Ecotoxicology and Environmental Safety 122 (2015) 159–170

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Low salinity enhances NI-mediated oxidative stress and sub-lethal toxicity to the green shore crab (*Carcinus maenas*)

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ARTICLE INFO

Article history:
Received 2 March 2015
Received in revised form
15 July 2015
Accepted 18 July 2015
Available online 31 July 2015

Keywords:
Oxidative stress
Invertebrate
Salinity
Metal
Catalase
Osmoregulation

ABSTRACT

Nickel (Ni) is a metal of environmental concern, known to cause toxicity to freshwater organisms by impairing ionoregulation and/or respiratory gas exchange, and by inducing oxidative stress. However, little is known regarding how nickel toxicity is influenced by salinity. In the current study we investigated the salinity-dependence and mechanisms of sub-lethal Ni toxicity in a euryhaline crab (*Carcinus maenas*). Crabs were acclimated to three experimental salinities – 20, 60 and 100% seawater (SW) – and exposed to 3 mg/L Ni for 24 h or 96 h. Tissues were dissected for analysis of Ni accumulation, gills were taken for oxidative stress analysis (catalase activity and protein carbonyl content), haemolymph ions were analysed for ionoregulatory disturbance, and oxygen consumption was determined in exercised crabs after 96 h of Ni exposure. Total Ni accumulation was strongly dependant on salinity, with crabs from 20% SW displaying the highest tissue Ni burdens after both 24 and 96-h exposures. After 96 h of exposure, the highest accumulation of Ni occurred in the posterior (ionoregulatory) gills at the lowest salinity, 20% SW. Posterior gill 8 exhibited elevated protein carbonyl levels and decreased catalase activity after Ni exposure, but only in 20% SW. Similarly, decreased levels of haemolymph Mg and K and an increased level of Ca were recorded but only in crabs exposed to Ni for 96 h in 20% SW. Oxygen consumption after exercise was also inhibited in crabs exposed to Ni in 20% SW. These data show for the first time the simultaneous presence of all three modes of sub-lethal Ni toxicity in exposed animals, and indicate a strong salinity dependence of sub-lethal Ni toxicity to the euryhaline crab, *C. maenas*, a pattern that corresponded to tissue Ni accumulation.

1. Introduction

Concentration alone does not determine the toxicity of metals to aquatic biota. The bioavailability of a metal is also important, a parameter largely driven by water chemistry and physiology. The importance of these factors has been recognised by the development of predictive modelling approaches, such as the Biotic Ligand Model (BLM; Paquin et al., 2002). One key factor that impacts metal bioavailability – by altering both metal speciation and the concentration of potentially protective natural cations, as well as the physiology of the organism – is salinity. For nickel (Ni), speciation changes only slightly with salinity (Pyle and Couture, 2012), but Ni uptake and toxic effects may be impacted by variations in levels of protective cations (Na^+, Mg^{2+}, Ca^{2+}, K^+) at different salinities, and also by physiological changes in the animal, such as the need for different ionoregulatory strategies as salinity changes. While our understanding of Ni uptake and toxicity is relatively well-developed for freshwater animals (Pane et al., 2003a,b, 2004a,b, 2005, 2006a,b), and has advanced significantly in the last few years for marine biota (Blewett and Wood, 2015; Blewett et al., 2015; Leonard et al., 2011; Tellis et al., 2014), mechanisms of Ni uptake and sub-lethal toxicity at intermediate salinities-such as those encountered by animals in estuarine or coastal settings-are largely unknown.

Ni is a metal of environmental concern. It is found in estuarine and coastal environments resulting from both natural (e.g. soil erosion) and anthropogenic sources (e.g. combustion of fossil fuels) (Eisler, 1998; ECB, 2008; NAS, 1975; WHO, 1991). Levels of Ni in the open ocean are up to 2.32 μg/L, but concentrations are much higher in estuaries and coastal regions (up to 100 μg/L) (Boyden, 1975). In industrially-intense regions, freshwater concentrations can reach as high as 1000 μg/L (Pyle and Couture, 2012).

The exact mechanism of Ni uptake into aquatic organisms is unknown, but it may involve ion mimicry, with transport of Ni through both calcium (Ca) and magnesium (Mg) pathways (Pane

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http://dx.doi.org/10.1016/j.ecoenv.2015.07.019
0147-6513/

et al., 2003a,b). Such a shared pathway of uptake would explain one putative mode of Ni toxicity to aquatic animals – ionoregulatory disruption. Ionoregulatory toxicity is considered the main mechanism of toxic effect in invertebrate species exposed to Ni. For example, in the freshwater cladoceran *Daphnia magna*, Ni inhibited unidirectional Mg influx, causing a large decrease in whole body Mg stores (Pane et al., 2003b). This was also seen in *Daphnia pulex*, together with comparable decreases in whole body sodium (Na) levels, and Ni-induced disruptions in Mg and Na homoeostasis were also reported in a snail, *Lymnaea stagnalis*, and an oligochaete, *Lumbriculus variegatus* (Leonard et al., 2011). In the marine environment, Leonard et al. (2011) observed a disruption in both Na and Mg homoeostasis in a shrimp, *Litopenaeus vannamei*, following Ni exposure at two different salinities. Tellis et al. (2014) reported a marked Ni inhibition of Ca uptake, Ca^{2+}ATPase activity, and whole body calcification in early life stages of the sea urchin *Strongylocentrotus purpuratus* in full strength sea water. Previous studies on the euryhaline crab *Carcinus maenas* have reported impairment of Na^+/K^+ ATPase activity in the posterior gills and disturbance in haemolymph ions after a 24-h Ni exposure (Blewett et al., 2015).

A second mode of Ni toxicity is respiratory impairment, which has been observed in aquatic vertebrates (Pane, 2003a, 2004a,b), and indirectly in aquatic invertebrates (Pane et al., 2003b), exposed to relatively high levels of Ni in fresh water. In rainbow trout, *Oncorhynchus mykiss*, levels of Ni in excess of 10 mg/L inhibited gas exchange, and ultimately oxygen consumption (Pane et al., 2003 a,b, 2004b). This was a consequence of gill ultrastructural damage, with fused lamellae and extensive swelling noted. Similar impacts on both gill morphology and respiratory performance of fish exposed to Ni had been observed previously (Hughes et al., 1979; Nath and Kumar, 1989).

A third mechanism of Ni toxicity is oxidative stress. In mammals, administration of Ni causes increased lipid peroxidation, inhibited glutathione peroxidase activity and altered tissue Fe levels (Stohs and Bagchi, 1995). The mechanism by which Ni induces oxidative damage is believed to be via displacement of Fe from its metal cofactor binding sites on important cellular proteins, leading to increased flux of this metal into the Fenton reaction, thereby generating hydroxyl radicals (Stohs and Bagchi, 1995). Ni may also have an effect on antioxidant mechanisms, thereby decreasing the cell's ability to scavenge reactive oxygen species (ROS), and leading to increased ROS-related damage. In the freshwater environment the relationship between Ni and oxidative stress has been investigated mainly in goldfish, *Carassius auratus* (Kubrak et al., 2012a,b, 2013, 2014). Ni has also been shown to affect antioxidant enzymes in both freshwater- and seawater-acclimated killifish, *Fundulus heteroclitus* (Blewett and Wood, 2015) while other metals (Cu, Zn) have been shown to induce oxidative stress in both freshwater and estuarine fish (e.g. Craig et al., 2007; Loro et al., 2012). Reduced salinity has been shown to exacerbate oxidative stress in metal-exposed killifish (Blewett and Wood, 2015; Loro et al., 2012). However, very few data exist in terms of the interactions between metals, salinity and oxidative stress in marine invertebrates (Sabatini et al., 2009; Vlahogianni et al., 2007).

The current study investigated the effect of salinity on Ni accumulation and mechanisms of sub-lethal Ni toxicity in the green shore crab (*Carcinus maenas*). This is a euryhaline decapod, previously shown to be sensitive to environmentally-relevant levels of Ni (Blewett et al., 2015) and is quickly becoming a model species in ecotoxicology (Leignel et al., 2014). We hypothesised that Ni bioaccumulation would be greater at lower salinities due to lower availability of protective cations. A related hypothesis was that the effectiveness of Ni in inducing oxidative stress would be similarly enhanced at low salinities. Finally, we also predicted the requirement of *Carcinus* to perform active ionoregulation at lower salinities would exacerbate ionoregulatory toxicity. The current investigation examined the salinity-dependence and time-dependence of a relatively high Ni exposure level (3 mg/L)-chosen because previous evidence has shown enzymatic and ionic disruption at this level in *Carcinus* (Blewett et al., 2015). Ni accumulation, and endpoints of ionoregulatory, respiratory and oxidative stress toxicity were examined over exposure periods of 24 and 96 h, at salinities of 20%, 60%, and 100% sea water. A particular focus was potential differences in responses between anterior (respiratory) and posterior gills (ionoregulatory), because of their different functions (Freire et al., 2008; Mantel and Farmer, 1983; McNamara and Lima, 1997; Onken and Riestenpatt, 1998; Péqueux, 1995).

2. Methods

2.1. Animal collection and maintenance

Male green crabs (*Carcinus maenas*) of mean mass 55.3 ± 7.8 g, and mean carapace width 5.04 ± 1.3 cm, were collected via traps under a licence from Fisheries and Oceans Canada in the summers of 2012 and 2013. The collection area was an uncontaminated site on the west coast of Vancouver Island, just outside of Pipestem Inlet (N 49°02.274 – W 125°20.710 and N 49° 01.749 – W 125°21.515) in Barkley Sound (BC, Canada). Crabs were transported back to Bamfield Marine Science Centre (Bamfield, BC; BMSC) and placed in constantly aerated 200-L tanks receiving flow-through seawater (~32 ppt SW) and exposed to a natural daylight cycle (10 h D:14 h L). Crabs were allowed to acclimate for a week in these conditions before they were equally distributed into 68- L aquaria (approximately 20 crabs per aquaria) containing one of three salinities: 20% (6.4 ppt), 60% (19.2 ppt), or 100% SW (32 ppt). 100% Bamfield SW was diluted with nanopure water to create the lower two salinities. Each aquarium was equipped with a recirculating carbon filtration system and constant aeration. Crabs were left in these aquaria for 10 days to acclimate, with water changes performed every 3 days. Throughout this acclimation period crabs were fed twice weekly with salmon fish heads (with water changed after feeding), but food was withheld 48 h prior to any experimentation. All procedures were approved by Bamfield Animal Research Ethics Committee and were in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2. Ni exposure and respirometry

After salinity acclimation, seven crabs were placed in individual aquaria, containing 10 L of water at each of the exposure salinities (20%, 60% or 100% SW). Ni (3 mg/L) from a $NiCl_2 \cdot 6H_2O$ (Sigma Aldrich) stock was added to exposure aquaria 24 h prior to crab addition to allow for equilibration, while radiolabelled ^{63}Ni (0.5 μCi/L; Amersham Biosciences, Inc., USA) was added to exposure aquaria 30 min prior to crab addition. Crabs were exposed to Ni for one of two exposure periods: 24 or 96 h. In parallel, controls crabs ($N=7$ at each salinity) were held under identical conditions but in the absence of Ni. At the conclusion of the 24-h exposure period, the Ni-exposed crabs were removed from the aquaria, rinsed in a high Ni solution (10 mg/L, $NiCl_2 \cdot 6\ H_2O$), and subsequently a 1 mM EDTA solution, to remove any loosely-bound radioisotope, before being placed on ice for anaesthesia. Control crabs were similarly anaesthetised.

A small preliminary experiment was run to determine the extent of Ni adsorption to both live and freshly dead crabs; the exposure followed the procedures as above. All crabs that were placed on ice were euthanized by a single spike to the ventral ganglion through the ventral wall. Briefly, Ni (3 mg/L) from a $NiCl_2 \cdot 6H_2O$ stock was added to exposure aquaria 24 h prior to

crab addition to allow for equilibration, while radiolabelled ^{63}Ni (0.5 μCi/L; Amersham Biosciences, Inc., USA) was added to exposure aquaria 30 min prior to crab addition. Exposures lasted for 3 h and at the end of this time Ni-exposed crabs were removed from the aquaria, rinsed in a high Ni solution (10 mg/L, $NiCl_2 \cdot 6H_2O$), and subsequently a 1 mM EDTA solution, to remove any loosely-bound radioisotope, before being placed on ice for anaesthesia. Only carapace was taken for tissue measurements described below.

The crabs in the 96-h exposure (control and Ni-exposed, $N=5$) were removed from their exposure chambers and exercised to exhaustion for 10 min by chasing, followed by repeatedly gently placing the crab on its back and allowing the animal to right itself (Booth and McMahon, 1985). This was performed in water of identical salinity and Ni concentration to that in which they had been previously exposed. Exhaustion was determined by the refusal of the crab to respond to a tactile stimulus, or an inability to right itself. At the conclusion of the exercise period, individual crabs were placed in 1.5-L respirometers containing the respective exposure salinity and Ni concentration. The respirometers were placed in a recirculating water bath maintained at 18 °C. Crabs were left in the respirometers for 1 h and water samples (5 ml) were taken at 0 and 1 h time points, for determination of the partial pressure of oxygen (PO_2) using a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas, TX, USA) connected to a AM Systems Polarographic Amplifier (Model 1900, Carlsburg, WA, USA) digital dissolved oxygen metre. The electrode was maintained and calibrated at the experimental temperature (18 °C). These 96-h exposed crabs were then rinsed and placed on ice, as described above for those crabs exposed for 24 h. At each salinity, there was also a group of resting control crabs ($N=5$) that were not exercised or dosed with Ni, but were tested in respirometers as above.

All crabs that were placed on ice were euthanized by a single spike to the ventral ganglion through the ventral wall. The following tissues were then excised and digested as described in Section 2.3 below: carapace, muscle, hepatopancreas (HP), haemolymph, heart, antennal gland, carcass, and gill pairs (2–9) (except 5 and 8, where a single gill was taken for digestion). The second gills of pairs 5 and 8 were taken for oxidative stress analyses, and once excised were placed immediately in liquid nitrogen and then transferred to a −80 °C freezer.

2.3. Tissue analyses

Thawed tissues were weighed and placed in 50-ml, 15-mL, or 2-mL plastic centrifuge tubes as appropriate, depending on mass. Gill, heart, and antennal gland were digested in 1N trace metal grade nitric acid while muscle, carapace and hepatopancreas were digested in 2N trace metal grade nitric acid (Sigma-Aldrich) at volumes 3–5 times the weight of the tissue. Once the acid was added, all tubes were sealed and placed into an incubator at 65 °C for 48 h, with vigorous vortexing at 24 h. After 48 h, the digested samples were centrifuged for 5 min at 3500 rpm at 18 °C. The following supernatant volumes were taken for analysis: 2 mL for all large tissues (carapace, hepatopancreas and muscle) and 1 mL for all other tissues (gill, antennal gland and heart). These volumes were added to 5 mL (for 1-mL tissue aliquots) or 10 mL (for 2-mL tissue aliquots) of scintillation fluor (Ultima Gold, Perkin Elmer, Waltham, WA). All tissue samples were counted for ^{63}Ni radioactivity on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer), using a quench curve that was constructed from various amounts of digest, and standardized to a common counting efficiency, that of the exposure water (see below).

Table 1
Water chemistry for exposures ($N=70$, 96 h and $N=21$ for 24 h exposures). Letters denote significant differences between salinities. Means are ± SEM.

Parameter	20% Seawater	60% Seawater	100% Seawater
pH	7.69 ± 0.09^a	7.74 ± 0.03^a	8.06 ± 0.03^b
Temperature (°C)	18	18	18
DOC (mg/L)	2.1 ± 0.2^a	2.7 ± 0.2^a	2.0 ± 1.0^a
Na^+ (mmol/L)	90.8 ± 2.3^a	337.4 ± 1.6^b	478.2 ± 5.7^c
Mg^{2+} (mmol/L)	7.8 ± 0.2^a	26.8 ± 0.2^b	46.7 ± 0.2^c
K^+ (mmol/L)	2.1 ± 0.02^a	6.2 ± 0.2^b	10.4 ± 0.3^c
Ca^{2+} (mmol/L)	1.62 ± 0.03^a	4.78 ± 0.13^b	9.78 ± 0.09^c
Cl^- (mmol/L)	91.6 ± 5.5^a	269.2 ± 26.6^b	514.3 ± 2.3^c
Control water (96-h), amount of Ni in μg/L	2.9 ± 0.7^a	1.7 ± 0.1^a	3.9 ± 0.8^a
3 mg/L of Ni (24-h exposure) in μg/L	3093 ± 327^a	3262 ± 113^a	3136 ± 253^a
3 mg/L of Ni (96-h exposure) in μg/L	2878 ± 204^a	3020 ± 125^a	3221 ± 162^a

2.4. Water and haemolymph ion analyses

For 24-h exposures, water Ni levels were monitored 3 times ($t=0$, $t=12$ h and $t=24$; $N=21$). For 96-h exposures water was taken at the start, before and after a water change, and at the conclusion of 96 h ($n=70$). For Ni analyses, both unfiltered and filtered (0.45 μm syringe filter; Acrodisc: Pall Life Sciences, Houston, TX, USA) samples were taken. Since there was less than a 5% difference between filtered and unfiltered samples, only filtered water Ni concentrations are reported in Table 1. Separate water samples were taken for ^{63}Ni radioactivity determination, to which scintillation fluid (Optiphase, Perkin Elmer) was added at a ratio of 2:1 (fluor:water) before these samples were counted on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer), as described above. Measurements of total Ni in water and control tissues were made on a Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS; Varian, SpectraAA- 220, Mulgrave, Australia) against certified atomic absorption standards (Sigma Aldrich Chemical Company, Oakville, ON, Canada). Ni recovery was 98.5 ± 4.7% as determined by Environment Canada certified reference materials, TM 25.3, TM 15, and DORT-1 lobster hepatopancreas. Ni concentrations were not corrected for recovery. Both water and haemolymph ions (K^+, Na^+, Mg^{2+} and Ca^{2+}) were measured via Flame Atomic Absorption Spectroscopy (FAAS; Varian SpectraAA FS-220, Mulgrave, Australia) (Tables 1 and 2). Water and haemolymph Cl^- was measured using a LabConco digital chloridometer (B5953; Kansas City, MO) (Tables 1 and 2). All ions were measured against reference standard solutions (Fisher Scientific, Ottawa, ON). Osmolality was measured via a Wescor VAPRO 5520 vapour pressure osmometer (Logan, UT). Water pH was measured by an Accumet Basic AB15 pH metre (Fisher Scientific, Ottawa, ON). Total DOC was measured using a Shimadzu TOC-Vcph/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). All water parameters for control and Ni exposures for all three salinities are displayed in Table 1.

2.5. Oxidative stress assays

Frozen gill tissues (gill numbers 5 and 8) were ground under liquid nitrogen with a chilled mortar and pestle. Gill samples for protein carbonyl analysis were then homogenised at a ratio of 20:1 (volume:weight) (Power Gen 125 homogenisation unit, Thermo Fisher Scientific, Toronto, ON, Canada) in a protein carbonyl determination buffer (50 mM MES, 1 mM EDTA; pH 6.7). Resulting homogenates were centrifuged at 10,000*g* for 20 min at 4 °C. Protein carbonyls were detected using a commercial kit (Sigma-Aldrich Protein Carbonyl Content Assay Kit; Sigma Aldrich, St.

Table 2
Internal ion concentrations in haemolymph (mmol/kg) in *C. maenas* under control conditions and after exposure to 3 mg/L Ni for 24 h and 96 h. Means ± SEM ($N=7$). Lower case letters indicate significant salinity differences between haemolymph values in control and Ni-exposed crabs.

24 h mmol/L	Na^+ – 24 h		Ca^{2+} – 24 h		K^+ – 24 h		Mg^{2+} – 24 h		Cl^- – 24 h	
Salinity	Control	Ni	Control	Ni	Control	Ni	Control	Ni	Control	Ni
20% SW	346.0 ± 6.1^a	332.2 ± 8.4^a	8.1 ± 0.3^a	8.6 ± 0.2^a	7.5 ± 0.3^a	6.8 ± 0.2^a	5.5 ± 0.1^a	5.6 ± 0.2^a	261.2 ± 11.2^a	275.1 ± 5.9^a
60% SW	449.3 ± 13.3^b	426.6 ± 4.9^b	12.5 ± 0.4^b	13.1 ± 0.3^b	9.2 ± 0.4^b	9.4 ± 0.2^b	10.2 ± 0.3^b	9.2 ± 0.4^b	382.4 ± 6.4^b	361.1 ± 7.5^b
100% SW	559.4 ± 23.3^c	504.3 ± 12.2^c	14.0 ± 0.3^c	15.4 ± 0.4^c	10.6 ± 0.2^c	10.9 ± 0.2^c	14.0 ± 0.7^c	14.2 ± 0.7^c	454.6 ± 15.3^c	465.2 ± 10.4^c
96 h mmol/L	Na^+ – 96 h		Cl^- – 96 h		Osmolality (mosmol/kg)					
Salinity	Control	Ni	Control	Ni	Control	Ni				
20% SW	326.6 ± 8.3^a	336.1 ± 6.1^a	260.2 ± 24.7^a	262.5 ± 5.9^a	585.5 ± 9.0^a	560.1 ± 3.3^a				
60% SW	436.21 ± 8.6^b	440.2 ± 3.6^b	378.2 ± 4.2^b	379.3 ± 7.0^b	800.4 ± 23.3^b	776.5 ± 12.9^b				
100% SW	514.4 ± 7.4^c	512.1 ± 14.6^c	458.6 ± 3.9^c	444.2 ± 15.2^c	940.3 ± 9.3^c	937.1 ± 5.4^c				

Louis, MO, USA) with the incorporation of a 1% streptomycin sulphate solution added to supernatants at 10 µL per 100 µL of homogenisation buffer. Streptomycin sulphate was used to remove nucleic acids as these can contribute to a higher estimation of protein carbonylation. Protein carbonyl contents are reported as nmol/mg protein. Catalase (CAT) activity was determined according to the methods described by Claiborne (1985). Briefly, gill 5 and 8 samples were homogenised (1:20, weight:volume) in a buffer containing 20 mM HEPES, 1 mM EDTA and 0.1% Triton X, adjusted to a pH of 7.2. The decrease in absorbance of hydrogen peroxide at a wavelength of 240 nm was measured at 21 °C using a quartz plate and expressed as U/mg protein where U is µmol/min. All assays were conducted in 96-well microplates (plastic for protein carbonyls, quartz for catalase) and read at 240 nm for catalase and 375 nm for protein carbonyls via a UV–visible spectrophotometer (SpectraMax 340PC, Sunnyvale, CA, USA). Readings were made at ambient temperature (~21 °C). Endpoints for both assays were expressed on a per mg of protein basis, with protein determined via the Bradford assay (Bradford, 1976), using bovine serum albumin as a standard.

2.6. Calculations

In order to determine total accumulation of Ni in a tissue (e.g. for tissues where a subsample, rather than the total tissue was taken), the relative percentage contribution of each tissue to total crab mass (see Blewett et al., 2015), was used. Briefly, subsample Ni radioactivity was extrapolated to total tissue accumulation based on the mean proportional masses (as % total crab mass) of each tissue.

For specific tissues, Ni accumulation data were calculated from the counts per minute (CPM) of the individual tissues divided by the mean specific activity (SA: based on measured water ^{63}Ni CPM concentrations and recorded water total Ni concentrations from AAS) and tissue weight (W). Data are expressed as µg/kg

$$Ni\ Accumulation = \frac{CPM}{SA} X \frac{1}{W}$$

2.7. Statistical analysis

Data have been expressed as means ± 1 SEM (N=number of crabs). For all treatments, $N=7$ was used, unless otherwise stated. Statistical analyses were performed with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA) and Sigma Stat 3.5 (Systat Software Inc., San Jose, CA, USA). When effects of salinity and Ni concentration were tested concurrently, a two-way ANOVA, with a Tukey's post hoc test, was used. For all other analyses, a one-way ANOVA was applied with a Tukey's post-hoc test. Significance for all statistical tests was accepted at $\alpha=0.05$. Any data that did not pass a normality test were transformed for normality. For Ni speciation analysis, the water chemistries recorded in Supplementary table 1 plus nominal values for anions were used to estimate the free ionic Ni (Ni^{2+}) concentrations using Visual MINTEQ software (ver. 3.1 beta, KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden). To estimate the effect of DOC, the NICA–Donnan model was used (Benedetti et al., 1995) (Supplementary table 1).

3. Results

3.1. Waterborne NI concentrations at different salinities for 24-h and 96-h exposures

Experimental Ni concentrations did not differ significantly across salinities. Control values ranged from 2.89 µg/L in 20% SW to 3.88 µg/L in 100% SW. Ni exposure values ranged from 2878 µg/L in 20% SW for the 96-h exposure to 3262 µg/L in 60% SW for the 24-h exposure (Table 1). Ni speciation changed only slightly between salinities where 20% SW had the highest free Ni^{2+} at 82% followed by 79% in 60% SW and finally 77% in 100% SW (Supplementary data, Table 1).

3.2. Salinity-dependent NI accumulation for 3 and 24 h

The 20% SW group displayed the highest total (i.e. whole body) Ni accumulation (12,527 ± 2030 µg/kg), a value that was significantly different from the total accumulation determined for the 100% SW group (5880 ± 533 µg/kg) (Fig. 1A).

There was a significant effect of gill number with respect to Ni accumulation ($P<0.001$), but the effects of salinity and interaction were not ($P=0.07$; $P=0.579$). Ni concentrations in the gills (Fig. 2B) were generally greater than in the whole body (Fig. 1A) at all salinities. Accumulation of Ni was significantly greater in gills 2 and 3 than it was in gills 6–8 (Fig. 1B). When average Ni accumulation values in all anterior gills (2–5) were compared with those in all posterior gills (6–9), the anterior gills had higher levels, but the difference was not significant (data not shown, but apparent from Fig. 1B).

Ni accumulation in the carapace was also generally greater than in the whole body, but followed a similar pattern with salinity as total accumulation. The 20% SW group accumulated the highest amount of Ni, a value significantly higher than that at 100% SW (Fig. 1C).

Heart and antennal gland displayed opposite accumulation patterns (Fig. 1D) from the carapace (Fig. 1C) and whole body (Fig. 1A), with the highest accumulation of Ni in the 100% SW group. Antennal glands displayed the highest levels of Ni accumulation among soft tissues (Fig. 1D), matched only by the gills (Fig. 1B). Muscle Ni accumulation was significantly greater by 2-fold in the 60% SW group than in the other two tested salinities

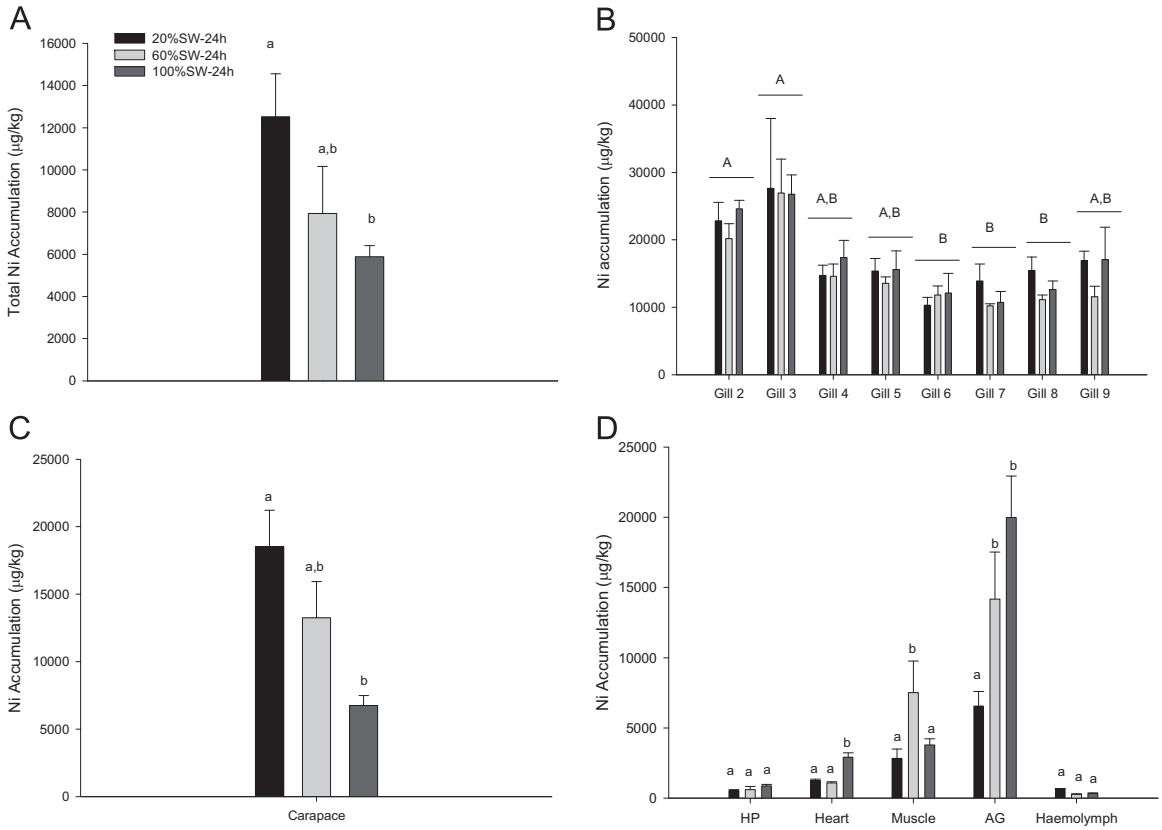

Fig. 1. Ni accumulation in tissues of Ni-exposed crabs (*Carcinus maenas*) after a 24 h exposure at a Ni concentration of 3 mg/L in 20%, 60% or 100% SW. (A) Total Ni accumulation, (B) Gill Ni accumulation, (C) Ni accumulation in carapace, and (D) Ni accumulation in hepatopancreas (HP), heart, muscle, antennal gland (AG), and haemolymph. Values are means ± SEM ($N=7$ per treatment). Lower case letters indicate significant differences between salinity groups ($P \leq 0.05$). Upper case letters denote significant changes between gill number. Means sharing the same letter are not significantly different.

(Fig. 1D). Relatively low levels of Ni accumulation in hepatopancreas and haemolymph were unaffected by salinity (Fig. 1D).

Ni accumulation into the carapace of both live and dead crabs was investigated and it was found that live crabs accumulate significantly 2-fold more Ni per unit weight of carapace than recently deceased crabs in the same exposure medium (Supplementary Fig. 3).

3.3. Haemolymph ions after 24-h exposure

As salinity increased, so did all haemolymph ion concentrations (Table 2). A two way ANOVA showed that there were significant effects of salinity on Na ($P<0.001$), but no effect of treatment ($P=0.231$) and no interaction between these two variables ($P=0.809$). Haemolymph *K* concentrations were significantly impacted by salinity ($P<0.001$) but not by Ni exposure ($P=0.160$), and interaction effects were not significant ($P=0.163$). Haemolymph Ca, Mg and Cl also displayed significant salinity-dependence (all $P \leq 0.001$), but no significant effects of Ni exposure were observed ($P=0.07$, $P=0.159$ and $P=0.874$, respectively). Haemolymph Ca, Mg and Cl all showed significant interactions between salinity and Ni exposure ($P<0.001$, $P<0.001$ and $P=0.05$, respectively) (Table 2).

3.4. Salinity-dependent accumulation over 96 h

Ni accumulation over 96 h was greater than the accumulation after 24 h in all tissues (Fig. 1*vs.* Fig. 2). Whole body Ni accumulation was the highest in the 20% SW group at 33, 901 ± 3751 μg/kg and was almost 3-fold greater than the accumulation in the 60% SW and 100% SW crabs (Fig. 2A). With respect to gill accumulation over 96 h, a two way ANOVA revealed that there were significant effects of both gill number ($P<0.001$) and salinity ($P<0.001$), but the interaction between these two factors was not significant ($P=0.116$). Specifically, at 96 h there was an effect of salinity on Ni accumulation in gills 2 and 3, with gills from crabs acclimated to 60% SW accumulating the highest amount of Ni relative to the other two salinities. In gills 5–9, the 20% SW crabs accumulated on average 2- to 3-fold higher concentrations of Ni than at the other two salinities. Gill 4 showed a pattern intermediate to the two described above, with levels of Ni accumulation in 20 and 60% SW gills approximate equal. Overall 100% SW gills always accumulated the least amount of Ni (Fig. 2B).

When average Ni accumulation levels in the anterior gills (2–5) versus posterior (6–9) gills were compared, there were significant differences (data not shown). In 20% SW, the posterior gills accumulated more Ni than posterior gills in 60% and 100% SW crabs and also accumulated more Ni than anterior gills at 20% SW. In 60% SW and 100% SW the posterior gills accumulated less Ni than anterior gills Again, Ni accumulation in the carapace mirrored the total accumulation (Fig. 2C). Salinity again had a marked effect on the accumulation of Ni, with the crabs in 20% SW exhibiting a significant 3-fold greater accumulation in the carapace than the other two salinity groups (Fig. 2C). In the remaining tissues there were significant salinity-dependentdifferences only in the hepatopancreas and haemolymph, where the 20% SW group accumulated more Ni relative to other salinities (Fig. 2D).

3.5. Haemolymph ions after 96-h exposure

A similar trend in ion concentrations was observed after a 96-h Ni exposure as after the 24-h exposure in terms of salinity. A two

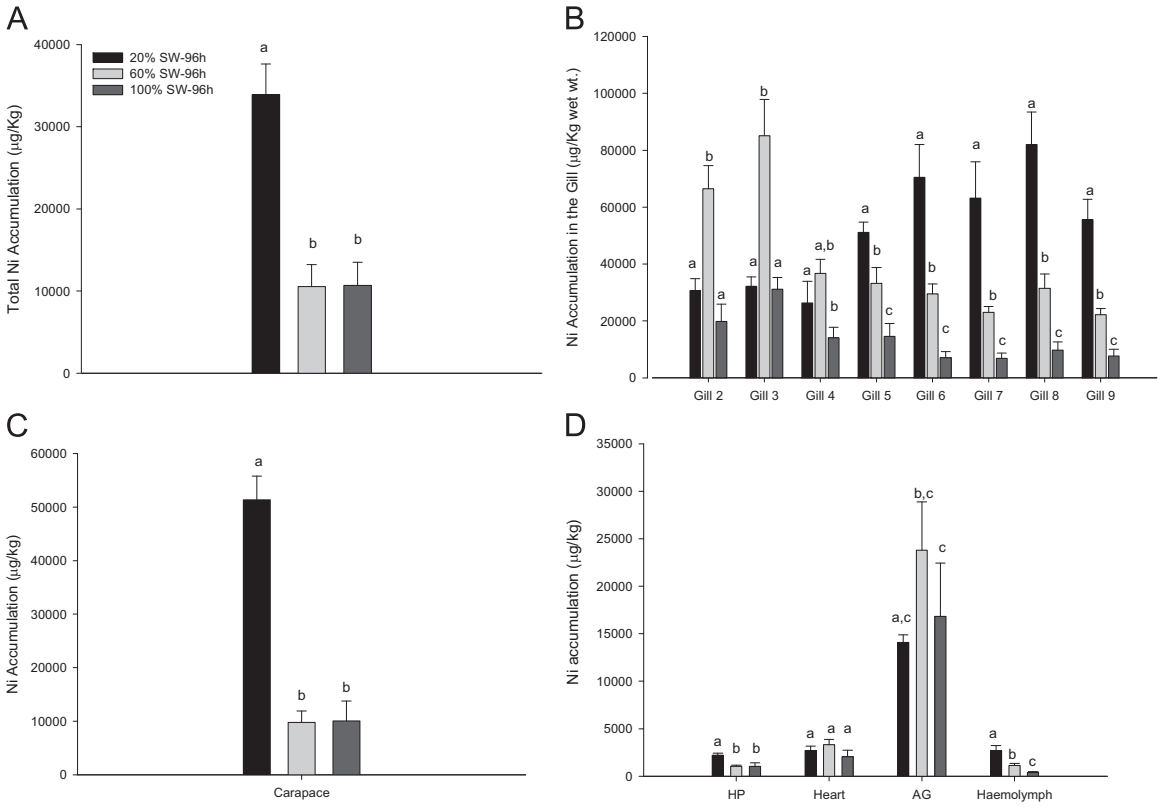

Fig. 2. Ni accumulation in tissues of Ni-exposed crabs (*Carcinus maenas*) after a 96 h exposure at a Ni concentration of 3 mg/L in 20%, 60% and 100% SW. (A) Total Ni accumulation, (B) Gill Ni accumulation, (C) Ni accumulation in the carapace and (D) Ni accumulation in hepatopancreas (HP), heart, muscle, antennal gland (AG), and haemolymph. Values are means ± SEM ($N=7$ per treatment). Lower case letters indicate significant differences between Ni concentrations between a salinity groups ($P \leq 0.05$). Means sharing the same letter are not significantly different.

way ANOVA showed an overall effect of salinity on haemolymph Na ($P < 0.001$), but no effect of treatment (control *vs.* Ni, $P=0.441$) and no significant interaction between these two variables ($P=0.613$). Haemolymph Cl concentration was affected by salinity ($P < 0.001$) but treatment and interaction effects were not significant ($P=0.490$, $P=0.723$). In general, as salinity increased so did osmolality and ion levels (Table 2, Fig. 3).

A two way ANOVA showed that Mg, osmolality and Ca were impacted by Ni exposure in the Ni-exposed crabs. Haemolymph Ca exhibited an overall increase ($P < 0.001$) with respect to Ni exposure across all salinities, but was significantly different only at 20% SW (Fig. 3). Overall, both Mg ($P=0.020$) and osmolality ($P=0.034$) decreased with exposure to Ni. Specifically, post-hoc tests showed that in 20% SW Mg and K concentrations decreased significantly with respect to control haemolymph values (Table 3). Haemolymph Ca, Mg, K and osmolality all differed significantly with respect to salinity- as salinity increased so did haemolymph values ($P \leq 0.001$, $P \leq 0.001$, $P \leq 0.001$, $P \leq 0.001$). Interaction effects (salinity *vs.* Ni exposure) were only significant for K and Ca ($P=0.031$, $P=0.005$), whereas Mg and osmolality did not show significant interaction effects ($P=0.385$, $P=0.413$).

3.6. Oxygen consumption after 96-h exposure

Resting oxygen consumption values did not vary significantly with salinity. However, oxygen consumption did vary with regards to Ni exposure and exercise. In control crabs, exercise elevated oxygen consumption only in 100% SW animals, but not at the lower two salinities (Fig. 4). The combination of Ni exposure with exercise had no additional effect in crabs at 60% or 100% SW, but in the 20% SW group, this treatment significantly lowered oxygen consumption relative to both exercised and resting control crabs (Fig. 4).

3.7. Indicators of oxidative stress after 96-h exposure

Both gills 5 and 8 were taken for oxidative stress analysis, as representative of anterior and posterior gills, respectively. A two way ANOVA showed that there were no significant effects of salinity ($P=0.377$) or interaction ($P=0.188$) on protein carbonylation in gill 8 but an overall effect of treatment, whereby Ni exposure tended to raise protein carbonyl levels ($P=0.05$). The post hoc test revealed that protein carbonyl content increased significantly in gill 8 of crabs exposed to Ni in both 20% SW and 60% SW relative to the unexposed controls (Fig. 5A) ($P=0.05$). There were no significant effects of any of those factors in gill 5 (salinity, $P=0.606$; Ni exposure, $P=0.191$; interaction $P=0.818$). Protein carbonyl concentrations in control tissues for both gills were not significantly different from each other. Overall a two way ANOVA showed that there were no significant differences with respect to salinity or interaction effects for either gill for CAT activity (gill 8, $P=0.353$ and $P=0.354$; gill 5, $P=0.694$ and $P=0.909$). However, a significant overall effect was determined with respect to Ni treatment, in gill 8 only, where Ni exposure tended to depress CAT activity ($P=0.05$) (Fig. 5B). A post hoc test determined that CAT activity was significantly decreased by 50% in the 20% SW Ni-exposed group relative to controls. This effect was not significant at the other two salinities. Gill 5 showed no significant differences with respect to Ni exposure ($P=0.191$). There were also no significant differences between control tissues of either gill number (5 or 8).

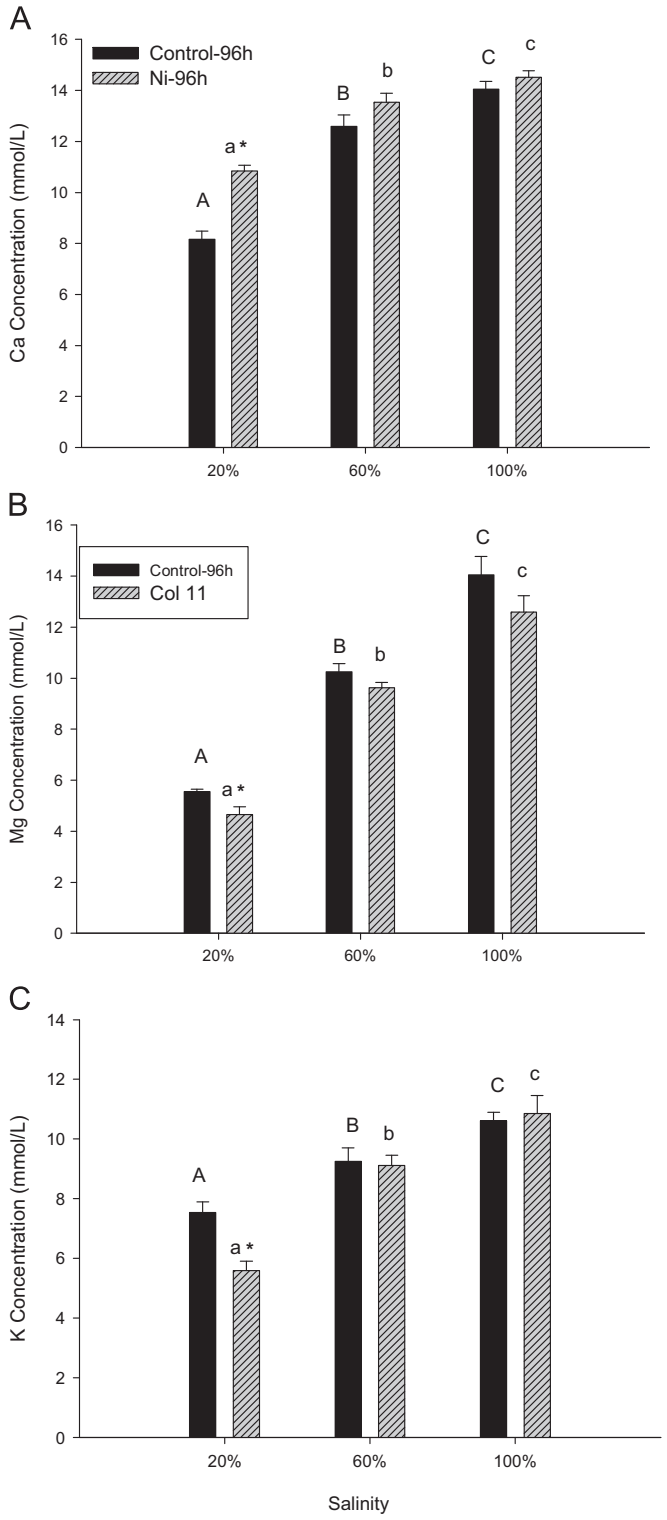

Fig. 3. Ionic composition (mmol/kg) in haemolymph of the green shore crab (*Carcinus maenas*) exposed to a Ni concentration of 3 mg/L for 96 h at three different salinities (20%, 60%, 100% SW). (A) Ca^{2+}, (B) Mg^{2+} and (C) K^{+}. All other ions and osmolality are displayed in Table2. Values are means ± SEM ($N=7$ per treatment). Asterisks indicate a significant difference from controls ($P \leq 0.05$). Upper case letters display significant salinity differences between haemolymph values in control crabs, lower case letters indicate significant salinity differences between haemolymph values in Ni-exposed crabs.

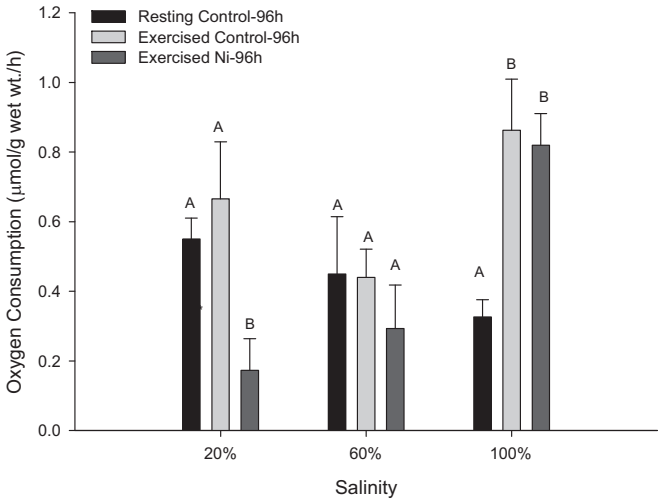

Fig. 4. Oxygen consumption values (μmol/g wet wt/h) in rest and after exercise in crabs exposed to Ni for 96 h. Values are means ± SEM ($N=7$ per treatment). Upper case letters denote significant differences within a salinity group ($P \leq 0.05$). Means sharing the same letter are not significantly different.

4. Discussion

In this study we have shown that salinity-dependent variations occur in Ni accumulation, as well as in all three areas of sub-lethal Ni toxicity investigated: ionoregulatory disturbance, respiratory interference, and oxidative stress, with all exacerbated at lower salinity. Changes in water chemistry (i.e. the protective effects of cations), and organism physiology are likely to explain this pattern. This study is the first to report oxidative stress responses with Ni in a marine invertebrate, and the first to demonstrate all three recognised modes of Ni toxicity in the same species. Finally, we have shown that Ni accumulation and sub-lethal toxicity depended on time of exposure, and have illustrated key differences in sub-lethal toxic responses in comparison to previous studies on this species exposed to lower Ni levels (Blewett et al., 2015).

4.1. Tissue- and salinity-specific NI accumulation after 24-h exposure

The ionoregulatory physiology of *Carcinus maenas* changes with salinity (Table 2, Fig. 3). *Carcinus* are isosmotic only in full seawater, and as salinity decreases, haemolymph ions are maintained at concentrations higher than those of the environment. This hyperosmotic regulation is achieved in part by changes in ion transport, whereby the crabs start to actively absorb NaCl from the external environment (Henry, 2005). The switch from being a passive osmoconformer in 100% SW to an active regulator in dilute waters is likely responsible for the observed salinity-dependence of Ni accumulation observed in the current study.

Following 24 h of Ni exposure, both total Ni accumulation and carapace tissue Ni increased with decreasing salinity (Fig. 1A and C). If a euryhaline crustacean is exposed to low salinity, the body fluids become hyper-osmotic to the external milieu, resulting in the osmotic entry of water. This in turn stimulates the production of urine, which in the case of crabs is usually iosomotic to blood, meaning that in addition to ridding the animal of excess water it also exacerbates salt loss (Mantel and Farmer, 1983). To rectify this loss of ions, energy-dependentuptake of ions increases across the gills. Ni is thought to be an ion mimic (see Section 1), and an increase in ion transport will also therefore result in an increase in potential pathways for Ni uptake into the animal, a hypothesis supported by the observation of higher Ni accumulation in lower salinities (e.g. Fig. 1A). This finding is consistent with previous studies reporting that euryhaline species are more sensitive to metal ions in freshwater (i.e. low salinity) when the organism is hyper-osmoregulating, than at a salinities which are closer to its

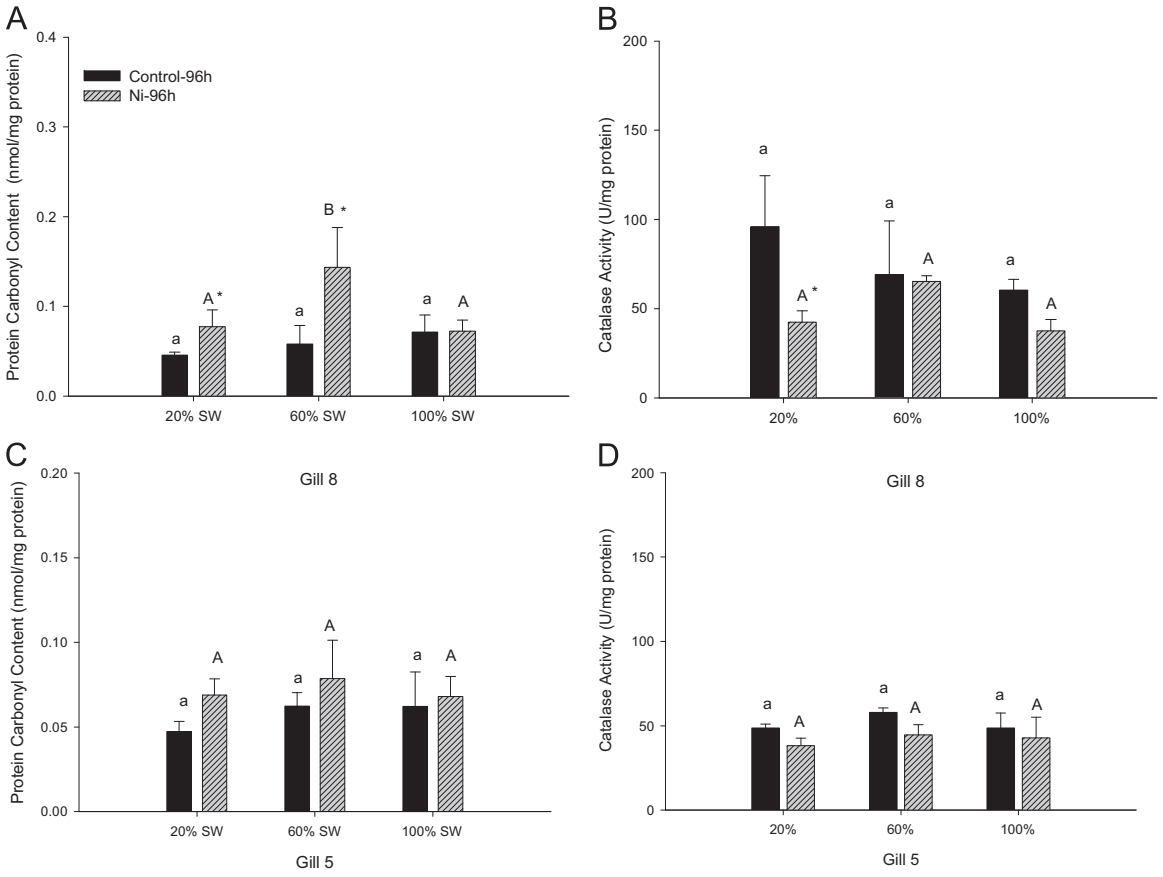

Fig. 5. Protein carbonyl content (A, gill 8; C, gill 5; nmol/mg protein) and catalase activity (B, gill 8; D, gill 5; U/mg protein) in gills of Ni-exposed and control crabs (*Carcinus maenas*) after a 96-h exposure at three different salinities (20, 60, 100% SW). Values are means ± SEM ($N=7$ per treatment), Asterisks denote significant difference from control tissue within a salinity ($P \leq 0.05$). Upper case letters denote significant differences between Ni treatments, while lower case letters denote significant differences between control treatments.

iso-osmotic point (Blewett et al., 2015; Hall and Anderson, 1995; Leonard et al., 2011, Martins et al., 2011; Wright, 1977). It is also consistent with our recent finding of greater Ni accumulation in isolated-perfused ionoregulatory gills (gill 8) of this species at 20% SW versus 100% SW (Blewett et al., 2015).

A role for water chemistry in influencing Ni accumulation is also likely. Changes in salinity result in only minimal changes in the proportion of the free Ni ion (Ni^{2+}; Supplementary table 1), generally thought to be the most bioavailable form, but more importantly reduced salinity will mean less competition from other cations, particularly the divalents (i.e. Mg^{2+}, Ca^{2+}). In higher salinities these will offer protection for an aquatic organism as they will compete with Ni for uptake (Paquin et al., 2002). Indeed there is a 5-fold increase in cation concentrations from 20 to 100% SW, and furthermore, as salinity increases the increase in total ionic strength will reduce free metal ion activity (Leonard et al., 2011; Martins et al., 2011). Overall this decrease in activity at the higher salinities will also likely decrease Ni uptake.

Although salinity-dependence in total body Ni and carapace Ni accumulation were observed, gills and other internal organs did not display a similar salinity-dependence. This is likely due to the fact that the carapace is the largest reservoir for accumulated Ni and thus best reflects the impacts of reduced salinity on Ni accumulation. Previous studies examining gill Ni handling in isolated perfused tissues have shown that even after two hours, Ni is capable of being transported into the haemolymph (Blewett et al., 2015), suggesting the ability of crabs to rapidly mobilize transport of Ni to the carapace for eventual elimination. Another possible explanation for these results could be changes in activity of the antennal gland. The antennal gland contributes to many volume-regulatory and associated processes, including: haemolymph volume control, excretion/reabsorption of organic compounds, fluids, sugars and amino acids (Mantel and Farmer, 1983). The antennal gland is also associated with detoxification of metals (Doughtie and Rao, 1984; Roldan and Shivers, 1987). It is therefore possible that the enhanced excretory activity of this gland in low salinities (as the crab increases production of urine to account for the passive inflow of water) may help to account for the lower Ni accumulation observed in dilute SW (Fig. 1D; Fig. 2D).

As mentioned, the carapace was the largest reservoir of accumulated Ni, particularly in the lowest tested salinity (20% SW). The carapace contains large stores of calcium carbonate and smaller stores of calcium phosphates and magnesium carbonate, where this large Ca demand is serviced from Ca^{2+} ion that is taken up from the haemolymph (Al-Sawalmih et al., 2008; Fabrius et al., 2012; Roer and Dillman, 1984). At least in lobster, this involves active transport via a transcellular pathway using Ca^{2+} pumps (e.g. Ca^{2+}ATPase), the Na^{+}/Ca^{2+} exchanger and other ion pumps such as H^{+}-ATPase and Na^{+}/K^{+}ATPase (Ahearn and Zhuang, 1996; Ahearn et al., 1999; Roer, 1980). In 20% SW, it may become difficult to maintain sufficient Ca for calcification, likely causing a compensatory increase in Ca flux through the Ca transport pathways. At the same time this will also aid in Ni transport into the carapace, as Ni^{2+} can potentially mimic Ca^{2+} pathways in several organisms (Deleebeeck et al., 2009, Eisler, 1998; Funakoshi et al., 1997; Pane et al., 2006a,b). This pattern of high Ni accumulation in the carapace with low salinity in *C. maenas* has been observed previously (Blewett et al., 2015), where it was proposed to be a

potential mechanism for Ni elimination during moulting. If this hypothesis is correct, then *Carcinus*, may be extremely sensitive to Ni following a moult, as during this period the newly forming carapace is rapidly calcifying (Travis and Friberg, 1963), and thus Ni uptake might be expected to be highest.

It is noteworthy that the *C. maenas* of the current study exhibit several differences in the sub-lethal ionoregulatory responses from those described by Blewett et al. (2015) under similar exposure conditions (24 h exposure to 8.2 μg/L). That study reported significant changes in haemolymph ions after 24 h, in contrast to the lack of changes in the current study. This is likely an effect of organism size. The *Carcinus* in the 20% SW exposure for the current study are 3–4 times larger than those used in Blewett et al. (2015). The higher surface area to volume ratios of smaller animals may exacerbate impairments to ionoregulation (Grosell et al., 2002), thus the greater prominence of this mode of toxicity in the smaller crabs.

4.2. Tissue- and salinity-specific Ni accumulation after 96-h exposure

Substantial differences in Ni accumulation were observed between 24-h and 96-h exposures. For example, there was a shift in Ni distribution from the carapace to soft tissues. This likely represents saturation of Ni binding in the carapace, and subsequent build-up of Ni in soft tissues. The pattern of Ni distribution after 24 h exposure to 3 mg/L in the current study was similar to that observed after 24 h exposure to 8.2 μg/L (Blewett et al., 2015), suggesting the relative distribution of Ni is not strongly influenced by exposure concentration. Comparisons of live versus very recently euthanized green crabs after Ni exposure were performed, and found that 50% of the Ni accumulation into the carapace from waterborne Ni exposure is likely due to adsorption to the carapace, while the other 50% of accumulation is due to active uptake processes (Supplemental Fig. 3).

There were also qualitative differences between 24-h and 96-h exposures, with the most prominent differences being the appearance of salinity-dependence of gill Ni accumulation following the longer exposure. This consisted of an elevated level of accumulation in anterior gills at 60% SW and in posterior gills in 20% SW (Fig 2C). The latter effect may relate to the mechanism of Ni toxicity. As described below, there was evidence of an ionoregulatory impairment following Ni exposure. The posterior gills (numbers 6–9) are generally considered to be specialized for ionoregulation, as opposed to the anterior gills that are more specialized for respiration (Freire et al., 2008; Mantel and Farmer, 1983; McNamara and Lima, 1997; Onken and Riestenpatt, 1998; Péqueux, 1995). If Ni was impairing the ability of the crab to maintain ionoregulatory homoeostasis, then mechanisms which would protect against this impairment might be induced. One such mechanism could be the induction of the metal-binding protein, metallothionein. The sequestration of Ni by metallothionein would minimise the bioreactivity of Ni, but could also trap the Ni in the posterior gills, thus increasing Ni burden. This effect would be greatest in 20% SW as this is the exposure condition where ionoregulation becomes most important, and is thus also where ionoregulatory toxicity might be most prevalent (see below and Blewett et al., 2015). Induction of metallothionein is not instantaneous, thus under our exposure conditions it may have taken longer than 24 h before sufficient metallothionein was induced to impact the gill Ni burden. Note that at 24 h, the same salinity-dependence was not as clearly seen (Fig. 1A). This period is consistent with metallothionein induction in other ectothermic animals (Amiard et al., 2006). Although Ni is not a well-described inducer of metallothionein, previous reports have shown induction of this metal-binding protein by Ni in crustaceans (Barka et al., 2001).

The increase in Ni burden in anterior gills in 60% SW (Fig. 2B) is more difficult to explain. Unlike the active regulation of ions across posterior gills, the anterior gills of crabs are thought to respond passively to changes in external salinity, with ion flux rates and epithelial permeability decreasing with decreasing salinity (Pequeux and Gilles, 1981). It would thus be expected that a general decrease in Ni accumulation would be observed as salinity reduced. The mechanism behind the peak in Ni accumulation in anterior gills at the intermediate salinity requires further investigation.

4.3. Ionoregulatory disruption after 96-h exposure

Ionoregulation disruption in response to Ni exposure has been described before in several invertebrate species (see Section 1). In this study, exposure of crabs to Ni in 20% SW resulted in changes in haemolymph Ca, Mg and K concentrations (Fig. 3).

In the current study Ni exposure increased haemolymph Ca. The mechanisms underlying this effect are unknown, but increases in haemolymph Ca are observed during the moult in crabs (e.g. Scott-Fordsmand and Depledge, 1997). Given that the majority of Ni accumulated in crabs accumulates in the exoskeleton, perhaps Ni exposure induces displacement of stored Ca from the carapace back into the haemolymph, an effect which was only significant in 20% SW. Indeed, it is intriguing to speculate whether Ni exposure may thereby induce moulting as a mechanism of eliminating Ni from the body. Alternatively the increase in Ca may relate to competitive effects with Ni for incorporation into the growing exoskeleton.

However, as alluded to above, differences in the Ca response were observed between the current study and that of Blewett et al. (2015), where crabs exposed to a lower Ni concentration (8.2 μg/L) showed an inhibitory effect of Ni on haemolymph Ca, which was attributed in that study to an inhibitory effect of Ni on gill Ca transport. Indeed, another explanation for the difference may be that Ni has been shown to induce hypoxia inducible factor (HIF), thus mimicking hypoxias (Salnikow et al., 1999; Yu et al., 2001). During the exposure to 3000 μg/L at 20% SW crabs were visibly more lethargic than control crabs and experienced significantly lower oxygen consumption rates after exercise (see below). Previous evidence has shown that hypoxia can cause Ca release from intracellular stores (Gelband and Gelband, 1997), and thus the higher exposure concentration of Ni of the current study may have caused a Ca homoeostasis effect quite different in mechanism from the interference of Ni with Ca transport pathways seen at lower exposure concentrations.

Ni exposure had an overall treatment effect to decrease haemolymph Mg levels, and this effect was significant in the 20% SW group (Fig. 3). Disturbances in Mg homoeostasis caused by Ni exposure have been reported a number of times in invertebrates. For example, in the freshwater cladoceran *Daphnia magna*, Ni inhibited unidirectional Mg influx, causing a large decrease in whole body Mg stores (Pane et al., 2003b). There were also disruptions in both Mg and Na homoeostasis in the euryhaline crustacean *Litopenaeus vannamei* (Leonard et al., 2011), and in a freshwater snail, *Lymnaea stagnalis*, and a freshwater oligochaete, *Lumbriculus variegatus* (Leonard et al., 2011) with Ni exposure.

Haemolymph K was also decreased by Ni exposure, but only in the 20% SW crabs, consistent with the appearance of other markers of Ni toxicity in lower salinities. A similar effect of Ni was observed on whole body K in sea urchins (Tellis et al., 2014). This was attributed to an inhibitory effect on the basolateral ion transporter Na^+/K^+ ATPase. Ni has been previously shown to inhibit Na^+/K^+ ATPase in *Carcinus maenas* gills in 20% SW (Blewett et al.,2015), suggesting this to be a conserved mechanism of Ni

effect in marine invertebrates.

4.4. Respiratory impairment after 96-h exposure

Respiratory impairment is considered the main mechanism of Ni toxicity to freshwater vertebrates (Pane et al., 2003a), but has only been indirectly noted in freshwater invertebrates (e.g. *Daphnia*; Pane et al., 2003b). However, in the current study greatly decreased post-exercise oxygen consumption was displayed in crabs exposed to Ni for 96 h in 20% SW (Fig. 4). This effect did not occur at higher salinities. In fish, Ni was shown to impair respiration by causing changes in gill epithelia, including the swelling and fusion of lamellae. These impacts diminished the effectiveness of the gill as a respiratory surface, and were accompanied by increases in ventilatory rates and volumes, and a decrease in oxygen extraction efficiency (Pane et al., 2003a). In invertebrates Ni has not been previously observed to directly impair respiration, but exposure of crabs to other metals (e.g. Cd, Zn) has been observed to cause ultrastructural damage to crab gills in dilute waters, with accompanying decreases in oxygen consumption (Silvestre et al., 2005; Spicer and Weber, 1992).

C. maenas is a very active forager. In fact, in a study where the spontaneous activity of *C. maenas* was compared with those of two fish species (blenny and scorpionfish), the crab spent the least time inactive (Burrows et al., 1999). Thus any factor that impairs activity is likely to have a significant ecological impact on this species.

4.5. Oxidative stress after 96-h exposure in representative anterior and posterior gills

Ni has been shown to cause oxidative stress in freshwater fish (Kubrak et al., 2012a,b, 2013, 2014; Loro et al., 2012), and metals are known to also cause oxidative stress in marine invertebrates (Sabatini et al., 2009; Vlahogianni et al., 2007). The present data are the first to show oxidative stress in response to Ni in marine invertebrates. Our results indicate two major trends occurring with respect to the oxidative stress responses to Ni – the first is an effect of salinity, and the second an effect of gill type (anterior-respiratory versus posterior-ionoregulatory). For example a decrease in catalase (CAT) activity in ionoregulatory gill 8 of crabs acclimated to 20% SW correlated with an increase in protein carbonyl formation in the same treatment (Fig. 5A and B). The restriction of these effects to the lowest salinity in ionoregulatory gills is likely a consequence of the higher Ni bioavailability in this water chemistry and the higher tissue Ni burden in these gills (see above).

CAT is a critical enzyme that decomposes hydrogen peroxide into water and oxygen, the second step in neutralizingreactive oxygen species (ROS). CAT is considered one of the most sensitive antioxidant enzymes with respect to Ni (Cartañá et al., 1992; Rodriguez et al., 1990). Generally, decreases in CAT activity in response to metal exposure, as observed here, are attributed to inhibition of the enzyme by the metal binding to enzyme histidine residues (e.g. Cu; Grosell, 2012). Ni has a high affinity for such histidine residues (e.g. Predki et al., 1992), which play an important role in CAT catalytic activity (Mate et al., 1999). CAT activity decreases were also observed in the euryhaline killifish (Blewett and Wood, 2015) where FW gills displayed a significant decrease in CAT compared to 100% SW gills, indicating again the sensitivity at lower salinities. The effects of Ni on oxidative stress in killifish corresponded to Ni accumulation; with higher accumulation observed in FW compared to 100% SW gills (Blewett and Wood, 2015). As mentioned above, there was an increase in protein carbonylation in gill 8 in 20% SW crabs. Protein carbonyls form when ROS directly attack proteins leading to the formation of a carbonyl (Bainy et al., 1996), causing non-reversible damage (Zhang et al., 2010). The exact mechanism by which Ni exerts oxidative stress is not clear, but there are two possible explanations. Firstly, Ni can interfere directly with ROS formation by displacing Fe in the Fenton/Haber Weiss reactions (with Ni^{2+}/Ni^{3+} rather than Fe^{2+}/Fe^{3+} redox coupling), ultimately increasing ROS production (Torreilles and Guerin, 1990). Secondly, Ni can affect antioxidant responses yielding a lower defence against ROS production. The current findings suggest that oxidative stress is a common conserved mechanism of Ni toxicity in aquatic animals, having previously been observed in fish (Blewett and Wood, 2015; Kubrak et al., 2012a,b, 2013, 2014).

4.6. Conclusions

This study indicates that several interrelated mechanisms may contribute to an overall pathological effect of Ni in the crab *C. maenas*.. The three modes of sub-lethal Ni toxicity displayed in this study are ionoregulatory impairment, respiratory toxicity, and oxidative stress. Furthermore, these effects are strongly time- and salinity-dependant, with greater toxicity noted following longer exposures and in low salinities which seem to favour Ni accumulation.

Acknowledgements

This research was supported by two NSERC CRD grants awarded to Scott Smith (Wilfrid Laurier University) and CMW (P.I.'s) with co-funding from the Nickel Producers Environmental Research Association, Inc., the International Zinc Association, the International Lead Zinc Research Organisation, the International Copper Association, the Copper Development Association, Teck Resources, and Vale Inco. Thanks to the Department of Fisheries and Oceans for help with animal collection, and the staff of Bamfield Marine Sciences Centre, especially the research co-ordinator Dr. Eric Clelland, for their assistance. CMW was supported by the Canada Research Chairs Programme.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.07.019.

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