



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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ARTICLE INFO

Article history:

Received 5 May 2011

Received in revised form 18 July 2011

Accepted 19 July 2011

Available online 24 July 2011

Keywords:

Nickel

Salinity

Acute

Chronic

Bioaccumulation

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 $\mu\text{mol L}^{-1}$ and 362 $\mu\text{mol L}^{-1}$ for *L. vannamei* and 278 $\mu\text{mol L}^{-1}$ and >1000 $\mu\text{mol L}^{-1}$ 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 $\mu\text{mol L}^{-1}$) 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.

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1. 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 $\mu\text{mol L}^{-1}$ in the open ocean and as high as 1.4 $\mu\text{mol L}^{-1}$ 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 cirrolanid 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, 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 cirrolanid 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

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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 intracellular 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) (Paquin et al., 2000; Di Toro et al., 2001; 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 (Deleebeck et al., 2007; 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 *Hyallolella 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 been 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 do 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.

2. Methods

2.1. Experimental organisms

2.1.1. *L. vannamei* and *E. 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 $\mu\text{mol Ni L}^{-1}$) and acclimated to laboratory conditions (i.e. water chemistry, photoperiod and temperature) for one week prior to salinity change.

2.1.2. 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 \approx 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^{-1}) for 5 ppt: Na^+ (71), Cl^- (85), Ca^{2+} (1.8), K^+ (1.4), Mg^{2+} (5.8), SO_4^{2-} (4.2), Ni ($<0.05 \times 10^{-5}$) with an alkalinity of 38 mg l^{-1} as CaCO_3 , dissolved organic carbon (DOC) of 0.13 mg C L^{-1} 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 ($<0.04 \times 10^{-5}$) with an alkalinity of 240 mg L^{-1} as CaCO_3 , DOC of 1.1 mg C L^{-1} and pH of 7.0. Temperature and photoperiod were fixed at 20 °C and 12 L: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 $\mu\text{mol kg}^{-1}$ dry wt.

2.2. 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^{-1} $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (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 5 days for measurement of pH and DOC. All samples were acidified to a final concentration of 1% HNO_3 (Suprapur, Merck) 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 h and then water was changed.

2.2.1. 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 $\mu\text{mol Ni L}^{-1}$.

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 $\mu\text{mol Ni L}^{-1}$. For *E. armata*, 0, 1, 10, 33, 56, 100, 333, 1000 $\mu\text{mol Ni L}^{-1}$ were the nominal Ni concentrations employed for acute and chronic LC50 values. Measured total and dissolved Ni concentrations 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; 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.

2.2.2. 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 $\mu\text{mol Ni L}^{-1}$. Chronic *L. vannamei* bioaccumulation was determined using nominal Ni concentrations of 0, 1, 3.3, 5.6 and 10 $\mu\text{mol Ni L}^{-1}$. 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.

2.3. 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, USA) against certified standards (Tritisol-Merck). The detection limit for measurement of Ni was 0.09 $\mu\text{g Ni mL}^{-1}$ (1.5 $\mu\text{mol L}^{-1}$). Water pH and DOC were measured using a Digimed DM 20 pH meter and a total organic carbon analyzer (VCPN series; Shimadzu, Japan), respectively.

2.4. 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, USA). 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 treat-

ment 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 $p < 0.05$.

3. Results

3.1. 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 $\mu\text{mol L}^{-1}$ and 362 $\mu\text{mol L}^{-1}$ as dissolved Ni, respectively; Table 1). This trend remained consistent when considering the nominal, total, dissolved, free ion and active fractions of the metal (Table 1). In contrast, chronic (30-d) LC50 values were not significantly different between 5 ppt (2.7 $\mu\text{mol L}^{-1}$) and 25 ppt (7.6 $\mu\text{mol L}^{-1}$), and this consistency was again independent of the Ni fraction being examined (Table 1). Note that acute LC50 values were approximately 15- and 50-fold higher than chronic LC50 values at 5 and 25 ppt, respectively (Table 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 $\mu\text{mol L}^{-1}$ and $>1000 \mu\text{mol L}^{-1}$ as dissolved Ni respectively Table 2). Note that organisms were not exposed to Ni concentrations above 1000 $\mu\text{mol L}^{-1}$ 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 $\mu\text{mol L}^{-1}$) and 25 ppt (23 $\mu\text{mol L}^{-1}$), and this trend was independent of the Ni fraction being examined (Table 2). Acute LC50 values were 35- and >45 -fold higher than chronic LC50 values at 5 and 25 ppt, respectively (Table 2).

3.2. 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. 1A, B). In contrast, Ni bioaccumulation continued to increase over time in *E. armata* (Fig. 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. 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 $\mu\text{mol g}^{-1}$ wet wt, not significantly different from the mean value in non-exposed control animals (Fig. 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. 2D). However, again, Ni burden did not differ within an exposure concentration at the two salinities (Fig. 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. 3A, B). It should be noted that 15-d mortality was used for *L. vannamei* in Fig. 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 ($\sim 0.1 \mu\text{mol Ni g}^{-1}$ 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. 3C).

Table 1
Acute (96-h) and chronic (30-d) LC50 values ($\mu\text{mol L}^{-1}$) for waterborne Ni toxicity in *Litopenaeus vannamei*.

Salinity	Nominal	Total	Dissolved	Ionic	Active
<i>Acute (96-h)</i>					
5 ppt	42 (25–69)	41 (25–67)	41 (25–66)	37 (23–61)	14 (8.61–23)
25 ppt	385* (270–540)	363* (255–510)	362* (254–508)	295* (207–415)	85* (59–119)
<i>Chronic (30-d)</i>					
5 ppt	2.9 (1.8–4.1)	2.9 (1.9–4.2)	2.7 (1.7–4.0)	2.5 (1.6–3.7)	0.9 (0.6–1.4)
25 ppt	8.3 (2.3–24)	8.0 (1.9–25)	7.6 (1.7–25)	6.1 (1.4–19)	1.8 (0.5–4.9)

Acute (96-h) and chronic (30-d) LC50 values ($\mu\text{mol L}^{-1}$) 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 $\mu\text{mol L}^{-1}$. * Indicates a significant difference in LC50 values between the two salinities.

Table 2
Acute (96-h) and chronic (15-d) LC50 values ($\mu\text{mol L}^{-1}$) for waterborne Ni toxicity in *Excirrolana armata*.

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

Acute (96-h) and chronic (15-d) LC50 values ($\mu\text{mol L}^{-1}$) for waterborne Ni toxicity in *Excirrolana 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 $\mu\text{mol L}^{-1}$. * Indicates a significant difference in LC50 values between the two salinities. Note organisms were not exposed to Ni concentrations above 1000 $\mu\text{mol L}^{-1}$ due to lack of environmental relevance.

3.3. Bioconcentration factors for Ni

Acute bioconcentration factors (BCFs) for *L. vannamei* were 40–60 l kg^{-1} wet wt. at 1 $\mu\text{mol L}^{-1}$, but at 10 $\mu\text{mol L}^{-1}$ significantly decreased by 85% and 80% in 5 ppt and 25 ppt, respectively (Fig. 4A). Between 10 and 333 $\mu\text{mol L}^{-1}$, acute BCFs did not change significantly, averaging approximately 5 l kg^{-1} wet wt., and were independent of salinity (Fig. 4A). Chronic BCFs in surviving animals at 30-d exhibited similar patterns to acute BCFs, and were again independent of salinity (Fig. 4B).

A similar trend was observed for *E. armata* where acute and chronic BCFs decreased with increasing exposure concentrations (Fig. 4C and D). At 1 $\mu\text{mol L}^{-1}$ acute BCFs were 153 and 336 l kg^{-1} wet wt. for 5 and 25 ppt, respectively and decreased by 75 and 94% by 10 $\mu\text{mol L}^{-1}$. In 5 ppt, the acute BCF continued to significantly decrease in the 33 $\mu\text{mol L}^{-1}$ exposure. Above this concentration, only slight variations in BCFs were observed (Fig. 4C). Chronic BCFs showed a similar, less marked pattern across exposure concentrations (Fig. 4D). At 5 ppt, the BCF significantly decreased from 70 l kg^{-1} wet wt. at an exposure concentration of 1 $\mu\text{mol L}^{-1}$ to 35 l kg^{-1} wet wt. at 33 $\mu\text{mol L}^{-1}$. At 25 ppt, the BCF significantly decreased by 75% from concentrations of 1 $\mu\text{mol L}^{-1}$ to 10 $\mu\text{mol L}^{-1}$, and remained constant over higher exposure concentrations (Fig. 4D).

3.4. Essential ions homeostasis

Average Na values were 180 and 243 $\mu\text{mol g}^{-1}$ wet wt for *L. vannamei* in 5 and 25 ppt, respectively (Fig. 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 $\mu\text{mol g}^{-1}$ wet wt in the controls to 50 $\mu\text{mol g}^{-1}$ wet wt in the 333 $\mu\text{mol Ni L}^{-1}$ treatment on day 4 (Fig. 5A). Mg whole body concentrations averaged 7.1 and 12 $\mu\text{mol g}^{-1}$ wet wt at 5 and 25 ppt respectively (Fig. 5C, D). However,

at 5 ppt, whole body Mg significantly decreased by 60% in the 333 $\mu\text{mol Ni L}^{-1}$ treatment on day 4 (Fig. 5C), and significantly decreased by 65% in the 10 $\mu\text{mol Ni L}^{-1}$ treatment on day 15 (Fig. 5C). At 25 ppt on day 4, there was a significant 70% decrease in whole body Mg in the 10 $\mu\text{mol Ni L}^{-1}$ exposure (Fig. 5D). All significant decreases in Mg could be directly correlated with significant increases in whole body Ni (Fig. 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. 5E, F). Whole body Ca concentrations averaged 91 and 138 $\mu\text{mol g}^{-1}$ wet wt for *L. vannamei* in 5 and 25 ppt, respectively (Fig. 5E, F).

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

4. Discussion

4.1. 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, *L. vannamei*, and the cirrolanid isopod, *E. armata*, at two salinities (5 and 25 ppt) were assessed. Salinity acts protectively against acute Ni toxicity with 96-h LC50 values of 41 $\mu\text{mol L}^{-1}$ and 362 $\mu\text{mol L}^{-1}$ for *L. vannamei* and 278 $\mu\text{mol L}^{-1}$ and >1000 $\mu\text{mol L}^{-1}$ 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; Tables 1 and 2). However,

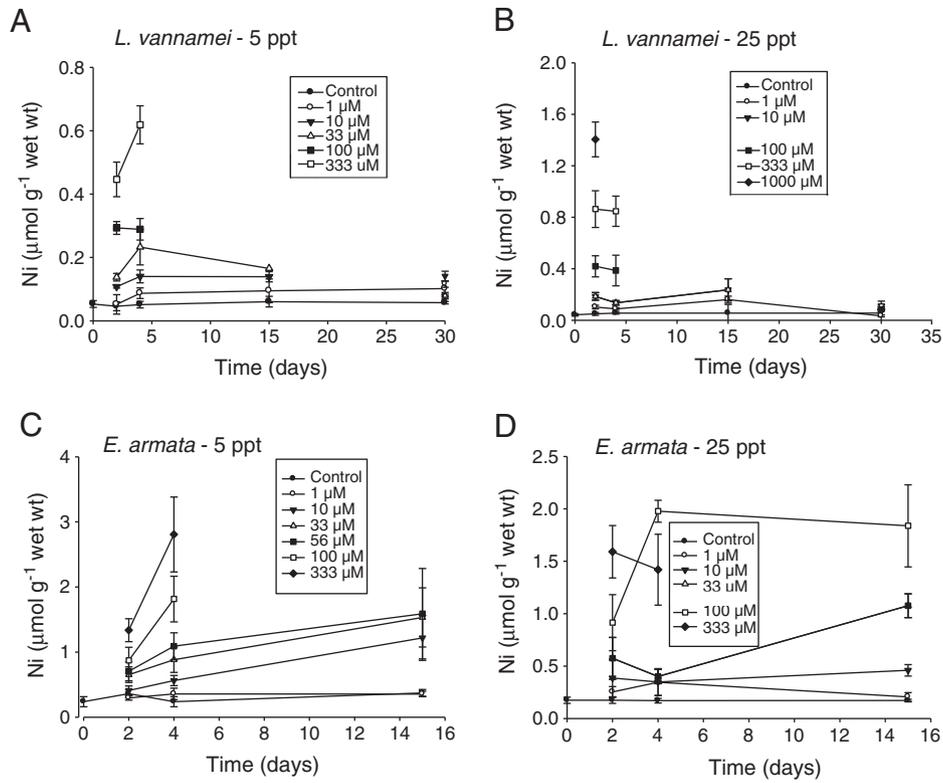


Fig. 1. Time dependent Ni bioaccumulation over a range of exposure concentrations from 1 to 333 µmol Ni L⁻¹ on days 0, 2, 4, 15 and 30 at (A, C) 5 ppt and (B, D) 25 ppt for *Litopenaeus vannamei* (A, B) and *Excirolana armata* (C, D). Values are means ± S.E.M.; n = 5–16 per treatment.

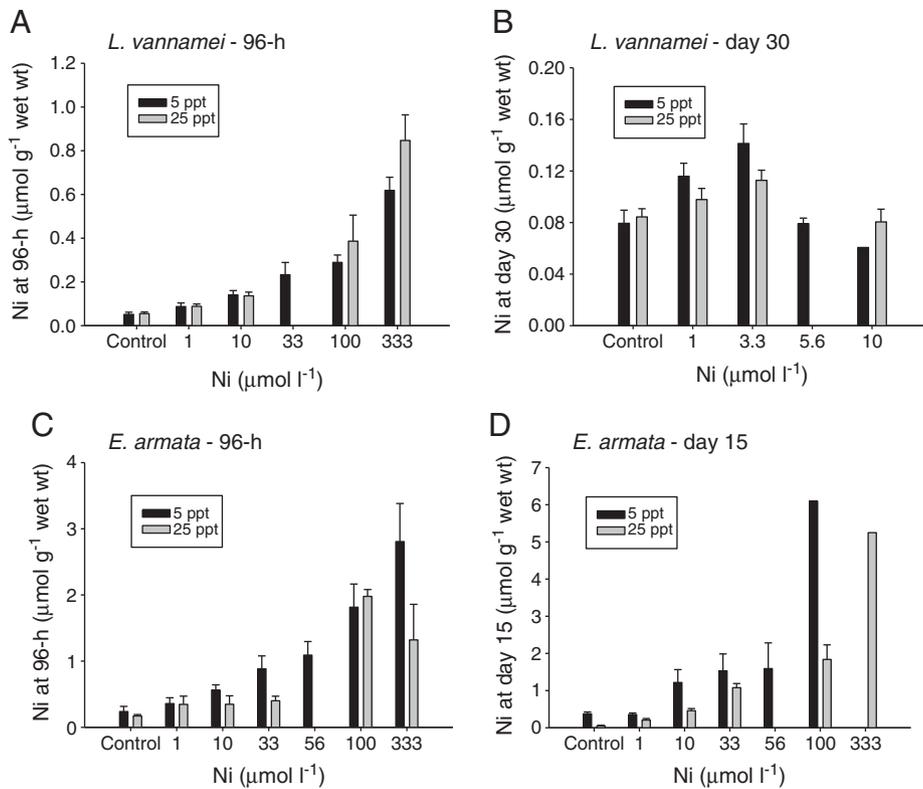


Fig. 2. Concentration-dependent Ni uptake over a range of exposure concentrations from 1 to 333 µmol Ni L⁻¹ at 96-h (A) and day 30 (B) for *Litopenaeus vannamei* and at 96-h (C) and day 15 (D) for *Excirolana armata*. Values are means ± S.E.M.; n = 5 per treatment (acute) and n = 7–16 per treatment (chronic). There was no significant difference in Ni bioaccumulation within the same exposure concentration between the two salinities.

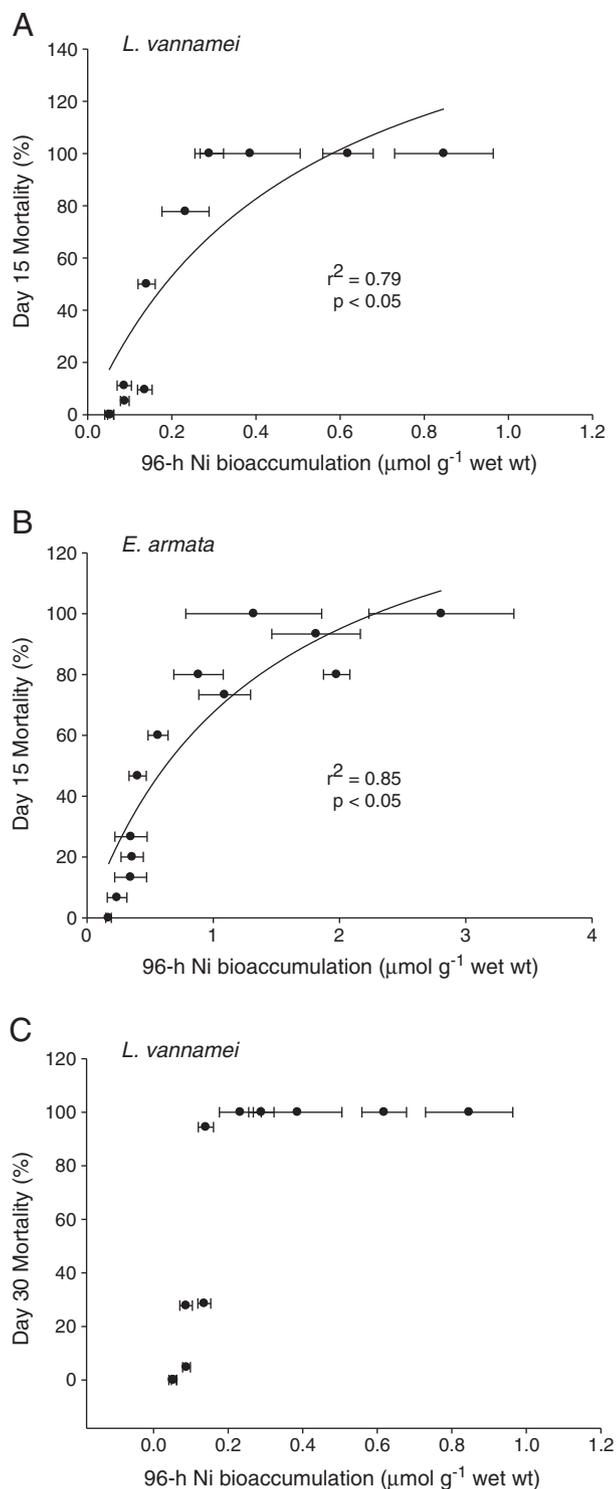


Fig. 3. Early (96-h) Ni bioaccumulation plotted against chronic (15 or 30-d) mortality at both salinities. (A) *Litopenaeus vannamei* – 15-d mortality (B) *Excirrolana armata* – 15-d mortality and (C) *L. vannamei* – 30-d mortality. Values are means \pm S.E.M.; $n = 5$ –16 per treatment.

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 $\text{Ni}^{2+} = 78\%$ in 25 ppt and 89% in 5 ppt) (Tables 1 and 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 1 and 2) with 30-d LC50 values of $2.7 \mu\text{mol L}^{-1}$ and $7.6 \mu\text{mol L}^{-1}$ (n.s.) for *L. vannamei* (Table 1) and 15-d LC50 values of $7.9 \mu\text{mol L}^{-1}$ and $23 \mu\text{mol L}^{-1}$ (n.s.) for *E. armata* at 5 ppt and 25 ppt, respectively (Table 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., 2003a,b). In addition, both Heijerick et al. (2005) and De Schampelaere 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, 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 $150 \mu\text{mol L}^{-1}$ (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

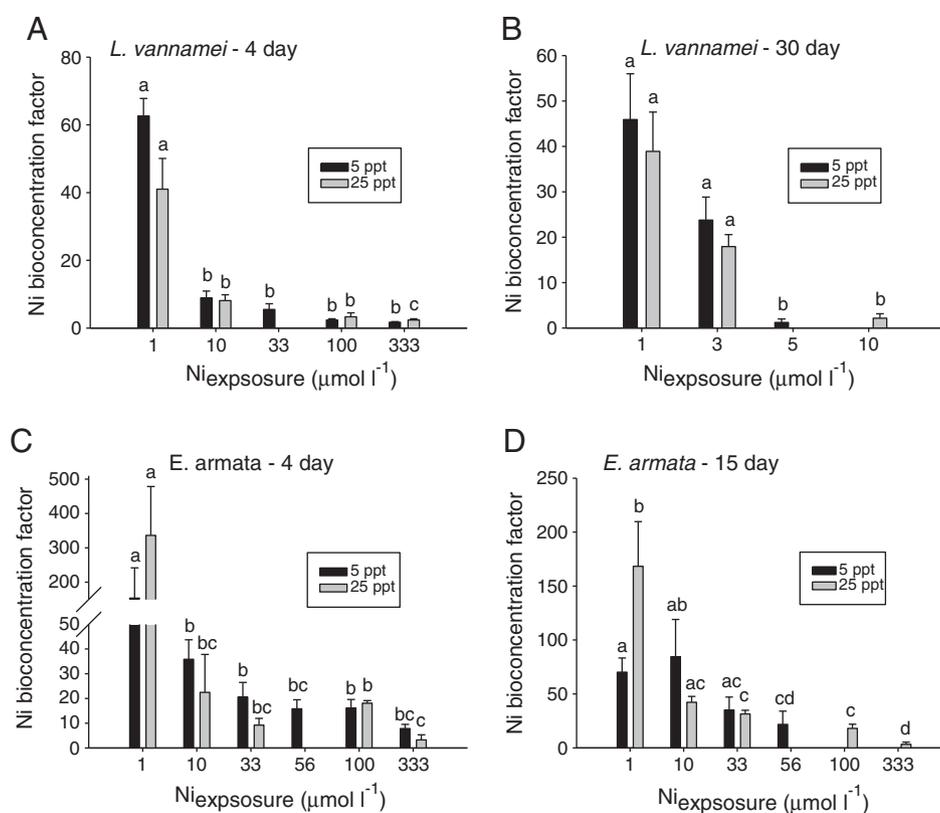


Fig. 4. Bioconcentration factor (BCF = concentration of Ni in organism expressed in $\mu\text{mol kg}^{-1}$ wet wt divided by the Ni concentration in exposure medium expressed in $\mu\text{mol l}^{-1}$) in *Litopenaeus vannamei* at 96-h (A) and day 30 (B) and in *Excirrolana armata* at 96-h (C) and day 15 (D). Values are means \pm S.E.M.; $n = 5$ –16 per treatment. Different letters denote significant differences in bioconcentration factors.

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 (1985) for dissolved Ni are $1.3 \mu\text{mol l}^{-1}$ and $0.14 \mu\text{mol l}^{-1}$, respectively. The Canadian Water Quality Guideline (chronic) by the CCME (2005) is $0.4 \mu\text{mol Ni l}^{-1}$. Therefore, *L. vannamei* and *E. armata* would be protected in both the U.S. and Canada against acute and chronic Ni toxicity (Table 1), though the margin of protection is considerably lower at 5 ppt than at 25 ppt.

4.2. Concentration-dependent Ni bioaccumulation and essential ion homeostasis

The significant difference between acute LC50 values at 5 and 25 ppt for *L. vannamei* (Table 1) cannot be explained by a difference in tissue Ni burden (Fig. 2A). However, one possible mechanism of acute toxicity may be the perturbation of the homeostasis of the essential ion, Mg (Figs. 5C, D and 6). All significant decreases in whole body Mg can be correlated with significant increases in whole body Ni concentration at both salinities (Fig. 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* (Snively 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 $333 \mu\text{mol l}^{-1}$ treatment for *L. vannamei* (Fig. 5C). However, in 25 ppt, Mg homeostasis was restored in *L. vannamei*, where this same 70% reduction ($10 \mu\text{mol l}^{-1}$ treatment) in whole body Mg returned to control values by day 15 of sampling time (Fig. 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 in 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 in rainbow trout at 24-h in freshwater, suggesting a trend for acute Na loss in freshwater or low salinity environments.

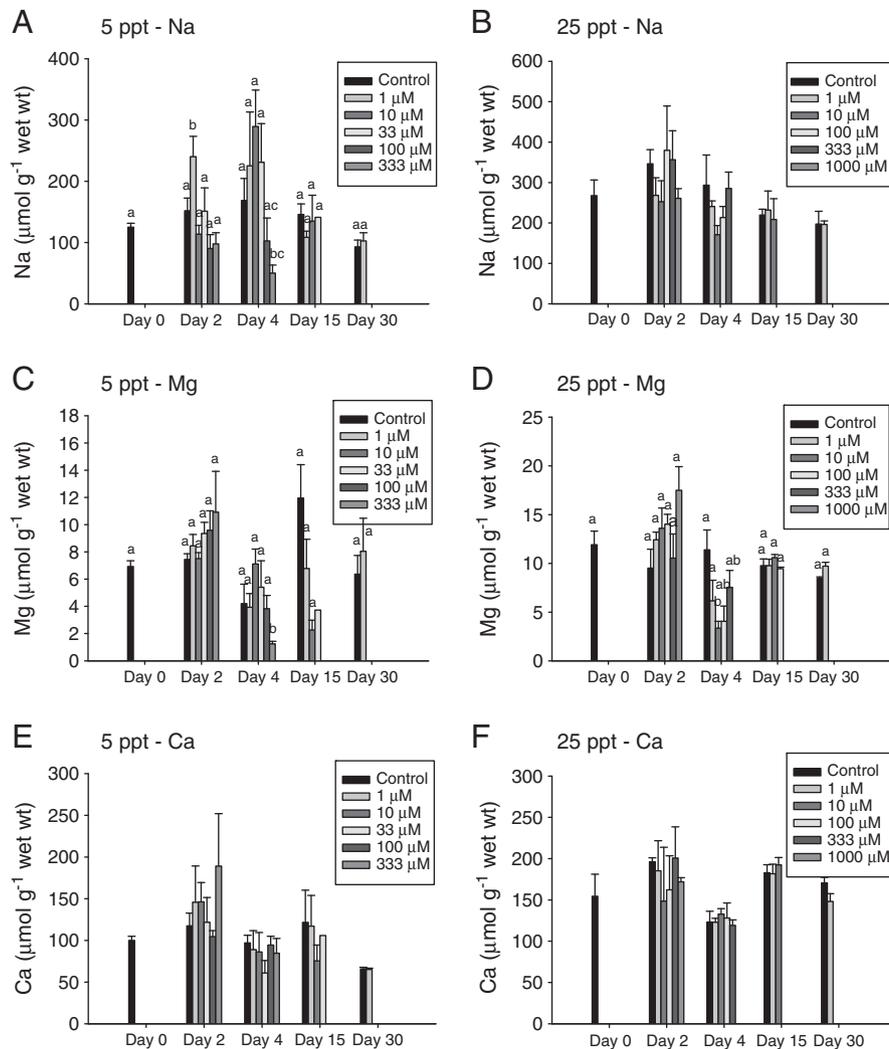


Fig. 5. Whole body ion concentrations in 5 ppt (A, C, E) and 25 ppt (B, D, F) for *Litopenaeus vannamei* over chronic (30-d) Ni exposure. Organisms were sampled on days 0, 2, 4, 15 and 30. Values are means \pm S.E.M.; $n = 5$ per treatment. Different letters denote significant differences in whole body ions within a sampling day. No letters denote no significant differences in whole body ions within a sampling day (B, E and F).

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 to 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, 1984). 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 Fleeger, 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, *O. mykiss*, fathead minnows, *Pimephales promelas* (Meyer et al., 1999; Deleebeeck et al., 2007) 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 molting of the exoskeleton to allow for growth (Greenaway, 1985). Therefore, regeneration of Ca throughout the molting cycle of these crustaceans may allow for rapid Ca replenishment (Huner et al., 1979), allowing internal Ca levels to be maintained.

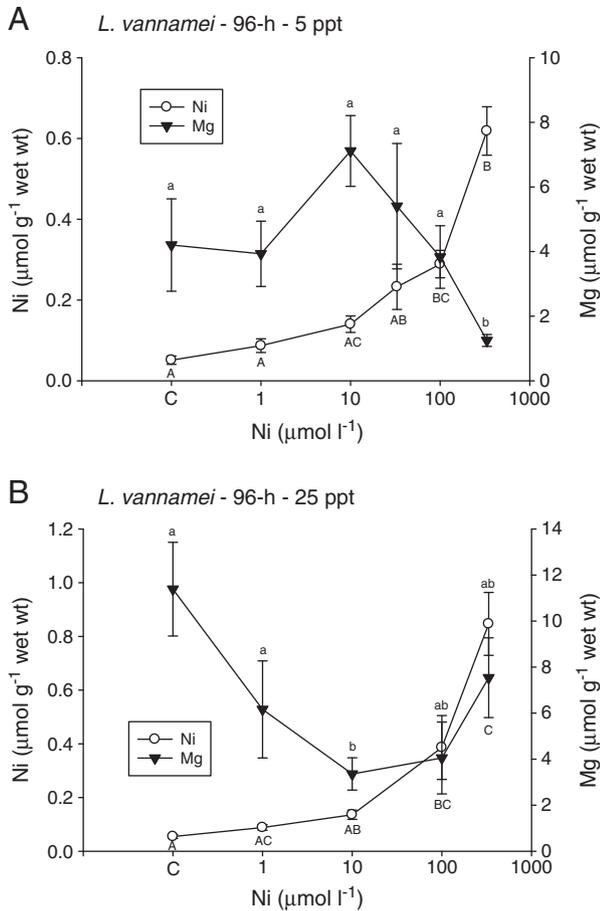


Fig. 6. Whole body Ni and Mg plotted against Ni exposure concentrations on day 4 at 5 ppt (A) and 25 ppt (B) of Ni exposure in *Litopenaeus vannamei*. No significant difference in whole body Mg was found in *Excirolana armata*. Values are means \pm S.E.M.; $n=5$ per treatment. Different letters denote significant differences in whole body ion concentration. Capital letters denote Ni and lower case letters denote Mg.

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 $\mu\text{mol L}^{-1}$ for 5 ppt and control, 1, 3 and 10 $\mu\text{mol L}^{-1}$ for 25 ppt; Fig. 2B as well as Fig. 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 $\sim 0.1 \mu\text{mol g}^{-1}$ wet wt. for chronic exposures (Fig. 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. 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.

4.3. 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. 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.

5. 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 $\sim 0.1 \mu\text{mol g}^{-1}$ 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.

Acknowledgments

We wish to thank Dr. Derek Alsop and Sunita Nadella (McMaster University, Hamilton, ON, Canada) for their expertise and help. This research was supported by an award from the International Development Research Centre (IDRC) and the Canada Research Chair Program to AB and CMW. CMW is supported by the Canada Research Chair Program. A. Bianchini is a research fellow from the Brazilian CNPq (Proc. #304430/2009-9) and is supported by the International Research Chair Program from IDRC.

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