and in the United States are 10.6 μmol Ni/L (acute) and 1.2 μmol Ni/L (chronic), respectively (U.S. EPA, 1995). By assessing Ni bioaccumulation patterns on both a whole organ level as well as a sub-cellular level we aimed to determine if differences in toxic response were associated with different strategies of metal compartmentalization.

Therefore, with this background in mind, our specific objectives were: (1) to assess survival over chronic (30-d) exposures to combined waterborne and dietary Ni at two exposure concentrations, (2) to measure and compare whole-organ gill and gut Ni concentrations as well as Ni in their sub-cellular fractions at several time points over these chronic exposures; (3) to assess the changes in BIM and BAM fractions in the two organs; and (4) thereby to evaluate the use of acute subcellular bioaccumulation as the residue indicator of chronic Ni toxicity in rainbow trout and round goby.

METHODS

Experimental organisms

Round goby, *N. melanostomus*, were collected during the weeks of Sept 26 – Oct 7th, 2011 from Hamilton Harbour at LaSalle Park (43°18'1" N, 79° 50'47"W). The background Ni concentration at this site averaged 0.31 ± 0.02 μmol/L. Commercial minnow traps baited with frozen corn and set at a depth of 1 m or less for 24 h were used to capture one hundred and eight round goby (mean body mass 11 ± 3 g). Fish were then transported back to the laboratory and acclimated for two weeks to laboratory conditions in 500-L containers served with flow-through, aerated, dechlorinated Hamilton (Ontario, Canada) tap water. PVC tubes were used for shelter and the round goby were fed *ad libitum* every second day with Big Al's Staple Flake Food (45 % protein, 5 % crude fat, 2 % crude fibre and 8 % moisture; Big Al's Aquarium Supercentres, Woodbridge, ON, Canada).

One hundred and eight rainbow trout, *O. mykiss*, 12 ± 1 g were purchased from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada. They were contained in 500-L tanks receiving flow-through dechlorinated Hamilton tap water. Trout were fed 2% body weight, every second day, with Martin's commercial dried pellet feed (Martin Mills Inc., Ontario, Canada).

Black worms (*Lumbriculus variegatus*) were purchased from Aquatic Foods (Fresno, CA, USA) and were kept in 80-L aquaria with a flow-through of continuously aerated dechlorinated Hamilton 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.

All organisms were kept in Hamilton dechlorinated tap water with an ionic composition of (in mmol/L) Na (0.9), Cl (1.0), Ca (1.0), K (0.4), Mg (0.4), and Ni ($<0.3 \times 10^{-5}$). Water hardness was ~140 mg/L as $CaCO_3$ equivalents; pH was 7.8, alkalinity 95 mg/L, water temperature was $12 \pm 2^{o}C$, dissolved organic carbon (DOC) was 2.2 mg/L and the photoperiod was 16:8 h light:dark.

Flow through exposure system

Ni stock solutions, made with $NiCl_2 \cdot 6 H_2O$ (Sigma Aldrich, St. Louis, Missouri, USA, CAS # 7791-20-0), were held in Mariotte bottles above the exposure tanks. Dechlorinated Hamilton tap water (750 ml/min) was mixed with 0.5 ml/min of Ni stock solutions from the Mariotte bottles in a mixing bucket before being administered to the exposure tanks containing the fish.

Mariotte bottle drip rates and flow rates of dechlorinated water were monitored daily. Water samples were taken every 24 h from each exposure to determine total and dissolved (0.45 µm filtration, see below) concentrations of Ni. Tanks were checked daily for impacted fish (defined as fish which had lost equilibrium and had turned upside down) which were removed immediately.

Thirty-six round goby and rainbow trout were transferred to each exposure tank (500-L) for 48 h prior to Ni exposure to allow time for acclimation to the new environment and were fed 2% body weight of black worms every second day throughout the 30-d trial. Nominal water exposure concentrations were 0, 10, 60 µmol Ni/L. Black worms were held in the out-flow from the fish exposure tanks for 48-h prior to being fed to fish. This allowed for the concentration of Ni in the prey to be constant throughout the experiment and attempt to better simulate the natural environment where both predators and prey are exposed to the same water chemistry. All black worms were either consumed or removed from the tank within 15 min.

Water chemistry

Mean water chemistry parameters for all experiments are shown in Supplementary Table 5.1. Measured total and dissolved Ni concentrations along with specific water chemistries were used to estimate the free ionic nickel (Ni^{2+}) concentrations and Ni^{2+} activity using Visual MINTEQ software (ver. 3.0, beta, KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden). The active fraction is a measure of the effective activity of Ni in these water chemistries, which is determined by concentration and by interactions (i.e. attraction or repulsion) of other molecules in solution. The NICA-Donnan model was used in the calculations to estimate the effect of DOC on Ni speciation.

Average Ni water concentrations of each fraction: nominal, total, dissolved, ionic and active fractions of the metal, taking into account the measured water chemistry from Supplementary Table 5.1, and are reported in the Supplementary Information Section Table 5.2. All Ni water concentrations presented in this study are reported as the dissolved fractions of the metal, which were 92 % of the total values (Supplementary Table 5.2).

Sub-cellular fractionation

The gills and gut of rainbow trout and round goby and the whole body of the prey, *Lumbriculus variegatus*, underwent tissue homogenization followed by a differential centrifugation procedure to separate the tissues into five operationally defined fractions: metal rich granules (MRG), organelles (ORG), heat-denaturable proteins (HDP), metallothionein proteins (MT) and cellular debris (CD). The sub-cellular fractionation protocol generally followed the procedure of Wallace et al. (2003), Lapointe et al. (2009), and Ng et al. (2011). Tissues were stored at -80°C and thawed on ice prior to subcellular fractionation. Tissues (required to be > 0.2 g (~20 black worms were amalgamated to obtain this weight)) were weighed, then homogenized in 3-5 volumes of homogenization buffer which includes TRIS-base 20 mM, pH 7.6, with 2 mM 2-mercaptoethanol and 0.2 mM phenylmethanesulfonylfluoride (PMSF). One third of the sample was used for measuring metal bioaccumulation for metal recovery. The remainder was then centrifuged at 1,450 g at 4 $^{\circ}$C for 15 min. The pellet was washed with buffer and re-centrifuged at the same speed to minimize the presence of cellular debris (unbroken cells, cell fragments and cell membranes).The pellet was washed with 2 ml of 1 N NaOH and heated in the water bath at 80 $^{\circ}$C for 15-30 min. The mixture was then spun at 5,000 g for 10 min to collect the cellular debris (CD; supernatant) and the metal rich granules (MRG; pellet). The supernatant collected after the 1,450 g spin was centrifuged at 100,000 g, 4°C, for 1 h for separation of the organelles (ORG; pellet) and cytosol (supernatant). The cytosol fraction was then heated at 80 $^{\circ}$C for 15 min and recentrifuged to separate the HDP (pellet) from the metallothionein (MT; supernatant) by centrifugation at 50,000 g for 10 min. Overall, recovery of Ni was 109 % ± 9 (sum of Ni in each fraction x 100% / Ni in homogenate).

In Figures, the BAM fractions (comprising HDP and ORG) are shown above the zero line, whereas the BIM fractions (comprising MRG and MT) are represented below the zero line. The CD fraction is not presented in the Figures as it represents the broken cellular fragments during fractionation and it is not classified as either BIM or BAM. On average, the CD fraction accounted for 35 % of the total organ Ni.

Tissue sampling

Six fish were sampled from each exposure concentration on days 0, 2, 4, 10, 20 and 30 of the exposure. Five sets of twenty black worms were sampled every other day for whole body Ni bioaccumulation (sub-cellular fractionation of prey was performed on the same days as the fish). Fish were euthanized with 0.80 mg/L of tricaine methanesulfonate (*MS-222*) (Syndel Laboratories Ltd. Nanaimo, BC, Canada; adjusted to pH 7.8 with NaOH) and rinsed briefly (1 min) in dechlorinated water. The body mass of each fish was measured and recorded. Gills and gut were surgically removed, rinsed with 0.9% NaCl solution, blotted dry and weighed and then stored in15-ml Falcon^{TM} tubes at -80°C for future analysis. Black worms were transferred to Hamilton dechlorinated water 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, patted dry, weighed and then stored in 2 ml bullet tubes at -80°C for future analysis.

Analytical techniques

Fish sub-cellular fractions and homogenate for Ni recovery were digested with 2 N HNO$_3$ (trace metal grade, Fisher Scientific, Ottawa, ON, Canada) with a volume of 3-5 times the weight of the tissue in sealed vials. These were incubated in a Precision Oven (Jouan Inc., Virginia, USA) at 60 °C for 48 h, with vortexing at 24 h. Tissues are then stored at 4 °C for later analysis. Black worm sub-cellular fractions and homogenate 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$_2$O$_2$ per mg of tissue wet wt) was added for 24 h to complete the digestion process (Croteau et al., 2002).

Ni in water samples and tissue fraction 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). Ni recovery in water samples was 93 ± 2.3 % as determined by Environment Canada certified reference materials, TM-24.3 (lot # 0310) and TM-25.3 (lot # 0809). National Research Council of Canada (Ottawa, ON, Canada) lobster hepatopancreas (TORT-1) was used to determine the recovery of Ni in the tissues. Quality control blanks were run every 20th sample to correct for background contamination. Background correction was not used and Ni concentrations were not corrected for recovery. 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 0.003 µmol l^{-1}.

Flame Atomic Absorption Spectroscopy (FAAS; Varian SpectrAA – FS-220, Mulgrave, Australia) was used to measure the concentrations of major cations (Na, Mg, Ca and K) in water samples. All water samples were diluted using 1% HNO_3 for Na analysis, 1% HNO_3 with 1% $LaCl_3$ for Ca and Mg analysis and 1% HNO_3 with 0.01% $CsCl_3$ for K analysis. Reference standard solutions for all ions were used to obtain standard curves (Fisher Scientific, Ottawa, ON, Canada). Water pH and DOC were measured using an 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.

As fish were sampled from the exposure tank at various time-points, overall percent survival for each exposure concentration was calculated as the product of percent survival at the time of sampling multiplied by the percent survival from previous sampling time as is stated in the ASTM – E1241-05 Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes (ASTM, 2012).

Statistical analyses

CBR50 values with 95% confidence intervals (C.I.) were calculated using ToxCalc – Toxicity Data Analysis Software ver.5.0.32 (Tidepool Scientific Software, McKinleyville, CA, U.S.A.). The CBR50 was the Ni bioaccumulation in an organ on a specific day of exposure that corresponded to 50% chronic mortality at 30-d of exposure. Raw (no background correction) bioaccumulation data were used against chronic (30-d) mortality to calculate CBR50 values in ToxCalc.

Data have been presented as means ± SEM (n), where n is the sample size. All data reached normality, and conformed to homogeneity tests, or were transformed as necessary before statistical analyses were performed. Significant differences between two groups were evaluated by unpaired Student's t tests (two-tailed). Comparisons among multiple treatment groups were assessed using a one-way analysis of variance (ANOVA) followed by Fisher LSD Method (Sigma Plot 10.0, Chicago, IL, USA). For all tests, statistical significance was allotted to differences with $p < 0.05$.

RESULTS

Water chemistry

Ni water concentrations for Ni exposures to rainbow trout and round goby expressed as nominal, total, dissolved, ionic and active fractions of the metal, taking into account this measured water chemistry, are reported in the Supplementary Table 5.2, whereas corresponding water chemistry data are reported in Supplementary Table 5.1. While nominal concentrations for both species were 10 and 60 µmol/L, actual measured mean total concentrations were 12.0 and 68.3 µmol/L for goby and 6.2 and 85.6 µmol/L for trout; total control concentrations averaged 0.05 µmol/L or less (Supplementary Table 5.2). All Ni water concentrations presented in this study are reported as the dissolved fraction of the metal, which averaged 92 % of the total values in the low (11.2 and 5.8 µmol/L) and high exposures (62.0 and 79.0 µmol/L for goby and trout respectively; Supplementary Table 5.2).

Survival over chronic exposure

Rainbow trout were more resistant to chronic waterborne and dietary nickel exposure than round goby. There was no mortality in the controls for either fish. There was no mortality at the lower exposure concentration and only 20% mortality in the higher exposure by day 30 for rainbow trout (Fig. 5.1A). In contrast, round goby mortality started on day 20 in the lower exposure with 38 % survival by day 30. In the higher exposure, survival dropped steeply by day 10 to 7 %, with no round goby surviving at the following two sampling times (Fig. 5.1B).

Comparison of whole-organ and sub-cellular fractional concentrations of Ni in gill and gut

Prior to Ni exposure in the laboratory, the gills and gut of round goby had ~ 2 x as much Ni bioaccumulation in comparison to rainbow trout and there was no significant difference in organ Ni bioaccumulation within a species (Fig. 5.2A). The BIM fractions (MRG and MT) of the gills bioaccumulated ~7 x more Ni in comparison to the BAM fractions (ORG and HDP) in both rainbow trout and round goby (Fig. 5.2B). A similar trend was observed in the gut where ~3 x and ~11 x more Ni was in BIM fraction of rainbow trout and round goby, respectively (Fig. 5.2B). Within the gills, round goby had ~2.4 x more Ni in both the BIM and BAM fractions in comparison to rainbow trout. The gut of round goby also had more Ni in the BIM (~2 x) fractions in comparison to rainbow

trout, whereas there was more Ni (~1.5 x) in the BAM fractions of the rainbow trout (Fig. 5.2B).

Dietary component of Ni exposure from prey

Fig. 5.3 shows the average Ni bioaccumulation both in the whole organism as well as the average sub-cellular distribution in the prey species, *Lumbriculus variegatus*. These worms were exposed for 48 h in the effluent of the fish exposure tanks before being fed to the fish. As the exposure concentration increased by either 13- fold or 6-fold in the rainbow trout and round goby exposure water, the Ni concentration within the *Lumbriculus* increased to a lesser extent with only a 3.4 and 1.9-fold increases, respectively (Fig. 5.3A,B). The bioaccumulations of Ni in the BIM vs. BAM fractions were generally about equal. Within the BIM fraction, there was no significant difference between the percent Ni in the MRG and MT fractions, however, within the BAM fraction, there was on average 7 x more Ni distributed to the ORG fraction in comparison to HDP fraction (Fig. 5.3C,D).

Whole organ Ni bioaccumulation at various time points

Gills: In the current study, gill Ni bioaccumulation in both species displays a biphasic relationship of Ni loading into the gills in the initial days of exposure followed by a period of stabilization above control values (Fig. 5.4A,B). Rainbow trout gill Ni bioaccumulation steadily increased from day 0 to day 4 in the 6 µmol/L exposure and until day 10 in the highest exposure concentration, after which there were no significant changes (Fig. 5.4A). Across all sampling days, there was 2-3.5 x more Ni in the gills of rainbow trout exposed to 79 µmol/L in comparison to 6 µmol/L, a 13 fold difference in exposure concentrations (Fig. 5.4A).

Gill Ni bioaccumulation in round goby was highest on day 2 followed by significant decreases of 70 % and 55 % by day 4 in the 11 µmol/L and 62 µmol/L exposure concentrations, respectively (Fig. 5.4B). In the 11 µmol/L exposure, gill Ni bioaccumulation remained constant until day 30 where it decreased by a further 60 % (Fig. 5.4B). On days 2 and 4, there was 3-4 x more Ni in the gills of round goby in the higher exposure in comparison to the lower exposure (Fig. 5.4B).

Gills of round goby bioaccumulated ~16 x and ~5 x more Ni by day 2 and day 4, respectively, in the higher exposure and an average of ~4 x more Ni in the lower exposure concentration in comparison to trout (Fig 5.4A, B).

Gut: Acute (days 2 and 4) Ni bioaccumulation in the rainbow trout gut remained constant then increased by day 10 (79 µmol/L) or day 20 (6 µmol/L) and plateaued again until day 30 (Fig. 5.4C). Similar to the gills, the gut did not bioaccumulate Ni in proportion to the differences in the concentrations in the water or the prey. There was only ~2 x more Ni in the gut of rainbow trout exposed to 79 µmol/L (83 µmol/kg wet wt in prey) in comparison to 6 µmol/L (24 µmol/kg wet wt in prey), a 13 fold difference in exposure water concentrations and a 3.4 fold difference in the prey concentration (Fig. 5.3A, 5.4C).

Gut Ni bioaccumulation of round goby spiked on days 10 and 4 in the 11 µmol/L and 62 µmol/L exposure concentrations, respectively (Fig. 5.4D). There was a ~10 fold increase in the higher exposure, whereas, in the lower exposure concentrations, gut Ni bioaccumulation decreased steadily by 80 % by day 30 (Fig. 5.4D).

In contrast to the gills, the gut Ni bioaccumulation did not differ as much between the two species with an average of ~2 x more Ni in round goby gut in comparison to rainbow trout gut , with the exception of day 4 in the highest exposure where there was ~6 x more in round goby (Fig. 5.4C,D).

Gill and gut subcellular Ni distribution

Gill: In the rainbow trout, there was 2-4 x more Ni in the BIM fraction in comparison to the BAM fraction of the gills at the lower exposure (Fig. 5.5A) and approximately 2 x more at the higher exposure concentration. Similarly, in the round goby, 2-4 x more Ni bioaccumulated in the BIM vs. BAM fraction of the gills (Fig. 5.5B,D) in both exposure concentrations.

A comparison between the Ni bioaccumulated in the BIM and BAM fractions of the two species, shows approximately 3 x more BIM Ni and 7 x more BAM Ni in the round goby gills vs. the rainbow trout, respectively, at most times, regardless of exposure concentrations. However, day 2 was an exception where there was 15 x and 40 x more Ni bioaccumulated in the BIM and BAM fractions of round goby gills, respectively (Fig. 5.5).

Gut: The gut of rainbow trout exposed to either Ni exposure concentration, bioaccumulated ~ 3 x more Ni in the BIM vs. BAM fraction (Fig. 5.6A,C). In contrast, the BIM fractions of the round goby gut bioaccumulated ~10 x more Ni in comparison to the BAM fraction (Fig. 5.6B,D).

In general, in the lower exposure concentration, similar amounts of Ni bioaccumulated in the BAM fractions of the rainbow trout and round goby (Fig. 5.6A,B). Similarly, there was about the same amount of Ni in the BIM fractions up until day 20 where there was ~4 x more Ni in the rainbow trout gut in

comparison to round goby. In the higher exposure concentration, more Ni bioaccumulated in the BIM and BAM fractions of rainbow trout on day 2, whereas by day 4, ~5 x and ~2 x more Ni was in the BIM and BAM fractions of round goby in comparison to rainbow trout, respectively (Fig. 5.6C,D).

How Ni bioaccumulation and sub-cellular fractions correlate to mortality

We examined relationships between Ni bioaccumulation at various exposure times versus chronic (30-d) mortality to look for predictive indicators. For simplicity, only day 4 and day 30 data are shown in Fig. 5.7, but data on intermediate days exhibited similar relationships. Acute gill Ni bioaccumulation at day 4 was just as predictive of chronic (30-d) mortality as bioaccumulation at day 30 in the round goby (Fig. 5.7A). As 4-d and 30-d Ni bioaccumulation increased, there was increasing chronic mortality. Ni bioaccumulation in both the BIM and BAM fractions were also predictive of chronic (30-d) mortality (Fig. 5.7C,E). In addition, there was no significant difference (95 % confidence intervals overlapped) between CBR50 values calculated either from 4-d or 30-d bioaccumulation for any of the fractions: whole gill or BIM or BAM fractions of the gills of round goby (Fig. 5.7A,C,E). Notably, in the gut, in contrast to the gills, there was no correlation between gut Ni bioaccumulation and chronic mortality with respect to the whole organ or BIM and BAM fractions (Fig. 5.7B,D,F).

The high survival of rainbow trout in both exposure concentrations did not permit a correlation to be made between early bioaccumulation and chronic mortality.

DISCUSSION

Overview

There are many benefits of acute mechanistic data on metal toxicity in terms of modeling approaches for protection of aquatic species; however, quite often the concentrations used in acute toxicity tests are not environmentally relevant and do not take into consideration dietborne metal exposure. In the current study we have employed a joint waterborne and dietary Ni exposure at two concentrations, the lower of which is typical of a Ni contaminated environment (<8.5 µmol Ni/L; Chau and Kulikovsky-Corderio, 1995; Eisler, 1998) and a higher level Ni exposure concentration which is not environmentally relevant but may give information on mechanisms of chronic Ni toxicity. To the best of our knowledge, only four other studies have assessed the subcellular distribution of Ni in fish tissues: the whole body of the fathead minnow,

Pimephales promelas (Lapointe and Couture, 2009), olfactory epithelium and nerve of the northern pike, *Esox lucius* (Tallkvist et al., 1998) and the liver of wild yellow perch, *Perca flavescens* (Giguère et al., 2006; Campbell et al., 2008). However none of these assessed the sub-cellular distribution of Ni in the gills or the gut – the two organs responsible for the exchange of nutrients and minerals, and eliminating waste as well as the organs considered to be biotic ligands of metal binding.

We have shown that round goby are more sensitive than rainbow trout to chronic Ni exposure. Earlier, we reported that round goby were also more sensitive to acute Ni exposure (Leonard et al., submitted = Chapter 4). Possibly, this greater sensitivity may be due to the round goby's pre-exposure to pollutants at their collection site, as evidenced by ~2-fold greater initial Ni bioaccumulation in both their gills and gut relative to trout. However, this was followed by ~2-16 x larger bioaccumulation in both the gills and the gut during the experimental exposure. On a subcellular level, ~ 3-40 x more Ni is associated with the BAM fraction of round goby in comparison to rainbow trout.

Assessing sub-cellular distribution of Ni in both the gills and gut of two fish of different habitat and lifestyles revealed two different strategies of Ni bioaccumulation and sub-cellular distribution. On the one hand, the rainbow trout exhibited an ability to regulate gill Ni bioaccumulation and maintain the majority of the Ni in the MT fraction as part of the BIM fraction. In contrast, the round goby exhibited large Ni spillovers to the BAM fraction of the gill, both to the HDP and ORG compartments. However, the same trend was not observed in the gut, where the potential acclimation of round goby to pollutants from their collection site may have aided their ability to regulate Ni spillover to the BAM fraction more so than the rainbow trout.

Gill Ni bioaccumulations at either 4-d or 30-d in the whole organ, or BIM or BAM fractions of the metal were predictive of chronic Ni toxicity in the round goby. There was not sufficient mortality in the rainbow trout to assess the Ni bioaccumulation which would be predictive of chronic mortality.

Survival over chronic exposure

Round goby are expected to be a rather pollution tolerant species because of their prevalence in highly contaminated areas (Pinchuk et al., 2003) including Hamilton Harbour (Marentette and Balshine, 2012). However, laboratory studies have demonstrated that this species is more sensitive to both acute Ni (Leonard et al., submitted = Chapter 4) and chronic Ni (present study) toxicity in comparison to rainbow trout, a species which is known to be one of the most sensitive teleosts to metal toxicity (USEPA, 1986; Brix et al., 2004). For example, the chronic no-

observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) for growth of trout are less than 0.6 µmol Ni/L (Nebeker et al., 1985). To the best of our knowledge, there are no previous studies which assess chronic Ni toxicity to round goby.

Comparison of whole-organ and sub-cellular fractional concentrations of Ni in the gill and gut

Prior to laboratory testing, the round goby had ~ 2 x more Ni in the gills and the gut than the rainbow trout purchased from a hatchery. As well, on a sub-cellular level, the round goby had ~ 3 x more Ni in the BIM and BAM fractions of the gills in comparison to rainbow trout. There was also ~ 7 x more Ni in the BIM fraction of the gut of round goby in comparison to rainbow trout. This suggests that the round goby were closer to reaching or had reached their threshold concentration in the BIM fraction leading to spill over into the BAM fraction (Adams et al., 2011), which potentially caused adverse physiological effects. In fact, this may help explain the higher sensitivity of the round goby. Although, the collection site (LaSalle Park on Hamilton Harbour) is considered to be a "clean site" (Marentette et al., 2010), the Harbour itself is in close proximity to industrial activities such as steel mills and many contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and metals including cadmium, arsenic, lead, iron, mercury, zinc, and nickel are known to be of concern in some areas of the Hamilton Harbour (Hamilton Harbour RAP, 1992, 2003; International Joint Commission, 1999). In fact, sediment Ni concentrations at LaSalle Park were 900 µmol/kg, a value which is above the lowest effect level according to the provincial sediment quality guidelines (Zeman, 2009) and liver concentrations of collected round goby were ~ 3 µmol/kg (Marentette et al., 2010), below levels of the gill and gut of the current study, however this may be due to low Ni bioaccumulation in the livers of teleosts (Leonard et al., submitted = chapter 4). Therefore, the residual influence of contaminants from Hamilton Harbour both via the diet and/or the water in addition to the laboratory Ni exposure may have overwhelmed the detoxifying strategies of the round goby and therefore reduced their overall tolerance.

Dietary component of Ni exposure from prey

Recently, the dietary transfer of metal to fish has been recognized as a critical exposure route which needs to be addressed in further detail as current WQC are based on waterborne exposures (Meyer et al., 2005; Béchard et al., 2009; Klinck et al., 2009). It has been suggested that dietary metal uptake from natural food (more typical prey) vs. artificial food may provide predators with a

form of the metal which is more trophically available (Meyer et al., 2005; Ng and Wood, 2008; Béchard et al., 2009; Klinck et al., 2009).

Black worms were held in the out-flow from the fish tanks for 48-h prior to being fed to the fish. A steady-state condition in the prey was therefore not achieved. Previous research with Zn and Cd has shown that steady-state conditions were not reached in the caddisfly, *Mystacicks spp.*, over 30 days of exposure (Timmermans et al., 1992). Additionally, in same black worm species used in the present study, steady state conditions were not achieved over a 7 week exposure to waterborne Pb (Derek Alsop, personal communication). Therefore, we chose to expose the predators to a constant Ni concentration from the diet over the course of the 30-d exposure by exposing the black worms in an acute manner.

While it is possible that Ni accumulation in the gut tissue of the predators occurred due to input from waterborne sources (drinking, transport from the gills via the plasma), it seems more likely that the bulk of the bioaccumulation would have originated from the prey. In trout gut, Ni concentrations by day 30 were approximately equal to those in the prey, suggesting that an equilibrium had been achieved. In round goby, at the lower Ni exposure concentration, Ni levels in the gut tissue were actually lower than in the prey, though the opposite occurred in the higher exposure concentration at day 4, the last sampling day before substantial mortality occurred. In the trout, at the lower Ni exposure concentration, there was significantly less of the total in the BAM fraction, and more in the BIM compartment than in the prey, suggesting that detoxification had occurred. However this was not true at the higher exposure concentration. In round goby, there was evidence of a greater detoxification capacity inasmuch as only about 10% of the accumulated Ni was partitioned into the BAM fraction, whereas 90% was stored in the BIM compartment at both exposure concentrations. These differences suggest that the round goby was more efficient at regulating dietary Ni uptake and detoxification in comparison to rainbow trout (see below section 4.6). However, based on our experimental design, we cannot accurately evaluate the trophic transfer efficiency of Ni in these species nor distinguish the contribution of the two Ni sources (waterborne and dietary) to Ni bioaccumulation in the predators. Future studies should investigate the aspect of contaminant pre-exposure on the trophic transfer efficiency of metals.

Whole organ Ni bioaccumulation at various time points

Gaining an understanding of Ni toxicokinetics during chronic exposures may establish links between toxicity and exposure (McGeer et al., 2000; McCarty and MacKay, 1993). In the current study, gill Ni bioaccumulation in both species displays a biphasic relationship of Ni loading into the gills in the initial days of exposure followed by a period of stabilization above control values (Fig. 5.4A,B).

This trend has been previously shown for Cu and Zn by Laurén and McDonald (1987), Grosell et al. (1997) and McGeer et al. (2000) and is suggestive of metabolic regulation characteristic of essential nutrients; it is not observed for Cd, a non-essential metal (McGeer et al., 2000). Ni essentiality has not been established in fish, however, homeostatic regulation of Ni has been shown in lake whitefish (Ptashynski and Klaverkamp, 2002), and rainbow trout (Chowdhury et al., 2008).

In general, the initial damage phase characteristic of chronic metal bioaccumulation occurs from bioaccumulation on or in the gills (McGeer et al., 2000; McDonald and Wood, 1993), which was observed in the present study where gill Ni bioaccumulation occurred at earlier time points than the gut Ni bioaccumulation (Fig. 5.4). In the gut, Ni bioaccumulation did not increase until day 4 or day 10 in either of the fish species, which correlates well to the commonly held view that diet-borne metals are unlikely to cause acute metal toxicity (Meyer et al., 2005) but play a more critical role in chronic metal toxicity.

Gill and gut subcellular Ni distribution

Gill

In general, similar trends were observed for both the lower, environmentally relevant Ni exposure and the higher, more toxic Ni concentrations, therefore, for simplicity; results will be discussed together for the two exposure concentrations.

In the gills of rainbow trout, the MT fraction was a major location of Ni bioaccumulation (Fig. 5.5A,C). This fraction is considered part of the BIM fraction and is therefore detoxified (Wallace et al., 2003). MTs are low molecular weight, cysteine-rich metal binding proteins which are induced from exposure to metals, namely Cu, Cd, and Zn (Roesijadi, 1992; Mason and Jenkins, 1995; Amiard, et al., 2006). The information on MT induction by Ni is much less robust; however, Ptashynski et al. (2002) showed MT induction in the intestine of lake whitefish (*Coregonus clupeaformis*) following a dietary exposure to Ni and Giguère et al. (2006) demonstrated MT induction in the liver at low chronic Ni exposures in the yellow perch, *Perca flavescens*. Mercury (Hg), Cd, Ag (silver) and Zn were also found to induce MT production in the gills of the carp, *Cyprinus carpio*, exposed for 7-d to each respective metal (Cosson, 1994), as well as Cd in the gills of rainbow trout following a 96-h waterborne exposure (Kamunde, 2009) and following a four week dietary Cd exposure from *L. variegatus* to rainbow trout (Ng and Wood, 2008). The binding of Ni by MTs appears be to a successful strategy for survival of this species (Fig. 1, Fig. 5.5A,C).

In general, the gills of round goby appear to use MT and MRG as mechanisms of detoxifying Ni (Fig. 5.5 B,D). MRG involve the precipitation of a

metal into insoluble concretions normally including Ca or Mg phosphate (Roesijadi 1980, Brown 1982; Vijver et al., 2004), rendering the metal detoxified. Other studies have suggested that MRG formation plays a role in chronic tolerance to metal exposure, while MT mainly acts to protect against acute metal exposure (Vijver et al., 2004). Within the 30 day Ni exposure, there was no defined pattern that either supports or rejects this theory. However, the use of both detoxifying strategies in round goby may suggest they were exhausting both detoxification strategies.

Nonetheless, Ni bioaccumulation in the gills of either species was not constrained to the BIM fraction. Even at sub-lethal levels of Ni exposure, both rainbow trout and round goby gills bioaccumulated more Ni than controls (Day 0) in the BAM fraction, suggesting that even at this low level of exposure metal detoxification was not entirely successful (Fig. 5.5). Previous research has shown complete metal detoxification by the BIM fraction (MTs and/or MRG) under conditions of low metal exposure, with partial detoxification (or spillover) into the BAM fraction under conditions of greater exposure (Wallace et al., 2003). However these studies have been primarily done on naïve organisms and the dietary component of metal exposure has not been taken into consideration. Kamunde (2009) also observed a similar trend for Cd in the gills and liver of rainbow trout where even background Cd bioaccumulation partitioned into all of the subcellular compartments analyzed. Simultaneous Ni bioaccumulation in both the BIM and BAM fractions suggests that detoxification strategies cannot entirely cope with the metal challenge.

A comparison between the two species demonstrates ~ 3-40 x more Ni in the BAM fraction of the round goby gills in comparison to the rainbow trout. Early spillover into this fraction occurred in HDP, which decreased and plateaued by later time points. This is in contrast to the liver of wild yellow perch where steady-state Ni partitioning showed greatest Ni bioaccumulation in the HDP fraction in comparison to any other fraction (Giguère et al., 2006). In the current study, Ni spillover to the ORG fraction of the gills occurs later in the exposure (Days 20 and 30; Fig. 5.5). Interpretation of the ORG fraction should be done with caution as there may be some functional overlap between BIM and BAM fractions. The inclusion of lysosomes as part of the BAM fraction is not ideal as Ni bioaccumulation in this organ may be indicative of either Ni storage for eventual elimination and detoxification (i.e. BAM classification incorrect) or Ni storage in a bioavailable form (i.e. BAM classification correct). The latter could occur if lysosomes become "leaky" following exposure to metals releasing hydrolytic enzymes into the cell (Viarengo et al., 1987). However, the other two components of the ORG fraction: mitochondria and microsomes are appropriately labelled as the BAM fraction; microsomes contain fragmented endoplasmic reticulum responsible for protein synthesis and transport (Fowler et al., 1989), and metal bound to the mitochondria has been shown to reduced metabolic capacities or increase oxidative stress (Silverberg 1976; Lapointe and Couture, 2009).

Therefore, round goby are not as adept at regulating gill Ni uptake and detoxification, leading to higher mortality in this species (Fig. 5.5).

Gut

Similar to the gills of rainbow trout, the majority of the Ni in the gut of trout was in the MT component of the BIM fraction; however, there was early spillover to the BAM fraction, mainly in the ORG in comparison to the HDP fraction (Fig. 5.6 A, C). A somewhat different trend was observed in the gut of round goby, where the Ni was mainly found in the MRG fraction in comparison to the MT fraction (Fig. 5.6 B,D). However, Ni in the BAM fraction was primarily associated with the ORG fraction. Therefore, it appears that spillover in the BAM fraction of the gut in both species occurs primarily in the ORG fraction, potentially leading to problems with protein synthesis and cellular respiration in the gut tissue.

The goby gut appears to be more efficient than the gills at regulating Ni bioaccumulation, whereas the opposite is true for the rainbow trout (Fig. 5.5, 5.6). Round goby, which were collected from Hamilton Harbour, may have fed on pollution-tolerant benthic invertebrates such as dipterans and oligochaetes known to bioaccumulate toxicants (Seidman et al., 1986). It has been shown that wild yellow perch collected from metal-contaminated lakes had lower rates of Cd absorption in the gut compared to control fish (Klinck et al., 2007). These findings suggest that physiological changes may occur along the gut of fish to allow for better coping strategies in polluted aquatic environments (Klinck et al., 2007). In the gills, it is well established that when fish are chronically exposed to sublethal metal concentrations there is an increase in the low-affinity, high capacity binding sites (Niyogi and Wood, 2003). Acute studies with Ni have shown that the gut of round goby display low affinity, high capacity transport systems, whereas, the gut of naïve rainbow trout exhibit high affinity, low capacity uptake parameters (Leonard et al., 2009; Leonard et al., submitted = Chapter 4). Therefore, the ability of the gut of round goby to better regulate gut Ni bioaccumulation and to detoxify the Ni to the BIM fraction may be due to the fish's previous exposure to contaminants via the diet. While this may have caused a change in the binding kinetics of the metal, however, it was not sufficient to protect this species against chronic mortality. Overall, chronic mortality observed in round goby (and not in rainbow trout) appears to be more closely related to the gill Ni bioaccumulation and not to the gut Ni bioaccumulation.

How Ni bioaccumulation and sub-cellular fractions correlate to mortality

One of the fundamental concepts of the BLM is that early (acute) metal bioaccumulation is predictive of chronic toxicity. For example, Meyer et al. (1999) demonstrated that 24-h gill Ni bioaccumulation (LA50) in the fathead minnow (*P. promelas*) was a constant predictor of 96-h LC50 regardless of water chemistry parameters. In the current study, round goby gill CBR50 values calculated from either 4-d or 30-d gill Ni bioaccumulation against chronic (30-d) mortality were not significantly different which strongly suggests that either acute (4-d) or chronic (30-d) gill Ni bioaccumulation can be used for tissue residue-based risk assessment, even if equilibrium has not yet been reached. This is supported by a study on Cd in *Tubifex tubifex* where 4- to 17-d CBR50 values appeared to be independent of exposure time (Redeker and Blust, 2004). In addition, Ng et al. (2012) showed a similar trend where 7-d Cu bioaccumulation and chronic (28-d survival) were qualitatively similar to those when mortality and Cu were measured at the same chronic endpoint of 28-days.

CBR50 values of the BIM fraction were ~ 2-4 x more than CBR50 values of the BAM fraction (Fig. 5.7). Therefore, the predicted 50 % mortality occurs at a lower Ni level in the BAM fraction than the BIM fraction, suggesting that round goby are more sensitive to Ni bioaccumulation in the BAM fraction. In addition, in the current study, BIM and BAM fractions of the goby gills are equally as predictive of chronic (30-d) mortality. This in contrast to Cu in the invertebrate, *L. variegatus*, where measurements in BIM, rather than BAM, gave a better indication of metal bioavailability on a cellular level (Ng et al., 2012).

There was no relationships between gut Ni bioaccumulation, or BIM and BAM fractions, at any time point with chronic (30-d) mortality, suggesting that the gut is not the site of key toxic action, thereby supporting our acute study on these two fish species which emphasized the gills as the main site of toxic action and the best organ for prediction of both acute and chronic mortality (Leonard et al., submitted = Chapter 4).

These data also support one of the main concepts of the BLM and TRA that the concentration of the toxicant within the target tissue, the gills, that produces a certain effect is independent of time. Future studies should expand more upon this to determine whether CBR50 values are also independent of exposure conditions, such as water chemistry. In rainbow trout, which were more resistant to Ni exposure, there was insufficient mortality (Fig. 5.1) to calculate CBR50 values; however, from Fig. 5.7 we observe that similar gill Ni concentrations in rainbow trout and round goby are associated with different chronic percent survivals. This same trend is observed in the BIM and BAM fractions, suggesting that CBR50 values of the two species may not be similar.

Earlier studies conducted in our laboratory demonstrated that CBR50 values were more consistent than exposure concentrations either between different water hardness values within a species or between different species (Leonard and Wood, 2013 = Chapter 3; Leonard et al., submitted = Chapter 4). Data from the current study show promise that gill tissue Ni residues can be used to predict chronic metal toxicity. Together, 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.

Conclusions

In this study, we have employed a joint waterborne and dietary Ni exposure at two concentrations over a chronic (30-d) time frame and assessed the sub-cellular distribution of Ni in the gills or the gut. Round goby were more sensitive than rainbow trout to chronic Ni exposure possibly due to their pre-exposure to pollutants at their collection site, their higher bioaccumulation of Ni in both the gills and gut during the laboratory exposure or the greater amount of Ni that associated with the BAM fraction of the gills.

Gill Ni bioaccumulations at either 4-d or 30-d in the whole organ, or BIM or BAM fractions of the metal correlated to chronic Ni toxicity in the round goby. There was not sufficient mortality in the rainbow trout to assess the Ni bioaccumulation which would be predictive of chronic mortality.

ACKNOWLEDGEMENTS

Fig. 5.1

Percent survival at each sampling time in both the lower and higher Ni exposure concentrations in rainbow trout (A) and round goby (B). Overall percent survival for each exposure concentration was calculated as the product of percent survival at the time of sampling multiplied by the percent survival from previous sampling time. $n = 1$ per treatment at each sampling time.
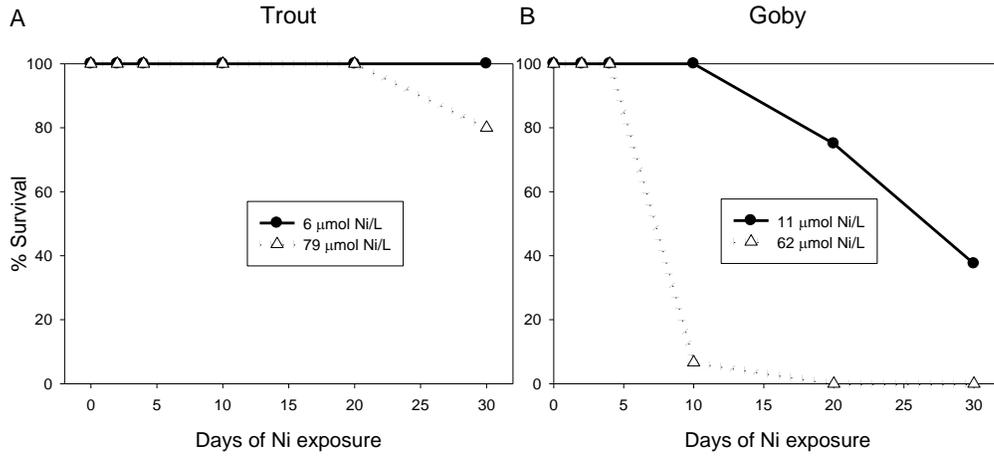
Fig. 5.2
Ni bioaccumulation in the gills and gut (A) and Ni bioaccumulation in the sub-cellular fractions (B) of rainbow trout and round goby at day 0 (i.e. prior to chronic Ni exposure in the laboratory) in control fish. HDP and ORG comprise the BAM fractions which are shown above the zero line, whereas, MRG and MT comprise the BIM fractions which are represented below the zero line. An asterisk * denotes a significant difference in the whole organ bioaccumulation between the two fish species, whereas different letters denote significant differences between organ bioaccumulation (A) or significant differences in either the BAM or BIM fractions of the organs (B; $p < 0.05$). Values are means $\pm$ S.E.M.; $n = 6$ per treatment.
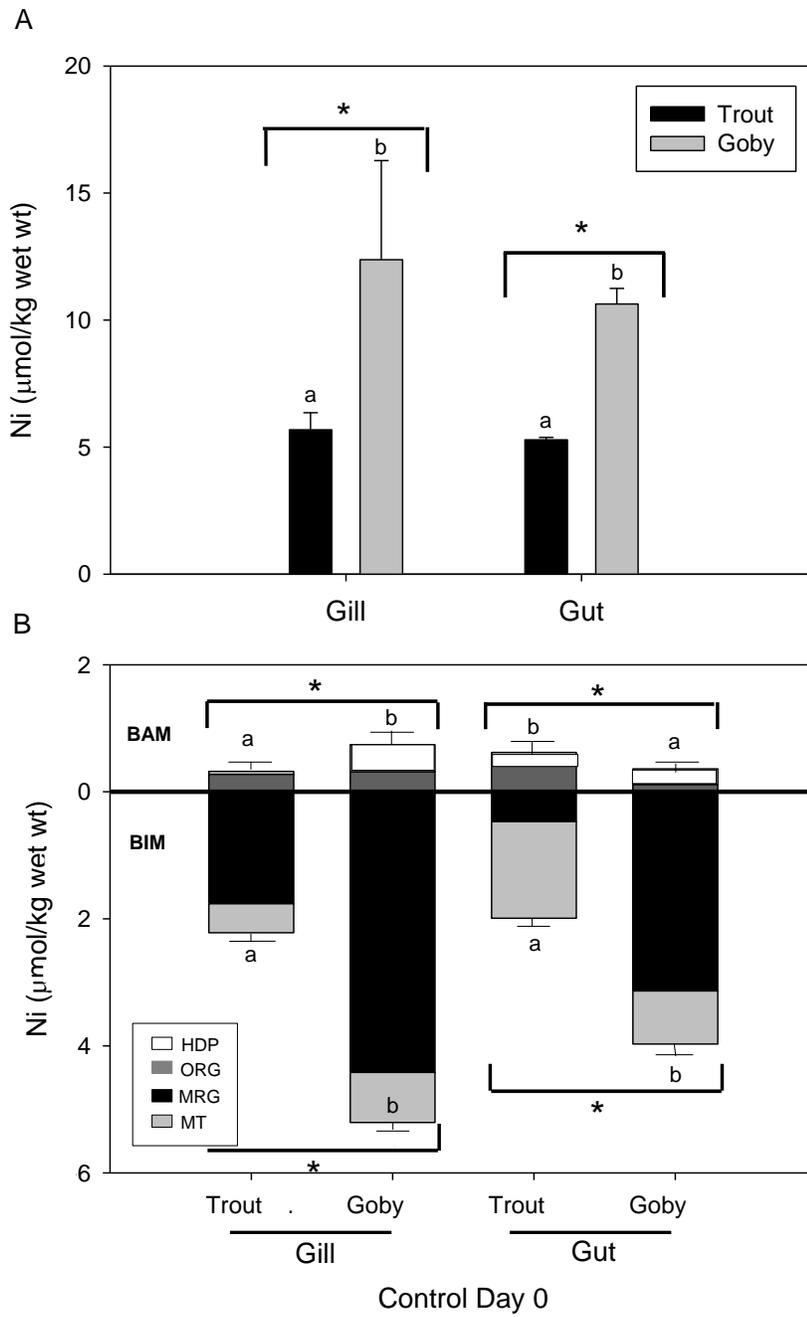
Fig. 5.3

Average whole body Ni bioaccumulation in prey (Lumbriculus; A, B) and
distribution of Ni in sub-cellular fractions of prey (C, D) following 48-h exposure
to Ni via effluent of rainbow trout (A, B) and round goby (C, D) exposure tanks.
HDP and ORG comprise the BAM fractions which are shown above the zero line,
whereas, MRG and MT comprise the BIM fractions which are represented below
the zero line. Values are means ± S.E.M.; $n = 6$ per treatment. Different letters
denote significant differences between organ bioaccumulation (panels A, B) or
significant differences in either the BAM or BIM fractions at different exposure
concentrations (panels C, D; $p < 0.05$).

Trout prey

Goby prey

A

B

C

D
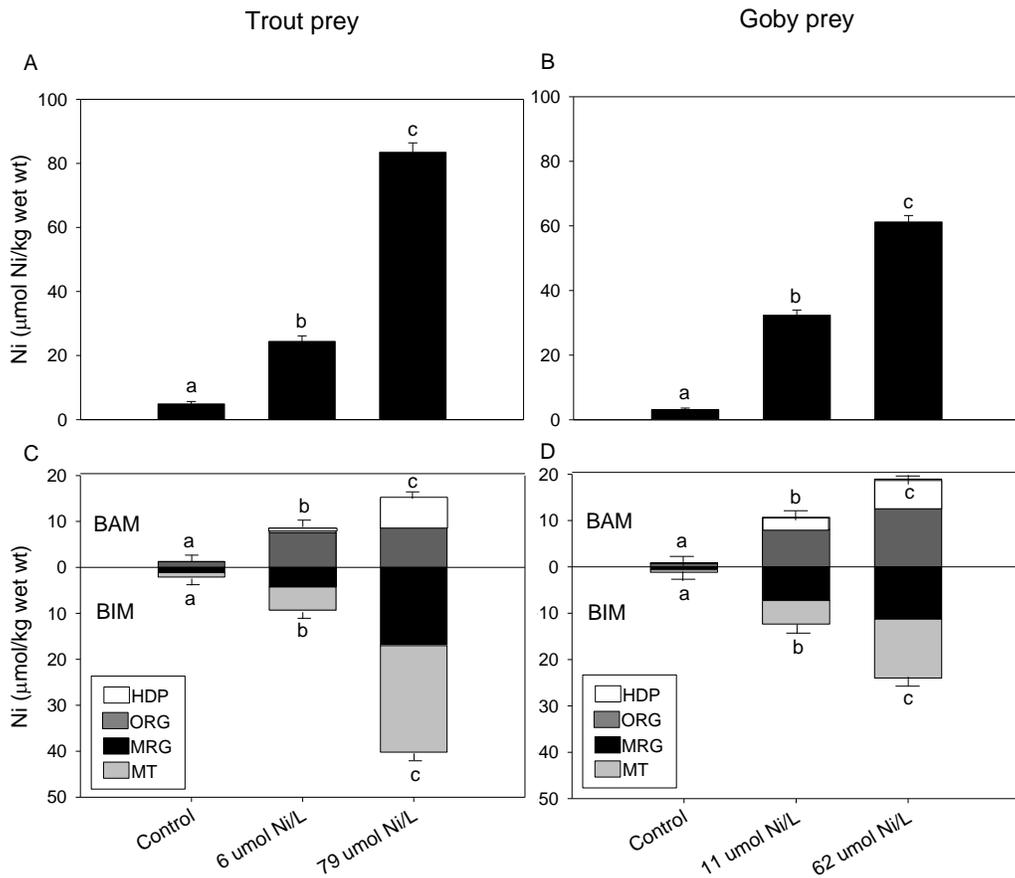
Fig. 5.4
Total Ni bioaccumulation in the gills and gut of rainbow trout (A,C) and round goby (B,D), respectively. Different letters denote differences within a concentration over time. An asterisk * denotes a concentration-dependent difference at a time point ($p < 0.05$). Values are means ± S.E.M.; $n = 6$ per treatment.
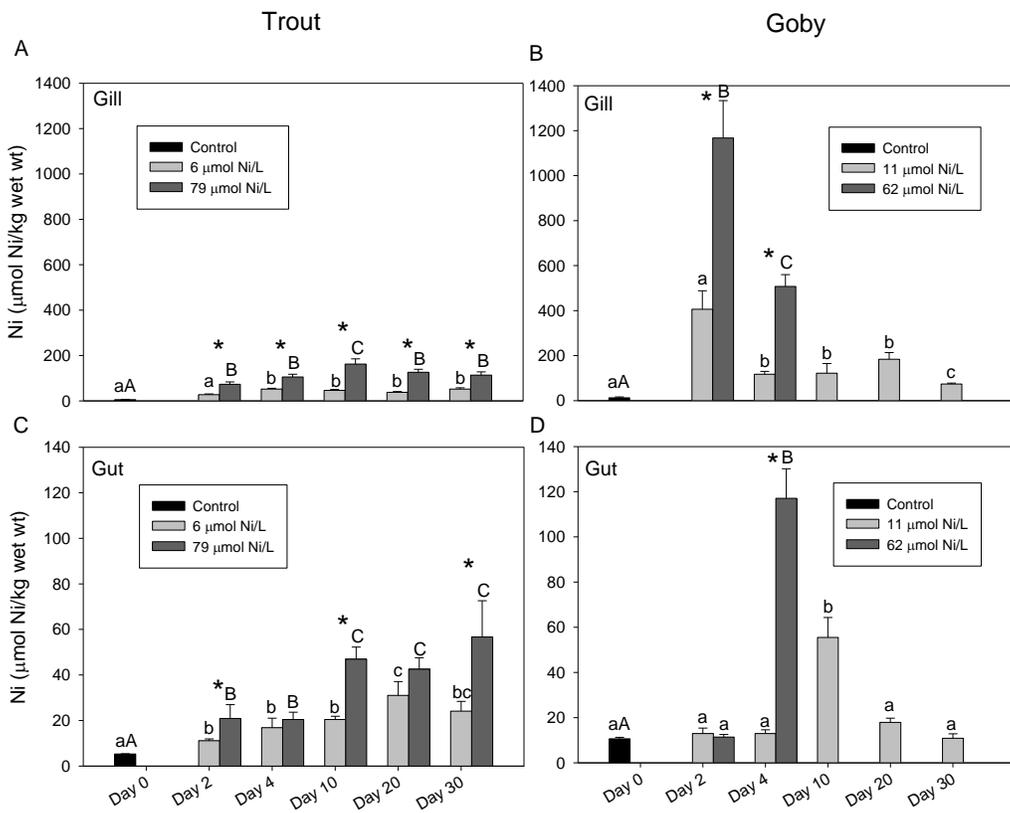
Fig. 5.5
Ni subcellular distribution in the gills of rainbow trout (A,C) and round goby (B,D). Dissolved Ni exposure concentrations are presented in the upper left quadrants. HDP and ORG comprise the BAM fractions which are shown above the zero line, whereas, MRG and MT comprise the BIM fractions which are represented below the zero line. Different letters denote significant differences in either the BAM or BIM among the six sampling times ($p < 0.05$). Values are means ± S.E.M.; $n = 6$ per treatment.

GILLS
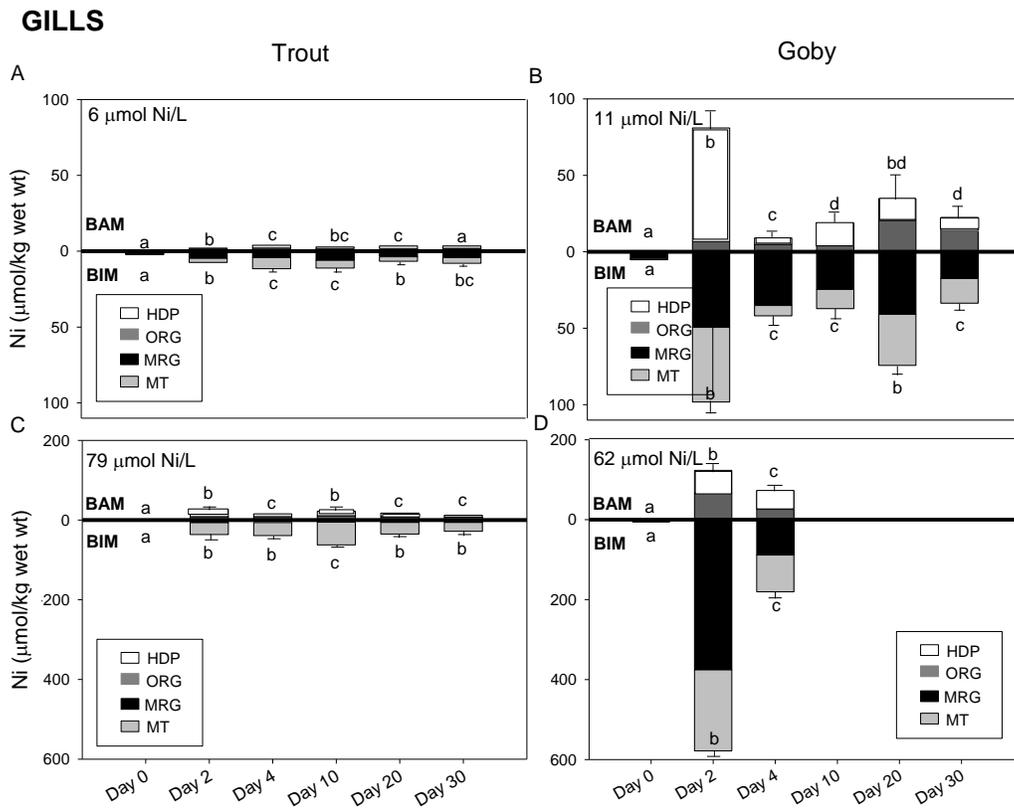
Fig. 5.6
Ni subcellular distribution in the gut of rainbow trout (A,C) and round goby (B,D). Dissolved Ni exposure concentrations are presented in the upper left quadrants. HDP and ORG comprise the BAM fractions which are shown above the zero line, whereas, MRG and MT comprise the BIM fractions which are represented below the zero line. Different letters denote significant differences in either the BAM or BIM among the six sampling times (p < 0.05). Values are means ± S.E.M.; $n = 6$ per treatment.

GUT
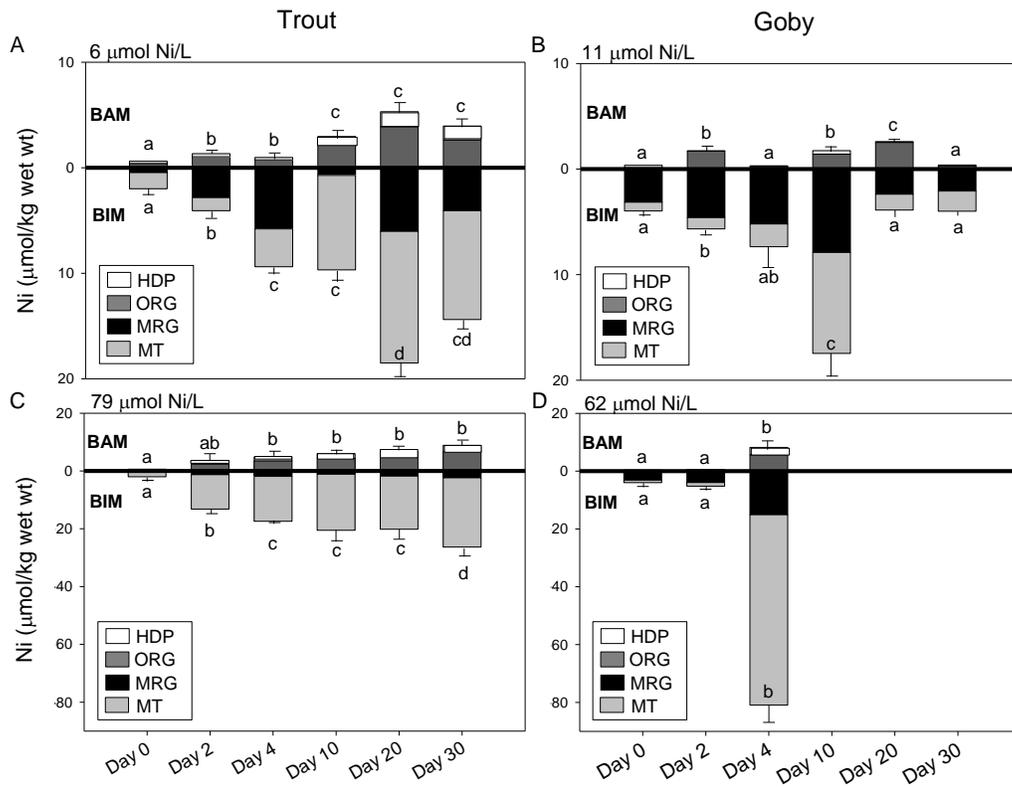
Fig. 5.7
Relationships between chronic (30-d) survival (%) and acute (4-d) and chronic (30-d) Ni bioaccumulation in the gills (A), BIM (C) and BAM (E) fractions of the gills, as well as in the gut (B), BIM (D) and BAM (F) fractions of rainbow trout and round goby. The lines at 50% survival intersect the bioaccumulation vs. mortality relationships at the CBR50 values, which are indicated on the Figure panel for round goby gill tissue only and were calculated using Toxcalc software. Note the lack of relationship for the gut tissue of round gobies, and also the high survival of rainbow trout at tissue burdens associated with 50% chronic mortality in round gobies. Values are means $\pm$ S.E.M.; $n = 6$ per treatment.

Gill

Gut

A

Whole organ

CBR50$_{(4-d)}$ = 68.6 μmol/kg (44.0-92.5)
CBR50$_{(30-d)}$ = 60.3 μmol/kg (54.1-65.5)

B

Whole organ

C

BIM

CBR50$_{(4-d)}$ = 25.2 μmol/kg (16.2-33.8)
CBR50$_{(30-d)}$ = 23.1 μmol/kg (0.1-135.4)

D

BIM

E

BAM

CBR50$_{(4-d)}$ = 5.6 μmol/kg (3.6-7.7)
CBR50$_{(30-d)}$ = 13.2 μmol/kg (4.4-25.1)

F

BAM

Day 4 - Trout
Day 30 - Trout
Day 4 - Goby
Day 30 - Goby

Chronic (30-d) survival %

Ni bioaccumulation (μmol/kg wet wt)
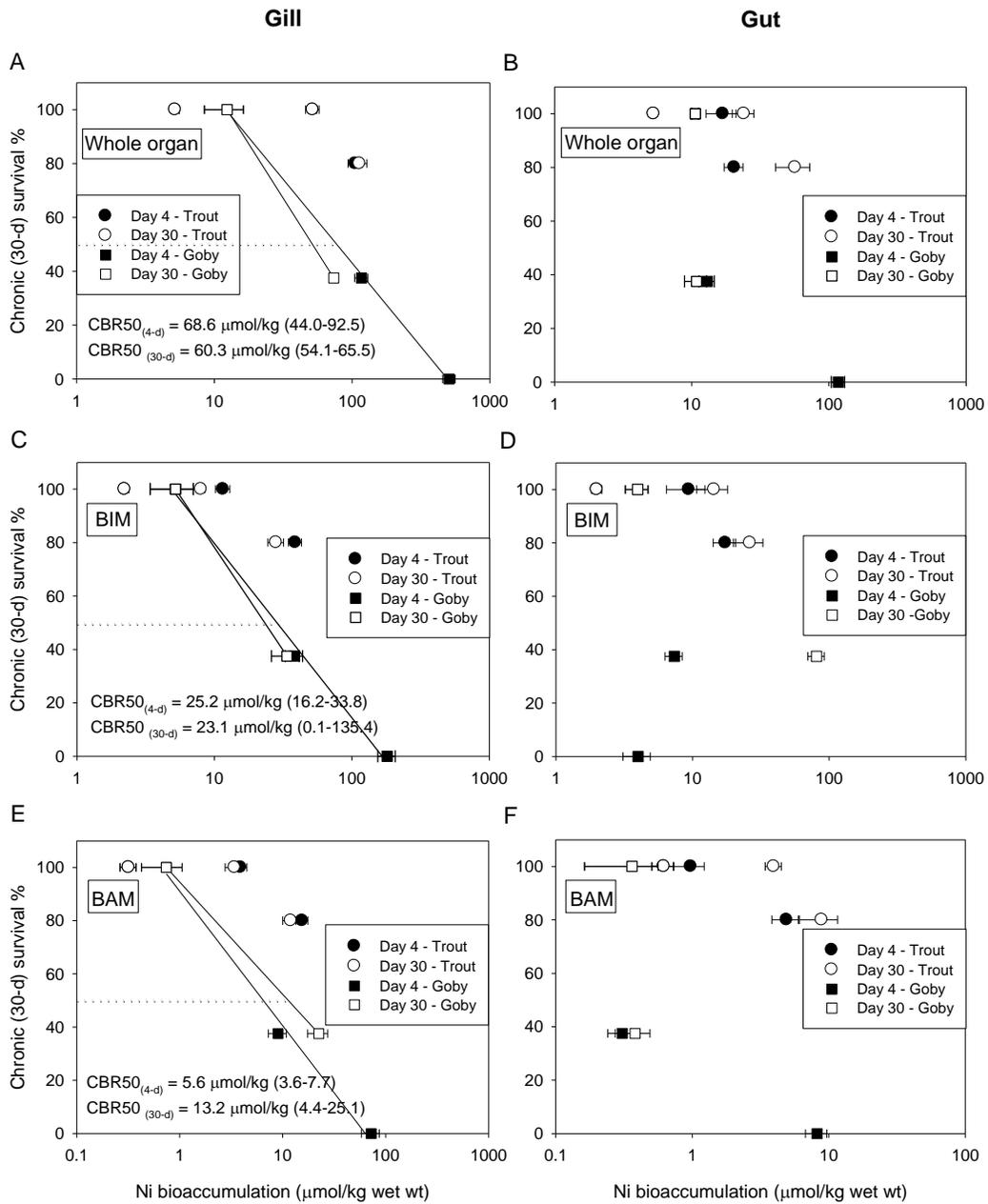
Supplementary Table 5.1
Water chemistry for Ni exposures. All ion concentrations are represented in
μmol/L with the exception of DOC (mg/L), hardness and alkalinity (mg/L as
CaCO3) and pH. Values are means ± S.E.M., n = 20-30 per value.

	Round goby	Rainbow trout
Na	911 ± 2.5	892 ± 3.9
K	39 ± 3.1	37 ± 1.9
Cl	992 ± 5.1	988 ± 6.0
Ca	1030 ± 2.6	1022 ± 3.6
Mg	395 ± 1.9	370 ± 5.8
Hardness	143 ± 2.5	139 ± 4.0
DOC	2.3 ± 0.3	2.2 ± 0.3
Alkalinity	95 ± 3	95 ± 4
pH	7.8 ± 0.04	7.7 ± 0.06

Supplementary Table 5.2
Average waterborne Ni exposure concentrations expressed as different fractions over the 96-h test period for each species. Means ± SEM (n = 30) are given for total Ni concentrations.

	Nominal (µmol Ni/L)	Total (µmol Ni/L)	Dissolved (µmol Ni/L)	Ionic (µmol Ni/L)	Active (µmol Ni/L)
Round goby					
	Control	0.05 ± 0.01	0.02 ± 0.01	0.01	0.01
	10	12.0 ± 1.1	11.2 ± 0.9	9.0	6.7
	60	68.3 ± 3.3	62.0 ± 2.9	49.7	37.2
Rainbow trout					
	Control	0.04 ± 0.01	0.03 ± 0.01	0.02	0.01
	10	6.2 ± 0.6	5.8 ± 0.5	4.7	3.5
	60	85.6 ± 2.8	79.0 ± 3.0	64.6	48.3

CHAPTER 6

GENERAL SUMMARY AND CONCLUSIONS

Over the past few years, the focus of metal toxicity has shifted from the metal concentration within the aqueous environment to the role of metal bioaccumulation on or within the organism. However, in comparison to other metals such as Cu, Cd, Zn and Pb, there was far less known regarding Ni bioaccumulation with respect to BLM and TRA approaches for both aquatic invertebrates and vertebrates in freshwater and saltwater environments. Therefore, the main objective of this thesis was to assess the use of bioaccumulation as a predictor of various Ni toxicity endpoints in different water chemistries (by varying salinity - saltwater or water hardness – freshwater) or comparing across species. The various endpoints that were assessed include Michaelis-Menten parameters (K_d and B_{max} values), BLM parameters (log K_{NiBL} values), critical-body residues (CBR50 values), and sub-cellular analysis.

Salt water

There is building evidence that salinity is protective against acute metal toxicity, namely for metals such as Cu, Cd, Zn and Pb. However, to the best of our knowledge **chapter 2** is the first study to illustrate this for Ni. Acute LC50 values were 4-9 x higher at high salinity vs. low salinity. Speciation calculations at the high salinity (25 ppt) demonstrated that complexation of Ni with anions such as SO_4^{2-}, HCO_3^- and Cl^- had a negligible effect in reducing the free Ni^{2+} ion component in comparison to a lower salinity (5 ppt). The salinity-dependent differences in acute Ni toxicity were not explained by differences in Ni bioaccumulation, suggesting that greater cation competition (by higher concentrations of Na^+, Ca^{2+}, and Mg^{2+}) are also not responsible for the difference in acute LC50 values. Therefore the differences in acute LC50 values can be mainly attributed to the physiology of the organisms at the two salinities. In contrast, chronic LC50 values were not significantly different between the two salinities, indicating that water chemistry and osmoregulatory strategy do not influence chronic toxicity and that acute and chronic toxicity mechanisms may be different.

The basis of the BLM is to understand the speciation of metals in different water chemistries, and to use this knowledge, in conjunction with binding constants on target surfaces of organisms, to determine whether sufficient metal will bind on or in the organism to cause acute toxicity. Short term metal burdens

are used to predict longer term toxicity. In **chapter 2**, acute (96-h) bioaccumulations in the two euryhaline species assessed were predictive of chronic (15-d) mortality, supporting one of the main concepts of the BLM. Therefore, these data may be used in the development of a marine BLM for Ni.

Fresh water

Michaelis-Menten parameters

Across four invertebrate and two vertebrate species, there were significant correlations between acute sensitivity to Ni and both kinetic parameters. In **chapter 3**, acute Ni LC50 values tended to increase with both K_d (Fig. 6.1A) and B_{max} values (Fig. 6.1B) - i.e. more sensitive species exhibited higher binding affinity (i.e. lower K_d) and lower binding capacity for Ni. Notably, however, for B_{max} the relationship was driven primarily by the extremely resistant *C. riparius*, whereas this was less true for the K_d vs. toxicity relationship. For the two vertebrate species studied in **chapter 4**, the relationship was strongest when assessing the kinetic parameters (predominantly K_d values) of the gills vs. other organs such as the gut, kidney and whole fish which also exhibited saturable Ni uptake. Therefore, in general, there was the strongest relationship with K_d values where lower K_d (higher affinity) was associated with higher Ni toxicity. This has implications for the BLM, especially with respect to invertebrates, as measurements of toxicity are used as alternatives to measurement of binding affinity in the derivation of the model (see next section).

Log K_{NiBL}

One of the main concepts of the BLM is that there is a strong overall correlation between log K values for gill binding and acute toxicity to the extent that measurement of binding affinity based on gill metal binding is an acceptable alternative to measurement of toxicity and vice versa. In **chapter 3** we evaluated whether this concept could be extended to Ni bioaccumulation in the whole body of invertebrates rather than bioaccumulation on a theoretical 'biotic ligand' (target site for toxicity) such as the gills in fish. And in **chapter 4**, we evaluated whether this concept could be extended beyond the biotic ligand of the gills to other organs such as gut, kidney, liver, brain, carcass, and whole fish.

In **chapter 3**, there was relatively good agreement between 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 – invertebrates). This suggests 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.

However, in **chapter 4**, for the gills of round goby the log K_{NiBL} values calculated from the ionic component of the K_d value were 0.8 log units higher than log K_{NiBL} values for the ionic component of the LC50 value (toxicity), whereas for the gills of rainbow trout there was a 0.4 log unit difference. These discrepancies suggest that a Ni BLM built on bioaccumulation would be more protective than one built on toxicity. The correlation between log K_{NiBL} values for binding and acute toxicity does not appear to extend to other organs (gut or whole fish) which are not considered the toxic site of action. To the best of our knowledge there are no other published comparisons of this nature.

CBR50

In **chapters 3 and 4**, LC50 values were compared against CBR50 values in invertebrates and fish species, respectively. Invertebrates were 3–3.5 times more tolerant to waterborne Ni in hard water (nominally 140 mg/L as $CaCO_3$) in comparison to soft water (nominally 40 mg/L as $CaCO_3$) whereas CBR50 values were not significantly different within a species at the two different water hardness values. A similar trend was observed in teleost fish, where CBR50 values between the two species varied much less than LC50 values between the two species. 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.

In this respect, Ni bioaccumulation may be used as a predictor of Ni toxicity. One of the main concepts of the BLM is that short term metal bioaccumulation can be used to predict longer term toxicity. In **chapter 5**, acute (96-h) gill Ni bioaccumulation was predictive of chronic (30-day) mortality in the round goby.

Sub-cellular Ni distribution

In **chapter 5**, freshwater acute (96-h) sub-cellular accumulations of Ni in either biologically active metal pools (BAM) or biologically inactive metal pools (BIM) were also predictive of chronic (30-d) mortality. There was greater sensitivity to BAM vs. BIM. Trout exhibited an ability to regulate gill Ni bioaccumulation and maintain the majority of the Ni in the MT fraction of the BIM. In contrast goby exhibited large Ni spillovers to both the HDP and ORG

fractions of the BAM in the gill. However, the same trend was not observed in the gut, where the potential acclimation of goby to pollutants from their collection site may have aided their ability to regulate Ni spillover to the BAM more so than in trout.

CONCLUSIONS

The objective of this thesis arose from the need for more knowledge on the use of Ni bioaccumulation as a predictor of toxicity. Although one of the main concepts of the BLM is the ability of short-term (24-h) binding constants to predict longer term survival (96-h), there had been no previous studies to show the direct relationship between Michaelis-Menten parameters and toxicity. In this manner, I showed a relatively good relationship between the Ni binding affinity (K_d values) and mortality where low K_d (high affinity) corresponds to higher toxicity of the metal.

I also assessed whether the current use of whole body bioaccumulation in invertebrates can serve as a surrogate for Ni binding to the theoretical 'biotic ligand' which causes toxicity. The results obtained further validated 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. This had not been previously assessed but the concept had been implemented into current BLMs for invertebrates.

There was very little information regarding the use of critical body residues (CBR50 values) as predictors of Ni toxicity. In this regard, I expanded the knowledge base to a suite of four invertebrates and two vertebrates in freshwater, and two invertebrates in seawater on acute and chronic time scales. As well, I extended this concept to sub-cellular fractions of Ni within the gills and gut of a teleost, which had not been previously done for Ni. This information shows promise for the use of both whole animal or whole tissue critical body residues as well as sub-cellular fractions in the understanding and prediction Ni toxicity.

Overall, this thesis advances the use of bioaccumulation as a predictor of Ni toxicity and may have implications for metal toxicity frameworks such as the BLM and TRA.

FUTURE DIRECTIONS

Future studies should employ co-exposures of two or more species to determine relationships of Ni bioaccumulation parameters between species and in

this manner explore the use of bioaccumulation parameters in a more tolerant species such as *Lumbriculus variegatus* to predict the toxicity in a more sensitive species such as *Lymnaea stagnalis*. These relationships could then be tested in environments of varying water chemistry to explore how robust they are, for example, by changing parameters such as Ca, Mg, pH and DOC which are known to affect Ni toxicity.

Another important future direction should be to explore dietary vs. waterborne routes of Ni exposures independently, as well as in combination, to gain a better understanding of mechanisms of Ni toxicity and how these different exposure routes affect Ni bioaccumulation parameters.

Long term objectives should address metal-mixtures and how they impact bioaccumulation parameters. The aforementioned studies would contribute to the body of literature that would inform metal toxicity frameworks such as the BLM and TRA for the derivation of WQC more suited to environmental exposure conditions in the real world.

Fig. 6.1

Correlation between Michaelis-Menten uptake parameters ((A) Bmax and (B) Kd) and LC50 values for Ni. Michaelis-Menten uptake parameters are means ± S.E.M.; n = 8-10 per treatment. n = 2 for the two teleost LC50 values and n = 3 for the four invertebrates LC50 values with 95% confidence intervals. For panel A, r^2 = 0.78, p = 0.017; for panel B, r^2 = 0.87, p = 0.003.
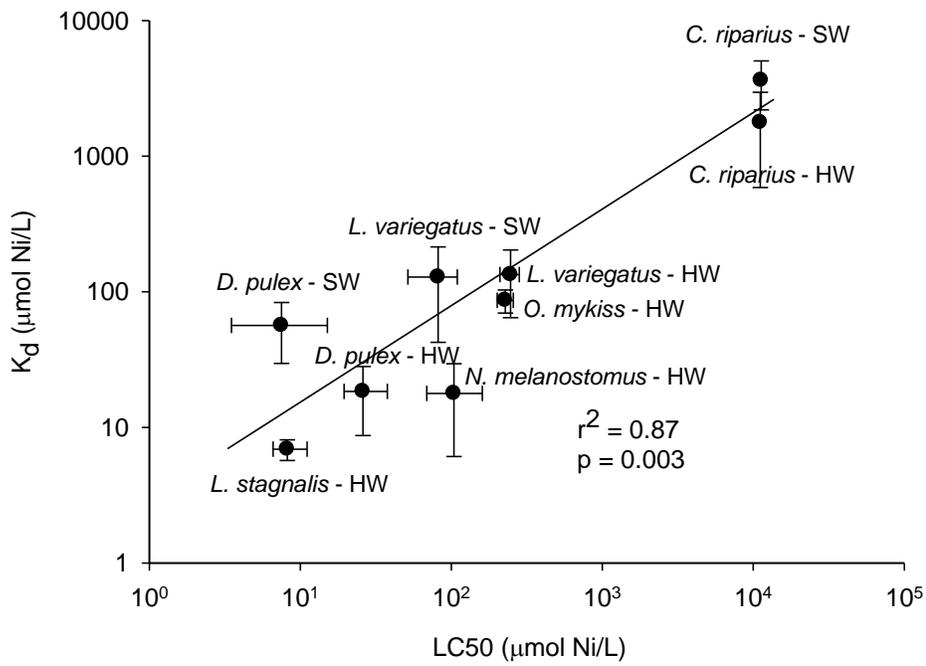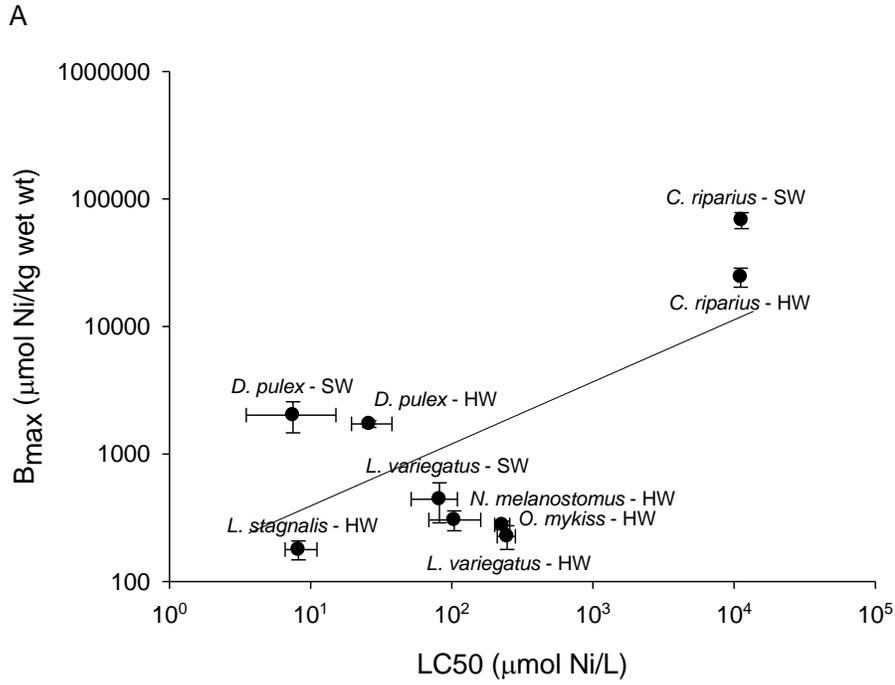
A

Fig. 6.2

Comparison between external effect concentrations (LC50 values) and internal effect concentration (CBR50 values) in freshwater across different species at two different water hardness values. LC50 values are found in Tables 3.2 and 4.4, whereas CBR50 values are in Tables 3.3 and 4.3. Gill CBR50 values derived using Toxcalc software was used for the two teleost species.
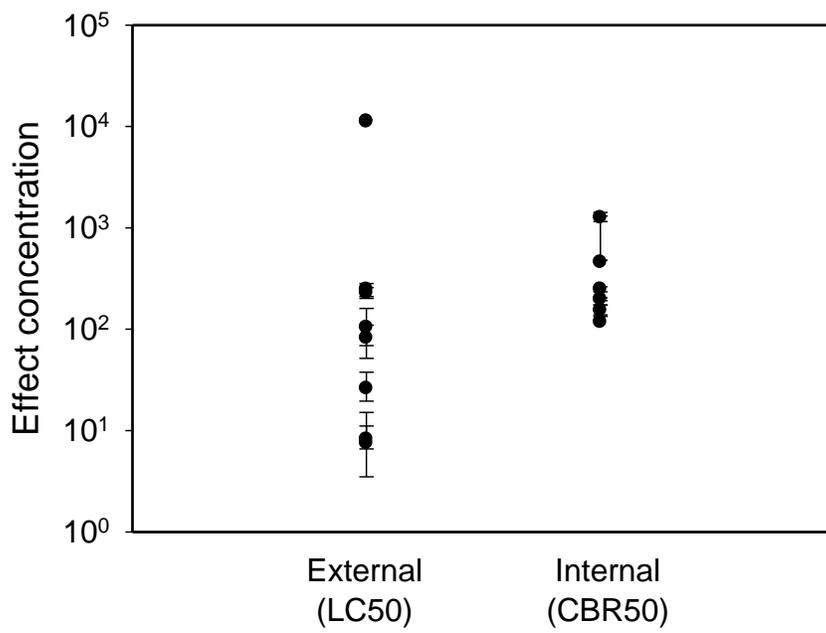
APPENDIX

Toxcalc methods

To calculate LC values using Toxcalc – Toxicity Data Analysis Software v5.0.32 (Tidepool Scientific Software, McKinleyville, CA, USA) average dissolved Ni exposure concentrations along with percent survival at each exposure concentration in each replicate were inputted into the program and arcsin squareroot transformed to obtain a dose-response curve. U.S. EPA protocol: EPAA 91-EPA/600/4-90/027F was used along with the test species of interest (e.g. *Oncorhynchus mykiss*, *Daphnia pulex*). In the cases where a species protocol was not available, the organism most representative of this species was used. The program utilizes Maximum Likelihood –probit to calculate LC values with their respective 95% confidence intervals.

In the instances where Toxcalc software was used to calculate CBR values, average Ni bioaccumulation was used instead of the dissolved Ni exposure concentrations. All other parameters were kept the same as described above.

Logit mortality vs. log bioaccumulation method

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 (Ng et al., 2013). Ni bioaccumulation and survival were corrected for control levels prior to analysis. Sample scatter plots are included in Fig. 7.1A and B along with sample calculation.

Appendix Fig. 1
Logit mortaility versus Log bioaccumulation method

A
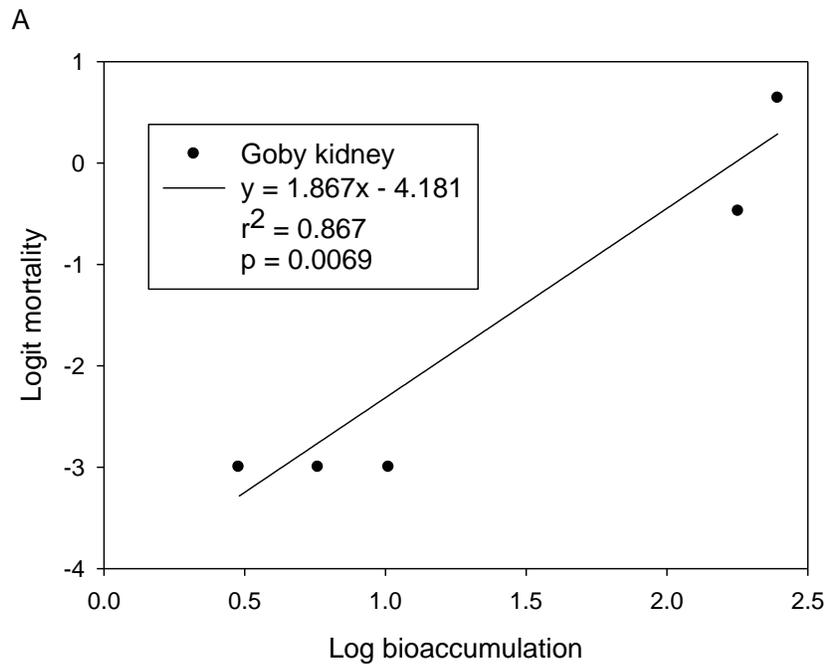
Solve for x at y=0
x = 2.239
The antilog of x is the CBR50 value = 173.38 μmol/kg wet wt.

  To determine 95 % confidence intervals (C.I.), re-plot graph with logit mortality on x-axis and log bioaccumulation on y-axis and insert CBR50 value (e.g. (0, 2.239)) as an added point. 95% C.I. are derived in Sigma Plot for each y-axis value.

B
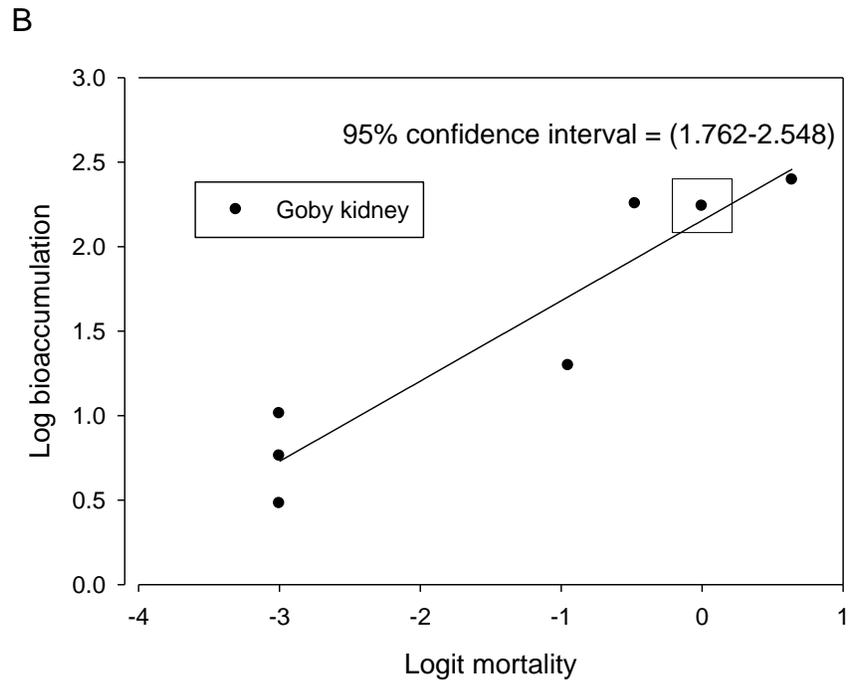
The antilog of 95% C.I. in this example derives a CBR50 value of 173. 38 μmol/kg wet wt (C.I.: 125.2-230.1).

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