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Investigating the mechanisms of Ni uptake and sub-lethal toxicity in the Atlantic killifish *Fundulus heteroclitus* in relation to salinity☆

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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 nickel (Ni). We investigated Ni accumulation and potential modes of Ni toxicity, in killifish, as a function of environmental salinity. Killifish were acclimated to 4 different salinities [0 freshwater (FW), 10, 30 and 100% seawater (SW)] and exposed to 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. Addition of Mg and Ca to FW protected 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_{2max}). Markers of oxidative stress showed either no effect (e.g. protein carbonyl formation), or 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.

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1. Introduction

Fundulus heteroclitus, the Atlantic killifish, is normally found in estuaries and salt marshes where it is subjected to daily variations in temperature, salinity and dissolved oxygen levels. Furthermore, they are often found near densely populated urban areas where exposure to pollution 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 tolerance to salinity (Griffith, 1974), temperature (Bulger and Tremaine, 1985) and hypoxia (Voyer and Hennekey, 1972). 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 environmental toxicology (Burnett et al., 2007).

Nickel (Ni) is a metal of 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 to 100 μg/L (Boyden, 1975). Estuarine habitats are particularly susceptible to elevated Ni as they directly receive freshwater (FW) Ni inputs and physico-chemical 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*). 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., 2004a,b, 2005). The main mode of Ni toxicity in FW trout is inhibition of respiratory gas exchange, primarily mediated by

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histological changes in the gill (Pane et al., 2004b). However, this mechanism does not hold for all FW animal; 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; Blewett et al., 2015a,b; Leonard et al., 2011) there is still much to learn regarding Ni uptake and toxicity in biota inhabiting saline waters.

Differences in water chemistry between FW and estuarine/marine environments are likely to have a substantial influence over Ni speciation and bioavailability, which in turn may impact uptake and toxicity (Di Toro et al., 2001; Paquin et al., 2002). The bioavailable form of Ni is considered to be the free Ni^{2+} ion (Niyogi and Wood, 2004; Wood, 2012), which can be taken up by the gill and is 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 Ni (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, metal affinities of gill binding sites, and resulting gill metal burdens 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 divalent cations and Ni has been shown (Pane et al., 2003a,b). Furthermore, as ion transport differs between FW and seawater (SW) 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.

Our previous work (Blewett and Wood, 2015a) indicated that oxidative stress occurs in the killifish 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. A second goal was to examine potential ionoregulatory and respiratory modes of toxicity in killifish. Finally, in order to better understand the mechanism of Ni accumulation, the concentration-dependence of branchial Ni uptake and the effects of water chemistry manipulation on gill Ni accumulation were explored. Our specific hypotheses were that salinity would be protective against Ni accumulation and sub-lethal toxicity due to both the complexation of Ni with anions and the protective effect of cations present in SW, and that similar to FW trout, respiratory toxicity would be the main mode of sub-lethal Ni toxicity in killifish at various salinities.

2. Materials and methods

2.1. Animal care

Atlantic killifish (*F. heteroclitus*; northern subspecies) (1–5 g) of both sexes were obtained from Aquatic Research Organisms Ltd. (Hampton, NH, USA). At McMaster University, fish were held in 10% SW, 12 h light:12 h dark at 18 °C, for several weeks in a 250-L recirculating system with charcoal filtration. Aquarium water was changed every 2–3 days. Fish were then acclimated to one of four different salinities (0%, 10%, 30% and 100% SW) under holding conditions for 7–14 days. Saline waters were made by the addition of Instant Ocean sea salt (Big Al's Aquarium Supercenter, Woodbridge, ON, Canada) to freshwater, considering 35 g per liter as 100% SW. Freshwater 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). 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), but 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.

2.2. Ni exposures at different salinities

The effect of Ni (5 mg/L) was investigated at each of the four salinities: 0 ppt (Hamilton freshwater; 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 per tank (biomass load ~1.5 g/L). 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. 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.

2.3. Ni bioaccumulation, ion and water analysis

Ni concentrations were monitored daily after every water change. Water samples for ions and dissolved Ni concentrations were passed 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 1–4. After the 96-h exposures, fish were terminally euthanized with a lethal dose of MS-222 (NaOH-neutralized, to pH 7; Syndel Laboratories Ltd., Vancouver, BC, Canada). A subset of gill, intestine, and liver were sampled, quickly frozen in liquid nitrogen, then transferred to −80 °C for eventual measurement of oxidative stress markers (see below). A second subset of tissues, including gill, intestine, liver, kidney, and carcass 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. Tissues were digested in 3–5 volumes (exact volume recorded) of trace metal grade nitric acid (1N; Sigma–Aldrich), except for the carcass which was digested in a 3–5 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. The resulting supernatants were then analyzed 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). 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

Table 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 (°C)	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 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 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 4
Water chemistry for high Ca and Mg exposures (means ± S.E.M; N = 8 per treatment, except temperature (N = 1).

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 (°C)	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.

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).

2.4. 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 was measured following its artificial generation from the thermal decomposition of a fluorescent dye [ABAP; 2,2′-azobis(2-methylpropionamidine) dihydrochloride, Sigma Aldrich], and was 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 Clairborne (1985). Samples were homogenized (20:1; v:w) 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), 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 Bradford (1976) using bovine serum albumin as a standard.

2.5. Critical swimming speed and oxygen consumption

In a separate exposure, conducted under identical conditions to those described in Section 2.2 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 determination 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 20 °C. Every 30 min, 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 again, and measurements repeated as above. Water samples (5 mL) 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 2.8.

2.6. Concentration-dependent kinetics of Ni uptake

Fish that had been acclimated to FW or to 100% SW (as described in Section 2.2 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 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 concentration (10 mg/L as $NiCl_2 \cdot 6H_2O$) of non-radioactive Ni, and subsequently a 1 mM EDTA solution, to remove any loosely-bound radioisotope, before euthanasia 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 placed in an incubator at 65 °C for 48 h, with vigorous vortexing at 24 h. The digests were then centrifuged at 3500 *g* for 5 min at 18 °C. Ultima Gold AB scintillation fluor (Perkin Elmer, Waltham, MA, USA) was then added to tissue digests to give a ratio of 1:5 (digest:fluor), and Optiphase 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.

2.7. Effects of Ca and Mg on Ni uptake in FW

Treatments with Ca (10 mM $CaCl_2 \cdot 2H_2O$, Sigma Aldrich) and/or Mg (45 mM $MgCl_2 \cdot 6H_2O$, Sigma Aldrich) were designed to raise the levels of Ca and Mg in FW to the levels of these ions in SW Killifish acclimated to FW (see Section 2.2 above), were placed in one of 4 different FW treatments (N = 5 for each): 5 mg/L Ni, 5 mg/L Ni + Ca + Mg, 5 mg/L Ni + Ca, and 5 mg/L Ni + Mg (Table 4). 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, 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 2.6. 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 2.6).

2.8. Calculations and statistics

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

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

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.

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:

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

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):

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

where U_i is the swim speed (bl/s) achieved for the 30 min 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.

Data have been expressed as means ± SEM (N = 5–7). 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; if data did not pass these tests, they were appropriately transformed prior to parametric testing. 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 1 and 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 3).

3. Results

3.1. Water chemistry and Ni speciation

Water chemistry analysis showed the expected pattern of an increase in ion concentrations as salinity increased (Table 1). 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 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 3). The next most dominant species in 100% SW were $NiSO_4$ and Ni-DOC, followed by $NiCl^+$, $NiCO_3^+$, and $NiCO_3$. The contributions of $NiSO_4$ and $NiCl^+$ declined as salinity decreased, but the carbonate complexes persisted at all salinities. Analysis confirmed elevations of Mg and Ca levels in the experiments where these ions were selectively raised in FW (Table 4).

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

Exposure of killifish to 5 mg/L Ni for 96 h resulted in increases in Ni burdens in all tissues examined (Fig. 1). Overall, levels of Ni accumulation were comparable in intestine, kidney, and whole body (8000–10,000 μg/kg), with somewhat lower concentrations in gill and liver (Fig. 1A,C). Levels of Ni in the tissues of control animals (not exposed to Ni) did not vary significantly with salinity, but background burdens of Ni differed among tissues (Fig. 1A–E). At all salinities, the Ni burden in the carcass quantitatively dominated, accounting for >95% of the whole body burden (Supplemental Table. S1). Ni exposure had no significant effects on whole body concentrations of K^+, Na^+, Mg^{2+} and Ca^2 (Supplementary Fig. S2), except for an elevation in K^+ in the freshwater treatment group.

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. 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. 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, there were significant effects of treatment ($P < 0.001$) and salinity ($P \leq 0.05$) but no interaction effect ($P = 0.077$). Ni accumulations in both liver and intestine were lowest in the 10% SW group, however all Ni treatments were significantly higher than controls.

In the kidney, there were significant effects 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; notably, the 100% SW group exposed to Ni displayed an accumulation that was only ~20% of those found in the other salinities (Fig. 1D).

Finally, for whole body Ni accumulation, there was a significant effect of treatment ($P \leq 0.05$), but not of salinity ($P = 0.08$) or interaction ($P = 0.112$) 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 in the intestine, this accumulation did not differ significantly among different salinities (Fig. 1E).

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

At 5 mg/L Ni, gill Ni accumulation in FW was significantly inhibited by about 70% ($P \leq 0.05$) by each of these two cations when added at their typical 100% SW concentrations (10 mM Ca, 45 mM Mg). This effect occurred to the same extent regardless of whether these ions were added together or separately (Fig. 2).

3.4. Concentration-dependence of Ni uptake kinetics in FW and SW

In both FW and SW, hyperbolic curves best fitted the raw data. For FW ($r^2 = 0.95$), a B_{max} of 9326 ± 1222 μg/kg and a K_m of 6942 ± 1335 μg/L were derived (Fig. 3A). In SW ($r^2 = 0.77$), the comparable kinetic constants were a B_{max} of 4587 ± 859 μg/kg, and a K_m of 2264 ± 899 μg/L (Fig. 3B). Both the B_{max} and K_m values were significantly lower in SW than in FW ($P = 0.005$ and $P = 0.009$, respectively).

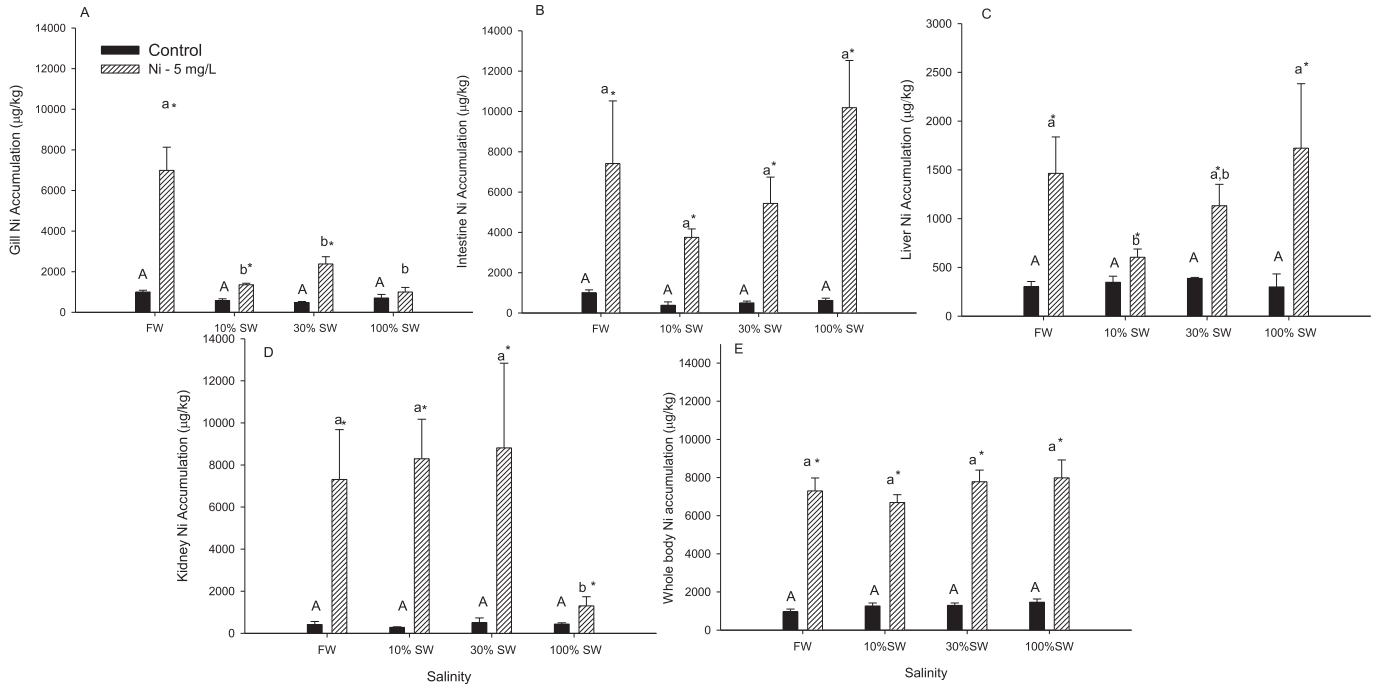

Fig. 1. Ni accumulation in: A) gills, B) intestine, C) liver, D) kidney, and E) whole body (µg/g wet wt.) of *Fundulus heteroclitus* after an exposure to 5 mg/L of Ni for 96 h, in one of four different salinity treatments (FW, 10%, 30% and 100% SW). Plotted points represent means ± S.E.M. (N = 6). Upper 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. Note scaling on the liver graph is lower than all other graphs. Asterisks denote significant differences between control and Ni-exposed treatments within a salinity.

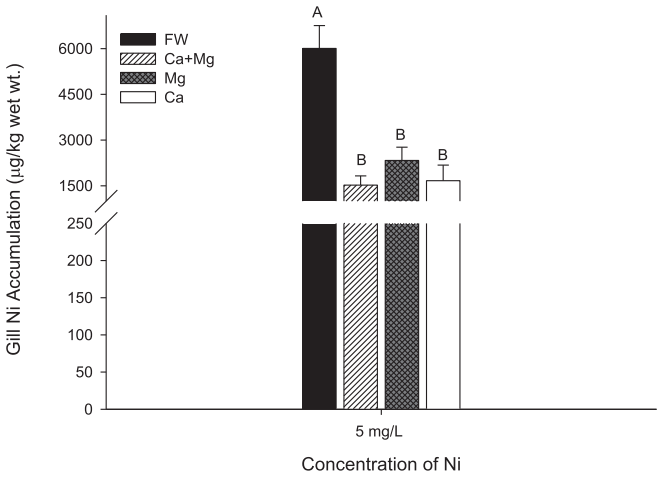

Fig. 2. Gill Ni accumulation in *Fundulus heteroclitus* at one concentration of Ni under 4 different water chemistry parameters (5 mg/L Ni, 5 mg/L Ni + Ca + Mg, 5 mg/L Ni + Ca, and 5 mg/L Ni + Mg) as detailed in Table 4. Plotted points represent means ± S.E.M. (N = 5). Upper case letters denote significant differences between experimental chemistries within a concentration. Means sharing the same letter are not significantly different.

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

Levels of ROS production, TOSC, and SOD activities were generally comparable among the three studied tissues (Fig. 4 and Supplemental Figs. S3, S4). In the gill (Fig. 4), 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. 4A, C). No other two-way ANOVA parameters were significant in gill tissue. More specifically, there was a significant 7-fold increase in ROS production in Ni-exposed gill tissue relative to controls in 10% SW (Fig. 4A). However, there were no differences in TOSC associated with either Ni or salinity (Fig. 4B). CAT activity displayed a significant 50% decrease in response to Ni treatments in both FW and 30% SW relative to unexposed control gills (Fig. 4C). 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. 4D).

In the intestine (Supplemental Fig. S3), 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 ($P = 0.53$, $P = 0.48$, $P = 0.112$, $P = 0.35$) (Supplemental Fig. S3A–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 (Supplemental Fig. S3B). 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 (Supplemental Fig. S3C). Intestinal SOD activity was significantly increased by Ni exposure but only in 100% SW (Supplemental Fig. S3D).

In the liver (Supplemental Fig. S4), 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 (Supplemental Fig. S4C) ($P \leq 0.05$). ROS production

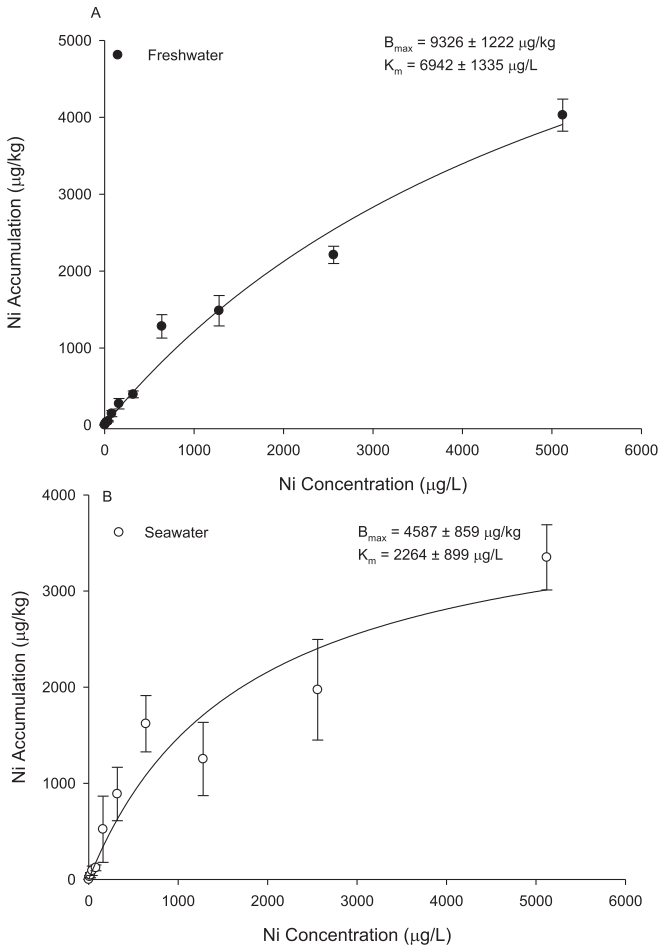

Fig. 3. FW (A) and SW (B) gill Ni accumulation (μg/g wet wt.) as a function of increasing Ni exposure concentrations (μg/L) following a 3-h exposure. Plotted curves and corresponding parameters (B_{max}, K_m) of the Michaelis–Menten equation, fitted using SigmaPlot. Both B_{max} and K_m parameters were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

was significantly elevated by Ni treatment ($P \leq 0.05$) but only in the 30% SW group (Supplemental Fig. S4A) 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 (Supplemental Fig. S4B–D).

3.6. U_{crit} and MO_2 after exposure to 5 mg/L Ni for 96 h

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. 5A; 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 their interaction ($P = 0.943$) 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. 5B).

4. 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.

4.1. 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. 1). This is similar to the response observed in FW fish exposed to elevated Ca and/or Mg in the exposure water (Fig. 2), so likely reflects competition between these two ions and Ni for uptake. The fact that similar effects were observed in salinity-acclimated animals and FW animals exposed acutely to elevated ions suggests that both water chemistry and physiology dictate Ni uptake at the gills. High salinity similarly reduced branchial Ni accumulation in the green shore crab (Blewett et al., 2015a), while gill Ni accumulation was also reduced in SW killifish relative to FW killifish in a previous study on this species (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 (Blewett et al., 2013; 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 (Wood et al., 2010). 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. These two intermediate salinities are relatively close to the killifish iso-osmotic point and so represent salinities where the need for drinking (and associated intestinal ion uptake) would be lowest, and therefore exposure of the gut to waterborne 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 (Blewett and Wood, 2015a).

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) as the liver is the main detoxifying organ in the body. 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.

Ni accumulation in the kidney displayed a very distinct pattern whereby in FW, 10% SW and 30% SW, it was significantly higher than in 100% SW (Fig. 1D). A large renal burden 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

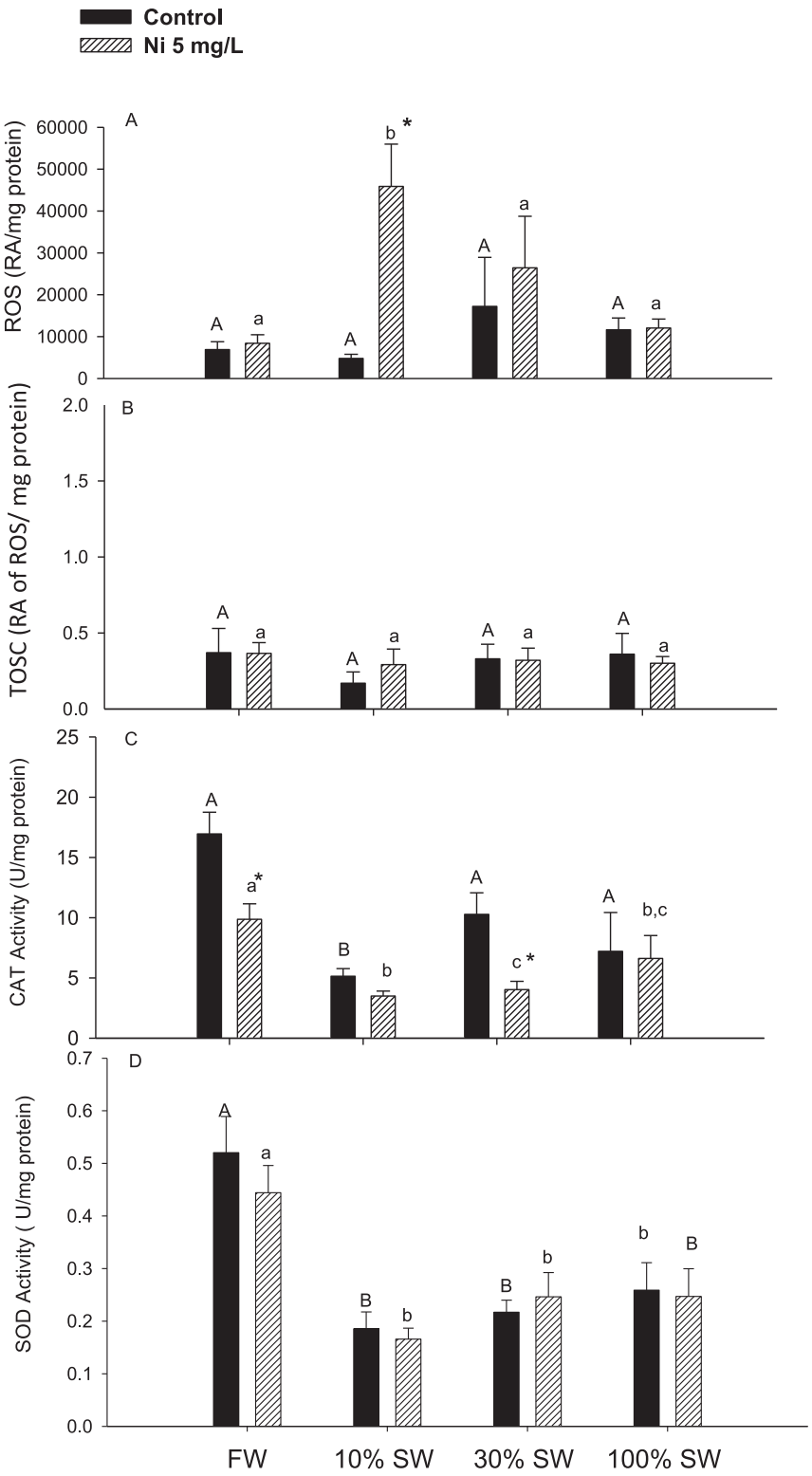

Fig. 4. Oxidative stress indicators in the gills of *Fundulus heteroclitus* after an exposure to 5 mg/L Ni for 96 h at one of 4 different salinities (FW, 10%, 30% and 100% SW): A) reactive oxygen species (ROS; RA = Relative Area/mg protein), B) total oxyradical scavenging capacity (TOSC; RA of ROS/mg 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.

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

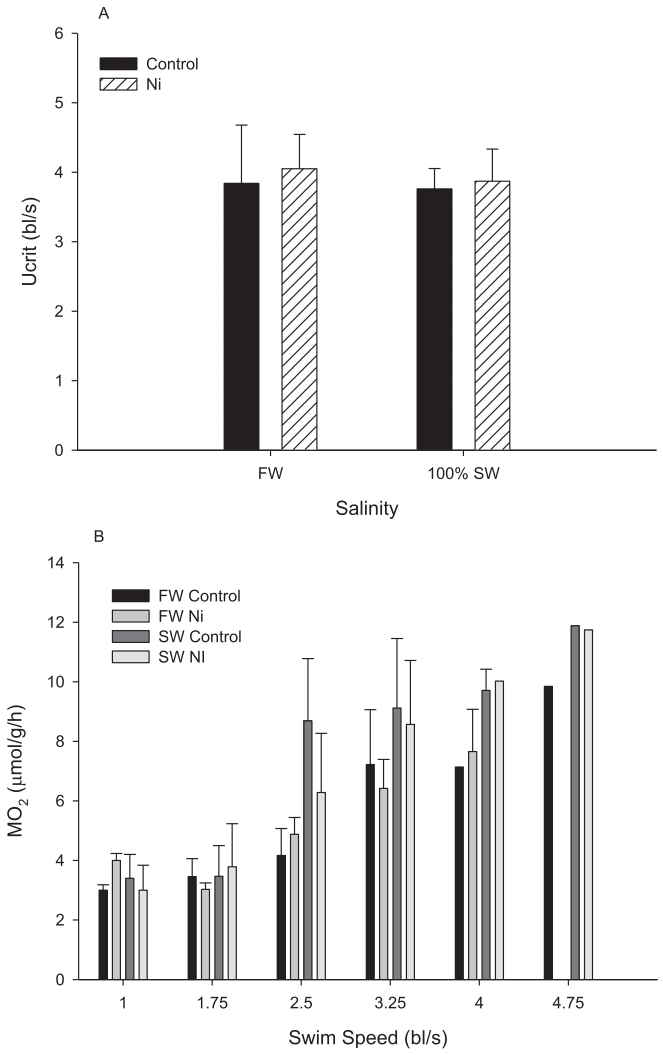

Fig. 5. Critical swimming speed (A; U_{crit}) and oxygen consumption (B; MO_2) in killifish (*Fundulus heteroclitus*) exposed to 5 mg/L of Ni for 96 h at two different salinities (FW and 100% SW). Plotted points represent means ± S.E.M. (N = 7, for all swim speeds up until 4 bl/s, thereafter N = 1–3).

salinities. 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.

4.2. The influence of Mg and Ca on acute Ni accumulation in FW-acclimated killifish

The Ca and Mg addition experiments support 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. 2). The magnitude of this effect was the same irrespective of whether Mg or Ca was added alone, or in combination. 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. 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., 2006a,b). For example, Ni is 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 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 reported in fish (Bijvelds et al., 1996; Shearer and Åsgård, 1992). Furthermore, Mg and Ca protected 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 M ratio (Pane et al., 2006a,b).

It is important to note that the addition of $CaCl_2$ and $MgCl_2$ in the current study would also increase water Cl levels (Table 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).

4.3. Concentration-dependent gill Ni accumulation kinetics in FW and SW

Both FW and SW killifish fish displayed saturable concentration-dependent kinetics for Ni uptake into the gills (Fig. 3). However, the SW fish displayed a lower B_{max} (i.e. fewer binding sites) and a lower K_m (i.e. higher binding site affinity).

The presence of saturation as exposure concentration increases is usually indicative of metal binding to specific transporters or channels. Similar the patterns have been observed for gill Ni accumulation in rainbow trout and round goby (Leonard et al., 2014). 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 9326 μg/kg in FW (160 μmol/kg) and 4587 μg/kg (79 μmol/kg) in SW, somewhat lower than the B_{max} values calculated for round goby (304 μmol/kg) and rainbow trout (278 μmol/kg), both of which are FW species. Similarly the K_m for killifish gill Ni accumulation in FW was 6942 μg/L (119 μmol/L), and in SW was 2264 μg/L (39 μmol/L). This FW value is higher than 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., 2014). 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 tolerant 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 tolerant 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 killifish decreases linearly with increasing salinity. However, these tests were performed on killifish larvae, in contrast to the adult animals used in the current study. To confirm the relationship between gill Ni binding affinity and lethality, experiments would need to be done concurrently on fish of an equivalent life stage.

The exact mechanisms of transcellular Ni uptake have yet to be characterized. However, as discussed in Section 4.2, 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). The saturable component of Ni uptake for killifish gill may be through 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 lower B_{max} and K_m values in SW killifish support the concept that FW- and SW-acclimated killifish may have different Ca transporting characteristics, which impact the kinetics of Ni accumulation.

Alternatively, Ni may be transported through metal-specific carriers such the divalent metal transporter (DMT1), which would also confer a saturable uptake pattern. DMT1 is known to occur 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 it may also transport Ni (Gunshin et al., 1997). For example, an increased uptake of Ni in the absence of Fe suggests a common carrier-mediated transport mechanism shared by these two ions (Tallkvist et al., 2003). 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.

The pattern of SW gill Ni accumulation differed between the 3-h kinetic study (Fig. 3B) and the 96-h exposure (Fig 1A). In Fig. 1A, the gills of SW killifish accumulated about 1000 μg/kg after 96 h, whereas after 3 h at an equivalent Ni exposure level (~5 mg/L), the accumulation was approximately 3000 μg/kg (Fig. 3B). A similar pattern was also reported by Blewett and Wood (2015a). 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.

4.4. 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 (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, including 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 (Supplemental Fig. S2A–C).

In general there was a lack of correlation between Ni accumulation in tissues (Fig. 1) and oxidative stress markers (Fig. 4 and Supplemental Figs. S3, S4), 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 (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 oxidative stress. For example, CAT activity in the gill was significantly impacted by Ni (in FW and 30% SW; Fig. 4C). Such an effect has been observed previously in killifish exposed to levels of Ni 2–3-fold higher than those used in the current study (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).

4.5. 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; critical swim speed (U_{crit}) and oxygen consumption (MO_2) were unaltered by Ni exposure (Fig. 5). 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 2.03 mg/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 may not have been 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 96-h exposure to Ni. It is possible that if the tests were run under continuous metal exposure conditions, that respiratory effects may have been observed. However, Wilson et al. (1994) reported 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.

It is possible that differences in Ni sensitivity between killifish and rainbow trout are also responsible for the different effects noted. The 96 h LC_{50} for juvenile killifish was 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 tolerance of killifish may have precluded the development of respiratory effects.

5. 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, very minor changes in ionoregulatory parameters and no respiratory toxicity effect. Ni accumulation did, however, vary with salinity, with effects of Ca and Mg on gill Ni uptake. The tested level of Ni was relatively high, so the current data suggest that acute exposures to Ni at environmentally relevant levels of Ni are unlikely to cause significant harm to killifish. However, because of their relatively low sensitivity and site-fidelity, killifish may be very useful as site-specific biomonitors (e.g. Weis and Candelmo, 2012). Linking exposure to effect is also important, and the present results suggest that salinity will impact bioaccumulation, and this should be accounted for under such risk assessment approaches. These experiments will lead to a better understanding of Ni bioaccumulation and toxicity in marine organisms, and thus may contribute essential information to the

eventual development of improved water quality guidelines for Ni in marine/estuarine environments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.01.002.

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