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Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossolus*) and the protective effect of dissolved organic carbon

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ABSTRACT

Marine water quality criteria for metals are largely driven by the extremely sensitive embryo–larval toxicity of *Mytilus* sp. Here we assess the toxicity of four dissolved metals (Cu, Zn, Ni, Cd) in the mussel *Mytilus trossolus,* at various salinity levels while also examining the modifying effects of dissolved organic carbon (DOC) on metal toxicity. In 48 h embryo development tests in natural seawater, measured EC50 values were 6.9–9.6 μg L^{-1} (95% C.I.=5.5–10.8 μg L^{-1}) for Cu, 99 μg L^{-1} (86–101) for Zn, 150 μg L^{-1} (73–156) for Ni, and 502 μg L^{-1} (364–847) for Cd. A salinity threshold of >20 ppt (~60% full strength seawater) was required for normal control development. Salinity in the 60–100% range did not alter Cu toxicity. Experimental addition of dissolved organic carbon (DOC) from three sources reduced Cu toxicity; for example the EC50 of embryos developing in seawater with 20 mg C L^{-1} was 39 μg Cu L^{-1} (35.2–47.2) a 4-fold increase in Cu EC50. The protective effects of DOC were influenced by their distinct physicochemical properties. Protection appears to be related to higher fulvic acid and lower humic acid content as operationally defined by fluorescence spectroscopy. The fact that DOC from freshwater sources provides protection against Cu toxicity in seawater suggests that extrapolation from freshwater toxicity testing may be possible for saltwater criteria development, including development of a saltwater Biotic Ligand Model for prediction of Cu toxicity.
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1. Introduction

Trace metals are natural components of the biosphere. Although some metals are essential for life (Engel and Sunda, 1979), all metals are toxic at sufficiently high concentrations; for some there is a narrow range of concentrations between what is essential and what is toxic. Elevated metal concentrations can cause a severe reduction or elimination of intolerant species, thereby having a significant effect on the diversity and trophic structure of the biological community (Peterson, 1986). However, the disruption of such systems is not determined merely by the concentration of the metal. A number of environmental and biological processes may influence the bioavailability of metals to organisms (Luoma, 1983). To date, most research has focused on the aspects of environmental water chemistry that modify the bioavailability of dissolved metals to freshwater species. This body of research has led to the development of the Biotic Ligand Model (BLM) which takes into account the protective effects of different components of water chemistry, either through competition with the metal or complexation of the metal. The BLM has proven to be effective in the prediction of toxicity for a variety of dissolved metals in freshwaters of diverse chemical composition making it a valuable resource for assessing environmental effects of metals in freshwater ecosystems (Di Toro et al., 2001; Paquin et al., 2002; Niyogi and Wood 2004).

Increased concerns regarding the use of oceans as a site for the disposal of anthropogenic wastes and the large scale use of copper and other metals in antifouling boat paint have prompted evaluation of metal toxicity to marine species (Apte and Day, 1998). Today, there is a considerable body of research outlining the toxicity of various metals to marine species. However, available data often exhibit wide variation in sensitivity. For instance mussel larvae of the genus *Mytilus* are widely accepted to represent one of the most sensitive marine organisms to Cu (e.g. Grosell et al., 2007) and therefore play a critical role in environmental regulations. Yet, the US EPA's (2003) ambient water quality criteria document reports mean acute values (EC50s in 48 h developmental tests) of 21.4 μg L^{-1} dissolved Cu (at 20 ppt salinity) for *Mytilus edulis* larvae but 6.1 μg L^{-1} dissolved Cu (at 28–30 ppt) salinity for *Mytilus* spp. respectively. This variation could reflect either the importance of water chemistry in influencing the

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Table 1
Measured water chemistry parameters for representative test solutions

	SW	60% SW	10 μg Cu L^{-1} 100% SW	100 μg Zn L^{-1} 100% SW	150 μg Ni L^{-1} 100% SW	500 μg Cd L^{-1} 100% SW	10 mg C L^{-1} NR 100% SW	10 mg C L^{-1} SW 100% SW	10 mg C L^{-1} LM 100% SW
pH	7.96	7.50	7.88	7.85	7.88	7.88	7.96	7.92	7.89
Na (mM)	492±13.1	282±7.5	497±12.5	503±8.7	511±11.4	507±12.7	499.5±20	502.5±22.3	497±20.5
K (mM)	9.1±1.5	5.3±0.5	9.0±1.1	9.8±0.1	9.5±0.5	9.5±1.2	9.1±0.7	9.0±1.3	9.1±0.8
Ca (mM)	11.6±0.3	6.6±2.5	11.2±0.1	11.5±0.1	11.5±1.2	11.3±0.9	11.5±1.1	11.8±2.1	11.8±1.0
Mg (mM)	50±0.8	32±9.1	48.8±1.7	56.3±1.5	53.7±3.4	56.4±2.5	50.1±4.1	52.0±3.1	49.4±1.1
Cl (mM)	539±10.1	343±22.5	539±18.5	545±10.5	544±32.5	544±22.2	534±17.1	533±15.7	539±20.1

(means±SEM).
Notes: 1. SW=seawater; NR=Nordic Reservoir NOM; SR=Suwanee River NOM; LM=Luther Marsh NO 2. All representative test solutions denoted by nominal values.

bioavailability of the metal, differences in physiological sensitivity of the organisms at different salinities, or true inter-species differences.

More knowledge about water chemistry effects could lead to the development of saltwater criteria adjusted for water chemistry, similar to the Biotic Ligand Model approach that has been used in freshwater. Collecting toxicity data from diverse sources, Arnold et al. (2005, 2006) have recently presented evidence that the toxicity of dissolved copper to *Mytilus* spp. is a function of the dissolved organic carbon (DOC) concentration of the test sample. However this analysis was based on correlations using natural variations in DOC concentration rather than experimental manipulation of DOC concentrations (Arnold, 2005; Arnold et al., 2006). Previous experimental studies in freshwater (Schwartz et al., 2004; De Schamphelaere et al., 2004; Glover et al., 2005) have shown that DOC from different sources can offer differential protection against toxicity of dissolved metals based on the heterogeneity of physicochemical properties of the DOC sample.

With this background in mind, we chose *Mytilus trossolus* to assess the toxicity of dissolved metals as it is a member of the most sensitive genus in the US EPA's database for saltwater copper quality criteria (WQC), but is a species for which copper toxicity has not been characterized previously. Current criteria are based on toxicity tests with *M. galloprovincialis* and *M. edulis* (e.g. US. EPA, 2003).We employed the standard 48 h embryo–larval development test (NIWA, 2005) to measure embryo survivorship after exposure to a toxicant. Very recently, Fitzpatrick et al. (2008) used *M. trossolus*, in this test and showed that the embryo–larval life-stage is 10 to 100-fold more sensitive than either sperm or eggs alone.

The main objectives of the study were (i) to quantify the toxicities of four dissolved metals, Cu, Zn, Ni and Cd to *M. trossolus* larvae; (ii) based on results of objective (i), to experimentally evaluate the effect of two potential modifying factors of water chemistry (salinity and DOC) on the toxicity of the most toxic one, which turned out to be Cu; and (iii) to determine whether DOC collected from natural sources will provide heterogeneous protection against the toxicity of dissolved Cu. The metals tested were selected because of their common occurrence in municipal and industrial effluents as well as their known toxicity at elevated concentrations to marine organisms.

2. Materials and methods

2.1. Collection and maintenance of adult mussels

Adult *M. trossolus* were collected from natural intertidal populations in the Broken Island Group, near Bamfield, B.C. (Canada) in June–July of 2006 and 2007. Heath et al. (1996) used PCR markers to investigate interaction between sympatric *Mytilus* species and reported that *M. trossolus* is the only bay mussel species found on the west coast of Vancouver Island. More recently, Arkester and Martel (2000) confirmed the bay mussel species in the Barkley Sound Inlet (the location of our study site on Vancouver Island) was indeed *M. trossolus*, based on shell morphology.

In the laboratory, animals were cleaned and transferred to aerated flowing seawater baths maintained at 11–13 °C and allowed to acclimate for 24 h. Representative seawater chemistry is given in Table 1.

2.2. Test solutions

Metals were tested for toxicity using the following inorganic salts (analytical grade): $CuCl_2\ 2H_2O$, $NiCl_2\ 6H_2O$, $CdCl_2\ 5H_2O$ (all Sigma) and $ZnSO_4$ (Anachemia). Stock solutions of these metals were made in deionized water and stored in airtight polyethylene containers. Metal stock solutions were serially diluted with filtered seawater (0.20 μm) to achieve required concentrations in the test vials prior to the day of testing and allowed to equilibrate overnight.

Similarly stock solutions of DOC from three sources—Luther Marsh (LM), Ontario, Canada, obtained by reverse osmosis (for details see Schwartz et al., 2004), Nordic Reservoir (NR), and Suwannee River (SR), the latter two purchased as freeze-dried powders from the International Humic Substances Society (St. Paul, MN. USA) were reconstituted in filtered seawater. DOC derived from marine waters is

Table 2
Nominal versus measured concentrations (means±SEM) for Cu, Zn, Ni and Cd in test solutions used to determine metal toxicity in developing embryos of *M. trossolus*

Copper		Zinc		Nickel		Cadmium	
Nominal μg L^{-1}	Measured μg L^{-1}	Nominal μg L^{-1}	Measured μg L^{-1}	Nominal μg L^{-1}	Measured μg L^{-1}	Nominal μg L^{-1}	Measured μg L^{-1}
0	1.5±0.1	0	5±1	0	BDL*	0	BDL*
0.32	1.1±0.1	50	65±3	50	25±4	50	60±7
1	2.3±0.3	100	108±10	100	40±6	100	115±4
3.2	4.6±0.6	125	153±7	150	75±19	150	250±12
10	11.6±0.2	150	204±5	200	120±19	200	300±15
32	27.4±0.1	200	300±12	500	415±25	500	800±9
100	71.0±6.9	300	576±10	1000	760±16	1000	1200±19

*BDL=Below Detection Limit.

Table 3
Nominal versus measured concentrations (means±SEM) of Cu and of DOC in test solutions used to measure Cu toxicity in the presence of various levels of DOC added to the test solutions

	Nordic Reservoir (mg C L^{-1})				Suwannee River (mg C L^{-1})				Luther Marsh (mg C L^{-1})			
Nominal DOC	2.5	5	10	20	2.5	5	10	20	2.5	5	10	20
Measured DOC	5.5±0.61	8.3±0.34	11.9±0.18	20.3±0.09	5.8±0.72	8.9±0.43	12.9±0.16	21.6±0.11	6.1±0.52	8.6±0.45	12.6±0.27	23±0.07
Nominal Cu µg L^{-1}	Measured Cu µg L^{-1}											
3.2	3.6±0.55	3.3±0.1			4±0.32	4.2±0.46			4.2±0.63	4.4±0.77		
10	11.8±0.73	12.2±1.04	9.9±0.11	14.1±0.63	9±0.25	9.4±0.77	12.5±0.08	8.6±0.53	8.9±0.09	11.4±1.3	8.7±0.32	9.8±0.01
20	15.7±0.1	18.0±1.6	15.9±0.9	17.0±2.1	17.1±1.8	18.0±0.8	19.9±1.1	18.1±2.5	14.9±0.3	15.8±0.7	23.8±1.6	22.8±0.9
32	22.1±0.1	22.0±2.4	24.6±1.9	22.2±2.1	25.4±0.3	26.4±1.2	24.1±1.0	25.0±1.5	23.9±2.7	23.9±1.2	34.2±0.8	34.0±1.4
100	52.8±7.2	60.0±11.5	56.0±9.0	50.4±5.4	76.0±6.1	72.0±8.1	60.0±12.5	48.0±3.4	60.0±10.6	63.0±2.5	60.0±8.8	72.0±7.9

Note that background DOC (in the absence of any additions) in these tests was 4.0 mg C L^{-1}.
Note: Blank cells were not tested.

not available commercially, and is difficult to collect because of the high salt content of seawater. Taking into account these practical and logistic problems involved in sourcing organic matter from marine waters, DOC derived from freshwater sources were used in the study as they are readily available commercially, and relatively easy to collect by reverse osmosis techniques. From each of these stock solutions, four nominal concentrations (2.5, 5, 10 and 20 mg L^{-1}) of DOC were added to a range of Cu solutions (final dilutions) prior to the day of testing and allowed to equilibrate overnight. This range was chosen to cover the range of DOC levels reported from site-specific samples (1.12–17 mg C L^{-1}) (Arnold and Warren-Hicks, 2007).

The salinity of the test solution was modified according to treatment by adding the required amount of deionized water to full strength seawater.

2.3. Toxicity tests

Tests were conducted in 15 mL polyethylene vials and 5 replicates were used per treatment. Each treatment vial contained 10 mL of the test solution. Salinity, pH (see Table 1) and dissolved O_2 were measured in each treatment prior to the tests and at termination. Dissolved O_2 was measured consistently as >5 mg L^{-1} in all test samples. In samples spiked with concentrated stock solutions of the metal and DOC, pH was adjusted back to the level in control seawater using dilute NaOH or HCl.

2.4. 48 h embryo test

Embryo development was assessed using a standard protocol (NIWA, 2005). Briefly, adult *M. trossolus* were transferred to a 10 L filtered (0.20 µm) seawater bath (15–20 adults/bath) maintained at 22–25 °C. The thermal shock induced spawning, and individuals releasing gametes were immediately moved to separate 250 mL beakers containing 200 mL of filtered seawater, for isolation and collection of gametes. Egg quality and sperm motility were assessed using a microscope at 200× magnification. Only round, non-transparent eggs and motile sperm were used for further testing. Subsequently, eggs from several individual females were pooled and homogenized by gentle stirring. An aliquot of sperm solution pooled from several male individuals was added to the eggs to initiate fertilization. A subsample of this mixture was periodically observed under the microscope until 80% or more of the eggs were fertilized. The test was initiated by adding 100 µL of fertilized embryos (approximately 600–1000 individuals) to each test vial containing 10 mL of test solution. Test vials were incubated in a biological incubator maintained at a constant temperature of 20 °C±1 and a photoperiod of 16-h light: 8-h dark. After 48 h, control test vials were examined to ensure more than 80% embryos developed into normal D-shaped prodissoconch larvae. Tests in which control development was less than 80% normal were discarded. The test was then terminated with the addition of 1 mL buffered formalin to each test vial to arrest development. Contents of each test vial were observed microscopically on a Sedgewick–Rafter slide to determine the percentage of embryos exhibiting normal development. At least 100 embryos in each replicate were assessed.

2.5. Analytical chemistry

As per US EPA (2001) recommendations water chemistry parameters critical to the toxicity tests were measured in treatments.

Dissolved Cu, Cd, Ni, and Zn concentrations were measured after passing samples through a 0.45 um filter, using a method modified from Toyota et al. (1982). Briefly, the representative metal was precipitated from 1 mL of sample by adding 1 µL of lanthanum oxide (10 mg La mL^{-1}) and 7.5 µL of 1 M Na_2CO_3, which brought the pH of the sample to approximately 9.8. The solution was gently stirred in a hot water bath maintained at 80 °C for 30 min to allow flocculation of precipitate (largely lanthanum hydroxide). The solution was centrifuged at about 3000 *g* for 15 min and the supernatant discarded. The remaining precipitate was dissolved in 1 mL of 1 N HNO_3 and metal concentration measured via graphite furnace atomic absorption spectroscopy for Cu, Cd, and Ni (220, Varian, Palo Alto, CA. USA).) or via flame atomic absorption spectroscopy for Zn (220FS; Varian). Fisher Scientific calibration standards were used before, during, and after every run. The detection limit was 0.2 µg L^{-1} for Cu, 0.1 µg L^{-1} for Cd, 3 µg $L^{-1}$for Ni, and 4.5 µg L^{-1} for Zn. Recovery was always ±10% as

Table 4
48 h EC 50 and EC20 values for abnormal development in embryos of *M. trossolus* for each of the metals tested and for Cu in the presence of potential modifying factors (altered salinity and DOC)

Test	EC50 µg L^{-1}	95% Confidence Interval	EC20 µg L^{-1}	95% Confidence Interval
Copper	9.6	7.8–10.8	6.6	5.2–9.2
Zinc	99	86–101	63.5	30.1–81.1
Nickel	150	73–156	88.2	57.3–112
Cadmium	502	364–847	239	108–332
Cu+100% seawater	6.9	5.5–8.2	2.7	0.8–4.1
Cu+70% seawater	7.1	5.6–8.5	2.9	1–4.5
Cu+60% seawater	7.1	4.9–8.6	2.9	0.9–4.3
Cu+0 mg L^{-1} DOC	9.4	7.7–10.5	6.4	5.1–8.2
Cu+2.5 mg C L^{-1} NR DOC	25.0	16.2–27.9	15.2	5.2–19.8
Cu+5 mg C L^{-1} NR DOC	28.0	26.3–31.5	21.8	14.4–25.1
Cu+10 mg C L^{-1} NR DOC	37.0	33.4–45.9	27.7	20.1–30.1
Cu+20 mg C L^{-1} NR DOC	39.0	35.2–47.2	27.5	15.6–31.3
Cu+2.5 mg C L^{-1} SR DOC	14.4	13.5–19.3	8.7	5.6–11.2
Cu+5 mg C L^{-1} SR DOC	20.2	18.1–22.9	12	5.5–15.5
Cu+10 mg C L^{-1} SR DOC	24.0	22.7–26.5	21.1	18.1–22.9
Cu+20 mg C L^{-1} SR DOC	3.1	0.37– 5.6	1.25	
Cu+2.5 mg C L^{-1} LM DOC	12.0	11.7–14.4	6.9	2.3–9.5
Cu+5 mg C L^{-1} LM DOC	16.1	12.7–23.5	10.3	5.2–13.3
Cu+10 mg C L^{-1} LM DOC	1.8	0.1–4.3	0.4	
Cu+20 mg C L^{-1} LM DOC	0.87	0.0–3.4	0.3	

*NR = Nordic Reservoir; *SR = Suwannee River * LM = Luther Marsh.

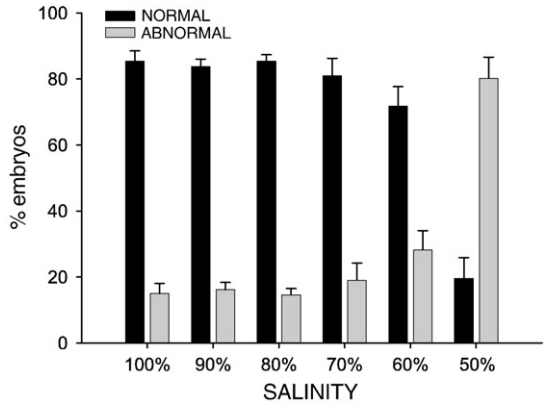

Fig. 1. The influence of salinity on percent normal versus abnormal development in embryos of *M. trossolus*, in the absence of added metals. Values are means±SEM of 5 replicates.

determined from similarly processed Analytical Reference material TM15 (Environment Canada, Natural Water Research Institute). Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations in seawater samples were determined by atomic absorption spectroscopy (Varian SpectrAA-1275FS) and Cl^- by coulometric titration on a chloridometer (CMT 10 Chloride Titrator; Radiometer, Copenhagen, Denmark; Cl^-). Reference standards were used for the measurement of all ions studied [Fisher Scientific and Radiometer].

DOC in the samples was analysed after passing the sample through a 0.45 μm filter. DOC was measured indirectly by subtracting measured Inorganic Carbon (IC) from measured total carbon (TC) using a Shimadzu TOC analyzer (5050A, Mandel Scientific, Canada). IC and TC Standards were prepared according to Shimadzu specifications.

Fluorescence excitation–emission matrix spectroscopy of DOC dissolved in seawater, was performed using a Varian Cary fluorescence spectrophotometer with 1 cm pathlength quartz cuvettes (Helma Canada Ltd. Concord, ON, Canada). Component peaks from the excitation–emission were also identified and quantified using parallel factor analysis (PARAFAC) as implemented in the PLS toolbox (Eigenvectors Research Inc, WA, USA). Application of PARAFAC to spectral resolution of total luminescence spectroscopy has been reported previously (Stedman and Markager, 2005). In brief, the technique involves consideration of an array or "stack" of matrices. Each matrix element contains the fluorescence intensity data for the corresponding excitation/emission wavelength pair. This complete data set is then resolved into component spectra and component concentrations. The resolved spectra have the same dimension as the original fluorescence surface and the resolved concentrations are not true concentrations but in fact numbers linearly proportional to the true component concentration. To convert these apparent concentrations to actual concentrations the fluorescence quantum efficiency (linear proportionality constant) would be required. Thus, the concentrations reported here can only be used for internal comparisons to determine relative trends within this data set. The number of fluorescent components is selected as the minimum number that described the data statistically well and resulted in spectroscopically reasonable components. A common "operational definition" of large molecular weight organic matter is separation into humic and fulvic acid (Wu et al., 2007). The tendency for larger molecular weight (humic) substances is to fluoresce at emission

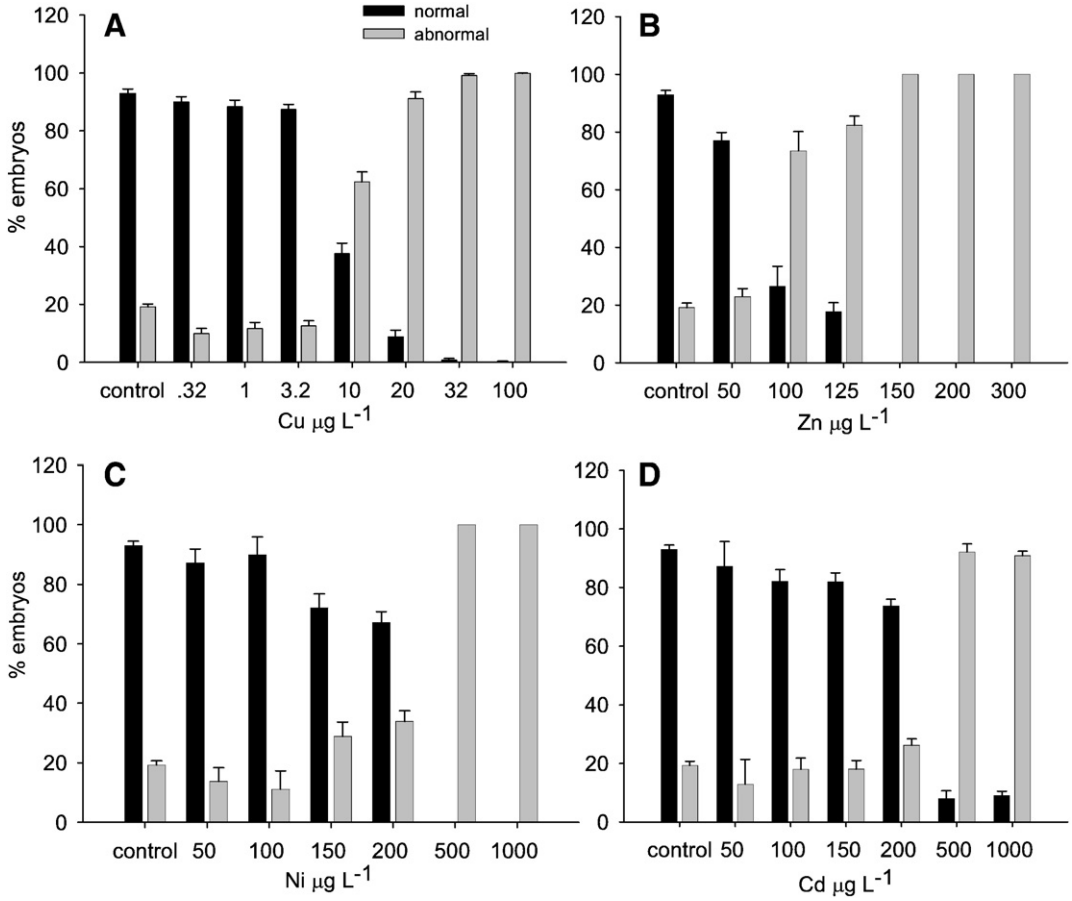

Fig. 2. Toxic responses (% abnormal development) of *M. trossolus* embryos exposed to various concentrations of metals in full strength seawater during 48 h development tests. A. Copper (Cu) B. Zinc (Zn) C. Nickel (Ni) D. Cadmium (Cd). Values are means±SEM of 5 replicates. Nominal metal concentrations are listed. Measured metal concentrations are tabulated in Table 2 and calculated EC50s and EC20s from the data in Table 4.

wavelengths >470 nm (nanometers). Thus, when two components were determined to satisfactorily describe the data (98% of variability explained), the longer wavelength component (>470 nm) was assigned the label humic and the shorter wavelength component (<470 nm) labeled as fulvic. These are operational definitions of humic and fulvic acid concentration based on fluorescence spectroscopy.

2.6. Statistical analysis

An Environmental Toxicity Data Analysis Software Tox Calc™ package(Tidepool Scientific Software) was used to estimate EC50 and EC20 with 95% confidence intervals (CI),[employing the responses and measured toxicant concentration data from all concentrations]. Responses were considered significantly different when their 95% CI did not overlap (Environment Canada, 2005). EC20 values were calculated because there is currently some conjecture that the 48 h embryo–larval development test could be used as a chronic test (see Discussion) and EC20 values can be used as chronic threshold concentrations providing a level of protection similar to the geometric mean of the LOEC and NOEC (US. EPA, 2007).

3. Results

Representative water chemistry data are summarized in Table 1. Measured dissolved metal concentrations for each nominal value are presented in Tables 2 and 3, and Table 3 also reports measured versus nominal DOC concentrations. For simplicity, nominal values have been used to illustrate Figs. 2, 3, and 4. Dissolved metal EC50 and EC20 values with 95% C.I. (calculated from measured concentrations) are listed in Table 4.

3.1. Salinity

Exposure to different salinities influenced embryo development (Fig. 1). Normal development of embryos was reduced (<80% normal) at salinity levels lower than 20 ppt or 65% of full strength seawater.

3.2. Metal toxicity

The responses of *M. trossolus* embryos to the four metals tested ranged over 3 orders of magnitude with toxicity values in the following order: Cu>Zn>Ni>Cd (Fig. 2). Copper was the most toxic with a 48 h EC50 value of 9.6 μg L^{-1} (7.8–10.8) for dissolved Cu. Zinc, Ni and Cd were less toxic at dissolved 48 h EC50s of 99 μg L^{-1} (86–101), 150 μg L^{-1} (73–156) and 502 (364–847) μg L^{-1} respectively. EC20 values representing possible chronic thresholds were 6.6 μg L^{-1} (5.2–9.2) for Cu and 63.5 (30.1–81.1), 88.2 (57.3–112) and 239 μg L^{-1} (108–332) for Zn, Ni and Cd.

3.3. Factors moderating Cu toxicity

These tests were limited to Cu as it proved to be the most toxic of the metals tested.

3.3.1. Salinity

There was no differential response in embryos exposed to a range of Cu concentrations at three different salinities (Fig. 3). Dissolved 48 h EC50 values for Cu were measured at 6.9, 7.1 and 7.1 μg L^{-1} for 100, 70, 60% seawater respectively. The slightly lower EC50 values in this series likely reflect the lower background DOC concentrations (1 mg C L^{-1}) in 2006 versus 2007 (4 mg C L^{-1}) (see Discussion).

3.3.2. Dissolved Organic Carbon (DOC)

In this series, performed in 2007, the background DOC concentration in control seawater samples was 4.0 mg C L^{-1} with a EC50 for dissolved Cu of 9.4 μg L^{-1}. The addition of DOC to test waters decreased

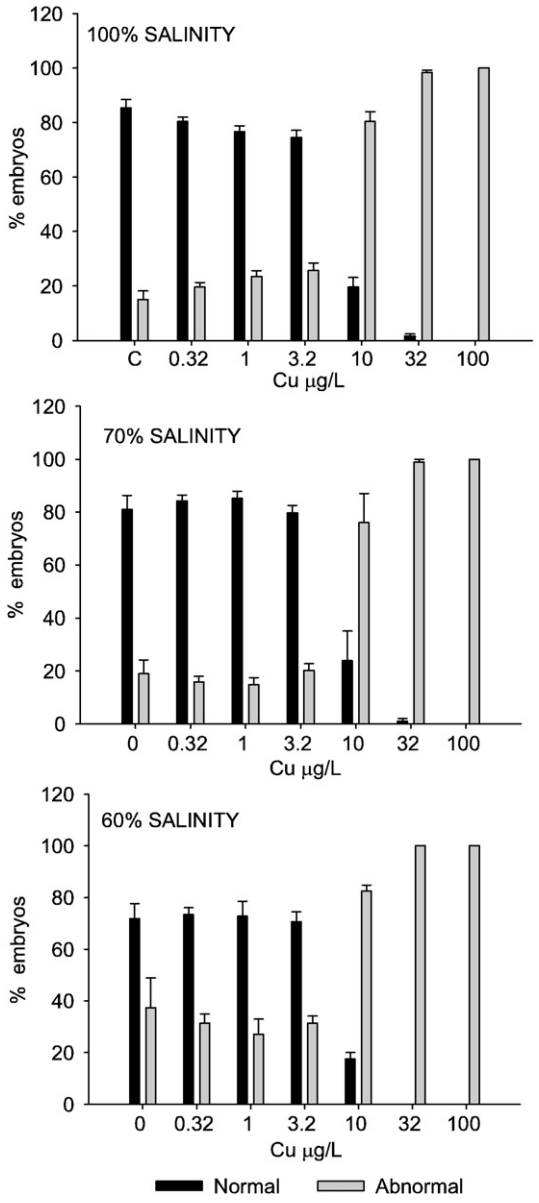

Fig. 3. The influence of salinity on toxic responses (% abnormal development) of *M. trossolus* embryos exposed to various concentrations of copper during 48 h development tests. Values are means±SEM of 5 replicates. Nominal Cu concentrations are listed. Measured Cu concentrations are tabulated in Table 2 and calculated EC50s and EC20s from the data in Table 4.

the toxicity of Cu in a concentration-dependent manner (Fig. 4). This effect was especially apparent for natural organic matter (NOM) from the Nordic Reservoir source which offered considerably greater protection with EC50 values for dissolved Cu ranging between 25 and 40 μg L^{-1} for 2.5–20 mg C L^{-1} added DOC (measured totals=4.5–20.4 mg C L^{-1}; Table 3), up from a control EC 50 value of 9.4 μg L^{-1}. NOM derived from SR and LM offered comparatively less protection. The protective effect of these two DOCs was prominent only at the lower added levels, up to measured totals of 8.9 mg C L^{-1} for SR, and up to 8.6 mg C. L^{-1} for LM, where the 48 h EC50 values ranged between 12 and 20 μg L^{-1}. Surprisingly NOM derived from the latter two sources appeared to be toxic to developing embryos at measured total levels >10–20 mg C L^{-1} (Fig. 4). A similar trend was also observed for the EC 20 data with measured values significantly higher than control for all exposures of NOM derived from NR but barely significant for SR and LM (see Table 3). In the range where the three sources showed

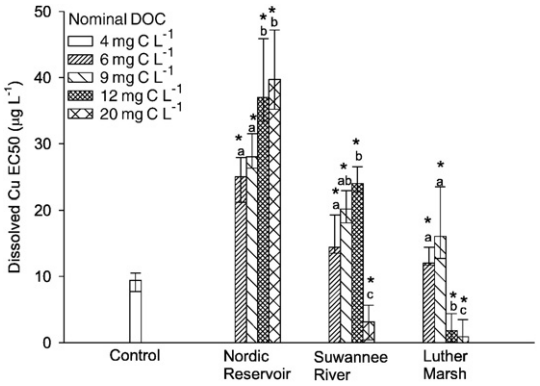

Fig. 4. The effect of DOC from three sources on Cu toxicity (EC50 values±95%C.I.) to embryos of *M. trossolus*. * denotes significant difference in EC50 from control, letters denote significant difference in EC50 within the treatment group. Note that protection changes to toxicity at high levels of SR and LM. DOC values represent nominal added concentrations. Actual measured DOC concentrations are listed in Table 3. Measured background DOC concentration was 4.0 mg C L^{-1}.

protective effects, NOM from Suwannee River and Luther Marsh were 40% and 60% less protective, respectively, when compared to NOM from the Nordic Reservoir, the rank order being NR>SR>LM.

Fluorescence excitation–emission matrix spectroscopy of these DOC samples from varied sources revealed subtle differences in their chemical composition. The overall fluorescence scans show very similar shape (Fig. 5A). In all three scans there are two peaks with emission around 450 nm and excitations at approximately 250 and 350 nm. The longer wavelength peak shows a shoulder in the region around 300 nm excitation and 400 nm emission. Differences can be quantified between these three samples by invoking PARAFAC component resolution. As discussed in the methods section, two components were deemed sufficient to satisfactorily describe all three fluorescence scans. These two spectroscopic components are shown in Fig. 5B. The top subplots (a, b) demonstrate the spectral shape and the bottom plots (c, d) show the relative concentrations. Based on wavelengths of fluorescence, shorter wavelength component 1 is labeled fulvic acid-like (a, c) and longer wavelength component two is labeled humic acid-like (b, d). Fluorescence is linearly proportional to concentration so even though the absolute concentrations are not known, relative comparisons between samples with the same component can be made. The low molecular weight fulvic acid-like component was highest in NOM from Nordic Reservoir, with 20% higher fluorescence (normalized to mg DOC) than for NOM from Suwannee River and almost 40% greater than NOM from Luther Marsh. Nordic Reservoir NOM also had the lowest concentration of the high molecular weight humic acid-like component. Suwanee River NOM had an elevated concentration of the fulvic-like component but a larger humic-like component. In comparison, the fulvic and humic-like components of Luther Marsh NOM were considerably lower.

4. Discussion

The present investigation records the toxicity of four dissolved metals (Cu, Zn, Ni, and Cd) commonly occurring in contaminated sites on the survival of the most sensitive life-stage of *M. trossolus*, a member of the genus which has not been previously studied in this regard, and shows that Cu is by far the most toxic. Our study demonstrates that salinity in the 60–100% seawater range does not affect Cu toxicity. The study also highlights the protective effects of DOC against the toxicity of dissolved Cu, thereby experimentally validating the correlational relationship between DOC and Cu toxicity described by Arnold et al. (2006). We also show that not all DOCs offer the same protective effects, with different NOM sources providing different degrees of protection. We argue for a closer examination of NOM properties, particularly with regard to their fulvic/humic acid contents, in the derivation of marine water quality criteria. Such information is critical to the development of a saltwater BLM.

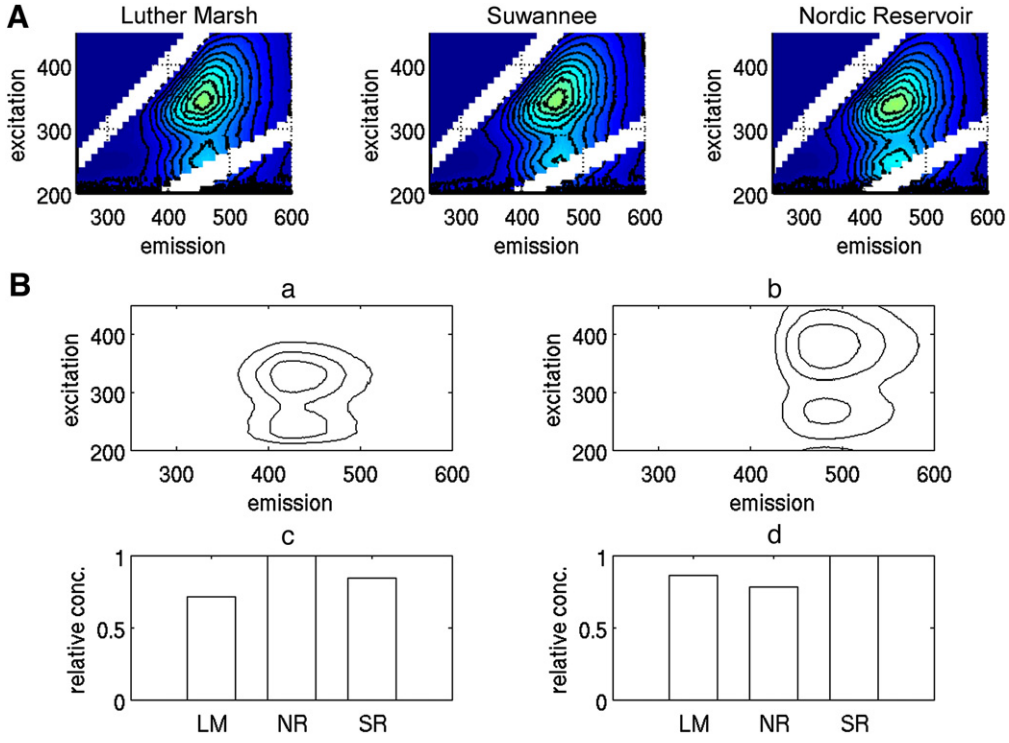

Fig. 5. A. Fluorescence excitation versus emission contour plots for each of the three sources of DOC dissolved in seawater. B. PARAFAC results from spectral resolution of the three measured scans from A. Subplot (a) and (b) are component spectra shape for fulvic-like and humic-like fluorphores and subplot (c) and (d) show the relative concentration of the same two components in each of the three samples.

The order of individual relative toxicities of the four dissolved metals in this study was Cu>Zn>Ni>Cd. These levels of relative toxicity compare well to a wide range of studies spanning the past four decades involving several species in similar early life-stage tests. Copper was also the most toxic dissolved metal tested to the embryos of the American oyster *Crassostrea virginica*, but dissolved Cd was less toxic than either dissolved Zn or Ni (Calabrese et al., 1973). For *M. edulis* embryos, Martin et al. (1981) reported a similar pattern demonstrating the following rank order of toxicity: Hg and Cu>Ag>Zn>Pb>Ni>Cd>Ar>Cr>Se. Assessing the influence of metals on the emergence and hatching of the brine shrimp *Artemia*, MacRae and Pandey (1991) found Cu and Pb to be about equally toxic at or below concentrations of 0.1 μM, Zn to be less toxic than Cu and Pb, and Ni to be least toxic. The EC50 values and the rank order of toxicity derived in the present study with *M. trossolus* embryos (Fig. 2, Table 4) also compare well to data available for the *M. edulis* 48 h embryo development test on the US EPA Acquire database (http://cfpub.epa.gov/ecotox/) with values of 5.8 μg L^{-1} for Cu, 175 μg L^{-1} for Zn, 891 μg L^{-1} for Ni and 1200 μg L^{-1} for Cd. However, Watling (1982) found a differential rank order for acute and sub-lethal effects in developing oyster larvae. Based on growth measurements, the order of toxicity was Cu>Zn>Cd; for 96 h LC50 values the order was Cu>Cd>Zn. Thus the results of this study should be further validated with chronic sub-lethal exposure to these metals in order to fully appreciate their environmental impact.

When the currently used saltwater copper criteria (U.S. EPA, 2003, 2007) were derived many years ago, the *Mytilus* embryo–larval development test was used by the US EPA as an acute test. It is currently being debated whether it can qualify as a critical life-stage estimate of chronic toxicity (Arnold, 2005) but the issue remains unresolved. However the EC50 values for dissolved Cu (6.9–9.6 μg L^{-1}), Zn (99 μg L^{-1}), Ni (150 μg L^{-1}) and Cd (502 μg L^{-1}) determined in this study (Table 4) are above the US EPA (2006, 2007) revision for Cu) recommended acute water quality criteria (3.1, 90, 74, 40 μg L^{-1}) for dissolved Cu, Zn, Ni and Cd respectively for the protection of aquatic life. Similarly, the EC20 values for dissolved Cu (6.6 μg L^{-1}), Zn (63.5 μg L^{-1}), Ni (88 μg L^{-1}) and Cd (239 μg L^{-1}) determined in this study (Table 4) are above the range recommended for chronic water quality criteria (1.9, 81, 8.2 and 8.8 μg L^{-1}) for these metals. These recommended levels indicate that while *M. trossolus* embryos are adequately protected against Ni and Cd, the protection against Cu and Zn will be marginal to these sensitive organisms under both acute and chronic conditions.

Salinity alone had an effect on normal embryo development in *M. trossolus*. Abnormal embryo development was observed at 20 ppt, or 60% seawater but not at 70% (Fig. 1), indicating that a salinity threshold of about 23 ppt exists for normal growth and development. In oyster embryos, Coglianese (1982) reported no significant effects on embryo development during exposure to salinities between 22.7–33 ppt but salinities below 22.7 ppt were highly deleterious. Bivalve molluscs are osmoconformers and can generally tolerate a wide range of salinities by regulation of body volume (Gilles, 1979). However, below a salinity threshold, the soft bodied larvae may lose the ability to volume regulate in response to hyposmotic shock. Responses such as suppression of protein synthesis at low salinities have been reported in the oyster (Tirard et al., 1996). Thus most standard operating procedures necessitate salinity levels of 32–34 ppt for standard embryo development tests.

There was no additive effect of salinity on dissolved Cu toxicity as indicated by comparable EC50 values obtained at each salinity, even at a low salinity level (60%, 20 ppt) where control development started to decline (Fig. 3). This result is encouraging as it supports the standard procedure used in mussel larvae testing where the salinity of natural water samples is first adjusted to 30 ppt before the test is conducted (e.g. Arnold et al., 2005). It also suggests that the differences in EC50 values of 21.4 μg dissolved Cu L^{-1} (at 20 ppt salinity) versus 6.1 μg dissolved Cu/L (at 28–30 ppt) in the US EPA (2003) data base (see Introduction) may not be caused by differences in salinity.

Nevertheless, the finding is surprising as there is evidence that Cu toxicity is influenced by changes in salinity (Grosell et al., 2007). This influence has been attributed to physiological factors and a change in the chemical form of Cu because of its affinity to form complexes with Cl and its ability to competitively interact with major cations for sensitive sites on the biological target (Mclusky et al., 1986; Grosell et al., 2007). Low salinity is known to increase Cu toxicity by decreasing the formation of Cu-chloro complexes and increasing therefore the concentration of Cu^{2+}. For example when oyster embryos were exposed to salinities ranging from 14.5–33 ppt and Cu concentrations between 0 and 10 μg L^{-1}, Coglianese (1982) found that low salinities when combined with relatively low Cu levels may represent a significant threat to the normal recruitment of oyster embryos. However other biological factors, including temperature and development stage can also interact with salinity to alter the magnitude of dissolved Cu toxicity. For example, MacInnes and Calabrese (1979) reported that the later oyster larval stage is less affected by the interactions of salinity and Cu concentrations than the early embryonic stage. More recently, Jose et al. (2005) showed that the effect of Cu was more pronounced in the rotifer *Brachonis rotundiformes* at 25 °C than at 20 °C at low salinities.

The toxicity of dissolved Cu to *M. trossolus* decreased as the concentrations of DOC from three different NOM sources was experimentally increased. This relationship is not without precedent and compares well (Fig. 6) to the correlation between these factors reported by Arnold et al. (2005, 2006). In a study which pooled dissolved Cu toxicity data from several field water samples with varying DOC levels, Arnold et al. (2006), based mainly on data for *M. edulis* and *M. galloprovincialis*, derived a relationship between dissolved Cu EC50 and DOC concentration which was quantified by the following equation

$$EC50 = 11.22\,DOC^{0.60} \quad (1)$$

where EC50 is in μg L^{-1} dissolved Cu, and DOC is in mg C L^{-1} (Fig. 6).

In comparison, log transformed data from the present study with *M. trossolus* describes this relationship as

$$EC50 = 5.27\,DOC^{0.68},\ r^2 = 0.70 \quad (2)$$

(Fig. 6).

Given this reasonably close relationship between the two data sources, data from *M. trossolus* larvae tested in the laboratory with

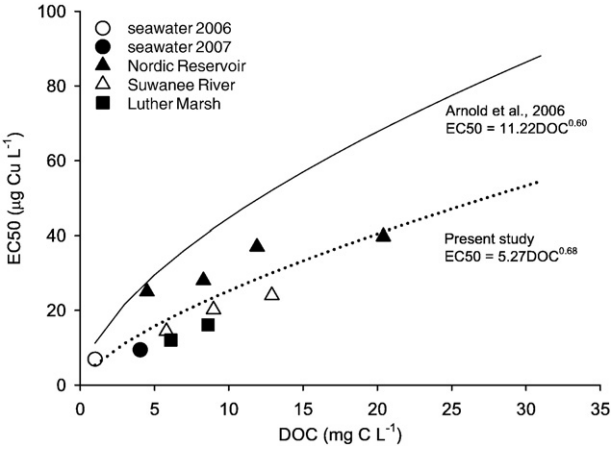

Fig. 6. Plot of dissolved Cu EC50s for embryos of *M. trossolus* as a function of DOC concentration. Solid line is plotted from the equation EC50 = 11.22 $DOC^{0.60}$ (Arnold et al., 2006). Dotted line represents data from the present study, with the equation EC50 = 5.27 $DOC^{0.68}$.

experimentally added DOC, and data from *M. edulis* and *M. galloprovincialis* tested in field-collected samples with natural DOC, the contention that ambient DOC concentrations can be used to adjust existing saltwater copper criteria on a site-specific basis (Arnold et al., 2006) is supported.

The weaker protective effect of DOC seen in the present relationship between DOC concentration and Cu EC50 (Fig. 6) could be attributed to the addition of DOC from different freshwater sources to the test samples. Arnold et al. (2006) used data derived from tests with natural seawater without additions of exogenous DOC. Several investigations have reported variations in metal complexing properties of different NOMs (De Schamphelaere et al., 2004; Schwartz et al., 2004; Glover et al., 2005). These studies concluded that biologically meaningful differences exist in NOM isolated from different sources, leading to differential binding abilities to the free metal ion. It is evident that the three sources of DOC used in the present study had distinct physicochemical properties, as indicated by the differential protection they provided against Cu toxicity (Fig. 4).

For protection against Cu toxicity in the marine environment, fulvic acids (FA) comprise the most relevant quantitative fraction of seawater DOC rather than humic acids (HA) according to Lorenzo et al. (2006). Approximately 50% of dissolved organic matter in natural waters is composed of HA (Guo and Santschi, 1994). Based on this observation we can reason that NOM derived from Nordic Reservoir with a higher FA content (operationally estimated by fluorescence) was more representative of the NOM from seawater sources used in the study by Arnold et al., (2006) and therefore more closely mirrored the protective effect observed in field-collected samples from the Arnold et al. (2005) study (see Fig. 4 for comparison). In contrast, the higher HA content of the NOM from Suwannee River and Luther Marsh probably caused a lower protective effect. Surprisingly, complete absence of protective effect was observed at concentrations higher than about 9 mg C L^{-1} for NOM from Luther Marsh and about 12 mg C L^{-1} for NOM from Suwannee River probably also due to their HA content. At these levels reduced survival of embryos was observed even in the absence of added Cu. Enhanced surfactant-like effects and humic acid induced membrane permeability have been reported in phytoplankton (Vigenault et al., 2000).This could lead to the passive uptake of low molecular weight lipophilic complexes which is further enhanced by the addition of species like Cu. Cu–HA complexes are reported to be partially available for uptake and therefore result in increased toxicity (Lorenzo et al., 2005). On the other hand Qiu et al. (2007) quantified the extent to which Cu–FA complexation affected Cu toxicity to the larvae of marine polychaete *Hydroides elegans* and found that FA was not toxic to the larvae even at the highest concentration tested (256 mg L^{-1}). They also reported that the Cu–FA complexation reduced labile Cu and was therefore protective against Cu toxicity. Similarly Lorenzo et al. (2006) did not observe any additional toxicity of Cu–FA complexes in sea urchin larvae.

In adults of the blue mussel *M. edulis*, Lorenzo et al. (2005) found that although the Cu–HA complexes were unavailable for direct uptake across the gills, other routes of uptake for this complex did exist at the whole-organism level, as the mussels accumulated more Cu in the presence of HA as the complexing medium. Similar results showing that HA does not have a protective effect against metal uptake have been reported for Cu (Lores et al., 1999), Cd (Pempkowiak et al., 1989) and Pb (Sanchez-Marin et al., 2007). These studies suggest that HA may facilitate the transfer of metal ions to the carrier proteins in the cell membrane, either through ingestion by the mussel or via pinocytosis. Cu–HA complexes may not be truly dissolved species but, rather, suspensions liable to forming colloidal particles with aggregation properties, and therefore potentially susceptible to pinocytosis (Lorenzo et al., 2005). It has also been suggested that some organisms may be endowed with active biological processes that counteract the sequestration of Cu by HA (Lores and Pennock, 1999). Lores and Pennock (1999) reported reduced Cu uptake, improved survival and reproduction in copepods in the presence of HA. In sea urchin larvae, Lorenzo et al. (2002) showed a clear protective effect of HA, reducing the toxicity of Cu. Lores and Pennock (1998) investigated the complex interaction between HA, salinity, pH and Cu and found decreased Cu binding at low salinities and low pH. Their study suggests that changes in environmental conditions such as pH, salinity, and the concentration of DOM can have dramatic effects on Cu bioavailability, and account for observed variations in protective effects. Risk assessment models have long recognized the importance of water chemistry parameters. The results of the present study further emphasize the importance of improving metal bioavailability models such as the BLM (Di Toro et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004) by accounting for the variability of natural DOC from different sources.

5. Conclusions

Considerable effort is now being directed towards the development of a saltwater BLM. The process is time-consuming and expensive. Thus the eventual acceptance of the BLM for use in marine systems maybe some years away (Arnold, 2005). If freshwater toxicity data are related to saltwater toxic effects in a systematic and predictable way, as indicated by preliminary application of the freshwater Cu BLM to marine mussel toxicity data (Arnold et al., 2005), the former can be used to predict the latter (Leung et al., 2001). In this context the present study gains significance as we show that DOC from freshwater sources provides protection against Cu toxicity in seawater. We have reported a 2–4 fold increase in Cu EC50 as DOC levels increase 2–5 fold, in comparison, for the glochidia larvae of freshwater mussels, Gillis et al. (2008) reported a 2–10 fold increase in Cu EC50 when augmented with DOC at levels proportionately comparable to the present study. This study therefore encourages use of the vast database of freshwater toxicity testing for extrapolation to saltwater copper criteria development.

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References

Apte, S.C., Day, G.M., 1998. Dissolved metal concentrations in the Torres Strait and Gulf of Papua. Mar. Pollut. Bull. 36, 298–304.
Arkester, R.J., Martel, A.L., 2000. Shell shape, dysodont tooth morphology and hinge-ligament thickness in the bay mussel *Mytilus trossolus* correlate with wave exposure. Can. J. Zool. 78, 240–253.
Arnold, W.R., 2005. Effects of dissolved organic carbon on copper toxicity: implications for saltwater copper criteria. Integr. Environ. Assess. Manag. 1, 34–39.
Arnold, W.R., Warren-Hicks, J.W., 2007. Probability-based estimates of site-specific copper water quality criteria for the Chesapeake bay, USA. Intgr. Environ. Assess. Manag. 3, 101–117.
Arnold, W.R., Santore, R.C., Cotsifas, J.S., 2005. Predicting copper toxicity in estuarine and marine waters using the Biotic Ligand Model. Mar. Pollut. Bull. 50, 1634–1640.
Arnold, W.R., Cotsifas, J.S., Corneillie, K.M., 2006. Validation and update of a model used to predict copper toxicity to the marine bivalve *Mytilus* sp. Environ. Toxicol. 1, 65–70.
Calabrese, A., Collier, R.S., Nelson, D.A., MacInnes, J.R., 1973. The toxicity of heavy metals to embryos of the American oyster *Crassostrea virginica*. Mar. Biol. 18, 162–166.

Coglianese, P.M., 1982. The effects of salinity on copper and silver toxicity to embryos of the Pacific oyster. Arch. Environ. Contam.Toxicol. 11, 297–303.
De Schamphelaere, K.A.C., Vasconcenlos, F.M., Filip, M.G., Tack, H.E., Allen, C.R., Janssen, 2004. Effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. Environ. Toxicol. Chem. 23, 1248–1255.
Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic Ligand Model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383–2396.
Engel, D.W., Sunda, W.G., 1979. Toxicity of cupric ion to eggs of the spot *Leiostomus xanthurus* and the Atlantic silverside *Menidia menidia*. Mar. Biol. 50, 121–126.
Environment Canada, 2005. Guidance document on statistical methods for environmental toxicity tests. Method Development and Applications Section, Environmental Technology Centre, Environment Canada. EPS 1/RM/46.
Fitzpatrick, J.L., Nadella, S., Bucking, C.P., Balshine, S., Wood, C.M., 2008. The relative sensitivity of sperm, eggs and embryos to copper in the blue mussel (*Mytilus trossulus*). Comp. Biochem. Physiol. C 147, 441–449.
Gillis, P.L., Mitchell, R.J., Schwalb, A.N., McNichols, K.A., Mackie, G.L., Wood, C.M., Ackerman, J.D., 2008. Sensitivity of the glochidia (larvae) of freshwater mussels to copper: assessing the effect of water hardness and dissolved organic carbon on the sensitivity of endangered species. Aquat. Toxicol. 88, 137–145.
Gilles, R., 1979. Mechanisms of Osmoregulation in Animals. Wiley Interscience, New York. 667 pp.
Glover, C.N., Playle, R.C., Wood, C.M., 2005. Heterogeneity of natural organic matter amelioration of silver toxicity to *Daphnia magna*: effect of source and equilibration time. Environ. Toxicol. Chem. 24, 2934–2940.
Grosell, M., Blanchard, J., Brix, K.V., Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquat. Toxicol. 84, 162–172.
Guo, L., Santschi, P.H., 1994. The distribution of colloidal and dissolved organic carbon in the Gulf of Mexico. Mar. Chem. 45, 105–119.
Heath, D.D., Hatcher, D.R., Hilbish, T.J., 1996. Ecological interaction between sympatric *Mytilus* species on the west coast of Canada investigated using PCR markers. Mol. Ecol. 5, 443–447.
Jose, G.F., Sarma, S.S., Nandini, S., 2005. Interaction among copper toxicity, temperature and salinity on the population dynamics of *Brachonis rotundiformes* (Rotifera). Hydrobiol. 546, 559–568.
Leung, K.M.Y., Morritt, D., Wheeler, J.R., Whitehouse, P., Sorokin, N., Toy, R., Holt, M., Crane, M., 2001. Can saltwater toxicity be predicted from freshwater data? Mar. Poll. Bull. 42, 1007–1013.
Lorenzo, J.I., Nieto, O., Beiras, R., 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquat. Toxicol. 58, 27–41.
Lorenzo, J.I., Beiras, R., Mubiana, V.K., Blust, R., 2005. Copper uptake by *Mytilus edulis* in the presence of humic acids. Environ. Toxicol. Chem. 24, 973–980.
Lorenzo, J.I., Neito, O., Beiras, R., 2006. Anodic stripping voltammetry measures copper bioavailability for sea urchin larvae in the presence of fulvic acids. Environ. Toxicol. Chem. 25, 36–44.
Lores, E.M., Pennock, J.R., 1998. The effect of salinity on binding of Cd, Cr, Cu and Zn to dissolved organic matter. Chemosphere 37, 861–874.
Lores, E.M., Pennock, J.R., 1999. Bioavailability and trophic transfer of humic-bound copper from bacteria to zooplankton. Mar. Ecol. Prog. Ser. 187, 67–75.
Lores, E.M., Snyder, R.A., Pennock, J.R., 1999. The effect of humic acid on uptake/adsorption of copper by a marine bacterium and two marine ciliates. Chemosphere 38, 293–310.
Luoma, S.N., 1983. Bioavailability of trace metals to aquatic organisms—a review. Sci. Total Environ. 28, 1–22.
MacInnes, J.R., Calabrese, A., 1979. Combined effect of salinity, temperature and copper on embryos and early larvae of American oyster, *Crassotrea virginica*. Arch. Environ. Contam. Toxicol. 8, 353–562.
MacRae, T.H., Pandey, A., 1991. Effects of metals on early life stages of the brine shrimp, Artemia: a developmental toxicity assay. Arch. Environ. Contam. Toxicol. 20, 247–252.
Sanchez-Marin, P., Lorenzo, I.I., Blust, R., Beiras, R., 2007. Humic acids increase dissolved lead bioavailability for marine invertebrates. Environ. Sci. Technol. 41, 5679–5684.
Martin, M., Osborn, K.E., Billig, P., Glickstein, N., 1981. Toxicities of ten metals to *Crassostrea gigas* and *Mytilus edulis* embryos and *Cancer magister* larvae. Mar. Poll. Bull. 12, 305–308.
Mclusky, D.S., Bryant, V., Cambell, R., 1986. The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. Oceanogr. Mar. Biol. Annu. Rev. 24, 481–520.
NIWA, 2005. Standard operating procedure 21.1 Blue mussel embryo (*Mytilus galloprovincialis*) acute toxicity protocol. National Inst. of Water and Atmos. Res. Ltd. Hamilton, New Zealand.
Niyogi, S., Wood, C.M., 2004. Biotic Ligand Model: a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.
Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.M., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comp. Biochem. Physiol. C 133, 3–35.
Pempkowiak, J., Bancer, B., Legezynska, E., Kulinsky, W., 1989. The accumulation and uptake of cadmium by four selected Baltic species in the presence of marine humic substances. Proceedings, 21st European Marine Biology Symposia, Gdansk, Poland, 1986, pp. 599–607.
Peterson, R.C.J., 1986. Population and guild analysis for interpretation of heavy metal pollution in streams. In: CairnsJr. Jr., J. (Ed.), Community Toxicity Testing. Spec. Tech. Publ., vol. 920. American Society for Testing and Materials, Philadelphia, pp. 180–198.
Qiu, J., Tang, X., Zheng, C., Li, Y., Huang, Y., 2007. Copper complexation by fulvic acid affects copper toxicity to the larvae of the polychaete *Hydroides elegans*. Mar. Environ. Res. 64, 563–573.
Schwartz, M.L., Curtis, J.P., Playle, R.C., 2004. Influence of natural organic matter source on acute copper, lead and cadmium toxicity to rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 23, 2889–2999.
Stedman, C.A., Markager, S., 2005. Resolving the variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using PARAFAC analysis. Limnol. Oceanogr. 50, 686–697.
Tirard, C.T., Grossfeld, R.M., Levine, J.F., Kennedy-Stoskopf, S., 1996. Effect of osmotic shock on protein synthesis of oyster hemocytes in vitro. Comp. Biochem. Physiol. A 116, 43–49.
Toyota, Y., Okabe, S., Kanamori, S., Kitano, Y., 1982. The determination of Mn, Fe, Ni, Cu and Zn in seawater by atomic absorption spectrometry after coprecipitation with lanthanum hydroxide. J. Oceanogr. Soc. Jap. 38, 357–361.
US EPA, 2001. Streamlined Water-effect Ratio Procedure for Discharges of Copper: US Environmental Protection Agency. Office of Water, Washington, DC. EPA 822-R-01-05.
US EPA, 2003. Draft Update of Ambient Water Quality Criteria for Copper. US Environmental Protection Agency. Office of Water, Washington, DC. EPA-822-R03-026.
US EPA, 2006. National recommended water quality criteria. Office of water, Office of science and technology. 4304T.
US EPA, 2007. Draft Update of Ambient Water Quality Criteria for Copper. US Environmental Protection Agency. Office of Water, Washington, DC. EPA-Copper 2007 Revision.
Vigenault, B., Percot, A., Lafleur, M., Campbell, P.G.C., 2000. Permeability changes in model and phytoplankton membranes in the presence of aquatic humic substances. Environ. Sci. Technol. 34, 3907–3913.
Watling, H.R., 1982. Comparative study of the effects of zinc, cadmium and copper on the larval growth of three oyster species. Bull. Environ. Contam. Toxicol. 28, 195–201.
Wu, F., Kothawala, D., Evans, R., Dillon, P., Cai, Y., 2007. Relationships between DOC concentration, molecular size and fluorescence properties of DOM in a stream. Appl. Geochem. 22, 1659–1667.