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The effect of marine dissolved organic carbon on nickel accumulation in early life-stages of the sea urchin, *Strongylocentrotus purpuratus*

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ABSTRACT

Dissolved organic carbon (DOC) is known to ameliorate the toxicity of the trace metal nickel (Ni) to aquatic animals. In theory, this effect is mediated by the capacity of DOC to bind Ni, rendering it less bioavailable, with the resulting reduction in accumulation limiting toxicological effects. However, there is a lack of experimental data examining Ni accumulation in marine settings with natural sources of DOC. In the current study, radiolabelled Ni was used to examine the time- and concentration-dependence of Ni accumulation, using naturally sourced DOC, on developing larvae of the sea urchin *Strongylocentrotus purpuratus*. Contrary to prediction, the two tested natural DOC samples (collected from the eastern United States, DOC 2 (Seaview park, Rhode Island (SVP)) and DOC 7 (Aubudon Coastal Center, Connecticut)) which had previously been shown to protect against Ni toxicity, did not limit accumulation. The control (artificial seawater with no added DOC), and the DOC 2 sample could mostly be described as having saturable Ni uptake, whereas Ni uptake in the presence of DOC 7 was mostly linear. These data provide evidence that DOC modifies the bioavailability of Ni, through either indirect effects (e.g. membrane permeability) or by the absorption of DOC-Ni complexes. There was some evidence for regulation of Ni accumulation in later-stage embryos (96-h) where the bioconcentration factor for Ni declined with increasing Ni exposure concentration. These data have implications for predictive modelling approaches that rely on known relationships between Ni speciation, bioavailability and bioreactivity, by suggesting that these relationships may not hold for natural marine DOC samples in the developing sea urchin model system.

1. Introduction

Nickel (Ni) is a transition metal with a number of important uses, including the manufacture of stainless steel, a process that accounts for more than 60% of Ni production (Eisler, 1998). However, an increase in industrial exploitation of Ni over the last century (Reck et al., 2008), has been accompanied by a rise in its environmental concentrations to the extent that it is now considered a contaminant of concern (Pyle and Couture, 2012). Indeed, Ni is ubiquitous in both freshwater and marine environments, a consequence of both natural geochemical and anthropogenic processes. For example, although background levels of Ni in the open ocean are in the high ng/L range, in near-coastal settings affected by Ni contamination, concentrations may reach above 250 μg/L (see Blewett and Leonard, 2017).

The toxicological effects of elevated Ni in freshwater biota are relatively well documented. In freshwater invertebrates the main mechanism of acute Ni toxicity appears to be the disruption of ionic homeostasis (specifically that of magnesium; Pane et al., 2003b; Leonard and Wood, 2013); whereas in fishes, disruption of respiratory gas transfer due to gill inflammation seems to occur (Hughes et al., 1979; Pane et al., 2003a, 2004). Oxidative stress appears to be an additional toxic effect that is conserved across different groups (Blewett and Wood, 2015a, 2015b). On the basis of our understanding of the relationship between Ni speciation and short-term Ni accumulation at a biologically-

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sensitive site (i.e., the locus at which the toxic effects are exerted), it has been possible to build models that can predict both acute and chronic Ni toxicity to freshwater organisms (e.g., Meyer et al., 1999; Deleebeeck et al., 2007, 2008, 2009). These biotic ligand models (BLM's) use known binding affinities of Ni with environmental ligands, and empirically-derived measurements of Ni binding affinity to biological surfaces (or whole organisms), to predict Ni bioavailability. As only ionic, unbound, Ni (i.e. Ni^{2+}) is bioavailable, and is thus capable of accumulating and generating a toxic effect, then knowledge of water chemistry can be used to predict toxicological impact. In the case of Ni, bioavailability is likely governed by uptake through trace metal transporters (e.g., DMT-1: Gunshin et al., 1997), or by virtue of the physicochemical similarity of their hydrated ions, via calcium and/or magnesium transport pathways (see Blewett and Leonard, 2017). Given conservation of these putative uptake pathways in both freshwater and marine species (Blewett and Leonard, 2017), BLMs based on principles established in freshwater data should be generally applicable to the prediction of Ni toxicity in marine waters.

A number of key factors have, however, precluded the development of BLMs for saline waters. For example, some marine species exhibit greater sensitivity to Ni toxicity than that predicted by Ni speciation. Indeed, early life-stages of some sea urchin species display median effect concentrations at Ni levels close to 15 μg/L (e.g. *Diadema antillarum* and *Evechinus chloroticus*; Bielmyer et al., 2005; Blewett et al., 2016). This is despite the lower bioavailability of Ni in sea water, mostly due to competition by cations for Ni uptake pathways (e.g. very high Mg^{2+} concentrations in sea water; Blewett and Leonard, 2017). An additional complication is that *Strongylocentrotus purpuratus* embryos and larvae showed an excellent ability to regulate whole body Ni burdens at or below control levels at Ni exposure concentrations that cause significant disruptions of ionoregulation (e.g., 40 μg/L; Tellis et al., 2014). Another critical knowledge gap is an understanding of how marine dissolved organic carbon (DOC) affects Ni accumulation. As a ubiquitous poly-anionic ligand, in natural waters DOC binds to Ni^{2+}. This has the effect of preventing its uptake by the organism, thus preventing toxicity (Gopalapillai et al., 2012). Based on biogeochemical models such as the Windermere Humic Acid Model (WHAM), it has been predicted that DOC will have a relatively minor influence on Ni toxicity in seawater settings (Stockdale et al., 2015). However, experimental studies suggest the opposite, with significant ameliorative effects of DOC reported (e.g., Blewett et al., 2016, 2018). One reason for this contradiction may be that our knowledge of Ni-DOC interactions is based on freshwater forms of DOC. However, there is evidence of the presence of high affinity Ni-binding ligands, specifically in marine settings (Martino et al., 2004), which would indicate that extrapolation of models from fresh water to sea water may not be entirely appropriate.

Further data indicating our limited knowledge of the role of DOC in modulating Ni toxicity in marine systems comes from studies examining Ni accumulation in the presence of a natural DOC source in sea urchin embryos (Blewett et al., 2016). In this study, a strong relationship between modelled free Ni^{2+} and sea urchin embryo Ni accumulation was identified. However, in the presence of a marine DOC the relationship between embryo Ni accumulation and toxic impact was not as clear (Blewett et al., 2016). Consequently, to adequately decipher the effect of DOC on Ni toxicity in marine systems, and in light of models that predict accumulation drives toxicity, it is necessary to expand our knowledge of how DOC influences Ni accumulation in marine systems.

The current work examines the concentration- and time-dependent uptake of Ni in the early life-stages of the purple sea urchin, *Strongylocentrotus purpuratus*. Although this species is among the least sensitive of studied sea urchins to Ni toxicity (EC_{50} ~ 160–340 μg/L; Phillips et al., 2003; DeForest and Schlekat, 2013; Blewett et al., 2018), it was the subject of recent research examining the relationship between natural marine DOC sources and Ni effects (Blewett et al., 2018). Our study, therefore, seeks to extend these previous observations by focussing on two natural marine DOC sources, both with protective effects against Ni toxicity, and determining their influence on sea urchin embryo Ni accumulation. We hypothesized that because of their reported amelioration of Ni toxicity, the tested marine DOC samples would significantly reduce Ni accumulation, consistent with the fundamental principles of predictive modelling approaches such as the BLM.

2. Methods

2.1. Animal care

Adult sea urchins (*Strongylocentrotus purpuratus*) were obtained from West Wind Sealabs Inc., in Victoria, B.C., Canada, and transported to McMaster University, Hamilton, Canada. Following their arrival, and during spawning, adults were kept in a constantly aerated 38-L recirculating glass tank containing artificial seawater (ASW; Kent Marine Reef Salt; Big Al's Aquarium, Kitchener, ON, Canada), maintained at 15.4 ± 0.3 °C, under a 14:10 light dark cycle. The salinity of this water was 33.1 ppt, with a pH of 8.1, and a DOC concentration of 0.9 mg C/L. Temperature and salinity were monitored using a handheld meter (YSI 30, YSI Inc., Yellow Springs), pH was measured using a pH probe (Accumet, Fisher Scientific, Ottawa, Canada), and DOC was determined via a Shimadzu TOC –L CPH/CPN analyzer (Shimadzu Corporation Kyoto, Japan), as detailed in Blewett et al. (2018). All procedures were approved by Wilfrid Laurier University Animal Care and McMaster University Animal Care committees.

2.2. Dissolved organic carbon

Collection, storage and analysis of the DOC samples used in the current study have been documented previously (Blewett et al., 2018). Briefly, grab samples of coastal marine waters were collected in October 2015, at several sites along the east coast of the United States from Rhode Island to Connecticut. Samples were filtered through a 1 μm string-wound cartridge filter (Filter Source, Hamburg, NY, USA) and collected in 2-L Nalgene bottles. Samples were then brought back to McMaster University and stored in 4-L polyethylene bottles at 4 °C in a dark room until use in assays. Our control sample constituted of ASW alone made from City of Waterloo dechlorinated tap water, with no added DOC. Of the different DOC samples documented in Blewett et al. (2018), two were selected for the current study (DOC 2 and DOC 7). These samples were chosen because they were among the most protective against Ni toxicity to sea urchin embryos. The DOC samples were salted up to a salinity of 32 ppt using ASW. The organic carbon concentrations of DOC 2 and DOC 7 were 4.5 ± 0.03 and 3.2 ± 0.05 mg C/L, respectively. The salinity of these samples was 32.4 ± 0.02 and 31.5 ± 0.05 ppt for DOC 2 and DOC 7, respectively. The studies described in the current work were conducted on the same samples as detailed in the previous study, albeit 2–3 months after the initial work was completed.

2.3. Fertilization

Fertilization followed methods previously described in Blewett et al. (2016, 2018). Briefly, spawning was induced by injection of 1 mL of 0.5 M KCl into the haemocoel of adult sea urchins. Eggs from individual spawning females were added to 50 mL aliquots of sea water in plastic Falcon tubes, and thereafter egg density was assessed using a Sedgewick-Rafter cell. Sperm was collected from five males, pooled, and stored on ice for up to 1 h while eggs were collected. To facilitate fertilization, ~200 μL of sperm was added to each egg-containing Falcon tube and stirred gently. Once at least 80% fertilization was achieved (<30 min), aliquots of eggs were added to 20-mL plastic scintillation vials containing fresh sea water with an appropriate water chemistry (i.e. Ni + DOC; see below) to a final density of 100 fertilized eggs per mL.

2.4. Ni accumulation

The uptake and accumulation of Ni was assessed in three water chemistries: ASW (no added DOC), DOC 2, DOC 7. The samples noted as DOC 2 and DOC 7 are the same grab samples identified by these codes as described in Blewett et al. (2018). In each water, fertilized eggs were exposed to six concentrations of Ni (0 (control, no added Ni), 50, 100, 200, 400, 800 μg/L), run in triplicate. To each treatment, radiolabelled Ni (^{63}Ni; Perkin Elmer; ~0.5 μCi/mL) was added as a tracer. Final Ni concentrations were achieved by addition from a $NiSO_4{\cdot}6H_2O$ stock solution. Concentrations were confirmed to be close to nominal via graphite furnace atomic absorption spectrometry as described in Blewett et al. (2018). All cold Ni solutions were made 24 h ahead of the addition of isotope and sea urchin embryos to allow for equilibration of water chemistry/Ni speciation, and aliquoted to 20-mL scintillation vials. Thirty minutes prior to the addition of fertilized eggs, isotope was added. At 4, 8, 48 and 96 h, water (1 mL in triplicate from all samples) and embryo (~100 individuals) samples were taken for an assessment of accumulated Ni (see below).

2.5. Water and embryo analysis

Embryo samples were filtered (0.45 μm) and subjected to a concentrated (10 mg/L) "cold" Ni ($NiSO_4$ $6H_2O$) wash to remove any loosely-bound (i.e. adsorbed not absorbed) radioisotope. This was followed by three separate ASW rinses, and finally a rinse in deionized (nanopure >18 MΩ) water. The filter was vacuumed dry and placed in a 20-mL plastic scintillation vial. Blank filters treated in an identical manner except with an embryo-free aliquot of exposure water were used to assess the effectiveness of the rinse protocol. Embryos and filter paper were digested in 2 N HNO_3 at 65 °C for 48 h, with vigorous vortexing at 24 h. The resulting digest was then diluted in nanopure water, before the addition Ultima Gold AB scintillation fluor (Perkin Elmer, Waltham, MA, USA), at an approximate a ratio of 1:5 (digest:fluor). Two-mL of Optiphase scintillation fluor (Perkin Elmer, Waltham, MA, USA) was added to each 1-mL water sample. All water samples and embryo digests were then analyzed for radioactivity on a Tri-Carb 2900 Liquid Scintillation Analyzer (Perkin Elmer, Waltham, MA, USA), correcting counts using a quench curve that was standardized to a common counting efficiency.

2.6. Calculations

Nickel accumulation was calculated as follows:

$$\text{Accumulation (pg/embryo)} = \text{CPM*1/SA*1/[n]}$$

where *CPM* is the counts in the embryos (quench corrected ^{63}Ni); *SA* is the specific activity (cpm/pg) of Ni; *n* is the number of embryos.

The accumulation of Ni as a function of exposure concentration, was graphed and fitted using regression analyses (Sigmaplot 11.2; Systat Inc., San Jose, CA, USA). The r^2 values of curves fitted to raw data (i.e. all replicates) were used to determine if the best fit relationship was linear, sigmoidal, or hyperbolic:

$$\text{Linear}: \quad y = mx + b$$

$$\text{Sigmoidal}: \quad y = \frac{B_{max}}{1 + e^{\left(-x - \frac{K_d}{m}\right)}}$$

$$\text{Hyperbolic}: \quad y = \frac{B_{max} \times x}{K_d + x}$$

where x is the Ni concentration; m is the slope (slope at the transition point for sigmoidal); b is the y-intercept; B_{max} is the maximal rate of Ni transport; K_d is the Ni concentration resulting in the half the maximal accumulation. Linear functions are characteristic of diffusive transport processes, hyperbolic relationships exist when transport pathways are saturable (i.e. governed by specific transport proteins), while sigmoidal patterns of uptake may be observed when accumulation is achieved via an allosterically-modified pathway, or by several hyperbolic pathways with distinct kinetics.

Bioconcentration factors (BCF) were calculated as follows:

$$\text{BCF} = \frac{Ni[accumulation] \text{ in } \frac{\mu g}{kg} \text{ dry wt.}}{Ni[exposure\ concentration] \text{ in } \frac{\mu g}{L}}$$

where BCF is the Bioconcentration factor (unitless); $Ni_{[accumulation]}$ is the measured Ni accumulation per embryo converted to Ni accumulation per kg dry weight calculated using the dry weights per embryo measured by Tellis et al. (2014) at the various timepoints of development; $Ni_{[exposure\ concentration]}$ is the nominal Ni concentration per L to which the sea urchins were exposed.

2.7. Statistics

Statistical analyses were performed using Sigma Plot version 11.2 (Systat Inc., San Jose, CA, USA). Normality and equality of variance was assessed using Shapiro-Wilk tests and Brown-Forsythe tests, respectively. When necessary, data were transformed to meet these assumptions. However, if data was not able to be transformed to meet the normality assumption, then tests proceeded in light of the consideration that ANOVA is robust to non-normality (Schmider et al., 2010). Time-dependent Ni accumulation and BCF were assessed by two-way ANOVAs with treatment (DOC) and time (h) as the two factors, with a Tukey's post hoc test to identify specific statistically distinct comparisons. The nature of the relationship between Ni exposure concentration and Ni accumulation was assessed via a curve-fitting process as described in Section 2.6, above. Significance for all statistical tests was accepted at $\alpha = 0.05$. All data have been expressed as means ± S.E.M.

3. Results

The accumulation of Ni over time, as a function of Ni exposure concentration, is illustrated in Fig. 1. In general, at all Ni exposure concentrations there was a rapid saturation of Ni accumulation. Indeed, only at 50 (Fig. 1A) and 100 (Fig. 1B) μg/L was there a significant effect of time noted ($p = 0.004$ and 0.022, respectively). For both concentrations, post hoc analysis showed that accumulation at 48-h was significantly greater than at 4-h. However, at Ni exposure concentrations of 200 μg/L and above, 4-h of exposure was sufficient to reach a sea urchin embryo Ni burden that was independent of duration of Ni exposure (i.e. no significant effect of time; p-value range 0.318–0.754; Fig. 1C–E). There were no significant effects of DOC treatment on Ni accumulation over time (p-value range 0.076–0.918), nor any significant interactions between the two tested factors (p-value range 0.337–0.608), for any of the tested Ni concentrations.

Fig. 2 uses the same data shown in Fig. 1, but instead plots the accumulation of Ni by sea urchin embryos as a function of Ni exposure concentration. This figure demonstrates relationships between accumulation and concentration that depend on exposure duration and treatment conditions. At the 4-h time interval, the best-fit relationship for Ni accumulation versus concentration in the absence of DOC (i.e. ASW alone), was hyperbolic (i.e. as Ni exposure concentration increased there was an increase in accumulation, until a point where increasing exposure Ni concentration only added minimally to embryo Ni burden; Fig. 2A). From this curve, affinity (K_d; Ni concentration required to give half maximal accumulation) and capacity (B_{max}; maximal Ni accumulation rate) kinetic constants were calculated at 624 μg/L and 4.2 pg/embryo, respectively (see Table 1). In the presence of both DOC samples, the relationship between Ni exposure concentration and Ni accumulation was sigmoidal at 4-h of exposure.

At 8-h, the hyperbolic relationship between seawater Ni

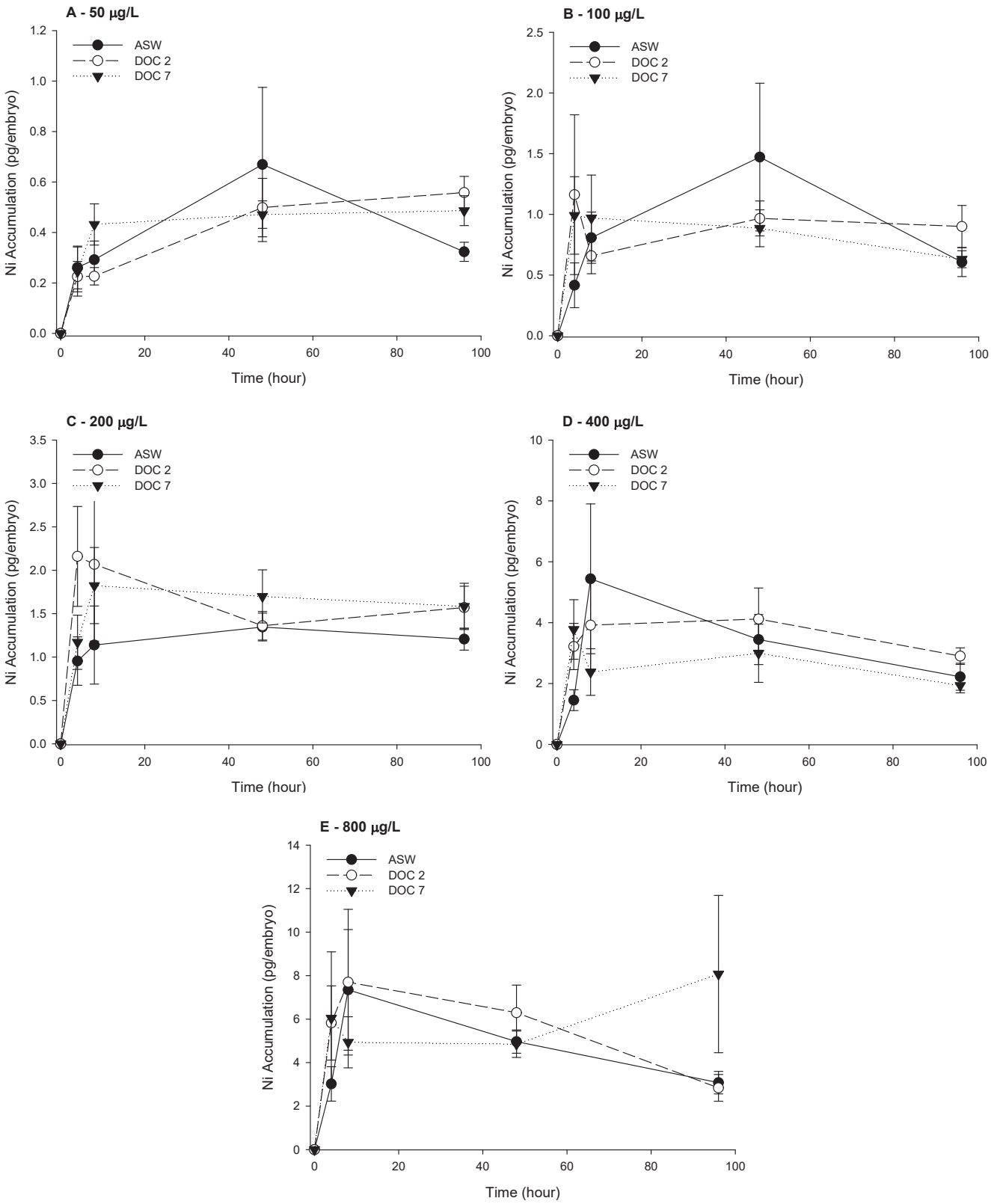

Fig. 1. Nickel (Ni) accumulation in artificial sea water (ASW) (black circles, solid line), or ASW in the presence of dissolved organic carbon, DOC 2 (white circles, dashed line) or DOC 7 (black triangles, dotted line) in sea urchin embryos over 96 h as a function of Ni exposure concentration (50 µg/L, A; 100 µg/L, B; 200 µg/L, C; 400 µg/L, D; 800 µg/L, E). Plotted values represent the means (±SEM) of 6 replicates.

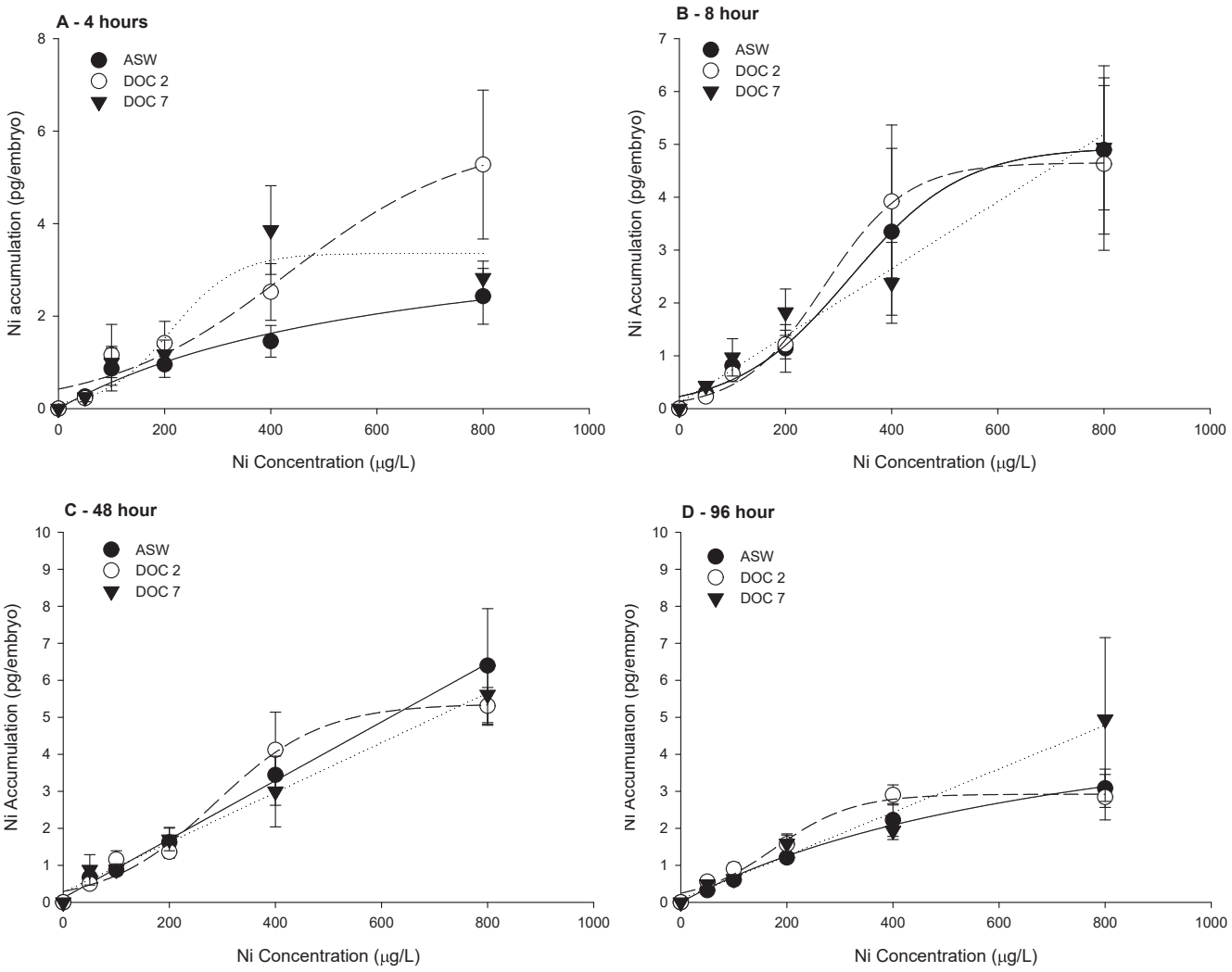

Fig. 2. Nickel (Ni) accumulation in artificial sea water (ASW) (black circles, solid line), or ASW in the presence of dissolved organic carbon, DOC 2 (white circles, dashed line) or DOC 7 (black triangles, dotted line) in sea urchin embryos over 96 h as a function of exposure time (4-h, A; 8-h, B; 48-h, C; 96-h, D). Plotted values represent the means (±SEM) of 6 replicates.

Table 1
Summary of Ni accumulation kinetics.

Time (h)	ASW				DOC 2				DOC 7			
	K_d (μg/L)	B_{max} (pg/embryo)	R^2	Type	K_d (μg/L)	B_{max} (pg/embryo)	R^2	Type	K_d (μg/L)	B_{max} (pg/embryo)	R^2	Type
4	624 ± 557	4.2 ± 2.1	0.34	H	438 ± 183	5.9 ± 1.9	0.33	S	211 ± 46	3.4 ± 0.4	0.47	S
8	321 ± 78	4.9 ± 0.9	0.48	S	273 ± 73	4.6 ± 0.9	0.49	S	–	–	0.58	L
48	–	–	0.64	L	287 ± 42	5.4 ± 0.5	0.73	S	–	–	0.66	L
96	271 ± 44	3.1 ± 0.3	0.74	S	177 ± 29	2.9 ± 0.2	0.72	S	–	–	0.44	L

Based on the data graphed in Fig. 2.
H = hyperbolic; S = sigmoidal; L = linear.
All regressions were statistically significant ($p < 0.001$).

concentration and Ni burden in the absence of DOC became sigmoidal and was similar in terms of its kinetic characteristics to Ni uptake in the presence of DOC 2 (Fig. 2B; Table 1). In the presence of DOC 7, however, Ni accumulation was linear with respect to exposure concentration.

At 48-h, the Ni accumulation curve for ASW was linear, increasing without saturation as exposure Ni increased to 800 μg/L (Fig. 2C). A linear trend was also observed for Ni in the presence of DOC 7. In the presence of DOC 2, Ni accumulation was sigmoidal.

At 96-h, embryo Ni accumulation was sigmoidal in relation to Ni exposure concentration for both the ASW control and DOC 2 (Fig. 2D). Similar to 8- and 48-h, after 96-h, embryo Ni burden was linear with respect to seawater Ni in the presence of DOC 7.

Sea urchins displayed BCF values that ranged from ~10 to 110, depending on exposure Ni concentration and time, demonstrating a strong capacity for Ni accumulation (Fig. 3). At 4-h of Ni exposure at 100 μg/L there was an apparent peak in BCF (Fig. 3A), however this was not significant (effect of Ni concentration, $p = 0.106$). Similarly, there was no significant effect of treatment ($p = 0.474$), nor was there a significant interaction between the two tested factors ($p = 0.980$). An identical statistical outcome was discerned for Ni BCF at 8 and 48-h of

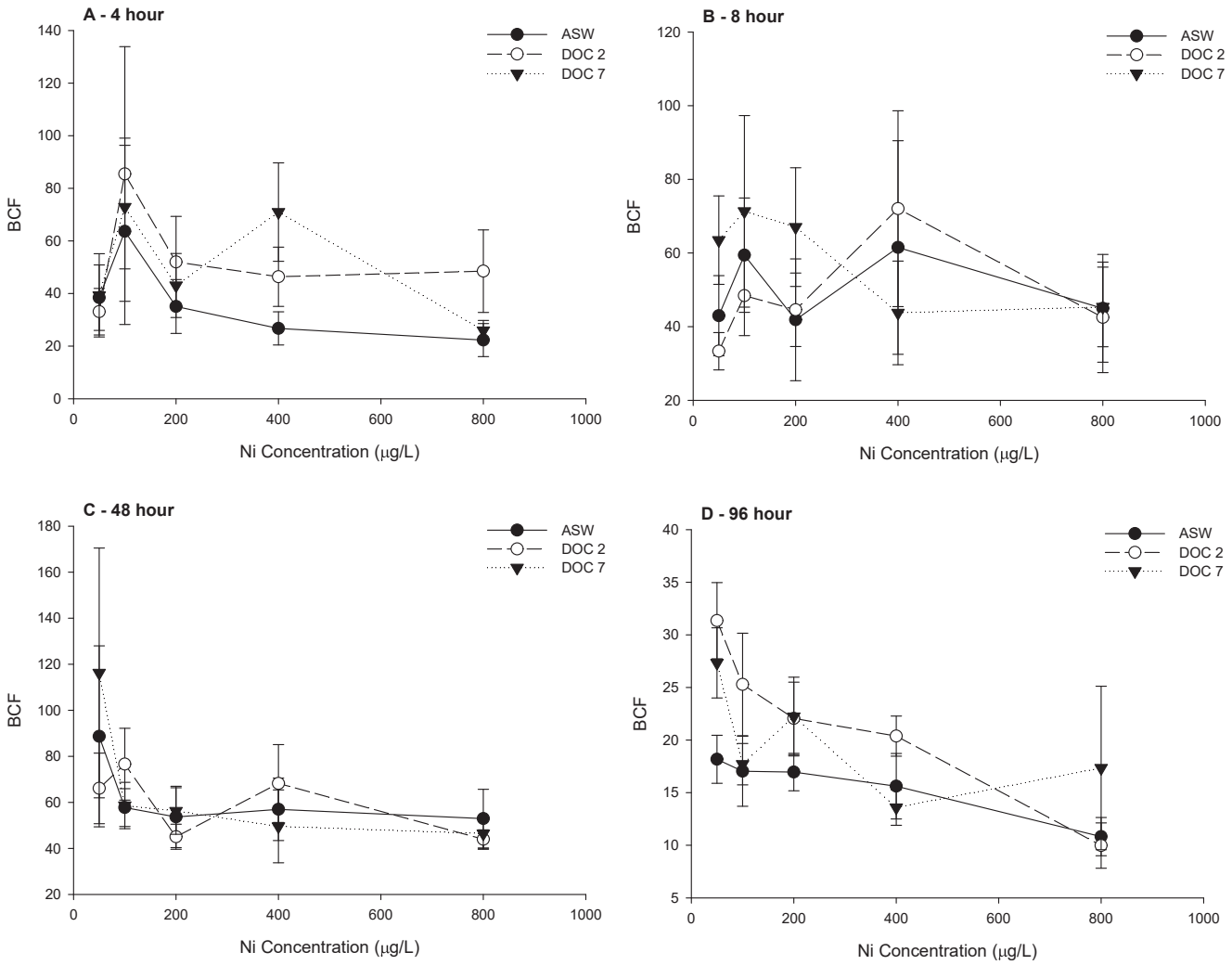

Fig. 3. Bioconcentration factors (BCF) of Ni in artificial sea water (ASW) (black circles, solid line), or ASW in the presence of dissolved organic carbon, DOC 2 (white circles, dashed line) or DOC 7 (black triangles, dotted line) in sea urchin embryos over 96 h as a function of exposure time (4-h, A; 8-h, B; 48-h, C; 96-h, D). Plotted values represent the means (±SEM) of 6 replicates.

exposure (p value range 0.090–0.915; Fig. 3B, C). At 96-h, BCF decreased significantly with Ni exposure concentration ($p = 0.001$), and embryos exposed to Ni in the presence of DOC 2 had an overall significant elevation in BCF relative to the ASW control ($p = 0.023$; Fig. 3D). There was no interaction between the effects of Ni concentration and treatment ($p = 0.394$).

4. Discussion

The primary goal of the current study was to assess Ni accumulation in the presence of DOC. Previous work has shown that natural marine DOC samples protect against Ni toxicity to sea urchin early life-stages (Blewett et al., 2016, 2018), a finding in line with the principles of biogeochemical models such as the BLM. According to the BLM approach, reduced toxicity promoted by the presence of DOC should also be accompanied by a reduction in Ni accumulation. This would result from the complexation of Ni by DOC, reducing the proportion of free Ni ion (Ni^{2+}), the bioavailable form of the metal. A decrease in bioavailability should translate into a decrease in bioaccumulation, and thus toxicity. In the current work, no reduction in whole embryo Ni body burden was noted in the presence of DOC, despite the reduction in Ni toxicity seen in our previous study using the same DOC samples, under near-identical exposure conditions (Blewett et al., 2018). This suggests that in the presence of marine DOC, Ni burden may not be predictive of toxicity, a key tenet of BLM approaches.

4.1. DOC and Ni body burden

Several previous freshwater studies have provided data that support the BLM approach, by demonstrating that DOC reduces Ni bioaccumulation (e.g., Gopalapillai et al., 2012; Chan, 2013; Custer et al., 2016). However, some literature supports the outcomes of the current work, by showing that a reduced Ni burden does not always occur in the presence of DOC. For example, in the freshwater crustacean *Hyallela azteca*, the presence of DOC did not reduce Ni body burden to the extent predicted by speciation analysis, at least for some exposure scenarios (Doig and Liber, 2006). A similar experimental outcome was noted in zebra mussels where, in the presence of DOC, Ni accumulation was greater than that predicted by the decline in free Ni^{2+} (Bourgeault et al., 2012). The accuracy of the model could be enhanced by accounting for the fact that DOC increased zebra mussel filtration rate, and therefore the amount of Ni in contact with the uptake surface. However, there was still a component of the accumulated Ni burden that could not be predicted through these modelling exercises. It was therefore hypothesized that DOC altered membrane permeability, thus creating a non-specific pathway for Ni uptake (Bourgeault et al., 2012). As the absorbed Ni is already bound, then this fraction of Ni body burden may not exert toxic effects (i.e. Ni is bioavailable but not bioreactive). Such a phenomenon

would explain the apparent contradiction between toxicity amelioration by DOC, and an unchanged Ni body burden, as was observed in the present study on bioaccumulation, and our previous work on toxicity (Blewett et al., 2018).

There is some recent evidence to further support the hypothesis that Ni^{2+} is not the only bioavailable form of this trace metal. Using the same model system as the current work, Sherman et al. (2021) showed that in the presence of the amino acids glutamate, histidine and the artificial ligand nitrilotriacetic acid, Ni accumulation in the purple sea urchin was greater than that predicted by speciation models where Ni^{2+} is considered the only bioavailable Ni form. If Ni bound to bioavailable components of DOC was accumulated, but Ni bound to other components of DOC was not able to be absorbed, then it is possible that these effects offset, resulting in no net change in Ni bioaccumulation, as we observed in the current work. However, in contrast to our previous study, where toxicity was ameliorated in the presence of marine DOC (Blewett et al., 2018), the complexes of Ni with specific organic ligands did enhance toxicity (Sherman et al., 2021). These differences in toxicological outcomes could relate to the relative binding affinities, and thus lability, of the tested ligands versus those found in natural DOC. The possibility of bound Ni uptake is further discussed in Section 4.2, below.

Finally, it is important to note that at early life stages in sea urchins the patterns of waterborne Ni accumulation are solely a consequence of waterborne absorption. Embryos of *S. purpuratus* do not develop the capacity to feed until 4 days post-fertilization, at the earliest (Smith et al., 2008). Consequently, interpretation of accumulation data is simplified as it does not need to consider the potential presence, and uptake, of Ni-DOC complexes in the developing sea urchin gut.

4.2. Kinetics of Ni accumulation

If Ni accumulation in sea urchin embryos was a specific, regulated process, then saturable kinetics (either hyperbolic or sigmoidal) should have been observed. Saturation occurs when increasing the Ni exposure concentration has no, or only a marginal, further effect on accumulation, thus facilitating the calculation of the kinetic parameters K_d and B_{max} (Leonard and Wood, 2013). A K_d represents the seawater Ni concentration at which half-maximal Ni accumulation is achieved and is therefore a measure of affinity. In contrast, a B_{max} is the maximum predicted Ni accumulation and is a measure of accumulation capacity. Under initial rate conditions these parameters will most strongly reflect the characteristics of uptake (i.e. the affinity and capacity of transporters achieving apical epithelial entry). In the current study, it is unlikely that even the first time interval at which accumulation was assessed (4-h) represented initial rate conditions. This is demonstrated by the very rapid saturation with time observed in Fig. 1. It is also supported by a previous study, which observed that the rate of Ni uptake by fertilized sea urchin embryos was rapid, achieving saturation after just 10 min of exposure (Timourian and Watchmaker, 1972). Consequently, in our study K_d and B_{max} will reflect a combination of apical Ni uptake, cellular Ni handling, and potentially, Ni excretion processes.

The patterns of Ni uptake varied with sea urchin age. In general, the affinity of an accumulation process is an indication of either the nutritional demand for the substrate, and/or the availability of the substrate to the animal. Fertilized embryos of *S. purpuratus* hatch into free swimming larvae between 16 and 22 h (Yager and Barrett, 1987). Consequently, the first two measures of Ni accumulation affinity in our study (4- and 8-h) were made when the embryos would most likely be in contact with sea-floor substrate in their natural settings. In contrast, our latter two time-points (48- and 96-h) were made on free-swimming larvae, more likely to be present in the water column. As sea water generally has lower Ni levels than sediment (see Blewett and Leonard, 2017), then this could drive changes in accumulation pathways. The essentiality of Ni to sea urchin early life stages is unknown, and so whether any changes represent a biologically beneficial change to meet increased Ni demand, or whether these might be a consequence of changes in other nutrient transport pathways that may facilitate incidental Ni uptake via mimicry or as part of an organic complex (see below), is not known. Previous reports have shown, for example, that transport of the amino acid alanine increases with developmental stage in *S. purpuratus* (Meyer and Manahan, 2009). Consequently, if Ni is taken up bound to components of DOC, such as amino acids, then this could explain changes in accumulation through development.

Changes in epithelial permeability occur during sea urchin development, and these could also modify Ni accumulation. For example, septate junctions are specific to non-chordate invertebrates and are associated with paracellular permeability (Itza and Mozingo, 2005). In *S. purpuratus* these junctions first appear in the early blastula stage (Spiegel and Howard, 1983). In the current study, the initial measurement of Ni accumulation at 4 h likely reflects the absence of septate junctions, whereas subsequent measures represent time-points where a permeability barrier has been established. Thus if Ni accumulation kinetics was a reflection of paracellular permeability then linear uptake at 4 h would be anticipated, to be superseded by saturable patterns of accumulation at later time-points. This pattern was not observed (Table 1). This indicates that Ni is unlikely to be paracellularly accumulated (see also below), and that changes in epithelial junction properties are unlikely to be important drivers of the patterns of accumulation observed.

Body mass of the developing sea urchin larvae does not increase appreciably during the ~96-h time frame of the current study (see Tellis et al., 2014). Therefore we would predict that Ni storage capacity would not change significantly. This is supported by BCF data. At 96-h, but not at earlier time points, there was a decline in BCF with increasing Ni concentration (Fig. 3D). One factor that may play an important role in cellular Ni regulation is metallothionein. This small metal-binding protein is induced by Ni exposure (Hwang et al., 2012), but even in the absence of trace metals has a characteristic increase in expression during early development in sea urchins (Ragusa et al., 2017). However, the specific mechanisms involved in regulating Ni burden, and how these may change over development, requires further investigation.

Importantly, in the presence of DOC 2, a distinct difference in Ni handling was observed relative to the ASW control. At 96-h, sea urchins exposed to Ni in the presence of DOC 2 exhibited a significantly elevated BCF (Fig. 3D). Consistent with the argument in Section 4.1, this would indicate either enhanced uptake of Ni, an enhanced cellular Ni binding pool, or impaired Ni excretion. Although the presence of DOC did not reduce Ni accumulation, the current study provides little evidence for enhanced Ni accumulation, while previous studies have indicated that the presence of DOC facilitates, rather than impedes, trace metal elimination from aquatic invertebrates (Glover and Wood, 2005). This indicates, therefore, that Ni may be absorbed bound to DOC and this complex has a slower cellular turnover, possibly because it is unavailable to the usual cellular Ni excretion pathways.

Further support for the concept of a bioavailable Ni-DOC species, which might explain an enhanced BCF but no toxicological impact (see Section 4.1), is provided by the presence of sigmoidal Ni accumulation kinetics. Sigmoidal curves are characteristic of multiple pathways with overlapping affinities and capacities contributing to uptake (Brauchi et al., 2005). Thus, the sigmoidal curves seen in the presence of DOC may represent the uptake of Ni through multiple pathways, some of which may be principally responsible for the uptake of dissolved organics. As mentioned above, one candidate group of dissolved organics are the amino acids. Amino acid transporters are known to facilitate amino acid uptake across the integument of developing sea urchin larvae (Manahan et al., 1989), and these could also mediate the transport of amino acid-bound Ni.

As exposure time progressed, Ni accumulation in the presence of DOC 7 became linear (Table 1). Linear accumulation is characteristic of diffusion, wherein accumulation occurs in a fixed ratio to Ni exposure concentration, and is not affected by potential regulatory factors. In terms of uptake, linear accumulation of Ni could be achieved

transcellularly or paracellularly. In the case of the former, a large hydrophobic component of DOC could traverse the epithelium, and in so doing could bring any complexed Ni with it (Turner and Williamson, 2005). Paracellular Ni uptake (i.e., that which occurs between epithelial cells), has been hypothesized as a potential pathway of Ni uptake in marine biota (Blewett and Leonard, 2017), but specific evidence for this is currently lacking. However, DOC-induced alterations in membrane permeability (see above) could facilitate uptake via a paracellular route.

4.3. Experimental considerations

One complicating factor is that the studies described in the current work were conducted 2–3 months following the toxicity assays reported in the previous study (Blewett et al., 2018), using the same marine DOC samples. In in the interim period, samples were stored in the dark at 4 °C to maintain sample integrity. Previous studies suggest that storage under these conditions for several months would have undetectable effects on DOC concentration, although small changes in non-aromatic components of the DOC could be induced (Peacock et al., 2015). Aromaticity, as measured by the specific absorption coefficient at a wavelength of 340 nm (SAC_{340}) was strongly correlated with protection against Ni toxicity in our previous work (Blewett et al., 2018). As these are the functional groups most resilient to degradation, it seems unlikely that short-term storage would be a factor explaining the lack of correlation between Ni toxicity amelioration and whole body Ni accumulation in this study system. Therefore more grab sampling and replication is needed to further the complex Ni-DOC story.

5. Summary

Predictive models are important regulatory tools facilitating the protection of aquatic systems against harm caused by the anthropogenic enrichment of metals. These tools, such as the BLM, rely on knowledge of the relationships between water chemistry, metal uptake and effect, and the mechanisms underlying these relationships (see Mebane et al., 2020). In recent years there has been a significant advancement in our knowledge of Ni toxicity in aquatic systems, including how toxicity changes with water salinity (Blewett and Leonard, 2017). However, the role of DOC in modifying Ni toxicity, especially in marine systems has remained somewhat elusive. Growing evidence suggests that marine DOC is protective against toxicity (e.g. Blewett et al., 2016, 2018), a finding that is contrary to model predictions (Stockdale et al., 2015). However, the relationship between metal accumulation and toxicity, a central tenet of BLM, remains unresolved. The current study confirms anomalies between predicted Ni speciation, its bioavailability and toxicity. Although natural marine DOCs protect against Ni toxicity, they appear to do so without decreasing Ni body burden, at least in the model sea urchin embryo system. Additional work is required to understand the bioavailability and bioreactivity of Ni-DOC species, in order to build marine BLMs that will adequately protect marine systems against Ni toxicity.

Declaration of competing interest

The authors declare no conflict of interest.

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