INFLUENCE OF WATER CHEMISTRY ON NICKEL ACCUMULATION AND SUB-LETHAL TOXICITY IN MARINE AND ESTUARINE ANIMALS

INFLUENCE OF WATER CHEMISTRY ON NICKEL ACCUMULATION AND SUB-LETHAL TOXICITY IN MARINE AND ESTUARINE ANIMALS

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TITLE: Influence of water chemistry on Ni accumulation and sub-lethal toxicity in marine and estuarine animals

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ABSTRACT

Nickel (Ni) is a metal that is anthropogenically enriched in aquatic settings. It has been reported as having three main modes of toxicity in freshwater animals (ionoregulatory disturbance, respiratory impairment, and the generation of oxidative damage), but there is little understanding of Ni toxicity in marine and estuarine environments. The mechanism(s) of Ni uptake and toxicity were investigated using three model species (adult green shore crab, *Carcinus maenas*; adult Atlantic killifish, *Fundulus heteroclitus*; early life-stages of the New Zealand sea urchin *Evechinus chloroticus*). In crabs, sea water protected against Ni accumulation and toxicity. In more dilute salinities, however, all three modes of Ni toxicity were identified at a sub-lethal level, with effects noted at Ni exposure levels as low as 8.2 µg/L, the US EPA environmental regulatory limit. In killifish, similar protective effects of SW were observed, however this species was much more resilient to Ni toxicity, with only minor changes in ionoregulation and oxidative stress noted, and no evidence of respiratory toxicity. Sea urchin larvae were found to be the most sensitive marine organisms to Ni toxicity yet reported, with a 96 h median effect concentration of 14.1 µg/L measured. Toxicity in this species was related to Ni impairment of calcium influx, consistent with proposed mechanisms of uptake observed in the other two models. Overall, the elevated ion levels associated with salinity were shown to be protective, suggesting a role for water chemistry in modifying Ni accumulation. However, physiology, which varies between species, developmental stages, and as a function of environmental salinity, also influenced organism sensitivity to Ni. These data contribute novel information regarding the relationships between water chemistry, Ni accumulation, and Ni toxicity, and as such, will be integral in the future development of predictive modelling tools for protecting marine and estuarine animals against environmental Ni.

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LIST OF ABBREVIATIONS

ºC	Degrees Celsius
µmol/L	Micromole per liter
ANOVA	Analysis of Variance
AG	Antennal gland
B_{max}	Binding capacity
Ca	Calcium
$CaCO_3$	Calcium carbonate
CAT	Catalase
Cd	Cadmium
Cl	Chloride
CPM	Counts per minute
Cu	Copper
DOC	Dissolved organic carbon
EC_{50}	Median effective concentration
FW	Fresh water
GFAAS	Graphite furnace atomic absorption spectroscopy
GPx	Glutathione peroxidase
h	Hour
HCO_3^-	Bicarbonate
HNO_3	Nitric acid
HP	Hepatopancreas
K_m	Binding affinity
L	Liter
LC_{50}	Median lethal concentration

Mg	Magnesium
Min	Minute
mmol/L	Millimole per liter
MO_2	Oxygen consumption
MS222	Tricane methane sulphonate
MRC	Mitochondria-rich cell
N	Sample size
Na	Sodium
Ni	Nickel
Pb	Lead
pH	Negative log of the hydrogen ion concentration
ppt	Parts per thousand
r^2	Coefficient of determination
ROS	Reactive oxygen species
SA	Specific activity
SEM	Standard error of the mean
SW	Sea water
SOD	Superoxide dismutase
TOSC	Total oxyradical scavenging capacity
Zn	Zinc
U_{crit}	Critical swim speed
V	Volume
W	Weight
WQC	Water Quality Criteria

THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format, as recommended and approved by members of my supervisory committee and approved by McMaster University. It consists of seven chapters. Chapter 1 is an overview of background material and hypotheses tested. Chapter 2 through 6 are manuscripts that are published, accepted or ready to be submitted for publication in a peer reviewed scientific journals. Chapter 2 is referred to as Blewett et al. (2015a), Chapter 3 is referred to as Blewett and Wood (2015b), Chapter 4 is referred to as Blewett et al. (2015b), Chapter 5 is referred to as Blewett and Wood (2015a), and Chapter 6 is referred to as Blewett et al. (2015c). Chapter 7 summarizes the major findings of this thesis, places these findings in the context of current knowledge, and indicates future directions that this research may take.

CHAPTER 1 **GENERAL INTRODUCTION**

CHAPTER 2 **MAKING SENSE OF NICKEL ACCUMULATION AND SUB-LETHAL TOXIC EFFECTS IN SALINE WATERS: FATE AND EFFECTS OF NICKEL IN THE GREEN CRAB *Carcinus maenas***

Authors: Tamzin A. Blewett, Chris N. Glover, Sandra Fehsenfeld, Michael J. Lawrence, Som Niyogi , Greg G. Goss and Chris M. Wood

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CHAPTER 3 **LOW SALINITY ENHANCES NICKEL-MEDIATED OXIDATIVE STRESS AND SUB-LETHAL TOXICITY TO THE GREEN SHORE CRAB *Carcinus maenas***

Authors : Tamzin A. Blewett and Chris M. Wood

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CHAPTER 4 **MECHANISMS OF NICKEL TOXICITY IN THE HIGHLY SENSITIVE EMBRYOS OF THE SEA URCHIN *Evechinus chloroticus* AND THE MODIFYING EFFECTS OF DISSOLVED ORGANIC CARBON**

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CHAPTER 5 **SALINITY-DEPENDENT NICKEL ACCUMULATION AND OXIDATIVE STRESS IN THE EURYHALINE KILLIFISH (*Fundulus heteroclitus*)**

Authors: Tamzin A. Blewett and Chris M. Wood

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CHAPTER 6 **INVESTIGATING THE MECHANISMS OF NICKEL UPTAKE AND SUB-LETHAL NICKEL TOXICITY IN THE ATLANTIC KILLIFISH *Fundulus heteroclitus***

Authors: Tamzin A.Blewett, Victoria E. Ransberry, Grant

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CHAPTER 7 **GENERAL DISCUSSION**

CHAPTER 1

GENERAL INTRODUCTION

1.0 Ni in the environment

Nickel (Ni) is a transition metal often thought to be an essential micronutrient (Eisler, 1998; Nielsen, 1993). Ni essentiality has only been shown in plants and bacterial species (Ragsdale, 1998) and also in terrestrial vertebrates such as pigs, cows and chickens (Phipps et al., 2002). While Ni is considered to be homeostatically regulated in fish (Chowdhury et al., 2008), its essentiality in aquatic vertebrates and invertebrates has yet to be proven. However, like other essential transition metals such as copper (Cu) and zinc (Zn), either an excess or a deficiency of Ni can decrease the overall health of aquatic organisms (Eisler, 1998; Muyssen et al., 2004).

Natural sources of Ni in the aquatic environment include erosion and weathering of Ni-containing minerals. Anthropogenic addition occurs from sources such as mining, emission of fossil fuels, fallout of air particulate matter, surface runoff near industrial and urban areas, smelting and alloy processing (Eisler, 1998; NAS, 1975; Schaumlöffel, 2005; WHO, 1991). Ni production and use has increased exponentially over the past century and continues through to today (Reck et al., 2008). As a consequence, Ni is a near ubiquitous component of marine and freshwater (FW) environments. In uncontaminated FW, Ni reaches concentrations of approximately 1- 10 μg/L (Eisler, 1998; USEPA, 1980), while in highly contaminated waters, levels approach 100 – 2000 μg/L (Eisler, 1998; Pyle and Couture, 2012). However, in areas with naturally-enriched Ni geology, waterborne Ni concentrations can reach as high as 10 mg/L (Chau and Kulikovsky-Cordeiro, 1995). In estuarine environments, Ni concentrations range from 1-100 μg/L, while marine values in uncontaminated areas are around 2 μg/L (Boyden, 1975).

Owing to its potential toxic effects, a number of regulatory limits are in place to protect aquatic ecosystems from Ni impacts. In Canada, the chronic exposure (defined as > 30 days for fish and 7-21 days for invertebrates) value considered to be potentially harmful is 25 µg/L, with this value based on a set water hardness value of 20 mg/L as Ca carbonate equivalents (CCME, 2007). There is no acute exposure value, and the regulation does not distinguish between FW and seawater (SW) environments. In the USA, the FW acute value (to protect 95% of aquatic biota, 95% of the time) is 120 µg/L, and using the same 95% criterion the chronic value is 13 µg/L (also based on a hardness of 20 mg/L). In SW the chronic Ni exposure guideline is set at 8.2 µg/L (USEPA, 2005). However, the toxicity of Ni is not solely dependent upon environmental concentration. There are also a number of important biotic and abiotic factors which contribute to its impact on aquatic animals.

1.1 Importance of water chemistry on Ni toxicity: a brief introduction to the BLM

For metals in the aquatic environment the free ion is generally considered to be the most toxic chemical species. This is the form that is most likely to be transported (through dedicated essential metal ion transporters or *via* ion mimicry) and is the form that is most bioreactive, with the capacity to inhibit or impair the function of sensitive cellular entities such as enzymes and other transporters (Niyogi and Wood, 2004; Wood, 2012). The chemistry of water will have a significant impact on the speciation of a particular metal, changing its redox state and/or its speciation. In turn, speciation will impact the bioavailability of the metal to an aquatic organism, and there is a well-established relationship between speciation/bioavailability and the toxicity of the metal (Paquin et al., 2002). This is the basic principle behind the Biotic Ligand Model (BLM). A BLM is a computer-based model that applies a quantitative method to evaluate the biological impact of a metal by accounting for its bioavailability, which is expressed as a function of site-

specific water chemistry and organism- or species-sensitivity (Paquin et al., 2002). The BLM aims to replace established, but sometimes flawed, methods of deriving water quality guidelines (based on total metal levels and often modified for chemistry parameters such as hardness) with more geochemically-relevant parameters (specific water ions chosen based on mechanism of metal uptake) and biological variables (e.g. gill metal burden) (De Schamphelaere et al., 2002). The predictions of toxicity generated by the BLM are based on knowledge of the specific sites to which a metal binds, the total number of binding sites (a parameter called B_{max}), and the affinity of these sites for the metal (K_m). These characteristics are determined for the "biotic ligand", the locus most associated with toxicity (usually the gills of aquatic organisms). In general a relationship exists between the affinity values for gill binding and toxicity, such that the higher the affinity, the greater the toxicity of the metal at lower concentrations. The combination of B_{max} and K_m values allows the prediction of the LA_{50} value, which is the amount of the metal accumulated by the biotic ligand required to cause 50% of the exposed population to eventually die. Thus the lower the LA_{50}, the more sensitive the organism. To date the BLM appears to be the best model for predicting both acute and chronic metal toxicity to aquatic organisms, and this model has now been adopted as the European Union Water Quality Standard for Cu, Zn and Ni in FW (ECB, 2008), with the US Environmental Protection Agency (US EPA) similarly implementing a BLM for FW Cu guidelines in 2007. In part the lack of a more widespread and comprehensive adoption of the BLM approach (e.g. for other metals and in SW) is due to the lack of research supporting the underlying BLM principles outside of the established model conditions (i.e. Cu, Zn and Ni in FW). For metals such as Ni, a lack of knowledge exists regarding accumulation and toxicity in estuarine and marine settings.

The BLM relies on the metal binding to specific sites on the biotic ligand. This is a process that will be strongly influenced by water chemistry. Key water chemistry factors include

the concentration and composition of dissolved organic carbon (DOC), pH, alkalinity, hardness, and salinity. These factors will alter the speciation, bioavailability and therefore also the toxicity of the metal. Consequently for Ni, as for other metals, toxicity will primarily be driven by the proportion of free Ni ion, the ability of this ion to bind to and be transported by sites of uptake (i.e. the gill), and the concentration of Ni to which the animal is exposed. These are factors that will be influenced by both water chemistry, and the physiology of the exposed organism. As the transition from FW to SW occurs, a whole host of water chemistry parameters will change, including cation and anion concentrations. These parameters will influence the speciation of Ni and ultimately its bioavailability to the organism being tested.

1.2 Anionic complexation

As the transition from FW to SW occurs, the key anionic species thought to be important in complexing Ni are SO_4^{2} and Cl^-, which increase in concentration with an increase in salinity. The formation of Ni complexes with these anions will theoretically reduce Ni bioavailability and toxicity (Leonard et al., 2011; Sadiq, 1989). In some modeling conditions, $NiCl_2$ accounts for approximately 15% of the total inorganic Ni in SW (Sadiq, 1989), while $NiSO_4$ comprises around 20% of total inorganic Ni in SW (Sadiq, 1989). The BLM only considers the free ion to be toxic. That $NiSO_4$ and NiCl are formed in SW highlights the need to determine if these anionic complexes of Ni are bioavailable and toxic to aquatic organisms. There is precedence for complexes having toxic effects, as $CuCO_3$ is reported to influence Cu toxicity in *Daphnia magna* (De Schamphelaere et al., 2002). However, to date, no complexes for Ni have been reported as toxic.

1.3 Protective cations

In SW the major cations, calcium (Ca), potassium (K), magnesium (Mg) and sodium (Na) are approximately 10-, 200-, 110-, and 724-fold greater, respectively, than in FW. As Ni is a divalent cation, it is likely that its uptake will be most greatly impacted by the presence of other similarly charged cations (e.g. Mg^{2+}, Ca^{2+}). These will offer possible protective effects to an aquatic organism as they will compete with Ni for the polyanionic biotic ligand, effectively reducing bioavailability relative to Ni in FW environments (Paquin et al., 2002). Previous evidence has shown that Ca and Mg may directly compete with Ni for a common uptake pathway, at least in invertebrates (Pane et al., 2003b; Section 1.7). Consequently, as the levels of these ions differ markedly between SW and FW environments, the bioavailability of Ni may change with salinity, and thus Ni uptake and toxicity may be altered.

1.4 Dissolved organic carbon (DOC)

Dissolved organic carbon (DOC) is found in all natural waters and is formed by the physical breakdown and/or microbial processing of plant and animal materials (Thurman, 1985). DOC plays a pivotal role in ameliorating the toxicity of metals in the aquatic environment, and is considered to have greater protective effects than "hardness" and alkalinity in model predictions for some metals. DOC concentrations in natural waters range from 1-15 mg C/L (Thurman, 1985). DOC is primarily thought to bind and complex metals, thereby decreasing their bioavailability to target surfaces such as the gill (Wood et al., 2011).

DOC's can be classified into two distinct types: allochthonous (terrigenous) – originating from the breakdown of leaves and wood or other land-based sources, and autochthonous - produced by algae within lakes and/or rivers often by degradation of allochthonous DOC. Autochthonous DOC's contain fewer aromatic ring structures, are composed of smaller molecules, and are optically lighter in comparison to allochthonous DOC's (for review see Wood

et al., 2011). Research to date indicates that the more optically dark a DOC, the more protective it is against metal toxicity (Pempkowiak et al., 1999; Van Genderen et al., 2003). Although some evidence does show that DOC protects against Ni toxicity to aquatic organisms (e.g. Doig and Liber, 2006), it is generally accepted that Ni-DOC complexes are less important than DOC complexes with other metals such as Cu and Ag. This is based on Ni-DOC binding constants being over an order of magnitude larger than the binding constants for Cu-DOC (i.e. being of lower affinity, meaning relatively less Ni bound by the DOC) (Niyogi and Wood, 2004).

However, DOC not only binds metals and thereby reduces bioavailability, it can also alter the physiology of the organisms themselves (for review, see Steinberg et al., 2006; Wood et al., 2011). DOC has been shown to affect Na uptake kinetics in rainbow trout (Matsuo et al., 2004) and *Daphnia magna* (Glover et al., 2005; Glover and Wood, 2005). McGeer et al. (2002) showed increased Na^{+}/K^{+} ATPase activity in rainbow trout chronically exposed to DOC, while altered transepithelial potentials across the gills *in vivo*, as well as in *in vitro* cultured trout gill epithelia, with DOC exposure has been observed (Galvez et al., 2009). Thus DOC could modify metal toxicity by altering membrane potential and some of the pathways by which the metal causes its effects.

Although FW toxicity of Ni has been the subject of substantial investigation (for review see Pyle and Couture, 2012), currently there is little information regarding the mechanisms of Ni toxicity to marine and estuarine species. In marine settings the major factor governing Ni toxicity is likely to be salinity. In estuarine settings where salinity will fluctuate, the concentrations of protective cations and complexing anions will vary significantly with factors such as tidal cycles and changes in FW inputs. These changes in water chemistry are likely to have a significant influence on bioavailability and toxicity of Ni.

1.5 Physiological changes with salinity

The most vulnerable site of metal toxicity to fish and crustacean species is the gill. The gills are exposed directly to the external medium, represent only a thin barrier between the animal and the environment, display a large surface area, and have high blood perfusion rates (Evans et al., 2005). These characteristics are ideal for important physiological processes such as gas and ionic exchange, but are also likely to promote metal uptake and toxicity (Wood, 2001). Because the gills are such a critical homeostatic tissue, when an animal is faced with environmental fluctuation the roles and properties of the gills may change, and along with this, metal uptake and toxicity may also be altered. For example, salinity changes will cause alterations in organism physiology, including the mechanisms for maintaining ion homeostasis at the gill. As a result this will likely alter sensitivity to Ni. This will be of particular relevance in estuarine environments as these settings are subjected to a high degree of variability. Over the course of hours, significant changes in factors such as oxygen, temperature and salinity may occur (Burnett et al., 2007). Of these factors, salinity (i.e. ionic composition) will likely have the largest effect on metal toxicity, in part due to speciation (see above) but also because of changes in gill physiology.

Marine teleosts are hypo-osmotic to the environment; thus they lose water by osmosis and gain ions (especially Na^+ and Cl^-) by diffusion. To counteract this active gain of ions and water loss, fish have specialized cells called mitochondria-rich cells (MRC's) or ionocytes. These cells help to maintain ionic homeostasis through active excretion of ions such as Na^+ and Cl^- across the gills (Evans et al., 1999; Evans et al., 2005; Hwang et al., 2011; Wood, 2001). In addition, marine teleosts drink SW to replace water lost by osmosis. Consequently, the gut may play an important role in the uptake of metals and metal toxicity. Overall, therefore, the MRC function in the SW gill is to minimize ionic stress by excreting ions that have fluxed in passively either via gill

diffusion or drinking. Within MRC's this is achieved by an integrated network of transmembrane transporters that allows the organism to achieve ionic homoeostasis. Among key transporters are the cystic fibrosis transmembrane conductance regulator (CFTR), which achieves transcellular excretion of Cl^- coupled with the excretion of Na^+ through a paracellular pathway (Evans et al., 2005; Hwang et al., 2011) and/or the basolaterally located $Na^{+/}K^+$ ATPase which provides an electrochemical gradient for movement of ions (Evans et al., 2005) .

In contrast to marine teleosts, FW teleosts are hyper-osmotic, so they actively take up salt from the environment to replace that lost *via* diffusion. In general, FW MRC's appear to be the active sites of uptake of ions such as Ca^{2+}, Na^+ and Cl^-. This is achieved via the actions of the basolateral Na^+/K^+ ATPase that energizes their movements, and contributes to basolateral Na^+ transport into the bloodstream (Evans et al., 1999; Evans et al., 2005; Hwang et al., 2011; Wood, 2001). However, recent evidence also suggests a role for ammonia excretion in driving apical Na uptake (Wright and Wood, 2009). FW teleosts also restrict drinking; otherwise this would lead to further accumulation of water. However, there are studies that suggest drinking does occur in FW, however, at rates that are much lower than in SW teleosts (Blewett et al., 2013; Scott et al., 2005). While this is a standard model for physiological function in osmoregulating animals in FW, there are subtle differences. For example, the Atlantic killifish (*Fundulus heteroclitus*) does not actively take up Cl at the gills in FW, and instead this occurs *via* the gut from the diet (Laurent et al., 2006; Patrick et al., 1997). Furthermore, as estuarine fish species are likely to switch between these hypo- and hyper-osmoregulating strategies, the potential pathways of Ni accumulation, and toxic impacts of this metal, may also differ with changes in environmental salinity.

In general, estuarine osmoregulating invertebrates use mechanisms similar to those of fish to balance salt and water, in that they regulate their internal ions through the actions of key tissues

and transmembrane transporters. However, there are some important differences. For example, marine invertebrate osmoregulators maintain their internal osmolarity around that of SW (1050 mOsm; Henry et al., 2012) unlike marine teleosts that regulate plasma osmolality at approximately 450 mOsm (Evans et al., 2005). This indicates that in normal full strength SW, regulating invertebrates are not faced with water exchange difficulties and thus do not have the need to drink like their fish counterparts to maintain osmotic balance (Henry et al., 2012). As the saline environment becomes more dilute (below approximately 26 ppt) hyperosmoregulators actively take up NaCl from the environment, however, above these levels they are physiologically similar to osmoconformers where net fluxes of Na^+ and Cl^- are low or absent (Henry et al., 2005). Furthermore, although the general concept of a gill epithelium that is responsible for regulating ion exchange holds in estuarine invertebrate regulators, there are subtle differences in the exact nature of the transporters that achieve this. For example, in the gills of marine invertebrates the Na^+/H^+ exchanger is electrogenic, transporting two Na^+ ions for each proton, in contrast to the matched exchange of one Na^+ ion for every proton in fish (Ahearn et al, 2001). It is thought that this stoichiometry also allows this transporter to function as a Ca^{2+} transporter (Zhuang and Ahearn, 1996), and as such it also becomes a potential pathway of Ni entry into the animal (see below), that would not exist in a vertebrate.

It is important to note that most marine invertebrates, including some that inhabit estuarine areas, osmoconform. These animals do not actively regulate or maintain an osmotic concentration different to their environment. Therefore when the environmental salinity changes so too does their internal osmolarity (Pierce, 1982). As SW becomes more dilute these organisms will experience haemodilution (Hardy and Roesijadi, 1982; Viarengo and Nott, 1993). Differences in physiological approaches to changing salinity will either subtly or substantially

alter potential pathways of Ni uptake into the animal, and thus may be expected to influence Ni toxicity.

1.6 Ca uptake in vertebrates and invertebrates

As in other fish, Ca^{2+} uptake from the environment in killifish occurs via the gills, which account for over 97% of total Ca^{2+} uptake. Some evidence has indicated that Ca^{2+} uptake across the gills of killifish is passive, supported by linear uptake with progressive acclimation to low Ca $^{2+}$ (Mayer-Gostan et al., 1983). However, more recent evidence suggests that killifish are similar to other teleost fish in that Ca^{2+} uptake is saturable, indicative of a specific Ca^{2+} transporter with a binding affinity similar to that of other teleost fish (Patrick et al., 1997). Uptake of Ca^{2+} across the ionocytes is facilitated by an apical epithelial Ca^{2+} channel (ECaC). Once in the cell Ca^{2+} is bound to calbindins. Calbindins facilitate diffusion of Ca^{2+} across the basolateral membranes via a Ca^{2+} ATPase and/or Na^+/Ca^{2+} exchanger (Hwang et al., 2011). Killifish movement from SW and FW has little impact on Ca^{2+} uptake mechanisms.

Similar to the killifish, Ca^{2+} uptake in the green shore crab also occurs via the gills. Overall *Carcinus maenas* is highly permeable to Ca^{2+} (Wright, 1977). Uptake of Ca^{2+} from SW occurs through apical verapamil-sensitive Ca^{2+} channels, amiloride-sensitive Ca^{2+}/H^+ exchangers and/or $Ca^{2+}/2H^+$ exchangers located on the posterior gills. Like killifish, Ca^{2+} is transported across the basolateral membrane via Ca^{2+} ATPases and Ca^{2+}/Na^+ exchangers energized by a basolateral Na^+/K^+ ATPase (Freire et al., 2008). Ca^{2+} transport varies with moult cycle in crabs, with much reduced levels of uptake during the intermoult, changes that are reflected in haemolymph Ca^{2+} levels (Wright, 1977). During both intermoult and premoult any Ca^{2+} loss is compensated by increased dietary Ca^{2+} uptake and by transbranchial Ca^{2+} absorption (Freire et al., 2008; Henry et al., 2012), while the antennal gland is also capable of reabsorbing Ca^{2+} from urine. During

exposure to dilute SW Ca^{2+} balance is maintained by upregulation of a basolateral transporters (Na^{+}/K^{+} ATPase increases 2.1-fold, Na^{+}/Ca^{2+} exchanger increases 1.7-fold) (Lucu and Flik, 1999).

Mechanisms of Ca^{2+} uptake in the sea urchin larvae are less well known, however, sea urchin embryos are known to obtain Ca^{2+} directly from the environment until they can feed. Ca^{2+} uptake is thought to occur through a relatively low affinity, high capacity transporter, thought to be a voltage-gated Ca^{2+} channel (De Araújo Leite and Marques-Santos, 2011). Once absorbed, Ca^{2+} is then precipitated intracellularly in vesicles (Wilt, 2002). Movement basolaterally occurs through Ca^{2+} ATPases (Wilt, 2002). The levels of Ca^{2+} ATPase activity change through development owing to varying Ca^{2+} demand (Tellis et al., 2014; Wilt, 2002).

1.7 Mechanisms of waterborne toxicity of Ni

Within the literature there is evidence for three proximate mechanisms of Ni toxicity. These are: 1) ionoregulatory disturbances; 2) respiratory disruption; and 3) induction of reactive oxygen species (ROS).

Most of our knowledge on aquatic Ni toxicity stems from FW species. Indeed, an extensive amount of research in this area has been carried out in model species such as the FW rainbow trout (*Oncorhynchus mykiss*). This is one of the most sensitive fish species to Ni exposure (Brix et al., 2004), is commercially available, and easily held in laboratory conditions. Among FW invertebrates, the water flea *Daphnia magna* has received considerable attention. Ni toxicity has been well documented in both acute and chronic studies in this species, which has also proven to be highly sensitive (Pane et al., 2003b). These models provide a mechanistic background for investigating waterborne Ni toxicity in estuarine organisms, particularly as the few current observations regarding euryhaline organisms suggest that they are the most

susceptible to metals at lower salinities (~ 20% SW) (Leonard et al., 2011). Based on FW studies, the general rule of thumb is that Ni is an ionoregulatory toxicant in invertebrates (Pane et al., 2003b, 2004a; Leonard et al., 2013), and a respiratory toxicant in vertebrates (Pane et al., 2003a, 2004b). However the exact mechanisms of toxicity in estuarine organisms have received only very limited study (Leonard et al., 2011).

1.8 Ionoregulatory toxicity

In the FW cladoceran *Daphnia magna*, Ni inhibits unidirectional Mg influx causing a large decrease in whole body Mg stores (Pane et al., 2003b). This seems to be Mg-specific because decreases in Na^+, Cl^- and Ca^{2+} did not occur. In accord with this finding, in trout elevated Mg^{2+} reduced the unidirectional uptake of Ni across the gastrointestinal tract (Leonard et al., 2009). Furthermore, an acute waterborne Ni exposure interfered with the normal regulation and reabsorption of Mg^{2+} in the rainbow trout kidney, ultimately causing decreased plasma Mg^{2+} (Pane et al., 2005). Together these data indicate an impact of Ni on Mg^{2+} metabolism.

However, toxicity related to impairment of Mg^{2+} homeostasis is sometimes accompanied by other ionoregulatory disturbances. For example, Leonard et al. (2011) observed a disruption in both Na^+ and Mg^{2+} homeostasis in the euryhaline crustacean *Litopenaeus vannamei*. A change in Na^+ handling with Ni exposure had been previously reported, in Brix et al. (2004) who observing a 15% reduction in plasma Na in FW rainbow trout after a 24 h exposure to Ni. These data suggest a trend for acute Na loss in FW or low salinity environments. Upon chronic exposure, Pane et al. (2004a) saw decreases in plasma Na^+, Cl^-, and Mg^{2+} in juvenile rainbow trout after 42 days of exposure to Ni, suggesting a similar mechanism for Ni toxicity. Thus, although evidence strongly supports the concept of ionoregulatory impacts of Ni in invertebrates (particularly of Na and Mg), this mechanism of Ni impairment cannot be discounted in the etiology of Ni toxicity in

fish. Ni is also considered to be a Ca antagonist impacting Ca homeostasis in other aquatic organisms (e.g. Deleebeeck et al., 2007; Eisler, 1998; Pane et al., 2006). In fact Ni is often used as a Ca channel blocker in pharmacological studies of Ca-mediated processes in mammalian systems (Funakoshi et al., 1997; Lee et al., 1999; McFarlane and Gilly, 1998; Todorovic and Lingle, 1998). In the aquatic environment both Mg and Ca have been shown to protect against acute waterborne Ni toxicity to the water flea *Daphnia pulex*, an effect explained by competition with the metal for transport sites (Kozlova et al., 2009). In rainbow trout Ca was shown to non-competitively inhibit Ni transport in the stomach of trout, and similar interactions were observed in the mid intestine with Ni and Mg (Leonard et al., 2009).

1.9 Respiratory toxicity

In the extensively-studied rainbow trout, exposure to Ni caused respiratory impairment at levels in excess of 10 mg/L (Pane et al., 2003a; 2004b). After 82 h of waterborne exposure, ultrastructural damage in the gill was clearly evident. Gill lamellae were completely fused due to extensive swelling. These impacts likely decreased the effectiveness of the gill as a respiratory surface, resulting in the observed increases in ventilatory rates and volumes, decreases in oxygen extraction efficiency, and decreases in arterial partial pressure of O_2. 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), confirming this as a mode of toxic action, albeit at high exposure levels. Respiratory distress with exposure to Ni has only been indirectly observed in the invertebrate *D. magna* (Pane et al., 2003b).

1.10 Induction of ROS

Nickel-induced oxidative stress in aquatic organisms has only recently been investigated in one freshwater species, the goldfish *Carassius auratus* (Kubrak et al., 2012a,b; Kubrak et al.,

2013; Kubrak et al., 2014). Other metals such as Cu, cadmium (Cd), and Zn (Faverney et al., 2001; Craig et al., 2007; Loro et al., 2012) have been widely reported to induce ROS and ROS-related antioxidant responses in aquatic organisms. The mechanism by which Ni induces ROS 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 negative effects on antioxidant mechanisms, decreasing the ability of the cell to scavenge ROS, leading to increased ROS-related damage (for review see Lushchak, 2011).

In goldfish, Ni exposure of 10-50 mg/L caused different antioxidant responses depending on the tissue tested. In the gill, superoxide dismutase (SOD) and glutathione peroxidase (GPx) decreased upon Ni exposure, while catalase (CAT) activity increased (Kubrak et al., 2013). In the liver CAT activity increased, but white muscle tissue displayed decreases in CAT activity relative to control tissue (Kubrak et al., 2012a). These results indicate different responses to Ni toxicity depending on the tissue tested. In mammals, administration of Ni causes increased lipid peroxidation (a marker of oxidative damage), inhibited GPx activity (a marker of antioxidant defense) and increased tissue Fe levels (Stohs and Bagchi, 1995).

Other markers of oxidative damage reflect the general and wide-ranging effects of ROS. In addition to lipid peroxidation, DNA damage and protein carbonylation may also occur. Protein carbonyls form when ROS directly attack proteins leading to the formation of a carbonyl moiety (Bainy et al., 1996), which results in non-reversible damage and thus inhibition of protein function (Zhang et al., 2010).

Perhaps of greatest relevance to Ni toxicity is the use of CAT as a marker of oxidative stress. Previous research has identified this enzyme as being impacted by Ni exposure (Cartañá et

al., 1992; Rodriguez et al., 1990, Kubrak et al., 2013). CAT appears to be a particularly sensitive marker of metal toxicity (Grosell, 2012) due to the presence of histidine residues in the active site which play an important role in its catalytic activity (Mate et al., 1999). Ni has a high affinity for such histidine residues (e.g. Predki et al., 1992), and thus the presence of Ni can have an adverse effect on CAT function.

Effects of salinity on Ni-induced oxidative stress in aquatic animals have not been investigated. However, increased salinity was noted as reducing key oxidative stress markers in Zn-exposed euryhaline killifish (Loro et al., 2012). This suggests that salinity has a potential protective role against this mode of toxicity in metal-exposed aquatic animals.

1.11 Test organisms

The killifish (*Fundulus heteroclitus*) is a euryhaline fish native to the east coast of North America. It is found in tidal shallows that experience both daily and seasonal fluctuations in temperature, salinity and dissolved oxygen levels. Killifish have a remarkable tolerance for salinity extremes, withstanding dilute FW to hypersaline waters (Griffith, 1974). As such they are an excellent model species for examining ionoregulation (Burnett et al., 2007). They have also been used as a model organism to study Cu toxicity in the marine environment (Grosell et al., 2007), and owing to their salinity resilience they are particularly valuable for examining the effects of salinity on metal toxicity. Little is known regarding Ni toxicity in killifish, although LC_{50} values in SW are extremely high (250 mg/L adult, Eisler, 1998; 66 mg/L larvae, Bielmyer et al., 2013), but mechanisms of toxicity have not been investigated.

The green shore crab (*Carcinus maenas*) is a crustacean that is native to the North East Atlantic and Baltic Sea. Due to its ability to withstand an extensive range of salinities (4 - 40 ppt) they are an invasive species, and inhabit similar habitats to killifish. They are considered a model

species for ecotoxicology studies (Leignel et al., 2014), and have been extensively used to determine impacts of metal contaminants and polycyclic aromatic hydrocarbons in the environment (Dissanayake et al., 2010; Elumalai et al., 2007; Martin-Diaz et al., 2004). They are iso-osmotic in full seawater, and unlike marine teleost fish do not drink. Consequently their exposure pathways for Ni may be different in marine settings than fish. Inherent differences in the physiology of euryhaline invertebrates and vertebrates are likely to result in different mechanisms of accumulation and toxicity with respect to Ni exposure. Of particular note is the very high Ca demand of these animals, particularly during reproduction and moulting (Travis and Friberg, 1963). Given the antagonism between metals and Ca this is a species that is of particular interest to metal ecotoxicologists (Dissanayake et al., 2008, 2010). Little is known regarding Ni toxicity in this species, but given their euryhalinity and general utility for studies of metal toxicity they are an excellent potential model for examining the effects of Ni and how these vary with salinity.

Sea urchin embryos are considered to be very sensitive to metal contaminants. In fact, the early life stages of sea urchins appear to be among the most sensitive of all tested organisms to Ni toxicity (DeForest and Schlekat, 2013). Although their sensitivity to Ni is well-characterised, little is known regarding the mechanisms of Ni toxicity in sea urchin larvae (Tellis et al., 2014). Applying the principal that makes *Daphnia* and rainbow trout model species in FW, the extreme sensitivity of sea urchin embryos makes them an excellent model species for marine toxicity studies of Ni. Further enhancing their role as an indicator species, studies have shown that accumulation of metals in sea urchin embryos correlates with toxicity (Nadella et al., 2013), a key tenet of environmental regulatory tools such as the BLM (Niyogi and Wood, 2004).

1.12 Thesis goals

The FW environment has been relatively well studied with regards to Ni bioavailability, accumulation and toxicity. However, far less is known regarding Ni in the marine environment and, in particular, estuarine settings where variable salinities may occur. In light of the three identified modes of Ni toxicity in FW, I sought to determine: a) if these toxic modes were observed in animals inhabiting marine and estuarine environments; b) how salinity affected uptake and accumulation in representative vertebrate and invertebrate species; and finally, c) if physiology played a relatively more important role in Ni toxicity and uptake than water chemistry.

1.13 Hypotheses tested

Overall there were five main hypotheses to be tested, as outlined below:

1) Salinity will be protective against Ni accumulation and sub-lethal toxicity due to the presence of protective cations and complexing anions present at higher concentrations in SW

2) Physiology will be more critical than the surrounding water chemistry in terms of shaping sub-lethal Ni toxicity

3) Marine and estuarine invertebrates will be more sensitive to Ni sub-lethal toxicity, than comparable vertebrates, conforming to the patterns of toxicity observed in FW

4) Toxicity will be related to ionoregulatory disruption in invertebrates, while respiratory toxicity will be the main mode of toxicity experienced by vertebrates

5) Oxidative stress will be a mechanism of Ni toxicity in both invertebrates and vertebrates

1.13 Chapter summaries

Chapter 2 has been published in *Aquatic Toxicology* (Blewett et al., 2015a) and investigated the effects of a range of Ni concentrations, including an environmentally-relevant

level (8.2 µg/L), on a euryhaline invertebrate, the green crab *Carcinus maenas*, at varying salinities. Bioaccumulation and ionoregulatory toxicity were examined after acute exposure to Ni (24 h). Posterior gill 8, hypothesized to have a primary role in ion regulation, was the most affected, with the highest accumulation of Ni in this tissue occurring in the lowest salinity tested (20% SW). Indeed the anterior gills, generally considered to be have primary roles in respiratory function, seemed largely unaffected by Ni toxicity, a result further confirmed by gill perfusion studies. Enzyme analysis showed decreases in Na^+/K^+ ATPase activity in gill 8, an ionoregulatory gill, in the higher two concentrations of Ni tested but only in the lowest salinity (20% SW). Furthermore, ionoregulatory disruption was also observed with respect to Ca and Na concentrations in the haemolymph.

Based on the findings of Chapter 2, Chapter 3 (submitted to *Ecotoxicology and Environmental Safety*; Blewett and Wood, 2015b) further investigated the mechanisms of Ni toxicity in green crabs. All three putative modes of toxicity identified in FW animals (respiratory, ionoregulatory and oxidative stress) were observed after an exposure to Ni (3 mg/L) for 96 h. Again, there were fundamental differences between gills, whereby the posterior (ionoregulatory) gills accumulated a greater amount of Ni in 20% SW, than did the anterior (respiratory) gills. Crab haemolymph showed alteration in ions observed for K, Mg and Ca at 20% SW. Respiratory impairment was also identified, again only in the lowest salinity (20% SW). Furthermore, impairment of CAT activity and enhanced protein carbonyl formation were noted, which varied depending on gill number and salinity. This was the first time all three modes of Ni toxicity were measured simultaneously in the same animal.

Chapter 4 explored Ni toxicity in a highly sensitive species, the early life stages of the sea urchin, *Evechinus chloroticus*. This species was found to be the most Ni-sensitive marine

organism recorded to date, with an EC_{50} of 14.1 µg/L. Morphological examination showed an impaired skeletal development, and a reduced Ca influx rate. Changes in whole body ions were also observed. Together these data indicated that the mechanism of Ni toxicity was ionoregulatory in nature. Further studies revealed that low levels of DOC were protective against Ni toxicity, but high levels exacerbated abnormal development, but only in the presence of Ni. This was suggestive of an interaction between DOC and Ni, possibly related to membrane physiology.

Chapter 5 has been published in *Archives of Environmental Contamination and Toxicology* (Blewett and Wood, 2015a). This study was the first to examine Ni bioaccumulation and oxidative stress responses in the model euryhaline teleost, *Fundulus heteroclitus*, as a function of environmental salinity and increasing Ni concentration. This study showed that both physiology and water chemistry are important in modifying the sub-lethal responses to Ni. Sea water was protective against both Ni accumulation and indicators of oxidative stress (protein carbonyl formation and CAT activity), implying a strong water chemistry effect preventing Ni accumulation. However in FW, the killifish liver showed high accumulation relative to the liver in SW-acclimated animals, but with a reduced CAT effect, indicating that organ-specific differences in Ni handling may also contribute towards toxicity.

Chapter 6 further examined the mechanisms of sub-lethal Ni toxicity in killifish, building on the findings in Chapter 5. Killifish were exposed to 5 mg/L of Ni for 96 h at four different salinities. Salinity was shown to be protective against Ni accumulation at the gill, but exacerbated uptake in the liver and intestine, likely due to drinking. Critical swimming speed was unchanged between FW- and SW–acclimated killifish. Confirming the lack of a respiratory effect, oxygen consumption rates also did not vary. Examination of the concentration-dependence of the kinetics of Ni uptake revealed saturable uptake for FW- and SW-acclimated killifish, although the latter

exhibited reduced Ni-binding capacity and higher Ni-binding affinity. Studies involving manipulation of water ion levels showed that Mg and Ca were protective against Ni accumulation, suggesting interaction of these ions with Ni uptake.

Chapter 7 summarizes the key findings of the experimental chapters of this thesis, and provides future directions with regards to the study of Ni toxicity in the marine and estuarine environment. It discusses the potential relevance of the thesis work for the development of a marine-based Ni BLM for fish and invertebrate species.

CHAPTER 2

MAKING SENSE OF NICKEL ACCUMULATION AND SUB-LETHAL TOXIC EFFECTS IN SALINE WATERS: FATE AND EFFECTS OF NICKEL IN THE GREEN CRAB, *Carcinus maenas*

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2.1 ABSTRACT

In freshwater invertebrates nickel (Ni) is considered an ionoregulatory toxicant, but its mechanism of toxicity in marine settings, and how this varies with salinity, is poorly understood. This study investigated Ni accumulation and physiological mechanisms of sub-lethal Ni toxicity in the euryhaline green crab *Carcinus maenas*. Male crabs were exposed to 8.2 µg/L (the US EPA chronic criterion concentration for salt waters) of waterborne Ni (radiolabelled with ^{63}Ni) at three different salinities, 20%, 60% and 100% seawater (SW) for 24 hours. Whole body Ni accumulation in 20% SW was 3-5 fold greater than in 60% or 100% SW, and > 80% of accumulated Ni was in the carapace at all salinities. Ni also accumulated in the posterior gill 8, which showed a higher accumulation in 20% SW than in other salinities, a pattern also seen at higher exposure concentrations of Ni (500 and 3000 µg/L). Gill perfusion experiments revealed that Ni was taken up by both anterior and posterior gills, but in 20% SW the posterior gill 8, which performs ionoregulatory functions, accumulated more Ni than the anterior gill 5, which primarily has a respiratory function. The sub-lethal consequences of Ni exposure were investigated by placing crabs in Ni concentrations of 8.2, 500, and 3000 µg/L at 20, 60 or 100% SW for 24 hours. In 20% SW, haemolymph Ca levels were significantly decreased by exposure to all Ni concentrations, whereas Na haemolymph levels were depressed only at 3000 µg/L. Na^+/K^+-ATPase activity was inhibited at both 500 and 3000 µg/L in gill 8 but only in 20% SW.

Haemolymph K, Mg, and osmolality were unaffected throughout, though all varied with salinity. These data suggest that Ni impacts ionoregulatory function in the green crab, in a gill- and salinity-dependent manner.

2.2 INTRODUCTION

For aquatic biota, such as decapod crustaceans, the gills perform a number of vital physiological functions. The gills are exposed directly to the external milieu, represent only a thin barrier between the animal and the environment, and have high blood perfusion rates (for review, see Henry et al., 2012). The above characteristics are ideal for gas and ionic exchange but in turn promote metal uptake and toxicity (Wood, 2001). As the major regulatory organ involved in homeostasis, the properties of decapod crustacean gills change with fluctuations in their environment, for example high ammonia (Martin et al., 2011), elevated pCO_2 (Fehsenfeld et al., 2011; Fehsenfeld and Weihrauch, 2013) and decreased salinity (Compère et al., 1989). Many marine crustaceans such as the green crab (*Carcinus maenas*) are osmoregulators maintaining their internal osmolality close to that of seawater (1050 mOsm; Henry et al., 2012). When faced with environmental dilution, a hyper-ionoregulating crab will attempt to maintain ionic balance, by utilizing the gills to absorb ions lost by diffusion. In *C. maenas*, the anterior gills (2-5) are primarily engaged in respiration and excretion, while the posterior gills (6-9) are the primary site of NaCl uptake and osmoregulation (Freire et al., 2008; Mantel and Farmer, 1983; McNamara and Lima, 1997; Onken and Riestenpatt, 1998; Péqueux, 1995). Due to metals being the most toxic in their ionic form (Wood, 2012), the posterior gills are the most probable targets of metal uptake and toxicity in this species. Furthermore, with higher rates of active ion uptake in the posterior gills in response to decreased salinities, metal uptake and toxicity might be exacerbated in dilute seawater. This has important implications for animals that are exposed to both metals and an estuarine setting.

One metal contaminant that has been shown to accumulate in estuaries is nickel (Ni). Ni occurs naturally in the environment, but levels in aquatic settings are anthropogenically enriched

due to combustion of fossil fuels, mining, and other industrial practices (Eisler, 1998; ECU, 2008; NAS, 1975; WHO, 1991). Ni concentrations in estuarine and marine environments range from 10-100 µg/L in highly polluted areas, whereas in unpolluted waters, Ni concentrations are approximately 2 µg/L (Boyden, 1975). Mechanisms of Ni toxicity to freshwater invertebrates are relatively well described. For example, it has been shown that in the freshwater cladoceran *Daphnia magna*, Ni inhibited unidirectional Mg influx causing a large decrease in whole body Mg (Pane et al., 2003). This ion mimicry mode of toxicity is common for metals. Ionic species of Cd , Zn and Pb can cross the gill epithelium of crabs *via* non-specific Ca channels, while in freshwater and brackish water crustaceans, Cu and Ag compete with Na for uptake at the gill via the Na^+/H^+ exchanger (Bianchini and Wood, 2003; Brooks and Mills, 2003; Glover and Wood, 2005; Martins et al., 2011). However, there is less known about Ni toxicity in the marine and estuarine environment, and specifically about the role salinity may have in modifying toxic effects. One study to date indicates that an ionoregulatory mode of toxicity for Ni may persist in waters of higher salinity, with a disruption in both Na and Mg homeostasis being observed in the shrimp *Litopenaeus vannamei* (Leonard et al., 2011).

Owing to differences in water chemistry and the distinct physiology of marine organisms, the mechanisms of Ni uptake and toxicity may differ significantly from those in freshwater animals. In marine environments, the major factor governing Ni toxicity, apart from dissolved organic carbon (DOC) (Paquin et al., 2002), is likely to be the increase in cations associated with increases in salinity (Pyle and Couture, 2012). In estuarine settings, where salinity will fluctuate, the composition of ions will therefore vary significantly with factors such as tidal cycles and changes in freshwater inputs. These changes in water chemistry are likely to have a substantial influence on the bioavailability and toxicity of Ni. In both freshwater and seawater systems it is thought that the dominant Ni species is the free ion form (Ni^{2+}; Pyle and Couture, 2012).

However, $NiCO_3$ and $NiSO_4$ cannot be discounted as significant chemical species of Ni at higher pH's (> 8) (Pyle and Couture, 2012). As Ni^{2+} is a divalent cation, it is likely that its uptake will be most greatly impacted by the presence of other divalent cations (e.g. Mg^{2+}, Ca^{2+}). These will offer possible protective effects as they may compete with Ni for the binding sites on the gill (Paquin et al., 2002).

The first objective of the present study was to characterize the tissue-specific pattern of accumulation of waterborne Ni in the green crab *Carcinus maenas*, a euryhaline hyper-osmoregulating decapod crustacean, with a particular emphasis on Ni handling by different gills (anterior *vs*. posterior). An exposure level of 8.2 µg/L was chosen as this is the US EPA (1995) Criterion Continuous Concentration for Ni in saltwater (the chronic guideline value), and is a value that is encompassed in the range of marine environment concentrations where site-specific toxicity testing is recommended (3.9-20.9 µg/L; DeForest and Schlekat, 2013). A second goal was to determine if the physiological mechanism of sub-lethal Ni toxicity is related to ion transport. Thirdly, impacts of salinity on Ni uptake were investigated. In the studies designed to examine these latter two goals, a range of Ni concentrations were chosen (an environmentally relevant level (8.2 µg/L), a mid-range level (500 µg/L), and a high level (3000 µg/L)) to investigate the concentration-dependence of Ni effects.

2.3 METHODS

2.3.1 Animal care

Male green crabs [*Carcinus maenas* (Linnaeus 1758); 76 ± 13.4 g; carapace width: 6.50 ± 0.45 cm] were obtained from two uncontaminated sites 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) via baited crab pots under a licence from Fisheries and Oceans Canada. Animals were then

transported back to Bamfield Marine Sciences Centre (Bamfield, BC, Canada) and held in outdoor tanks (~200-L) maintained with flow-through seawater (~32 ppt) under constant aeration and under a natural photoperiod (10 h D:14 h L). Crabs were allowed to acclimate to the holding conditions in 100% seawater (32 ppt; SW) for 7 days. Crabs were then randomly distributed into one of three salinity exposure groups (20% SW (6.4 ppt), 60% SW (19.2 ppt), 100% SW (32 ppt)), where intermediate salinities were made by diluting Bamfield sea water with nanopure water. They were then held for a 10-day acclimation period. Each group (N = 21) was maintained in a plastic container (68-L) with aeration and filtration. Water changes were made every 3 days to avoid the accumulation of deleterious nitrogenous waste (Regnault, 1987). Crabs were fed twice a week with salmon fish heads (followed by a water exchange the next day), but were starved 48 h prior to any experimentation. All procedures were approved by Bamfield Animal Research Ethics Board and were in accordance with the Guidelines of the Canadian Council on Animal Care.

2.3.2 Ni exposure at 8.2 µg/L

In the first experiment, Ni exposures were conducted in large plastic containers (~10-L) fitted with aeration devices and containing sea water of the appropriate salinity with a final nominal waterborne [Ni] of 8.2 µg/L that was made from concentrated $NiCl_2 \cdot 6H_2O$ stock. Radiolabelled nickel (^{63}Ni; 0.5 µCi/L; Amersham Biosciences, Inc., USA) was added to each exposure chamber. All Ni was added to the containers prior to the addition of crabs ("cold" Ni at t = 24 h, to allow for Ni to reach equilibrium and "hot" Ni at t = 0.5-h to avoid loss of the radioisotope caused by adsorption). Seven crabs were placed individually into these containers and exposed for 24 h. Upon termination of the exposure, crabs were washed in a high Ni ($NiCl_2 \cdot 6H_2O$) solution (10 mg/L) for "cold displacement" of ^{63}Ni, and then transferred to a 1 mM

EDTA solution for 1 min to remove any remaining loosely bound ^{63}Ni isotope before terminal sampling. For terminal tissue sampling, crabs were anesthetized (~15 min) on ice and then quickly euthanized by a single spike to the ventral ganglion through the ventral wall of the carapace. Gill 1 is considered to be a gill bailer and not a true gill so gill pairs 2-9 were used. Carapace, muscle, hepatopancreas (HP), haemolymph, heart, and antennal gland (AG) were excised and placed in the appropriate amount of nitric acid (described below).

2.3.3 Concentration series and enzyme activity

In the second experiment, using a similar approach to that detailed above, green crabs were subjected to one of four different nominal Ni concentrations: 0, 8.2, 500 or 3000 µg/L. These series were repeated in each salinity group (20, 60 or 100% SW) in the same manner as the 8.2 µg/L exposures using a fixed [^{63}Ni] (0.5 µCi/per exposure chamber) and a 24-h exposure timeframe. Animals were euthanized as above and haemolymph and gills 5 and 8 were excised and snap frozen in liquid nitrogen before being stored at -80°C for further analysis. Branchial Na^+/K^+ ATPase (*EC 3.6.3.9*) activity was determined using methods previously described (McCormick, 1993). Briefly, frozen gill 5 and gill 8 were ground to a fine powder under liquid nitrogen with a chilled mortar and pestle. This powder was suspended in 20 volumes of an imidazole buffer (50 mM imidazole, 125 mM sucrose, 5 mM EGTA; pH=7.5) and then homogenized on ice with a Power Gen 125 homogenization unit (Thermo Fisher Scientific, Toronto, ON, Canada). Briefly, a 10 µl sample was plated with 200 µl of 2.8 mM of phosphoenolpyruvate (PEP), 3.5 mM ATP, 0.22 mM NADH, 4 U/ml of lactate dehydrogenase (LDH) and 5 U/ml of pyruvate kinase (PK), 189 mM NaCl, 10.5 mM MgCl, 42 mM KCl and 50 mM of imidazole. Additional samples were run with the above solutions with the addition of 0.65 mM of ouabain to inhibit the Na^+/K^+ ATPase. Assays were read at 340 nm using a UV-visible 96-

well plate reader (SpectraMax 340 PC, Sunnyvale, CA, USA). A Bradford assay (Sigma Aldrich, Canada) was used to normalize activity to total protein content (Bradford, 1976).

2.3.4 Gill perfusion

In the third experiment, crabs were taken from salinity acclimation tanks and placed on ice for 15 min to ensure anesthesia. Crabs were then euthanized as above. The carapace was lifted and removed before gills 5 and 8 were selected and placed in a petri dish on ice containing the respective salinity for the exposure (see below). Gill perfusion experiments were then performed as described by Siebers et al. (1985) with some modifications. Gills 5 and 8 were taken as they are the largest of the true anterior and posterior gills. Briefly, the excised gill was perfused with an artificial haemolymph based on previous literature (Lignon, 1987; Mantel and Farmer, 1983; Winkler, 1987) for 100% SW crabs [in mM]: 470 NaCl, 12 $CaCl_2$, 12 $MgCl_2$, 11 KCl, 9 $NaHCO_3$, 0.1 NH_4Cl, 0.3 glucose, 0.1 glutathione, 0.5 glutamine; pH 7.9. For 60% SW and 20% SW crabs the artificial haemolymph was based on recorded values in *C. maenas* from Fehsenfeld and Weihrauch (2013); in 60% SW crabs [in mM]: 332 NaCl, 5.3 $CaCl_2$, 10.2 $MgCl_2$, 8 KCl, 4.0 $NaHCO_3$, 0.3 glucose, 0.1 glutathione, 0.5 glutamine; pH 7.9, and in 20% SW crabs [in mM]: 260 NaCl, 5 $CaCl_2$, 7 $MgCl_2$, 7 KCl, 7 $NaHCO_3$, 0.1 NH_4Cl, 0.3 glucose, 0.1 glutathione, 0.5 glutamine; pH 7.9. Perfusion was achieved using a peristaltic pump (Sci 323 Watson-Marlow Bredel Pump, Falmouth Cornwall, England) and elastic tubing that was inserted into afferent and efferent blood vessels of the gill. Perfusion of the afferent vessel occurred at a rate of 127 ± 0.1 μL/min, for a maximum of 2 h, following methods of Fehsenfeld and Weihrauch, 2013. During perfusion, the gills were suspended in a 50-mL bathing solution that included ^{63}Ni (0.5 μCi/per container) and a total nominal concentration of 8.2 μg/L Ni in the respective seawater solution (i.e. 20, 60 or 100% SW). At the conclusion of the exposure, gills were taken for digestion and

analysis of ^{63}Ni radioactivity as described below. Collected perfusates were analyzed for the movement of Ni from the bathing solution into the perfusate, while samples of the bathing solution were also taken for determination of specific activity.

2.3.5 Tissue analyses

Each tissue was weighed, and depending on mass, was then placed in a 50-mL, 15-mL, or a 2-mL centrifuge tube. Tissues were digested with 1N trace metal grade nitric acid (Sigma-Aldrich) at volumes of 3-5 times (exact volume recorded) the weight of the tissue, except for the hepatopancreas, carapace and muscle which were digested in 3-5 volumes of 2N trace metal grade nitric acid. All tubes were tightly sealed and placed in an incubator at 65°C for 48 h, with vigorous vortexing at 24 h. The digested samples were then centrifuged for 5 min at 3500 rpm at 18°C. Volumes of the resulting supernatant (all 2 mL, except for haemolymph, heart, gill and antennal gland with 1 mL) were mixed with scintillation fluor (Ultima Gold, Perkin Elmer, Waltham, MA) in a 5:1 ratio (fluor:supernatant) for assay of radioactive beta-emission counts (Tri-Carb 2900TR Liquid Scintillation Analyzer; Perkin Elmer, Waltham, MA). Samples were standardized to a common counting efficiency (the same as that of water samples, see below) using a quench curve constructed from various amounts of tissue digest.

2.3.6 Water and haemolymph ion and Ni analyses

Water Ni concentrations were monitored at 0.5, 12 and 24 hours and average values are reported in Table 2.1. Water samples for ions and dissolved Ni concentrations were collected via passage through a 0.45 µm syringe filter (Acrodisc; Pall Life Sciences, Houston, TX, USA). Unfiltered (total) samples were also taken for total Ni measurements. Radioactive water samples were analyzed by the addition of scintillation fluid (Optiphase, Perkin Elmer in a ratio of 1:1 water:fluor) and counted on the same liquid scintillation analyzer as used for the tissue samples.

Measurements of 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 97.3 ± 2.7 % as determined by Environment Canada certified reference materials, TM 25.3 and TM 15 and DORT-1 lobster hepatopancreas. Final reported 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) (Table 2.2). Water and haemolymph Cl^- were measured using a Labconco digital chloridometer (B5953; Kansas City, MO; Table 3.2). All ions were measured against reference standard solutions (Fisher Scientific, Ottawa, ON) using standard curves. Osmolality was measured via a Wescor VAPRO 5520 vapour pressure osmometer (Logan, Utah, USA). Water pH was measured by an Accument Basic AB15 pH meter (Fisher Scientific, Ottawa, ON). Dissolved organic carbon (DOC) was measured using a Shimadzu TOC-Vcph/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan).

2.3.7 Calculations

A pilot study was conducted to determine the relative percentage of weight that the carapace and muscle contributed to the whole crab. This was performed so that total Ni accumulation could be calculated, as only a small sample of each of these tissues was analyzed for total Ni concentration. Seven whole crabs were dissected into their component tissues. Each tissue was then weighed and expressed as a percentage of total crab weight (Table 2.4). These proportions were then used to extrapolate from the subsample Ni concentrations to total tissue Ni burdens for carapace and muscle (by multiplying proportional mass contribution by measured Ni concentration in each tissue). These were then used to calculate total Ni accumulation.

Ni accumulation data were calculated from the counts per minute of the individual tissues (CPM), mean specific activity (SA; based on the CPM and measured water concentrations from the GFAAS) and tissue weight (W). Data are expressed as µg/kg.

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

For gill perfusion experiments, the rate of gill tissue uptake (µg/kg/h) was calculated as follows:

$$Ni\ uptake\ rate = \frac{CPM}{SA}X\frac{\mathbf{1}}{W}\ X\ \ \frac{\mathbf{1}}{\mathbf{\Delta}T}$$

where CPM is the total counts per minute in the individual gill tissue, SA is the specific activity of the external seawater bath in that experimental group, W is the weight of the gill and T is the time of exposure.

To determine the rate of uptake of Ni into the perfusate (µg/kg/h), an identical equation was used except CPM represented the total accumulated CPM in the perfusate over the 2-h perfusion, where W indicates the volume of the perfusate.

2.3.8 Statistical analysis

Data have been expressed as means ± SEM (N). 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). All gill 24-h exposure data were analyzed via two-way ANOVA where salinity and Ni concentration were the two factors of interest. Where significance ($P < 0.05$) was found, a Tukey's post-hoc test was applied. For all other analyses, a one-way ANOVA model was applied with a Tukey's post-hoc test. Significance for all statistical tests was accepted at $\alpha = 0.05$. For Ni speciation analysis the water chemistries recorded in Tables 2.1 and 2.2 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 determine the specific effect of DOC on speciation the NICA-Donnan model was followed (Benedetti et al., 1995) (Table 2.3).

2.4 RESULTS

2.4.1 Ni concentrations, speciation, and water chemistry in all exposures

All Ni concentrations were consistent between salinities and reasonably close to nominal values (Table 2.1). All reported levels are dissolved (i.e. passed through a 0.45 µm filter) as there was less than 10% difference between all filtered and unfiltered Ni measurements. Ni concentrations in control SW ranged from 2.53 to 2.95 µg/L. Water ion concentrations and osmolality followed a linear drop with decreasing salinity from 100% SW to 20% SW (Table 2.2). The pH varied from 7.64, 7.88 and 8.12 in 20, 60 and 100% SW, respectively. Speciation was similar between salinities, with 20 SW% having the highest proportion of free Ni (82%), and 100% SW having the lowest proportion (77%) at a Ni concentration of 8.2 µg/L (Table 2.3). The proportion of different Ni species did not vary significantly with Ni exposure concentration, nor did they vary much with salinity. Speciation was relatively independent of Ni concentration.

2.4.2 Salinity-dependent Ni accumulation and tissue distribution at different Ni exposure concentrations for 24 hours

At 8.2 µg/L Ni, whole body Ni accumulation over 24 h was substantially higher (624 µg/kg) in the 20% SW treatment in comparison to the two other salinities (75 µg/kg at 60% SW; 198 µg/kg at 100% SW), which were not significantly different from one another (Fig. 2.1A). Overall a two way ANOVA determined that for the gills there were no significant differences in

terms of salinity (P > 0.05), treatment (Ni *vs*. control, P > 0.05) or interactive effects (P > 0.05). There were no significant differences in Ni accumulation within salinities across the 9 gill pairs, with average values of 180 μg/kg. Only gill 8 showed significant differences in accumulation between different salinities, with the 20% SW gill 8 accumulating significantly more Ni (418 μg/kg) than the 60% SW gill 8 (188 μg/kg), which was also significantly higher than the 100% SW gill 8 (106 μg/kg) (Fig. 2.1B). This overall trend of salinity-based differences in accumulation continued within the remaining tissues. The hepatopancreas, heart and muscle showed a consistent significant effect, whereby the 20% SW group accumulated by far the most Ni (Fig. 2.1C). Both haemolymph and antennal gland accumulation data for the 100% and 60% treatment groups were very low, not significantly different from zero, reflecting negligible uptake into these compartments. Overall, the carapace exhibited the highest concentrations of Ni in view of its large relative size (77% of the whole body mass; Table 2.4). The 20% SW carapace accumulated almost 3-fold more Ni than the 100% SW carapace and 12-fold more than the 60% SW carapace (Fig. 2.1D).

The relative (%) tissue distribution profile was generally similar across all salinities. However, the relative proportion of Ni accumulated in the gill was the highest in 60% SW, despite the absolute gill accumulation in gill 8 being greatest in 20% SW (Fig. 2.1B; Fig. 2.2). This accumulation seemed to come mainly at the expense of the carapace, which exhibited the smallest relative and absolute accumulation in the 60% exposure group (Figs. 2.1D and 2.2). In all three groups, the carapace accounted for > 80% of the total Ni accumulation (note Fig. 2.2 y-axis scale).

2.4.3 Salinity-dependent accumulation by perfused gills at a Ni exposure concentration of 8.2 μg/L for 3 hours

The highest gill Ni uptake rate (35 μg/kg/h) occurred in the 20% SW gill 8 at 8.2 μg/L (Fig. 2.3A). This uptake rate was significantly different from that in gill 5 in the same salinity treatment, and was also significantly higher than the Ni accumulation observed in the 100% SW treatment (Fig. 2.3A). Rate of transport into the perfusate was only 3-10% of the gill uptake rate. There were no salinity-dependent differences in the rate at which Ni accumulated in perfusates, however the highest perfusate accumulation rate was found in the 20% SW group (Fig. 2.3B).

2.4.4 Salinity dependent Ni accumulation in concentration series

When comparing the different levels of Ni exposure (0, 8.2, 500, 3000 μg/L), posterior gill 8 accumulated significantly more Ni than anterior gill 5, but only in the 20% SW acclimated crabs (Fig. 2.4A). For example, in both 8.2 and 3000 μg/L concentrations gill 8 accumulated 2-fold more Ni than gill 5, while at 500 μg/L, gill 8 displayed a 67% increase in Ni accumulation compared to gill 5 (Fig. 2.4A). In contrast, there were no differences between gills 5 and 8 in crabs acclimated to 60% or 100% SW at any exposure concentration (Fig. 2.4 B,C). In every salinity tested, as the concentration of Ni in the water increased, so did the accumulation in both the anterior and the posterior gill (Fig. 2.4 A,B,C). When these data were plotted on quantitative concentration co-ordinates (not shown), uptake displayed a curvilinear relationship but saturation did not occur.

2.4.5 Salinity-dependent Na^+/K^+ATPase activity in gills exposed to 4 different concentrations of Ni

Regardless of salinity, gill 8 had markedly higher Na^+/K^+ ATPase activity than gill 5 (Fig. 2.5), but there were no significant differences across salinities under control conditions. However, in the 20% SW group, there were significant decreases in Na^+/K^+ ATPase activity upon Ni exposure but only at the two highest exposure concentrations (500 and 3000 μg/L). In the 500

μg/L concentration in the 20% SW group, Na^+/K^+ ATPase activity was the lowest with a 95% decrease from control activity levels. The 3000 μg/L group also displayed a 50% decrease in activity from control values (Fig. 2.5A). In contrast, Na^+/K^+ ATPase activity in gill 5 were unaffected by Ni at any exposure concentration. In the other two salinity treatments Na^+/K^+ ATPase activity remained fairly consistent within a gill, regardless of the level of Ni exposure (Fig. 2.5B,C).

2.4.6 Salinity-dependent haemolymph ion and osmolality in crabs exposed to 3 different concentrations of Ni

Salinity had a marked effect on all haemolymph ions and osmolality, with decreasing salinity resulting in significant declines in all measured parameters. Overall, however, haemolymph ions were always kept above the external water ion concentrations (Table 2.2, Table 2.5, Figure 2.6). Ni exposure had effects on only two ions (Na and Ca), but only at 20% SW. Na concentration decreased significantly by about 10% from 329 mmol/L in the control haemolymph to 296 mmol/L in the haemolymph of 20% SW crabs exposed to the highest concentration of Ni (3000 μg/L)(Fig. 2.6A, B). Ni effects on Ca were more pronounced, whereby all Ni concentrations decreased haemolymph Ca concentration in 20% SW crabs to levels significantly below those of the control animals (Fig. 2.6B). In the 8.2 and 500 μg/L Ni exposures, Ca concentration decreased by 12% and 13% respectively, while in the highest concentration of Ni (3000 μg/L), Ca decreased by 26% with respect to controls (Fig. 2.6B). Two way ANOVA's showed that there was no effect of Ni concentration for Na ($P = 0.199$), K ($P = 0.714$), Cl ($P = 0.121$), Ca ($P = 0.257$), Mg ($P = 0.960$) and osmolality ($P = 0.152$) (Table 2.5). Na ($P = 0.895$), K ($P = 0.467$), Cl ($P = 0.147$) and osmolality ($P = 0.752$) displayed no interaction effects (salinity *vs*. concentration of Ni), however Mg ($P = 0.004$) and Ca ($P = 0.007$), both showed interaction effects

(Table 2.5). All ions and osmolality were significant with respect to differences in salinity (all $P < 0.001$) (Table 2.5).

2.5 DISCUSSION

Ni accumulation and sub-lethal toxicity (pathological, but non-fatal, disturbances in physiology) in the green shore crab, *Carcinus maenas,* were salinity-dependent, with the major effects being an increase in uptake of Ni and evidence of ionoregulatory disturbance as salinity decreases. There was also evidence suggesting that branchial handling and sub-lethal toxicity were gill-arch-dependent.

2.5.1 Salinity-dependent tissue-specific patterns of Ni accumulation

Total Ni accumulation in *C. maenas* at 8.2 µg/L (the US EPA (1995) Criterion Continuous Concentration) was higher at 20% SW than in either 60 or 100% SW (Fig. 2.1A). Salinity is considered to be protective due to the increased concentrations of cations in a saline environment, competing with Ni for uptake sites present at the gill (Paquin et al., 2002). However, there was also a slight speciation change as salinity increased, with free Ni ion declining from 82% (20% SW) to 77% in 100% SW (Table 2.3), which may also have had a minor effect on the uptake of Ni. Consistent with the current finding, several studies have reported a protective effect of salinity against acute waterborne metal toxicity in marine invertebrates (Lee et al., 2010; Leonard et al., 2011; Martins et al., 2011a,b; Pedroso et al., 2007).

There was not a linear relationship between Ni accumulation and salinity, with no significant differences in whole body Ni accumulation between 60% SW and 100% SW observed (Fig. 2.1A). This was consistent with findings by Wright (1977a) in green crabs exposed to cadmium (Cd), where only at the low salinities was a significant increase in Cd accumulation

detected. This is likely due to the osmoregulatory strategies employed in dilute SW. *C. maenas* is considered to be a weak to moderate hyper-osmoregulator. Consistent with the finding of Siebers et al. (1982), haemolymph osmolality in the current study was always above SW osmolality (except in full SW where crabs were iso-osmotic), with the higher the salinity, the smaller the difference (Table 2.5). Many euryhaline hyper-osmoregulators have a critical salinity where transbranchial uptake of NaCl is activated. This critical salinity is often around 26 ppt (~74% SW) but can be higher or lower depending on the crustacean (Henry, 2005). Crabs in both 100% SW and 60% SW displayed similar total Ni accumulation patterns, possibly because similar osmoregulatory strategies were employed in both these salinities, in contrast to 20% SW where increasing active transbranchial regulation of ions seems to occur (Henry et al., 2012). Supporting this, metabolic rates of green and mud crabs increase at low exposure salinities indicating an increase in the workload associated with acquiring ions at these salinities to hyper-osmoregulate (Normant and Gibowicz, 2008; Wallace, 1972). Assuming that Ni is taken up through ion-specific pathways in the gill (see below), then the increase in active uptake of ions at the lowest tested salinity would likely increase the ability of Ni to gain access to the animal through "ionic mimicry" (see Introduction) and would result in the higher accumulation observed here. Thus, even though the drop in salinity between 100% and 60% SW is similar to that between 60% and 20% SW, the change in physiology that is induced at the lowest salinity likely drives the accumulation patterns observed.

In general, all tissues displayed a similar pattern of accumulation to that observed for total Ni accumulation (i.e. Ni accumulation inversely related to salinity). However among the gills, only gill 8 displayed a pattern of increasing Ni accumulation with decreasing salinity (Fig. 2.1B). Gill 8 is considered to be a "true" posterior gill. Posterior gills (6-9) are the primary sites of active NaCl uptake in euryhaline marine crabs (Mantel and Farmer, 1983; McNamara and Lima, 1997;

Onken and Riestenpatt, 1998; Pequeux, 1995). As these are the main sites for active ion movement, it is not surprising that the Ni accumulation was the highest in this posterior gill, given that the likely pathways of Ni uptake into the animal are *via* ion transport mimicry (see below).

The special role of gill 8 in terms of gill accumulation of Ni was also verified in isolated gill perfusion experiments (Fig. 2.3A), where Ni accumulation in gill 8 was salinity-dependent. However, there was no salinity-dependent effect of Ni movement into the perfusate (Fig. 2.3B). Nevertheless, the presence of Ni in the perfusate indicates that Ni is capable of being transported across the gills into the haemolymph within the 2 h exposure, although the bulk (> 90% of Ni) remains trapped in the gill. It may be that Ni transport into the haemolymph would be facilitated by the presence of potential binding ligands such as haemocyanin, the crustacean respiratory pigment, and thus the artificial perfusate used in the present study may underestimate gill-to-haemolymph transport. Alternatively, the time scale of the perfusion may have been too short to see significant movement into the haemolymph. Evidence from the tissue accumulation (Fig. 2.2) suggests Ni is effectively transported to other tissues *via* the haemolymph, at least over 24 h.

2.5.2 Effect on Ni on Ca homeostasis

The tissue that accumulated the most Ni was the carapace (Fig. 2.1D). The carapace consists of several layers (the epicuticle, the endocuticle and the membranous layer; Luquet and Marin, 2004; Roer, 1980). Ca transport into the cuticle layers occurs internally, from the haemolymph, not externally, from the sea water. In the lobster this involves active transport via a transcellular pathway using Ca pumps (e.g. Ca^{2+} ATPase), the Na^{+}/Ca^{2+} exchanger and other common ion pumps such as H^{+}-ATPase and Na^{+}/K^{+}ATPase (Ahearn and Zhuang, 1996; Ahearn et al., 1999; Roer, 1980) however not all of these have been characterized in crabs. As the medium becomes more dilute, it becomes more difficult for the animal to attain sufficient Ca for

calcification, likely causing a compensatory increase in the activities and/or numbers of Ca transporters contributing towards haemolymph-to-carapace Ca transport. This, however, may also aid in Ni uptake into the carapace. Given that Ni can interact with Ca transport pathways it is possible that the changes in the accumulation of Ni in the carapace may be a consequence of altered Ca homeostasis. Such a pathway may exist as a mechanism for Ni elimination because during a moult crustaceans replace the old carapace thus potentially eliminating metal burden in this tissue (Martins et al., 2011a; Pourang, 2004; Steenkamp et al., 1994). Altered Ca homeostasis was also observed in *C. maenas* exposed to Cd at several different salinities (Wright, 1977b).

Crab carapace has been shown to be effective at binding waterborne Ni, reflected in the use of the exoskeleton for metal bioremediation (Pradhan et al., 2005). Recently, through comparisons of live *versus* recently euthanized green crabs, it was found that approximately 50% of the Ni accumulation into the carapace from waterborne Ni exposure was due to an "adsorptive-type" process at the surface of the carapace, while the remaining 50% of accumulation was due to active processes (Chapter 3, Blewett and Wood, 2015b). In this study the live and dead crabs were subjected to rinses with high non-radioactive metal concentrations (shown to effectively desorb radiolabelled metals; Martins et al., 2011a), and with EDTA (shown to desorb Ni from crab carapace; Vijayaraghavan et al., 2005), prior to accumulation measurements. Nevertheless 50% of the accumulation remained in dead crabs without a functional circulatory system.

Ni has been implicated in alterations of Ca homeostasis in other aquatic organisms (e.g. Deleebeeck et al., 2007; Eisler, 1998; Pane et al., 2006), and is considered to be an effective blocker of several different types of Ca channels in vertebrate preparations (Funakoshi et al., 1997; Lee et al., 1999; McFarlane and Gilly, 1998; Todorovic and Lingle, 1998). Indeed, both Mg

and Ca have been shown to protect against acute waterborne Ni toxicity to *Daphnia pulex* likely by competing with Ni at transport sites (Kozlova et al., 2009). Interactions with Ca and Mg have also been observed in the stomach and mid-intestine of ranbow trout with regards to non-competitive inhibition with Ni (Leonard et al., 2009). A decrease in Ca haemolymph concentration was observed in every Ni concentration tested in the current study, lending support to antagonism between Ca and Ni (Fig. 2.6B).

2.5.3 Gill-dependent salinity effects on Ni accumulation and Na^+/K^+ATPase activity

Ni had a marked effect on gill Na^+/K^+ATPase activity that was both gill and salinity dependent. The 20% SW group displayed a marked decrease in Na^+/K^+ATPase activity from controls at two Ni concentrations (500 and 3000 μg/L)(Fig. 2.5A). There are documented inhibitory effects of other metals on Na^+/K^+ATPase in marine crustaceans (Canli and Stagg, 1996; Martins et al., 2011b; Postel et al., 1998), however Ni is not normally considered to be an inhibitor of Na^+/K^+ATPase activity. Ni is, however, a strong Mg antagonist (see above). Na^+/K^+ATPase has Mg binding sites to which Ni may also bind, resulting in blockade of activity. This has been proposed as the mechanism for Cu impairment of Na^+/K^+ATPase in rabbit kidney (Li et al., 1996).

This effect on Na^+/K^+ATPase may also affect Ca homeostasis. Indeed, when Na^+/K^+ATPase is inhibited there is a direct decrease of Ca uptake into the hypodermis tissue in *Carcinus maenas* (Roer, 1980), an effect that could alter Ni metabolism, given the interactions between Ca and Ni (see above).

The effects of Ni on Na^+/K^+ATPase activity were not observed in gill 5 (Fig. 2.5A,B,C). It has been noted that there are two types of epithelia in the gills of crabs, a thin respiratory epithelium and an ion transporting epithelium characterized by thick cells. As mentioned above,

Na^+/K^+ATPase activity is higher in posterior gills, and the transporter has been directly linked to basolateral infoldings of the thick cell epithelia (Towle and Kays, 1986). In the anterior gills the thick cell types (termed chloride or mitochondria-rich cells in fish and crustaceans; Zadunaisky, 1984) are noticeably absent or severely reduced. Green crabs in low salinity appear to exhibit a small increase in these types of cells on the anterior gills, however they still only occupy about 30% of the total lamellar area (Compère et al., 1989; Péqueux et al., 1989). Posterior gills, however, are fully covered in chloride cells. This suggests that gill 8 is more susceptible to the impacts of Ni owing to the greater number of chloride cells, and again supports the idea that ionoregulatory impairment is a significant mode of Ni toxicity in *C. maenas*.

2.5.4 Concentration- and salinity–dependent effects of Ni on haemolymph ions

There were significant effects of Ni on Na and Ca concentrations in the haemolymph in the 20% SW treatment only (Fig. 2.6). This was not surprising as previous evidence supports Ni as an ionoregulatory toxicant in acute studies (Pane et al., 2003; Deleebeeck et al., 2007; Eisler, 1998; Pane et al., 2006). The interactions between divalent cations and Ni have been discussed above, but there is also evidence from the literature for an impact of Ni on Na homeostasis. For example, Leonard and Wood (2013) reported changes in Na in Ni-exposed *Lymnaea stagnalis*, while a study in the marine crustacean *Litopenaeus vannamei* also showed an impact on Na, but only in 14% SW (Leonard et al., 2011). It is likely that the impairment of Na haemolymph levels stems from the effect of Ni on Na^+/K^+ATPase. Situated on the basolateral surface of ion transporting cells, Na^+/K^+ATPase is directly responsible for pumping Na into the haemolymph, and at the same time also generates electrochemical gradients that help to power the transport of Na across the apical cell surface and the movements of other ions across the cell (for review see Henry et al., 2012). An impairment of Na^+/K^+ATPase activity may therefore be anticipated to

impact haemolymph Na, as observed in the current study. The effect of Ni on Na^{+}/K^{+}ATPase may also contribute to the alterations in Ca haemolymph levels, owing to this disruption in electrochemical gradient.

2.5.5 Conclusion

The results of the current study have shown that Ni accumulation is strongly salinity-dependent, with a lower salinity (20% SW) resulting in higher accumulation than at 60% or 100% SW. This was likely the effect of competition between divalent cations and Ni for uptake. Consistent with this mode of uptake, the mechanisms of Ni toxicity to *Carcinus maenas* appear to be ionoregulatory in nature and are also salinity-dependent, with greater impacts at lower salinities. Branchial handling and impacts of Ni are also gill-dependent, with ionoregulatory gills more susceptible than respiratory gills. Sub-lethal Ni toxicity was observed in a Ni concentration of 8.2 µg/L, which is of environmental and regulatory significance. As Ni concentrations increased, physiological disturbances were also amplified. However, given that estuaries are systems where the highest Ni levels are likely to occur (Eisler, 1998) and that these are also the environments where salinities will be lowest (and thus susceptibility the greatest), the results of the current study indicate that these are also the habitats where aquatic biota will be most at risk from Ni.

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2.6 TABLES AND FIGURES

Table 2.1: Dissolved Ni exposure concentrations (µg/L) in both 24-h concentration series and 24-h 8.2 µg/L exposure experiments in 20, 60 and 100% SW (N=21).

Salinity	Control	8.2 µg/L (24h single exposure)	8.2 (µg/L)	500 (µg/L)	3000 (µg/L)
20%	3.0 ± 0.9	9.5 ± 0.6	8.6 ± 0.7	493 ± 21	3182 ± 226
60%	2.9 ± 0.7	9.0 ± 0.9	8.7 ± 1.0	495 ± 29	2900 ± 232
100%	2.5 ± 0.6	8.1 ± 1.3	8.8 ± 0.7	498 ± 26	2804 ± 286

Table 2.2: Water chemistry for experimental salinities. Reported values represent means ± SEM, (N = 21). Reported values sharing lowercase letters are not significantly different between salinities ($P \leq 0.05$).

Parameter	20% Seawater	60% Seawater	100% Seawater
pH	7.64 ± 0.30^{a}	7.88 ± 0.50^{a}	8.12 ± 0.70^{b}
Temperature (ºC)	13	13	13
DOC (mg/L)	2.5 ± 0.7	2.3 ± 0.8	2.9 ± 0.6
Na (mmol/L)	92.9 ± 1.4^{a}	332.4 ± 10.4^{b}	475.4 ± 10.4^{c}
Mg^{+} (mmol/L)	7.1 ± 0.2^{a}	27.4 ± 0.7^{b}	46.8 ± 0.3^{c}
K (mmol/L)	1.9 ± 0.1^{a}	6.0 ± 0.4^{b}	10.9 ± 0.2^{c}
Ca (mmol/L)	1.6 ± 0.1^{a}	4.9 ± 0.2^{b}	9.7 ± 0.1^{c}
Cl (mmol/L)	94.5 ± 3.6^{a}	279.2 ± 23.1^{b}	514.7 ± 1.9^{c}
Osmolality (mmol/kg)	586.0 ± 18.2^{a}	740.0 ± 13.2^{b}	923.0 ± 9.3^{c}

Table 2.3: Ni speciation (% of total Ni) as calculated by Visual MINTEQ based on measured and nominal water chemistry. Numbers are based on a Ni concentration of 8.2μg/L.

Species of Ni	20% SW	60%SW	100%SW
Ni^{2+}	81.57	78.79	76.86
Ni-DOC	5.41	3.33	4.87
$NiOH^{+}$	0.13	0.4	0.38
$Ni(OH)_2$ (aq)	0.001	0.03	0.03
$NiCl^{+}$	0.98	0.05	0.04
$NiSO_4$ (aq)	5.34	6.46	7.31
$NiCO_3$ (aq)	4.47	4.49	3.47
$NiCO_3^{+}$	2.1	6.46	7.04

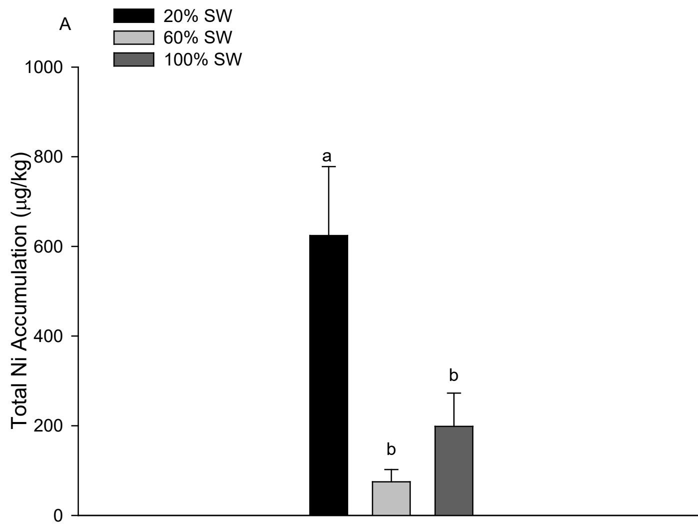
Speciation analysis was also run at a Ni concentration of 3000 μg/L, differences were only detected in Ni^{2+} and SO_4 where species were only slightly higher, and $NiCO_3^{+}$ species were lower.

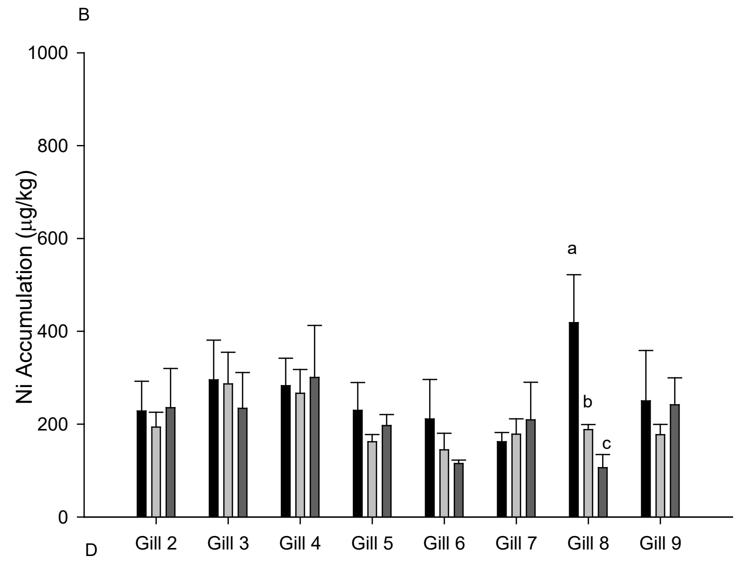
Table 2.4: Relative proportion of the tissue compartments (means ± SEM, N=7) within the whole body of the green shore crab (*Carcinus maenas*) where "gill" represents all gill pairs in control crabs.

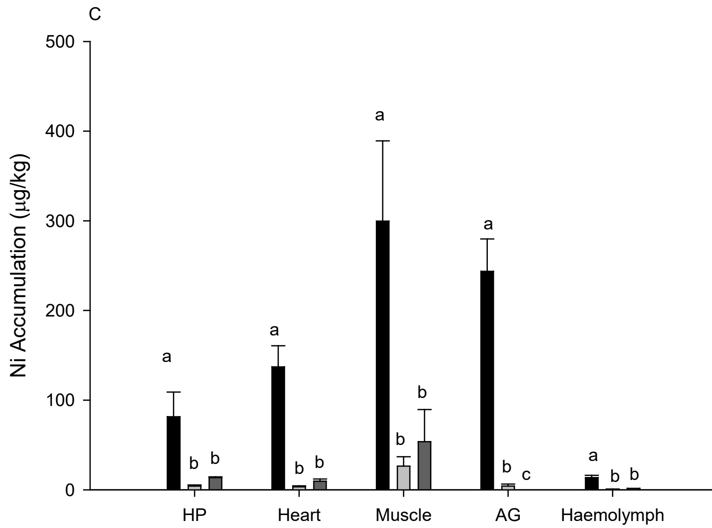
Tissue	Relative proportion of crab (%)
Carapace	77.08 ± 0.61
Gill	1.65 ± 0.25
Hepatopancreas	4.20 ± 0.69
Heart	0.19 ± 0.01
Muscle	9.50 ± 0.07
Antennal Gland	0.01 ± 0.01
Haemolymph	7.37 ± 0.18

Table 2.5: Ion (mmol/L) and osmolality (mosmol/kg) concentrations in haemolymph of green shore crabs (*Carcinus maenas*) exposed to four different concentrations of Ni (controls (0), 8.2, 500, 3000 µg/L) at one of three salinities (20%, 60. 100% SW) for 24 h. Reported values sharing letters within a measure (i.e. ion or osmolality) are not significantly different. Reported values represent means ± SEM, N=7.

	Potassium (K; mmol/L)			Magnesium (Mg; mmol/L)			Chloride (Cl; mmol/L)			Osmolality (mosmol/kg)		
Salinity	20%	60%	100%	20%	60%	100%	20%	60%	100%	20%	60%	100%
Control	6.6 ± 0.5[a]	8.0 ± 0.3[b]	10.4 ± 0.4[c]	4.9 ± 0.3[a]	8.6 ± 0.3[b]	13.7±0.5[c]	278 ± 8[a]	375 ± 6[b]	480 ± 1[c]	586 ± 18[a]	740 ± 13[b]	923 ± 9[c]
8.2 µg/L	6.1 ± 0.2[a]	8.7 ± 0.3[b]	10.4 ± 0.7[c]	5.2 ± 0.3[a]	9.0 ± 0.5[b]	12.6 ± 0.4[c]	287 ± 10[a]	379 ± 4[b]	470 ± 13[c]	618 ± 9[a]	769 ± 22[b]	922 ± 6[c]
500 µg/L	6.2 ± 0.4[a]	9.0 ± 0.5[b]	10.3 ± 0.2[c]	4.8 ± 0.1[a]	9.1 ± 0.4[b]	13.4 ± 0.4[c]	300 ± 6[a]	396 ± 15[b]	478 ± 17[c]	613 ±13[a]	760 ± 28[b]	922 ± 14[c]
3000 µg/L	5.9 ± 0.4[a]	9.0 ± 0.3[b]	10.4 ± 0.1[c]	4.2 ± 0.2[a]	8.0 ± 0.3[b]	15.1 ± 1.0[c]	275 ± 9[a]	383 ± 16[b]	455 ± 15[c]	557 ± 17[a]	776 ± 16[b]	900 ± 9[c]


A
20% SW
60% SW
100% SW
Total Ni Accumulation (µg/kg)
1000
800
600
400
200
0
a
b
b


B
Ni Accumulation (µg/kg)
1000
800
600
400
200
0
a
b
c
Gill 2
Gill 3
Gill 4
Gill 5
Gill 6
Gill 7
Gill 8
Gill 9


C
Ni Accumulation (µg/kg)
500
400
300
200
100
0
HP
Heart
Muscle
AG
Haemolymph

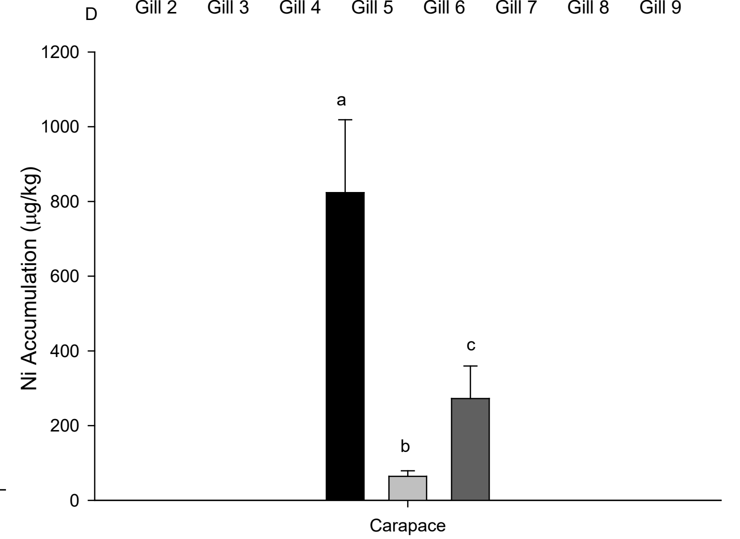
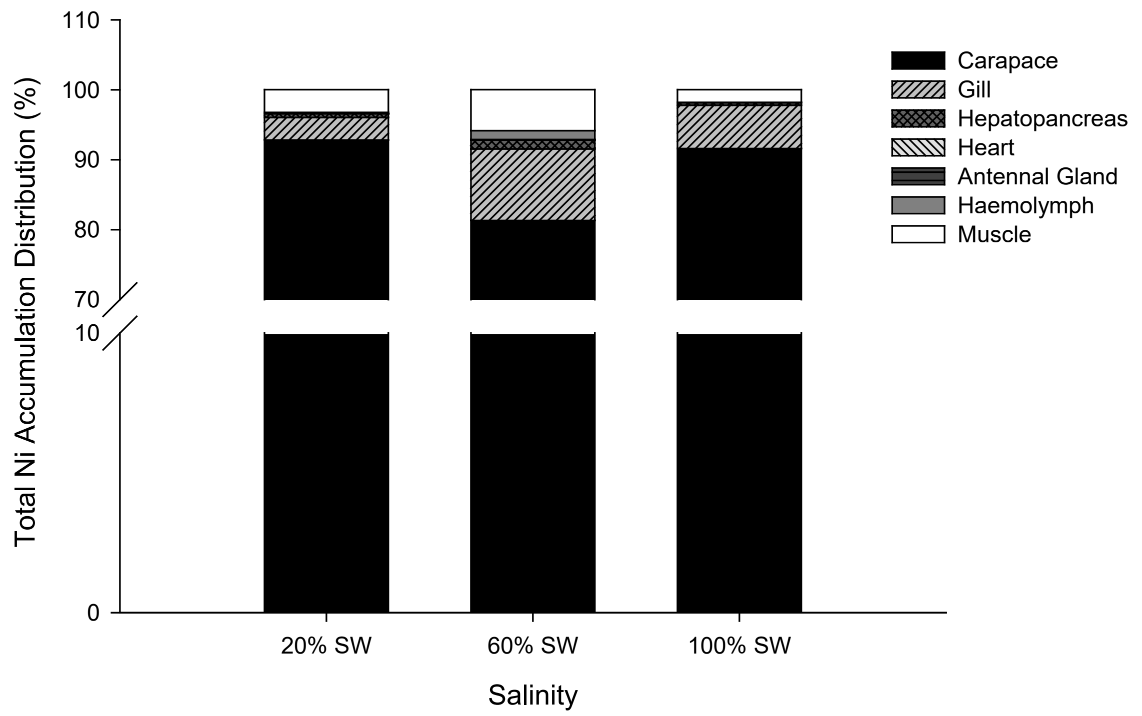

D
Ni Accumulation (µg/kg)
1200
1000
800
600
400
200
0
a
b
c
Carapace

Figure 2.1. Ni accumulation in tissues of Ni-exposed crabs (*Carcinus maenas*) after a 24-h exposure at a Ni concentration of 8.2 µg/L in 20% SW, 60% SW and 100% SW. A) Total Ni accumulation in the whole body, B) Ni accumulation in individual gills, C) Ni accumulation in hepatopancreas (HP), heart, muscle, antennal gland (AG), and haemolymph, and D) Ni accumulation in carapace. Lower case letters indicate significant differences ($P \leq 0.05$) in Ni accumulation among salinity groups. Means sharing the same letter are not significantly different. Values are means ± SEM (N = 7 per treatment).



Total Ni Accumulation Distribution (%)
110
100
90
80
70
10
0
20% SW
60% SW
100% SW
Salinity
Carapace
Gill
Hepatopancreas
Heart
Antennal Gland
Haemolymph
Muscle

Figure 2.2. Percent distribution of whole body Ni accumulation in the various tissues of the green shore crab (*Carcinus maenas*) exposed to 8.2 µg/L Ni in 20% SW, 60% SW or 100% SW for 24 h. Note that the Y-axis scale starts at 70%. Values are means (N = 7 per treatment).

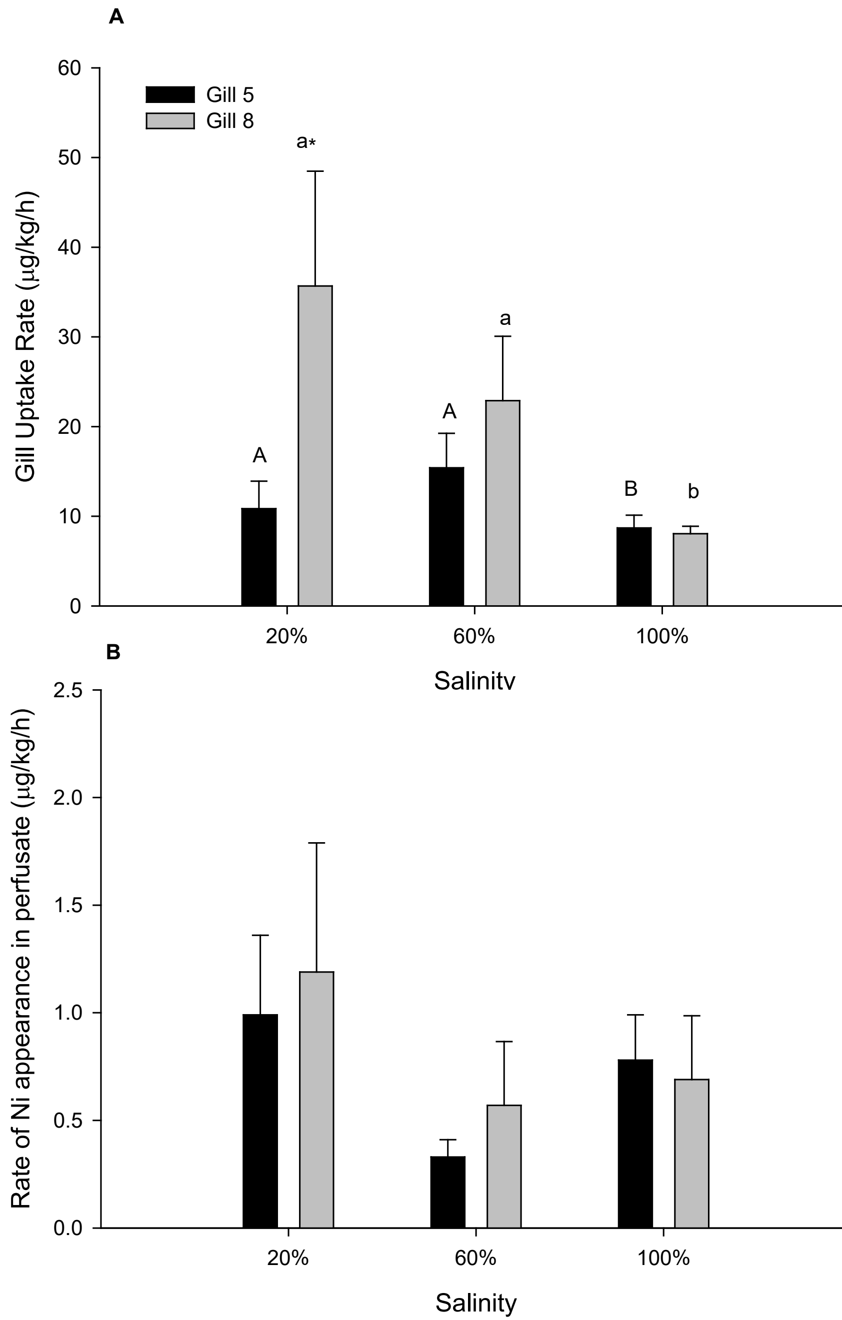

A
Gill 5
Gill 8
Gill Uptake Rate (µg/kg/h)
a*
a
A
A
B
b
20%
60%
100%
Salinity
B
Rate of Ni appearance in perfusate (µg/kg/h)
20%
60%
100%
Salinity

Figure 2.3 A) The rate of accumulation of Ni in isolated-perfused gills 5 and 8 of the green shore crab (*Carcinus maenas*). B) The rate of accumulation of Ni in the perfusate in these same isolated-perfused gill experiments. Asterisks indicate significant differences ($P \leq 0.05$) between the two gills within a salinity. Upper case letters denote significant differences in gill 5 between salinities, while lower case letters denote significant differences in gill 8 between salinities. Plotted values are means ± SEM, where N=7.

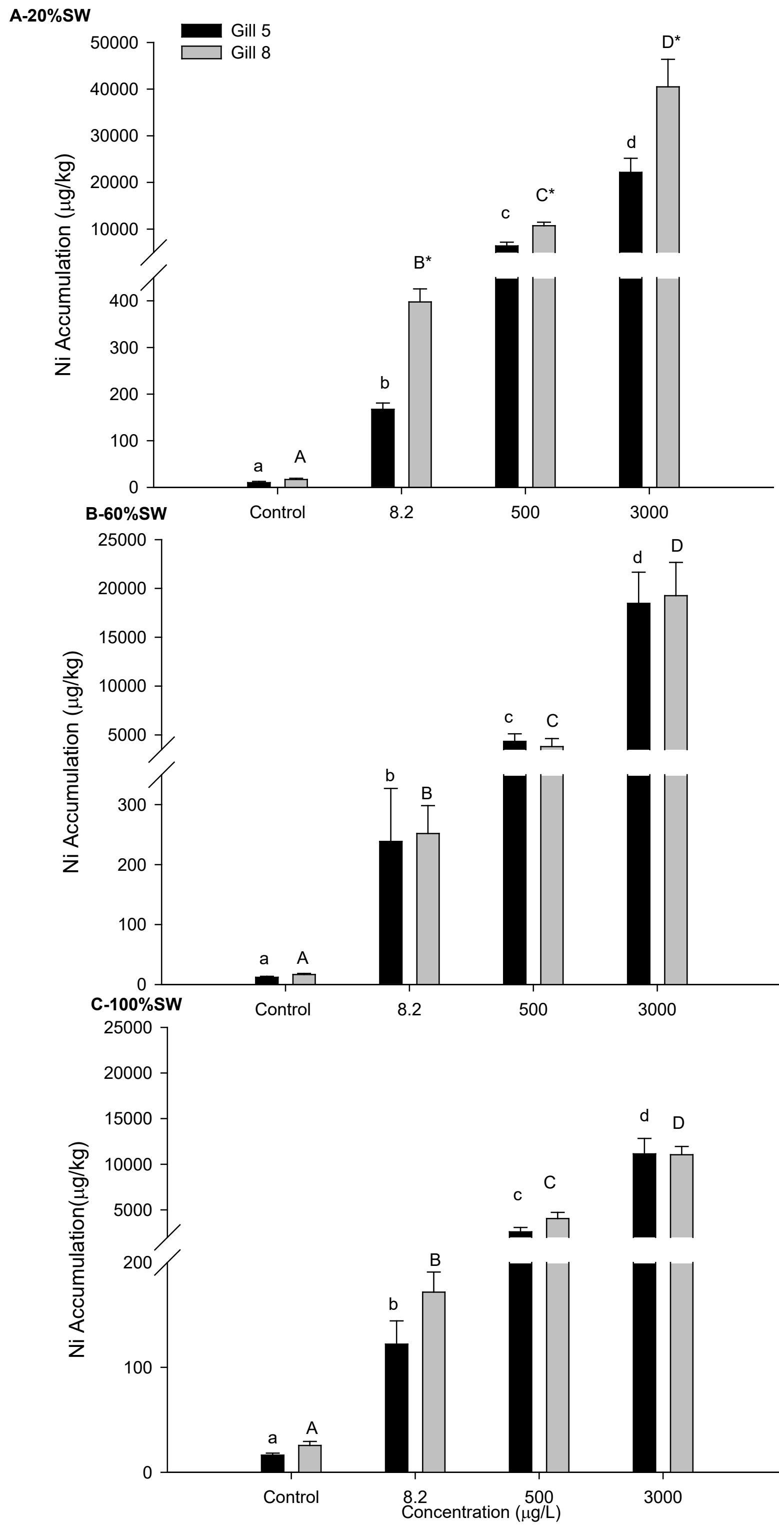

A-20%SW
Gill 5
Gill 8
Ni Accumulation (μg/kg)
50000
40000
30000
20000
10000
400
300
200
100
0
a
A
b
B*
c
C*
d
D*
Control
8.2
500
3000
B-60%SW
25000
20000
15000
10000
5000
300
200
100
0
a
A
b
B
c
C
d
D
C-100%SW
25000
20000
15000
10000
5000
200
100
0
a
A
b
B
c
C
d
D
Concentration (μg/L)

Figure 2.4. Ni accumulation in gills 5 and 8 in the green shore crab (*Carcinus maenas*) exposed to four different concentrations of Ni (control (0), 8.2, 500, 3000 µg/L) at one of three salinities: A) 20% SW, B) 60% SW and C) 100% SW, for 24 h. Upper case letters denote significant differences ($P \leq 0.05$) in Ni accumulation in gill 8 among different salinities and lower case letters denote significant differences in Ni accumulation in gill 5 among different salinities. Asterisks denote significant differences between gill 8 and gill 5 within a concentration. Plotted values are means ± SEM (N = 7 per treatment).

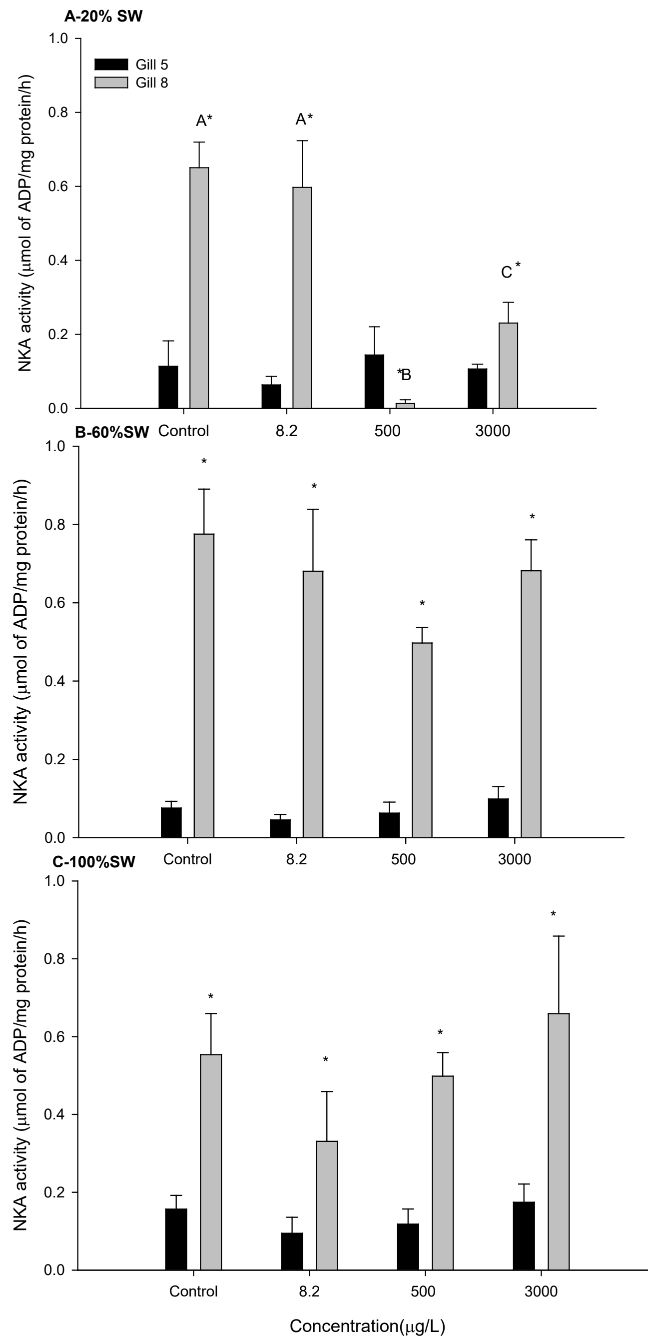

A-20% SW
Gill 5
Gill 8
NKA activity (µmol of ADP/mg protein/h)
A*
A*
C*
*B
Control
8.2
500
3000
B-60%SW
C-100%SW
Concentration(µg/L)

Figure 2.5. Na^+/K^+ ATPase activity in gills 5 and 8 in green crabs (*Carcinus maenas*) exposed to 4 different concentrations of Ni (control (0), 8.2, 500 and 3000 µg/L) at one of three salinities: A) 20% SW, B) 60% SW, C) 100% SW for 24 h. Upper case letters denote a significant differences ($P \leq 0.05$) in gill 8 among different salinities. There were no significant differences in gill 5 among different salinities. Asterisks denote a significant difference between gill types within a concentration. Plotted values are means ± SEM (N = 7 per treatment).

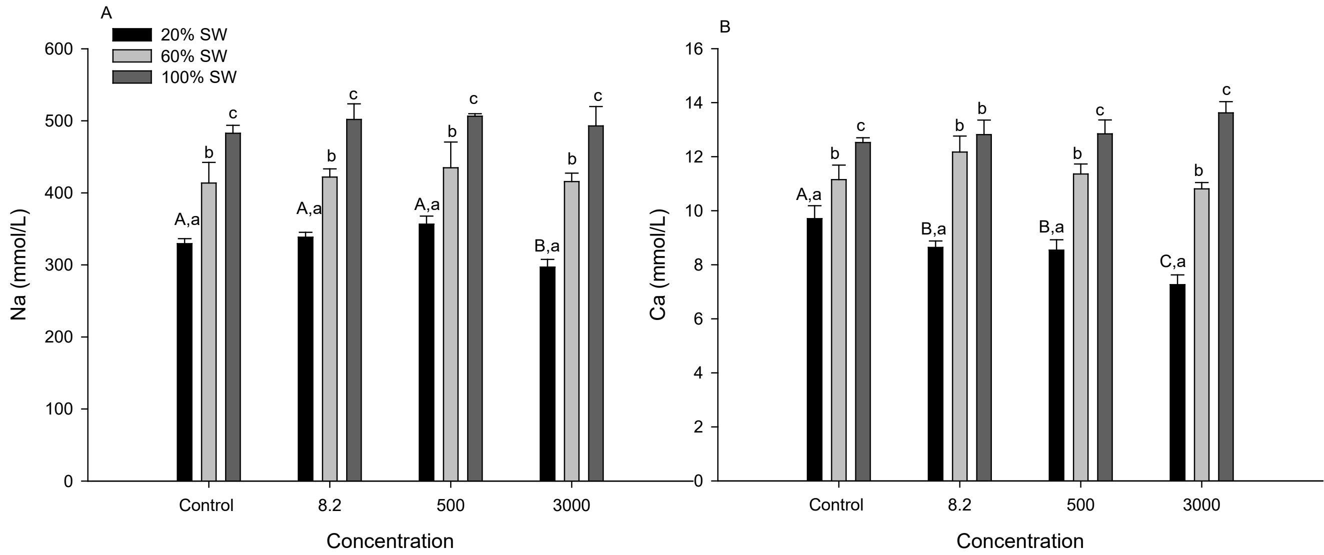

A
20% SW
60% SW
100% SW
Na (mmol/L)
Concentration
Control
8.2
500
3000
B
Ca (mmol/L)
Concentration
Control
8.2
500
3000

Figure 2.6. Haemolymph ion composition (A: Na, B: Ca; mmol/L) of the green crab (*Carcinus maenas*) exposed to four different Ni concentrations (control (0), 8.2, 500, and 3000 µg/L) at three different salinities: 20%, 60%, 100% SW, for 24 h. Upper case letters denote a significant difference ($P \leq 0.05$) across Ni concentrations. Lower case letters denote changes within a salinity. Values are means ± SEM (N = 7 per treatment).

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CHAPTER 3

LOW SALINITY ENHANCES NICKEL-MEDIATED OXIDATIVE STRESS AND SUB-LETHAL TOXICITY TO THE GREEN SHORE CRAB *Carcinus maenas*

3.1 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 Ni toxicity is influenced by salinity. In the current study the salinity-dependence and mechanisms of sub-lethal Ni toxicity in a euryhaline crab (*Carcinus maenas*) were investigated. 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 hours of Ni exposure. Total Ni accumulation was strongly dependent 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.

3.2 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 recognized by the development of predictive modelling approaches, such as the Biotic Ligand Model (BLM; Paquin et al., 2002). Salinity is one key factor that impacts metal bioavailability, by altering metal speciation and the concentration of potentially protective natural cations, and the physiology of the organism. 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., 2003 a,b; 2004a,b; 2005; 2006 a,b), and has advanced significantly in the last few years for marine biota (Chapter 5, Blewett and Wood, 2015a; Chapter 2, Blewett et al., 2015a; 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 suggested (Pane 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 homeostasis were also reported in a snail, *Lymnaea stagnalis*, and an oligochaete, *Lumbriculus variegatus* (Leonard et al., 2013). In the marine environment, Leonard et al. (2011) observed a disruption in both Na and Mg homeostasis in a shrimp, *Litopenaeus vannamei*, following Ni exposure at two different salinities. 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-hour Ni exposure (Chapter 2, Blewett et al., 2015a).

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., 2003a,b; 2004b). Previous evidence has shown impacts on respiratory performance in fish exposed to Ni, including gill ultrastructral damage, with fused lamellae and extensive swelling (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). One way that Ni may induce oxidative damage is to displace Fe from its cofactor binding sites on cellular proteins. This in turn will then cause an increased flux of Ni into the Fenton reaction, ultimately resulting in hydroxyl radicals (Stohs and Bagchi, 1995). The second process by which Ni induces oxidative stress may be by affecting antioxidant mechanisms, thereby decreasing a cell's ability to scaenge 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., 2012 a,b, 2013, 2014). Ni has also been shown to affect antioxidant enzymes in both freshwater- and seawater-acclimated killifish, *Fundulus heteroclitus* (Chapter 5, Blewett and Wood, 2015a) while other metals (Cu, Zn) induce oxidative stress in freshwater and estuarine fish (Craig et al., 2007; Loro et al., 2012). Reduced salinity has been shown to exacerbate oxidative stress in metal-exposed killifish (Chapter 2, Blewett and Wood, 2015a; Loro et al., 2012). However, very little 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 crab species, previously shown to be sensitive to environmentally-relevant levels of Ni (Chapter 2, Blewett et al., 2015a) and is a model species in ecotoxicology (Leignel et al., 2014). It was 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, it was also predicted that 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* (Chapter 2, Blewett et al., 2015a). 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).

3.3 METHODS

3.3.1 Animal collection and maintenance

Male green crabs (*Carcinus maenas*) (Linnaeus, 1758) of mean mass 55.3 ± 7.8 g, and mean carapace width 5.04 ± 1.30 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 acclimated 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). Bamfield SW was diluted with nanopure water to create the lower two salinities. Ten crabs were also acclimated at 80% and 40 % SW to determine the osmoregulatory pattern in this species (see Section 3.1). 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.

3.3.2 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 anaesthetized.

A small pre-exposure (following the procedures described above) was run to determine Ni absorption characteristics in both live and dead crabs. 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. Dead crabs had been previously been euthanized by a ventral ganglion puncture (Blewett et al., 2015a). 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, $NiCl_2 \cdot 6\,H_2O$) and subsequently a 1 mM EDTA solution, to remove any loosely-bound radioisotope, before being placed on ice for anaesthesia (in the case of the live crabs). Carapace was taken for tissue Ni measurements, as 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 the 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 meter. The electrode was maintained and calibrated at the experimental temperature (18°C). These crabs were then rinsed and placed on ice, as described above for 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 were placed on ice and 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 3.3.3 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.

3.3.3 Tissue analyses

Thawed tissues were weighed and placed in 50-mL, 15-mL, or 2-mL plastic centrifuge tubes, as needed, 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).

3.3.4 Water and haemolymph ion analyses

For 24-h exposures, water Ni levels were monitored 3 times (t = 0, t = 12 and t = 24 h; N = 21). For 96-h exposures water was taken at the start, before and after a water change, and at the conclusion of 96 hours (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 3.1. 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) (Table 3.2). Water and haemolymph Cl^- was measured using a LabConco digital chloridometer (B5953; Kansas City, MO) (Table 3.2). 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 meter (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 3.2.

3.3.5 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 homogenized at a ratio of 20:1 (volume:weight) (Power Gen 125 homogenization 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. Louis, MO, USA) with the incorporation of a 1% streptomycin sulfate solution added to supernatants at 10 µL per 100 µL of homogenization buffer. Streptomycin sulfate 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, gills 5 and 8 were homogenized (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.

3.3.6 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 Chapter 2, Blewett et al., 2015a), 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 GFAAS) and tissue weight (W). Data are expressed as μg/kg:

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

3.3.7 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 Tables 1 and 2 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) (Table 3.3).

3.4 RESULTS

3.4.1 Haemolymph osmolality

In unexposed green crabs, the osmolality of the haemolymph decreased as external salinity decreased (Fig. 3.1). This decrease in the haemolymph was not, however, proportional to the decrease in SW, and at all salinities haemolymph was maintained at a higher osmolality than the corresponding medium, except at 100% SW where haemolymph and water osmolality were equal. Thus the crab osmoconformed in 100% SW, but exhibited hyperosmotic regulation at all lower salinities. The deviation above the isosmotic line increased at lower salinities, indicating that the degree of active osmoregulation (and ionoregulation) was greater at lower salinities. This was little affected by Ni exposure (see Sections 3.4.5 and 3.4.9).

3.4.2 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 3.1). 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 (Table 3.3).

3.4.3 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. 3.2A).

There was a significant effect of gill number with respect to Ni accumulation ($P < 0.001$), but the effect of salinity and interaction was not significant ($P = 0.07$; $P = 0.579$). Ni concentrations in the gills (Fig. 3.2B) were generally greater than in the whole body (Fig. 3.2A) at all salinities. Accumulation of Ni was significantly greater in gills 2 and 3 than it was in gills 6, 7 and 8 (Fig. 3.2B). 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 except for 20% SW, but the difference was not significant (data not shown, but apparent from Fig. 3.2B).

Ni accumulation in the carapace was also generally greater than in the whole body, but followed a pattern with salinity similar to that of total accumulation. The 20% SW group accumulated the highest amount of Ni, a value significantly higher than that at 100% SW (Fig. 3.2C).

Heart and antennal gland displayed opposite accumulation patterns (Fig. 3.2D) from the carapace (Fig. 3.2C) and whole body (Fig. 3.2A), with the highest accumulation of Ni in the

100% SW group. Antennal glands displayed the highest levels of Ni accumulation among soft tissues (Fig. 3.2D), matched only by the gills (Fig. 3.2B). Muscle Ni accumulation was significantly greater (by 2-fold) in the 60% SW group than in the other two tested salinities (Fig. 3.2D). Relatively low levels of Ni accumulation in hepatopancreas and haemolymph were unaffected by salinity (Fig. 3.2D).

Ni accumulation in the carapace of both live and dead crabs was investigated. Live crabs accumulated significantly (2-fold) more Ni than recently deceased crabs in the same exposure medium (Fig. 3.3).

3.4.4 Tissue-specific Ni partitioning after 24-h exposure

At all three salinities, the highest proportion of Ni accumulation occurred in the carapace where over 80% of the Ni was partitioned (Fig. 3.4). Muscle was the next highest contributor to total Ni accumulation. The proportion of Ni accumulated in the muscles of 20% SW crabs was significantly lower than that of the other two salinities. Gills were the next highest proportional accumulators of Ni, followed by the hepatopancreas and haemolymph. The antennal gland and heart contributed less than 1% of the total Ni in all salinities (Fig. 3.4).

3.4.5 Haemolymph ions after 24-h exposure

As salinity increased, so did all haemolymph ion concentrations (Table 3.4). 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 $P < 0.001$), 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 3.4).

3.4.6 Salinity-dependent Ni accumulation over 96 h

Ni accumulation over 96 h was greater than the accumulation after 24 h in all tissues (Fig. 3.5 *versus* Fig. 3.2). Whole body Ni accumulation was the highest in the 20% SW group at 33, 901 ± 3751 µg/kg, almost 3-fold greater than the accumulation in the 60% SW and 100% SW crabs (Fig. 3.5A). With respect to gill accumulation over 96 h, a two way ANOVA revealed that there was a significant effect 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 Ni (Fig. 3.5B).

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, but apparent from Fig 3.5B). 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. 3.5C). Salinity also 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. 3.5C). In the remaining tissues there were significant salinity-dependent differences only in the hepatopancreas and haemolymph, where the 20% SW group accumulated more Ni relative to other salinities (Fig 3.5D).

3.4.7 Tissue-specific Ni partitioning after 96-h exposure

In the 20% SW exposure group, the carapace contribution at 96 h was 87% (Fig. 3.6) , somewhat less than the 95% seen after 24 h (Fig. 3.4), and the muscle contribution was 8% (Fig. 3.6), substantially higher than the 2% seen after 24 h (Fig. 3.4). Ni accumulation also increased in the gill between 24 h and 96 h, rising from 1% to 3% (Figs.3.4, 3.6).

In 60% SW, after 96 h the carapace was still the highest contributor to proportional Ni accumulation (81%), with gills accounting for 5%, haemolymph 2%, and muscle 9%. This was again different from the pattern displayed after 24 h, where the carapace displayed 90% of the total Ni accumulation, the muscle accounted for 7%, and the gills accounted for less than 3% of total Ni accumulation (Figs. 3.4, 3.6).

The 100% SW groups displayed the least amount of change in Ni accumulation pattern between exposure times (96 h *versus* 24 h). Overall, after 96 h the carapace accounted for 87% of the total Ni accumulation while the gills and muscle only accounted for 4% and 9% respectively (Fig. 3.6). At 24 h the carapace accumulated 89%, a mere 2% more than after 96 hours, and the muscle Ni concentration remained unchanged from the 96 -h exposure (Figs. 3.4, 3.6).

3.4.8 Haemolymph ions after 96-h exposure

A similar salinity-dependent trend in ion concentrations was observed after a 96-h Ni exposure as after the 24-h exposure. A two 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 3.5).

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 differentfrom it's control only at 20% SW (Fig. 3.7A). Overall, both Mg (P = 0.020) and osmolality (P = 0.034) decreased with exposure to Ni at 20% SW but not at 60% SW or 100% SW (Fig 3.7). Specifically, post-hoc tests showed that in 20% SW Mg and K concentrations decreased significantly with respect to control haemolymph values (Fig. 3.7B,C). Haemolymph Ca, Mg, K and osmolality all differed significantly with respect to salinity- as salinity increased so did haemolymph values (P <0.001, P <0.001, P = 0.001, P <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).

3.4.9 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. 3.8). 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. 3.8).

3.4.10 Indicators of oxidative stress after 96-h exposure

Both gill 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)(Fig. 3.9A). 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. 3.9A) (P = 0.05). There were no significant effects in gill 5 (salinity, P = 0.606; Ni exposure, P = 0.191; interaction P = 0.818) (Fig. 3.9C). 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)(Fig. 3.9 B,D). 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). 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 either of 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. 3.9 B,D).

3.5 DISCUSSION

In this study it has been shown that salinity-dependent variations occur in Ni accumulation, as well as in all three modes of sub-lethal Ni toxicity investigated: ionoregulatory disturbance; respiratory interference; and oxidative stress. In all cases, the effects were 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 recognized modes of Ni toxicity in the same species. Finally, it was shown that Ni accumulation and sub-lethal toxicity depended on time of exposure, and key differences in sub-lethal toxic responses were illustrated in comparison to previous studies on this species exposed to lower Ni levels (Chapter 2, Blewett et al., 2015a).

3.5.1 Tissue- and salinity-specific Ni accumulation after 24-h exposure

The ionoregulatory physiology of *Carcinus maenas* changes with salinity (Fig. 3.1). *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 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. 3.2A,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-dependent uptake of ions increases across the gills. Ni is thought to be an ion mimic (see Introduction), 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. 3.2A). 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 iso-osmotic point (Chapter 2,Blewett et al., 2015a; Hall and Anderson, 1995; Leonard et al., 2011; Martins et al., 2011; Wright, 1977). It is also consistent with the recent finding of greater Ni accumulation in isolated-perfused ionoregulatory gills (gill 8) of this species at 20% SW *versus* 100% SW (Chapter 2, Blewett et al., 2015a).

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+}; Table 3.3), 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 after two hours, Ni is transported into the haemolymph (Chapter 2, Blewett et al., 2015a), suggesting the ability of crabs to rapidly transport Ni to the carapace for 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, and 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 3.2D; Fig.3.5D).

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 Ca carbonate and smaller stores of Ca phosphates and Mg carbonate, where this large Ca demand is serviced from Ca^{2+} ions that are taken up from the haemolymph (Al-Sawalmih et al., 2008; Fabritius et al., 2012; Roer and Dillman, 1984). At least in the 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 aid in Ni transport into the carapace, as Ni^{2+} can potentially mimic Ca^{2+} in several pathways (Deleebeeck et al., 2009, Eisler, 1998; Funakoshi et al., 1997; Pane et al., 2006 a,b). This pattern of high Ni accumulation in the carapace with low salinity in *C. maenas* has been observed previously (Chapter 2, Blewett et al., 2015a), where it was proposed as a potential mechanism for Ni elimination during moulting. If this hypothesis is correct, then *Carcinus* may be extremely sensitive to Ni following a moult, due to the fact that during this period a 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. (2015a, Chapter 2) 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. (2015a, Chapter 2). 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.

3.5.2 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 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 (Chapter 2, Blewett et al., 2015a), 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 was likely due to absorption to the carapace, while the other 50% of accumulation was due to active uptake processes (Fig. 3.3).

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. 3.5B). 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 homeostasis, 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 minimize 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 Chapter 2; Blewett et al., 2015a). Induction of metallothionein is not instantaneous, thus under the current studies 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. 3.2A). 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. 3.5B) 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 (Péqueux 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.

3.5.3 Ionoregulatory disruption after 96-h exposure

Ionoregulation disruption in response to Ni exposure has been described in several invertebrate species (see Introduction). In this study, exposure of crabs to Ni in 20% SW resulted in changes in haemolymph Ca, Mg and K concentrations (Fig. 3.7 A,B,C).

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. (2015a; Chapter 2), 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. There is, however, an alternative explanation. Ni has been shown to induce hypoxia inducible factor (HIF), thus mimicking hypoxia (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 (Fig. 3.8). 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 homeostasis 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. 3.7B). Disturbances in Mg homeostasis 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 homeostasis in the euryhaline crustacean *Litopenaeus vannamei* (Leonard et al., 2011), in a freshwater snail, *Lymnaea stagnalis*, and a freshwater oligochaete, *Lumbriculus variegatus* (Leonard and Wood, 2013) 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 (Chapter 2, Blewett et al., 2015a), suggesting this to be a conserved mechanism of Ni effect in marine invertebrates.

3.5.4 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. 3.8). 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 lamella. 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.

3.5.5 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; Kubrak et al., 2013; Kubrak et al., 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. These results indicate two major trends 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 3.9 A, 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.

CAT is a critical enzyme that decomposes hydrogen peroxide into water and oxygen, a critical part of the detoxification cascade responsible for neutralizing reactive 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 histidine residues (e.g. Predki et al., 1992), which play an important role in CAT catalytic activity (Mate et al., 1999). Decreases in CAT activity were also observed in the euryhaline killifish (Chapter 5, Blewett and Wood, 2015a), 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 (Chapter 2, Blewett and Wood, 2015a). 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 with Fe and move through the Fenton/Haber Weiss reactions (with Ni^{2+} /Ni^{3+} rather than Fe^{2+}/Fe^{3+} redox coupling), resulting in increased ROS production (Torreilles and Guerin, 1990). Secondly, Ni can affect antioxidant responses yielding a lower defense 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 (Chapter 2, Blewett and Wood, 2015a; Kubrak et al., 2012a,b; Kubrak et al., 2013; Kubrak et al., 2014).

3.5.6 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 were: ionoregulatory impairment, respiratory toxicity, and oxidative stress. Furthermore, these effects were strongly time- and salinity-dependent, with greater toxicity noted following longer exposures and in low salinities, which seemed to favour Ni accumulation.

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3.6 TABLES AND FIGURES

Table 3.1. Dissolved Ni exposure concentrations (µg/L) in experimental water for both the 24 hour and the 96 hour exposures to 3 mg/L Ni in 20, 60 and 100% SW (N = 70, 96 hour and N = 21 for 24 hour exposures).

Salinity	Control (96- h exposure) in µg/L	3 mg/L (24-h exposure) in µg/L	3 mg/L (96-h exposure) in µg/L
20%	2.9 ± 0.7	3093 ± 327	2878 ± 204
60%	1.7 ± 0.1	3262 ± 113	3020 ± 125
100%	3.9 ± 0.8	3136 ± 253	3221 ± 162

Table 3.2. Water chemistry for exposures (N = 70, 96 hour and N = 21 for 24 hour exposures). Letters denote significant differences between salinities. Values represent means ± 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 (ºC)	18	18	18
DOC (mg/L)	2.1 ± 0.2^{a}	2.2 ± 0.2^{a}	2.7 ± 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 ± 0.0^{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}

Table 3.3. Ni speciation (% of total Ni) as calculated by Visual MINTEQ based on recorded and nominal water chemistry. Values are based on a Ni concentration of 3000 µg/L at 96 hours.

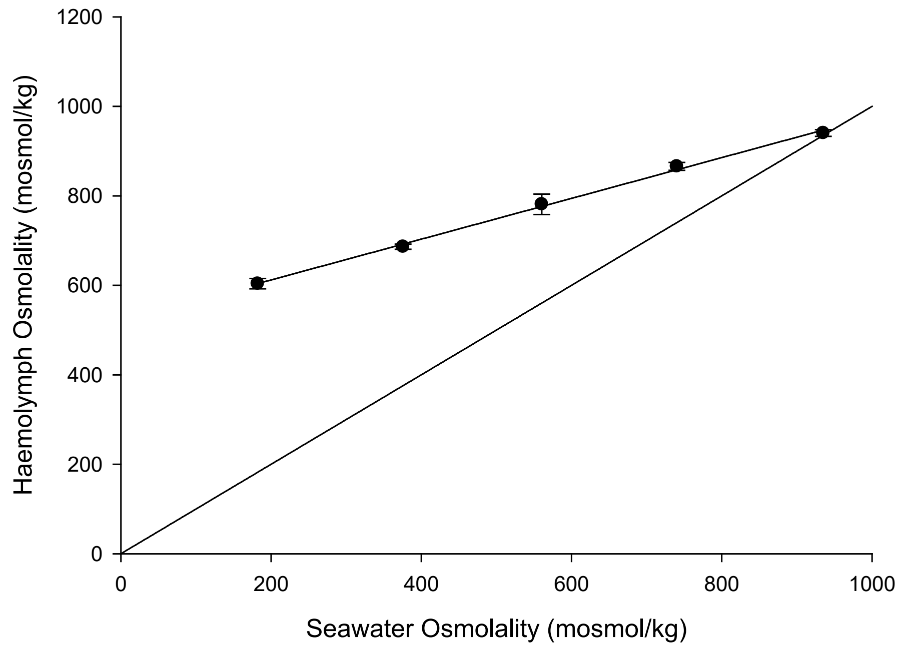
Ni Speciation	20% SW	60%SW	100%SW
Ni^{2+}	82.78	79.90	77.32
Ni-DOC	3.56	4.11	5.03
$NiOH^{+}$	0.13	0.19	0.19
$Ni(OH)_2$ (aq)	0.001	0.04	0.03
$NiCl^{+}$	1.03	0.91	0.60
$NiCl_2$ (aq)	0.88	0.03	0.04
$NiSO_4$ (aq)	5.09	6.93	7.50
$NiCO_3$ (aq)	2.10	3.93	3.83
$NiCO_3^{+}$	4.43	3.96	5.49

Table 3.4. Haemolymph ions (mmol/L) in *C.maenas* under control conditions and after exposure to 3 mg/L Ni for 24 hours. Reported values are means ± SEM (N=7). Values sharing letters are not significantly different within an ion.

	Na^{+} (mmol/L)		Ca^{2+} (mmol/L)		K^{+} (mmol/L)		Mg^{2+} (mmol/L)		Cl^{-} (mmol/L)	
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]

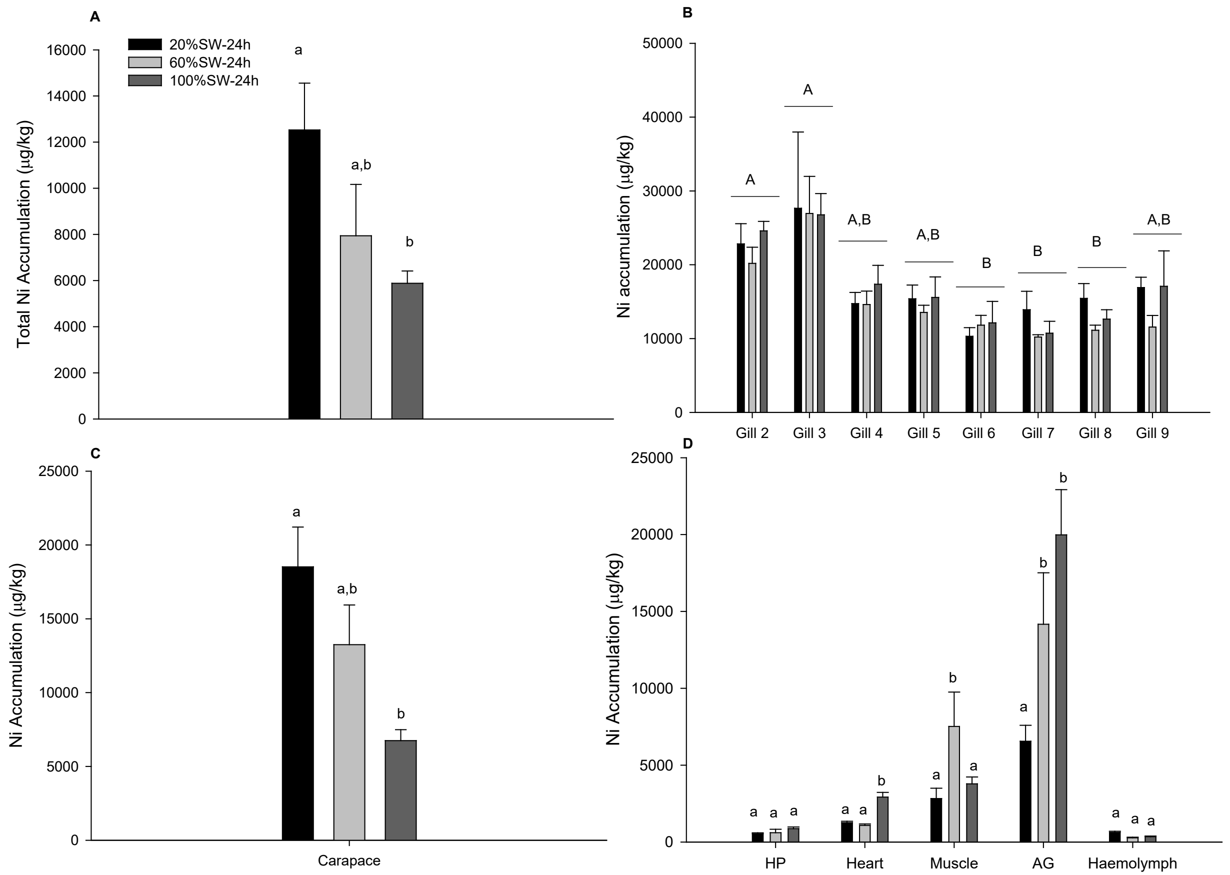
Table 3.5. Haemolymph ions (mmol/L) and osmolality (msomol/kg) in *C. maenas* under control conditions and after exposure to 3 mg/L Ni for 96 hours. Reported values are means ± SEM (N=7). Values sharing letters are not significantly different within a measurement (ion or osmolality).

	Na^+ (mmol/L)		Cl^- (mmol/L)		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	436.2 ± 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



1200
1000
800
600
400
200
0
Haemolymph Osmolality (mosmol/kg)
0
200
400
600
800
1000
Seawater Osmolality (mosmol/kg)

Figure 3.1. Haemolymph osmolality (mosmol/kg) of the green shore crab (*Carcinus maenas*) as a function of seawater osmolality (mosmol/kg). Reference line represents a hypothetical true osmoconformer (i.e. isosmotic line).



A
20%SW-24h
60%SW-24h
100%SW-24h
Total Ni Accumulation (µg/kg)
a
a,b
b
B
Ni accumulation (µg/kg)
A
A
A,B
A,B
B
B
B
A,B
Gill 2
Gill 3
Gill 4
Gill 5
Gill 6
Gill 7
Gill 8
Gill 9
C
Ni Accumulation (µg/kg)
Carapace
D
Ni Accumulation (µg/kg)
HP
Heart
Muscle
AG
Haemolymph

Figure 3.2. 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.

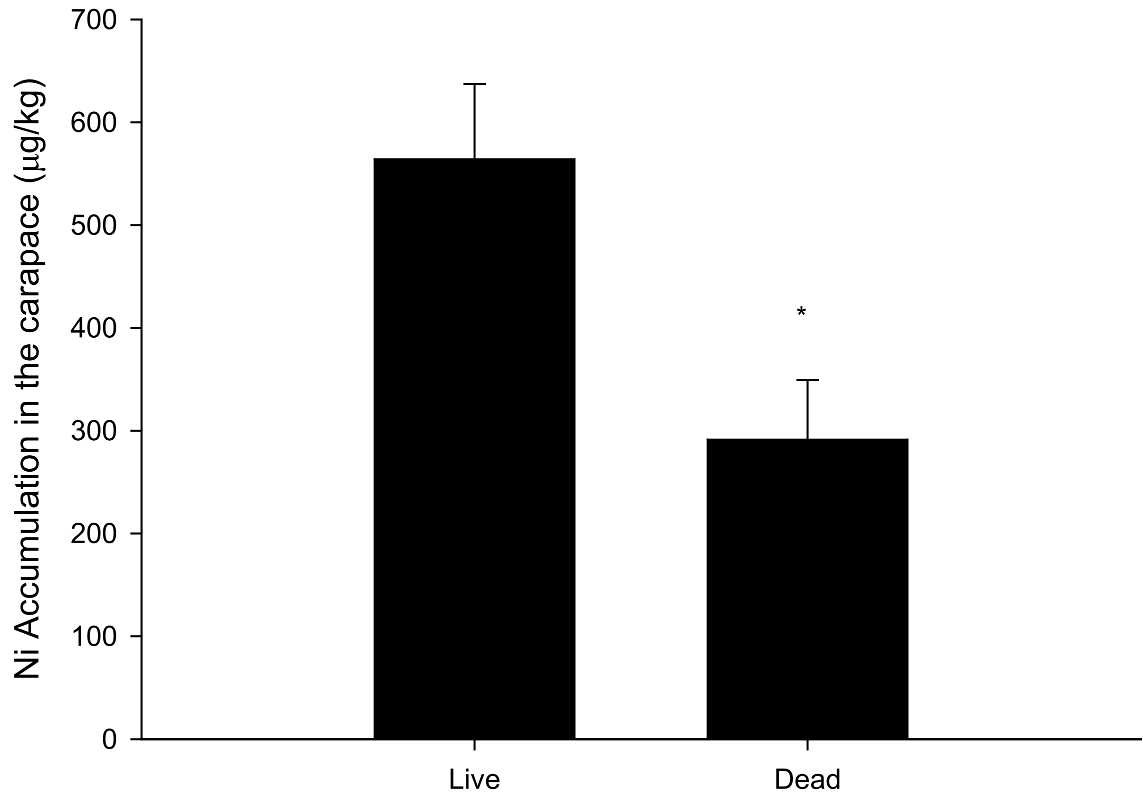
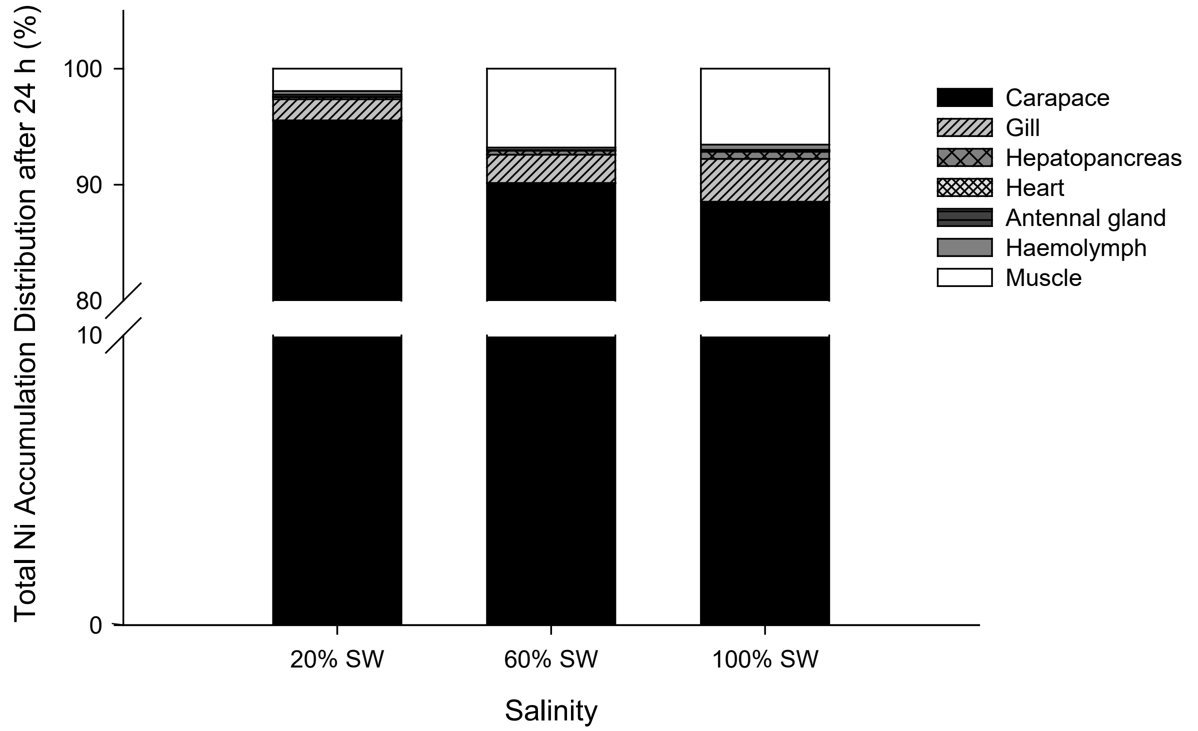

Ni Accumulation in the carapace (μg/kg)
700
600
500
400
300
200
100
0
*
Live
Dead

Figure 3.3. Ni accumulation (µg/kg) in live crabs and recently deceased crabs after a 3 hour exposure to 3 mg/L of Ni at a salinity of 100% SW. Values are means ± SEM (N = 4 per treatment). Asterisk denotes significant difference between live and dead crabs ($P < 0.05$).



Total Ni Accumulation Distribution after 24 h (%)
100
90
80
10
0
Carapace
Gill
Hepatopancreas
Heart
Antennal gland
Haemolymph
Muscle
20% SW
60% SW
100% SW
Salinity

Figure 3.4. Percent distribution of Ni accumulation in the various tissues of the green shore crab (*Carcinus maenas*) after an exposure to 3 mg/L for 24 h. Values are means ± SEM (N=7 per treatment).

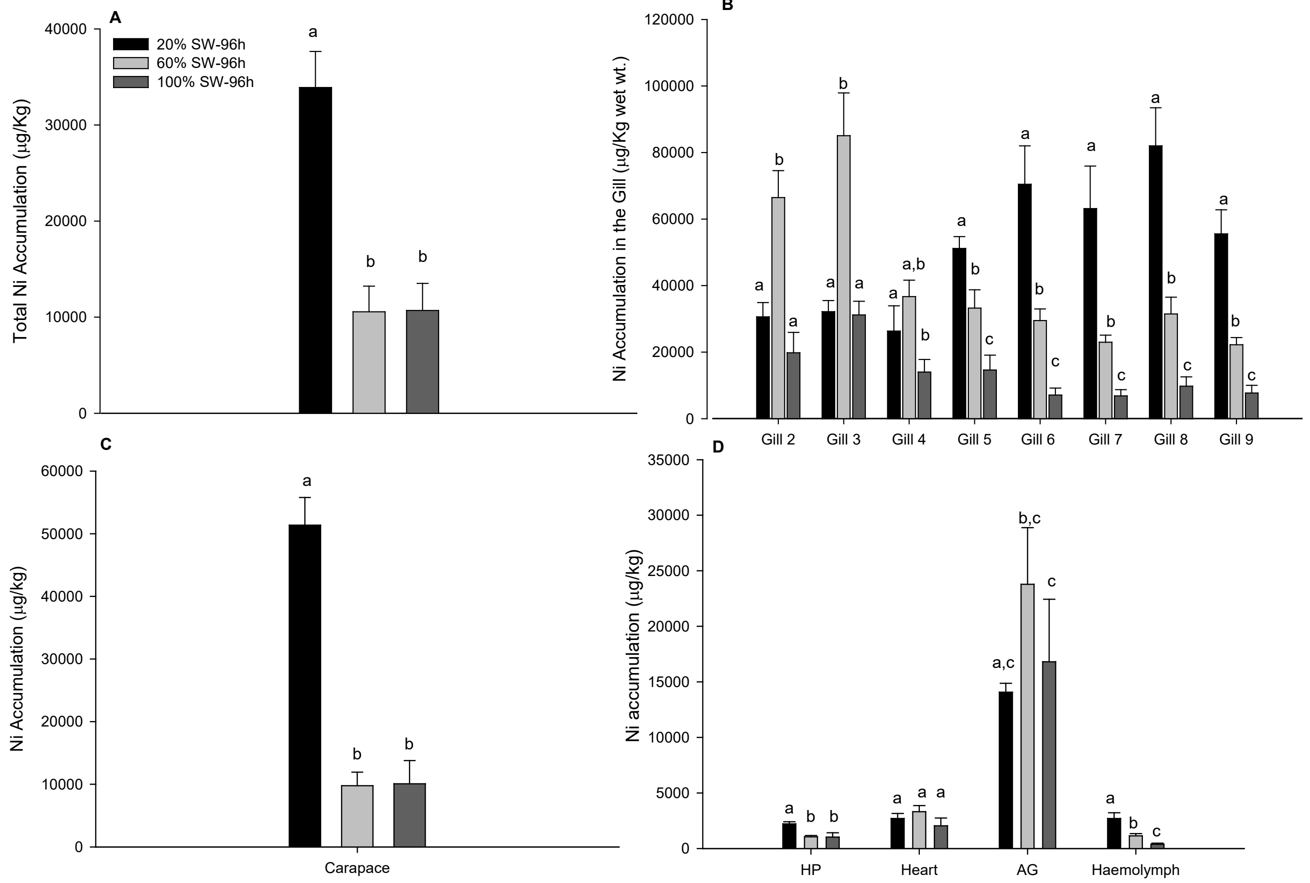

A
20% SW-96h
60% SW-96h
100% SW-96h
Total Ni Accumulation (µg/Kg)
B
Ni Accumulation in the Gill (µg/Kg wet wt.)
Gill 2
Gill 3
Gill 4
Gill 5
Gill 6
Gill 7
Gill 8
Gill 9
C
Ni Accumulation (µg/kg)
Carapace
D
Ni accumulation (µg/kg)
HP
Heart
AG
Haemolymph

Figure 3.5.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 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 in Ni concentrations between salinity groups ($P \leq 0.05$). Means sharing the same letter are not significantly different.

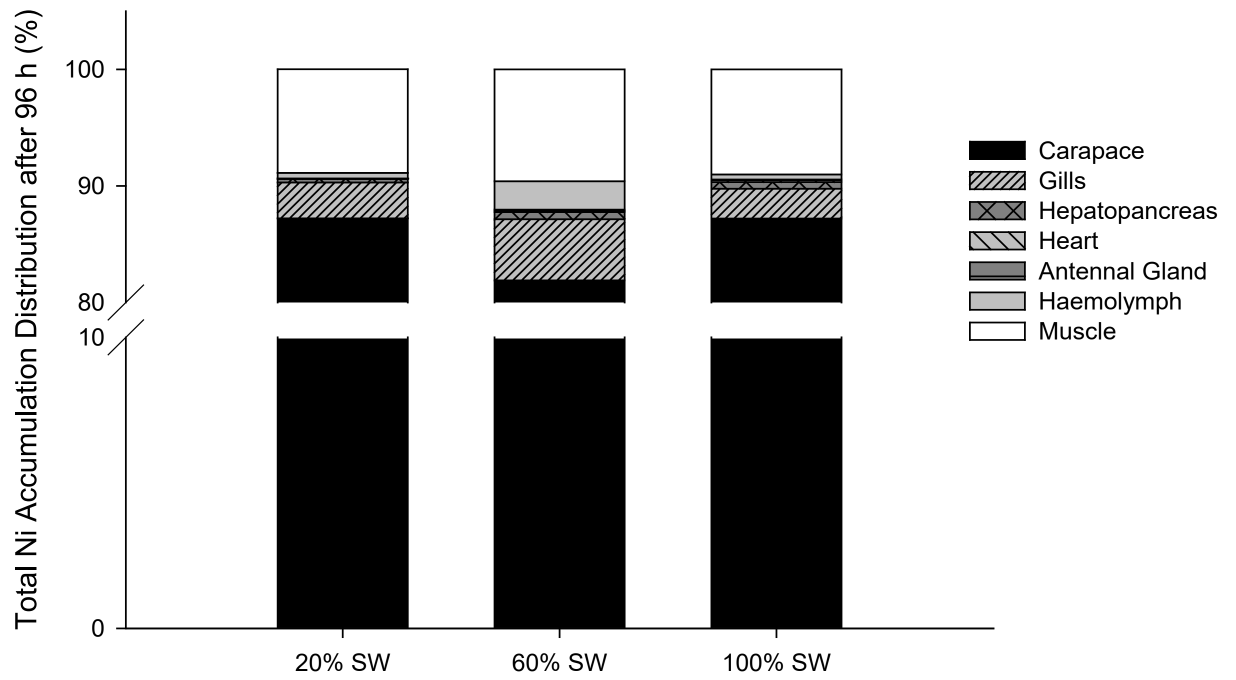

Total Ni Accumulation Distribution after 96 h (%)
100
90
80
10
0
20% SW
60% SW
100% SW
Carapace
Gills
Hepatopancreas
Heart
Antennal Gland
Haemolymph
Muscle

Figure 3.6. Percent distribution of Ni accumulation in the various tissues of the green shore crab (*Carcinus maenas*) after an exposure to 3 mg/L for 96 h. Values are means ± SEM (N=7 per treatment).

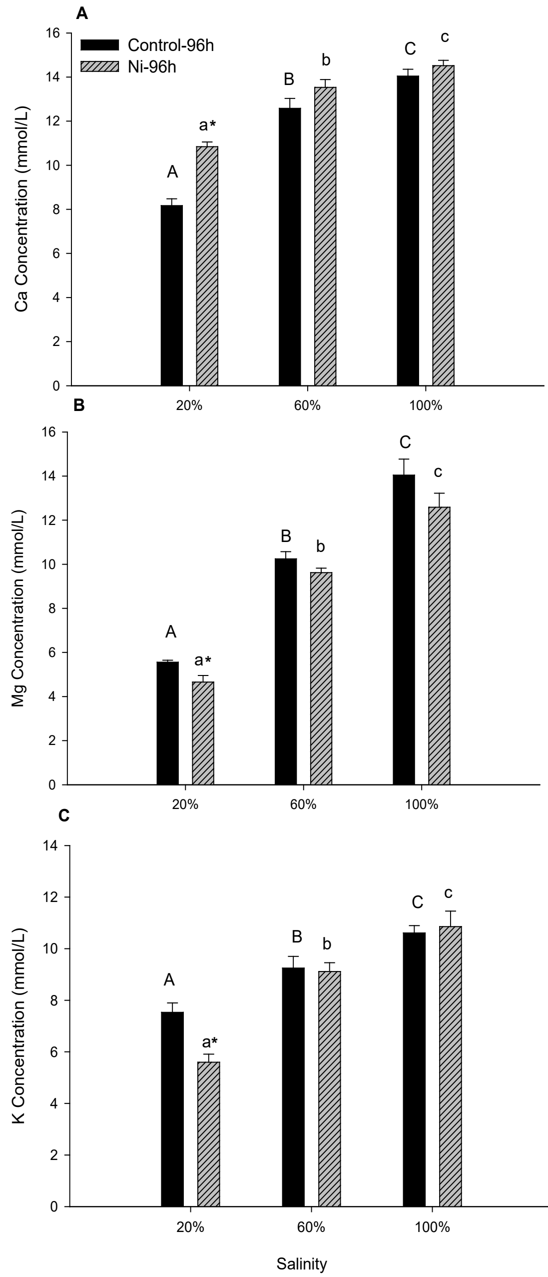

A
Control-96h
Ni-96h
Ca Concentration (mmol/L)
A
a*
B
b
C
c
20%
60%
100%
B
Mg Concentration (mmol/L)
A
a*
B
b
C
c
20%
60%
100%
C
K Concentration (mmol/L)
A
a*
B
b
C
c
20%
60%
100%
Salinity

Figure 3.7. Ionic composition (mmol/L) 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+}, C) K^{+}. All other ions and osmolality are displayed in Table 3.5. Values are means ± SEM (N = 7 per treatment). Asterisks indicate a significant difference from controls ($P \leq 0.05$). Upper case letters indicate significant differences in haemolymph values between control crabs, lower case letters indicate significant differences in haemolymph values between Ni-exposed crabs. Means sharing the same letter are not significantly different.

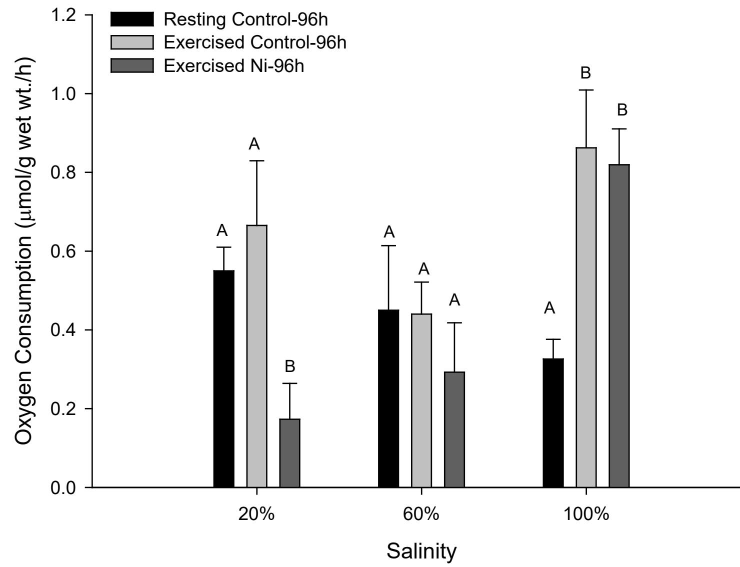

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Oxygen Consumption (µmol/g wet wt./h)
Resting Control-96h
Exercised Control-96h
Exercised Ni-96h
A
A
B
A
A
A
A
B
B
20%
60%
100%
Salinity

Figure 3.8. Oxygen consumption values (µmol/g wet wt./h) in rest and after exercise in crabs exposed to Ni for 96 hours. 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.

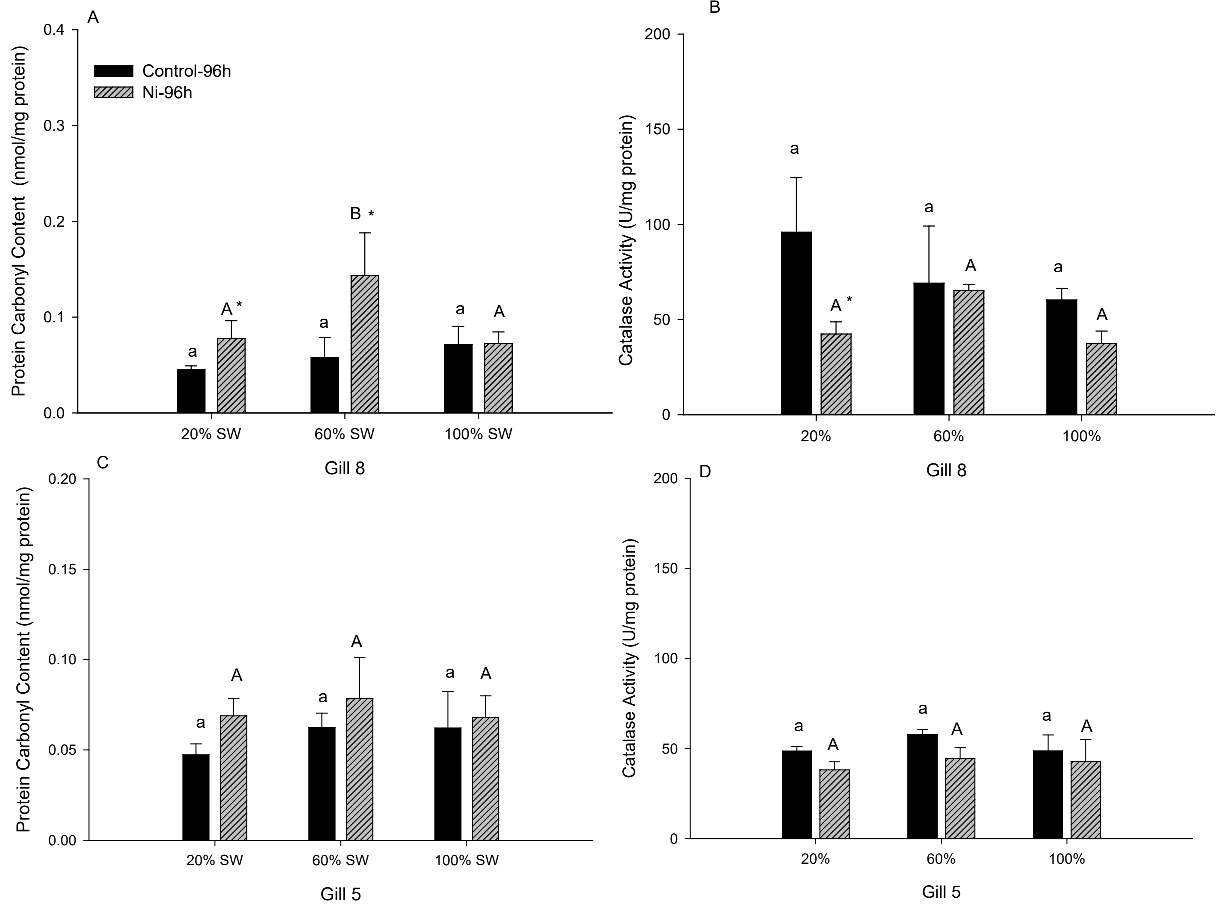

A
Control-96h
Ni-96h
Protein Carbonyl Content (nmol/mg protein)
0.0
0.1
0.2
0.3
0.4
a
A *
a
B *
a
A
20% SW
60% SW
100% SW
Gill 8
B
Catalase Activity (U/mg protein)
0
50
100
150
200
a
A *
a
A
a
A
20%
60%
100%
Gill 8
C
Protein Carbonyl Content (nmol/mg protein)
0.00
0.05
0.10
0.15
0.20
a
A
a
A
a
A
20% SW
60% SW
100% SW
Gill 5
D
Catalase Activity (U/mg protein)
0
50
100
150
200
a
A
a
A
a
A
20%
60%
100%
Gill 5

Figure 3.9. 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 within Ni treatments, while lower case letters denote significant differences within control treatments. Means sharing the same letter are not significantly different.

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CHAPTER 4

MECHANISMS OF NICKEL TOXICITY IN THE HIGHLY SENSITIVE EMBRYOS OF THE SEA URCHIN *Evechinus chloroticus* AND THE MODIFYING EFFECTS OF DISSOLVED ORGANIC CARBON

4.1 ABSTRACT

Early life stages of aquatic organisms are considered the most sensitive to metal toxicants and are therefore of significant relevance for environmental risk assessment. A 96-h toxicity test showed that the embryos of the New Zealand sea urchin are the most sensitive of all studied marine species to waterborne nickel (Ni), with a EC_{50} for the development of fully formed pluteus larvae at 14.1 µg/L. Failure to develop a standard larval shape suggested impairment of skeletal development. Whole body ions (Na, Mg) increased with Ni exposure and calcium influx was depressed. The effects of dissolved organic carbon (DOC) on Ni accumulation and toxicity were also examined in three different seawater sources (nearshore, offshore, and near the outlet of a "brown water" stream). At low DOC concentrations the brown water source was protective against Ni toxicity; however at higher DOC levels it exacerbated developmental toxicity in the presence of Ni. These results show that sea urchin development is highly sensitive to Ni *via* a mechanism that involves ionoregulatory disturbance, and that Ni toxicity is influenced by environmental factors such as DOC. These data will be critical for the development of water quality guidelines for Ni in the marine environment.

4.2 INTRODUCTION

Sea urchin embryos are considered to be excellent indicators of marine pollution (Hernández et al., 2010; Silva et al., 2013), based on their wide distribution in temperate, tropical and polar waters, their ecological roles (Uthicke et al., 2009), and, their high sensitivity to environmental contaminants. In fact, the early life stages of sea urchins appear to be among the most sensitive of all tested organisms to metal toxicity (DeForest and Schlekat, 2013). Further enhancing their role as an indicator species, studies have shown that metal accumulation in sea urchin embryos correlates to the toxicity (Nadella et al., 2013), a key tenet of environmental regulatory tools such as the biotic ligand model (BLM; Niyogi and Wood, 2004).

Metal contamination is of growing concern in aquatic settings owing to inputs from increasing industrial activity and fossil fuel production during the last century (Lander and Reuther, 2004). In a recent survey of coastal sediments of New Zealand's South Island, nickel (Ni) was the only metal that exceeded sediment quality guidelines (Chandurvelan et al., 2015), with Ni also identified as a metal of concern in other studies of marine sediments worldwide (e.g. Arienzo et al., 2014; Li et al., 2014). In seawater, Ni concentrations range from 0.2 to 100 μg/L, with the highest levels resulting from anthropogenic inputs (Boyden, 1975).

Ni has three main mechanisms of toxicity to aquatic biota: ionoregulatory disturbance (Chapter 2, Blewett et al., 2015a; Chapter 3, Blewett and Wood, 2015b; Leonard et al., 2011, 2013; Pane et al., 2003b, 2004a), respiratory impairment (Chapter 3, Blewett and Wood, 2015b; Pane et al., 2003a, 2004b), and oxidative damage (Chapter 3, Blewett et al., 2015b; Chapter 5, Blewett and Wood, 2015a; Kubrak et al., 2012). The dominant mechanism of toxicity differs depending on organism. For example, respiratory toxicity is generally considered the main mode

of action in vertebrates (Pane et al., 2003a, 2004b), while ionoregulatory toxicity is the most reported mechanism of toxicity in invertebrates (Leonard et al., 2011, 2013).

Water chemistry is an important factor influencing Ni toxicity. For example, dissolved organic carbon (DOC), a ubiquitous but variable component of natural waters, can detoxify metals such as Ni through its ability to bind them, thereby reducing their bioavailability to the organism (Doig and Liber, 2006). However, the composition and concentration of DOC will vary among water sources, and therefore, so too will its protective effects. For example, it has been shown that the more optically dark the DOC, a factor usually associated with increased phenolic rings of allochthonous origin (Pempkowiak et al., 1999), the more protective it is against metal toxicity (Wood et al., 2011). As many predictive modelling approaches (e.g. the BLM) assume that toxicity is primarily associated with the free metal ion (the bioavailable form) (Niyogi and Wood, 2004), an understanding of the role of DOC on Ni bioavailability and toxicity is of significant regulatory value.

Given their potential roles as indicators of metal pollution, a number of studies have investigated Ni toxicity to sea urchin early life stages. Reported EC_{50}'s (concentration required to generate a given adverse effect in 50% of the exposed population) vary from 15 (Bielmyer et al., 2005) to 341 (Phillips et al., 2003) µg/L. These lower values are well within the range of environmental Ni concentrations, and are therefore of significant environmental relevance. For example, regulatory values in Canada, the USA, and New Zealand fall close to or within this range (8.2 to 75 µg/L; ANZECC/ARMCANZ, 2000; CCME, 2007; USEPA, 2005). The cause of the variation between studies remains poorly understood, in part because the mechanisms of Ni toxicity in sea urchin embryos remain relatively unexplored. A recent study by Tellis et al. (2014) found Ni toxicity in sea urchin embryos was related to perturbation of calcium (Ca) homeostasis,

albeit at Ni exposures significantly below previously reported EC_{50} values for this species (*Strongylocentrotus purpuratus*; Phillips et al., 2003).

The current study aimed to determine the sensitivity of early life-stages of the New Zealand sea urchin *Evechinus chloroticus* to waterborne Ni. *Evechinus* is endemic to New Zealand, where it is of significant cultural and socioeconomic value (Barker, 2007). However, very little is known regarding the sensitivity of this species to metal toxicants. Following assessment of lethal Ni exposure levels, the potential role of ionoregulatory disturbance in generating embryo-larval Ni toxicity was investigated at Ni levels where abnormal development occurred, and finally the effects of DOC on Ni toxicity were examined.

4.3 MATERIALS AND METHODS

4.3.1 Animal Care

Adult sea urchins (*E. chloroticus*) were collected from Lyttleton Harbour, Canterbury, New Zealand and transported (~1 h) in aerated seawater from the site of collection to the aquarium facility at the University of Canterbury. They were held in a recirculating seawater (~32 ppt) system that was constantly aerated and maintained at 15°C. All subsequent experimental procedures were performed in a temperature-controlled room set to 15°C.

4.3.2 Collection and fertilization of gametes

To induce spawning, 10 mL of 0.5 M KCl was injected into the haemocoel of the adult sea urchins (Hinegardner, 1975). Sex was determined by visual inspection of the gametes. Sperm was collected from three males (pooled for each set of experiments) and stored on ice (4°C) for up to an hour until eggs were collected. Spawning females (N = 3) were inverted over a 100-mL beaker filled with filtered seawater. The aboral surface was in contact with the seawater, allowing

spawned eggs to fall to the bottom of the beaker for collection. Eggs were then removed from this beaker and added to 50 mL of seawater in plastic flasks until a density of 100 eggs/mL was obtained. Approximately 200 µL of sperm was added to each egg-containing flask and stirred gently to facilitate fertilization, which was normally achieved within 0.5 h. Fertilization success was confirmed under a microscope by the elevation of a fertilization envelope around each egg. Once at least 80% fertilization was achieved, eggs were gently agitated to ensure homogeneity of the eggs in suspension and egg density was determined by counting a 1-mL sample in a Sedgewick-Rafter cell. Aliquots of eggs were then added to 500-mL acid-washed glass beakers containing fresh seawater with an appropriate water chemistry (i.e. Ni and/or DOC; see below) to achieve a final density of 20 fertilized eggs per mL. Full strength (100%) seawater was used in all exposures (~32 ppt), which were run at 15ºC, under a 14:10 hour light:dark cycle, with dawn and dusk transitions.

4.3.3 EC_{50} determination

Fertilized eggs (1 hour post-fertilization) were added to 500-mL solutions containing Ni (from a stock solution of $NiCl_2 \cdot 6H_2O$; Sigma Aldrich). Each Ni concentration (nominally: 0 (no added Ni), 15, 30, 60, 120, 240, 480 and 960 µg/L; N = 3) was prepared 24 h in advance of the start of the experiment to allow equilibration. Measured (see below) Ni concentrations are displayed in Table 4.1. Embryo development was scored at 48 and 96 h after embryos were added to the exposure medium. Abnormal development was defined as an embryo that did not display typical gastrula (48 h) or pluteus (96 h) form, with reference to control embryos as a guide to the appropriate developmental stage. At the end of 96 h, a 15 mL aliquot of water, containing ~ 300 embryos, was removed and development was terminated by the addition of 1% neutral buffered formalin for later microscopic observation. At 96 h, remaining embryos were taken for metal

analysis by filtering solutions through a pre-weighed 8 μm polycarbonate filter (Whatman Nucleopore Track-Etch Membrane PC MB). These isolated embryos were analysed for ions and total metal burden. Water samples were taken at the start (time 0) and the end of the exposure (time 96). 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 4.1 and Table 4.2.

4.3.4 DOC collection, preparation and analysis

All EC_{50} exposures were conducted in natural seawater collected from Lyttelton Harbour (GPS co-ordinates: -43.604343, 172.714833; hereafter referred to as University of Canterbury DOC or UC DOC). To determine the effect of DOC composition and concentration, a further two DOC sources were collected. Offshore DOC (OS DOC) was collected approximately two kilometers offshore from Banks Peninsula in the Pacific Ocean, in line with the entrance to Lyttelton Harbour (-43.588257, 172.835396). West Coast DOC (WC DOC) was collected from Kumara on the West Coast region of the South Island, where a brown water stream, draining native beech forest, entered the Tasman Sea (-42.572311, 171.116101). This brackish sample (13 ppt at collection) was returned to the University of Canterbury and concentrated by evaporation *via* gentle heating until the salinity reached 32 ppt. Treatments of 10 and 50% WC DOC were made by diluting the 100% WC DOC (that which had been concentrated to 32 ppt) with West Coast seawater, collected at the same time as the WC DOC, but 1 km south of the river inlet. A subsample of each DOC was shipped to Wilfrid Laurier University (Waterloo, ON, Canada) for measurement of DOC concentration and molecular structural information *via* fluorescence-excitation-emission measurements (FEEM). Total DOC was measured using a Shimadzu TOC-

Vcph/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). For FEEM analysis a small aliquot of each DOC sample was first passed through a 0.45 µm GMF GD/C membrane filter. Fluorescence surface scans of emission wavelengths were recorded from 250 to 600 nm in a Varian Cary Eclipse Fluorescence spectrophotometer (Varian, Mississauga, ON) using a 1 cm quartz cuvette. During the scan, fluorescence moved in 1 nm increments, for every 10 nm excitation wavelength increment between 200 nm and 450 nm. Scan speed was set at 400 nm/min and the photomultiplier tube was set to high detection (800 V). The excitation and emission monochromator slit widths were both set to 5 nm for all measurements.

Fluorescence indices (FI) were calculated, following the methods of McKnight et al. (2001) and DePalma et al. (2011), using the following calculation:

$$Flex_{370} = \frac{Em450}{Em500}$$

where FI_{ex370} is the fluorescence index at 370 nm and em450 and em500 are the emission intensities at 450 nm and 500 nm, respectively.

4.3.5 DOC exposures

Fertilized eggs were separated into 5 different DOC treatments (UC DOC, OS DOC, WC DOC 10%, WC DOC 50%, WC DOC 100%). Within each treatment there was a control (0 µg/L of Ni), and two concentrations of Ni (15 and 30 µg/L; i.e. one level at the EC_{50} and the other above). Each exposure was replicated five times. After 96 h of exposure the test was terminated by the addition of 1% neutral buffered formalin, which stopped development and preserved the embryos for microscopic observation (see above). For each assessment of development at least 100 embryos from each replicate were scored, while water samples for metals analysis were also taken, as described above.

4.3.6 Ion influx

In a separate set of fertilized embryos, unidirectional Ca and Na influx assays were performed every 24 h over the course of the first 96 h following fertilization. Influx was assessed in four groups: 1) Control (no added Ni, in system seawater; i.e. UC DOC), 2) Ni (30 μg/L), 3) DOC (10% WC DOC), and 4) Ni + DOC (30 μg/L in 10% WC DOC). Fertilization and exposure was conducted as described above, and at each assay time-point 1250 embryos were removed (following counting on Sedgewick-Rafter cell), and placed in 5 ml screwtop plastic vials. A matching volume (0.5 to 2.5 mL, depending on embryo density) of water, (identical water chemistry as the exposure chamber) was added to each vial, which contained 0.5 μC_i of either ^{22}Na or ^{45}Ca (Perkin-Elmer). After 20 minutes of incubation (Tellis et al., 2014), the contents of the vials were emptied onto a flasktop vacuum filter apparatus, and embryos were collected on a 0.45 μm membrane filter (Schleicher and Schuell). The vial and the embryos on the filter were then rinsed with 20 mL of seawater. Filters containing embryos were counted directly for ^{22}Na analysis using a gamma counter (Wizard Wallac 1470; Perkin-Elmer). Filters for ^{45}Ca were digested in 2 mL of 2N HNO_3 before the addition of 15 mL of scintillation fluor (Ultima-Gold) and counting via a liquid scintillation analyzer (TriCarb 2910TR: Perkin-Elmer). Triplicate 1-mL water samples (of spiking rather than exposure solutions to avoid harvesting of embryos) were taken for determination of specific activity. There was no quenching in gamma counting of ^{22}Na. Quenching of ^{45}Ca samples was accounted for using the external standards ratio approach. Influx was calculated as:

$$J_{in}\ (\text{nmoles/embryo/h}) = \frac{cpm / SA}{n * T}$$

where *cpm* is the counts in the embryos (quench corrected in the case of ^{45}Ca); *SA* is the specific activity (cpm/nmol); *n* is the number of embryos (1250) and *T* is time in hours.

4.3.7 Analytical chemistry

Embryos and filter paper were digested in 2N HNO_3 at 65ºC for 48 h, with samples vortexed at 24 h to aid digestion. Embryo digests were then diluted appropriately in nanopure water (milli-Q: > 18 MΩ), and analyzed for metals and ions *via* inductively coupled plasma mass spectrometry (ICPMS; Agilent 7500cx). QA/QC involved the addition of ^{103}Rhodium as an internal standard and the inclusion of a blank and spiked standards (2 and 20 ppb) every 20 samples. Filter paper blanks were also run to determine the effect of the filter paper on the ionic composition of the embryos. These blanks contributed negligible concentrations of Na, K, Ca, Mg, and metals, however these blank values were subtracted from reported values of Ni accumulation and ion concentrations.

4.3.8 Statistical and speciation analysis

All 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). Ion influx (with water chemistry and time as the two variables) and the effects of DOC on development (with DOC source and Ni concentration as the two variables) were tested *via* two-way ANOVA, with a Tukey's post hoc test. EC_{50} values and 95% confidence intervals were determined from a sigmoidal logistic curve in SigmaPlot. For all other analyses, a one-way ANOVA model was applied with a Tukey's post-hoc test. Significance for all statistical tests was accepted at $\alpha = 0.05$. All data have been expressed as means ± S.E.M. (N = number of replicates).

Ni speciation analysis for the recorded water chemistries in Tables 4.1 and 4.2 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), which utilizes the NICA-Donnan model to estimate the effect of DOC on speciation (Benedetti et al., 1995) (Table 4.4).

4.4 RESULTS

4.4.1 Water chemistry

Dissolved Ni concentrations were close to nominal for all Ni exposures (Tables 4.1 and 4.2). Mean control values ranged from 1.7 µg/L in the OS DOC exposure to 3.7 µg/L in UC DOC exposures. Salinity was constant at 32 ppt in all tests, whereas measured DOC concentration was lowest for OS, slightly higher in UC, and higher again in 10%, 50%, and 100% WC tests, with the latter increasing proportionately as expected (Table 4.3). Speciation analysis determined differences in ionic Ni across all DOC exposures. The highest level of ionic Ni^{2+} was in the OS DOC exposure series (77%). The 100% WC DOC treatment had the lowest proportion of ionic Ni at 46%. The Ni-DOC complex was the highest in this exposure at 28% (Table 4.4).

4.4.2 Determination of EC_{50} for *Evechinus chloroticus*

In a 96 h embryo-larval toxicity test the EC_{50} of Ni was determined to be 14.1 µg/L (95% confidence intervals: 12.1-16.5 µg/L) (Fig. 4.1). An estimated 96 h EC_{10} value of 6.6 µg/L was also calculated. There were no signs of abnormal development in sea urchin embryos after 48 h (Fig. 4.1).

4.4.3 Ni accumulation and embryo-larval ions

As the external water concentrations of Ni increased, so did Ni body burden. However, only at 240 µg/L and greater were these increases significant relative to the Ni accumulation in

control embryos. At the highest external water concentration of Ni (960 µg/L) a 25-fold increase in Ni accumulation was observed in comparison to control embryos (Fig. 4.2).

Larval ion concentrations at the end of the 96 h exposure revealed significant effects from Ni exposure (Fig 4.3A-D). Both Mg and Na displayed significant increases with increasing Ni exposure; Fig 4.3A,C). The effects of Ni on Ca were close to significance (P = 0.052), with an increasing Ca accumulation pattern displayed as external Ni water concentrations increased (Fig. 4.3B). Larval K levels were not significantly impacted by Ni (Fig. 4.3D).

4.4.4 Na and Ca influx rates in Ni-exposed embryos over 96 h

Na influx was not significantly altered over the course of 96 h in either control or Ni exposed (30 µg/L) embryos (Fig. 4.4A). A two way ANOVA showed an overall treatment effect, with Ni causing decreased Ca influx (P = 0.044), but this could not be attributed to a specific time-point *via* post-hoc analysis. Although there was no overall effect of time (P = 0.236), there was a significantly higher Ca influx at 96 h than at 24 h (P = 0.044; Fig. 4.4B).

4.4.5 DOC effects on sea urchin embryos exposed to Ni

A two way ANOVA highlighted overall significant effects of DOC treatment (P <0.001), Ni concentration (P <0.001) and the interaction between these two factors (P = 0.04) with respect to normal development of sea urchin embryos (Fig. 4.5). There were no significant differences across control treatments. Post hoc tests revealed that the 10% WC DOC exposure resulted in a higher percentage of normal development at 52% (15 µg/L) and 41% (30 µg/L) relative to the reference DOC (UC DOC; 42% in 15 µg/L and 13% in 30 µg/L) at the same Ni concentrations. In both the 15 and 30 µg/L Ni treatments, but not the control, there was a significant effect of 100% WC DOC on development. In this water chemistry normal development was reduced from 82% in the control treatment to 12% in 15 µg/L and just 1% in 30 µg/L exposed embryos.

4.4.6 Ni accumulation and larval ion content with respect to DOC

There were significant effects of DOC source ($P < 0.001$), Ni concentration ($P = 0.004$) and interaction effects ($P = 0.047$) on Ni accumulation in sea urchin embryos (Fig. 4.6). A post hoc test revealed that in the absence of added Ni, embryos exposed to UC DOC seawater accumulated significantly higher levels of Ni than all the other “Ni-free” DOC treatments except WC 50%, reflecting the higher levels of Ni in this source (Table 4.2). In general, Ni accumulation increased with respective increases in Ni concentration, but there were no significant differences among different DOC’s. After 96 h exposure to different DOC treatments and Ni concentrations, there were no significant effects detected with respect to ion concentrations in sea urchin embryos (Table 4.5).

4.4.7 Na and Ca influx rates in embryos exposed to Ni and 10% WC DOC over 96 h

A two way ANOVA showed that neither effects of DOC ($P = 0.621$), time ($P = 0.112$) nor the interaction between these two factors ($P = 0.537$) significantly affected Na influx rate (Fig. 4.7A). Ca influx rate into sea urchin embryos was also not significantly affected by DOC ($P = 0.236$), but was significantly influenced by time ($P = 0.028$). Indeed, a significant increase in Ca influx rate was observed at 96 h in both control and Ni exposed embryos (Fig. 4.7B).

4.4.8 DOC characterization

All three DOC sources were found to have similar optical components but with varying contributions. Figure 4.8 shows FEEM contour maps for these samples. The lines where emission equals excitation, and emission equals two times the excitation are scattered light, and not fluorescence, and have been omitted for plotting purposes. The linear features offset from these scattering lines correspond to the Raman peaks for water and are not related to DOC fluorophores.

WC DOC samples had the highest fluorescence intensity reflecting their relatively high DOC concentrations. Structurally, the WC fluorophores contain more humic and fulvic acid-like fluorescence with emissions in the range 400 to 450 nm (Fig. 4.8 C-D). The OS DOC and UC DOC also exhibited fluorescence in the humic/fulvic acid range, but also displayed fluorescence in the range normally attributed to proteinaceous substances (wavelengths between 280-350 nm; Fig. 4.8 A,B).

4.5 DISCUSSION

4.5.1 Ni toxicity to developing *E. chloroticus*

Early life stages of *E. chloroticus* are the most sensitive of any tested marine organism to Ni toxicity, with an EC_{50} of 14.1 µg/L. This value is well below the Australian/New Zealand marine trigger value for protection of 95% of species (70 µg/L; ANZECC/ARMCANZ, 2000) and the USEPA's acute ambient water quality guideline (75 µg/L) for Ni in marine waters (USEPA, 2005). The value is above the USEPA chronic guideline marine value of 8.2 µg/L (USEPA, 2005). Previous research has shown that the sea urchin *Diadema antillarum* exhibits a similar sensitivity to that noted here (15 µg/L; Bielmyer et al., 2005). However, toxicity for other sea urchin species shows significant variation, with EC_{50} values as high as 341 µg/L being reported (Table 4.6). The sensitivity does not appear to correspond to obvious phyletic groupings, with other *Diadema* species shown to be significantly less sensitive than *D. antillarum* (Kobayashi, 1994; Rosen et al., 2015).

One factor that could influence reported toxicity values is the salinity of the exposure medium, which varies between studies (Table 4.6). Nadella et al. (2013) showed that normal development in *S. purpuratus* dropped from 95% in 35 ppt to < 60% in 28 ppt, confirming similar findings on three other species (Kobayashi, 1971). DOC is likely to be another important factor

influencing toxicity. Although DOC has not always been measured in Ni toxicity tests, the values reported here for EC_{50} assays (0.5 mg C/L) are the lowest recorded, and there is an apparent trend in other studies whereby higher DOC levels result in lower toxicity (Table 4.6). The possible influence of DOC on Ni toxicity is discussed in more detail below.

Ni-exposed embryos developed normally for the first 48 hours; it was only after this period where notable abnormal development occurred (Fig. 4.1). Similar findings have been reported previously, with Ni exposure at early stages of development having no noticeable effect relative to the severe effects observed at later developmental stages (Hardin et al., 1992; Kobayashi, 1990; Novelli et al., 2003). Although developmental speed varies with temperature, after 48 hours, sea urchin embryos are typically entering gastrulation. This phase includes the formation of primary mesenchyme cells, which will eventually form the skeleton/spicule of the pluteus larvae (Farach-Carson et al., 1989). During this period, the skeleton, which is composed largely of calcium carbonate, undergoes biomineralization, which is dependent on Ca uptake from the environment (Wilt, 1999). Ni has been previously shown to impact skeletal development. Specifically, it causes failure of the primary mesenchyme cells to aggregate into two ventrolateral clusters, resulting in embryos that develop spicules that are shorter and missing critical side branches (Hardin et al., 1992). It has been speculated that Ni prevents appropriate cell migration and commitment during development, potentially *via* an inhibition of intercellular communication (Armstrong et al., 1993; Hardin et al., 1992; Wilt et al., 1995). Interestingly, manganese, which like Ni is a Ca channel blocker, impacts the signalling of ERK-mediated signalling pathways (Pinsino et al., 2011), which perform critical roles in primary mesenchyme cell processes in sea urchins (Fernandez-Serra et al., 2004). This effect is likely mediated by an impact on Ca uptake and results in impaired skeletogenesis in sea urchins (Pinsino et al., 2011). These morphological and functional changes are similar to those observed in the current study, indicating that this is

likely the pathway by which Ni impairs development. It is of note that homologous developmental pathways are differentially regulated between different species of sea urchins (Franks et al., 1988; Wessel et al., 1989), which may help explain the variation in sensitivity from species to species (Table 4.6).

After a 96 hour exposure to increasing concentrations of Ni, the ion content (specifically Na and Mg, with Ca narrowly eluding statistical significance) of sea urchin embryos increased (Fig. 4.3), indicating a disruption of ion homeostasis. Ni is considered an ionoregulatory toxicant in invertebrates, and has been previously reported to disturb Mg, Na, Ca and K homeostasis *via* ionic mimicry and/or inhibition of transporters (Chapter 2, Blewett et al., 2015a; Chapter 3, Blewett and Wood, 2015b; Leonard et al., 2011; Pane et al., 2003b; Tellis et al., 2014). This may indicate an inhibition of Na^{+}/K^{+} ATPase, as this basolateral transporter generates gradients for cellular transport of all ions. Previous evidence has shown that Na^{+}/K^{+} ATPase activity can be disrupted at an exposure concentration of 8.2 μg/L of Ni in adult crabs, resulting in perturbed haemolymph Na levels (Chapter 2, Blewett et al., 2015a).

Notably, in developing embryos of another sea urchin, *Strongylocentrotus purpuratus*, Tellis et al. (2014) reported that while whole body accumulation of Ca was inhibited at early time points by a comparable level of Ni exposure (40 μg/L) , this effect had disappeared, and in fact reversed, by 84 h. This observation is in accord with the current data showing inhibition of unidirectional Ca influx rate (Fig. 4.4B) yet a tendency for higher whole body Ca concentrations at 96 h in *Evechinus chloroticus* (Fig. 4.3B). It is also important to note that whole body accumulation reflects the net difference between unidirectional influx and efflux rates, but only the former was measured in both the present study and in Tellis et al. (2014).

Further evidence for an effect on Ca metabolism was provided by ion influx experiments. Over the course of 96 hours, an overall inhibition of Ca influx by Ni was observed. Ni effects on

Ca homeostasis in invertebrates have been previously described in a range of organisms (e.g. Deleebeeck et al., 2009; Eisler, 1998; Funakoshi et al., 1997; Pane et al., 2006 a,b) including developing sea urchin embryos (Tellis et al., 2014). Effects on Ca influx are likely mediated by interference with Ca channels (e.g. Funakoshi et al., 1997). The elevation in whole body Ca indicates that the impairment in skeletal development was likely not solely a consequence of an impaired influx. However, it instead suggests that there may also have been an issue with internal Ca distribution. Ni has previously been shown to impact the activity of the basolateral Ca-ATPase in the early life-stages of another sea urchin species (*S. purpuratus*; Tellis et al., 2014). Such inhibition may restrict the movement of Ca from ectodermal tissue to the primary mesenchyme cells, which are primarily responsible for spicule formation (Vidavsky et al., 2014).

4.5.2 Effects of different DOC sources on Ni accumulation and toxicity

Sea urchins were exposed to five different DOC treatments**.** Normal development was observed across all control (Ni-free) exposures regardless of the source or concentration of the DOC (Fig. 4.5). In the presence of Ni, 10% WC DOC led to an increase in normal development relative to reference DOC's exposed to the same Ni level. This finding is in agreement with the protective role of DOC against Ni toxicity (e.g. Doig and Liber, 2006). DOC displays multiple binding sites for cations and therefore functions as a multi-site complexing ligand for metals. This decreases the availability of the metal to the organism in the aquatic environment. Speciation analysis showed that in 100% WC DOC, only 46% of the total Ni was present as the free ion (Table 4.4), generally considered the bioavailable form (Pyle and Couture, 2012). This relationship between metal toxicity and DOC has been well established in freshwater settings, however, few studies have been performed in the marine environment, and those that do exist are heavily weighted towards Cu-DOC interactions (e.g. Arnold et al., 2010 a,b; Cooper et al., 2014;

Nadella et al., 2009). These nevertheless show that DOC is protective against Cu toxicity in seawater, while a recent study also showed moderate protection by DOC against Pb toxicity to sea urchin embryos (Nadella et al., 2013). Confirming the protective effects of DOC, the overall inhibitory effect of Ni on Ca influx rate (Fig. 4.4B) was not observed with 10% WC DOC (Fig. 4.7B), lending support to the evidence for ionoregulatory impairment as an important underlying mechanism of Ni toxicity to *E. chloroticus* embryos.

The protective effects of 10% WC DOC are likely attributable to its higher concentration (1.5 mg C/L) relative to UC DOC (0.5 mg C/L) and OS DOC (0.2 mg C/L). However, the DOC composition may also be playing a role. FEEM's for WC DOC displayed emissions in the 400-450 nm range with excitation peaks at 250 nm and 350 nm (Fig. 4.8C-E), indicating humic-like and fulvic-like acids of an allochthonous-derived DOC (DePalma et al., 2011). The measured FI indices are also in accordance, with the low value calculated for WC DOC (1.2), indicating a greater fulvic/humic source (McKnight et al., 2001). DOC that is rich in humic and fulvic acids has been shown to have a greater protective effect against metal toxicity (e.g. De Schamphelaere et al., 2004; Glover et al., 2005 a,b; Nadella et al., 2009), consistent with the data shown here.

In the 50% and 100% WC DOC exposures there was an unexpected decrease in normal development of embryos in both the 15 µg/L and 30 µg/L Ni treatments, with almost a ~10 fold decrease from the normal development observed in the 10% WC DOC treatment in the absence of added Ni (Fig. 4.5). Sea urchin larvae are known to have the ability to take up organic matter directly from seawater (e.g. Manahan, 1990). Thus the higher levels of DOC could represent an increase in potential ligands for Ni uptake, representing a route of Ni exposure distinct from that of ion mimicry. However, if this hypothesis were true, Ni accumulation would be increased in the higher DOC exposures. This was not observed (Fig. 4.6), thus the relationship between DOC and Ni toxicity seems to be more complex.

DOC is known to have a direct effect on membrane function, thought to be manifested by alterations in membrane fluidity (e.g. Galvez et al., 2009; Glover et al., 2005; Glover and Wood 2005b; Vigneault et al., 2000; Wood et al., 2011). Such an effect would be most prevalent at high DOC levels and may explain the observed decrease in normal embryo development in the high WC DOC exposures. However, this is an effect that was only observed in the presence of Ni, suggesting that the mechanism of effect relies on the presence of the metal. Previous research has shown that changes in Ca metabolism may impact membrane fluidity (e.g. Kumar and Prasad, 2003; Matthews, 1986). Given the impact of Ni on cellular Ca handling, the presence of DOC at high levels may have an additive or synergistic effect, leading to impaired cellular transport functions that depend on membrane fluidity (e.g. Le Grimellec et al., 1992).

4.5.3 Conclusion

Evechinus chloroticus is the most sensitive marine organism yet tested with regards to Ni toxicity. Toxicity was likely manifested through a perturbance of Ca homeostasis, resulting in improper skeletal formation. As a highly sensitive organism, it will likely drive environmental regulations for the protection of marine species against Ni contamination (DeForest and Schlekat, 2013). Further research should investigate whether this species also exhibits high sensitivity to other metal contaminants, in particular known Ca antagonists such as Zn.

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Environmental Research Association, the International Copper Association, the Copper Development Association, Teck Resources, and Vale Inco. CMW was supported by the Canada Research Chairs Program

4.7 TABLES AND FIGURES

Table 4.1. Dissolved Ni concentrations in EC_{50} exposure. Reported values are means ± S.E.M. (N = 6 for each treatment).

Nominal (µg/L)	Measured (µg/L)
0	4.24 ± 2.11
15	18.9 ± 2.2
30	29.9 ± 4.4
60	52.6 ± 7.7
120	119 ± 28
240	298 ± 62
480	599 ± 110
960	1304 ± 199

Table 4.2. Ni concentrations (µg/L) in DOC exposures. Reported values represent means ± S.E.M. (N = 5).

Treatment	Control	Ni at 15 µg/L	Ni at 30 µg/L
UC DOC	3.7 ± 0.6	14.0 ± 0.3	21.7 ± 0.4
OS DOC	1.7 ± 0.1	14.4 ± 0.3	27.1 ± 0. 7
West Coast 10%	3.1 ± 0.1	14.2 ± 0.5	27.3 ± 1.5
West Coast 50%	2.1 ± 0.3	14.0 ± 0.2	24.6 ± 0.2
West Coast 100%	2.0 ± 0.1	13.3 ± 0.3	24.8 ± 0.6

Table 4.3. Water chemistry in DOC exposures.

Parameter	UC DOC	OS DOC	WC DOC 10%	WC DOC 50%	WC DOC 100%
Salinity	32.0	31.7	31.9	32.0	32.1
DOC (mg C/L)	0.5	0.2	1.5	4.6	11.7
pH	8.1	8.0	8.1	8.1	8.1
Temperature (°C)	15	15	15	15	15
Fluorescence index	1.5	2.1	1.2	1.2	1.2

Table 4.4. Ni speciation (% of total Ni) as calculated by Visual MINTEQ based on measured and nominal water chemistry and a Ni concentration of 30 μg/L.

Ni Speciation	UC DOC	OS DOC	WC DOC 10 %	WC DOC 50%	WC DOC 100%
Ni^{2+}	76.57	77.20	75.46	62.54	46.03
Ni-DOC	0.86	0.57	1.08	12.79	28.50
$NiOH^{+}$	0.22	0.22	0.22	0.20	0.15
$Ni(OH)_2$ (aq)	0.03	0.03	0.03	0.03	0.02
$NiCl^{+}$	4.63	4.74	3.96	5.62	4.81
$NiCl_2$ (aq)	0.04	0.04	0.03	0.03	0.03
$NiSO_4$ (aq)	12.54	11.78	12.11	12.66	12.83
$NiCO_3$ (aq)	2.11	1.90	1.66	2.33	3.03
$NiCO_3^{+}$	3.00	3.52	5.45	3.80	4.60

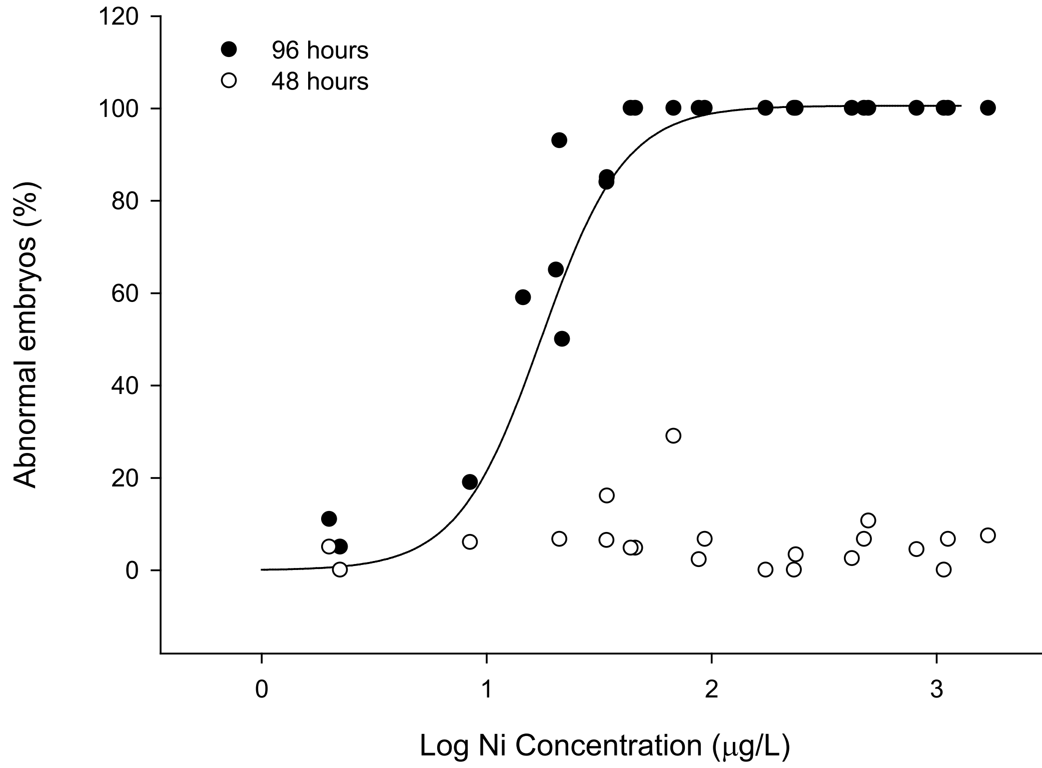
Table 4.5**.** Ion concentrations (nmol/embryo) in sea urchin embryos exposed to different Ni concentrations and different DOC concentrations. Values represent means ± S.E.M., N=3.

	Mg			Na			Ca			K		
	Control	*12.5 µg/L*	*25 µg/L*	*Control*	*12.5 µg/L*	*25 µg/L*	*Control*	*12.5 µg/L*	*25 µg/L*	*Control*	*12.5 µg/L*	*25 µg/L*
UC DOC	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.2	7.0 ± 0.7	8.3 ± 0.6	9.3 ± 1.4	0.15 ± 0.02	0.18 ± 0.01	0.20 ± 0.03	0.19 ± 0.02	0.23 ± 0.01	0.25 ± 0.03
OS DOC	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	8.3 ± 1.6	8.0 ± 1.0	8.6 ± 1.4	0.16 ± 0.03	0.17 ± 0.02	0.18 ± 0.03	0.22 ± 0.03	0.22 ± 0.02	0.24 ± 0.03
10%WC DOC	1.0 ± 0.0	1.2 ± 0.2	1.2 ± 0.2	7.1 ± 0.2	8.8 ± 1.4	9.0 ± 1.2	0.15 ± 0.01	0.18 ± 0.03	0.18 ± 0.03	0.19 ± 0.01	0.23 ± 0.03	0.23 ± 0.03
50%WC DOC	1.1 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	7.6 ± 1.2	9.2 ± 0.5	9.5 ± 1.0	0.15 ± 0.03	0.19 ± 0.01	0.19 ± 0.03	0.20 ± 0.03	0.25 ± 0.01	0.25 ± 0.03
100% WC DOC	1.5 ± 0.1	1.2 ± 0.2	1.6 ± 0.1	10.8 ± 0.4	8.8 ± 1.6	11.3 ± 0.7	0.22 ± 0.01	0.17 ± 0.04	0.24 ± 0.02	0.28 ± 0.01	0.23 ± 0.04	0.30 ± 0.02

Table 4.6. Toxicity of Ni to sea urchin embryo-larval development

Species	Toxicity measure	Concentration (µg/L)	Exposure duration (h)	Temperature (°C)	Nominal (N) or measured (M) Ni	DOC (mg C/L)	Salinity (‰)	Reference
Anthocidaris crassispina	EC†	1200	12	28	N	-	34	Kobayashi, 1971
Diadema antillarum	EC_{50}	15	40	20	M	-	33	Bielmyer et al., 2005
Diadema savignyi	EC_{50}	94	48	25	M	0.8	34	Rosen et al., in press
Diadema setosum	EC†	500	48	27	N	-	-	Kobayashi, 1994
Evechinus chloroticus	EC_{50}	14	96	15	M	0.5	32	This study
Hemicentrotus pulcherrimus	EC†*	500	48	18	N	-	-	Kobayashi, 1990
Hemicentrotus pulcherrimus	EC_{50}	34	64	16	N	-	32	Hwang et al., 2012
Paracentrotus lividus	EC_{50}	320	72	18	N	-	35	Novelli et al., 2003
Paracentrotus lividus	EC_{10}	217	72	16	M	1.0	38	DeForest and Schlekat, 2013
Strongylocentrotus purpuratus	EC_{50}	341	96	15	N	-	34	Phillips et al., 2003
Strongylocentrotus purpuratus	EC_{10}	335	48	16	M	1.2	30	DeForest and Schlekat, 2013

† "Effect concentration": lowest tested exposure concentration at which embryo development was abnormal.
* Represents exposure where fertilization was also performed in the presence of Ni.



120
100
80
60
40
20
0
Abnormal embryos (%)
96 hours
48 hours
0
1
2
3
Log Ni Concentration (μg/L)

Figure 4.1. Toxicity of Ni (determined using the endpoint of abnormal development) to sea urchin (*Evechinus chloroticus*) embryos after 48 h (open circles) and 96 h (closed circles), at 15 °C (N = 3). Plotted curve represents the best-fit sigmoidal logistic regression to 96 h data.

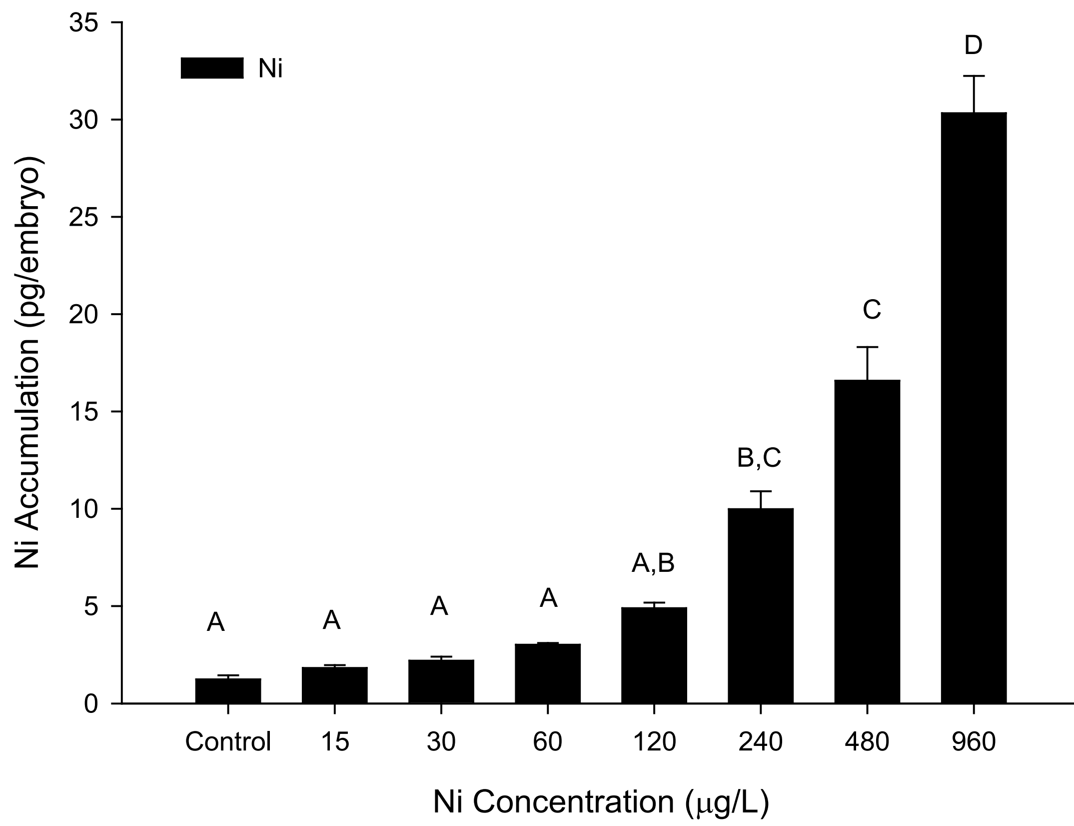

Ni
D
C
B,C
A,B
A
A
A
A
35
30
25
20
15
10
5
0
Ni Accumulation (pg/embryo)
Control
15
30
60
120
240
480
960
Ni Concentration (μg/L)

Figure 4.2. Ni accumulation (pg/embryo) in sea urchin (*Evechinus chloroticus*) embryos exposed to Ni for 96 hours at 15ºC. Plotted points represent means ± S.E.M. (N = 3). Bars sharing letters are not significantly different, as determined by a one-way ANOVA and Tukey post hoc test (α = 0.05).

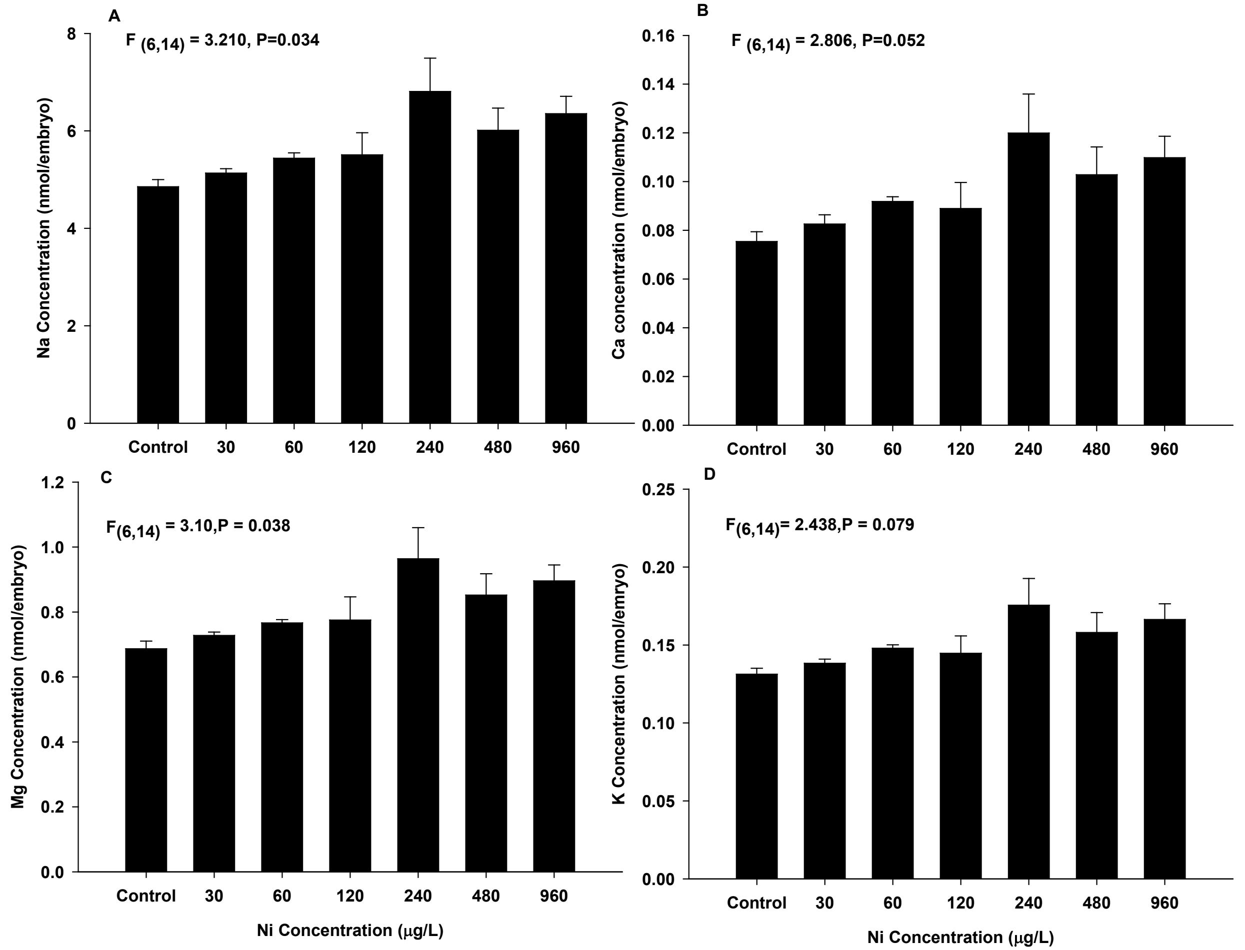
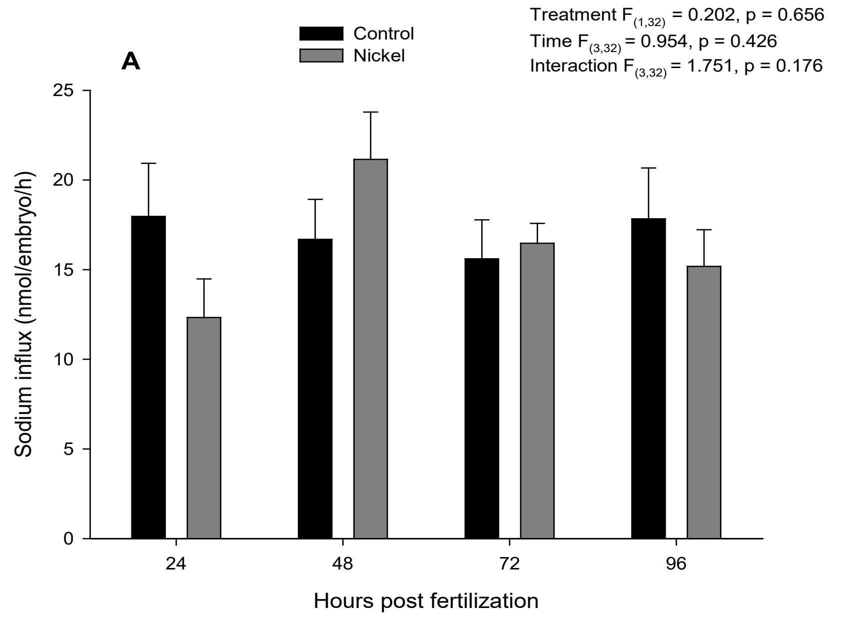
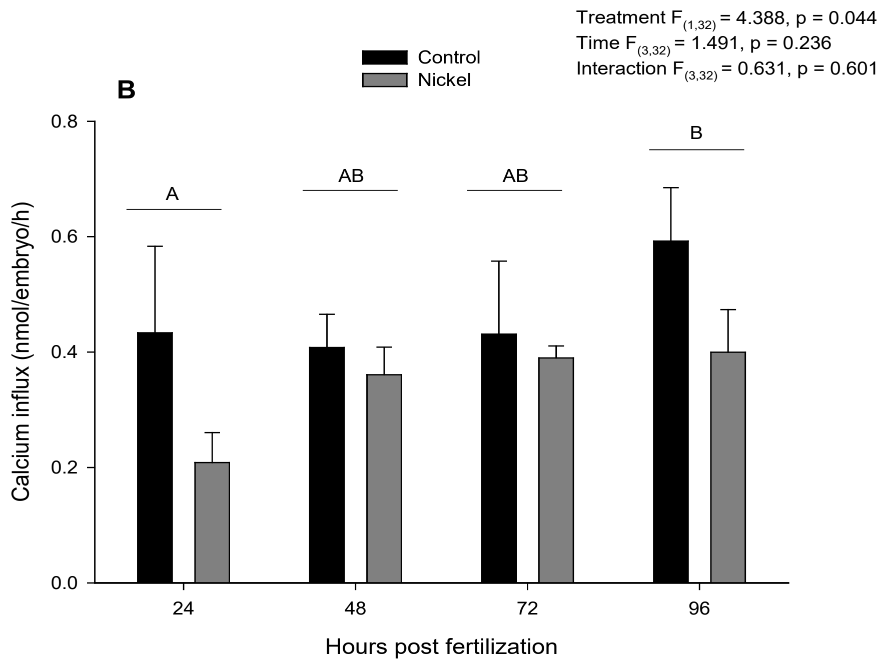

A
F (6,14) = 3.210, P=0.034
Na Concentration (nmol/embryo)
8
6
4
2
0
Control 30 60 120 240 480 960
B
F (6,14) = 2.806, P=0.052
Ca concentration (nmol/embryo)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Control 30 60 120 240 480 960
C
F(6,14) = 3.10,P = 0.038
Mg Concentration (nmol/embryo)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control 30 60 120 240 480 960
Ni Concentration (µg/L)
D
F(6,14)= 2.438,P = 0.079
K Concentration (nmol/emryo)
0.25
0.20
0.15
0.10
0.05
0.00
Control 30 60 120 240 480 960
Ni Concentration (µg/L)

Figure 4.3. Whole sea urchin (*Evechinus chloroticus*) embryo ion contents (nmol/embryo) for Na (A); Ca (B); Mg (C) and K (D) following Ni exposure for 96 hours at 15ºC. Plotted points represent means ± S.E.M. (N = 3). Statistical significance was determined by a one-way ANOVA and Tukey post hoc test ($\alpha = 0.05$).



A
Control
Nickel
Treatment $F_{(1,32)}$ = 0.202, p = 0.656
Time $F_{(3,32)}$ = 0.954, p = 0.426
Interaction $F_{(3,32)}$ = 1.751, p = 0.176
Sodium influx (nmol/embryo/h)
0
5
10
15
20
25
24
48
72
96
Hours post fertilization



B
Control
Nickel
Treatment $F_{(1,32)}$ = 4.388, p = 0.044
Time $F_{(3,32)}$ = 1.491, p = 0.236
Interaction $F_{(3,32)}$ = 0.631, p = 0.601
A
AB
AB
B
Calcium influx (nmol/embryo/h)
0.0
0.2
0.4
0.6
0.8
24
48
72
96
Hours post fertilization

Figure 4.4. Unidirectional Na (A) and Ca (B) influx (nmol/embryo/h) in sea urchin embryos (*Evechinus chloroticus*) exposed to 0 and 30 µg/L of Ni over the course of 96 hours. Plotted points represent means ± S.E.M. (N = 5). Statistical significance was determined by a two-way ANOVA and Tukey post hoc test (α = 0.05).Time intervals sharing letters are not statistically different (α = 0.05).

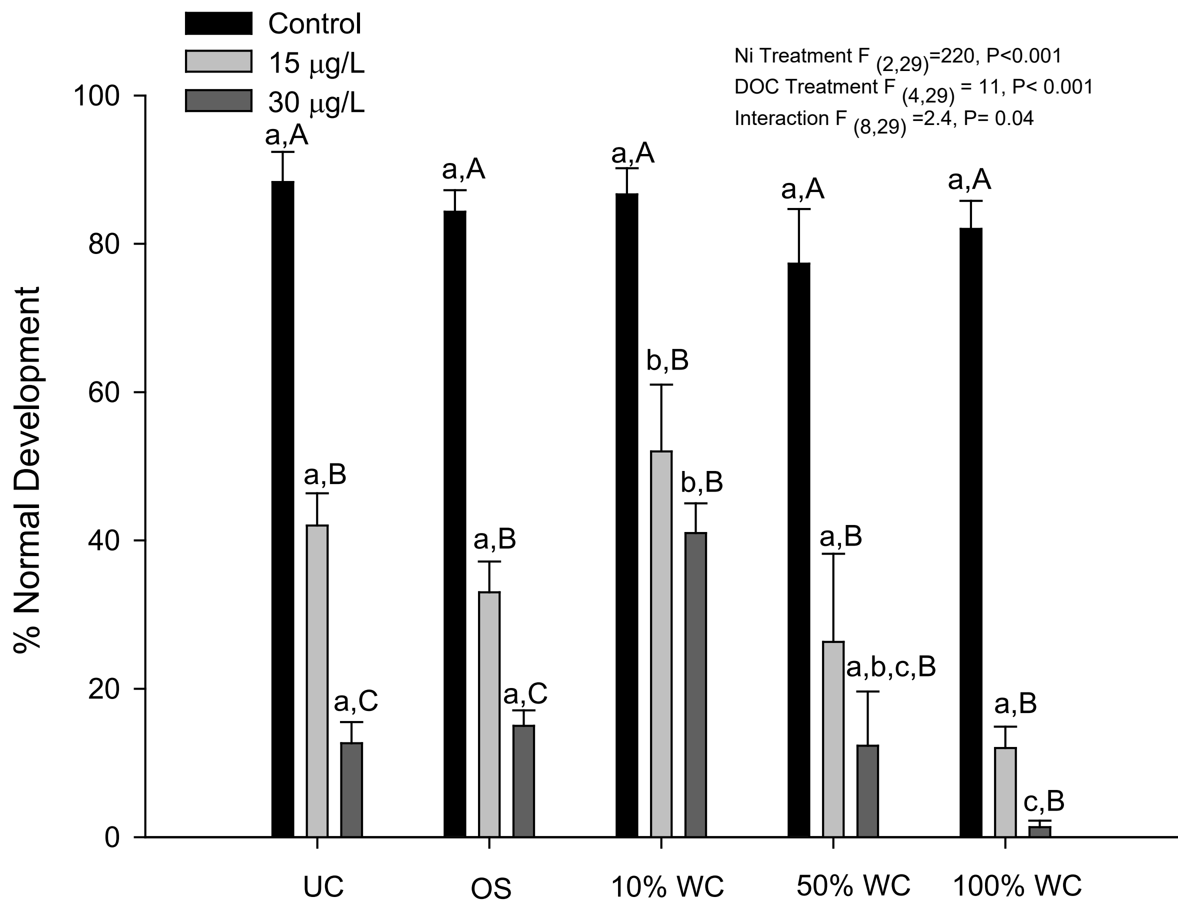

Control
15 μg/L
30 μg/L
Ni Treatment F (2,29)=220, P<0.001
DOC Treatment F (4,29) = 11, P< 0.001
Interaction F (8,29) =2.4, P= 0.04
% Normal Development
100
80
60
40
20
0
a,A
a,B
a,C
a,A
a,B
a,C
a,A
b,B
b,B
a,A
a,B
a,b,c,B
a,A
a,B
c,B
UC
OS
10% WC
50% WC
100% WC

Figure 4.5. Impact of Ni on the development of sea urchin (*Evechinus chloroticus*) embryos exposed to one of 5 different DOC treatments (UC DOC, OS DOC, 10% WC DOC, 50% WC DOC and 100% WC DOC) and 3 different concentrations of Ni (0, 15 and 30 µg/L) for 96 hours at 15ºC. Plotted points represent means ± S.E.M. (N = 5). Statistical significance was determined by a two-way ANOVA and Tukey post hoc test ($\alpha = 0.05$). Bars sharing lower case letters are not significantly different across DOC treatments within Ni concentration. Bars sharing upper case letters are not significantly different between Ni concentrations within a DOC treatment.

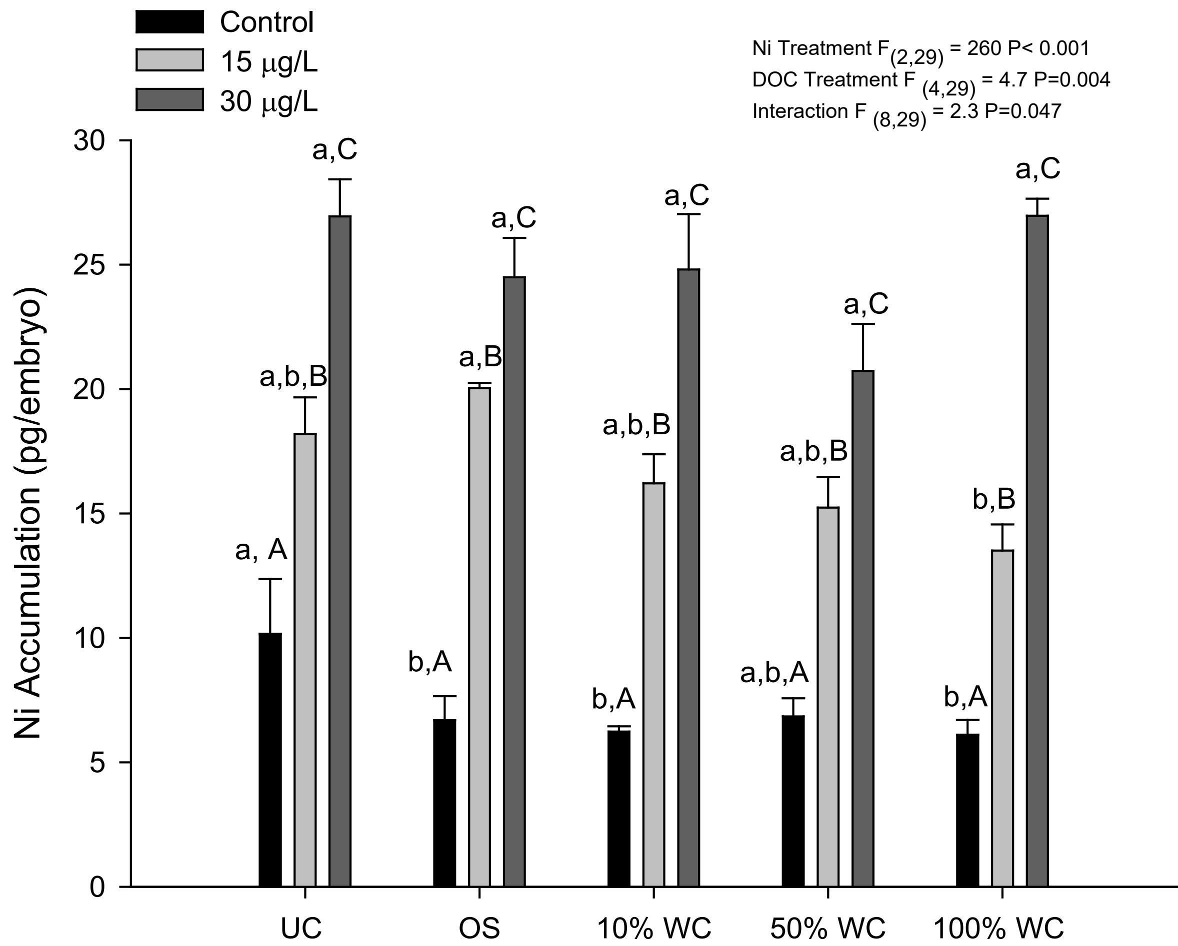

Control
15 μg/L
30 μg/L
Ni Treatment F(2,29) = 260 P< 0.001
DOC Treatment F (4,29) = 4.7 P=0.004
Interaction F (8,29) = 2.3 P=0.047
Ni Accumulation (pg/embryo)
0
5
10
15
20
25
30
a, A
a,b,B
a,C
b,A
a,B
a,C
b,A
a,b,B
a,C
a,b,A
a,b,B
a,C
b,A
b,B
a,C
UC
OS
10% WC
50% WC
100% WC

Figure 4.6. Ni accumulation (pg/embryo) in sea urchin (*Evechinus chloroticus*) embryos exposed to 5 different DOC treatments (UC DOC, OS DOC, 10% WC DOC, 50% WC DOC and 100% WC DOC) and 3 different concentrations of Ni (0, 15 and 30 µg/L) for 96 hours at 15ºC. Plotted points represent means ± S.E.M. (N = 5). Statistical significance was determined by a two-way ANOVA and Tukey's post hoc test ($\alpha = 0.05$). Bars sharing upper case letters are not significantly different between DOC treatments within Ni concentration. Bars sharing lower case letters are not significantly different between Ni concentrations within a DOC treatment.

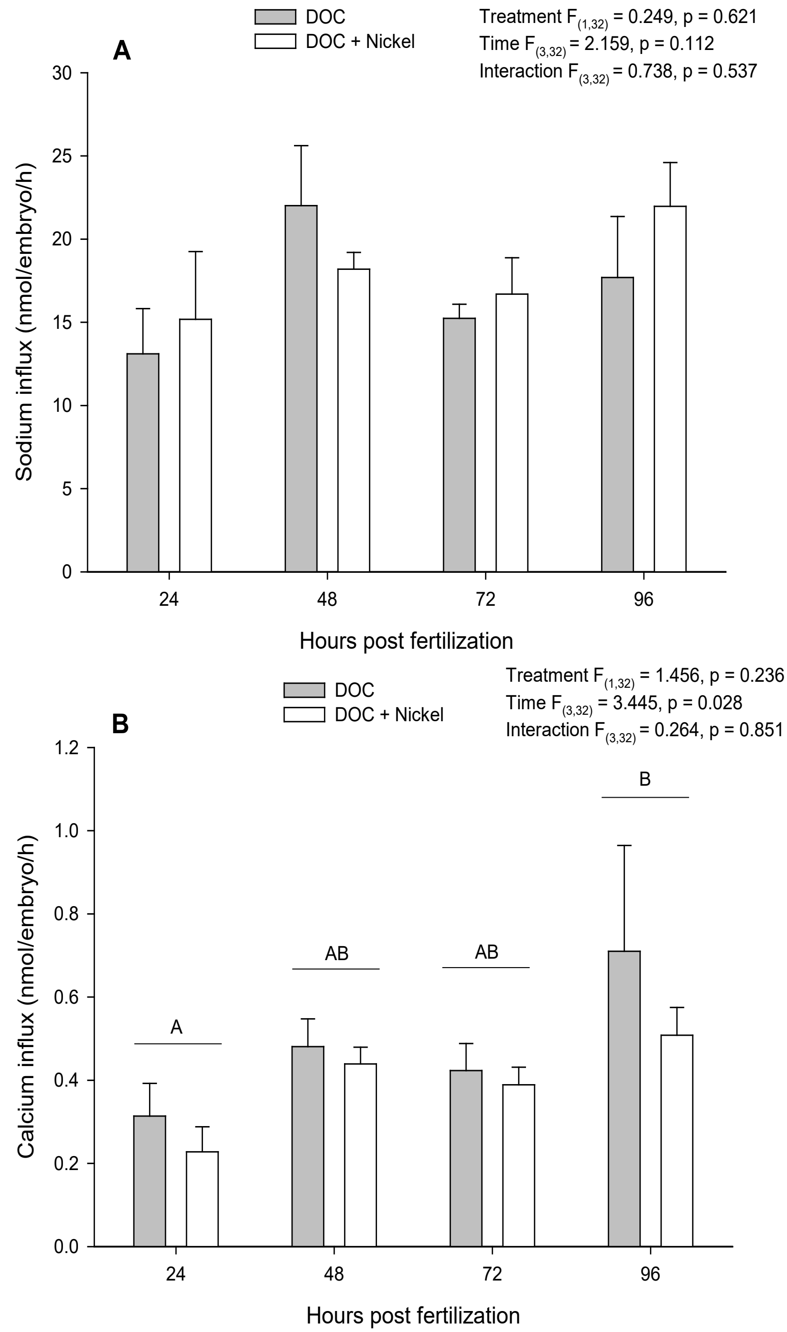
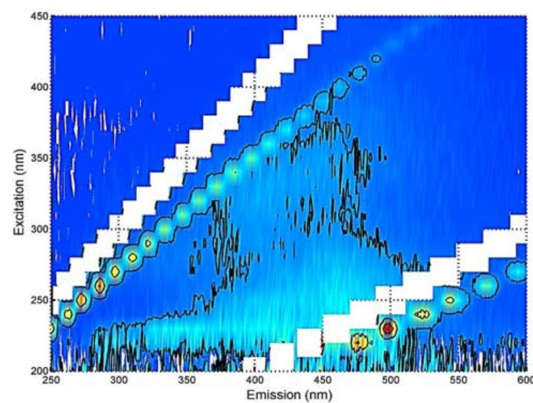
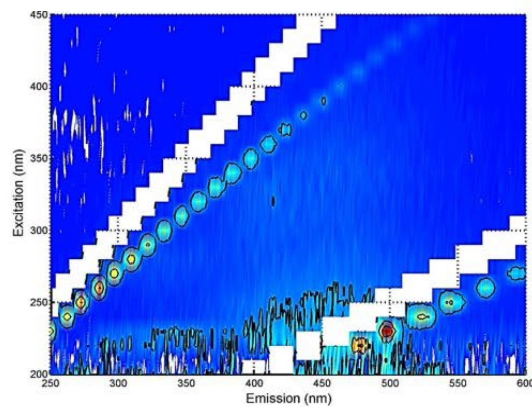
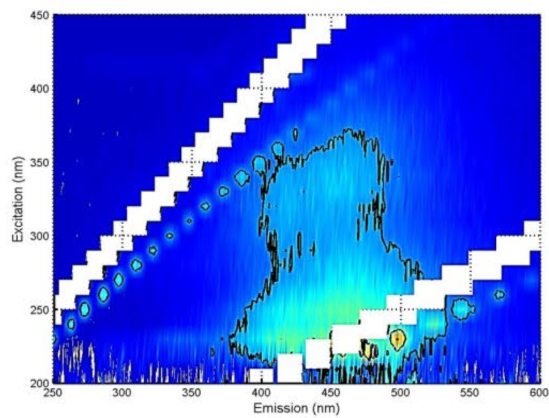
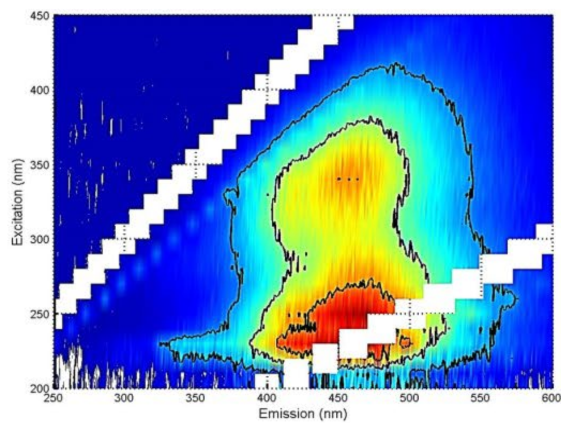

A
DOC
DOC + Nickel
Treatment F(1,32) = 0.249, p = 0.621
Time F(3,32) = 2.159, p = 0.112
Interaction F(3,32) = 0.738, p = 0.537
Sodium influx (nmol/embryo/h)
0
5
10
15
20
25
30
24
48
72
96
Hours post fertilization
B
DOC
DOC + Nickel
Treatment F(1,32) = 1.456, p = 0.236
Time F(3,32) = 3.445, p = 0.028
Interaction F(3,32) = 0.264, p = 0.851
Calcium influx (nmol/embryo/h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
A
AB
AB
B
24
48
72
96
Hours post fertilization

Figure 4.7. Unidirectional Na (A) and Ca (B) influx (nmol/embryo/h) in sea urchin embryos (*Evechinus chloroticus*) exposed to 10% WC DOC and Ni concentration of 30 µg/L for 96 hours at 15 ºC. Plotted points represent means ± S.E.M. (N = 5). Statistical significance was determined by a two-way ANOVA and Tukey post hoc test ($\alpha = 0.05$). Time intervals sharing letters are not statistically different ($\alpha = 0.05$).


Excitation (nm)
Emission (nm)


Excitation (nm)
Emission (nm)


Excitation (nm)
Emission (nm)


Excitation (nm)
Emission (nm)

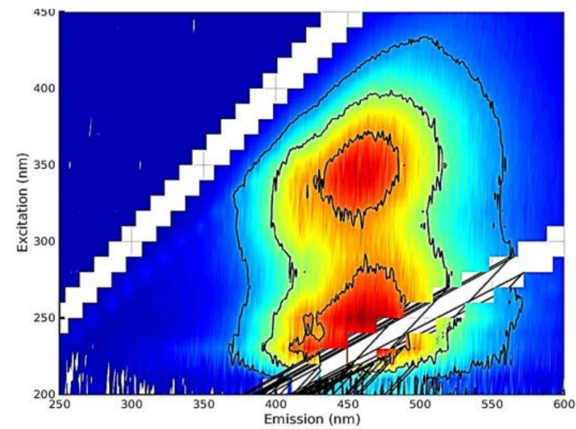

Excitation (nm)
Emission (nm)

Figure 4.8. Fluorescence excitation-emission matrices of UC DOC (A), OS DOC (B), 10% WC DOC (C), 50% WC DOC (D), 100% WC DOC (E). Contour lines are indicated as the fluorescence emission intensity.

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CHAPTER 5

SALINITY-DEPENDENT NICKEL ACCUMULATION AND OXIDATIVE STRESS RESPONSES IN THE EURYHALINE KILLIFISH (*Fundulus heteroclitus*)

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5.1 ABSTRACT

The mechanisms of nickel (Ni) toxicity in marine fish remain unclear, although evidence from freshwater fish suggests that Ni can act as a pro-oxidant. This study investigated the oxidative stress effects of Ni on the euryhaline killifish (*Fundulus heteroclitus*), as a function of salinity. Killifish were exposed to sub-lethal levels (5, 10, 20 mg/L) of waterborne Ni for 96 h in FW (0 ppt) and 100% SW (35 ppt). In general, SW was protective against both Ni accumulation and reduced oxidative stress (protein carbonyl formation and catalase (CAT) activity). This effect was most pronounced at the highest Ni exposure level. For example, FW intestine showed an elevated Ni accumulation relative to SW intestine at 20 mg/L Ni, and this was accompanied by a significantly higher protein carbonylation and CAT activity in this tissue. There were exceptions, however, in that although liver of FW killifish at the highest exposure concentration showed a higher Ni accumulation relative to SW liver, the levels of CAT activity were greatly reduced. This may relate to tissue- and salinity-specific differences in oxidative stress responses. The present study suggests Ni-induced oxidative stress in killifish, and the effects of salinity, depend upon differences in the physiology of the fish in FW *versus* SW, and that the elevated levels of cations (Na, Ca, K and Mg) and anions (SO_4 and Cl) in SW are likely protective against Ni accumulation in tissues exposed to the aquatic environment.

5.2 INTRODUCTION

The chemical speciation of a metal may impact its bioavailability. To this extent, therefore, water chemistry will have a significant influence on the ability of aquatic biota to take up a metal, which in turn will impact toxicity (Paquin et al., 2002). The free metal ion is generally considered the most bioavailable chemical species, and thus also the most toxic, likely owing to its ability to react with sensitive cellular sites such as enzymes and transporters (for reviews see Niyogi and Wood, 2004; Wood, 2012). This relationship between the speciation of the metal and the toxicity of the metal is one of the key principles underlying the Biotic Ligand Model (BLM). A BLM applies a quantitative method to evaluate the biological impact by accounting for the metal bioavailability, which is expressed as a function of site-specific water chemistry and organism species-sensitivity (Paquin et al., 2002). Water chemistry parameters of importance include dissolved organic carbon (DOC), pH, hardness, temperature and salinity, each of which will have different influences on bioavailability and toxicity, in a metal-specific manner. One metal of environmental concern, and for which there has been initial work relating toxicity to water chemistry, is nickel (Ni).

Ni is a transition metal often thought to be an essential micronutrient to plants and certain prokaryotes, however, the evidence of this essentiality for animals is less clear (Nielsen, 1993; Eisler, 1998). Like other essential transition metals such as copper (Cu) and zinc (Zn), either an excess or deficiency of Ni can decrease the overall fitness of aquatic invertebrates and vertebrates (Pane et al., 2003a; 2003b). Natural sources of Ni in the aquatic environment include erosion and weathering, while anthropogenic addition of Ni occurs from industrial practices such as combustion of fossil fuels mining, smelting and alloy processing (ECB, 2008; NAS, 1975; WHO, 1991; Eisler, 1998).

Most of our knowledge of aquatic Ni toxicity is from freshwater (FW) species. In particular an extensive amount of research has been performed in FW rainbow trout (*Oncorhynchus mykiss*). This is one of the most sensitive fish species to chronic Ni exposure (Nebeker et al., 1985; Brix et al., 2004), it is commercially available, and there is significant understanding of the toxic mechanisms of Ni in this species (Chowdhury et al., 2008; Deleebeeck et al., 2007; Pane et al., 2004a; 2004b; 2005). There is, however, surprisingly little information regarding the toxicity of Ni in marine and estuarine settings, even though these waters may be subject to elevated Ni exposures. For example, although Ni concentrations in uncontaminated marine waters are around 2 μg/L (0.04 μmol/L), in highly polluted coastal waters and estuarine environments Ni can be found in the range of 10 -100 μg/L (0.17 -1.7 μmol/L) (Boyden, 1975; Jiann et al., 2005; Martino et al., 2004; Paucot and Wollast, 1997; Wells et al., 2000).

An important factor that will affect bioavailability and toxicity in marine waters is salinity. Salinity not only impacts metal speciation and bioavailability, but also the physiology of aquatic organisms. In general, the levels of free Ni ion may be reduced in high salinity environments, due to the increased presence of complexing anions (e.g. SO_4^{2-} and Cl^-) (Sadiq, 1989). Furthermore, bioavailability will likely be reduced relative to FW because of higher concentrations of cations (Ca^{2+}, K^+, Na^+, Mg^{2+}). This is important because a key putative mechanism of Ni entry into aquatic organisms involves initial binding at cation binding sites, and thus a higher level of cations in the water increases competition for uptake (for review see Pyle and Couture, 2012). For osmoregulating animals, such as fish, salinity also significantly alters ion transport physiology. A FW fish maintains a higher body osmolarity than its surroundings and thus is faced with ion loss. To compensate, epithelial surfaces such as the gill are focused on ion uptake (Evans et al., 2005). The opposite occurs in seawater (SW), where the fish is faced with

ion influx, and the gill is primarily an ion excretion tissue. For these reasons the nature and magnitude of potential uptake pathways for cations, and therefore Ni, will likely differ between FW and SW environments.

Evidence has led mammalian researchers to suggest that Ni, like metals such as Cu and lead (Pb), is not only a respiratory toxicant (Eisler, 1998), but also has the potential to impair cellular respiration and oxidative balance (Stohs and Bagchi, 1995). Pro-oxidant toxicants are thought to generate enhanced production of reactive oxygen species (ROS), such as the superoxide radical. These are highly unstable molecules with unpaired electrons that react readily with components of cells, including DNA, proteins and lipids, resulting in physiological impairment, cell death and severe detriment to the organism. Induction of ROS species and oxidative damage by other metals such as Cu, Pb, cadmium (Cd), arsenic (As) and Zn (Stohs and Bagchi, 1995; Faverney et al., 2001; Craig et al., 2007; Loro et al., 2012) have been widely reported in aquatic organisms. Notably, increased salinity was highly protective against Zn-induced oxidative stress in the euryhaline killifish (Loro et al., 2012). However, the ability of Ni to induce oxidative stress in aquatic organisms has been only sparsely investigated. One group of researchers has studied a single species, the goldfish (*Carassius auratus*) (Kubrak et al., 2012a,b; Kubrak et al., 2013; Kubrak et al., 2014), while the potential impact of salinity on these euryhaline organisms has not yet been studied.

The goal of the current study was to investigate the impact of salinity (FW *versus* 100% SW) on Ni accumulation and oxidative stress (a sub-lethal measure of toxicity) on a tissue-specific basis. The aim was to determine if Ni was a pro-oxidant and if so, whether the mechanism was specific to ROS production and damage (as assessed by protein carbonyl formation – Bainy et al., 1996), or *via* inhibition of antioxidant enzymes (as indicated by catalase

(CAT) activity – for review see Lushchak, 2011). For this study the Atlantic killifish (*Fundulus heteroclitus*) was chosen, as this species is a model organism for estuarine toxicity studies (Burnett et al., 2007) and has been studied previously with respect to Zn-induced oxidative stress as a function of salinity (Loro et al., 2012). In the present study we hypothesized that Ni would induce oxidative stress and that decreased salinity would increase Ni bioavailability, thereby further stimulating indices of oxidative stress.

5.3 MATERIALS AND METHODS

5.3.1 Animal care

Atlantic killifish *Fundulus heteroclitus* (weight 3.87 ± 0.83 g) were obtained from Aquatic Research Organisms (ARO) Ltd. (Hampton, NH, U.S.A). These animals were collected *via* beach-seining methods, transported to McMaster University, and placed in 250-L aquaria. The water was maintained at a salinity of 3.5 ppt (10% SW), which was recirculated through charcoal filters. Aquarium water was changed every 2 to 3 days. Fish were keep in constant temperature (20˚C) and photoperiod (12 h light:12 h dark) conditions. Fish were held at 3.5 ppt for several weeks prior to experimentation, and then acclimated to the two salinities (0 ppt (FW) and 35 ppt (100% SW)) under these temperature and photoperiod conditions (acclimation of 7 days for SW and 14 days for FW). SW was made by the addition of Instant Ocean sea salt (Woodbridge, ON, Canada) to FW, considering 35 grams per liter as 35 ppt (100%) salinity. FW was dechlorinated City of Hamilton tap water (moderately hard: [Na^+] = 0.6 mequiv/L, [Cl^-] = 0.8 mequiv/L, [Ca^{2+}] = 1.8 mequiv/L, [Mg^{2+}]= 0.3 mequiv/L, [K^+] = 0.05 mequiv/L; titration alkalinity 2.1 mequiv/L; pH ~8.0; hardness ~140 mg/L as $CaCO_3$ equivalents; see Table 5.1 for full water chemistry). During acclimation fish were fed once a day to satiation with commercial fish flakes (Wardley

Total Tropical Gourmet Flake Blend, Hartz Mountain Corp., Secausus, NJ, U.S.A). Fish were fasted for 48 hours prior to the start of all experiments. All procedures were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

5.3.2 Ni exposures at different salinities

After the acclimation period ended, fish were divided into one of 12 treatments (N = 6 fish per treatment, unless otherwise stated), conducted in separate 8-L aquaria. The biomass load was approximately 3 g per L. There were a total of 3 Ni exposure concentrations (5, 10 and 20 mg/L) and 3 respective controls run per salinity (FW and 100% SW) for 96 h. All Ni was added from a concentrated stock of $NiCl_2 \cdot 6H_2O$ (trace metal grade, Sigma Aldrich, St. Louis, Missouri, USA) at the beginning of each exposure. Tanks were dosed 24 hours before fish introduction to ensure equilibrium was reached between Ni and exposure water. Over the course of the 96 h, 80% water changes occurred daily (with water pre-equilibrated with the appropriate level of Ni), and no water filters were used during this time to prevent the loss of Ni. At the end of 96 hours fish were terminally euthanized with MS-222 (NaOH neutralized pH 7). Tissues (gill, intestine and liver) were then taken for analysis of Ni (below) and oxidative stress measurements (Section 5.3.4).

Each tissue was weighed, and then placed in 2-mL centrifuge tube. Tissues were digested with 1N trace metal grade nitric acid (Sigma-Aldrich) at volumes of 3-5 times (exact volume recorded) the weight of the tissue. Tubes were tightly sealed and placed in an incubator at 65°C for 48 h, with vigorous vortexing at 24 h. The digested samples were then centrifuged for 5 min at

3500 rpm at 18°C. Volumes of the resulting supernatant, all 1 mL, were taken for analysis, as detailed below.

5.3.3 Ni bioaccumulation, ion and water analyses

Ni concentrations were monitored daily and after every water change, to ensure that metal exposure remained constant. Water samples for ions and dissolved Ni concentrations were collected *via* passage through a 0.45 µm syringe filter (Acrodisc; Pall Life Sciences, Houston, TX, USA). Unfiltered samples were also taken for total Ni measurements. However, since total Ni (N=10) was less than 3% different from dissolved Ni, only the latter measurements have been shown. All Ni measurements 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 95 ± 1% as determined by Environment Canada certified reference materials, TM 24.3 and TM 25.3 and DORT-1 dogfish liver for tissue measurements. Ni concentrations were not corrected for recovery. Water ions (K^+, Na^+, Mg^{2+} and Ca^{2+}) were measured *via* Flame Atomic Absorption Spectroscopy (FAAS; Varian SpectraAA FS-220, Mulgrave, Australia)(Table 5.1). All ion measurements were measured against reference standard solutions (Fischer Scientific, Ottawa, ON) using standard curves. Water pH was measured by an Accumet Basic AB15 pH meter (Fisher Scientific, Ottawa, ON). Total DOC was measured using a Shimadzu TOC-Vcph/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan).

5.3.4 Oxidative stress assays

All assays were performed on gill, intestine and liver tissue. Tissue samples were prepared using two different homogenization buffers (each 1:20 weight:volume). For protein

carbonyl determination a buffer containing 50mM MES and 1 mM EDTA at a pH of 6.7 was used. A MES-based buffer was preferred over a Tris-based buffer as the latter can alter protein carbonyl content. Resulting homogenates were centrifuged at 10, 000 g for 20 min at 4°C. Thereafter, protein carbonyl content was determined in two ways. The first method was via a commercial kit (Protein Carbonyl Colorimetric Assay Kit by Cayman Chemicals, Ann Arbor, MI, USA) with the incorporation of a 1% streptomycin sulfate solution, which was added to supernatants to remove nucleic acids, as these can contribute to a higher estimation of carbonyls. The second method used a different commercial kit (Sigma-Aldrich Protein Carbonyl Content Assay Kit; Sigma Aldrich, St. Louis, MO, USA), also using streptomycin. Because two different methods were used to determine protein carbonyls, the data were normalized against common reference samples. Protein carbonyl contents are reported as nmol/mg protein.

CAT activity was determined according to the methods described by Claiborne (1985). Briefly, samples were homogenized in a buffer containing 20 mM HEPES, 1 mM EDTA and 0.1% Triton, 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. To enable expression of endpoints on a per mg protein basis, protein content was assayed according to the Bradford Reagent assay (Bradford, 1976) using bovine serum albumin as a standard.

5.3.5 Calculations and statistical analysis

Statistical tests were performed with SigmaPlot 10.0 for linear and non-linear curve fitting, and Sigma Stat 3.5 or Statistica 10 (Statsoft) for comparisons of means. All data have been expressed as means ± SEM (N = number of fish) and for all treatments, N = 6 was used, unless

otherwise stated. All Ni exposure data were initially analyzed via two-way ANOVA where salinity and Ni concentration were the two factors of interest. Where significance ($P < 0.05$) was found, a Fisher post-hoc test was applied to delineate which data points differed from each other. In addition, due to the presence of time-matched controls, a Student's unpaired two-tailed t-test was used to assess statistical differences between Ni-exposed and control values within a tissue at a given Ni exposure level. The water chemistries recorded in Tables 5.1 and 5.2 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 on speciation the NICA-Donnan model was used (Benedetti et al., 1995) (Table 5.3).

5.4 RESULTS

5.4.1 Dissolved Ni exposure concentrations

Measured dissolved Ni concentrations were close to nominal values, and FW and SW exposures were closely matched in terms of their Ni concentrations (Table 5.2). Mean control values ranged from 2.0 µg/L in FW to 2.5 µg/L in SW treatments. Dissolved Ni levels were closely matched to total Ni (< 3% difference for all exposures), and thus only dissolved measured Ni values are reported here. Speciation analysis determined that there was a modest difference in the speciation between FW and SW with regards to free Ni ion (FW = 85%; SW = 77 %; Table 5.3). Ni^{2+} was the dominant species in both FW and SW while the second most dominant species of Ni was $NiCO_3^+$ in FW and $NiSO_4$ in SW.

5.4.2 Concentration-dependent Ni accumulation in gill, intestine and liver in FW and SW

The results of the two-way ANOVA show that there was an overall effect of salinity on gill Ni accumulation ($P < 0.001$), but no overall effect of Ni concentration ($P = 0.082$), or of the interaction between these two factors ($P = 0.114$). The FW gill tissue showed an increasing pattern of Ni accumulation with increasing concentrations of Ni. The highest Ni accumulation (223 µmol/kg) occurred in the highest Ni exposure concentration (Fig. 5.1A), with this value being significantly elevated with respect to the Ni accumulation at 5 mg/L. In SW gill, Ni accumulation was of a reduced magnitude relative to that in FW (significantly so for the 5 and 20 mg/L exposures; Fig. 5.1A). Notably, Ni accumulation did not vary significantly with exposure concentration in the SW fish.

Overall, Ni concentration ($P = 0.019$), salinity ($P < 0.001$) and the interaction between these two factors ($P = 0.003$) had a significant effect on intestine Ni accumulation. Generally, however, a similar pattern to that of the FW gill occurred in the FW intestinal tissue with the 20 mg/L Ni exposure leading to a significantly greater Ni accumulation than in the two other exposure groups (Fig. 5.1B). In the SW intestine accumulation of Ni was significantly less than that of FW for the 20 mg/L exposure, but there were no significant SW *versus* FW differences in accumulation at the other two Ni concentrations, despite the fact that background Ni concentrations in the controls were lower in SW fish than in FW fish.

Liver Ni accumulation was significantly affected by Ni concentration ($P < 0.001$), salinity ($P = 0.002$), and the interaction between concentration and salinity ($P < 0.001$). The liver tissue also displayed a pattern of increasing Ni accumulation with increasing Ni exposure concentration in FW. For example, the 20 mg/L fish accumulated approximately 3- and 17-fold more Ni than the 10 and 5 mg/L groups, respectively (Fig. 5.1C). The SW group exposed to Ni displayed elevated liver Ni concentrations relative to controls at all exposure concentrations, with the

highest accumulation occurring in the 10 mg/L group (Fig. 5.1C). Only at 20 mg/L was there a significant difference between salinities: Ni accumulation in FW was 7- fold higher than for the same exposure concentration in SW.

5.4.3 Concentration-dependent protein carbonyl formation in gill, intestine, and liver in FW and SW

A two-way ANOVA showed that salinity had an overall significant effect on gill protein carbonyl content (P = 0.001), but that Ni concentration itself was not a significant factor influencing this endpoint (P = 0.734) and there was no significant interaction between the two factors (P = 0.598).

More specifically, the gill tissue displayed no change in protein carbonyl formation in any Ni treatment with respect to controls in either FW or SW groups (Fig. 5.2A). However, both the SW 5 mg/L and 10 mg/L Ni-exposed gills displayed 2-fold higher protein carbonyl content than the corresponding group in FW (Fig 5.2A). Protein carbonyls also tended to be higher in control gill tissue in SW *versus* FW.

The degree of protein carbonylation in intestinal tissue of Ni-exposed killifish was significantly influenced by Ni (P = 0.012), salinity (P = 0.001), and the interaction between Ni and salinity (P = 0.015). In the FW intestine there was a significant 2-fold increase (relative to lower Ni exposure concentrations) in protein carbonyl production following exposure to 20 mg/L Ni (Fig.5.2B). This value was also significantly higher than the level of protein carbonylation measured in the 20 mg/L group exposed in SW. Similarly, at 10 mg/L Ni there was a higher level of protein carbonyl production in FW *versus* SW. Protein carbonyls also tended to be higher in control intestinal tissue in FW *versus* SW, in contrast to the trend seen in the gills.

Ni concentration ($P = 0.002$), and salinity ($P = 0.014$) were shown by two-way ANOVA to have overall significant impacts on liver protein carbonyl content in Ni-exposed killifish. However, there was not a significant interaction between the two factors ($P = 0.068$). There were no significant differences in protein carbonyl formation among Ni-exposed treatments in FW liver (Fig. 5.2C). However, the SW liver tissue displayed a significant decrease in protein carbonyl formation after exposure to both 5 and 10 mg/L Ni, and a significant increase from control values in the 20 mg/L exposure (Fig. 5.2C). The level of carbonylation in the SW liver tissue at 5 mg/L and 10 mg/L was significantly less than that observed in the corresponding FW liver tissue, but the SW control values also tended to be lower.

5.4.4 Concentration-dependent catalase activity in gill, intestine, and liver in FW and SW

The levels of CAT activity in the gill of killifish were significantly impacted by Ni concentration ($P < 0.001$) and salinity ($P = 0.041$), while no significant interaction was detected between the two ($P = 0.080$). In FW a significant decrease relative to the unexposed control was observed in response to 5 mg/L Ni only (Fig. 5.3A). The level of CAT activity in SW gill was significantly higher than in FW when the fish were exposed to 20 mg/L Ni. However, there was also some significant variation in the controls in the SW fish.

Overall, Ni concentration ($P <0.001$), salinity ($P <0.001$), and the interaction between these two factors ($P <0.001$) were all shown to have a significant impact on intestinal CAT activity. No change in intestinal CAT activity occurred in fish exposed to 5 and 10 mg/L Ni in FW, relative to their time-matched controls. Mirroring the effect of protein carbonyl formation there was, however, a 10-fold increase in CAT activity relative to control tissue in the 20 mg/L

exposure, a value that was also significantly different from the other two Ni FW exposure concentrations and significantly higher than the SW 20 mg/L Ni group (Fig. 5.3B).

For liver CAT activity, a two-way ANOVA illustrated an overall effect of the interaction between Ni concentration and salinity (P = 0.001) but no significant effect was obtained with either Ni concentration or salinity alone (P = 0.057, P = 0.318 respectively). In FW liver tissue there were no significant differences in CAT activity in the Ni-exposed liver relative to their respective controls (Fig. 5.3C). The only significant impact of Ni in SW was an elevation in CAT activity in the 20 mg/L exposure group, relative to the unexposed control (Fig. 5.3C). This value was also significantly elevated relative to the CAT activity in liver exposed to 5 mg/L in SW. In both the 5 mg//L and 20 mg/L concentrations there were significant differences between FW and SW. In the 5 mg/L group CAT activity was significantly lowered in SW compared to the FW counterpart, while the opposite effect was displayed in the 20 mg/L grouping (Fig. 5.3C). However, these same differences, though less pronounced, were also seen in the respective controls.

5.5 DISCUSSION

Salinity clearly altered both Ni accumulation and oxidative stress responses in killifish. As Ni exposure levels increased, SW reduced Ni accumulation in all tissues, such that it tended to lose dose-dependency. However, the effect of this reduced accumulation on the oxidative stress response was strongly tissue dependent. Much lower CAT activity and protein carbonyl production were observed in the intestine of SW *versus* FW animals at the highest Ni exposure levels, whereas in the liver, responses were concentration-dependent such that SW reduced oxidative stress markers at lower Ni exposure levels, and increased CAT activity at 20 mg/L of

Ni. Overall, this study provided evidence that Ni exposure can induce oxidative stress in *Fundulus heteroclitus*.

5.5.1 Salinity-dependent and tissue-specific patterns of Ni accumulation

There are two key factors that will affect the accumulation of Ni in the gills of the killifish. The first factor is the difference in chemical composition between FW and SW environments. Speciation analysis (Table 5.3) indicated that there were some modest differences in the species of Ni present between FW and SW (more SO_4^{2-} and Cl^- complexes, less carbonate complexes in SW *vs.* FW). However, more importantly, increases in salinity result in greatly increased concentrations of cations, which will compete with Ni for binding and/or transport sites present at the gill (Pyle and Couture, 2012). The lower overall Ni accumulation in SW gills relative to FW gills supports the concept that the differences in accumulation could be driven by this cation competition effect (Fig. 5.1A).

The second factor that will influence accumulation in the gill is physiology. The gills are a critical homeostatic tissue, whose structure and function will change with salinity. Consequently, metal uptake and toxicity may also be altered. In FW, killifish are hyperosmotic, and in response to diffusive ion loss and water gain, they recover lost ions *via* active uptake at the gills and excrete large volumes of dilute urine (Evans et al, 1999; Evans et al, 2005; Hwang et al., 2011; Wood, 2001). In SW, the gill serves as an excretion pathway for major ions. If Ni is transported into the fish *via* mimicry of an ion transport pathway (Bury et al., 2003; Büsselberg, 1995; Clarkson, 1993), then it would be expected that Ni uptake would be greater in FW than in SW, the pattern which is observed in Figure 5.1A. It is not possible under the current exposure conditions to determine whether the reduced cation competition or the active ion absorbing

phenotype of FW fish is most responsible for the higher gill Ni accumulation in FW killifish. It is also unclear why the Ni accumulation pattern tends to lose dose-dependency in SW.

Regardless of the factors responsible for the salinity-dependence of Ni accumulation at the gill, it is likely that the gill, as the primary uptake pathway, plays a significant role in influencing accumulation patterns in other key tissues. Notably, both the liver (Fig. 5.1C) and the intestine (Fig. 5.1B) exhibited similar, though not identical, Ni bioaccumulation patterns relative to those of the gill (Fig. 5.1A). Once Ni has been accumulated at the gill it can be transported through the blood as either the free ion or bound to albumins (Glennon and Sarkar, 1982) and will end up in the liver for storage and excretion as for other toxicants. The liver has been previously characterized as a tissue of Ni accumulation in fish (Sreedevi et al., 1992). This route of detoxification ends with the liver "dumping" the toxicant *via* the bile into the anterior intestine for excretion *via* the gut (Blom et al., 2000; Hauser-Davis et al., 2012). Because of this system, transport from the gill to the liver and then to gut, a similar pattern of accumulation in tissues "downstream" of the gill is observed.

However, there is likely to also be a significant direct influence of physiology on accumulation in the gut. Levels of Ni in the gut (Fig. 5.1B) were higher than those of the gill (Fig. 5.1A) in both SW and FW, particularly as exposure Ni concentrations increased. This is likely to represent uptake of waterborne Ni *via* drinking, as well as accumulation *via* the bile. There are studies that suggest drinking does occur in FW, however, at rates that are much lower than in SW teleosts (Blewett et al., 2013; Scott et al., 2005), which are known to drink to support intestinal water uptake in light of osmotic losses at the gills. It might therefore be expected that in SW, elevated drinking would lead to higher levels of Ni accumulation in the intestine. This was not observed in the current study, and indeed at a Ni exposure concentration of 20 mg/L, SW killifish

displayed a significantly lower intestinal Ni accumulation (Fig. 5.2B). This might indicate a cation competition effect limiting Ni absorption, as occurs in the gill, and/or Ni-binding by gastrointestinal mucus that, following sloughing, might limit Ni uptake. It is known, for example, that other metals at high levels stimulate intestinal mucus production in fish, and this is thought to be effective in limiting metal absorption (Glover and Hogstrand, 2002; Khan and McGeer, 2013).

5.5.2 Tissue-specific patterns of protein carbonyl formation as a function of salinity and Ni exposure

Protein carbonyls form when ROS directly attack proteins leading to the formation of a carbonyl moiety (Bainy et al., 1996). This non-reversible damage can cause decreased catalytic activity of enzymes and will result in the eventual breakdown of the protein by proteases (Zhang et al., 2010). In the present study protein carbonyls in the gill were only elevated in response to increasing salinity, and not in response to waterborne Ni (Fig. 5.2A). The gill is a critical tissue mediating physiological changes associated with altered salinity, and undergoes significant morphological transformationwith changes in environmental salinity (Evans et al., 2005; Perry, 1997). Consequently, this can lead to increased ROS and enhanced oxidative damage in this tissue. For example, sturgeon gradually acclimated from FW to SW (35 ppt), showed elevated cortisol levels, antioxidant enzymes (including catalase and superoxide dismutase), and levels of lipid peroxidation relative to those observed in FW (Martínez-Alvarez et al., 2002). This indicates that salinity caused physiological changes in the antioxidant system, an observation consistent with salinity-dependent changes in protein carbonyl levels in the gill, intestine and liver of killifish (Fig. 5.2). In the intestine (Fig. 5.2B) and the liver (Fig. 5.2C), protein carbonyl levels tended to be lower in SW controls than in FW controls, the opposite pattern from that at the gills

(Fig. 5.2A). However, these salinity-dependent differences in control killifish were not seen by Loro et al. (2012) in the same species. The reason for this discrepancy is not known.

Unlike the gill, the intestine and liver protein carbonyl levels were affected by Ni exposure. More specifically, in the intestine of FW killifish, levels of protein carbonylation were ~2-fold higher than in control fish (Fig. 5.2B). This increase corresponds well with intestinal Ni accumulation in this group (Fig. 5.1B) and indicates that Ni is having a pro-oxidant effect in this tissue. The exact mechanism by which Ni exerts oxidative stress is not clear. Ni could directly increase ROS formation by replacing Fe in the Fenton/Haber Weiss reactions (where Ni^{2+}/Ni^{3+} rather than Fe^{2+}/Fe^{3+} redox coupling occurs leading to the production of ROS) (Torreilles and Guerin, 1990). Alternatively, it could inhibit the antioxidant response, thereby impairing defense against ROS production. There are two proposed mechanisms by which Ni could impair antioxidant defense. Ni is known to suppress antioxidant enzymes, with CAT in particular being highly sensitive to Ni toxicity (Cartañá et al., 1992; Rodriguez et al., 1990, Kubrak et al., 2013). However, this clearly did not occur in the 20 mg/L FW exposure group, because CAT activity was greatly elevated (Fig. 5.3B). Secondly, Ni is thought to reduce non-enzymatic ROS scavenging by creating Ni complexes with reduced scavenging ability (e.g. Ni-glutathione (GSH) complexes) (Kręźel et al., 2003, Salnikow et al., 1994). Regardless, it seems likely that the enhanced levels of Ni accumulation leads to Ni-induced oxidative stress in FW intestine, and effects are not observed in SW owing to the reduced bioavailability of Ni in this tissue at this salinity. This is consistent with the findings of Loro et al. (2012) in the same species acutely exposed to high waterborne Zn.

In the killifish liver the main effects of Ni exposure were a significant decrease in protein carbonylation at low Ni exposure levels, and an increase in this endpoint at the highest Ni

exposure level tested. These effects were observed only in SW, indicating that salinity was a major factor in shaping this response. A lack of oxidative damage in liver is perhaps not surprising. Although this tissue accumulates Ni, it also contains the highest levels of antioxidant defenses (e.g. Trenzado et al., 2006), which would act to limit toxic impacts.

5.5.3 Tissue-specific patterns of catalase activity as a function of salinity and Ni exposure

Catalase is an important antioxidant enzyme that aids in the decomposition of hydrogen peroxide (formed by the action of superoxide dismutase from superoxide radicals) to water and oxygen. Catalase exists in all tissues of fish, but generally the liver has the highest baseline activity (Trenzado et al., 2006), a finding also seen in the present study. Catalase was chosen as a representative antioxidant defense enzyme as it is considered to be the most sensitive to Ni (Cartañá et al., 1992; Rodriguez et al., 1990).

Catalase responses seemed to be tissue-dependent. Gill CAT displayed a significant decrease from controls only in the 5 mg/L group in FW (Fig. 5.3A). Generally, decreases in CAT activity in response to metal exposure are attributed to inhibition of the enzyme by the metal (e.g. Cu; Grosell, 2012). Previous evidence has shown that CAT catalytic activity is largely dependent on protein histidine residues, which Ni has a high affinity for (e.g. Predki et al., 1992; Mate et al., 1999). It is noteworthy that branchial Ni accumulation levels were higher in the FW exposure group at 5 mg/L than in the corresponding SW group (Fig. 5.1A), which might support such an effect. Arguing against this, however, is that greater levels of accumulation in higher Ni exposure groups did not lead to CAT inhibition. A previous study exposing goldfish to Ni showed that at levels of 10 mg/L, Ni caused increased branchial CAT activity, but this effect diminished slightly as Ni concentration increased (Kubrak et al., 2013). An increased activity may be attributed as an

effective response to greater ROS associated with pro-oxidant accumulation. It could be that the exact impact of branchial Ni (no effect or direct inhibition of activity by Ni or indirect upregulation of defense pathway) is concentration-dependent. In the case of the gill, Ni accumulation levels were insufficient to lead to an upregulation of CAT activity, whereas in the intestine at 20 mg/L, a marked stimulation was observed. It is possible that at some levels of accumulation these two effects may have counteracted each other leading to no change.

In the liver at the 5 mg/L Ni exposure level, FW and SW animals accumulated similar levels of Ni (Fig. 5.2C). Similarly, although the FW liver at 20 mg/L accumulated significantly more Ni and showed no change in CAT response relative to its control, the SW tissue, which accumulated less Ni, showed a significant upregulation of CAT. As discussed above this may relate to salinity-dependent antioxidant profiles (i.e. differing levels of other antioxidant defense enzymes, and non-enzymatic defenses). Furthermore differences in Ni bioavailability could play a role. For example, subcellular fractionation of Ni can have an important role in toxicological outcomes depending on whether Ni partitions in metal-sensitive or metal-insensitive fractions within cells of a given tissue. Indeed, subcellular Cu distribution has an important impact on sensitivity to this metal in FW fish (Eyckmans et al., 2012). Ni does appear to bind principally to metal-insensitive subcellular fractions in the liver (Lapointe and Couture, 2009), but once a certain threshold is reached it can associate with metal-sensitive cellular components (Campbell et al., 2008; Lapointe et al., 2009). This situation was observed in both the gill and gut of trout and round goby, where Ni was found to be in the metal-sensitive component (Leonard et al., 2014). This may be a factor that dictates whether relationships exist between Ni exposure, Ni accumulation, and Ni effects for given exposure concentrations and tissues of interest.

Consequently, the response of a tissue to Ni will depend on a number of factors asides from simply water chemistry and whole animal physiology.

5.6 CONCLUSIONS

These data suggest that Ni causes oxidative stress to killifish and that this indicator of Ni toxicity is salinity-dependent. Killifish are estuarine fish and therefore inhabit areas where effluent outflows may occur. It is important in terms of risk assessment to understand sub-lethal endpoints such as oxidative stress under conditions of changing salinity to develop tools that will accurately determine potential damage and regulate water quality accordingly.

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5.7 TABLES AND FIGURES

Table 5.1. Water chemistry for exposures, (N = 10). Reported values are means ± SEM. Values sharing letters are not significantly different between the two salinities.

Parameter	Freshwater (0 ppt)	100% Seawater (35 ppt)
pH	7.78 ± 0.06[a]	8.02 ± 0.04[a]
Temperature (°C)	20	20
DOC (mg/L)	2.17 ± 0.35[a]	2.48 ± 0.45[a]
Na^+ (mmol/L)	0.65 ± 0.03[a]	471.4 ± 5.3[b]
Mg^{2+} (mmol/L)	0.33 ± 0.00[a]	44.67 ± 0.76 [b]
K^+ (mmol/L)	0.056 ± 0.001[a]	11.03 ± 0.60[a]
Ca^{2+} (mmol/L)	0.84 ± 0.01[a]	9.56 ± 0.11[b]

Table 5.2. Dissolved Ni exposure concentrations (µg/L) in both FW and SW (N = 10).

Concentration	Freshwater (0 ppt) (mg/L)	Seawater (35 ppt) (mg/L)
Control	0.002 ± 0.000	0.002 ± 0.000
5 mg/L	5.3 ± 0.0	4.8 ± 0.1
10 mg/L	10.2 ± 0. 2	9.4 ± 0.2
20 mg/L	19.4 ± 1.1	19.3 ± 0.4

Table 5.3. Ni speciation (% of total Ni) as calculated by Visual MINTEQ based on recorded and nominal water chemistry.

Species of Ni	Freshwater (0 ppt) (%)	Seawater (35 ppt) (%)
Ni^{2+}	84.83	76.95
Ni-DOC	2.65	4.50
$NiOH^{+}$	0.46	0.16
$Ni(OH)_2$	0.04	0.00
$NiCl^{+}$	0.02	3.39
$NiCl_2$	0.00	0.06
$NiCO_3$	4.03	2.80
$NiCO_3^{+}$	4.24	4.54
$NiSO_4$	3.73	7.60

These values were calculated on the basis of a Ni concentration of 5 mg/L, although there was only minimal difference in proportional speciation between different Ni concentrations.

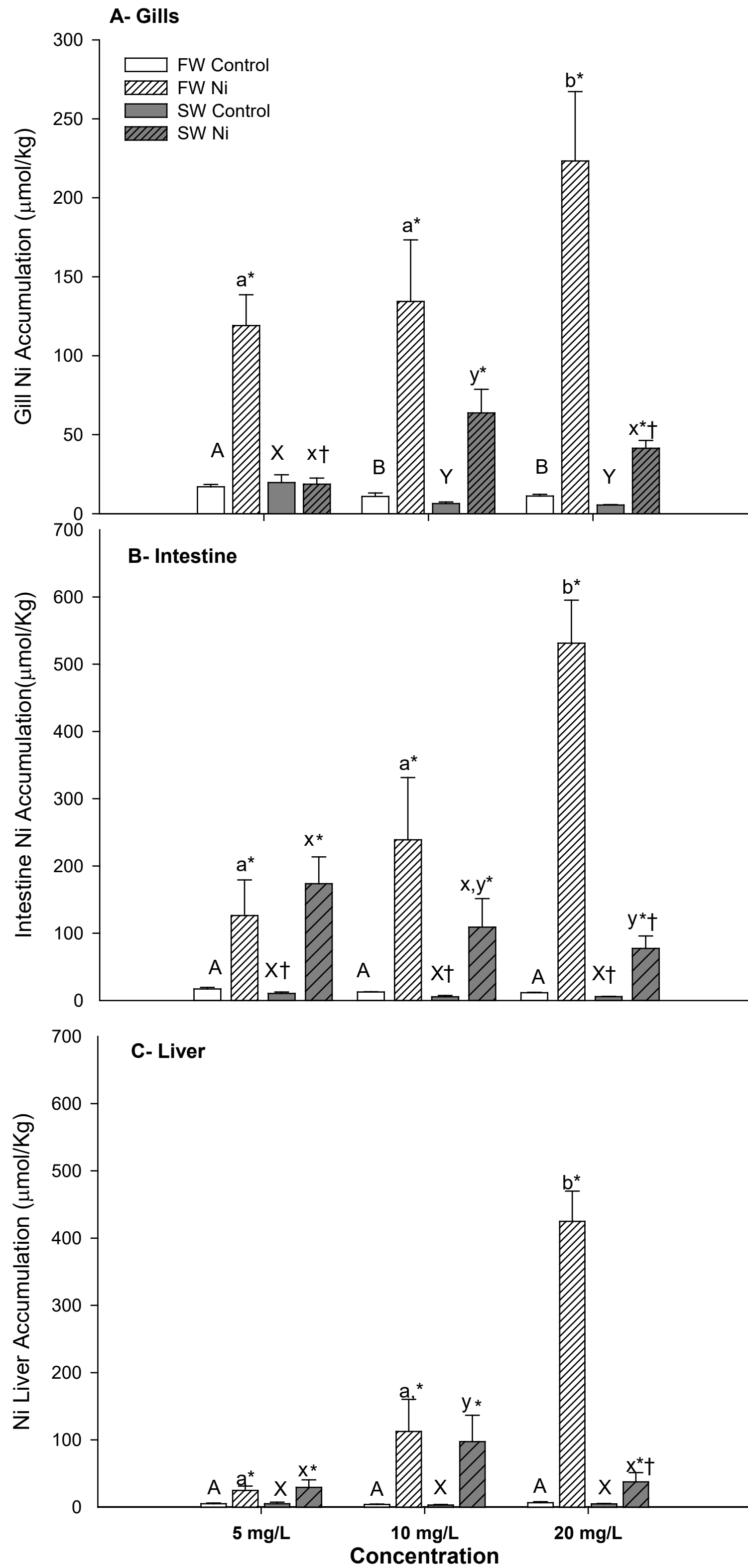
A- Gills
FW Control
FW Ni
SW Control
SW Ni
Gill Ni Accumulation (μmol/kg)
0
50
100
150
200
250
300
A
a*
X
x†
B
a*
Y
y*
B
b*
Y
x*†
B- Intestine
Intestine Ni Accumulation(μmol/Kg)
0
100
200
300
400
500
600
700
A
a*
X†
x*
A
a*
X†
x,y*
A
b*
X†
y*†
C- Liver
Ni Liver Accumulation (μmol/Kg)
0
100
200
300
400
500
600
700
A
a*
X
x*
A
a,*
X
y*
A
b*
X
x*†
5 mg/L
10 mg/L
20 mg/L
Concentration

Figure 5.1. Ni accumulation in tissues of Ni-exposed and control killifish (*Fundulus heteroclitus*) after a 96 h exposure at three different Ni concentrations (5, 10 and 20 mg/L) in either FW or SW, where A) gill, B) intestine and C) liver. Plotted values are means ± SEM (N = 6 per treatment except the 5 mg/L group where N= 5). Asterisks indicate significant differences within a concentration compared to the respective control. Lower case letters (a,b,c for FW; x,y,z for SW) indicate significant differences between Ni concentrations within a salinity group. Upper case letters (A,B,C for FW; X,Y,Z for SW) indicate significant differences between controls within a salinity group. Means sharing the same letter are not significantly different. Daggers indicate differences between salinities (FW *versus* SW) within a concentration series.

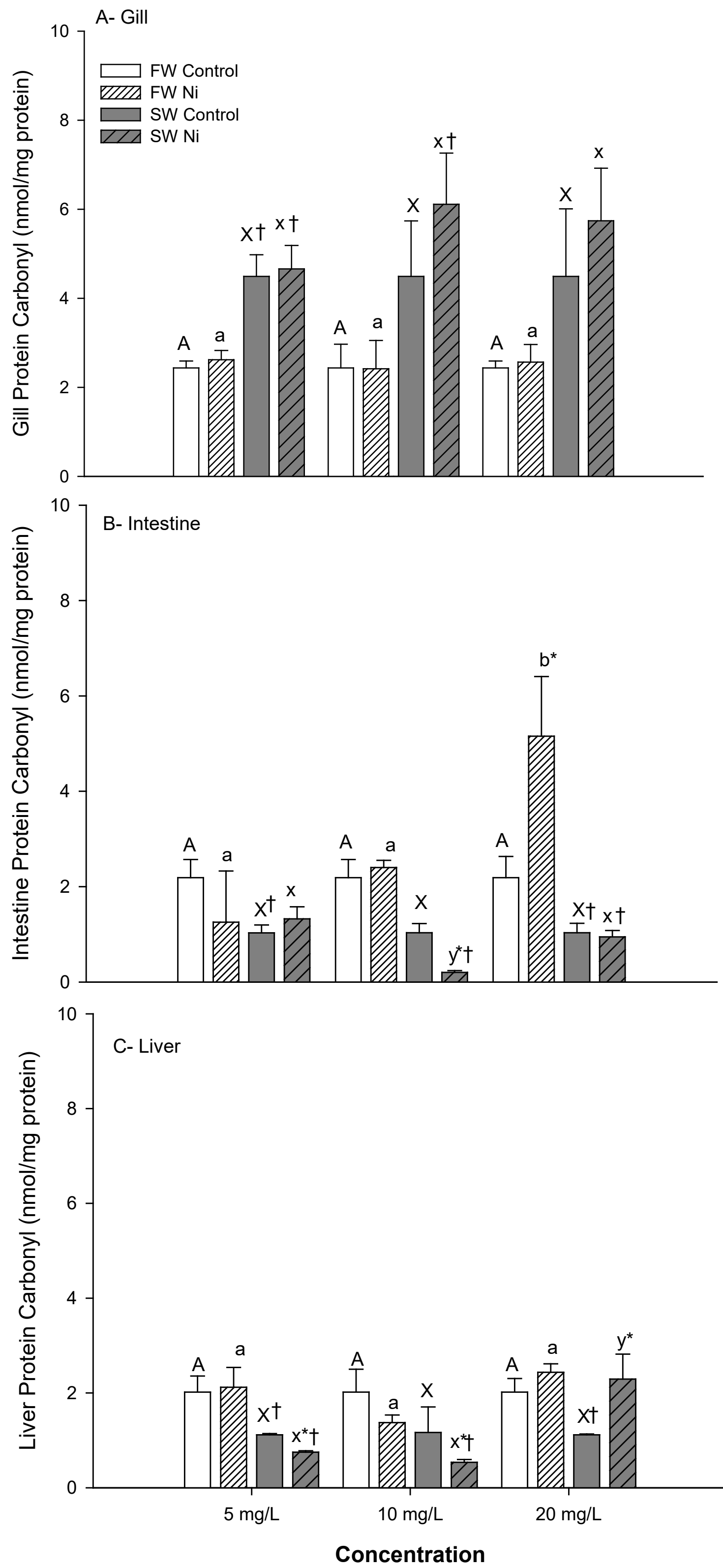
A- Gill
FW Control
FW Ni
SW Control
SW Ni
Gill Protein Carbonyl (nmol/mg protein)
B- Intestine
Intestine Protein Carbonyl (nmol/mg protein)
C- Liver
Liver Protein Carbonyl (nmol/mg protein)
5 mg/L
10 mg/L
20 mg/L
Concentration

Figure 5.2. Protein carbonyl content expressed as nmol/mg protein in tissues of Ni-exposed and control killifish (*Fundulus heteroclitus*) after a 96 h exposure at three different concentrations (5, 10 and 20 mg/L) in either FW or SW, where A) gill, B) intestine and C) liver. Plotted values are means ± SEM (N = 6 per treatment except the 5 mg/L group where N= 5). Asterisks indicate significant differences within a concentration compared to the respective control. Lower case letters (a,b,c for FW; x,y,z for SW) indicate significant differences between Ni concentrations within a salinity group. Upper case letters (A,B,C for FW; X,Y,Z for SW) indicate significant differences between controls within a salinity group. Means sharing the same letter are not significantly different. Daggers indicate differences between salinities (FW *versus* SW) within a concentration series.

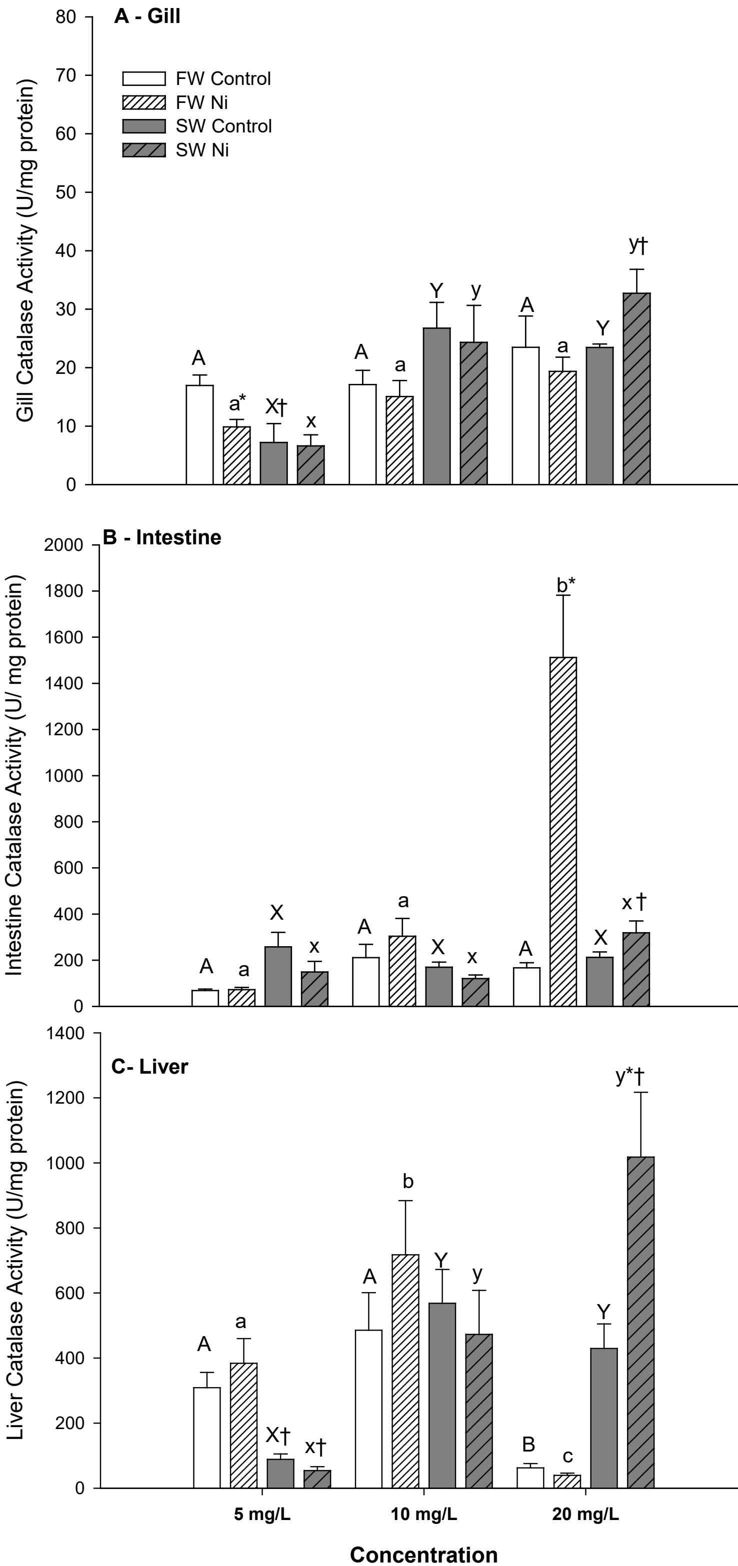
A - Gill
FW Control
FW Ni
SW Control
SW Ni
Gill Catalase Activity (U/mg protein)
B - Intestine
Intestine Catalase Activity (U/ mg protein)
C- Liver
Liver Catalase Activity (U/mg protein)
5 mg/L
10 mg/L
20 mg/L
Concentration

Figure 5.3. Catalase activity expressed as U/mg protein in tissues of Ni-exposed and control killifish (*Fundulus heteroclitus*) after a 96 h exposure at three different concentrations (5, 10 and 20 mg/L) in either FW or SW, where A) gill, B) intestine and C) liver. Plotted values are means ± SEM (N = 6 per treatment except the 5 mg/L group where N= 5). Asterisks indicate significant differences within a concentration compared to the respective control. Lower case letters (a,b,c for FW; x,y,z for SW) indicate significant differences between Ni concentrations within a salinity group. Upper case letters (A,B,C for FW; X,Y,Z for SW) indicate significant differences between controls within a salinity group. Means sharing the same letter are not significantly different. Daggers indicate differences between salinities (FW *versus* SW) within a concentration series.

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CHAPTER 6

INVESTIGATING THE MECHANISMS OF NI UPTAKE AND SUB-LETHAL NI TOXICITY IN THE ATLANTIC KILLIFISH *Fundulus heteroclitus*

6.1 ABSTRACT

The Atlantic killifish (*Fundulus heteroclitus*) is a resilient estuarine species that may be subjected to anthropogenic contamination of its natural habitat, by toxicants such as the metal nickel (Ni). This study aimed to investigate Ni accumulation, and potential modes of Ni toxicity, in killifish, as a function of environmental salinity. Killifish were acclimated to 4 different salinities [0, 10, 30 and 100% seawater (SW)] at an exposure level of 5 mg/L of Ni for 96 h. Tissue Ni accumulation, whole body ions, critical swim speed and oxidative stress parameters were examined. SW was protective against Ni accumulation in the gills and kidney. Studies where Mg and Ca were added to FW showed a protective effect of these cations against gill Ni accumulation, suggesting competition with Ni for uptake. Concentration-dependent Ni accumulation in the gill exhibited saturable relationships in both FW- and SW-acclimated fish. However SW fish displayed a lower B_{max} (i.e. lower number of Ni binding sites) and a lower K_m (i.e. higher affinity for Ni binding). No effect of Ni exposure was observed on critical swim speed (U_{crit}) or maximum rate of oxygen consumption (MO_2). Markers of oxidative stress showed either no effect (e.g. protein carbonyl formation), or had variable effects that appeared to depend more on salinity than on Ni exposure. These data indicate that the killifish is very tolerant to Ni toxicity, a characteristic that may facilitate the use of this species as a site-specific biomonitor of contaminated estuaries.

6.2 INTRODUCTION

Fundulus heteroclitus, the Atlantic killifish, is subjected to environmental conditions that vary significantly in temperature, salinity and dissolved oxygen levels. This is by virtue of their habitation of tidal marshes and estuaries. Furthermore, they are often found near densely populated urban areas where exposure to polluted water is not uncommon (Burnett et al., 2007). Due to the extensive environmental variability that they may face, killifish must be highly adaptable, a trait that is reflected in their salinity (Griffith, 1974), thermal (Bulger and Tremaine, 1985) and hypoxia (Voyer and Hennekey, 1972) tolerance. This ability to withstand extreme conditions, habitation of impacted environments, and site-fidelity (Skinner et al., 2005), coupled with the growth in basic biological knowledge of these fish in recent decades, has made the killifish a model organism in the field of environmental toxicology (Burnett et al., 2007).

Nickel (Ni) is a metal of considerable and growing toxicological interest. It is found naturally in waters, albeit it at low levels. However anthropogenic inputs such as mining and fossil fuel emissions (ECB, 2008; NAS, 1975; WHO, 1991; Eisler, 1998) can result in concentrations of Ni in marine environments that range from 1- 100 μg/L (Boyden, 1975). Estuarine habitats, such as those inhabited by killifish, are particularly susceptible to elevated Ni as they directly receive freshwater (FW) Ni inputs and physicochemical conditions can trap and concentrate metals such as Ni (Flegal et al., 1991).

Although estuaries are the aquatic settings most likely to be impacted by elevated Ni, little is known regarding the toxic effects of Ni on marine and estuarine biota. This contrasts with FW species, where Ni uptake pathways and mechanisms of toxicity have been extensively investigated. In particular, considerable research has been conducted on the FW rainbow trout (*Oncorhynchus mykiss*). Rainbow trout are considered to be one of the most sensitive fish species

to Ni exposure (Brix et al., 2004), and an understanding of toxic mechanisms in this species is reasonably well developed (Chowdhury et al., 2008; Deleebeeck et al., 2007; Pane et al., 2004 a,b; 2005). The main mode of Ni toxicity in FW rainbow trout is *via* inhibition of respiratory gas exchange, primarily mediated by histological changes in the gill (Pane et al., 2004b). This mechanism does not hold for all FW animals though, as in the model invertebrate *Daphnia magna*, Ni toxicity manifests mainly as changes in ionoregulatory status (Pane et al., 2003b). There is also evidence that oxidative stress may be an important mode of Ni toxicity in FW fish (Kubrak et al., 2012a,b; Kubrak et al., 2013; Kubrak et al., 2014; Palermo et al., 2015). Despite recent efforts (e.g. Bielmyer et al., 2013; Blewett and Wood, 2015a,b (Chapters 5,3); Blewett et al., 2015a,b (Chapters 2,4); Leonard et al., 2011) there is still much to learn regarding Ni uptake and toxicity in biota inhabiting waters of elevated salinity.

One of the significant differences between FW and estuarine/marine environments is water chemistry. This is likely to have a substantial influence over Ni speciation and bioavailability, which in turn impacts uptake and toxicity (Di Toro et al., 2001; Paquin et al., 2002). The bioavailable form of Ni is considered to be the free Ni ion (Niyogi and Wood, 2004; Wood, 2012), which is able to be taken up by the gill and thought to be the most toxic to organisms (Niyogi and Wood, 2004). The amount of Ni absorbed will depend on water chemistry factors that complex (e.g. anions such as Cl^- or SO_4^{2-}) or compete (e.g. Ca^{2+} or Mg^{2+}) with Ni, preventing uptake, and the physiological characteristics of the gill (i.e. numbers and affinities of transport pathways). This is the basis of the biotic ligand model (BLM), which utilizes knowledge of water chemistry and metal burdens at the gill (a reflection of gill physiology) to predict toxicity (Paquin et al., 2002).

When salinity increases, the higher levels of cations will likely compete with Ni for uptake, at least by analogy with FW biota where antagonism between Ni and divalent cations has been shown (Pane et al., 2003 a,b). Furthermore, as ion transport differs between FW and sea water (SW) (at least in osmoregulating animals such as fish), the availability and binding characteristics of putative Ni uptake pathways will also likely differ. Together the combined effects of water chemistry and organism physiology are likely to significantly impact Ni accumulation, and ultimately modes of Ni toxicity.

Previous work with killifish by Blewett and Wood (2015a, Chapter 5) indicated that oxidative stress occurs in response to Ni exposure, and that the magnitudes of responses are both salinity- and Ni-dependent. However, this study only examined these endpoints at salinity extremes (FW and 100% SW). One goal of the current study was to examine oxidative stress responses to Ni in killifish acclimated to a range of salinities. Furthermore, to date no study has examined ionoregulatory or respiratory modes of toxicity in killifish, both of which were investigated in the current investigation. Finally, in order to better understand the mechanism of Ni accumulation in more detail, the concentration-dependence of branchial Ni uptake and the effects of water chemistry manipulation on gill Ni accumulation were explored. These experiments will lead to a better understanding of Ni bioaccumulation and toxicity mechanisms in marine organisms, and thus may contribute essential information to the eventual development of improved water quality guidelines for Ni in marine/estuarine environments. It is hypothesized that due to both the complexation of Ni with anions and the protective effect of cations present in SW, salinity will be protective of Ni accumulation and sub-lethal toxicity, and that similar to FW trout, respiratory toxicity will be the main mode of sub-lethal Ni toxicity in killifish at various salinities.

6.3 METHODS

6.3.1 Animal care

Atlantic killifish (*Fundulus heteroclitus*; northern subspecies) (1-5 g) of both sexes were obtained from Aquatic Research Organisms Ltd. (Hampton, NH, USA). Following transport to McMaster University, fish were placed in a 250-L re-circulating system, where water was pumped through charcoal filters. Aquarium water was changed every 2 to 3 days, and fish were held in a photoperiod of 12 h light:12 h dark at 18˚C. Fish were maintained in 10% SW for several weeks prior to salinity acclimation. Saline waters were made by the addition of Instant Ocean sea salt (Big Al's Aquarium Supercenter, Woodbridge, ON, Canada) to fresh water, considering 35 grams per liter as 100% SW. Fresh water was dechlorinated City of Hamilton tap water **(**moderately hard: [Na^+] = 0.6 mequiv/L, [Cl^-] = 0.8 mequiv/L, [Ca^{2+}] = 1.8 mequiv/L, [Mg^{2+}] = 0.3 mequiv/L, [K^+] = 0.05 mequiv/L; titration alkalinity = 2.1 mequiv/L; hardness ~140 mg/L as $CaCO_3$ equivalents, [Ni^{2+}] = 4 µg/L). Fish were acclimated to one of four different salinities (0%, 10%, 30% and 100% SW) under holding conditions for 7-14 days. During acclimation fish were fed once a day to satiation with commercial fish flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain Corp., Secausus, NJ, USA). Fish were fasted for 48 h prior to the start of all experiments. All procedures were approved by the McMaster University Animal Research Ethics Board and were in accordance with the Guidelines of the Canadian Council on Animal Care.

6.3.2 Ni exposures at different salinities

The effect of Ni (5 mg/L) was investigated at each of the four salinities: 0 ppt (Hamilton fresh water; FW), 3.5 ppt (10% SW), 10.5 ppt (30% SW) and 35 ppt (100% SW). At each salinity

there was also a control group (no added Ni), resulting in a total of 8 exposure groups (2 replicates), each with N = 6 (biomass load ~1.5 g/L).

For all exposures Ni was added from a concentrated stock of $NiCl_2 \cdot 6H_2O$ (Sigma Aldrich, St. Louis, MO, USA) at the beginning of each exposure. Each aquarium was pre-cleaned with 10% HNO_3. All tanks were dosed 24 h before fish were added to ensure equilibrium was reached between Ni and exposure water. Over the course of the 96-h exposure, 80% water changes occurred daily to maintain Ni levels, and no water filtering was used during exposures to prevent the loss of Ni.

6.3.3 Ni bioaccumulation, ion and water analysis

Ni concentrations were monitored daily after every water change. Water samples for ions and dissolved Ni concentrations were collected *via* passage through a 0.45-µm syringe filter (Acrodisc syringe filter; Pall Life Sciences, Houston, TX, USA). Unfiltered samples were also taken, however since total Ni concentrations differed from dissolved Ni concentrations by less than 5%, only dissolved Ni measurements are reported. All water chemistry parameters are shown in Tables 6.1, 6.2 and 6.3. After the 96-h exposures had concluded, fish were terminally euthanized with a lethal dose of MS-222 (NaOH-neutralized, pH of 7; Syndel Laboratories Ltd., Vancouver, BC, Canada). A subset of gill, intestine, and liver were sampled and quickly frozen in liquid nitrogen, before being transferred to -80˚C for eventual measurement of oxidative stress markers (see below). A second subset of tissues, including gill, intestine, kidney was taken for Ni analysis. The reported whole body measurements represent all tissues + carcass.

For Ni analysis, tissues were weighed, and depending on mass, placed in a 15-mL, or a 2-mL centrifuge tube. Trace metal grade nitric acid (1N; Sigma-Aldrich) was used to digest the

tissue at volumes of 3-5 times (exact volume recorded) the weight of the tissue, except for the whole body which was digested in a 3-5 times volume of 2N trace metal grade nitric acid. All tubes were tightly sealed and placed in an incubator at 65°C for 48 h, with vortexing after 24 h. The digested samples were then centrifuged for 5 min at 3500 rpm at 18°C. Samples of the resulting supernatant were then analysed for Ni on the Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS; Varian, SpectraAA- 220, Mulgrave, Australia; see below).

Measurements of Ni in water were made on the same GFAAS against certified atomic absorption standards (Sigma Aldrich Chemical Company, Oakville, ON, Canada). Ni recovery for both water and tissue was 91.0 ± 2.2 % as determined by Environment Canada certified reference materials, TM 15.1 and TM 25.3, and DORT-1 dogfish liver. Ni concentrations were not corrected for recovery. Ions (K^+, Na^+, Mg^{2+} and Ca^{2+}) in both water samples and whole body tissue were measured *via* Flame Atomic Absorption Spectroscopy (FAAS; Varian SpectraAA FS-220, Mulgrave, Australia). Reference standard solutions (Fisher Scientific, Ottawa, ON, Canada) were used to generate standard curves. Water pH was measured by an Accumet Basic AB15 pH meter (Fisher Scientific). Chloride in water samples was determined *via* a mercury thiocyanate-based colorimetric assay (Zall et al., 1956). DOC measurements were conducted using a Shimadzu TOC-Vcph/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan).

6.3.4 Oxidative stress assays

All oxidative stress assays were performed on gill, intestine and liver tissue. Tissue samples were prepared using a homogenization buffer (1:20 w:v) containing 10 mM Tris HCl , 2 mM EDTA and 5 mM $MgCl_2$ at a pH of 7.75. Homogenates were centrifuged at 10, 000 g for 20 min at 4°C. Reactive oxygen species (ROS) and total oxyradical scavenging capacity (TOSC)

were determined using fresh supernatant after the method described by Amado et al. (2009). Briefly, ROS is measured following its artificial generation resulting from the thermal decomposition of a fluorescent dye [ABAP; 2,2'-azobis(2-methylpropionamidine) dihydrochloride, Sigma Aldrich], and is expressed as relative area of ROS per mg protein. TOSC was estimated as the difference in ROS-generating area with *versus* without the ABAP added, relative to the fluorescence registered without ABAP. This provides an index which is inversely proportional to the total oxyradical scavenging capacity, and therefore high values represent an overall reduced capacity for scavenging. Change in fluorescence was measured on a Spectra Max Gemin XPS fluorimeter (Sunnyvale, CA, USA).

Protein carbonyl content was determined using a commercial kit (Protein Carbonyl Colorimetric Assay Kit; Cayman Chemicals, Ann Arbor, Michigan, USA), according to manufacturer instructions with some modifications. Tissues were homogenized in a buffer containing 50 mM MES, 1 mM EDTA, pH 6.7, and then centrifuged at 13, 000 g for 5 min. A 1% streptomycin sulfate solution was added to supernatants at a concentration of 10 µL per 100 µL of homogenization buffer to remove nucleic acids, as they contribute to an over-estimation of carbonyls (Reznick and Packer, 1994). Protein carbonyl contents are reported as nmol/mg protein.

Catalase activity (CAT) was determined according to the methods described by Claiborne (1995). Samples were homogenized in a buffer containing 20 mM HEPES, 1 mM EDTA and 0.1% Triton, adjusted to a pH of 7.2 and centrifuged at 13, 000 g for 5 min. Briefly, CAT activity was measured by the disappearance of the absorbance of hydrogen peroxide at a wavelength of 240 nm and a temperature of 21°C, using a quartz plate and a UV-visible spectrophotometer (SpectraMax 340 PC, Sunnyvale, CA, USA), and expressed as U/mg protein where U is µmol/min. The same supernatant was used for determination of superoxide dismutase

activity (SOD). SOD was quantified using a commercially available kit (Sigma Aldrich, St. Louis, MO, USA), based on the fact that SOD will inhibit xanthine oxidase as ROS are produced. Measurements were made by spectrophotometer (as above) at a wavelength of 440 nm. In this assay, a 50% inhibition of xanthine oxidase is considered one U of SOD, and values were expressed per mg protein. Protein content was assayed according to the Bradford (1976) assay using bovine serum albumin as a standard.

6.3.5 Critical swimming speed and oxygen consumption

In a separate exposure, conducted under identical conditions to those described in Section 6.3.2 above, killifish acclimated to either FW or 100% SW were exposed to 5 mg/L Ni for 96 h. Control groups were held under identical conditions but in the absence of added Ni. Fish (N = 7) were gently removed from the exposure chambers, measured for length (cm) and transferred to Blazka-type swim respirometers (~3.2 L) for measurement of critical swimming speed (U_{crit}). All swimming experiments were performed in the absence of Ni. Chambers were supplied with a water flow of ~300 mL/min and fish were allowed to settle for 30 min prior to experimentation. Temperature was maintained throughout the experiment by submersing the respirometers in a 60-L recirculating water bath on a wet table receiving a constant flow of water at 18°C. As the respirometers themselves heated the water, the temperature was maintained at 20ºC. Every 30 minutes, starting at a velocity based on individual fish length, water flow rate was increased by 0.75 body lengths per second (bl/s). Oxygen consumption (MO_2) was determined using closed-system respirometry, with PO_2 measurements taken at the start and end of each 30-min time point. After this time, respirometers were opened to flowing water for 10 min to allow for flushing of the chambers with fully air-equilibrated water. While the respirometers were open, the speed of flow was increased. After 10 min, respirometers were sealed, and measurements repeated as

above. Five-mL water samples were taken for analysis of water PO_2 using a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas, TX, USA) connected to an AM systems Polarographic Amplifier (Model 1900 Carlsberg, WA, USA) digital dissolved oxygen meter. The electrode was maintained and calibrated at the experimental temperature. A fish was determined to have reached U_{crit} when it stopped swimming, lay against the mesh screen at the end of the respirometer, and did not respond to light stimuli or gentle tapping. Both U_{crit} and oxygen consumption (MO_2) calculations are detailed in Section 6.3.8.

6.3.6 Concentration-dependent kinetics of Ni uptake

Fish that had been acclimated to FW or to 100% SW (as described in Section 6.3.2 above) but not previously exposed to Ni were taken for determination of the concentration-dependent kinetics of Ni uptake. Individual fish (N = 5) were exposed to nominal Ni concentrations increasing in a geometric sequence (0, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120 µg/L). Individual fish were exposed to each concentration in 250-mL containers that had been equilibrated with the appropriate "cold" Ni concentration for 24 h (to allow waterborne Ni to reach equilibrium) from a stock $NiCl_2$ solution (10 g/L; Sigma Aldrich). Approximately 0.5 h before killifish were added, radiolabelled ^{63}Ni (~ 6.4 µCi/250 mL, Amersham Biosciences, Inc. Mississauga, ON, Canada) was added. Two-mL water samples were then taken to determine radioactivity, and another 10-mL sample was removed for analysis of total Ni. At time 0, killifish were added individually to exposure chambers, where they remained for 3 h. Thereafter, killifish were removed from exposures, placed in a high non-radioactive 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 in a lethal dose of MS-222 (NaOH neutralized). Gills were excised, weighed, and placed in 20-mL scintillation vials, and 1 mL of 1N HNO_3 was added to digest tissues.

Tissues were vigorously vortexed and placed in an incubator at 65ºC for 48 h, with vortexing at 24 h. The digests were then centrifuged at 3500 g for 5 min at 18ºC. Ultima Gold AB scintillation fluor (Perkin Elmer, Waltham, MA) was then added to tissue digests to give a ratio of 1:5 (digest:fluor), and Optiphase (Perkin Elmer, Waltham, MA) was added to water samples to give a ratio of 1:10 (water:fluor). Both tissue and water 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.

6.3.7 Effects of Ca and Mg on Ni uptake in FW

Killifish that had been previously acclimated to FW (see Section 6.3.2 above), were placed in one of 6 different FW treatments (N = 5 for each): 0.5 mg/L Ni, 0.5 mg/L Ni + Ca/Mg, 5 mg/L Ni, 5 mg/L Ni + Ca/Mg, 5 mg/L Ni + Ca, and 5 mg/L Ni + Mg (Table 6.4). Treatments with $CaCl_2{\cdot}2H_2O$ (at 10 mM, Sigma Aldrich) and/or $MgCl_2{\cdot}6H_2O$ (at 45 mM, Sigma Aldrich) were designed to raise the levels of Ca and Mg in FW to the levels of these ions in SW. All exposures were conducted using individual fish in 250-mL containers with constant aeration. Ni, as $NiCl_2{\cdot}6H_2O$ (Sigma Aldrich), was added 24 h in advance to allow for Ni to reach equilibrium, and approximately 0.5 h before the addition of fish, radiolabel (^{63}Ni ~ 6.4 µCi/250 mL) was added. Total Ni and radiolabelled Ni were analyzed as described in Section 6.3.6. Fish were left in experimental containers for 3 h and then euthanized as before, with gills then dissected and counted for Ni accumulation as described above (Section 6.3.6).

6.3.8 Calculations and statistics

Tissue accumulation measured by ^{63}Ni was calculated as:

Tissue accumulation (μg/g) = $\frac{CPM/SA}{mass}$

where CPM are the quench corrected counts per minute, SA is the measured specific activity (i.e. CPM/μg Ni) in the exposure medium, and tissue mass is in g.

To determine the relative proportions of total Ni bioaccumulation on a tissue-specific basis, a pilot study was performed to determine the relative proportion (as a % of body mass) of each tissue within the killifish. These proportions were then applied to the tissue-specific Ni concentrations to achieve the relative Ni tissue distribution shown in Fig. 6. 4

Curve-fitting for the concentration-dependent kinetics of Ni accumulation in the gill was performed using SigmaPlot (Systat Inc. Chicago, IL USA). The r^2 values were used to determine whether data best fitted a linear, or a hyperbolic (Michaelis-Menten) relationship:

$$\textit{Specific Binding} = B_{max} * [G]/([G] + K_m)$$

where [G] is the Ni concentration on the ligand (gill), B_{max} is the binding site density for the ligand (μg/g wet wt.) and K_m is the binding affinity (μg/L).

U_{crit} was calculated using the formula of Brett (1964):

$$U_{crit} = U_i + (\frac{T_i}{T_{ii}} x U_{ii})$$

where U_i is the swim speed (bl/s) achieved for the 30 minute period prior to the one in which exhaustion occurred, U_{ii} is the incremental speed increase in bl/s, while T_i is the time the fish swam at the final swim speed (min), and T_{ii} is the incremental period of swimming (30 min).

Oxygen consumption (MO_2) was calculated according to Boutilier et al. (1984):

$$MO_2 = \frac{(\Delta PO_2) \times (\alpha O_2) \times (V)}{(M) \times (t)}$$

where ΔPO_2 is the change in the environmental partial pressure of oxygen over the experimental time (mmHg), αO_2 is the oxygen solubility co-efficient for oxygen (μmol/L/mmHg) at the given experimental temperature and salinity, V is the volume of the water in L, M is the mass of the fish in g, and t is the time in h.

Throughout the chapter data have been expressed as means ± SEM (N). Statistical analyses were performed with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA) and SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA). All data were initially assessed for normality and homogeneity of variance, and if these criteria were passed parametric analyses were performed. If data did not pass normality, data were log-transformed prior to analysis to fit parametric testing approaches. All Ni exposure data were analyzed via two-way ANOVA where salinity and Ni concentration were the two factors of interest. Where significance was found, a Tukey's post-hoc test was applied. For all other analyses, a one-way ANOVA model was applied with a Tukey's post-hoc test. Significance for all statistical tests was accepted at $\alpha = 0.05$. The significance of differences between kinetic curves was assessed using the methods of Glover and Wood (2005).

All speciation analysis was performed using Visual MINTEQ software (ver. 3.1 beta, KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden) where the water chemistries recorded in Tables 6.1 and 6.2 were used in addition to nominal values for other

anions. The NICA- Donnan model was used to estimate the effect of DOC on Ni speciation within each experimental treatment (Benedetti et al., 1995) (Table 6.5).

6.4 RESULTS

6.4.1 Water chemistry and Ni speciation

Water chemistry analysis showed the expected pattern of an increase in ion concentrations as salinity increased (Table 6.1). The contributions of $NiSO_4$ and $NiCl^+$ declined as salinity decreased, but the carbonate complexes persisted at all salinities. Measured dissolved Ni concentrations were reasonably close to nominal ranging from 3.8 mg/L in 10% SW to 5.3 mg/L in FW (Table 6.2). Speciation analysis indicated a decline in the concentration of the free ion (Ni^{2+}) as salinity increased, though it remained by far the dominant species (Table 6.3). The next most dominant species in 100% SW were $NiSO_4$ and Ni-DOC, followed by $NiCl^+$, $NiCO_3^+$, and $NiCO_3$. Analysis confirmed elevations of Mg and Ca levels in the experiments where these ions were selectively raised in FW (Table 6.4).

6.4.2 Tissue and salinity-dependent Ni accumulation after exposure to 5 mg/L for 96 h's

Exposure of killifish to 5mg/L Ni for 96 h resulted in increases in Ni burdens in all tissues examined (Fig. 6.1). Overall, levels of Ni accumulation were comparable in intestine, kidney, and whole body (8000 – 1000 µg/kg), with somewhat lower concentrations measured in gill and liver (Fig. 6.1A,C). Levels of Ni in the tissues of control animals (not exposed to Ni) did not vary significantly with salinity, but tissue burden of Ni differed among tissues (Fig. 6.1A-E).

A two-way ANOVA determined significant effects of Ni exposure, acclimation salinity and their interactions (Ni *vs.* salinity) (all $P \leq 0.05$) in gill tissue. Ni-exposed gills displayed lower

values of Ni accumulation at salinities greater than FW (Fig. 6.1A). However, at all salinities Ni exposure led to an increase in Ni burden relative to control fish, with 100% SW displaying the lowest Ni accumulation overall.

Ni accumulation in the intestine was independent of salinity (Fig. 6.1B). A two-way ANOVA determined a significant effect of treatment ($P \leq 0.001$), but not of salinity ($P = 0.119$), or interaction effect ($P = 0.343$). In the liver a two-way ANOVA revealed significant effects of treatment ($P < 0.001$) and salinity ($P \leq 0.05$) but no interaction effect ($P = 0.077$). Ni accumulation in both liver and intestine was lowest in the 10% SW group, however all Ni treatments were significantly higher than controls.

A two-way ANOVA showed that in the kidney there was a significant effect of Ni exposure ($P \leq 0.001$), salinity ($P \leq 0.05$) and their interaction ($P \leq 0.05$). In every salinity treatment the Ni burden in kidney tissue was significantly higher than in controls, but the 100% SW group exposed to Ni displayed an accumulation that was only ~20% of those found in the other salinities (Fig. 6.1D).

Finally, a two-way ANOVA determined that there was a significant effect of treatment ($P \leq 0.05$), but not of salinity ($P = 0.08$) or interaction effects ($P = 0.112$) on whole body Ni accumulation. As with all other tissues the whole body accumulated significantly more Ni in every salinity treatment relative to the controls, but in this case, similar to the pattern shown in the intestine, this accumulation did not differ significantly among different salinities (Fig. 6.1E).

6.4.3 The influence of Mg and Ca on acute 3 h Ni accumulation in FW-acclimated killifish

At the lower tested concentration of Ni (0.5 mg/L) no effects of Ca/Mg addition on FW killifish gill Ni accumulation were observed in acute (3-h) exposures (Fig. 6.2). However at 5

mg/L Ni, gill Ni accumulation in FW was significantly ($P \leq 0.05$) inhibited by each of these two cations when added at their typical 100% SW concentrations (10 mM Ca, 45 mM Mg). This effect occurred regardless of whether these ions were added together or separately (Fig. 6.2).

6.4.4 Concentration-dependence of Ni uptake kinetics in FW and SW

In FW a hyperbolic curve fitted to raw values was a better fit to the data than a linear curve ($r^2 = 0.95$ *vs.* 0.94). From this a B_{max} of 9326 ± 1222 µg/kg and a K_m of 6942 ± 1335 µg/L were derived (Fig. 6.3A). In SW, the data also fitted more strongly to a hyperbolic relationship than to a linear one ($r^2 = 0.77$ *vs.* 0.74), and a B_{max} of 4587 ± 859 µg/kg, and a K_m of 2264 ± 899 µg/L were derived (Fig. 6.3.B). Both the B_{max} and K_m values were significantly lower in SW than in FW ($P = 0.005$ and $P = 0.009$, respectively).

6.4.5 Tissue-specific Ni partitioning after 96 h exposure to 5 mg/L Ni

At all three salinities, the highest proportion of Ni accumulation occurred in the carcass where over 90% of the Ni was partitioned (Fig. 6.4). In FW the next highest proportion of Ni was in the gill (2.0%) and the intestine (1.8%). After 100% SW exposure the gill only accounted for 0.8% of the total Ni accumulation, while the intestine contributed 4.5% (Fig. 6.4).

6.4.6 Whole body ions after 96 h exposure to 5 mg/L Ni

Concentrations of ions in killifish whole bodies revealed a single significant effect of Ni exposure, an increase in K ion concentration in FW (Table 6.4). No other ions (Na, Mg, Ca) were impacted by Ni exposure, or salinity (Table 6.5).

6.4.7 Oxidative stress and antioxidant enzymes after exposure to 5 mg/L Ni for 96 hs

Protein carbonyl concentrations were comparable among gill, liver, and intestine. Two way ANOVA analysis indicated that there were no significant differences associated with either salinity ($P = 0.340$) or Ni exposure ($P = 0.110$) or interaction effects ($P = 0.90$) (Fig. 6.5 A,B,C).

Levels of ROS production, TOSC, and SOD activities were generally comparable among the three studied tissues, but CAT activity was notably about 10-fold higher in intestine and liver than in gill tissue (Figs. 6.6, 6.7, 6.8). In the gill a two-way ANOVA revealed that salinity had a significant effect on CAT and SOD activity ($P \leq 0.05$), while ROS and CAT activity were also significantly impacted by Ni treatment ($P \leq 0.05$) (Fig. 6.6A, C). No other two-way ANOVA parameters were significant in gill tissue. More specifically, a significant 7–fold increase in ROS production in Ni-exposed gill tissue relative to controls was measured in samples from 10% SW (Fig. 6.6A). However, there were no differences in TOSC associated with either Ni or salinity (Fig. 6.6B). CAT activity displayed a significant 50% decrease in response to Ni treatments in both FW and 30% SW relative to unexposed control gills (Fig. 6.6C). Significant salinity-dependence of control CAT activity was also observed. There were no other Ni-specific effects in the gill, although SOD activity in the gill was significantly higher in FW in both Ni-exposed and control tissues relative to the other salinities (Fig. 6.6D).

In the intestine, two way ANOVA's determined that there was an effect of salinity on CAT activity and TOSC (both $P \leq 0.05$) but not on SOD or ROS ($P = 0.078$, $P = 0.88$) and an effect of Ni treatment on SOD activity ($P < 0.001$). There were no treatment effects on ROS, CAT or TOSC ($P = 0.067$, $P = 0.97$, $P = 0.44$) and no significant interaction effect for ROS, CAT, TOSC, or SOD ($P = 0.53$, $P = 0.48$, $P = 0.112$, $P = 0.35$) (Fig. 6.7A-D). No other effects in the intestine were identified. Specifically, Ni exposure increased TOSC in the intestine by two-fold from control tissue, but only in FW (Fig. 6.7B). There was no significant effect of Ni on CAT

activity in the intestine, but activity was significantly influenced by salinity, with FW values approximately half of those in higher salinity treatments for both control and Ni-exposed tissue (Fig. 6.7C). Intestinal SOD activity was significantly increased by Ni exposure but only in 100% SW (Fig. 6.7D).

In the liver, two way ANOVA's showed that there were no overall effects of salinity, treatment or interaction for TOSC ($P = 0.458$, $P = 0.771$, $P = 0.887$) or SOD ($P = 0.334$, $P = 0.09$, $P = 0.077$). CAT was not affected by Ni treatment, or interaction ($P = 0.10$, $P = 0.781$). However CAT activity was significantly affected by salinity whereby both Ni-exposed and control tissues in FW exhibited 5-fold higher values than in the other salinities (Fig. 6.8C) ($P \leq 0.05$). ROS production was significantly elevated by Ni treatment ($P \leq 0.05$) but only in the 30% SW group (Fig. 6.8A) and no significant effects were noted in either salinity or interaction effects ($P = 0.44$, $P = 0.78$). There were no other Ni-dependent effects on any other oxidative stress markers in liver tissue (Fig. 6.8B-D).

6.4.8 U_{crit} and MO_2 after exposure to 5 mg/L Ni for 96 hours

Critical swimming speed (U_{crit}) was close to 4 bl/s and did not differ with Ni exposure, or between fish acclimated to FW *versus* 100% SW (Fig. 6.9A; two way ANOVA: treatment ($P = 0.908$), salinity ($P = 0.771$), interaction ($P = 0.765$)). MO_2 values followed a similar pattern wherein Ni exposure ($P = 0.692$), salinity ($P = 0.129$) and the interaction between these two factors ($P = 0.943$) were not significant influences. MO_2 values ranged from resting values of 3-4 μmol/g/h to 12 μmol/g/h at the highest velocity tested (Fig. 6.9B).

6.5 DISCUSSION

Ni uptake at the gill exhibited Michaelis-Menten type concentration-dependent kinetics in both FW and SW, with higher affinity (lower K_m) and lower B_{max} in the latter. In accord with one proposed hypothesis, salinity protected against Ni accumulation in the kidney and in the gill. However, this was not seen in other tissues. The effect at the gill appeared to be mediated by the elevated levels of Ca and Mg present in SW, as addition of these elements to FW significantly reduced Ni accumulation in FW-acclimated fish. Overall, despite the high levels of Ni used, there were few effects of Ni on sub-lethal toxicity endpoints, with the largest effects observed on oxidative stress markers, although these effects of Ni exposure were not as marked as those induced by salinity. There was no evidence of respiratory toxicity, in opposition to the second proposed hypothesis.

6.5.1 Tissue and salinity-dependent Ni accumulation after exposure to 5 mg/L Ni for 96 h

Ni accumulation patterns differed significantly among different tissues and with salinity. For example, as salinity increased, Ni accumulation in the gill was significantly inhibited (Fig. 6.1). This is similar in nature to the response observed in FW fish exposed to elevated Ca and/or Mg in the exposure water (Fig. 6.2), so likely reflects competition between Ni and these two ions for uptake. That similar effects were observed in salinity-acclimated animals and FW animals exposed acutely to elevated ions, suggests that water chemistry, not physiology, is the main driver of this effect at the gills. Similar to the results of the current study, high salinity reduced branchial Ni accumulation in the green shore crab (Chapter 2, Blewett et al., 2015a), while gill Ni accumulation was reduced in SW killifish relative to FW killifish in a previous study on this species (Chapter 5, Blewett and Wood, 2015a).

The gut of killifish accumulated a significant amount of Ni, even though the exposure was of a waterborne nature. In SW, killifish, like other marine teleosts, drink as part of their osmoregulatory strategy (Scott et al., 2005). This will expose the intestinal epithelial surface to Ni, thus facilitating absorption. However, FW killifish also drink (Blewett et al., 2013; Scott et al., 2006), with the gut playing an important role in Cl ion balance (Laurent et al., 2006). Although the drinking rate in FW killifish is considerably lower than that of SW fish (Blewett et al., 2013), it may still be an important contributor to Ni burden in this tissue. This is supported by the low Ni accumulation observed in the 10% and 30% SW groups. Although Ni accumulation at these salinities was not significantly different from those at the salinity extremes, they did trend towards lower Ni burdens. These two intermediate salinities are relatively close to the killifish iso-osmotic point (the point where the plasma osmolarity equals that of external medium) and so represent salinities where the need for drinking (and associated intestinal ion uptake) would be lowest, and therefore exposure of the gut to Ni would be lowest (Burnett et al., 2007). This has been speculated as the mechanism behind a similar salinity-dependent gut accumulation pattern in the gulf toadfish exposed to waterborne silver (Wood et al., 2004). The lack of significant differences between FW and 100% SW gut at an exposure level of 5 mg/L Ni is consistent with the findings of a previous study (Chapter 5, Blewett and Wood, 2015a). In that study, however, differences in gut accumulation were observed at higher Ni exposure concentrations (10 and 20 mg/L), indicating that accumulation patterns in the gut are driven by dose.

It is also noteworthy that in terms of the relative contribution to total body Ni burden, the intestine becomes increasingly important as salinity increases (Fig. 6.4). In FW the intestine accounted for 1.8% of total Ni, but in 100% SW this value was 4.5%. Gill Ni accumulation showed the opposite trend, decreasing in relative importance from 2.0% in FW to 0.8% in 100%

SW. This pattern is consistent with the dual concepts of cations protecting against waterborne Ni absorption at the gill, and an increase in drinking rate in SW facilitating Ni uptake *via* the gut.

Ni is known to accumulate in the liver of fish (Sreedevi et al., 1992), and this was observed in the current study for killifish. Hepatic Ni accumulation likely occurs because the liver is a major tissue for metal storage (thanks to high levels of metallothionein in this tissue) and eventual elimination (*via* bile). In fact the elimination of Ni *via* bile into the intestine could also be a significant source of gut Ni (Hauser-Davis et al., 2012), and may lead to the overall similarities in accumulation patterns seen in these tissues. Alternatively, Ni accumulation in the liver could be due to plasma trapping. Once Ni is absorbed it is transported in either the free ion form or bound to plasma albumins (Glennon and Sarkar, 1982). In rainbow trout the plasma was shown to be a large sink for Ni (Pane et al., 2004a). If a similar scenario exists for killifish then the presence of blood in well-perfused tissues such as the liver, may influence tissue Ni burdens. Unfortunately plasma was not taken for Ni analysis in the current study.

Ni accumulation in the kidney displayed a very distinct pattern with respect to salinity. In FW, 10% SW and 30% SW Ni accumulation was significantly higher than accumulation in 100% SW (Fig. 6.1 D). A large renal accumulation of Ni is perhaps not surprising as Ni has been shown to preferentially accumulate in this tissue (Pane et al., 2005). However, the relatively low Ni accumulation in the kidneys of killifish acclimated to 100% SW is curious. This may reflect physiological differences in the use of the kidney as an excretion pathway. In FW rainbow trout 98% of Ni was filtered and reabsorbed by the glomeruli (Pane et al., 2005), while in the marine gulf toadfish (*Opsanus beta*) 30% of infused Ni was excreted *via* the kidneys (Pane et al., 2006b). This suggests the kidney accumulates Ni in FW, but at high salinities excretes Ni.

Ni accumulation into the whole body was similar across all salinities. Furthermore this compartment accumulated by far the most Ni (>90%), across all salinities (Fig. 6.4). This may again represent plasma trapping (see above), with this compartment being the largest and likely to have the greatest amount of trapped blood. Alternatively, or additionally, it has been shown that Ni preferentially deposits in the bone and muscle of fish (Pyle and Couture, 2012), which would also support the large relative accumulation of Ni in this compartment. The tissue distribution patterns obtained here for killifish are similar to previous work in the green shore crab, where Ni mostly accumulated in the carcass and shifts in distribution were observed with salinity (Blewett et al., 2015a, Chapter 2; Blewett and Wood, 2015b, Chapter 3).

6.5.2. Experimental determination of the influence of Mg and Ca on acute Ni accumulation in FW-acclimated killifish

The results of the Ca and Mg addition experiments lend some support to the idea that Ni accumulation is at least partly mediated by pathways shared with these ions. The addition of seawater concentrations of Mg or Ca to FW significantly decreased Ni accumulation (Fig. 6.2). The magnitude of this effect was the same irrespective of whether Mg or Ca was added alone, or whether they were added together. This would suggest that Ca, Mg and Ni are all being transported by a single pathway, and that when high levels of Ca or Mg are present they outcompete Ni for accumulation.

There is literature support for such a phenomenon. For example, Ni is considered to be an effective blocker of several different types of Ca channels (Lee et al., 1999; McFarlane and Gilly, 1998; Todorovic and Lingle, 1998), and there is evidence from studies in mammalian pulmonary tissue suggesting that Ni competes with Mg (Kasprzak and Poirier, 1985). Mg is usually obtained

via the intestine (Flik et al., 1993), however, indirect evidence of active branchial Mg transport pathways (maintenance of internal Mg balance following a Mg-deficient diet) has been indicated in fish (Bijvelds et al., 1996; Shearer and Åsgård, 1992). Furthermore, Mg and Ca have been shown to protect against acute waterborne Ni toxicity to *Daphnia pulex*, likely by competing with Ni at transport sites (Kozlova et al., 2009). In fish, *in vitro* Ni uptake into renal brush-border membrane vesicles of the trout kidney was inhibited by Mg at a 100:1 Mg to Ni molar ratio, and by both Mg and Ca at a 1000:1 molar ratio (Pane et al., 2006 a,b). Finally, non-competitive inhibition between Ni and Ca was also observed in the stomach of trout, and similar interactions were observed in the medial intestine with Ni and Mg (Leonard et al., 2009).

It is important to note that the addition of $CaCl_2$ and $MgCl_2$ in the current study would also increase Cl levels in the exposure medium (Table 6.4), which could theoretically also modify Ni transport through alternative mechanisms. This should be checked in future experiments on killifish, though one study on FW daphnia detected no effect of elevated Cl on Ni uptake (Kozlova et al., 2009).

6.5.3 Concentration-dependent gill Ni accumulation kinetics in FW and SW

The relationships between Ni exposure level and branchial Ni accumulation differed significantly between FW- and SW-acclimated killifish (Fig. 6.3). Both groups of fish displayed saturable kinetics, however the parameters of B_{max} (number of binding sites) and K_m (binding site affinity) derived from the curves differed. The SW fish displayed a lower B_{max} (i.e. fewer sites) and a lower K_m (i.e. higher affinity).

The presence of saturation as Ni exposure level increases is usually indicative of a metal binding to specific transporters or channels, and is similar to the patterns observed for gill Ni

accumulation in rainbow trout and round goby (Leonard et al., 2014b). The Michaelis-Menten constants of B_{max} and K_m for Ni accumulation in the gill, were in the same general ranges between killifish and other tested fish species. The B_{max} for killifish gill was calculated to be 9326 µg/kg in FW (160 µmol/kg) and 4587 µg/kg (79 µmol/kg) in SW, somewhat lower than the B_{max} calculated for round goby (304 µmol/kg) and rainbow trout (278 µmol/kg). Similarly the K_m for killifish gill FW Ni accumulation was 6942 µg/L (119 µmol/L), and in SW was 2264 µg/L (39 µmol/L). This FW value is higher than those for round goby (17.8 µmol/L) but closer to rainbow trout (86.4 µmol/L). In general the lower the K_m (i.e. the higher the affinity for Ni), the more sensitive the species is to Ni (Leonard and Wood 2013; Leonard et al., 2014b). This relationship is central to the basis of the BLM approach, allowing toxicity to be predicted from accumulation characteristics (Niyogi and Wood, 2004). However, although lethality was not assessed in the current study, killifish are reported to be quite resilient to Ni exposure with a 96-h LC_{50} (concentration to cause 50% mortality) in FW of 150 mg/L for adult animals (Eisler and Hennekey, 1977), much higher than the value of 15.6 mg/L in rainbow trout (Pane et al., 2003a). Although the magnitude of this difference is not reflected in the respective K_m values, the general pattern is consistent, with the more resilient killifish displaying the lower affinity (i.e. higher K_m). However, these data also suggest that SW killifish, by virtue of a higher affinity for Ni, would be more sensitive to Ni than FW killifish. This does not, however, seem to be the case, as Bielmyer et al. (2013) have reported that Ni toxicity to larval killifish decreases linearly with increasing salinity. However, these toxicity tests were performed in larval fish, compared to the adult animals used in the current study for measurement of accumulation. To confirm the relationship between gill Ni binding affinity and lethality, experiments would need to be done concurrently in fish of an equivalent life stage.

As noted above, saturation of Ni uptake indicates a role for a specific transport pathway. The exact mechanisms of transcellular Ni uptake have yet to be characterized. However there is evidence that Ni is able to gain access to the gill *via* ion transport pathways designed to take up nutrient ions, a common mode of metal ion uptake (Bury et al., 2003). For example, Ni has been shown to impact Ca and Mg transport systems in a wide range of organisms, from algae to rats (e.g. Deleebeeck et al., 2009; Eisler, 1998; Funakoshi et al., 1997; Pane et al., 2006 a,b). It is therefore possible that the saturable component of Ni uptake described here for killifish gill is through the transcellular pathways dedicated to Ca and/or Mg.

Ca transporters are known to be responsive to changes in environmental Ca levels, with both the numbers of transporters (Hsu et al., 2014), and the specific isoform (Liao et al., 2007) changing. The former effect would lead to a decrease in B_{max} and the latter a change in K_m. The data presented here of decreases in both B_{max} and K_m support the concept that FW- and SW-acclimated killifish may have different Ca transporting characteristics, which impact the kinetics of Ni accumulation.

Alternatively, rather than a Ca transporter, Ni may be transported through metal-specific carriers such the divalent metal transporter (DMT1). There is a large amount of molecular and physiological evidence for the occurrence of DMT1 in fish (Bury et al., 2001, 2003; Cooper et al., 2006; Donovan et al., 2002; Dorschner and Phillips, 1999; Nadella et al., 2007). This transporter is best characterized as an iron (Fe) transporter, but evidence indicates that it will also transport Ni (Gunshin et al., 1997). For example, an increased uptake of Ni in the absence of Fe indicates a common carrier-mediated transport mechanism shared by these two ions (Tallkvist et al., 2003). Transport through DMT1 would also confer a saturable pattern to Ni uptake. This explanation is, however, less likely than Ni uptake through ion mimicry. If uptake were through DMT1 then it

would be anticipated that the K_m value would be in the environmental range, instead the measured K_m values for both FW and SW were well in excess of normal environmental levels.

It is important to note that the pattern of SW gill Ni accumulation differed between the 3 h kinetic study (Fig. 6.3) and the 96 h Ni exposure (Fig 6.1A). In Figure 6.1A (and also shown previously by Blewett and Wood, 2015a; Chapter 5), the gills of killifish acclimated to SW accumulated 1000 µg/kg after 96 h, whereas the level of accumulation in SW gills after 3 h at an equivalent Ni exposure level (~5 mg/L) was approximately 3000 µg/kg. This likely reflects the different time-frames of these two exposures. In killifish whole body Ca levels decrease with increasing salinity, but Ca influx rates actually increase (Prodocimo et al., 2007). This is similar in nature to the differences observed here for gill Ni, with the initial uptake (after 3 h), reflecting influx, being elevated, but overall "steady state" accumulation (after 96 h) being lower than that after 3 h. Given the proposed interactions between Ca and Ni it is intriguing to suggest that the time-dependent patterns observed are driven by changes in Ca transport pathways.

6.5.4 Whole body ions after exposure to 5 mg/L Ni for 96 h

The only effect of Ni on whole body ions was an elevation in K in FW-acclimated killifish (Table 6.4). Ionoregulatory impairment, while sometimes reported in fish, is not generally considered to be the major mode of toxicity (Pane et al., 2003a). However when it does exist it is most frequently observed in physiologically active tissues such as the gill (Pane et al., 2003a).

Effects of Ni on K levels may be manifested by inhibition of the basolateral sodium pump, Na^+/K^+ ATPase. Previous studies have shown that, at least in crabs, Ni is capable of inhibiting Na^+/K^+ ATPase (Chapter 2, Blewett et al., 2015a). However impairment of this transporter would be expected to also result in changes in other ions, as this pump indirectly

drives the cellular transport of all ions. Instead it is possible that the effects of Ni on K represent a more specific mechanism. Intriguingly, it has been shown that Ni interferes with voltage-sensitive K channels in the toad (*Bufo arenarum*) (Bertrán and Kotsias, 1997). Given that the bulk of the whole body is muscle, a tissue with heavy reliance on electrical conduction, it is possible that such an effect underlies the observed response. However, why this would only be present in FW-acclimated fish remains unclear.

6.5.5 Oxidative stress and antioxidant enzymes after exposure to 5 mg/L Ni for 96 h

Ni exposure has been previously shown to cause oxidative stress in fish (Chapter 5; Blewett and Wood, 2015a; Kubrak et al., 2012a,b; Kubrak et al., 2013; Kubrak et al., 2014 Palermo et al., 2015). Oxidative stress can be manifested in a number of ways. Changes can occur in terms of upregulation of anti-oxidant defence enzymes, and/or depletion of non-enzymatic defences in response to increased ROS. If these changes fail to successfully ameliorate the oxidative stress, then oxidative damage can occur. One marker of oxidative damage is protein carbonylation (Bainy et al., 1996). Even though there were significant elevations in Ni accumulation in the gill, intestine and liver of Ni-exposed killifish this did not translate into protein carbonyl formation (Fig. 6.5 A,B,C). A general lack of Ni-dependent changes in protein carbonylation has been observed previously in killifish exposed to Ni (Chapter 5, Blewett and Wood, 2015a). Such a finding indicates that either Ni did not cause significant generation of ROS, or that defence mechanisms were successful in scavenging any ROS and thus preventing damage.

In general there was a lack of correlation between Ni accumulation in tissues and oxidative stress markers, which suggests that Ni itself may not have been the main inducer of ROS and resulting effects. In fact salinity seemed to have a more significant overall impact on

oxidative stress than Ni exposure. For example, in the gill both SOD and CAT activity were strongly salinity-dependent. Such salinity-dependence in oxidative stress responses has been previously observed in the gills of killifish exposed to higher levels of Ni than those used in the current study (Chapter 5, Blewett and Wood, 2015a). This was explained by the relatively high metabolic demand of the gill tissue. As mitochondria are the main sources of ROS in a cell, and mitochondria-rich cells drive ion transport across gill epithelia, the changes in oxidative stress with salinity are perhaps unsurprising (Lushchak, 2011).

Although salinity seems to be important, there were some effects of Ni on ROS. For example, CAT activity in the gill was significantly impacted by Ni (in FW and 30% SW; Fig. 6.6C). Such an effect has been observed previously in killifish exposed to levels of Ni 2 to 3-fold higher than those used in the current study (Chapter 5, Blewett and Wood, 2015a). The proposed mechanism of this effect is the binding of Ni to protein histidine residues (e.g. Predki et al., 1992), which perform key roles in the active site of CAT (Mate et al., 1999).

In the liver and intestine only minor changes in measures of Ni-impacted oxidative stress were noted. This may reflect the relatively minor changes in Ni accumulation that occurred in these tissues as a function of salinity. However, perhaps more important than total Ni is the amount of bioreactive Ni. Because the intestine and liver are storage tissues for Ni, they may be well prepared in terms of mechanisms that limit Ni bioreactivity. For example, elevated levels of metallothionein would bind Ni, not affecting tissue burden but lessening Ni reactivity, thus minimizing the generation of ROS in these tissues. This has support in the literature. In both rainbow trout and round goby, the vast majority of accumulated Ni in the gut of these fish was found in the “biologically inactive metal” fraction, comprising Ni in metal-rich granules and that bound to metallothionein (Leonard et al., 2014a). Furthermore, there were no relationships

between metal accumulation in the gut of these fish and eventual chronic toxicity, indicating that Ni in the gut was not toxicologically-relevant (Leonard et al., 2014a).

6.5.6 U_{crit} and oxygen consumption after exposure to 5 mg/L Ni for 96 h

Ni-exposed killifish did not display any evidence of respiratory toxicity, with maximal prolonged swim speed (U_{crit}) and oxygen consumption (MO_2) being unaltered by Ni exposure (Fig. 6.9). The values of these parameters were in accordance with previous measurements in killifish (Fangue et al., 2008). Although respiratory impairment in response to Ni (i.e. change in U_{crit}) has been reported in rainbow trout, this was only after 12 days of exposure to 2034 µg/L of Ni (Pane et al., 2004b). More subtle respiratory effects (decreased arterial oxygen tension) were, however, observed in rainbow trout exposed to 11.6 mg/L of Ni for 117 h (Pane et al., 2003a), levels and durations that are still significantly higher than those used in the current study. In the current study the exposure period was only 96 h, which was likely not long enough to cause any structural damage to the gill (the underlying driver of respiratory impairment in the trout study; Pane et al., 2004b). Of note, however, is that respiratory toxicity tests in the current study were run in clean water following exposure to Ni for 96 hour. It is possible that if the tests were run under continuous metal exposure conditions, that respiratory effects may have been observed. However, previous work has shown that both U_{crit} and MO_2 remained the same regardless of whether fish were swum in the presence *versus* absence of aluminum after pre-exposure (Wilson et al., 1994).

It is possible that differences in Ni sensitivity between killifish and rainbow trout are also responsible for the different effects noted. Killifish are extremely tolerant to Ni in comparison to rainbow trout, with the 96 h LC_{50} for juvenile killifish an order of magnitude higher (i.e. less

sensitive) than that for juvenile rainbow trout (Brix et al., 2004; Bielmyer et al., 2013). Consequently, the resilience of killifish may have precluded the development of respiratory effects.

6.5.7 Conclusions

Overall, killifish are relatively insensitive to Ni toxicity. At the level of Ni tested in the current study (5 mg/L) there was some evidence for oxidative stress, minor changes in ionoregulatory parameters and no respiratory toxicity effect. Ni accumulation does, however, vary with salinity, with effects of Ca and Mg on gill Ni uptake. The tested level of Ni represents a very high environmental exposure, so the data from the current study suggest that acute exposures to Ni are unlikely to cause significant harm to killifish. However, because of their relatively low sensitivity they have some utility as site-specific biomonitors. For example, killifish from contamination-impacted sites display specific behavioural impairments that can be used to characterise environmental risk (Weis and Candelmo, 2012). However linking exposure to effect is also important, and the results of the current study suggest that salinity will impact bioaccumulation, and thus would need to be accounted for under such risk assessment approaches.

Acknowledgements

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6.7 TABLES AND FIGURES

Table 6.1.Water chemistry parameters for 96 h Ni exposure. Reported values represent means ± S.E.M. (N = 8), except temperature (N = 1).

Parameter	FW (0 ppt)	10% SW (3.5 ppt)	30 % SW (10.5 ppt)	100% SW (35 ppt)
pH	7.70 ± 0.06[a]	7.80 ± 0.02[a,b]	7.90 ± 0.08[a,b]	8.02 ± 0.04[a,b]
Temperature (ºC)	20	20	20	20
DOC (mg/L)	2.1 ± 0.3[a]	1.8 ± 0.7[a]	1.7 ± 0.8[a]	2.5 ± 0.5[a]
Na^+ (mmol/L)	0.65 ± 0.03[a]	45.0 ± 1.7[b]	127.5 ± 0.8[c]	471.4 ± 5.3[d]
Mg^{2+} (mmol/L)	0.33 ± 0.01[a]	4.70 ± 0.09[b]	14.50 ± 0.20[c]	44.67 ± 0.70[d]
K^+ (mmol/L)	0.05 ± 0.00[a]	1.12 ± 0.02[b]	3.26 ± 0.05[c]	11.0 ± 0.6[d]
Ca^{2+} (mmol/L)	0.84 ± 0.01[a]	1.76 ± 0.03[b]	3.80 ± 0.05[c]	9.6 ± 0.1[d]

Values sharing letters across rows are not significantly different.

Table 6.2. Dissolved Ni exposure concentrations (µg/L) in both FW and SW (mean ± SEM; N = 10).

Salinity	Control (mg/L)	Ni (mg/L)
Freshwater	0.0021 ± 0.0009	5.3 ± 0.2
10% SW	0.0034 ± 0.0003	3.8 ± 0.2
30% SW	0.0047 ± 0.0003	4.3 ± 0.5
100% SW	0.0039 ± 0.0004	4.8 ± 0.6

Table 6.3. Ni speciation (% of total Ni) as calculated by Visual MINTEQ based on recorded and nominal water chemistry.

Species of Ni	Freshwater (0 ppt)	10% SW (3.5 ppt)	30% SW (10.5 ppt)	100% SW (35 ppt)
Ni^{2+}	84.83	83.12	81.79	76.95
Ni-DOC	2.65	1.25	1.28	4.50
$NiOH^{+}$	0.46	0.24	0.22	0.16
$Ni(OH)_2$	0.04	0.01	0.01	0.00
$NiCl^{+}$	0.02	0.57	1.19	3.39
$NiCl_2$	0.00	0.00	0.01	0.06
$NiCO_3$	4.03	4.92	7.51	2.80
$NiCO_3^{+}$	4.24	5.23	4.23	4.54
$NiSO_4$	3.73	4.66	3.76	7.60

Table 6.4. Water chemistry for high Ca and Mg exposures (Means ± S.E.M; N = 8 per treatment).

Parameter	Freshwater	Freshwater, High Mg	Freshwater High Ca	Freshwater, High Mg + Ca
pH	7.60 ± 0.04[a]	7.50 ± 0.03[a]	7.60 ± 0.05[a]	7.80 ± 0.07[a]
Temperature (ºC)	20	20	20	20
DOC (mg/L)	1.9 ± 0.3[a]	2.1 ± 0.7[a]	1.8 ± 0.9[a]	2.0 ± 1.0[a]
Ni (mg/L)	5.5 ± 0.3[a]	4.4 ± 0.5[a]	4.6 ± 0.8[a]	5.3 ± 0.7[a]
Na^+ (mmol/L)	0.63 ± 0.03[a]	0.63 ± 0.02[a]	0.62 ± 0.03[a]	0.71 ± 0.08[a]
Mg^{2+} (mmol/L)	0.42 ± 0.10[a]	44.0 ± 9.1[b]	0.30 ± 0.07[a]	53.2±9.0[b]
K^+ (mmol/L)	0.05 ± 0.01[a]	0.06 ± 0.00[a]	0.05 ± 0.00[a]	0.06 ± 0.00[a]
Ca^{2+} (mmol/L)	0.9 ± 0.1[a]	0.8 ± 0.0[a]	8.5 ± 0.3[b]	9.3±0.1[b]
Cl^- (mmol/L)	0.9 ± 0.1[a]	86.3 ± 3.0[b]	19.2 ± 1.3[c]	102. 1 ± 2.4 [d]

Values sharing letters across rows are not significantly different.

Table 6.5. Whole body ions in killifish exposed to 5 mg/L of Ni for 96 h. Reported values are means ± S.E.M, N = 6. Asterisk indicates a significant difference between Ni-exposed and control fish for a given ion, within a salinity

Salinity	Na^+ (mmol/L)		Ca^{2+} (mmol/L)		K^+		Mg^{2+}	
	Control	Ni	Control	Ni	Control	Ni	Control	Ni
FW	67.6 ± 14.0	89.6 ± 18.6	358.3 ± 71.7	404 ± 77.0	72.4 ± 6.9	119.0 ± 14*	26.3 ± 7.0	31.2 ± 2.5
10% SW	97.3 ± 7.4	97.6 ± 13.4	437 ± 25.9	492.8 ± 46.9	82.3 ± 2.3	83.2 ± 3.5	27.5 ± 2.3	26.8 ± 1.5
30% SW	95.6 ± 16.3	93.9 ± 11.6	555.5 ± 30.1	431.5 ± 48.5	96.4 ± 6.3	91.0 ± 7.0	32.0 ± 6.3	28.0 ± 3.5
100% SW	77.2 ± 22.7	126. ± 9.17	493.8 ± 53.6	494.2 ± 30.1	88.3 ± 20.2	84.1 ± 4.3	36.8 ± 2.6	32.8 ± 1.4

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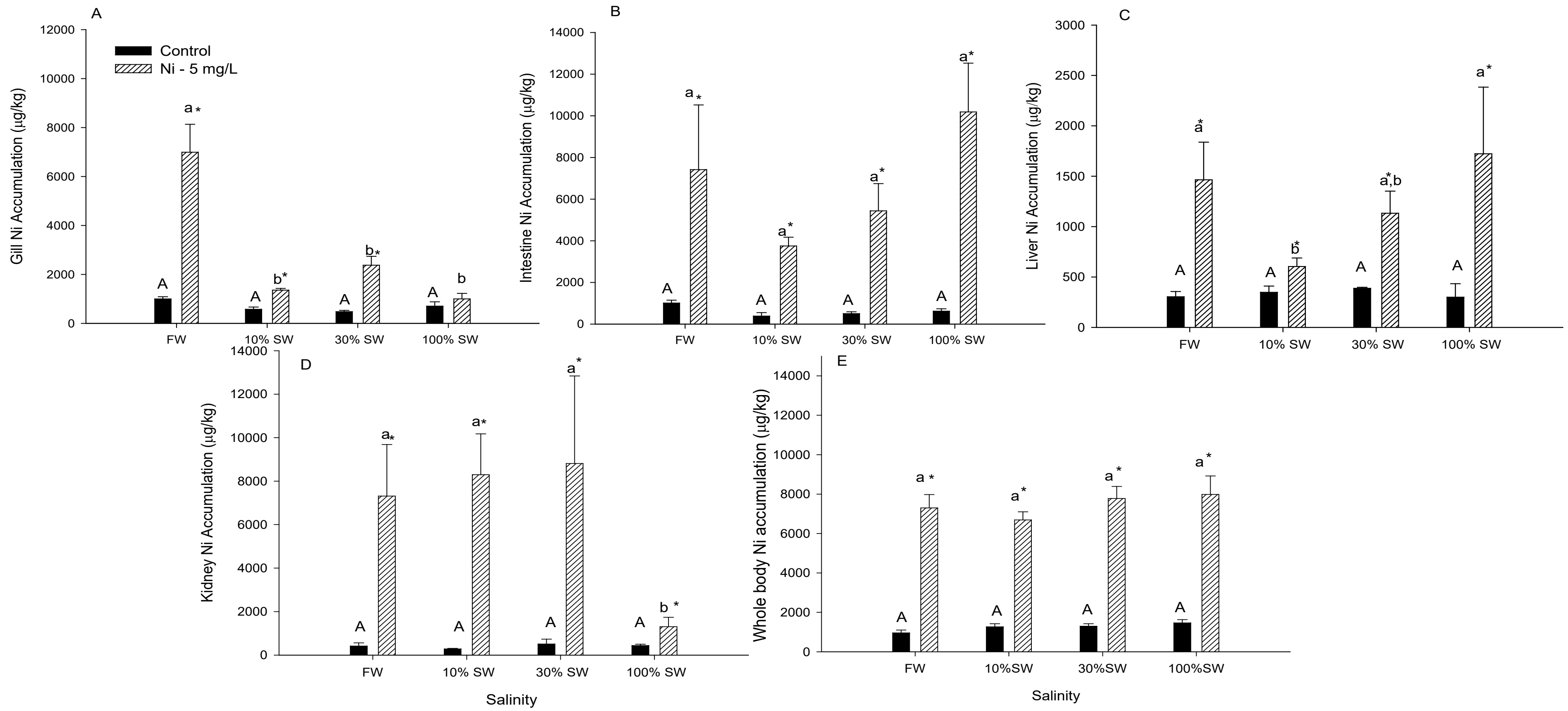
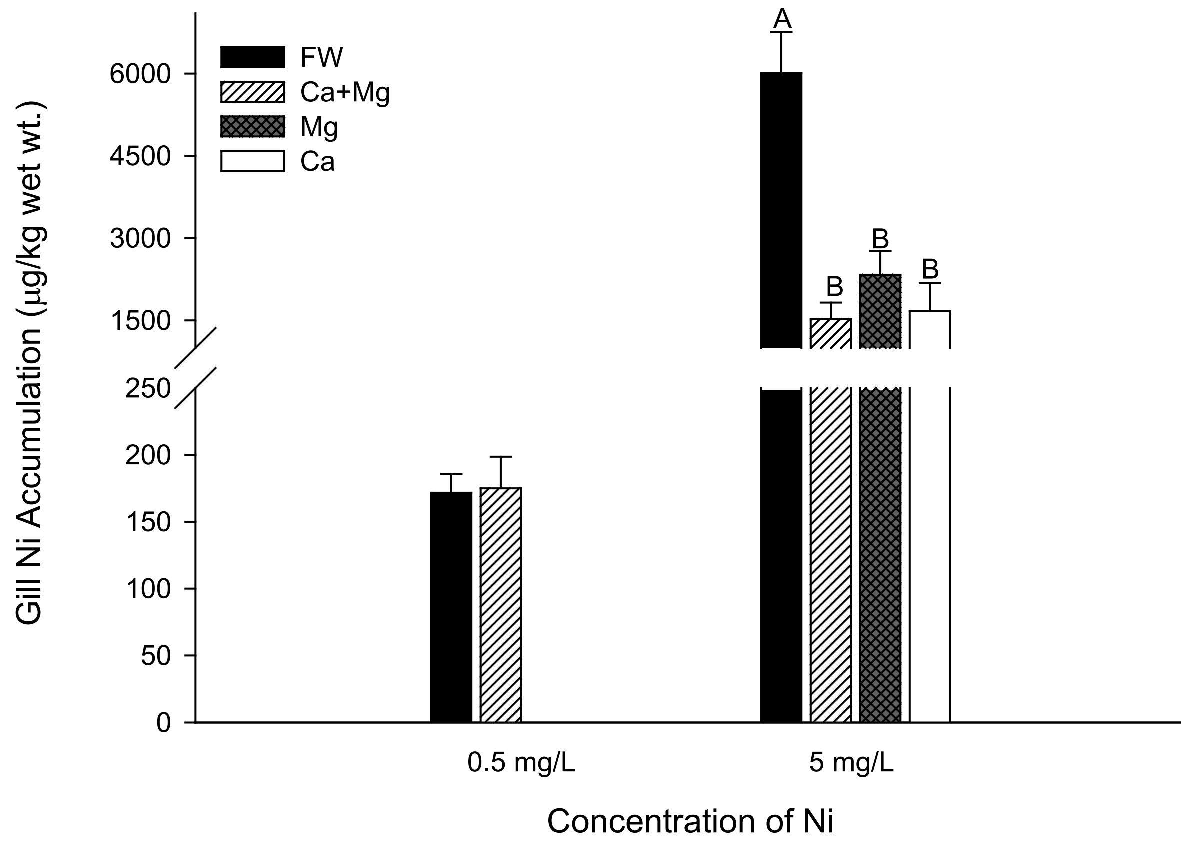

A
Control
Ni - 5 mg/L
Gill Ni Accumulation (µg/kg)
FW
10% SW
30% SW
100% SW
B
Intestine Ni Accumulation (µg/kg)
C
Liver Ni Accumulation (µg/kg)
D
Kidney Ni Accumulation (µg/kg)
Salinity
E
Whole body Ni accumulation (µg/kg)
10%SW
30%SW
100%SW
Salinity

Figure 6.1. Ni accumulation in: A) gills, B) intestine, C) liver, D) kidney, and E) whole body (µg/g wet wt.) of *Fundulus heteroclitus* after an exposure to 5 mg/L of Ni for 96 h, in one of four different salinity treatments (FW, 10%, 30% and 100% SW). Plotted points represent means ± S.E.M. (N = 6). Upper case letters denote significant differences in control tissues across salinities. Lower case letters denote significant differences in the tissues of Ni-exposed fish across salinities. Means sharing the same letter are not significantly different. Asterisks denote significant differences between control and Ni-exposed treatments within a salinity.



FW
Ca+Mg
Mg
Ca
A
B
B
B
Gill Ni Accumulation (µg/kg wet wt.)
6000
4500
3000
1500
250
200
150
100
50
0
0.5 mg/L
5 mg/L
Concentration of Ni

Figure 6.2. Gill Ni accumulation in *Fundulus heteroclitus* at two different concentrations of Ni under different water chemistry parameters. Plotted points represent means ± S.E.M. (N=5). Uppercase letters denote significant differences between experimental chemistries within a concentration. Means sharing the same letter are not significantly different.

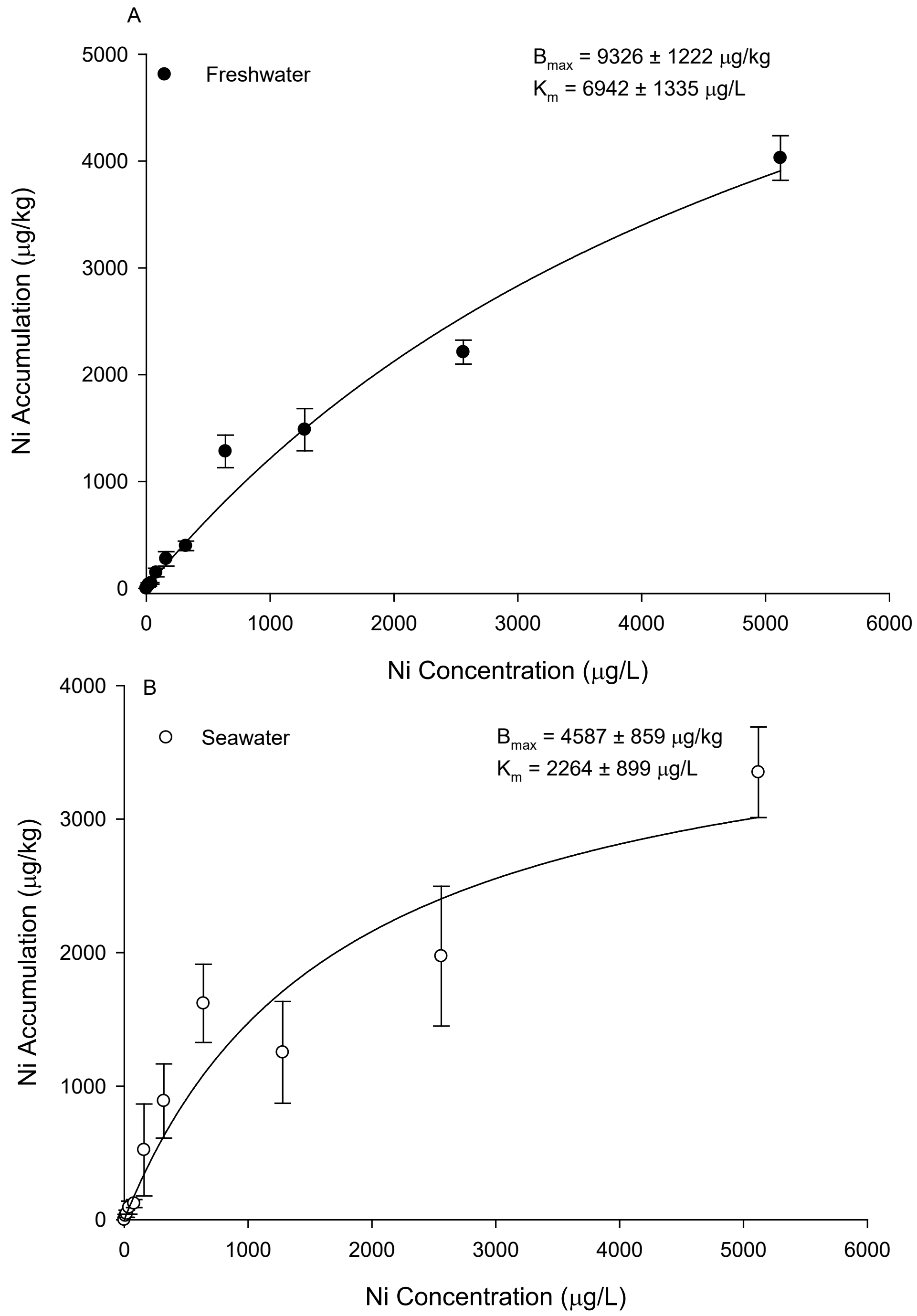

A
Freshwater
B_{max} = 9326 ± 1222 μg/kg
K_m = 6942 ± 1335 μg/L
Ni Accumulation (μg/kg)
Ni Concentration (μg/L)
0
1000
2000
3000
4000
5000
6000
B
Seawater
B_{max} = 4587 ± 859 μg/kg
K_m = 2264 ± 899 μg/L
Ni Accumulation (μg/kg)
Ni Concentration (μg/L)

Figure 6.3. FW (A) and SW (B) gill Ni accumulation (µg/g wet wt.) as a function of increasing Ni exposure concentrations (µg/L) following a 3 h exposure. Plotted curves and corresponding parameters (B_{max}, K_m) of the Michaelis-Menten equation, fitted using SigmaPlot. Both B_{max} and K_m parameters were significantly different between FW and SW, as tested according to Glover and Wood (2005).

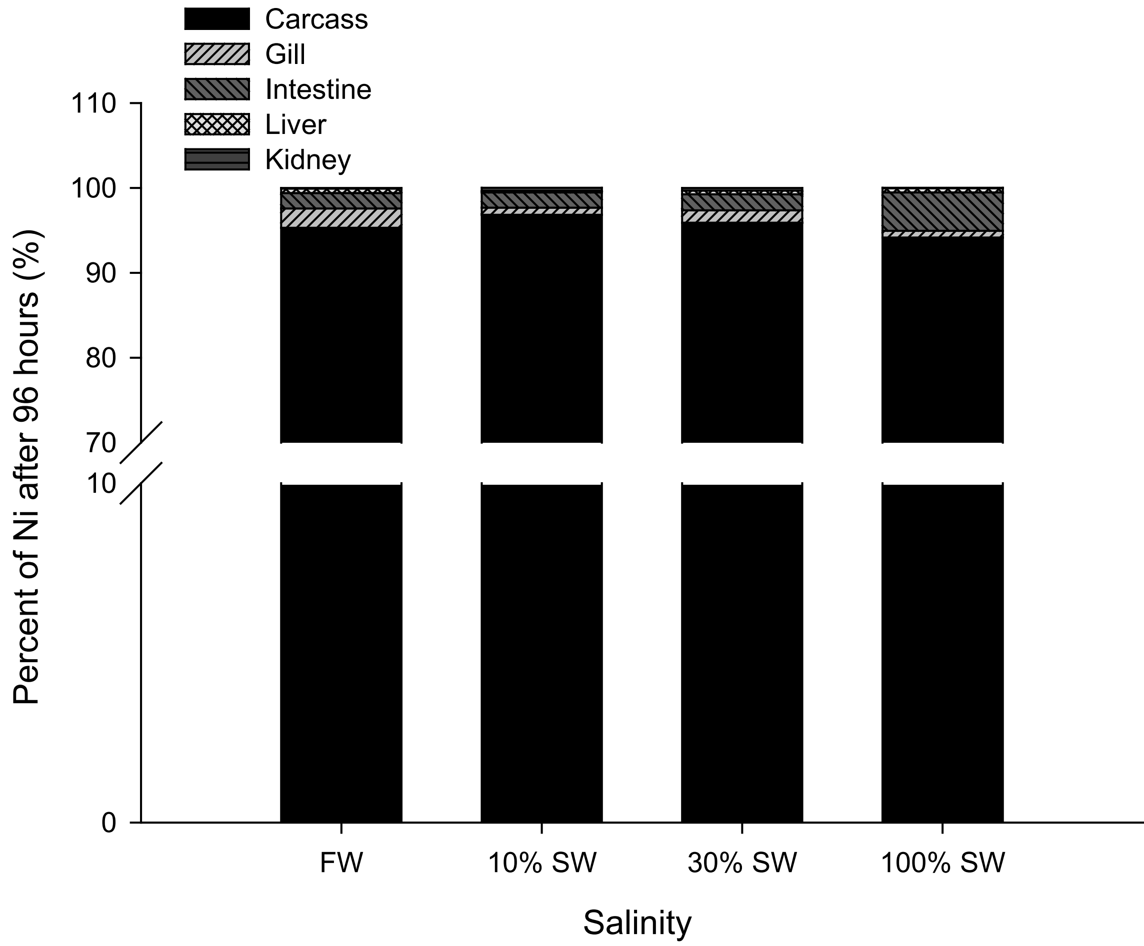

Carcass
Gill
Intestine
Liver
Kidney
110
100
90
80
70
10
0
Percent of Ni after 96 hours (%)
FW
10% SW
30% SW
100% SW
Salinity

Figure 6.4. Percent distribution of Ni accumulation in the various tissues of the killifish (*Fundulus heteroclitus*) after an exposure to 5 mg/L Ni for 96 h at 4 different salinities (FW, 10%, 30% and 100%SW). Plotted values are means (N=7 per treatment).

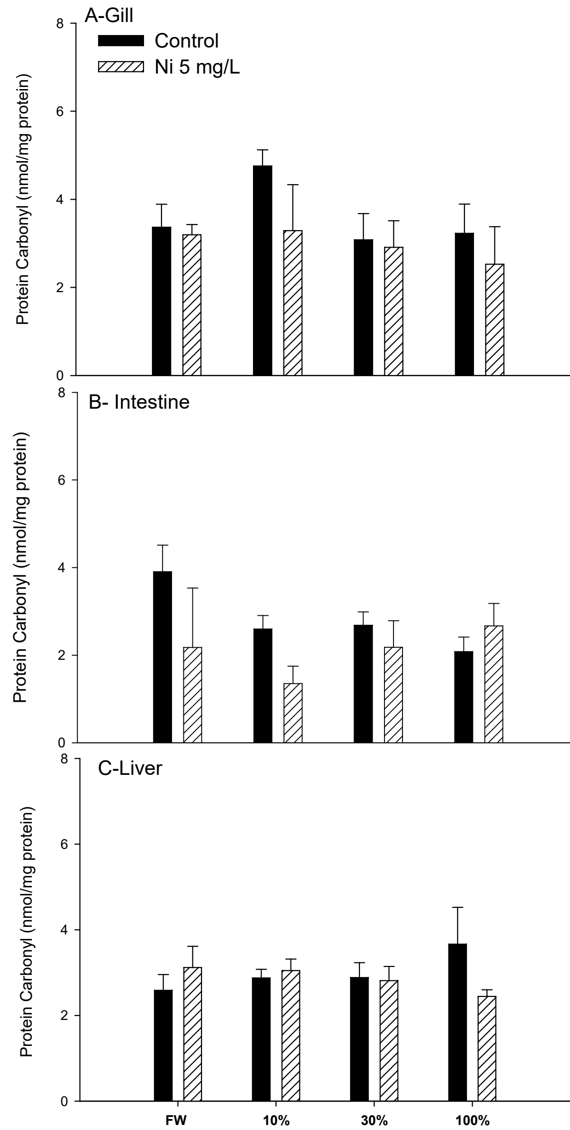

A-Gill
Control
Ni 5 mg/L
Protein Carbonyl (nmol/mg protein)
B- Intestine
Protein Carbonyl (nmol/mg protein)
C-Liver
Protein Carbonyl (nmol/mg protein)
0
2
4
6
8
FW
10%
30%
100%

Figure 6.5. Protein carbonyl content (nmol/mg protein) in gill (A), intestine (B), and liver (C) of *Fundulus heteroclitus* exposed to one of 4 different salinities (FW, 10%, 30% and 100% SW). Plotted points represent means ± S.E.M. (N = 5). No significant differences were noted *via* a two –way ANOVA.

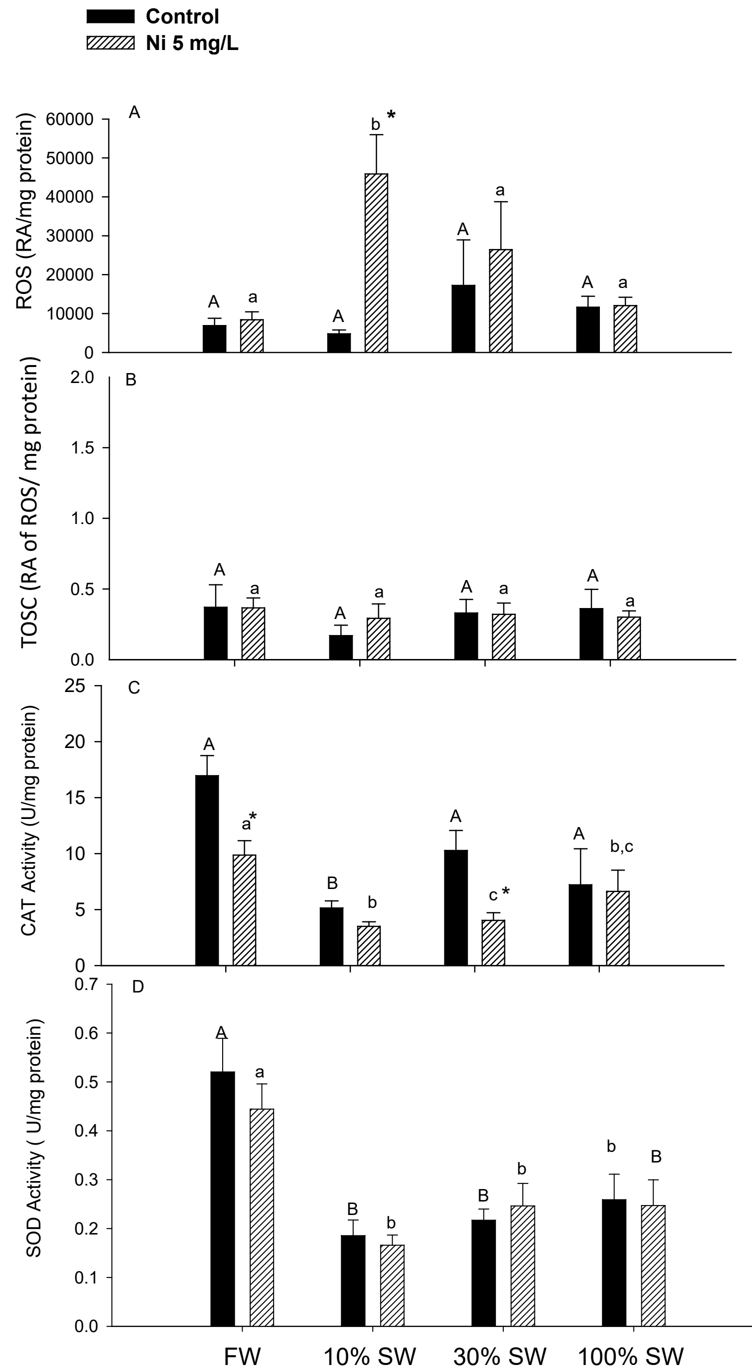

Control
Ni 5 mg/L
A
ROS (RA/mg protein)
B
TOSC (RA of ROS/ mg protein)
C
CAT Activity (U/mg protein)
D
SOD Activity (U/mg protein)
FW
10% SW
30% SW
100% SW

Figure 6.6. Oxidative stress indicators in the gills of *Fundulus heteroclitus* after an exposure to 5 mg/L Ni for 96 h at one of 4 different salinities (FW, 10%, 30% and 100% SW): A) reactive oxygen species (ROS; RA = Relative Area/mg protein), B) total oxyradical scavenging capacity (TOSC; RA of ROS/mg protein), C) catalase (CAT) activity (U/mg protein), D) superoxide dismutase (SOD) activity (U/mg protein). Plotted points represent means ± S.E.M. (N=5). Upper case letters denote significant differences in control tissues across salinities. Lower case letters denote significant differences in the tissues of Ni-exposed fish across salinities. Means sharing the same letter are not significantly different. Asterisks denote significant differences between control and Ni-exposed treatments within a salinity.

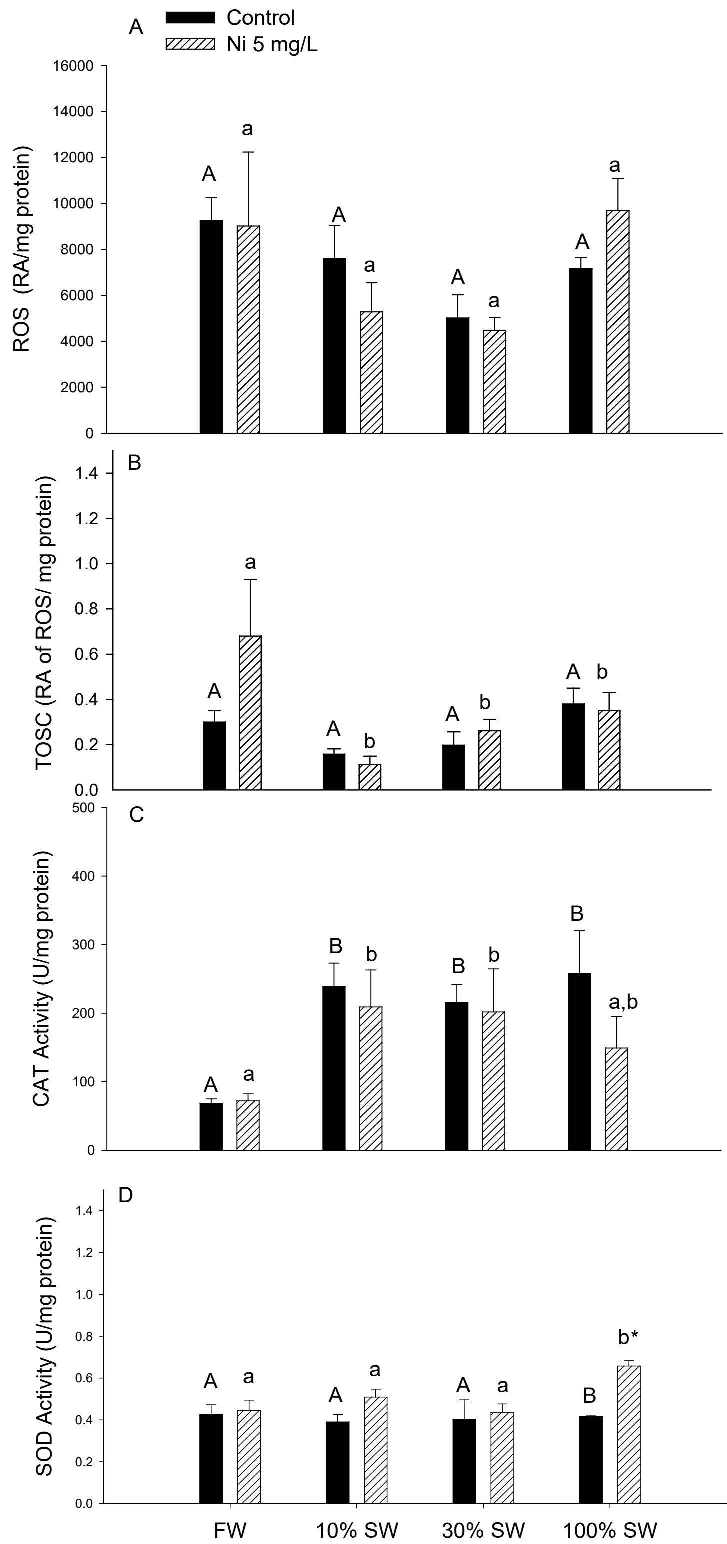

A
Control
Ni 5 mg/L
ROS (RA/mg protein)
B
TOSC (RA of ROS/ mg protein)
C
CAT Activity (U/mg protein)
D
SOD Activity (U/mg protein)
FW
10% SW
30% SW
100% SW

Figure 6.7. Oxidative stress measurements in the intestine of *Fundulus heteroclitus* after an exposure to 5 mg/L Ni for 96 h at one of 4 different salinities (FW, 10%, 30% and 100% SW): A) reactive oxygen species (ROS; RA = Relative Area/mg protein), B) total oxyradical scavenging capacity (TOSC; RA of ROS/mg protein), C) catalase (CAT) activity (U/mg protein), D) superoxide dismutase (SOD) activity (U/mg protein), Plotted points represent means ± S.E.M. (N = 5). Upper case letters denote significant differences in control tissues across salinities. Lower case letters denote significant differences in the tissues of Ni-exposed fish across salinities. Means sharing the same letter are not significantly different. Asterisks denote significant differences between control and Ni-exposed treatments within a salinity.

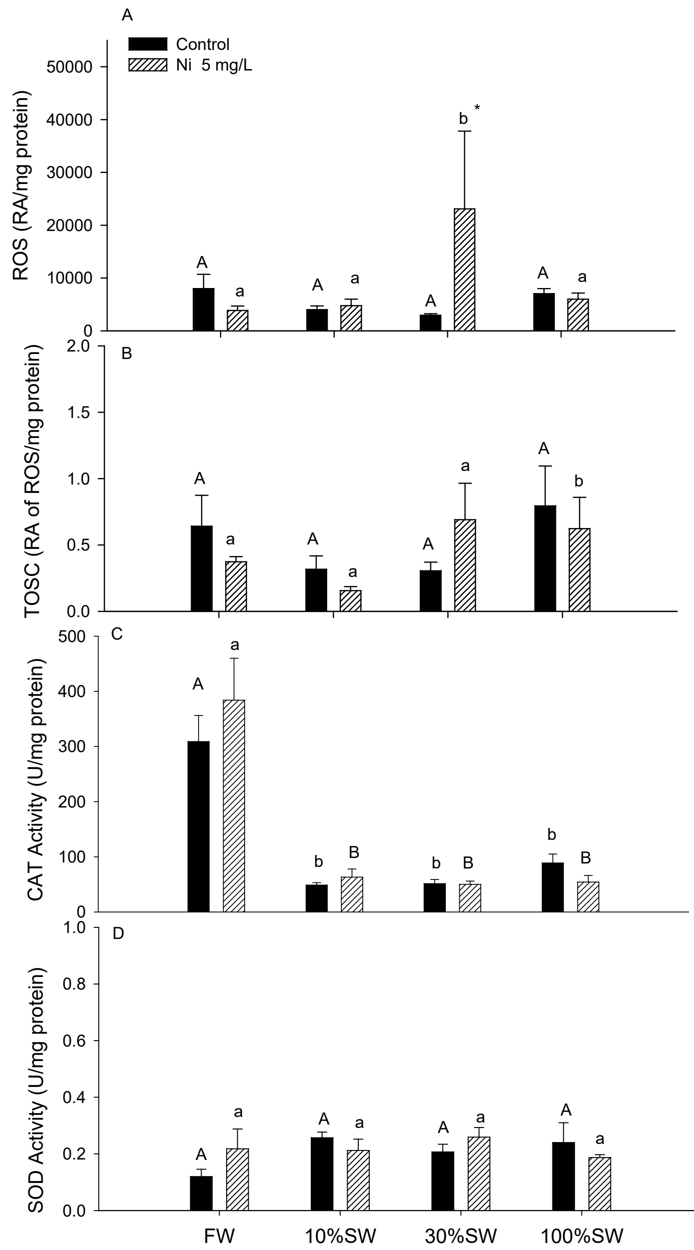
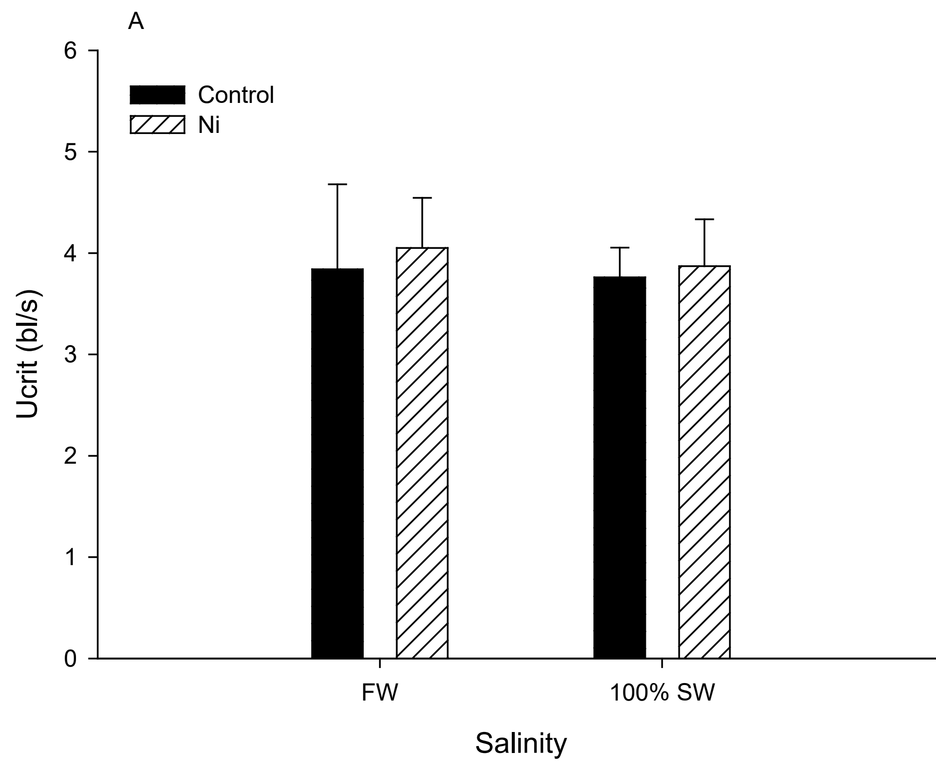

A
Control
Ni 5 mg/L
ROS (RA/mg protein)
0
10000
20000
30000
40000
50000
A
a
A
a
A
b *
A
a
B
TOSC (RA of ROS/mg protein)
0.0
0.5
1.0
1.5
2.0
A
a
A
a
A
a
A
b
C
CAT Activity (U/mg protein)
0
100
200
300
400
500
A
a
b
B
b
B
b
B
D
SOD Activity (U/mg protein)
0.0
0.2
0.4
0.6
0.8
1.0
A
a
A
a
A
a
A
a
FW
10%SW
30%SW
100%SW

Figure 6.8. Oxidative stress measurements in the liver of *Fundulus heteroclitus* after an exposure to 5 mg/L Ni for 96 h at one of 4 different salinities (FW, 10%, 30% and 100% SW): A) reactive oxygen species (ROS; RA = Relative Area/mg protein), B) total oxyradical scavenging capacity (TOSC; RA of ROS/mg protein), C) catalase (CAT) activity (U/mg protein), D) superoxide dismutase (SOD) activity (U/mg protein), Plotted points represent means ± S.E.M. (N = 5). Upper case letters denote significant differences in control tissues across salinities. Lower case letters denote significant differences in the tissues of Ni-exposed fish across salinities. Means sharing the same letter are not significantly different. Asterisks denote significant differences between control and Ni-exposed treatments within a salinity.


A
Control
Ni
Ucrit (bl/s)
0
1
2
3
4
5
6
FW
100% SW
Salinity

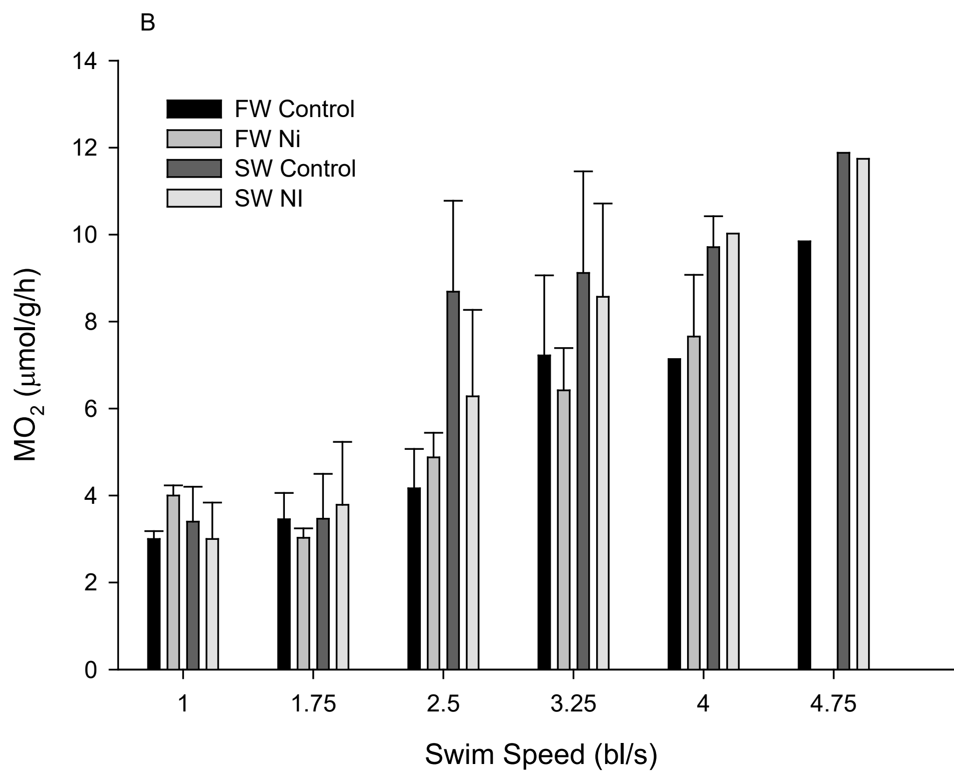

B
FW Control
FW Ni
SW Control
SW NI
MO_2 (µmol/g/h)
0
2
4
6
8
10
12
14
1
1.75
2.5
3.25
4
4.75
Swim Speed (bl/s)

Figure 6.9. Critical swimming speed (A; U_{crit}) and oxygen consumption (B; MO_2) in killifish (*Fundulus heteroclitus*) exposed to 5 mg/L of Ni for 96 h at two different salinities (FW and 100% SW). Plotted points represent means ± S.E.M. (N=7, for all swim speeds up until 4 bl/s, thereafter N = 1-3).

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CHAPTER 7

GENERAL DISCUSSION

7.1 SUMMARY OF FINDINGS

Utilizing estuarine and marine vertebrate (killifish) and invertebrate (green crab, sea urchin) models, I sought to investigate the relationships between water chemistry, organism physiology, tissue Ni accumulation and Ni toxicity across a range of environmental salinities. Specifically, there were five hypotheses tested in the thesis, each of which is addressed below.

7.1.1 Salinity will be protective against Ni accumulation and sub-lethal toxicity, due to the protective cations and complexing anions present at higher concentrations in SW

I predicted that salinity would protect against Ni toxicity as the increased levels of divalent cations (Mg and Ca) would probably outcompete Ni for binding sites on the anionic biotic ligand (the gill in killifish and crab species; Paquin et al., 2002). In addition, Ni speciation will be affected in high salinity waters by the elevated presence of anions, which will result in an increase in chemical species such NiCl and $NiSO_4$. This will effectively reduce the levels of the free ionic form of Ni (Ni^{2+}), decreasing Ni bioavailability and thus potentially reduce toxicity (Niyogi and Wood, 2004).

The proportion of free Ni ion changes depending on salinity. In FW approximately 86% of the Ni is present as the free Ni ion, while in 100% SW this value is 74-76%. The loss of free ion results from an increase in anion complexation, with $NiCl^+$ and $NiSO_4$ being the most prominent species formed. This decrease of free Ni did correlate with lower Ni accumulation in the gills of killifish (Chapters 5, 6) and lower total Ni accumulation in the green shore crab

(Chapters 2, 3) in 100% SW. However, it is difficult to attribute changes in bioaccumulation to changes in in free Ni alone, because as salinity increases so too do the levels of protective cations, which have a higher relative change in terms of magnitude than variations in Ni speciation. However, the magnitude of changes supports the idea that anion complexation is less important. In Chapter 3, there was a 2.9% change in free Ni ion between 20 and 60% SW, but between these two salinities there was a 32% reduction in total Ni accumulation in the crab (at 3 mg/L Ni, for 24 h). Similarly, in killifish gill exposed to 5 mg/L for 96 h, the change in Ni speciation between FW and 10% SW was only 1.7%, but Ni accumulation fell by more than 70% (Chapter 6). This suggests the changes related to anion complexation are of insufficient magnitude to cause such dramatic decreases in Ni bioaccumulation.

It is likely that the greatest protection against Ni accumulation, and therefore Ni toxicity, in SW is due to the increase in cations (Na, Ca, Mg, K). At 100% SW cations increase by 10- (Ca), 200- (K), 110- (Mg) and 724-fold (Na) relative to FW. Previous evidence has shown that Mg and Ca may directly compete with Ni for a common uptake pathway in FW cladocerans (Pane et al., 2003b). Chapter 6 also investigated this relationship in FW-acclimated killifish, and showed protective effects when Mg and Ca in FW were raised to the levels that these ions are found in SW. This effect correlates well with the increased toxicity in dilute SW seen in the green shore crab (Chapter 3). Consequently, the results of this thesis suggest that the increased environmental levels of cations, particular Mg and Ca, are likely to protect marine and estuarine animals against Ni toxicity.

DOC is also thought protective against metal toxicity owing to its ability to bind and decrease bioavailability (Paquin et al., 2002), a finding confirmed in the current study. In Chapter 4, 1.5 mg/L of DOC was sufficiently protective to significantly decrease developmental toxicity

of Ni-exposed sea urchin larvae, relative to those exposed to SW in the nominal absence (actually 0.2 mg C/L) of DOC. However, toxic effects of DOC on sea urchin development were also noted, albeit only in the presence of Ni, possibly mediated through an effect on membrane fluidity. Similar toxicity effects of DOC have been previously observed in sea urchin larvae (Nadella et al., 2013)

7.1.2 Physiology will be more critical than the surrounding water chemistry in terms of shaping sub-lethal Ni toxicity

Water chemistry probably protects against Ni toxicity primarily *via* a cation competition effect, but physiological changes associated with salinity also shaped responses to Ni. For example, crabs are iso-osmotic at 100% SW and do not actively start regulation of ions until salinity falls below 60% SW (Henry et al., 2012). In Chapter 2 and 3, total Ni accumulation followed this theoretical pattern of ion regulation well, with little change between 60 and 100% SW, but a much larger change between 20% and 60% SW, suggesting a physiological driver to this effect.

The importance of physiology in Ni toxicity was also apparent in the responses of crab gills to Ni exposure. The anterior gills, which are specialized for respiration showed very few toxicological impacts, whereas the posterior gills, specialized for ionoregulation, exhibited elevated Ni accumulation, decreased Na^+/K^+ ATPase activity, decreased CAT activity and increased protein carbonylation (Chapters 2, 3). This strongly indicates that the different physiological roles of the gills influenced Ni toxicity.

Physiology also made a significant contribution to Ni accumulation patterns in killifish. SW-acclimated killifish drink more than do FW killifish (Blewett et al., 2013; Scott et al., 2005).

Consequently, as salinity increases the gut becomes a source of Ni accumulation, reflected by the increase in intestinal Ni burden in SW *versus* FW killifish (Chapter 6).

Further emphasizing the role of physiology, the kidney of killifish exhibited a very characteristic pattern of Ni accumulation in killifish, with a much reduced level of Ni relative to that observed in other salinities (Chapter 6). This difference was probably explained by salinity-dependence of kidney excretory function, in agreement with similar findings previously observed between different FW and SW fish species (Pane et al., 2003a; Pane et al., 2006b).

The most sensitive organism tested in this thesis was the developing sea urchin embryo. Over the course of hours, rapid changes occur in terms of growth and development, which can translate seemingly minor changes in physiology, into major impacts. Owing to the importance of Ca for skeletal formation (Carson et al., 1985), there is a high demand for Ca uptake in sea urchin embryos, particularly close to gastrulation (~48 h; Tellis et al., 2013). In Chapter 4, Ni was shown to impair Ca influx, and this effect was only observed during the period of highest Ca demand (see Fig. 4.1). Therefore it is likely that the physiological characteristics of Ca uptake played a critical role in the high sensitivity observed.

Overall, both physiology and water chemistry will alter the uptake, accumulation and toxicity of Ni. Physiological differences associated both with responses to salinity, and also with changes in developmental stage, will play an important role in organismal responses to Ni. However, to adequately delineate between the relative importance of water chemistry and organism physiology, further research is required. It is clear, however, that the interplay between these two factors is critical to the understanding of toxicological mechanisms.

7.1.3 Marine and estuarine invertebrates will be more sensitive to Ni sub-lethal toxicity than vertebrates, conforming to the patterns of toxicity observed in FW

In FW, Ni is generally regarded as being more toxic to invertebrate species (Kozlova et al., 2009; Leonard et al., 2011) than to vertebrates (Pane et al., 2003c, 2004a; Eisler, 1998). For example, the acute LC_{50} for the water flea, *Daphnia magna*, is 71.9 µg/L (Pane et al., 2003b), while in rainbow trout the LC_{50} is 15.6 mg/L (Pane et al., 2003b, Eisler, 1998), close to three orders of magnitude higher. Size may be a significant factor explaining these differences, with the higher surface area to volume ratios of smaller invertebrates leading to greater Ni accumulation, and thus toxicity. However, these two groups also exhibit distinctly different major modes of toxicity in FW. Ionoregulatory disturbance is the main mechanism of Ni toxicity in *Daphnia* (Pane et al., 2003b), and other studied aquatic invertebrates (Chapter 2, Blewett et al., 2015a; Chapter 4, Blewett et al., 2015b). Respiratory toxicity (Pane et al., 2003a) is the main toxic mechanism in rainbow trout. However, it is clear that more data on a wider range of vertebrate and invertebrate organisms will be necessary to test the validity of generalizations regarding toxic mechanisms of taxonomic groupings.

In the current study, the most sensitive species examined was the New Zealand sea urchin embryo *Evechinus chloroticus*. This is not surprising as toxicity was examined in early life stages which tend to be more sensitive to metals (DeForest and Schlekat, 2013). However, the EC_{50} of 14.1 µg/L was still significantly more toxic relative to early life stages of vertebrates. For example, larval killifish exposed to Ni show an LC_{50} of 66 mg/L (Bielmyer et al., 2013), while the most sensitive vertebrate to Ni are topsmelt larvae (*Atherinops affinis*), with an EC_{10} of 3.6 mg/L, following a chronic 40 day exposure (Hunt et al., 2002; DeForest and Schlekat, 2013). The exact mechanisms underlying this difference between sensitive invertebrates and insensitive vertebrates

are unknown, but could again relate to relative size. For example, 96- h *E. chloroticus* larvae are around 500 µm in total length (Sewell et al., 2004) , while 7-d old *F. heteroclitus* larvae are in the order of 7 mm long (Radtke and Dean, 1979). However, it is also likely that size alone does not explain the differences in sensitivity. Ni has also been shown to have a specific effect on key developmental genes, such as LVS1, in sea urchins, a transcript that is responsible for dorsoventral axis formation, and which could underpin the high sensitivity of these animals to Ni (Hardin et al., 1992).

The hypothesis of high relative sensitivity to Ni in invertebrates *versus* vertebrates is probably best tested by examining effect concentrations in adult crabs and killifish, as sea urchins may be considered an extreme outlier for Ni toxicity (DeForest and Schlekat, 2013). Adult crabs were overall more sensitive to Ni toxicity than adult killifish. At 8.2 µg/L, ionoregulatory disruption and decreases in Na^+/K^+ ATPase activity were observed in the green shore crab (Chapter 2), and at 3 mg/L Ni sub-lethal toxic effects included impacts on ionoregulation, oxidative stress and respiratory function (Chapter 3). Conversely, killifish displayed no evidence of respiratory toxicity, a single effect on ionoregulatory toxicity (disturbance of whole body K concentration in FW), and only a few changes in oxidative stress parameters that did not correlate with Ni accumulation, after a 96 h exposure to 5 mg/L of Ni (Chapter 6). As crabs used in this thesis (20-50 g) were larger than killifish (1-5 g), this also indicates that, at least in this particular comparison, taxonomic grouping is likely to be more important in determining Ni sensitivity than size of the tested animal.

7.1.4 Toxicity will be related to ionoregulatory disruption in invertebrates, while respiratory toxicity will be the main mode of toxicity experienced by vertebrates.

The invertebrates tested (sea urchin and green crab) in this thesis both displayed ionoregulatory toxicity following Ni exposure. For example, the sea urchin showed impaired Ca influx rates (Chapter 4), likely the underlying cause of skeletal malformation. In the green crab altered levels of haemolymph Na, Mg and Ca were observed following Ni exposure (Chapters 2, 3). This effect on crab ionoregulation was attributed to Ni inhibition of Na^+/K^+ ATPase activity. Both ionoregulatory disruption and inhibition of Na^+/K^+ ATPase occurred at concentrations of Ni (8.2 μg/L, 500 μg/L) that were lower than the level of Ni exposure that caused respiratory toxicity or oxidative stress (3 mg/L).

In contrast to the proposed hypothesis, there was no evidence of respiratory toxicity in killifish. After an exposure to 5 mg/L of Ni for 96 h in both 100% SW and FW, no effects on swim speed or oxygen consumption were observed. Previous reports of respiratory toxicity have linked such effects to changes in gill ultrastructure (Pane et al., 2003a, 2004b), suggesting that branchial damage is a prerequisite to decreased oxygen consumption and swimming performance. Gill histology was not performed in the current study so the absence of changes in gill ultrastructure could not be confirmed, but the outcomes suggest such changes were unlikely.

Killifish are extremely hardy fish, and in the wild are exposed to varying dissolved oxygen levels, temperature and salinity on a daily and seasonal basis (Burnett et al., 2007). Their ability to cope with multiple environmental stressors may make them resilient to toxicants. Indeed, these fish are well recognized as being among the least sensitive aquatic organisms to toxic compounds (Burnett et al., 2007; Eisler, 1998).

7.1.5 Oxidative stress will be a mechanism of Ni toxicity in both invertebrates and vertebrates.

In FW fish the general mechanism by which Ni induces ROS 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). The secondary mechanism is through Ni effects on antioxidant mechanisms, decreasing the ability of the cell to scavenge ROS, leading to increased ROS-related damage (for review see Lushchak, 2011). In the current thesis, a number of endpoints of oxidative stress were measured, including catalase activity and protein carbonyls (Chapters 3, 5, 6), as well as ROS and TOSC (Chapter 6).

Green crabs exposed to Ni displayed increased protein carbonylation and decreased CAT activity levels (Chapter 3). This was the first study to show an oxidative stress response in a marine invertebrate exposed to Ni. This effect was only observed when the crabs were exposed to Ni levels of 3 mg/L, only in animals acclimated to 20% SW, and only in the ionoregulatory posterior gill 8 (Fig. 3.9). These two effects are likely related, with the decrease in CAT activity suggesting an impaired ability to detoxify ROS, leading to protein carbonylation. This suggests that the main mechanism of Ni-induced oxidative stress in crabs is through inhibition of oxidative defence markers, possibly *via* Ni binding to the histidine groups critical for CAT activity (Predki et al., 1992).

Killifish acclimated to FW and exposed to Ni showed decreased levels of CAT activity in the gill (Chapters 5, 6). These results confirm those previously shown in goldfish following Ni exposure, with oxidative stress noted, albeit it in a tissue- and endpoint-dependent manner (Kubrak et al., 2012a,b; Kubrak et al., 2013; Kubrak et al., 2014). Unlike crabs, where a clear link could be made between a decline in oxidative defence and an increase in oxidative damage, no such clear relationship was observed for killifish. This may indicate a different mode of Ni-

induced oxidative stress in this species. Nevertheless this is the first record of oxidative stress in a marine fish resulting from Ni exposure.

Oxidative stress was thus seen in both invertebrates and vertebrates in the current study, confirming the hypothesis. Of note, however, was the significant impact that exposure salinity had on oxidative stress endpoints (Fig. 3.9, Fig. 5.2, Fig. 5.3, Figs. 6.6-6.8). This suggests that at least some endpoints of oxidative stress (SOD, ROS and TOSC) may not be good markers of Ni toxicity in animals that naturally inhabit waters that vary in salinity.

7.2 METHODOLOGICAL CONSIDERATIONS

One notable feature of the thesis was the variability between identical exposures conducted at different times. For example, killifish in Chapter 6 exhibited no changes in protein carbonylation after a 96 h exposure to 5 mg/L Ni, whereas a different batch of killifish tested for the same endpoint after the same length of exposure to the same concentration showed a significant salinity-dependent decrease (Fig. 5.2 C). These exposures were generally identical in nature, except were conducted at different times of the year, in different batches of experimental animals.

Seasonal effects may play an important role in shaping responses to Ni, particularly with respect to reproduction. Spawning killifish have been shown to express higher levels of metallothionein than non-spawning fish (Van Cleef et al., 2000). This would have an obvious impact on tissue Ni accumulation and bioreactivity, and could underlie the batch-related differences observed in killifish. Similarly, markers of oxidative stress (CAT, lipid peroxidation) were shown to increase in *Carcinus maenas* during summer months, unrelated to winter increases in environmental metals (Pereira et al., 2011). As these are important oxidative stress markers,

this indicates that at certain times of the year animals may have a better ability to deal with metal exposure than in other periods.

The moult cycle of crabs is likely to be another important variable. Moulting induces physiological, biochemical and behavioural changes (Chang, 1995) that can influence metal toxicity to an organism. For example, the sensitivity of shrimp to Cd toxicity varies with moulting stage (De Lisle and Roberts, 1994), while metabolism, and thus toxicological impact of pyrene, was shown to vary with moulting frequency in *C. maenas* (Dam et al., 2006). The duration of the intermoult is a function of body size (Caddy, 2003), and therefore the differences in crab sizes between Chapters 2 and 3 may have contributed to differences in responses between these two chapters.

7.3 ENVIRONMENTAL AND REGULATORY CONSIDERATIONS

In crabs, sub-lethal physiological disturbance was observed at a Ni exposure level of 8.2 μg/L (at 24 h in 20% SW), where a decrease in haemolymph Ca and Na was noted. In Chapters 5 and 6, killifish first showed signs of toxicity (decreased levels of CAT activity) at 5 mg/L in FW after a 96 h exposure, while little evidence of toxicity in 100% SW was observed under similar conditions. Given that environmental levels of Ni rarely, if ever, exceed 1 mg/L (Pyle and Couture, 2012), it is unlikely that toxic effects of Ni will be observed in killifish, although effects on crabs are more likely. Whether these sub-lethal effects would significantly impact survival, or other long-term indicators of individual fitness such as growth or reproduction, remains to be determined. It is, however, likely that such physiological disturbances will incur at least a short-term cost in terms of impacts associated with loss of homeostasis.

At exposure levels where Ni causes effects, are current environmental regulations likely to be adequate? For chronic exposure, the US EPA recommended guideline values for SW are 8.2 µg/L, and for acute exposures, 74 µg/L (US EPA, 2005). These values are unlikely to cause any harmful effects to killifish, but for the shore crab these limits are within the range where sub-lethal impacts were seen (albeit mostly in more dilute waters).

Lethal effects of Ni on early development of the sea urchin *E. chloroticus* were also observed at low Ni levels. The EC_{50} of 14.1 µg/L is well below the regulatory guidelines for protection of 95% of species in New Zealand's marine waters (70 µg/L; ANZECC/ARMCANZ, 2000). Regulatory limits for Ni are regularly exceeded in some coastal regions of New Zealand, owing to deposits from rivers that drain areas naturally rich in Ni (Forrest et al., 2007). This indicates that the effect level of Ni is likely to be environmentally relevant for *E. chloroticus*. Again, however, the long-term impacts of the morphological effects observed in the skeleton of developing embryos are unknown, but it is likely that such impairment would impact locomotion and viability of embryos (Hardin et al., 1992).

In the case of all the organisms studied in the current investigation, Ni toxicity could be mitigated by behavioural avoidance of metals (assuming Ni contamination was localized and escapable). For example, rainbow trout have been shown to avoid Ni (Giattina et al., 1982), although this is the only record of a Ni avoidance response by an aquatic animal in the literature. It therefore remains to be seen whether this is a response that is more widespread and could be an effective mechanism for alleviating potential Ni exposure in estuarine and marine species.

The basis of the BLM is to understand the speciation of metals in varying water chemistries and use this knowledge, in accordance with binding constants on target surfaces of

organisms, to predict whether sufficient metal binding will occur to cause acute toxicity (Niyogi and Wood, 2004). For the development of a BLM in a marine environment, it would seem insufficient to simply adopt FW models, as the differing physiology of marine organisms causes changes to the relationships between speciation, accumulation and toxicity. The osmoregulation-dependent decreases in Ni accumulation observed in the green shore crabs (Chapter 2, 3), and the drinking-related differences in accumulation in the killifish (Chapter 5, 6), are two such examples. Thus more research must be performed in marine and estuarine environments to attain an accurate depiction of biotic ligand metal burdens, how these change with water chemistry, and what impacts these burdens have on organismal health.

7.4 FUTURE DIRECTIONS

Applying Krogh's principle ("For many problems there is an animal on which it can be most conveniently studied"; Krebs, 1975), the high Ni sensitivity of *E.chloroticus* imbues it with the properties of a model species. There is consequently much additional work that could be conducted in this species, particularly with respect to delineating mechanisms of Ni toxicity. For example, conducting a full concentration-dependent kinetic curve for Ni would allow assessment of the transport characteristics of the uptake pathway (as seen with killifish in Chapter 6; Leonard and Wood, 2013). This, coupled with experimental manipulations of Ca and/or Mg levels in the SW, would potentially allow further determination of the roles played by these ions in Ni uptake. Furthermore, the use of pharmacological agents to block putative pathways (e.g. Ca channel blockers such as verapamil and lanthanum; Rogers and Wood, 2004) would aid identification of the Ni uptake pathway and also the potential mechanisms of Ni's toxic effect in this model species. Unfortunately, the poor tolerance of sea urchin larvae to lowered salinity (Nadella et al.,

2013) indicates that it would have limited utility for examining impacts of salinity on Ni uptake and toxicity.

Although the sea urchin is small, in recent decades methodological advances mean this is no longer as significant an experimental drawback as it has been in the past. ICP-MS techniques (Chapter 4) are very sensitive and are able to easily provide measures of Ni burdens in relatively small numbers of pooled embryos. Furthermore, there is much scope for the investigation of molecular endpoints in order to delineate toxic mechanisms (Neumann and Galvez, 2002). For example, Craig and colleagues (2010) identified clusters of genes that changed in response to Cu exposure in zebrafish, and which corresponded to changes in physiological markers of exposure. These authors suggested that gene endpoints could be incorporated into BLM's. In yeast, Ni exposure impacts the expression of around 1300 genes, indicating a strong effect on transcriptional processes (Takumi et al., 2010). Measures of gene expression are very sensitive, relatively easily performed in sea urchin larvae, and there is an increasing understanding of how gene expression changes relate to functional endpoints, particularly in development (Lyons et al., 2012). Changes in sea urchin gene expression have already been used to develop an understanding of toxic mechanisms in response to waterborne Cd (Ragusa et al., 2013). Consequently, there is great scope for the use of the sea urchin as a model organism for examining mechanisms of Ni toxicity in marine settings, particularly *via* the incorporation of gene expression endpoints.

There will be value in the future investigation of Ni speciation in marine environments, particularly with regard to refining current geochemical programs (e.g. Visual MINTEQ, MINEQL+; Geochemists Work Bench; PHREEQC) to better capture the influence of alterations in salinity, and the effects of different types and concentrations of DOM. It will also be important

to determine if more than just the free, ionic species of Ni is bioavailable to marine and estuarine biota. Ni species that may be of significant interest include $NiCl_2$, $NiSO_4$, and $NiCO_3$, which become quantitatively more important in SW owing to higher concentrations of the relevant anions in this environment (Chapters 2-6). Previous research has suggested that neutral metal complexes could be important in bioaccumulation and toxicity. For example, $CuCO_3$ is considered an important species in Cu toxicity, and its inclusion in Cu BLM's improves predictive success (De Schamphelaere et al., 2002).

Regulations are currently written as though all salinity is equal to 100% SW (i.e. one guideline fits all environmental salinities). However, the work from this thesis indicates that not all saline waters are the same or should be treated equally. This is an important point for the future development of a marine-estuarine BLM for Ni, especially since the brackish waters of river mouths are often the sites of metal discharge.

The majority of the current thesis focussed on accumulation of Ni and its toxic effects over acute time-frames. Although development was examined in sea urchins (Chapter 4), and this may be considered a chronic toxicity endpoint (DeForest and Schlekat, 2013), this was only followed for 96 hours, with no assessment on how the morphological changes observed translated to long-term effects. Studies of toxicity over acute time-frames often involve exposure of animals to levels of toxicants beyond those commonly found in the environment. While environmental relevance may be compromised, acute studies facilitate the investigation of toxic mechanism, a critical component of environmental regulations (Paquin et al., 2002). Nevertheless, chronic exposures are likely to be more environmentally realistic. A number of studies have examined chronic toxicity to Ni in marine settings (e.g. Hunt et al., 2002; DeForest and Schelkat, 2013), but there is little mechanistic understanding of chronic Ni toxicity in SW species. Studies of acute

versus chronic mechanisms of toxicity have been conducted in FW organisms. In *Daphnia*, ionoregulatory toxicity (impact on Mg regulation) was observed in both acutely- and chronically-exposed animals, however only during chronic exposure was a respiratory mode of action also evident (Pane et al., 2003a). Differences in toxic mechanisms between acute and chronic studies indicate predictive modelling of chronic toxicity may not be as simple as adopting and modifying acute models. Studies examining the effects of long-term Ni exposure on endpoints such as reproduction and growth in marine organisms would be an important future step.

7.5 Combined References

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