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The role of dissolved organic carbon concentration and composition on nickel toxicity to early life-stages of the blue mussel *Mytilus edulis* and purple sea urchin *Strongylocentrotus purpuratus*

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ABSTRACT

Nickel (Ni) emissions resulting from production and transportation raise concerns about the impact of Ni exposure to marine ecosystems. Ni bioavailability models are established for FW systems, but the influence of chemical parameters (e.g. dissolved organic carbon (DOC)) on Ni toxicity within marine systems is less well understood. To examine the effects of DOC concentration and composition on Ni toxicity, acute toxicity tests were conducted on early life-stages of blue mussels (*Mytilus edulis*) and sea urchin embryos (*Strongylocentrotus purpuratus*) in full strength sea water (32 ppt). Nine different field collected samples of water with varying concentration (up to 4.5 mg C/L) and composition of DOC were collected from the east coast of the United States. Organic matter compositional analysis included molecular fluorescence and absorbance spectroscopy. The different DOC sources had different protective effects against embryo toxicity. The control (no DOC) Ni 48 h-EC_{50} for *Mytilus* embryos was 133 μg/L (95% confidence interval (C.I.) of 123–144 μg/L), while *Strongylocentrotus* embryos displayed control 96-h EC_{50} values of 207 μg/L (167–247 μg/L). The most significantly protective sample had high humic acid concentrations (as determined from fluorescence spectroscopy), which yielded an EC_{50} of 195 μg/L (169–222 μg/L) for *Mytilus*, and an EC_{50} of 394 μg/L (369–419 μg/L) for *S. purpuratus*. Among all samples, protection was related to both DOC quantity and quality, with fluorescence-resolved humic and fulvic acid concentrations showing the strongest correlations with protection for both species. These data suggest that DOC is protective against Ni toxicity in *M. edulis* and *S. purpuratus*, and that accounting for a DOC quality factor will improve predictive toxicity models such as the biotic ligand model.

1. Introduction

Dissolved organic matter (DOM) is a ubiquitous component of all natural waters, and is almost universally expressed in terms of its carbon concentration (DOC). DOC varies in concentration from 1 to 15 mg C/L in fresh water (FW) (Thurman, 1985), between 0.5 and 1 mg C/L in open ocean, and in estuaries is found in concentrations intermediate to these two values (0.1–10 mg/L; Nelson and Siegel, 2002). Consisting of bacterial-, animal-, algal- or plant-derived breakdown products, DOC has numerous important roles in aquatic systems. For example, DOC is important in nutrient cycling, and acts as an ultraviolet screen in FW ecosystems (Petersen, 1991). Among the best characterized and most critical roles of DOC is its ability to alter the toxicity of metals in the aquatic environment. In fact, it is increasingly evident that DOC has greater protective effects for some metals than "hardness" and alkalinity, previously considered key factors responsible for shaping toxic impacts of metals in freshwaters (Paquin et al., 2002). DOC is primarily thought to bind and complex metals, thereby decreasing their bioavailability to sensitive target surfaces such as the gill (Wood et al., 2011).

DOCs can be classified on the basis of their principal origins: allochthonous (terrigenous) which originate from the breakdown of plant matter or other land-based sources, and autochthonous which are produced by algae and bacteria within lakes, mangroves and/or rivers. Autochthonous DOCs contain fewer aromatic ring structures, are composed of smaller molecules, and are optically lighter in comparison to allochthonous DOCs (for review see Wood et al., 2011). Research to date indicates that the more optically dark a DOC, the more protective

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it is against metal toxicity, at least in fresh water (e.g. Van Genderen et al., 2003; Glover et al., 2005).

DOC is now considered to be one of the most important variables in modelling approaches used to predict metal toxicity on a site-specific basis (e.g. the biotic ligand model; BLM). The use of BLMs to establish water quality guidelines can be seen as an advancement of the science over other established approaches such as hardness corrections, which do not adequately account for the important complexing effects of DOC (De Schamphelaere et al., 2002). The BLM relies on the relationship between metal binding to specific sites on the biotic ligand (e.g. gills of a fish) and toxicity. Specifically, it considers the ability of dissolved ions to compete with a given metal for uptake, and ligands in the water that may bind metals and affect their bioavailability (Paquin et al., 2002). While DOC is a key factor affecting metal binding, only absolute DOC concentration, and in some cases only humic and fulvic acids, are taken into account in the current available BLMs. However, there is growing evidence that suggests that DOCs exhibit differential metal binding capacity depending on their composition (Playle, 1998; Glover et al., 2005; Arnold et al., 2005, 2006; Al-Reasi et al., 2012; Blewett et al., 2016). To date, no study has systematically examined the impacts of marine DOC composition and concentration on the toxicity of the important aquatic metal contaminant, nickel (Ni).

Nickel is a transition metal which is an essential micronutrient in plant and bacterial species (Ragsdale, 1998), but at elevated concentrations in aquatic environments it can be toxic. Elevation of environmental Ni occurs from production, use in downstream industrial processes (e.g., alloy manufacture), and emission of fossil fuels (Eisler, 1998). The continued production of Ni (Reck et al., 2008) has led to it becoming almost ubiquitous in marine and FW environments. Total water Ni concentrations can reach up to 2000 μg/L downstream of industrial activity (Pyle and Couture, 2012). The majority of aquatic Ni toxicity research has focussed on FW settings, and Ni toxicity in the marine environment is less commonly measured. Nevertheless, previous evidence has shown that certain marine invertebrates (e.g. early life stages of some sea urchins) are very sensitive to Ni toxicity at low concentrations (< 15 μg/L; *Diadema antillarum,* and *Evechinus chloroticus*; Bielmyer et al., 2005; Blewett et al., 2016).

Evidence confirms DOC protects against Ni toxicity to FW organisms (e.g. Deleebeeck et al., 2007, 2008, 2009). However, there is less certainty about the role of DOC in ameliorating toxicity in marine settings. For example, the Windermere Humic Aqueous Model (WHAM), which was developed for fresh water, and not for salt water applications, predicts that for Ni in the marine environment, there is weak binding except at low salinities – suggesting poor protective effects of DOC against Ni toxicity (Stockdale et al., 2015). However WHAM has not been calibrated with marine specific ligands. Martino et al. (2004) used voltammetry to measure Ni speciation in an estuary and found a class of very strong (logK $\approx$ 18) but dilute Ni binding, capacities were measured in the low nM range. These sites would likely be saturated at levels of Ni where toxicity is observed (μM range of total Ni), but this finding highlights the fact that WHAM predictions might not provide a complete perspective when it comes to Ni-DOC interactions in marine systems.

Embryonic stages of invertebrates are considered to be among the most sensitive of marine organisms to environmental toxicants (Jha et al., 2000; Bielmyer et al., 2005; DeForest and Schlekat, 2013; Blewett et al., 2016). Blue mussels (*Mytilus edulis*) are sessile bivalves native to the North Atlantic (Thompson, 1979) and have been extensively utilized as tools for examining the effects of a wide range of aquatic contaminants (e.g. Jha et al., 2000). There have been studies focussed on understanding Ni bioaccumulation and depuration in adult blue mussels (Millward et al., 2012), but, to our knowledge, there has been no research performed examining the effects of Ni, and the protective role of DOC, to blue mussel embryos. Previous research in other mussel species have shown *M. galloprovincialis* and *M. trossolus* embryos are of intermediate sensitivity to Ni, relative to other invertebrates, with 48 h EC_{50}s ranging from 61 to 350 μg/L (DeForest and Schlekat, 2013).

The purple sea urchin, *Strongylocentrotus purpuratus* is endemic to the Pacific Ocean, and embryos of this species have been previously utilized to investigate the toxicity of metals such as lead and zinc, copper and Ni (Tellis et al., 2014a, 2014b). In general, the early life-stages of sea urchins have been better studied than their mussel counterparts, with Ni EC_{50} values ranging from 14 to 341 μg/L (DeForest and Schlekat, 2013; Blewett et al., 2016).

Thus, the current study had two goals. The first was to investigate the protective effect of natural marine dissolved organic matter (DOM) with regards to Ni toxicity to both *Mytilus edulis* and *Strongylocentrotus purpuratus* early life-stages. The second was to determine the dependence of protection, if any, on the properties of DOM that co-vary with optical characteristics of the DOCs.

2. Methods

2.1. Animal collection

Adult mussels (*Mytilus edulis*) were obtained from Aquatic Research Organisms (ARO, Hampton, NH, USA) and adult purple sea urchins (*Strongylocentrotus purpuratus*) were obtained from WestWind SeaLabs Supplies (Victoria, BC, Canada), and transported to Wilfrid Laurier University Animal Care facility where they were placed in separate 38-L glass tanks containing aerated, recirculating salt water (made by the addition of Kent Marine Reef Salt (Big Al's Aquarium, Kitchener, ON, Canada)) to City of Waterloo tap water (see Cunningham and McGeer, 2016 for composition). Nickel concentrations in this water were below detectable limits (see Sections 2.6 and 3.1). Salinity (32.1 ± 0.7 ppt) and temperature (15.4 ± 0.3 °C) were kept constant and monitored daily using a handheld meter (YSI 30, YSI Inc., Yellow Springs). A light:dark cycle of 12:12 h was maintained.

2.2. Water collection, storage and DOC analysis

Samples of coastal marine waters were collected in July of 2015, at nine sites along the east coast of the United States from Rhode Island to Connecticut. The sites are detailed in Supplemental Table 1. Note that Site 7 and 10 are the same site, but the two samples were collected at different periods of the tidal cycle (sample 7 was taken during low tide and sample 10 during high tide). During collection, samples were filtered through a 1-μm filter (String Wound Cartridge filter, Filter Source, Hamburg, NY, USA) and collected in 2-L Nalgene bottles (pre-acid washed before use with 10% trace metal grade nitric acid (Sigma Aldrich, Oakville, ON). Samples were then brought back in coolers to Wilfrid Laurier University and stored in 4-L polyethylene bottles at 4 °C in a dark room until further analysis or use in toxicity assays. Samples for analysis of DOC concentration (50 mL) were taken and filtered through a 0.45-μm filter (Acrodisc HT tuffryn membranes, Pall Corp., Ann Arbor, MI, USA). Three readings were taken for each DOC sample from each collection. As in previous studies (DePalma et al., 2011a, 2011b), polyethylene bottles, instead of glass, were used for collection and storage of samples to protect from sample loss due to container breakages. In addition to the field-collected samples, a sample was made from an artificial sea water (ASW) source (Kent Marine Reef Salt dissolved in City of Waterloo tap water) and acted as a control (no added DOC). DOC concentrations in all samples were measured using a Shimadzu TOC –L CPH/CPN analyzer (Shimadzu Corporation Kyoto, Japan). Standard concentrations were prepared from potassium hydrogen phthalate (Mandel Scientific, Guelph, ON) at 5, 10 and 15 mg of C/L. All saltwater samples were filtered and acidified with 10 μL of concentrated HCl to ensure conversion of dissolved inorganic carbon into volatile CO_2. In addition, Milli-Q water rinses were made after every sample. All samples were measured well above the instrument detection limit of approximately 0.1 mg C/L. All samples were salted up to maintain a consistent salinity for toxicity testing, pH and water

chemistry were made at the start of the toxicity testing (see below).

To identify molecular and structural composition, fluorescence excitation-emission matrices (FEEM) were generated from samples before and after salt addition to 32 ppt (Kent Marine Reef Salt), shown in Fig. 2. Briefly, FEEM plots were generated by scans of emission wavelengths at 250–600 nm, in 1 nm increments, for excitation wavelengths between 200 nm and 450 nm, at intervals of 10 nm, using a Varian Cary Eclipse Fluorescence spectrophotometer (Varian, Mississauga, ON, Canada). The photomultiplier tube was set to high detection (800 V) and the scan speed was set at 400 nm/min. Two-dimensional FEEMs were created using MATLAB (MathWorks, Natick, MA, USA) and in-house scripts.

The aromatic composition of DOCs was estimated by examining the absorbance of samples at a wavelength of 340 nm (specific absorption coefficient; SAC340), based on the method described in Curtis and Schindler (1997). Briefly SAC340 (cm^2/mg) was calculated as:

$$SAC340 = \frac{(2.303X\ Abs340)\ /\ (pathlength)}{[DOC]\ /1000}$$

The fluorescence index (FI) was used as an indicator of DOC composition, and was calculated by previously described methods (McKnight et al., 2001):

$$ex370 = \frac{Em450}{Em500}$$

where ex370 is the FI index at 370 nm and Em 450 and 500 refer to emission intensities at 450 nm and 500 nm. Note, however, that there are no FI indices or SAC340 values for ASW, as there was not sufficient measured fluorescence or absorbance.

Parallel factor analysis (PARAFAC) modelling was performed using the PLS Toolbox (Eigenvectors Inc, WA, USA) in MATLAB as described by DePalma et al. (2011a, 2011b). In brief, the method *a priori* assumes four fluorescent components: humic-like, fulvic-like, tyrosine-like and tryptophan-like. Then, using scans of pure tyrosine and tryptophan as weighting factors, this approach recovers the relative fractions of each of the four fluorophores contributing to the measured total fluorescence of each sample.

For comparisons between optical properties and effects concentrations (i.e., EC_{50}), it is necessary to convert the relative indices (intensive variables) to absolute quantities (extensive variables). To this end, percent of each fluorophore, was multiplied by DOC concentrations. Fulvic and humic acid have been estimated from fluorescence analysis in previous publications (Al-Reasi et al., 2012).

2.3. Mytilus edulis fertilization protocol

To induce spawning, adult mussels were placed in 10-L containers with sea water (SW, 32 ppt) that were gently heated to 22–24 °C over the course of 2 h to induce thermal shock. Spawning individuals were immediately removed to 100-mL containers filled with filtered SW. Males were identified by the release of sperm within the water column, and females were identified by the appearance of orange eggs. Both egg quality and sperm motility were assessed on an EVOS compound microscope (Fisher Scientific, Toronto, ON) at 200× magnification. Sperm from several individuals was pooled and stored on ice until 3 spawning females were identified. Eggs were removed from spawning containers and placed in fresh 100-mL glass beakers containing filtered SW. To initiate fertilization, pooled sperm (~ 200 µL) was added to eggs of three individual females and checked over the course of 30 min for fertilization. Once 80% fertilization occurred (counted using a Sedgewick-Rafter slide) eggs were diluted to a concentration of 1000 eggs per mL into 50-mL plastic tubes which contained filtered SW. The test was initiated by adding 2 mL of fertilized embryos (~ 2000 eggs) to 20-mL glass scintillation vials containing the respective Ni + DOC test solution. Both filtered (0.45 µm syringe filter) and unfiltered water samples were taken for Ni analysis (see below), at test initiation and at the end of 48 h. Tests were conducted in a recirculating water bath maintained at 15 °C. At the end of 48 h, controls were observed to ensure that > 80% had developed into normal D-shaped prodissoconch larvae (Thompson, 1984). At the end of 48 h, the addition of 1 mL of 5% buffered formalin (Sigma Aldrich) was used to stop development and preserve embryos for later scoring. One hundred embryos were assessed for each replicate. If the embryo did not display the normal "D-shaped" prodissoconch larvae form, it was considered impaired.

2.4. Strongylocentrotus purpuratus fertilization protocol

To induce spawning, 1 mL of 0.5 M KCl (Sigma Aldrich) was injected into the hemocoel of adult sea urchins. Eggs and sperm were collected from spawning adults. Sperm was stored “dry” without sea water on ice at 4 °C until fertilization (n = 3), while eggs from spawning females were collected in 250-mL beakers containing filtered SW (n = 3). Eggs were washed 3x with filtered SW before initiating fertilization. After washing, eggs were placed into 50-mL Falcon™ tubes at a density of 200 eggs /mL (all densities determined with a Sedgewick-Rafter slide). Approximately 200 µL of sperm was added to each egg-containing Falcon™ tube and stirred gently to facilitate fertilization, and after 30 min, fertilization success was determined. Fertilization success was confirmed under a microscope by the elevation of a fertilization envelope around each egg. Once 80% fertilization was achieved, aliquots of embryos were added to 20-mL glass scintillation vials containing the respective Ni + DOC test solution (concentrations as *Mytilus* tests as above) at a density of 100 eggs per mL or ~ 2000 eggs per container. Tests were conducted in a recirculating water bath maintained at 15 °C. Full strength SW was used in all exposures (32 ppt), which were run at 15 °C, under a 14:10 light to dark cycle. At the end of 96 h, the test was terminated by the addition of 1 mL 5% neutral buffered formalin for later microscopic observation. One hundred embryos were assessed for each replicate. If the embryo did not display the normal "pluteus" larvae form, it was considered impaired.

2.5. Mytilus edulis and Strongylocentrotus purpuratus toxicity assay

Assessment of mussel and sea urchin embryo toxicity was performed according to standard protocols (ASTM, 1994). Nickel concentration tests were based off of previous work in Blewett et al. (2016). Tests on mussels were conducted for 48 h, while tests on sea urchins were over 96 h. Twenty-four hours before toxicity tests commenced, experimental waters were filtered through 0.45-µm filters (Cellulose Nitrate Membrane filters, Buckinghamshire, UK) and were brought to the same salinity (full-strength SW; 32 ppt) using Kent Marine Salt, pH was measured at this point. Ni stock solution was made by the addition of $NiSO_4{\cdot}6H_2O$ to a 0.5% nitric acid solution (Trace metal grade,-TraceCERT, Sigma Aldrich, Oakville, ON, Canada) made up in MilliQ water and stored in a 2-L Nalgene plastic bottle. For each of the ten water samples, eight different nominal Ni concentrations were tested in triplicate: 0, 25, 50, 100, 200, 400, 800, 1600 µg/L and were then measured by GFAAS see below (S.I Fig. 1). However, for sea urchin toxicity testing only three of the collected water samples were tested (see Results). Each DOC had a control with no Ni added that ran in parallel. Nickel was added at the time when water samples were salted up (24 h prior to toxicity assessment) to allow solutions to equilibrate. Sites are named as follows 1. ASW = Kent Marine Salt (Artificial Seawater); 2. SVP = Seaview Park, RI; 3. BTP = Barbara Tuffs Playground, RI; 4. PCA = Perry Creek Access, RI; 5.BPP = Beebe Pond Park, CT; 6. ELM = Elm Street, CT; 7. CC1 = Audubon Coastal Center, CT Low Tide; 8. IR = Indian River, CT; 9. WB = Walnut Beach, 10. CC2 = Audubon Coastal Center; CT, High Tide. Additional water chemistry (Ni, DOC, pH and salinity) replicates (i.e. no embryos added; n = 3) were run in parallel with test exposures.

2.6. Ni measurement

Nickel concentration in water was determined by Graphite Furnace

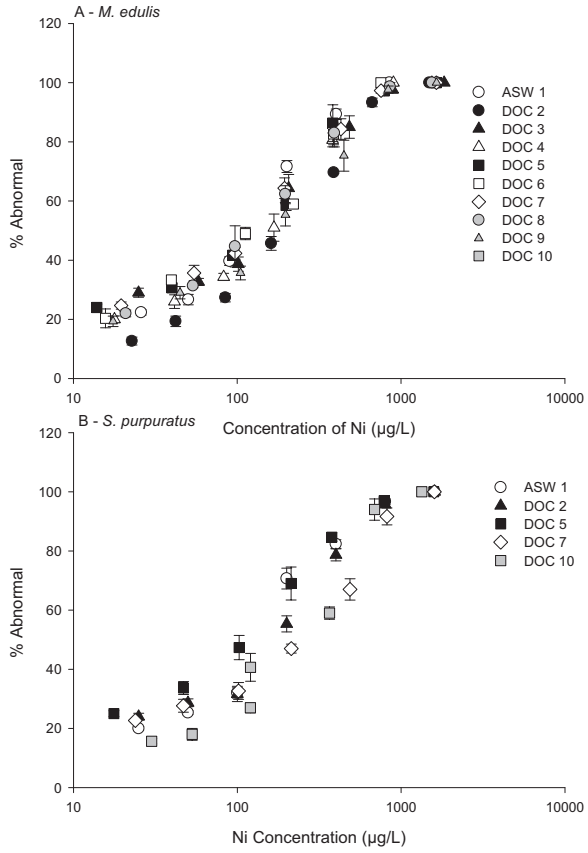

Fig. 1. % Abnormality across Ni concentrations of A) *Mytilus edulis* and B) *Strongylocentrotus purpuratus* as a function of DOC source. Plotted values represent means ± S.E.M (n = 3).

Atomic Absorption Spectroscopy (GFAAS) at both the start and end of the development tests. The specific instrument used was a Perkin Elmer PinAAcle 900 T, which includes Zeeman background capabilities allowing for direct injection of saltwater samples. There was < 5% difference between filtered and unfiltered water samples, and thus only filtered (dissolved) Ni concentrations are reported in Fig. 1. All field and control exposure waters that contained no added Ni were below the detection limit (0.7 µg/L). All exposure water was measured against certified Environment Canada freshwater reference materials, TM 24.2 (5.0 µg/L Ni) and TM 15 (18.1 µg/ Ni), Ni recovery was 98.5 ± 1.5% (S.E.M = standard error of the mean). Nickel measurements for sea urchin embryonic tests were all within 7% of those measured for *Mytilus* tests, and thus only water chemistry for the mussel trials is shown.

2.7. Statistics

The 48-h median and 96-h median effective concentrations (EC_{50}) and confidence intervals were determined from a sigmoidal logistic curve using MATLAB (Version 2013a, Mathworks Inc, Natick, MA, USA). These values were then fitted to a logistic model with EC_{50} as the fitting parameter, as previously described by Meyer and Adams (2010). Linear regression analysis to determine the relationship between DOC composition and toxicity was carried out using SigmaPlot version 11 with SigmaStat (version 3.5) integration (Systat Software Inc. San Jose CA, USA). To determine if EC_{50} values were significantly different from each other, the first step was to determine that the confidence intervals did not overlap. If they did not, then standard Environment Canada protocols were used following the Litchfield –Wilcoxon methodology described in Wheeler et al. (2006). All data have been expressed as means ± SEM (standard error of the mean for 3 replicates). Significance for all tests was accepted at $\alpha = 0.05$.

3. Results

3.1. Water chemistry

DOC concentrations were 0.88 mg C/L in the ASW control, and ranged from 2.3 mg C/L at site 6 (ELM) to 4.5 mg C/L at Site 2 (SVP). The ASW also displayed the lowest FI and SAC340 values, whereas Site 10 (CC2) and Site 5 (BPP) displayed the highest FI (CC2 - 1.58) and SAC340 (BPP - 14.87) values, respectively. Salinity and pH was also monitored and was not significantly different between sites after the addition of salt (Supplemental Table 1).

All water samples had natural Ni levels that were below the limit of detection (i.e., less than 0.7 µg/L). For toxicity assays, measured Ni concentrations did not differ between water samples, and were close to nominal Ni concentrations (Fig. 1).

3.2. DOC composition

Field-collected water sources were generally similar in terms of sharing optical components (SAC340, and FI), but the relative importance of each fluorescent component did vary (Fig. 2). Fluorescence spectrophotometry data indicated measureable fluorescence for all 10 DOC samples. Sites 2 (SVP), 3 (BTP), 5 (BPP), 7 (CC1), 10 (CC2) showing the highest fluorescence intensity (Fig. 2. B, C, E, G, J). These sites also displayed fluorophores that contained more humic- and fulvic- acid-like fluorescence, as exhibited by the emissions observed in the range of 400–450 nm (Fig. 2). Low fluorophore peaks for tyrosine-like and tryptophan-like substances (emissions in the 300 nm range) were also displayed in all field-collected samples, except Site 8 (IR) (Fig. 2H) and the ASW control where no peaks were observed (Site 1.Fig. 2A).

3.3. DOC source effect on Ni toxicity

Acute toxicity of Ni to *M. edulis* embryos was assessed in each of the ten DOC samples (9 samples and one ASW control). Within each sample, eight different concentrations of Ni (0, 25, 50, 100, 200, 400, 800, 1600 µg/L) were used to determine a 48-h EC_{50} (concentration of Ni where 50% of the population showed impaired development). The control sample (ASW with no added DOC) generated an EC_{50} of 133 µg/L (95% C.I. of 123–144) of Ni, while collected SW samples containing natural DOC exhibited toxicity values that were either greater (more protective) or similar to this value. DOC samples collected from Sites 2 (SVP), 3 (BTP), 4 (PCA), 7 (CC1), and 10 (CC2) were significantly more protective with respect to ASW with EC_{50}'s at 195 (95% C.I. 174–219 µg/L), 180 (95% C.I. 159–204 µg/L), 172 (95% C.I. 151–197 µg/L), 170 (95% C.I. 150–193 µg/L), 194 (95% C.I. 170–222 µg/L) µg Ni/L respectively, while the lowest EC_{50} (most toxic; excluding control), were waters collected from Site 8 (IR) at 139 µg/L (95% C.I. 120–158 µg/L) (Fig. 3). Samples were considered significantly different from the control if 95% confidence intervals did not overlap, according to the methodology of Wheeler et al. (2006). Samples, 5, 6, 8 and 9 were not significantly different from the control ASW sample 1.

Acute toxicity (96 h) of Ni to *S. purpuratus* embryos was assessed in the presence of the most protective samples from *Mytilus* tests (2:SVP, 5:BPP, 7:CC1, and 10:CC2). Sea urchin embryos had ASW EC_{50} values of 207 µg/L (95% C.I. 167–247 µg/L). Two collected SW sites provided protection against Ni toxicity. Site 2 (SVP) and site 10 (CC2) displayed significant increases in the EC_{50} from ASW at 394 µg/L (95% C.I. 369–419 µg/L) and 334 µg/L (95% C.I. 298–371 µg/L) respectively. Site 2 (SVP) was also significantly different from site 5(BPP: EC50 205 95% C.I 156–254 µg/L) (Fig. 3).

The effect of DOC concentration on Ni toxicity was investigated by plotting DOC concentration against EC_{50} values for *Mytilus* and *Strongylocentrotus* (Fig. 4). Although the *Mytilus* relationship was not

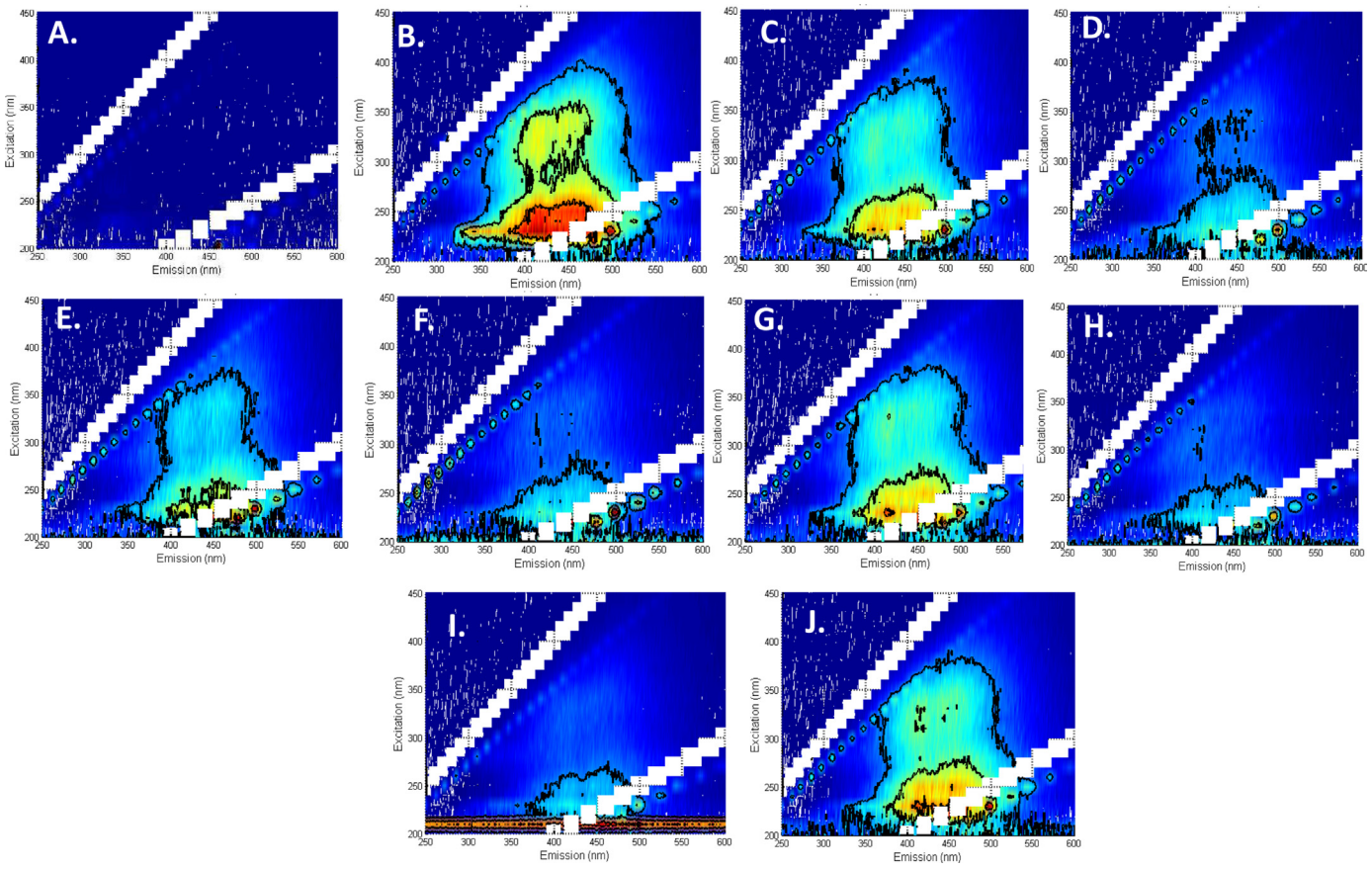

Fig. 2. Fluorescence excitation-emission matrices for the collected water samples. Panel A represents artificial seawater (32 ppt) while Panels B-J correspond to collection sites 2–10 (see Table S1).

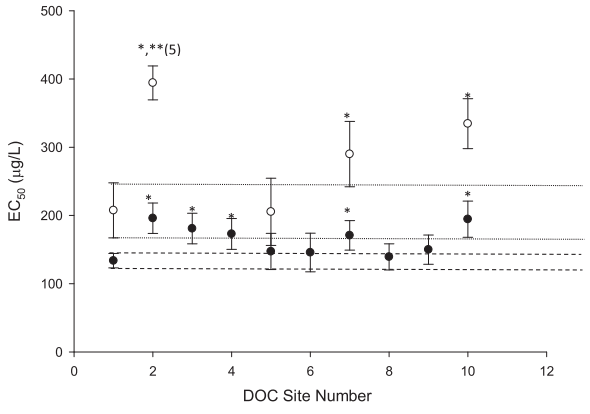

Fig. 3. Ni toxicity (EC_{50}; µg/L) to *Mytilus edulis* embryos (solid symbols) and *Strongylocentrotus purpuratus* (open symbols) as a function of DOC source. Site 1 represents artificial seawater with no DOC added (measured DOC of 0.88 mg of C/L), other sites are named in Table S1. Plotted values represent EC_{50} values and surrounding 95% confidence intervals (n = 3). Dashed lines indicate confidence intervals associated with the control (Site 1), and any values that have an asterisk indicate significant differences from control (ASW –dashed lines) ($p < 0.05$) (see methods), two asterisks indicate significant differences between sites 2 and 5 for sea urchin only.

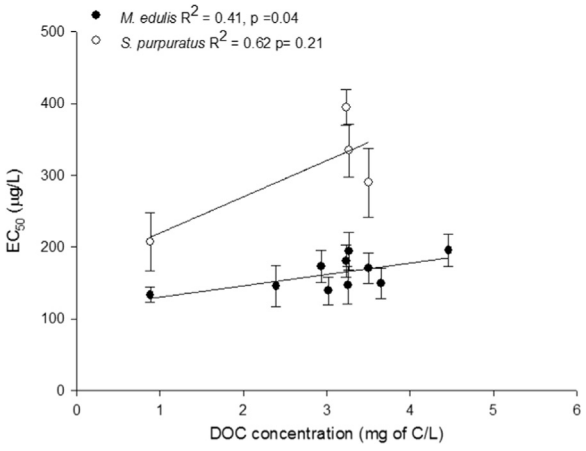

Fig. 4. Nickel toxicity (EC_{50}; µg/L) to *Mytilus edulis* embryos (solid symbols) and *Strongylocentrotus purpuratus* embryos (open symbols) as a function of DOC concentration (mg of C/L). Plotted values represent EC_{50} values with 95% C.I. (n = 3).

strong ($R^2 = 0.41$), it was significant ($p = 0.04$) and the relationship was positive (i.e. as DOC concentration increased the EC_{50} also increased) (Fig. 4). For *Strongylocentrotus* the R^2 value was 0.62 but not significant ($p = 0.21$; Fig. 4). All regression line diagnostics are present in Supplemental Table 3 for each species.

Water sample absorbance at 340 nm was significantly related to EC_{50} values for both species. This relationship was best described by a linear fit, yielding R^2 values of 0.52 ($p = 0.02$) and 0.97 ($p = 0.01$), for *M. edulis* and *S purpuratus*, respectively (Fig. 5). Note that simple absorbance rather than SAC340 values were plotted here in order to remove the normalization to absolute DOC concentration.

From FEEM plots (e.g. Fig. 2), parallel factor analysis (PARAFAC) analysis determined the percentage of the total fluorescence explained by each of the four identified fluorophores (Supplemental Table 2). To convert this relative estimate to a quantitative estimate, the measured

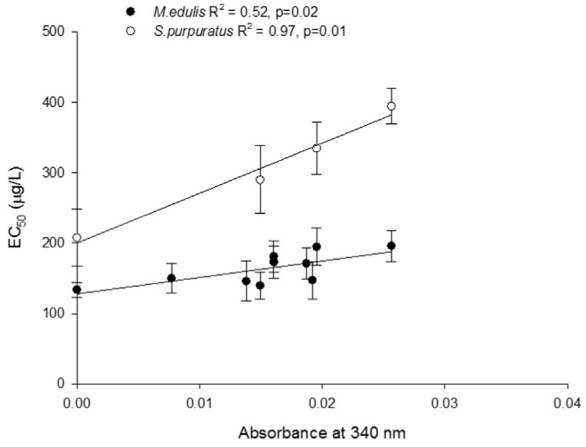

Fig. 5. Nickel toxicity (EC_{50}; µg/L) to *Mytilus edulis* embryos (solid symbols) and *Strongylocentrotus purpuratus* embryos (open symbols) as a function of absorbance at 340 nm (SAC_{340}). Plotted values represent EC_{50} values with 95% C.I. (n = 3).

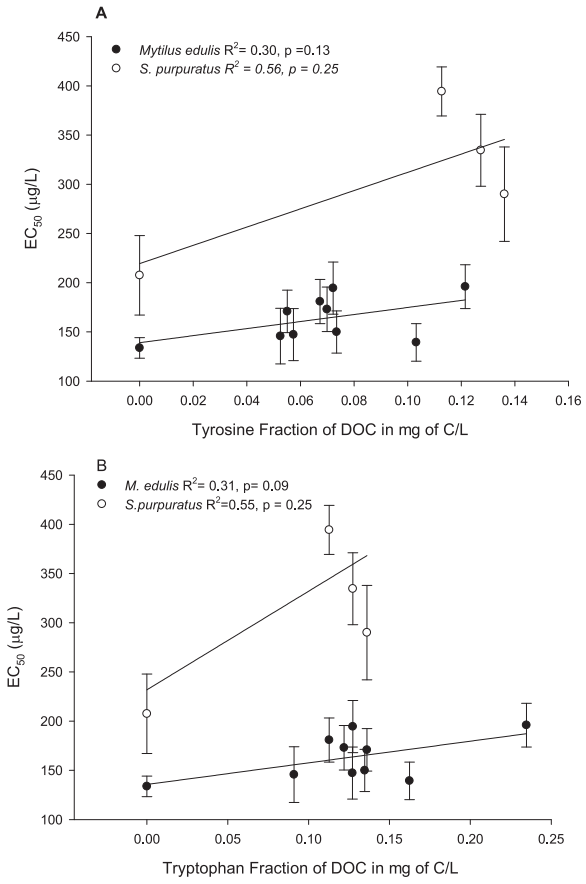

Fig. 6. Nickel toxicity (EC_{50}; µg/L) to *Mytilus edulis* embryos (solid symbols) and *Strongylocentrotus purpuratus* embryos (open symbols) as a function of (A) tyrosine fraction of the collected DOC's) and (B) tryptophan fraction of the collected DOC's. Plotted values represent EC_{50} values with 95% C.I. (n = 3).

DOC was multiplied by each fluorescence fraction to determine the mg C/L concentration of humic-, fulvic-, tryptophan- and tyrosine-like components. To determine which fraction of DOC correlated with observed protective effects against Ni toxicity, correlational analysis was performed. There were no significant relationships between *Mytilus* toxicity and tyrosine (R^2 = 0.31, p = 0.09) or tryptophan (R^2 = 0.30, p = 0.13) relative concentrations (Fig. 6A,B). Similarly, for *Strongylocentrotus* the relationships were present but not significant for both

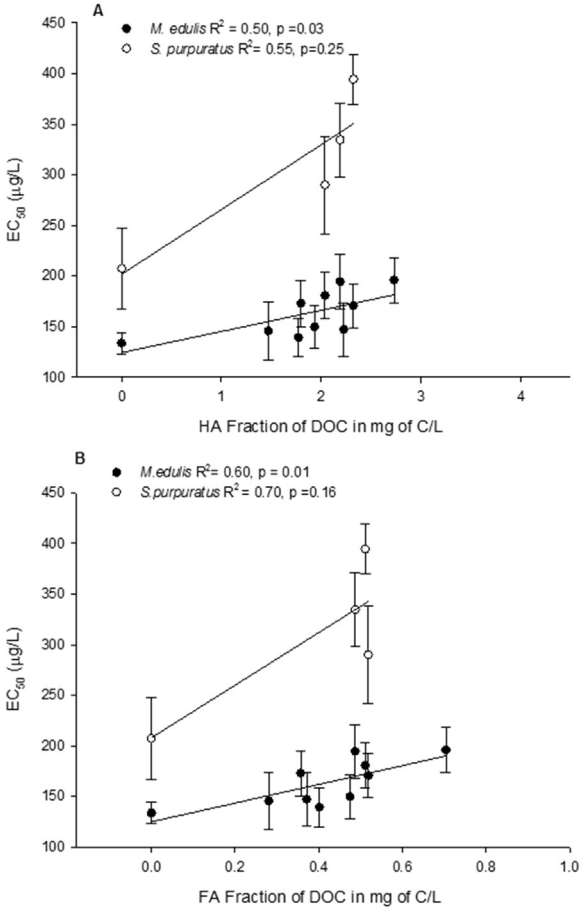

Fig. 7. Nickel toxicity (EC_{50}; µg/L) to *Mytilus edulis* embryos (solid symbols) and *Strongylocentrotus purpuratus* embryos (open symbols) as a function of both (A) humic and (B) fulvic acid fraction of the collected DOC's. Plotted values represent EC_{50} values with 95% C.I. (n = 3).

tyrosine (R^2 = 0.56, p = 0.25) and tryptophan components (R^2 = 0.55, p = 0.25) (Fig. 6A,B).

The humic acid fraction displayed a clear protective effect against Ni toxicity for both invertebrate species, with a significant relationships between humic acid concentration and toxicity protection for both *Mytilus* (Fig. 7A, R^2 value of 0.50, p < 0.03), and *Strongylocentrotus* (R^2 = 0.84, p = 0.02; Fig. 7A). Fulvic acid fractions were also protective with a strong relationship in *Mytilus* (R^2 = 0.60, p = 0.01), but not in *Strongylocentrotus* (R^2 = 0.70, p = 0.16 Fig. 7B)

4. Discussion

DOCs from different marine sources varied in their ability to protect against Ni toxicity. Toxicity of Ni towards developing *Mytilus edulis* and *Strongylocentrotus purpuratus* was dependent upon the DOC composition and concentration of the water in which the test was conducted. Among the chemical components of the DOC, the concentrations of the humic and fulvic acid components as defined by SAC340 had the strongest influence on EC_{50}s.

4.1. DOC characteristics

Field-collected DOCs displayed different chemical characteristics. These differences are likely due to the influence of distinct input sources (i.e., terrestrial or sewage inputs), local algal populations, and oceanographic factors such as wave action and ocean currents (e.g. Maie et al., 2012). Samples ranged from those with high terrestrial

(allochthonous) inputs (e.g. Sites 7 (CC1) and 10 (CC2)), to more open ocean-like sites (e.g. Sites 4 (PCA) and 9 (WB)), which displayed more autochthonous-like characteristics (Supplemental Table 2). Autochthonous DOCs tend to be microbiologically-derived, formed in the water column, and characterized by optically light properties with low aromatic group concentrations. In contrast, allochthonously-derived DOCs, which come from the breakdown of terrestrial plant matter, are optically darker, tend to have more phenolic groups, and have higher concentrations of humic and fulvic acids (Al-Reasi et al., 2011). Allochthonous DOCs have been shown to be more protective against metal toxicity in FW (Wood et al., 2011). Waters collected from Sites 2, 3, 5, 7 and 10 showed evidence of humic- and fulvic- like fluorophores (DePalma et al., 2011a), with emissions at both 250 nm and in the 400–450 nm range. The most intense peaks occurred at Sites 2, 7 and 10 respectively, indicating a higher concentration of humic and fulvic components (Fig. 2B, G, J; Supplemental Table 2). The FI index and the SAC340 measurements support the finding that Sites 8 and 9 are more allochthonous in nature (Supplemental Table 1, Supplemental Table 2). In general, the higher the FI, the more humic/fulvic acid-rich, and the more allochthonous the DOC (McKnight et al., 2001). SAC340 values are a measure of aromaticity, with lower values indicating a more autochthonous DOC, while higher numbers indicate a more allochthonous DOC (Curtis and Schindler, 1997). The highest SAC340 values occurred at Site 2, 5 and Site 10, in accordance with the FI (Supplemental Table 1) and the values correspond to sites surrounded by terrestrial vegetation. Conversely, the sites with the lower FI or SAC340 values were more open ocean or harbour collection sites, thus associated with autochthonous generation of DOCs with a more proteinaceous composition. Therefore, together FI and SAC340 provide insight into the source/origin of the DOC isolate (Al-Reasi et al., 2011). While these optical characteristics and their ability to discriminate DOC origin, are based on FW DOCs, the current study does suggest applicability to marine DOCs.

4.2. DOC source effect on Ni toxicity

To our knowledge this is the first study to examine the effects of DOC composition and Ni toxicity in this species. The 48-h Ni EC_{50} for *Mytilus edulis* in ASW was 133 µg/L (Fig. 3). This toxicity value is, however, in accordance with values for Ni toxicity in other, related, species. For example, Nadella et al. (2009) reported a measured 48-h EC_{50} for Ni of 150 µg/L in early life-stages of the west coast native, *Mytilus trossolus.* However, *Mytilus galloprovincialis*, the Mediterranean mussel, was less sensitive to Ni toxicity with an EC_{10} value of 259 µg/L (DeForest and Schlekat, 2013). The differences between *M. edulis* and *M. galloprovincialis* may be due to species sensitivity differences to water chemistry. Arnold et al. (2009) examined the sensitivity of *M. edulis* and *M. galloprovincialis* to copper, and found that most of the variation in literature data could be explained by differences in the water chemistry of the test solution.

For the purple sea urchin *Strongylocentrotus purpuratus* the 96-h Ni EC_{50} was 207 µg/L (Fig. 3; DOC of 0.88 mg of C/L). While this value is higher (i.e Ni is less toxic) than some other sea urchin species (e.g. *Diadema antillarum* and *Evechinus chloroticus* with EC_{50}values below 15 µg/L; Bielmyer et al., 2005; Blewett et al., 2016) this value is lower than that of previously published work on this species. Phillips et al. reported an *S. purpuratus* EC_{50} of 341 µg/L. However, a recent study reported significant physiological disturbances in developing embryos of this species at only 33–40 µg/L (Tellis et al., 2014b). Key differences in experimental conditions may explain the variation in these toxicity values. For example, in both of the previous studies, natural SW was used to test effects, whereas in the current study artificial SW was used. As demonstrated in our data, natural SWs are likely to decrease toxicity, owing to components such as DOC. It is also important to note that the study of Phillips et al. (2003), did not measure actual Ni exposure concentrations, which could also influence the accuracy of the calculated EC_{50}.

The present study showed that DOC is protective with respect to Ni toxicity, however the extent of the protection was source–dependent. Based on 95% confidence intervals the field collected samples are not different from each other, although our results indicate that samples 2,3,4,7 and 10 are significantly protective compared to our ASW *Mytilus* control (Fig. 3). However, in our sea urchin exposure, toxicity of Ni in DOC collected from site 2 (SVP) was significantly lower (i.e. higher EC_{50}) from that in waters collected from site 7 (CC1) (Fig. 3), in addition to being significantly different from the ASW control. This indicates that a component of the naturally-collected waters is protective, relative to the ASW control samples. One possibility is that natural SW provides a micronutrient component that is lacking in ASW. The presence of high survival and normal development in the ASW controls, suggests, however, that this is unlikely. Instead, the most likely explanation is that DOC in the natural samples is complexing Ni and decreasing its toxicity. DOCs are thought to be protective against metal toxicity due to the formation of metal complexes, rendering the metal less bioavailable to the organism. Therefore, the lower the concentration of DOC, the fewer binding sites for metals (Wood et al., 2011), with the caveat that to be protective the DOC must be of the appropriate (source-dependent) molecular nature to bind Ni. This is in accordance with our data, as the control (ASW) had the lowest concentration of DOC (0.88 mg/L; Supplemental Table 1) and also the lowest EC_{50} value for both *Mytlius* (Fig. 3A) and *Strongylocentrotus* (Fig. 3B). The correlation between absolute DOC concentration and Ni EC_{50} was significant for *Mytilus* (R^2 = 0.41, p = 0.04; Fig. 4A) and the relationship was reasonably strong for *Strongylocentrotus* (R^2 of 0.62, p = 0.21). Previous evidence has shown a correlation between concentration of DOC and protective effect against other metals. For example, Arnold et al. (2005) demonstrated that Cu toxicity was significantly inversely related to DOC concentration. A similar finding has been shown in other test systems for Ag (Glover et al., 2005), and Cu (Nadella et al., 2009).

However, DOC concentration is not the only important factor potentially influencing Ni toxicity. The composition of DOC varies both temporally and spatially in marine environments (Blewett et al., 2016; Wood et al., 2011). Changes in composition influence the relative concentrations of potential metal binding groups, which will in turn influence toxicity. Evidence of DOC composition variability in the current study was provided by the SAC340 values. Notably, absorbance at 340 nm displayed significant strong correlations with protection against Ni toxicity for both species (*Mytilus* R^2 = 0.52, p = 0.02) and (*Strongylocentrotus,* R^2 = 0.97, p = 0.01) (Fig. 5). As simple indicators of the ability to alter Ni toxicity in marine invertebrate early life-stages, absorbance at 340 nm proved useful, and inclusion of these optical parameters did improve correlations compared to DOC concentration alone. Our data are of particular interest in that they show a relationship between DOC concentration and Ni toxicity. As previously explained, the WHAM model predicts only a weak DOC-Ni binding relationship in full strength SW (Stockdale et al., 2015), but the experimental data presented here indicates that the binding is sufficiently strong to exhibit altered toxicity.

Parallel factor (PARAFAC) analysis of excitation-emission fluorescence spectroscopy data (Fig. 2) can potentially be used for greater molecular discrimination of DOC composition, and represents a more integrated measure than simple colour-based indices such as SAC340. PARAFAC is a multivariate statistical technique that resolves underlying moieties identified by their corresponding emission wavelength pair and relative concentration. From this analysis we can identify relative humic acid-like, fulvic acid-like and proteinaceous components of the DOC samples (DePalma et al., 2011a). Using PARAFAC, the relative concentrations of humic- and fulvic-like acids for each DOC were plotted against EC_{50} concentrations. This analysis revealed that humic and fulvic acid concentrations of the DOCs correlated more strongly with protection than DOC concentration alone and simple indices (HA -*Mytilus*, R^2 = 0.50, p = 0.03, FA-R^2 = 0.60, p = 0.01;

Strongylocentrotus HA-R^2 = 0.55, p = 0.03; FA-R^2 = 0.70, p = 0.16 respectively, Fig. 7A,B). Our results are in agreement with other measured SW DOC input sources from the east coast. Previous PARAFAC characterization of samples from the estuarine and marine DOC's from the coast of North America displayed similar tyrosine and tryptophan DOC values (DePalma et al., 2011a, 2011b). However, our humic and fulvic acid values were much higher than previously collected DOC's. This result was not surprising as previous studies have shown that DOCs of allochthonous origin, which have higher levels of these components, tend to be more protective against metal toxicity (Nadella et al., 2009; Wood et al., 2011; Blewett et al., 2016).

Humic acids themselves are complex mixtures, containing carboxylic and phenolic groups. It is these groups, that are negatively charged in natural waters, which are the sites of metal binding. Fulvic acids are considered to be less protective, because they are of lower molecular weight and have fewer potential binding groups (Hayes, 1998). This, however, is the first study to demonstrate protective effects of humic and fulvic acids against Ni toxicity by correlating these moieties versus protection over a wide range of DOCs of marine origin. Previous studies have utilized FW DOC samples salted up to SW salinities, and not true marine samples (Blewett et al., 2016).

Recent research has suggested that certain amino acid-like ligands in DOC may also have important modifying impacts on Ni toxicity amelioration. Observations by Cooper and colleagues (Cooper, Nasir, Mori, McGeer and Smith, unpublished) showed that elevated tyrosine concentrations in field-collected DOC samples contributed towards a protective effect against Ni toxicity in saline waters (adult Mysid shrimp - LC_{50} for mortality value of humic-rich DOC 194 μg/L *vs*. Tyrosine-rich DOC 443 μg/L). In the current study, a protective effect of amino acids was not as great as that for humic and fulvic acid-like fluorophores. Tyrosine- and tryptophan-like concentrations in our field-collected samples did not correlate significantly with EC_{50} values for Ni (*Mytilus*: tryptophan R^2 = 0.31, p = 0.09; tyrosine R^2 = 0.30, p = 0.13; *Strongylocentrotus*: tryptophan R^2 = 0.55, p = 0.25; tyrosine R^2 = 0.56, p = 0.25; Fig. 6). However, the discrepancy between these two studies is likely due to total concentrations of tyrosine, location and time of collection. In the study of Cooper and colleagues one of the DOC field collected samples was 50% tyrosine by concentration, whereas in all of our samples less than 1% of the fluorescence of DOC was made up of tyrosine. The reason for such significant differences between the amino acid contents of DOCs in naturally-collected samples is unknown, but it could relate to the source location and/or the time between collection and testing.

There is some debate about the protectivity of DOCs against metal toxicity, and how this relates to DOC composition. For example, as previously stated, the WHAM prediction suggests that for Ni in the marine environment, there is only weak binding, indicating poor protective effects of DOC against Ni toxicity (Stockdale et al., 2015). Studies using FW DOCs suggest that for Cu in fathead minnows (Ryan et al., 2004) and rainbow trout (Schwartz et al., 2004), humic acid-like fluorophores offer more protection than fulvic-acid like fluorophores. Furthermore, in some cases fulvic-acid like fluorophores are associated with greater toxicity, at least for Ag (Van Genderen et al., 2003). Currently the BLM allows the user to change the percentage of humic and fulvic acid, but in application the predictions from the BLM do not follow the pattern displayed by our measured values (De Schamphelaere et al., 2004). Indeed, Al-Reasi et al. (2012) were able to improve BLM predictions with *D. magna* by taking into account DOM quality. Specifically, the authors used measured SAC340 to adjust the % humic acid in the standard BLM model. Ultimately, understanding of protective quality via composition and concentration in laboratory studies will strengthen the BLM and help reduce variability.

5. Conclusions

The relationship between marine Ni toxicity and DOC is complex. Our study is the first to examine the composition of DOCs in an effort to identify what particular constituent of DOC is truly protective against Ni toxicity to early life-stages of marine invertebrates. This study indicates that real world samples are protective against Ni toxicity, thus modelling approaches, such as WHAM, may not adequately predict the complex relationships between DOC and organism health. Furthermore, this has implications for a marine BLM, in terms of what variables are considered for DOC quality and composition. This study shows that concentration alone is unable to fully explain protective effects of DOC against Ni toxicity to developing marine invertebrates. Future work building on reproducing results of the current study and expanding the range of natural DOC's could be adequate in order to generate a more refined understanding of the relationship between composition and protection. Other valuable future studies should include chronic timelines.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.05.029.

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