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Salinity-dependent nickel accumulation and effects on respiration, ion regulation and oxidative stress in the galaxiid fish, *Galaxias maculatus*☆

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ABSTRACT

Inanga (*Galaxias maculatus*) are a euryhaline and amphidromous Southern hemisphere fish species inhabiting waters highly contaminated in trace elements such as nickel (Ni). Ni is known to exert its toxic effects on aquatic biota via three key mechanisms: inhibition of respiration, impaired ion regulation, and stimulation of oxidative stress. Inanga acclimated to freshwater (FW), 50% seawater (SW) or 100% SW were exposed to 0, 150 or 2000 μg Ni L^{-1}, and tissue Ni accumulation, metabolic rate, ion regulation (tissue ions, calcium (Ca) ion influx), and oxidative stress (catalase activity, protein carbonylation) were measured after 96 h. Ni accumulation increased with Ni exposure concentration in gill, gut and remaining body, but not in liver. Only in the gill was Ni accumulation affected by exposure salinity, with lower branchial Ni burdens in 100% and 50% SW inanga, relative to FW fish. There were no Ni-dependent effects on respiration, or Ca influx, and the only Ni-dependent effect on tissue ion content was on gill potassium. Catalase activity and protein carbonylation were affected by Ni, primarily in FW, but only at 150 μg Ni L^{-1}. Salinity therefore offsets the effects of Ni, despite minimal changes in Ni bioavailability. These data suggest only minor effects of Ni in inanga, even at highly elevated environmental Ni concentrations.

1. Introduction

Inanga (*Galaxias maculatus*) are one of the few truly amphidromous fish species (McDowall, 2007). After hatching on spring tides from aerially-exposed eggs laid on riparian margins, larval inanga spend three to six months at sea, before swimming back to freshwater (FW) as juveniles. As adults, inanga usually occupy FW near the saltwater wedge and may encounter salinity fluctuations on a daily basis (McDowall, 2007). The presence of inanga in near-coastal and estuarine waters exposes these fish to environments that are impacted by anthropogenic stressors. In addition to urban and industrial contaminants which are prevalent in such waters, some streams that function as important inanga habitat on New Zealand's West Coast are also impacted by acid mine drainage, and contain elevated levels of trace metals (Greig et al., 2010). For example, concentrations of nickel (Ni) may reach in excess of 150 μg L^{-1} in these waters (Harley et al., 2016). Although these levels are lower than some reported in waters in the vicinity of industrial sites in Canada (upper ranges 2–6 mg L^{-1}; Chau and Kulikovsky-Cordeiro, 1995), they are still significantly higher than regulatory guidelines (level required to protect 95% of aquatic species in New Zealand are 11 μg L^{-1} in FW and 70 μg L^{-1} in seawater (SW); ANZECC/ARMCANZ, 2000). In fact, trace element pollution has been suggested as a factor contributing to the decline in New Zealand's "whitebait" fishery (McRae et al., 2016), which involves the harvest of juvenile inanga as they return to FW. Despite the elevated levels of Ni in key inanga habitats, nothing is known regarding the sensitivity of inanga to this metal.

Although there is some limited evidence (e.g. Chowdhury et al.,

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2008) that Ni is an essential trace element for fish at low concentrations, there is clear evidence that Ni has toxic effects at higher concentrations (Pyle and Couture, 2012). In general, three modes of Ni toxicity to aquatic organisms have been identified. In invertebrates (e.g. Blewett et al., 2015; Blewett et al. 2016b; Blewett and Wood, 2015a; Pane et al., 2003b), and to a lesser extent in fish (e.g. Alsop and Wood, 2011), Ni is believed to impair ion homeostasis. Effects of Ni have been observed on calcium (Ca), magnesium (Mg), and sodium (Na) regulation. In the case of the divalent ions, these effects are likely mediated by ion mimicry, with Ni acting as a blocker of key Ca and/or Mg-regulated cellular processes (Blewett et al., 2016b; Pane et al., 2003b). In the case of Na, effects are most likely mediated by the inhibitory actions of Ni on the basolateral sodium pump (Blewett et al., 2015), which is a key entity responsible for the cellular homeostasis of Na and other ions. Conversely, in fish the main mode of Ni toxicity appears to be respiratory disruption. Ni causes gill inflammation resulting in decreased diffusive distance and impaired oxygen uptake (Pane et al., 2003a, 2004b). The third mechanism of Ni toxicity is the promotion of oxidative stress (Blewett and Wood, 2015a, 2015b; Kubrak et al., 2012, 2013). It is thought that Ni acts to inhibit antioxidant scavenging defences or has a pro-oxidant effect, possibly by displacing iron from enzyme active sites and increasing reactive oxygen species (ROS) production via the Fenton reaction (Lushchak, 2011).

Understanding how toxicity varies as a function of environmental salinity has been identified as a current challenge for environmental trace metal regulations (Bielmyer and Grosell, 2011; de Polo and Scrimshaw, 2012). For example, the biotic ligand model (BLM) approach is a regulatory tool that relies on the relationship between trace metal speciation, and accumulation at the site of toxicity (the "biotic ligand") to predict risk (Paquin et al., 2002). However, in marine settings both water chemistry and physiology may change, affecting this relationship. To appropriately calibrate BLM approaches for settings that vary in salinity, more data are required to establish the relative importance of these changing factors in modifying toxic impacts. Key to such studies are euryhaline organisms, such as inanga, which are capable of inhabiting waters of variable salinity (Chessman and Williams, 1975; Urbina et al., 2013). Inanga are among the most widespread of all FW fish species in the Southern hemisphere (McDowall, 2007), but the regulatory tools utilised to protect this species are based on research performed on a few Northern hemisphere species (ANZECC/ARMCANZ, 2000). The validity of this regulatory approach is potentially compromised by a number of unusual physiological characteristics that may impact mechanisms of toxicity, and thus endow inanga with different sensitivities to metal toxicants. For example, inanga skin is a physiologically-active surface responsible for ~40% of the total respiration rate (Urbina et al., 2014). If, as in other fish, Ni affects respiration by impairment of the gill epithelia of inanga, this may have a limited toxic impact in a species that is capable of utilising the cutaneous surface to meet a significant proportion of its oxygen requirements.

The current study aimed to examine the effect of salinity on Ni accumulation and its impacts on respiration, ion regulation and oxidative stress, the three modes of Ni toxicity identified in other aquatic biota. Inanga acclimated to FW (0‰), 50% SW (16‰) or 100% SW (32‰) were exposed to one of three Ni exposure concentrations: control (no added Ni), 150 μg L^{-1} (an elevated environmental level recorded in inanga habitats; Harley et al., 2016) or 2000 μg L^{-1} (a "worst case" scenario representing a heavily impacted environmental level; Chau and Kulikovsky-Cordeiro, 1995) at their acclimation salinities. After 96 h, tissue Ni burden, tissue ion concentration, Ca influx, metabolic rate (MO_2), antioxidant defence status (catalase activity), and oxidative damage (protein carbonylation) were assessed.

2. Materials and methods

2.1. Animal collection and holding

Adult inanga (n = 90; 1.4 ± 0.1 g), were collected by seine netting near-coastal streams in the Canterbury region of New Zealand, and transported to the aquarium facility at the University of Canterbury. There they were held in aerated, flow-through FW (pH 7.1; total hardness 0.7 mmol L^{-1}; total alkalinity 0.519 mmol L^{-1}; electrical conductivity 18.8 mS m^{-1}; Ca 0.57 mmol L^{-1}; Mg 0.14 mmol L^{-1}; K 0.29 mmol L^{-1}; Na 0.37 mmol L^{-1}; Cl 0.31 mmol L^{-1}; dissolved organic carbon <0.2 mg C L^{-1}), at 12–14 °C, under a 12:12 h light:dark cycle, for two weeks prior to salinity acclimation. Fish were fed ad libitum on commercial flake food on a daily basis during holding and salinity acclimation, but food was with-held for two days prior to, and during, Ni exposures. All procedures were approved by the University of Canterbury Animal Ethics Committee.

2.2. Salinity acclimation

Inanga were split into three groups, each of which was housed in an 80-L plastic container, and held under static water conditions. Fish designated as 100% SW (n = 30; 1.2 ± 0.2 g) were subjected to a step-wise increase in salinity (by 6 or 7‰ every three days) until 100% SW was reached (32‰), at day 15 of acclimation. Inanga were held at this salinity for 7 days prior to experimentation. Fish designated as the 50% SW group (n = 30; 1.1 ± 0.2 g), underwent a similar step-wise acclimation (steps of 6, 7 and 3‰ respectively every three days) to reach an acclimation salinity of 16‰ after 9 days. These inanga were held at this salinity for 13 further days before experimentation. In both the 50 and 100% SW groups, the SW used was freshly collected from Lyttelton Harbour (pH 8.1; Na 520 mmol L^{-1}; Ca 14 mmol L^{-1}; Mg 59 mmol L^{-1}; K 12 mmol L^{-1}; dissolved organic carbon 0.5 mg C L^{-1}), and where necessary was diluted with aquarium FW. Water in these two acclimation salinities was changed every three days. Fish in the FW group (n = 30; 1.7 ± 0.2 g) were subjected to water changes with fresh aquarium FW every three days.

2.3. Ni exposure

At each salinity inanga (n = 10) were divided into one of three exposure concentrations of Ni, nominally 0 (no added Ni), 150, or 2000 μg L^{-1}, added as $NiCl_2 \cdot 6H_2O$ (Sigma Aldrich). Exposure chambers consisted of 4-L acid-washed plastic containers, filled with 2 L of water adjusted to the appropriate salinity (FW = 0‰; 50% SW = 16‰; or 100% SW = 32‰), and constantly aerated. Ni was added to each chamber 24 h prior to fish to allow for equilibration. These exposures were conducted in a temperature-controlled room held at 15 °C and an identical light cycle to that during acclimation (12 h light: 12 h dark). After 48 h, fish were transferred into fresh containers with identical water chemistry that had also been equilibrated for 24 h. Water samples for measurement of Ni exposure concentration were taken at 0, 48 (both before and after water change) and 96 h. Both total (unfiltered) and dissolved (passed through a Millex 0.45 μm filter; Millipore) samples were taken at each time-point.

2.4. Respirometry and tissue dissection

At the conclusion of the 96-h exposure, oxygen consumption rate (MO_2) was measured following a protocol similar to that used

for inanga previously (McRae et al., 2016). Individual fish (n = 5) were placed in 250-mL glass chambers containing control (i.e. Ni-"free") water at the exposure salinity, and held in a temperature bath at 15 °C. Respirometry chambers were sealed with a rubber bung, through which was pushed a needle connected to a valve, which facilitated water sampling. At times 0 and 1 h, water samples were removed, and oxygen partial pressure (PO_2) was determined using a water-jacketed O_2 electrode connected to an O_2 meter (Strathkelvin). This set-up was calibrated at regular intervals using 0.01 M sodium tetraborate and air-saturated water. The decline in PO_2 as fish depleted the oxygen in the chamber was converted to MO_2 (μmoles O_2 g^{-1} h^{-1}), via the following equation:

$$MO_2 = \frac{\Delta PO_2 \times C \times V}{W \times T}$$

where ΔPO_2 is the change in O_2 partial pressure, C is temperature- and salinity-adjusted O_2 capacitance (Battino et al., 1983), V is the respirometer volume (L), W is fish mass (g), and T is time (h). Control respirometers (without fish) were run to account for any microbial contribution to MO_2 (there was none), and no fish depleted PO_2 below 80 mmHg over the course of oxygen consumption measurement.

Following respirometry fish were euthanised by anaesthetic overdose (100 mg L^{-1} 3-amino benzoic acid ethylester, followed by severance of the spinal cord), and the gill, gut and liver were removed. Approximately half of each of these tissue samples was snap-frozen in liquid nitrogen for later analysis of oxidative stress markers (see below), and the remaining sample was transferred to microcentrifuge tubes for tissue elemental analysis (see below). The remaining body (i.e. all tissues except gill, gut and liver), was transferred to a 15 mL tube for subsequent digestion and elemental analysis.

2.5. Ca influx assays

Following salinity acclimation, separate groups of inanga were subjected to an identical Ni exposure to that described above. The exception was that the intermediate group (150 μg L^{-1} Ni) was not tested, owing to limited fish availability. Fish were removed from exposure chambers and immediately placed in 4-L plastic bags with the same chemistry as in the exposure chambers. Bags were held in a water bath, at a temperature of 15 °C, and aerated continuously. Fish were left for two hours to minimize the impacts of handling stress (Harley and Glover, 2014), before each bag was inoculated with 20 μC_i of ^{45}Ca (Perkin-Elmer). Water samples were taken at time 0 and 1 h for analysis of specific activity. After 1 h, fish were removed, rinsed in a high Ca solution (1 M $Ca(NO_3)_2$) and two tap water rinses, to remove adsorbed but not absorbed isotope. Fish were then euthanised by severing the spinal cord. After weighing, gills were dissected and placed into 1.5-mL centrifuge tubes, and the remaining fish was placed in a 15-mL tube. Gills were acid digested (2 N HNO_3, 72 h at 70 °C), before being transferred to a scintillation vial where 5 mL of scintillation fluor was added (Ultima Gold AB, Perkin-Elmer). The remaining body was treated similarly (2 N HNO_3, 72 h, 70 °C), before a 2-mL subsample was removed to a scintillation vial, and 15 mL of scintillation fluor were added. For water samples, 5 mL of scintillation fluor water were added to 1 mL of water. All samples were then counted using a scintillation counter (TriCarb 2910 TR). Quench was corrected via the external standards ratio method. Influx of Ca into the gill and the remaining body was determined as follows:

$$J_{in} = \frac{CPM}{SA \times W \times t}$$

where CPM is the quench-corrected gill or body counts per minute, SA is the measured mean specific activity of ^{45}Ca in the water (cpm μM^{-1}), W is fish mass (g), and t is time (h).

2.6. Oxidative stress markers

Tissues (gill, gut and liver) were ground to a fine powder (using a mortar and pestle over liquid nitrogen to prevent thawing) and placed in a homogenization buffer (1:20 w:v) containing 20 mM HEPES, 1 mM EDTA and 0.1% Triton X (Sigma Aldrich) at a pH of 7.2. Homogenates were then centrifuged at 13,000 g for 5 min at 4 °C. Catalase activity (U mg $protein^{-1}$ where a unit (U) is 1 μmol min^{-1}) was determined as the decrease in absorbance at a wavelength of 240 nm due to hydrogen peroxide decomposition (Claiborne, 1985; Blewett and Wood, 2015b), via a microplate reader. Protein concentrations in homogenized tissues (used for both catalase activity and protein carbonylation) were determined according to the Bradford (1976) assay using bovine serum albumin (Sigma Aldrich) as a standard.

Protein carbonyl content was assessed using a commercial kit (Protein Carbonyl Colorimetric Assay Kit; Cayman Chemicals). Tissues were prepared as described above and placed in a homogenization buffer (50 mM MES, 1 mM EDTA, pH 6.7), followed by centrifugation at 13,000*g* for 5 min. A 1% streptomycin sulfate solution was added to the resulting supernatants at a final concentration of 10 μL per 100 μL of homogenization buffer, to minimize the effect that nucleic acids have on overestimation of carbonyl content (Reznick and Packer, 1994). Protein carbonyls were normalized to protein content and are reported as nmol mg $protein^{-1}$.

2.7. Elemental analysis

Gill, gut and liver samples were digested in 1 mL, and remaining body (rest of tissues) was digested in 10 mL, of 2 N trace metal grade HNO_3 at 70 °C for 72 h. Samples were vortexed every 24 h to aid digestion. Both exposure water and tissue samples were then diluted to bring them into range for analysis via inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500c). QA/QC involved the addition of $^{103}Rhodium$ as an internal standard and the inclusion of a blank and spiked standards (2 and 20 μg L^{-1}) every 20 samples. A certified reference material (DORM-3; National Research Council Canada) was also included, and Ni recovery averaged 91%. Blanks were always below the limit of detection, and thus presented data were not blank-corrected. The limit of detection for Ni in water was 0.50 μg L^{-1} and for tissue samples was 0.01 μg g^{-1}.

2.8. Statistical analysis

Following tests for normality and homogeneity of variance, tissue Ni, tissue ions, and oxidative stress markers were assessed via two-way ANOVA (with salinity and Ni concentration as the two factors), with *post-hoc* Tukey analysis. Unidirectional Ca influx was assessed via one-way ANOVA, followed by Tukey's test. Statistical significance was determined at $\alpha = 0.05$, and all analyses conducted using SigmaPlot ver. 11.2. All data are expressed as mean ± SEM.

3. Results

There were no significant differences between total and

dissolved (0.45 μm filtered) water Ni concentrations, and thus only dissolved Ni levels are reported here. Measured Ni levels in exposures were close to nominal (Table 1). Aquarium FW had Ni concentrations lower than the detection limit (0.50 μg L^{-1}), while natural SW did have a low baseline level of Ni (2 μg L^{-1}).

The results of two-way ANOVA statistical tests showed that Ni exposure concentration had a significant effect on Ni accumulation in gill, gut and remaining body (tissue remaining after dissection of gill, gut and liver; Fig. 1). For each of these tissues, exposure to 2000 μg Ni L^{-1} resulted in levels that were elevated relative to the Ni-free control, with the magnitude of this effect ranging from a 4-fold increase in 50% SW gill to a 25-fold increase in 100% SW gut. The only exceptions to this were for gut and remaining body Ni in 50% SW, where the elevation in Ni burden was approximately 10-fold relative to control, but this was not statistically significant. The only significant effect of salinity on Ni accumulation was in the gill, where at the highest Ni exposure level (2000 μg L^{-1}) fish in FW accumulated significantly more Ni than fish in 100% SW, which in turn exhibited a significantly greater tissue accumulation than fish in 50% SW. No significant effects of either exposure concentration or salinity were detected for liver Ni accumulation. Overall, the relative concentrations of Ni accumulation were: gut > gill = liver > remaining body. In general, inanga exposed to the highest Ni exposure concentration exhibited the greatest variability in Ni tissue concentration.

Exposure of inanga to Ni had no significant effect on MO_2 (Fig. 2). Similarly, there was no significant effect of salinity on this parameter.

The only statistically-significant effect of Ni exposure concentration on tissue ion concentration was for K^+ in the gill, where an overall significant effect was described, but this could not be ascribed to a specific treatment via *post-hoc* analysis. There were no other significant effects of Ni exposure concentration on tissue ion concentration for gill, nor for gut, liver or remaining body (Tables 2 and 3). There were, however, some salinity-related effects. The tissue concentrations of Na and Mg in the gut and the gill increased significantly with increasing salinity.

The rates of unidirectional Ca influx into the gill and the remaining body were unaffected by Ni exposure concentration (Fig. 3A–C). Rates of Ca influx on a "whole body" basis (i.e. body tissues excluding the gills) in FW were approximately 30 nmol g body weight^{-1} h^{-1}, with rates in 50% and 100% SW ranging between 870 and 1580 nmol g body weight^{-1} h^{-1}. Although there were no significant differences in fish mass within salinities (i.e. between control and Ni treatments), the fish used in the FW salinities were significantly larger than those in the more saline waters (~2.3 vs. 0.9 g). This applies only to fish used for Ca influx. There were no statistically-significant differences in the mass of fish used for other end-points which averaged about 1.3 g.

Table 1
Nominal and measured Ni (μg L^{-1}) in exposures (mean ± S.E.M., n = 4).

Salinity	Nominal	Measured
FW	0	< LOD
	150	120 ± 10
	2000	1966 ± 56
50% SW	0	< LOD
	150	129 ± 6
	2000	2216 ± 39
100% SW	0	2 ± 0
	150	140 ± 2
	2000	2385 ± 51

Measured values represent dissolved (<0.45 μm filtered) values, as these did not statistically differ from total (unfiltered) values. < LOD = less than limit of detection (0.50 μg L^{-1}).

Effects of Ni exposure concentration on catalase activity were observed (Fig. 4A–C), but only in FW inanga. In both gill and liver the 150 μg L^{-1} exposure concentration resulted in significantly elevated catalase activity relative to the unexposed control. In both tissues, a further increase in Ni exposure concentration to 2000 μg L^{-1} resulted in a decrease in catalase activities, such that these were not statistically distinguishable from control values. There were also strong effects of salinity, especially in the gill, where catalase activity was significantly elevated in FW relative to gill tissues of fish in 50% SW and 100% SW. Similar effects of salinity, albeit of lesser magnitude, were observed for gut and liver tissue.

Effects of Ni exposure concentration on tissue protein carbonylation were also largely restricted to FW inanga (Fig. 5A–C). In gill tissue, exposure to 150 μg L^{-1} Ni resulted in a significant increase in protein carbonylation, an effect that did not occur at elevated Ni exposure level - similar to the response in catalase activity. In gut, the opposite effect was observed with a significant decrease in protein carbonylation at the intermediate, but not the higher Ni exposure concentration. In liver the only significant effect of Ni exposure concentration was an elevation in protein carbonylation in fish exposed to 150 μg L^{-1} in 50% SW. As for catalase activity, salinity impacted protein carbonylation, with a similar pattern of higher levels in FW inanga, relative to fish exposed to higher salinities.

Correlation analyses were conducted to ascertain any significant relationships between the level of tissue Ni accumulation and the impact on oxidative stress markers. These analyses all displayed r^2 values less than 0.05, and p values between 0.09 and 0.85, indicating a lack of correlation.

4. Discussion

4.1. Ni accumulation

Following exposure to a "worst case scenario" concentration of waterborne Ni (2000 μg L^{-1}), tissue Ni burdens increased in the gill, gut and remaining body of inanga. However, only the pattern of accumulation in the gill was salinity-dependent. The free ion (Ni^{2+}) is considered to be the bioavailable and toxic form of Ni to aquatic organisms (Niyogi and Wood, 2004). Previous chemical speciation analysis has shown that when moving from FW to 100% SW, the proportion of free Ni ion drops from around 87% to 75% (Blewett and Wood, 2015b). Furthermore, the relatively elevated levels of Ca and Mg in SW would increase competition with Ni for access to the divalent cation absorption pathways that are believed to mediate its uptake into aquatic biota (Blewett et al., 2016a). Thus, if water chemistry was the key factor driving Ni accumulation in the gill, it would be predicted that the lowest levels of Ni accumulation would be found in the 100% SW fish. However, among fish exposed to 2000 μg L^{-1}, the lowest levels of Ni accumulation in gill, gut and remaining body were found in inanga exposed in 50% SW. Only in the gill was the accumulation of Ni significantly lower in 100% SW than in FW. This indicates that physiological factors are more important than water chemistry in influencing Ni accumulation patterns, a finding generally consistent with other studies examining the salinity-dependence of Ni tissue burdens in euryhaline aquatic biota (Blewett et al., 2015; Blewett and Wood, 2015a, 2015b).

The U-shaped relationship between Ni tissue accumulation and salinity (high accumulation at salinity extremes, low at intermediate salinities) in elevated Ni exposure concentrations, suggests that the main driver of Ni uptake is ion homeostasis. The isosmotic point in inanga (i.e. where body osmolality equals environmental osmolality) is around 9‰ (= 28% SW; Urbina and Glover, 2015). At salinities lower than this inanga are hyperosmotic. Faced with net

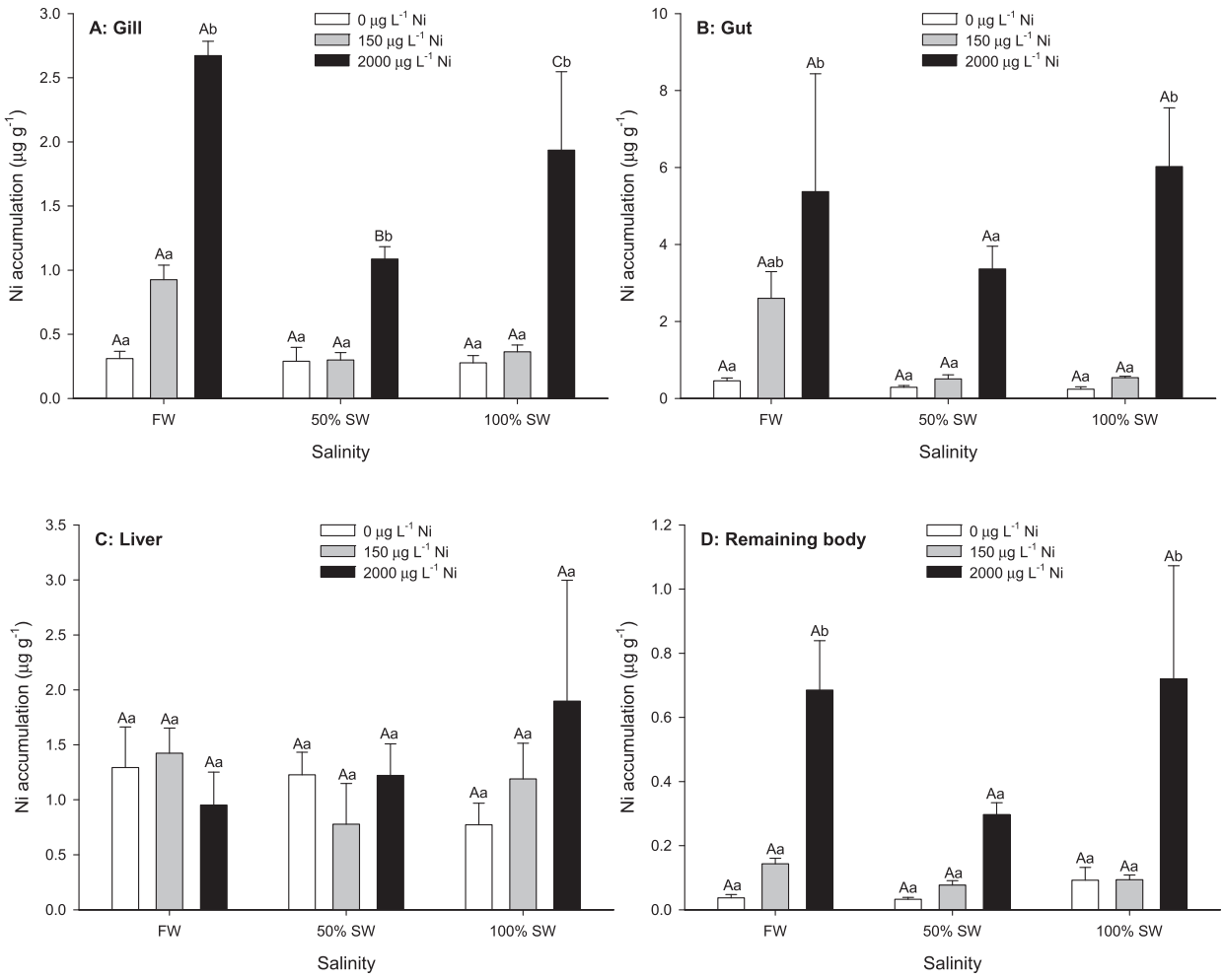

Fig. 1. Ni accumulation in gill (A), gut (B), liver (C), and remaining body (tissue remaining after excision of gill, gut and liver; D) of inanga exposed to Ni (0, 150 or 2000 μg L^{-1}) for 96 h in one of three salinities (FW, 50% SW, 100% SW). Plotted points represent the means ± SEM of 3–5 replicates. Bars sharing upper case letters are not significantly different with respect to other exposure salinities within a Ni concentration, while bars sharing lowercase letters are not significantly different relative to other Ni exposure concentrations within an exposure salinity. Statistical significance was determined by two-way ANOVA, followed by Tukey's *post-hoc* test at $\alpha = 0.05$.

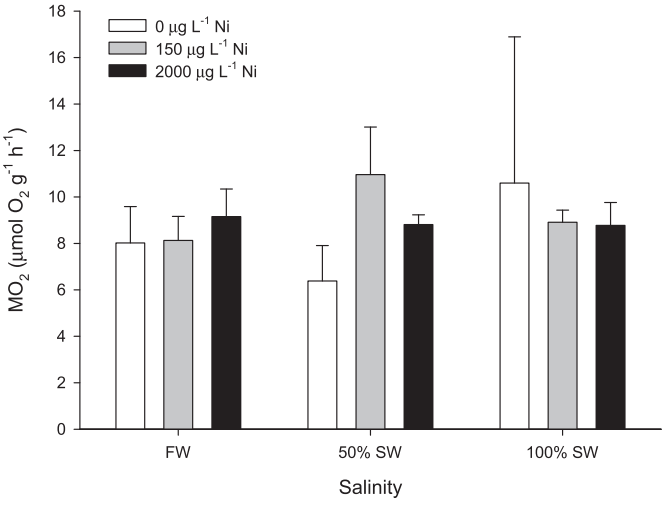

Fig. 2. Oxygen consumption rate (MO_2; μmol O_2 g^{-1} h^{-1}) of inanga exposed to Ni (0, 150 or 2000 μg L^{-1}) for 96 h in one of three salinities (FW, 50% SW, 100% SW). Plotted points represent the means ± SEM of 3–5 replicates.

diffusive ion losses, they rely on rapid and efficient ion uptake mechanisms to balance body osmolality. At concentrations higher than the isosmotic point, inanga are hypo-osmotic and must cope with net diffusive ion loading; thus their physiology is focussed on active ion efflux mechanisms (Urbina et al., 2013). These ion transport processes mediating homeostasis are principally gill-based, and the main driver of ion transport is the sodium pump on the basolateral membranes of the ionocytes. Around the isosmotic point the activity of the sodium pump is at its lowest (Urbina and Glover, 2015). As Ni likely traverses the gill by mimicry through ion transport pathways, this suggests that the general decrease in ion transport activity decreases the accessibility of Ni to the gill, leading to reduced Ni accumulation.

Ni handling in fish appears to be species-dependent. For example, in the current study the only measured tissue to exhibit no changes in Ni accumulation irrespective of Ni exposure concentration or salinity was the liver. Previous work in FW rainbow trout (Leonard et al., 2014b; Pane et al., 2003a) support this observation, by also showing no significant hepatic Ni accumulation following acute waterborne exposures (even at levels more than 5-fold in excess of those studied here). Contrastingly, in the neotropical FW fish species *Prochilodus lineatus*, Palermo et al. (2015) showed that within 24 h of exposure to 2000 μg L^{-1} Ni,

Table 2
Inanga gill and gut ion concentrations (μmol g^{-1}) as a function of salinity and Ni concentration following a 96 h exposure.

Salinity	Ni (μg L^{-1})	Gill				Gut			
		Na^+	K^+	Ca^{2+}	Mg^{2+}	Na^+	K^+	Ca^{2+}	Mg^{2+}
FW	0	22 ± 4[a]	41 ± 6	55 ± 8	4 ± 0[a]	20 ± 3[a]	53 ± 8	4 ± 1	4 ± 1[a]
	150	31 ± 3[a]	48 ± 3	50 ± 5	4 ± 0[a]	20 ± 1[a]	53 ± 1	7 ± 1	4 ± 0[a]
	2000	28 ± 3[a]	41 ± 1	62 ± 8	4 ± 0[a]	17 ± 1[a]	47 ± 3	4 ± 1	3 ± 0[a]
50% SW	0	39 ± 3[b]	46 ± 3	64 ± 5	5 ± 0[a]	24 ± 1[a]	59 ± 3	6 ± 2	6 ± 1[a]
	150	42 ± 3[b]	54 ± 4	57 ± 4	5 ± 0[b]	26 ± 3[ab]	63 ± 7	5 ± 2	6 ± 1[a]
	2000	36 ± 1[a]	44 ± 1	64 ± 9	4 ± 0[a]	21 ± 1[a]	52 ± 1	4 ± 1	6 ± 1[a]
100% SW	0	50 ± 2[c]	46 ± 3	75 ± 11	7 ± 0[b]	35 ± 3[b]	51 ± 1	6 ± 1	13 ± 2[b]
	150	48 ± 4[b]	48 ± 6	52 ± 4	6 ± 1[b]	29 ± 3[b]	56 ± 2	5 ± 2	11 ± 1[b]
	2000	50 ± 3[b]	40 ± 1	90 ± 19	7 ± 0[b]	29 ± 4[b]	52 ± 4	10 ± 4	11 ± 2[b]

Reported values represent mean ± SEM (n = 3–5). Values sharing letters are not significantly different relative to other salinities at the same Ni exposure concentration as determined by two-way ANOVA, followed by Tukey's *post-hoc* test ($\alpha = 0.05$). For ions lacking letters there were no significant differences.

Table 3
Inanga liver and remaining body (tissue remaining following excision of gill, gut and liver) ion concentrations (μmol g^{-1}) as a function of salinity and Ni concentration following a 96 h exposure.

Salinity	Ni (μg L^{-1})	Liver				Remaining body			
		Na^+	K^+	Ca^{2+}	Mg^{2+}	Na^+	K^+	Ca^{2+}	Mg^{2+}
FW	0	16 ± 1	45 ± 3	10 ± 3	4 ± 0	19 ± 3	38 ± 3	80 ± 14	5 ± 0
	150	16 ± 1	61 ± 1	14 ± 6	5 ± 0	18 ± 1	47 ± 5	82 ± 9	5 ± 0
	2000	12 ± 3	42 ± 11	3 ± 1	3 ± 1	21 ± 5	39 ± 4	85 ± 10	5 ± 0
50% SW	0	35 ± 6	93 ± 6	10 ± 2	7 ± 1	17 ± 1	54 ± 3	84 ± 10	5 ± 0
	150	50 ± 31	180 ± 120	10 ± 5	10 ± 7	19 ± 1	52 ± 3	80 ± 7	5 ± 0
	2000	23 ± 2	74 ± 8	17 ± 5	5 ± 1	15 ± 1	48 ± 1	77 ± 5	5 ± 0
100% SW	0	21 ± 3	61 ± 10	9 ± 4	5 ± 1	22 ± 4	60 ± 14	75 ± 15	6 ± 1
	150	25 ± 3	71 ± 8	10 ± 6	6 ± 1	19 ± 2	47 ± 4	72 ± 4	5 ± 1
	2000	20 ± 4	62 ± 13	13 ± 8	6 ± 2	23 ± 4	46 ± 5	81 ± 12	6 ± 1

the liver accumulated significantly elevated levels of this metal. Killifish, a euryhaline fish species, also displays accumulation of Ni in the liver after acute waterborne exposure (Blewett and Wood, 2015b). Although factors such as size and exposure temperature vary between the studies, it is most likely that species differences in Ni handling are responsible. In the case of the "non-accumulators" such as inanga, Ni may be trapped in the epithelial tissues. For example, in crab gill perfusion studies only 3–10% of accumulated gill Ni made it into efferent perfusates suggesting basolateral transport of Ni may be rate-limiting for some aquatic biota (Blewett et al., 2015). Alternatively, Ni could remain trapped in blood, thus reducing Ni bioavailability to internal tissues such as liver. In FW rainbow trout, a species that lacks significant liver Ni burden, the plasma was found to be the major Ni sink attributed to the presence of high affinity ligands therein (Pane et al., 2004a).

A notable finding of the current study was that the gut of inanga displayed the highest concentrations of Ni. There are two explanations for this. The bile is proposed to be an important route of Ni excretion in fish (Blewett and Wood, 2015b), and coupled with the relatively low and constant hepatic Ni concentrations, this may suggest that Ni was effectively passed from the liver to the gut via bile. Alternatively, gut Ni may result from drinking. Fish at high salinities drink in order to replace water lost via diffusion, but there is evidence that fish in FW also drink, albeit at much reduced rates (Scott et al., 2006). The higher rate of drinking in SW would be predicted to lead to higher gut Ni in this salinity, a pattern that was not observed in the current study (Fig. 1B). In euryhaline killifish, gut Ni concentration also failed to follow the expected pattern of accumulation with respect to salinity, an effect attributed to higher cation competition and/or enhanced sloughing of mucus-bound Ni (Blewett and Wood, 2015b).

4.2. Effects on respiration, ion regulation and oxidative stress

In the current study no effect of Ni exposure was noted on MO_2. Previous research has shown that Ni leads to inflammation of the fish gill, resulting in an increased diffusive distance and impaired respiration (Hughes et al., 1979; Pane et al., 2004b). The lack of such an effect in inanga could be explained by a Ni exposure concentration that was insufficient to cause changes in gill structure. In FW rainbow trout, respiratory effects of Ni have been observed at levels (3200–10,000 μg L^{-1}; Hughes et al., 1979; Pane et al., 2004b) significantly higher than the highest concentration in the current study (2000 μg L^{-1}). However, it is notable that no effects of Ni on MO_2 or maximum sustained swimming speed were observed in killifish exposed to Ni concentrations of 5000 μg L^{-1} for 96 h (Blewett et al., 2016a), indicating that respiratory toxicity may be species-dependent. In the case of inanga, the lack of effect may be a consequence of cutaneous respiration. This species meets around 40% of its oxygen requirements via the skin (Urbina et al., 2014). Thus even if the level of Ni exposure in the current study led to changes in gill structure, it is possible that the skin surface was able to compensate, allowing MO_2 to be maintained at control levels.

Although respiratory effects are considered the main mode of Ni toxicity in fish, some studies have previously shown effects on ion regulation. For example, zebrafish exhibit a decrease in whole body Na in response to Ni exposure levels of 9 mg L^{-1} for 40 h in FW (Alsop and Wood, 2011), while waterborne Ni exposures in excess of 12.5 mg L^{-1} resulted in a decrease in plasma Ca in the marine gulf toadfish (Pane et al., 2006). However, in the current study, the only ionoregulatory effect of Ni exposure was on gill K concentration, an effect that could not be attributed to a specific exposure condition by *post-hoc* analysis. Again this may be a consequence of the Ni exposure levels used in the current study, which although high in terms of environmental exposure scenarios, were lower than those

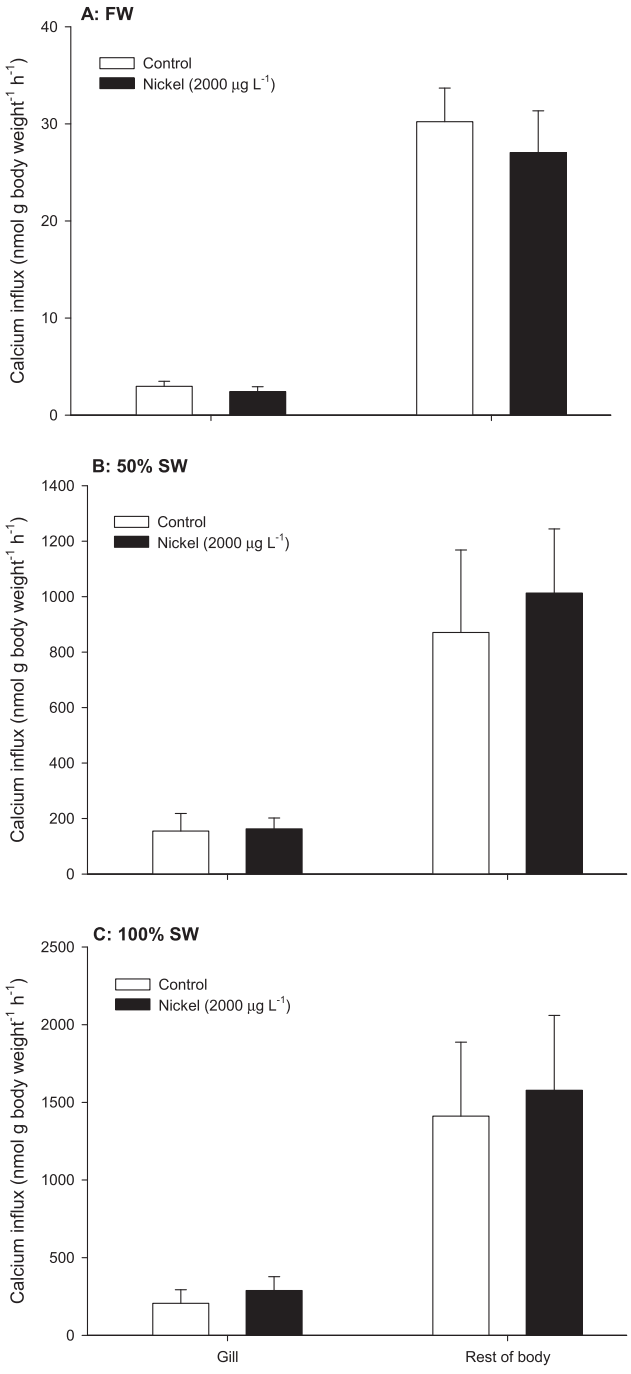

Fig. 3. Calcium influx (nmol g body weight^{-1} h^{-1}) in gills or remaining body of inanga exposed to Ni (0, 150 or 2000 μg L^{-1}) for 96 h in one of three salinities: FW (A), 50% SW (B), 100% SW(C). Plotted points represent the means ± SEM of 6–8 replicates.

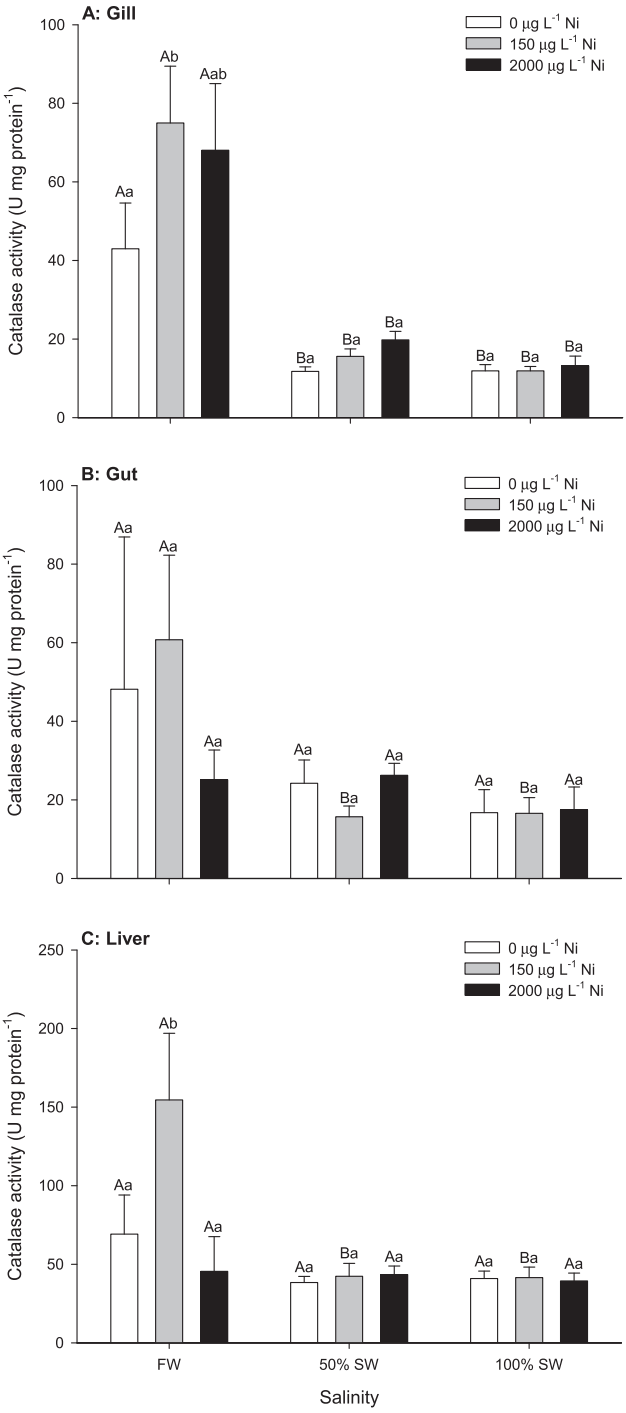

Fig. 4. Catalase activity (U mg protein^{-1}) in gill (A), gut (B), and liver (C) of inanga exposed to Ni (0, 150 or 2000 μg L^{-1}) for 96 h in one of three salinities (FW, 50% SW, 100% SW). Plotted points represent the means ± SEM of 3–5 replicates. Bars sharing upper case letters are not significantly different with respect to other exposure salinities within a Ni concentration, while bars sharing lowercase letters are not significantly different relative to other Ni exposure concentrations within an exposure salinity. Statistical significance was determined by two-way ANOVA, followed by Tukey's *post-hoc* test at $\alpha = 0.05$.

used by authors identifying ionoregulatory effects in other fish species.

In fish there is evidence that Ca can protect against Ni uptake (Blewett et al., 2016a), an effect also observed in FW invertebrates (Komjarova and Blust, 2009). However, in inanga exposed to 2000 μg L^{-1} the presence of Ni was insufficient to impair Ca influx. A similar lack of effect of Ni on Ca influx has been observed in FW brown trout fry exposed to 50 μg L^{-1} Ni (Sayer et al., 1991). The relative affinities of transport pathways for the 'natural' ion versus the metal mimic, coupled with their relative concentrations in exposure waters, means that the effect of the "natural" ion on metal transport is usually much greater than the effect of the metal on "natural" ion transport (see Hogstrand et al., 1998).

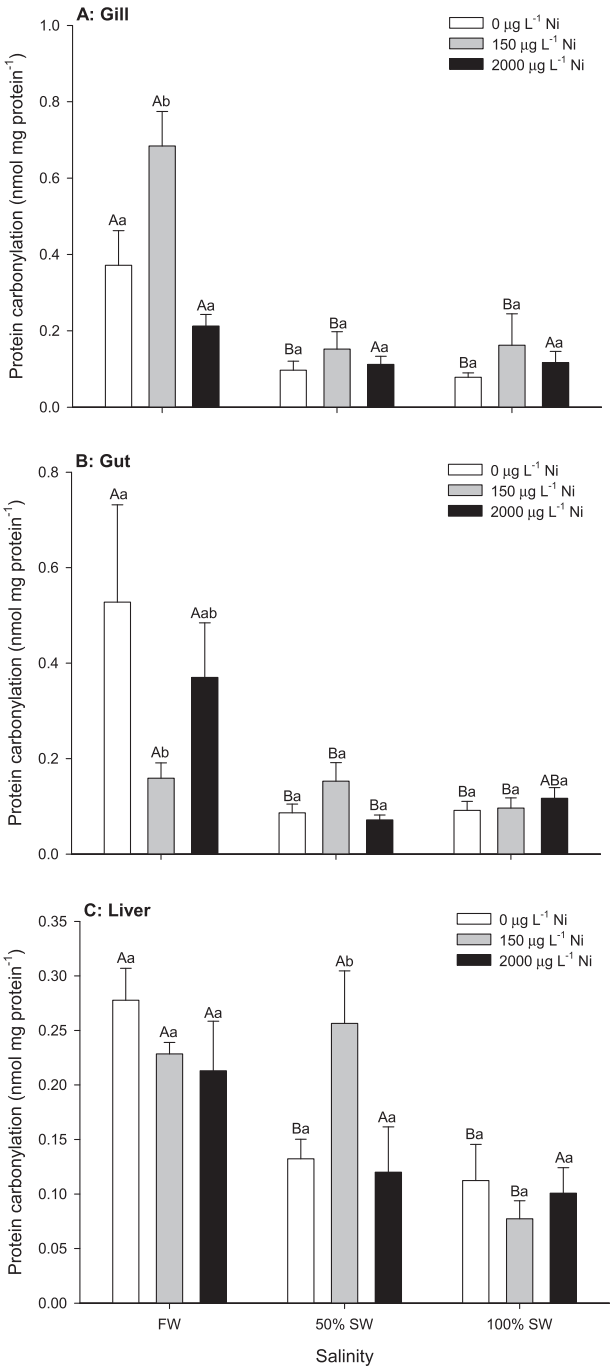

Fig. 5. Protein carbonylation (nmol mg protein^{-1}) in gill (A), gut (B), and liver (C) of inanga exposed to Ni (0, 150 or 2000 µg L^{-1}) for 96 h in one of three salinities (FW, 50% SW, 100% SW). Plotted points represent the means ± SEM of 3–5 replicates. Bars sharing upper case letters are not significantly different with respect to other exposure salinities within a Ni concentration, while bars sharing lowercase letters are not significantly different relative to other Ni exposure concentrations within an exposure salinity. Statistical significance was determined by two-way ANOVA, followed by Tukey's *post-hoc* test at $\alpha = 0.05$.

The rates of Ca influx in inanga exposed to saline waters (50 and 100% SW) were more than 30-fold higher than those in FW. This trend is consistent with previous studies on the impact of salinity on Ca influx in fish (e.g. Prodocimo et al., 2007). However, the magnitude of this effect is larger than that previously described, with Prodocimo and colleagues noting an 8-fold difference in influx rates in rainbow trout. The much larger differences in inanga are likely a consequence of the size disparity between the salinity groups, with the fish used in the 50% and 100% SW treatments in the current study, much smaller (~0.9 g) than those used for FW influx determination (2.3 g).

The most prominent effects of Ni on inanga related to oxidative stress. Changes in catalase activity and protein carbonylation were observed in gill, gut and liver, with effects mainly shown in FW. Ni may exert oxidative stress effects in a number of different ways. It could impact anti-oxidant defences by impairing the activity of key scavenging enzymes, or it could promote pro-oxidant reactions in tissues, by potentially displacing iron from enzyme active sites resulting in an increase in ROS via the Fenton reaction (Lushchak, 2011). Catalase activity was increased in gill and liver, but only at the low Ni exposure concentration (150 µg L^{-1}). This is likely a response to an inhibitory increased level of ROS, leading to an upregulated catalase activity. It is possible that as Ni exposure concentrations and tissue burdens increased, Ni "spillover" led to an effect on the catalase enzyme, thus a decrease in catalase activity back to control levels. It is known that Ni is capable of binding strongly to histidine residues (Predki et al., 1992), and that these are key components of the active site of catalase (Mate et al., 1999).

Previous studies examining Ni effects on catalase show a variety of different responses. For example, in muscle tissue of FW perch, Defo et al. (2014) saw a biphasic effect of concentration similar to that in the current study, although in their work catalase activity decreased at low exposure concentrations (70 µg L^{-1}), before returning to control levels at higher exposures (540 µg L^{-1}). A similar pattern (decrease at low exposure concentrations, back to control at high) was seen in FW killifish gill exposed to Ni (Blewett and Wood, 2015b). However, in both perch and killifish liver catalase activity increased, albeit only at the highest exposure level, and in the case of killifish, only in SW-acclimated animals (Defo et al., 2014; Blewett and Wood, 2015b). Other studies have found no effect (gill, Palermo et al., 2015), or an increase (liver, Palermo et al., 2015; gill, Kubrak et al., 2013), in tissue catalase activity following Ni exposure. It is clear that although catalase activity is affected by Ni exposure, the nature of the impact varies significantly as a consequence of exposure salinity, concentration and species.

Markers of anti-oxidant activity would be predicted to correspond with markers of oxidative damage, such that an increase in protection against ROS would lead to a reduction in protein carbonylation. This was not the case in the current study. In inanga gill, protein carbonylation increased at a Ni exposure concentration of 150 µg L^{-1}, despite an increase in gill catalase activity. This suggests the increased activity was insufficient to protect against an increase in ROS at this exposure concentration. Similarly, despite no change in catalase activity, there was a significantly decreased gut protein carbonyl content at the same exposure concentration. This suggests that the activities of other anti-oxidant defence mechanisms are likely having a significant influence on oxidative damage markers. For example, activity of the anti-oxidant enzyme superoxide dismutase was not measured in the current study, but has previously been shown as highly responsive to Ni exposure in fish (Palermo et al., 2015).

Except for an increase in protein carbonylation in the liver of inanga in 50% SW, effects of Ni on oxidative stress were confined to FW. This is consistent with the BLM approach, which suggests that Ni is most bioavailable in FW, and thus will have strongest effects on FW biota. However, this conclusion is inconsistent with Ni accumulation data. There were no effects of salinity on gut and liver Ni accumulation, and although there were changes in gill Ni burden, these did not correlate with impacts on oxidative stress. This may reflect differences in Ni bioreactivity. For example, studies have identified that the subcellular localisation of Ni can vary in a

tissue- and species-specific manner, such that the greater determinant of toxicity may be the particular cellular component (i.e. biologically active vs. biologically inactive fractions) with which Ni associates (Leonard et al., 2014a).

5. Conclusion

These data indicate that even extreme environmental levels of Ni have limited acute biochemical and physiological effects in inanga, and those that are present are largely restricted to FW, suggesting that salinity is protective against Ni toxicity. Physiology is likely more important than water chemistry in shaping organism sensitivity to Ni, based on patterns of accumulation that correspond to changes in ion transport, and the distinct tissue-specific responses. Overall these data suggest that inanga are relatively tolerant to Ni exposure, and are likely to be protected under existing regulatory guidelines. This conclusion is generally consistent with recent studies of inanga sensitivity to other metal toxicants (Cu, Glover et al., 2016; Zn; McRae et al., 2016). Future work in inanga should investigate the toxicity of Ni in exposure scenarios reflecting water chemistries in natural settings (e.g. low pH, metal mixtures), which could alter Ni bioavailability and toxic impacts.

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