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Influence of dissolved organic matter (DOM) source on copper speciation and toxicity to *Brachionus plicatilis*

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Environmental context. Organic matter dissolved in water can mitigate toxic effects of copper, which should be taken into account when estimating risks of copper pollution. The composition of this organic matter, however, can vary widely, and these variations might also need to be taken into account. This work addresses the question of organic matter quality and demonstrates that only the amount and not the source influences copper toxicity – good news for risk analysis because it simplifies predictions of the effects of copper in specific receiving waters.

Abstract. The toxicity of copper in marine systems is dependent on its speciation and bioavailability. Dissolved organic matter (DOM) can complex copper, resulting in decreased bioavailability and hence decreased toxicity. The purpose of this study was to measure acute copper LC_{50} values (concentration lethal to 50 % of the organisms) in natural marine waters in a sensitive organism, and identify the relationships between DOM quality and copper toxicity and speciation. Static acute copper toxicity tests (48-h LC_{50}) were performed using the euryhaline rotifer *Brachionus plicatilis*. Ion-selective electrode measurements of free copper were performed at the LC_{50} concentrations to determine the influence of DOM source on copper speciation. LC_{50} values ranged from 333 to 980 nM (21.1 to 62.3 μg L^{-1}) with DOC concentrations ranging from 0.55 to 7.57 mg C L^{-1}. DOC was found to be protective ($R^2 = 0.72$, $P = 0.016$); however, the degree of protection decreased as DOC increased. This suggests salt-induced colloid formation could be occurring, resulting in a decrease of binding sites available to complex free copper. Free copper remained fairly constant between each sample site, with an average pCu of 10.14. Overall, this study is consistent with other studies that suggest free copper is the best species for predicting toxicity. Additionally, no significant correlation between DOM source and copper toxicity was observed as compared with total DOC concentration and copper toxicity, suggesting that DOM quality does not need to be taken into account for copper toxicity modelling in salt water.

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Introduction

Copper is a trace element that is essential for proper functioning of plants, animals and microorgansims owing to its requirement in many specific metabolic processes.[1] However, only low amounts are necessary for normal metabolic functioning and at increased concentrations, copper can be toxic. In fish and invertebrates, toxicity is usually due to copper interference with ion transport, most notably interference of sodium transport causing electrolyte imbalance and ionoregulatory failure.[2,3]

With over 53 % of the United States population living along coastal regions[4] and Canada having the longest marine coastline of any country,[5] there is an increased concern of contamination of the ocean due to anthropogenic input of metals. Typical total copper concentrations range from 0.12 to 0.38 μg L^{-1} in areas of open ocean[6] to levels over 6 μg L^{-1} in heavily affected areas such as San Francisco Bay.[7] Current US Environment Protection Authority (EPA) criteria for copper limits in seawater are a dissolved copper criterion continuous concentration (CCC) of 3.1 μg L^{-1} and a criteria maximum concentration (CMC) of 4.8 μg L^{-1}.[8] Currently, there is no copper concentration limit for marine systems set by the Canadian government[9] but provinces, like British Columbia, have their own provincial limits, with a total copper CCC of less than or equal to 2 μg L^{-1} and a CMC of 3 μg L^{-1}.[10]

The Biotic Ligand Model (BLM) is a predictive tool used to estimate site-specific bioavailability and subsequent toxicity of metals.[11–13] Modelling is based on bulk water chemistries and the interaction between the metal and the site of toxic action, which is called the biotic ligand (e.g. the gills of a fish). The freshwater BLM has been adopted as a regulatory tool by the US

EPA[7] for copper; however, there is need for a BLM in saltwater environments. Investigations pertaining to salt water are currently under way for application of a marine BLM; however, more information is needed on copper speciation and dissolved organic matter (DOM) source before the BLM is accepted for regulatory use.[3,14] The focus of the current study was to characterise each of these parameters for further development of the marine BLM.

The bioavailability of copper is influenced by the species of copper present in the system.[15–18] Copper can exist in many different forms in aquatic environments and factors within these environments can affect its toxicity to organisms. Most copper is found in the form of inorganic and organic complexes.[19,20] Organic ligands have been found to play a larger role on copper speciation and are generically classified as DOM.[18] From a BLM perspective, the copper in both organic and inorganic complexes is generally thought to be unavailable to interact with organisms to cause toxicity. As such, free copper, Cu^{2+}, is often used as an indicator for toxicity because it is the species most available to be taken up by an organism.[15,16,18]

The concentration of DOM is usually measured as dissolved organic carbon (DOC). DOC is operationally defined as organic carbon that passes through a 0.45-μm filter. Typical concentrations of DOC in marine systems range from 0.5 to 10 mg C L^{-1} from open ocean to coastal waters.[21] Increased DOC concentrations have been shown to be protective in marine organisms such as the blue mussel *Mytilus* sp.,[22] the rotifer *Branchionus plicatilus*[23] and the sea urchin *Parecentrotus lividus*.[24] However, variation in DOM source may influence copper toxicity. Different DOM sources show variation in copper complexing capacities that could have an overall effect on DOM protection.[25] This was seen by Nadella et al.,[26] in which three different sources of exogenous DOM spiked into sea water resulted in different levels of protection against copper toxicity in *Mytilus trossolus*. The most protective DOM source was found to contain 20 and 40 % higher fulvic substance content than the two less-protective DOM sources. This corresponded to 40 and 60 % less protectivity to the blue mussel.[26]

DOM can be broadly classified into two groups, allochthonous (terrigenous) and autochthonous.[27] Terrigenous organic matter is terrestrially derived from the decomposition and leaching from soil and plant materials such as lignin, tannins and detritus and typically contains a higher humic and fulvic substance content. Autochthonous organic matter is microbially derived organic matter from bacterial and algal processes occurring in the water column and usually contains a higher proteinaceous content.[27,28] Typically, terrigenous organic matter is associated with a darker colour and relatively high amounts of aromatic and phenolic compounds, whereas autochthonous organic matter is lighter in colour and contains relatively low amounts of aromatic and phenolic groups.[29] This colour can be described by the specific absorption coefficient of the DOC at 340 nm (SAC_{340}). Darker, terrigenous organic matter (higher SAC_{340}) has been found to be more protective to copper toxicity than ligher, microbially derived organic matter (lower SAC_{340}).[30–33] DOM origin can also be approximated using fluorescence indices, as proposed by McKnight et al.[27] A fluorescence index (FI) of ~1.4 and 1.9 indicates terrestrially derived (allochthonous) and microbially derived (authochthonous) DOM respectively.

Fluorescence spectroscopy can be used to distinguish different fluorescent molecules (fluorophores) within a heterogeneous system. The compilation of data from simultaneously measuring excitation (Ex) and emission (Em) wavelengths results in a fluorescence excitation–emission matrix (FEEM). The intensity and position of the fluorophores within the matrix provide information on the chemical composition of DOM. Terrigenous components (humic and fulvic acids) fluoresce at longer wavelengths than proteinaceous components.[34] Fulvic- and humic-like components can be detected in the Ex–Em ranges of 300–350 nm to 400–450 nm and 250–390 nm to 460–520 nm respectively.[27,34–37] Tyrosine and tryptophan can be detected in the Ex–Em ranges of 225–275 nm to 350 nm and 225–270 nm to 300 nm respectively, representing microbially derived carbon sources.[35,36] DOM characterisation using this method has been used to identify components in aquatic systems ranging from fresh water[38,39] to sea water.[38,40]

Parallel factor analysis (PARAFAC) can be used to determine the relative quantities of the humic-, fulvic-, tryptophan- and tyrosine-like components observed by fluorescence. Through spectral deconvolution of a stack of FEEMs, PARAFAC is able to quantify the minimum number of components to describe each FEEM.[41] In the present study, natural seawater water 'grab' samples from nine different sites in North America were evaluated for Cu toxicity to the rotifer *Brachionus plicatilis* and were assayed for DOC content and DOC characterisation by the optical methods described above. Four discrete components, humic-, fulvic-, tyrosine- and tryptophan-like fractions, were analysed for correlations with copper toxicity. In an earlier study, De Palma et al.[22] looked at the same components for correlations with copper toxicity to *Mytilus* sp.; however, no significant correlations were found, suggesting that DOC was predictive of toxicity independently of DOM quality.

The objectives of the present study were to (1) measure acute toxicity of copper in the rotifer *Brachionus plicatilis* in natural marine waters; (2) identify relationships between LC_{50} (concentration lethal to 50 % of organisms) in rotifers and the free Cu, concentrations of humic-, fulvic-, tyrosine- and tryptophan-like components of DOC, SAC_{340} and FI; (3) identify the relationship between DOC source and free copper; and (4) evaluate whether DOM quality should be included as an input parameter into a marine BLM for copper.

Methods

Sampling, storage and selection

Ambient water samples were collected from marine and estuarine sites along the coasts of Canada and the USA. Samples were collected in high-density polyethylene bottles while submerged to ensure no airspace in the bottle. Samples were transported to Wilfrid Laurier University (Waterloo, ON) in coolers. The bottles were stored at 4 °C. After subsampling, argon (Ar) was used to fill the headspace. In total, 28 samples were collected. DOC quality was used to determine a subset of samples in which to measure copper toxicity (refer to *Fluorescence measurement and analysis* section). Samples were chosen to encompass low and high concentrations (and combinations within) of each of the four parameters (humic, fulvic, tryptophan and tyrosine content). The locations of the nine sites that were selected for toxicity assays are described in Table 1.

Dissolved organic carbon analysis

DOC concentrations of all ambient and salinity-adjusted grab samples were measured using a Shimadzu $TOC\text{-}L_{CPH/CPN}$ (Shimadzu Scientific Instrumentations Inc., Columbia, MD,

Table 1. Description of sampling sites used for toxicity assays

Sample	Location	Coordinates (lat., long.)	Collection date
Bouctouche (BT)	NB	46°28′17.5152″N, 064°43′02.2188″W	Nov 2011
Petit Rocher (PR)	NB	47°47′00.7224″N, 065°42′30.9816″W	May 2012
Major Kollock Creek (MKC)	NB	46°48′48.4884″N, 064°54′44.7876″W	May 2012
Naufrage Harbour (NH)	PEI	46°28′03.4680″N, 062°25′02.4348″W	May 2012
Rathtrevor Beach (RB)	BC	49°19′18.4548″N, 124°15′52.8624″W	Aug 2012
Hawke's Bay (HB)	NFLD	50°36′58.1112″N, 057°10′56.8740″W	Aug 2012
Blackberry Bay (BB)	BC	49°09′06.4476″N, 125°53′52.8720″W	Nov 2012
Chesterman Beach (CB)	BC	49°06′48.0960″N, 125°53′52.8720″W	Nov 2012
Jimbo's Bar (JB)	Miami, FL	25°46′28.9560″N, 080°08′43.4400″W	Jan 2013

USA). Filtered (0.45-μm cellulose nitrate membrane filter; Whatman, Germany) samples (20 mL) were acidified with 2–3 drops of concentrated OmniTrace HCl (EMD Chemicals, Gibstown, NJ, USA). Standard total carbon solutions of 5, 10 and 20 mg C L^{-1} prepared from potassium hydrogen phthalate (BDH, West Chester, PA, USA) and dissolved in artificial sea water (ASW, see below) were measured with samples to allow for an external calibration. Ultrapure water (MilliQ A10, resistance >18 MΩ; EMD-Millipore, Darmstadt, Germany) rinses were performed after every sample analysis to ensure removal of any salt deposits from the analyser syringe.

Fluorescence measurement and analysis

Daily standards of 2.4 μM tyrosine, 1.0 μM tryptophan and 5 mg C L^{-1} (Nordic Reservoir) were prepared. These solutions were prepared from stock solutions of reagent grade L-tyrosine (1.0×10^{-3} M) (>98 % pure, Sigma–Aldrich, St Louis, MO, USA) and L-tryptophan (1.0×10^{-2} M) (>98 % pure, Sigma–Aldrich) prepared using ultrapure water (18.2 MΩ, MilliQ). For standardisation, organic matter from a terrestrial reverse-osmosis organic matter isolate, Nordic Reservoir DOM (IHSS, St Paul, MN, USA) was utilised. This daily standard was used to determine relative fluorophore component concentrations using a one-point calibration and PARAFAC (see below).

An aliquot of each water sample was passed through a 0.45-μm cellulose nitrate membrane filter (Whatman) and the filtrate measured in a 1-cm quartz cuvette using a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Mississauga, ON, Canada). Fluorescence emission wavelengths were measured from 250 to 600 nm in 1-nm increments for every 10-nm excitation wavelengths between 200 and 450 nm. Both the excitation and emission monochromator slit widths were set to 5 nm for all measurements. The photomultiplier tube was set to high sensitivity (800 V). When sample fluorescence intensity was very low, the photomultiplier tube was set to 1000 V and the daily standard was diluted by a factor of 10 in these cases. The use of daily standards (see above) at the same operating conditions as the samples allowed relative concentrations of each component to be determined corrected for instrument settings, thereby allowing a comparison between samples measured at different instrument settings.

A Varian Cary 50 UV-Vis spectrophotometer (Varian) was used to measure absorbance on each sample following fluorescence measurements. Absorbance (*Abs*) was measured from 250 to 600 nm in order to correct for inner-filter effects (if needed) as well as to determine SAC_{340} for each sample. SAC_{340} was calculated based on the following equation[32]:

$$SAC_{340} = 2303\left(\frac{Abs_{340}}{DOC}\right) \quad (1)$$

Fluorescence indices to estimate DOM source were calculated for each water sample as in McKnight et al.[27] using the following equation:

$$FI_{ex370} = \frac{em_{450}}{em_{500}} \quad (2)$$

where FI_{ex370} is the fluorescent index at 370-nm excitation, and em_{450} and em_{500} are the respective emission intensities at 450 and 500 nm.

MATLAB (MathWorks Inc., Natick, MA, USA) was used to generate a three-dimensional FEEM for each sample. Rayleigh scattering was removed (replaced with NaN ('not a number') values) from the spectra during preprocessing to prevent mathematical interferences in later spectral analysis. For sample measurements in which the absorbance at 254 nm was less than 0.3 units, inner-filter effect corrections were not necessary.[42] For data that required inner-filter corrections, these were performed using Eqn 3 from Larsson et al. [43] where F is the corrected fluorescence intensity, F_o is the observed fluorescence, b is the path length, A_{ex} is the absorbance at the excitation wavelength and A_{em} is the absorbance at the emission wavelength.

$$F = F_o(10^{-b(A_{ex}+A_{em})}) \quad (3)$$

Fluorescence intensities were expressed in arbitrary fluorescence units (counts) to avoid propagation of errors and additional assumptions in data analysis. The same instrument was used for all fluorescence measurements. The FEEMs were resolved using PARAFAC and constrained to four fluorescent components. Pure spectra of tyrosine and tryptophan were processed to recover pure tyrosine and tryptophan as components during analysis.[22] These made up the proteinaceous component of the resolved components. The spectra of the terrigenous material were labelled as humic-like and fulvic-like components. These spectra were based on the observation that higher-molecular-weight material fluoresces at longer wavelengths.[22,37] Therefore, after data processing, tyrosine-like, tryptophan-like, humic-like and fulvic-like components were defined. The concentrations of these components within each sample were determined using the resolved component concentrations from the daily standards.

Toxicity assay

All toxicity tests on the nine samples were conducted using the euryhaline rotifer *Brachionus plicatilis* purchased from Florida Aqua Farms Inc. (Dade City, FL). Static acute toxicity tests (48-h) were performed following ASTM (American Society for the Testing of Materials) (2004) guidelines[44] with modifications from Arnold et al. [23] A summary of these recommendations can be seen in Table S1. *B. plicatilis* resting cysts were hatched in six-well tissue culture plates (Falcon, Becton Dickinson, Mississauga, ON) in ASW[44] at 30 ppt and a pH of 8.0 ± 0.1. Hatching took place under continuous light (2500 lx) at 25 °C. Newly hatched rotifers (<6 h old) were transferred to the exposure chamber, a 24-well tissue culture plate (Falcon) in which each well contained 2 mL of the test solution. Six replicates of 10 rotifers per exposure concentration (control + five concentrations in the range 0 to 300 μg L^{-1} depending on water chemistry) were performed for each sea water sample. The test chamber was in continuous darkness and maintained at 25 ± 1 °C. The rotifers were observed at the end of the 48-h exposure under a microscope. Individual rotifers were considered dead if there was no movement of body or body parts within 5 s of observation.[44] The test acceptability criterion was less than 10 % mortality in the control.[44]

The ASW[44] was made using the following concentrations of salts (trace metal grade or better (Sigma–Aldrich)): 11.31 g of NaCl, 0.36 g of KCl, 0.54 g of $CaCl_2$, 1.97 g of $MgCl_2 \cdot 6H_2O$, 2.39 g of $MgSO_4 \cdot 7H_2O$ and 0.17 g of $NaHCO_3$ dissolved in 1 L of ultrapure water (18.2 MΩ, MilliQ, EMD-Millipore, Darmstadt, Germany). Salinity was adjusted to 30 ± 0.1 ppt using ultrapure water.

The nine samples used for the toxicity assays were adjusted to a salinity of 30 ± 0.1 ppt using a mixture of the ASW salts or ultrapure water. The sample was then filtered through a 0.45-μm cellulose nitrate filter (Whatman, Germany). The filtrate pH was adjusted to 8.0 ± 0.1 using 0.1 M NaOH (Orion 91–57BN pH electrode and Orion 420A+ meter, Thermo Electron Corp., Gormley, ON, Canada). Subaliquots of the filtrate were distributed into six separate Teflon (Nalgene) containers. A 1.0-mM solution of $CuSO_4 \cdot 5H_2O$ (BioShop Canada Inc., Burlington, ON, Canada) was prepared in ultrapure water and was used to spike the test concentrations into the appropriate containers. Concentrations ranged from zero added copper (control) up to 300 μg L^{-1} Cu depending on the sample water tested. These solutions were allowed to equilibrate for 24 h in darkness before rotifer transfer to the test solutions was initiated. At the end of the assay, pH values were remeasured and confirmed to be within the initial target of 8.0 ± 0.1.

The LC_{50} values and confidence intervals for the toxicity data were determined using probit analysis as prescribed by the US EPA.[45] Effects concentrations were determined based on measured final copper concentrations at the end of the assay. If the 95 % confidence intervals did not overlap, the LC_{50} values were considered significantly different.

Total copper analysis

Total copper measurements were made on subaliquots of the control sample and samples spiked with copper for the toxicity assay as well as solutions containing copper concentrations equivalent to the 48-h LC_{50}. According to manufacturer (Metrohm) recommendations, UV digestion of each sample to remove organic components was required before total copper analysis using differential pulse anodic-stripping voltammetry (DPASV). UV digestion was performed using a 705 UV Digester (Metrohm, Mississauga, ON, Canada). A volume of 100 μL of 30 % H_2O_2 was added to 10 mL of sample within the sample vessel. Irradiation time was 60 min at a temperature of 89 ± 2 °C.

DPASV analyses were carried out with a static mercury drop electrode (SMDE) and a glassy carbon rod counter-electrode (Metrohm) held in a Metrohm 663 VA stand coupled to a computer-controlled AutoLab PGSTAT128N potentiostat–galvanostat (Eco Chemie, Metrohm). *Nova 1.7* software (Eco Chemie, Metrohm) was used for analysis of peaks. The experimental conditions followed manufacturer recommendations (Metrohm Application Bulletin number 231/2e). Therefore, 1 mL of KCl–sodium acetate buffer (1.5 mol L^{-1} KCl, 0.5 mol L^{-1} CH_3COONa and 50 mL 30 % w/v NaOH L^{-1}) was added to 10 mL of sample. The pH was then adjusted to 6.400 (±0.005). The sample was purged for 5 min with Ar after which copper was accumulated on a mercury drop for 90 s with stirring. The equilibration time was 10 s and the differential pulse ranged from −1.250 to 0.000 V. The standard addition solution was prepared daily at a concentration of 157 μM from a 1000 mg L^{-1} copper standard solution (Assurance grade, SPEXCertiPrep, Metuchen, NJ, USA). Standard addition analysis by linear regression was then used to determine the original concentration of copper in the sample.

Free copper analysis

Solutions containing total copper concentrations equivalent to the 48-h LC_{50} for all marine samples were prepared and allowed to equilibrate for 24 h before analysis. Free copper was measured using the internal calibration flow-through method described in Tait et al.[46] All measurements were conducted in a flow-through system using an Orion copper electrode (Model 94–29, Boston, MA) contained within an ion selective electrode (ISE) micro-Flowcell (FIAlab, Bellevue, WA). The flow cell was contained within a Faraday cage made from wire wrapped to electrical ground (a water tap). An Orion double junction Ag/AgCl reference electrode (Model 900200, Boston, MA) using ASW prepared using OECD Annex 10, *Guidance on transformation/dissolution of metals and metal compounds in aqueous media* (https://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev04/English/14e_annex10.pdf, accessed 13 November 2015) as the outer filling solution was located in a separate beaker at the end of the system to avoid interference of the Cu ISE by silver. Each electrode was connected to a potentiometer (Tanager, model 9501, Ancaster, ON, Canada). A valveless metering pump, the Cerampump FMI 'Q' Pump (GQ6, Fluid Metering Inc., Syosset, NY, USA) was used to deliver the test solution through the system at a flow rate of 10 mL h^{-1}. The Cu ISE was polished weekly using aluminum oxide (<10 μm, 99.7 %, Sigma–Aldrich) followed by silver electrode polish (Corning Inc., Tewksbury, MA, USA). Following polishing and for overnight storage the Cu ISE was left in the flow cell in the copper ion buffer (15 mM ethylenediamine; ReagentPlus ≥99 %, Sigma–Aldrich), 1 mM $CuSO_4 \cdot 5H_2O$ (BioShop Canada Inc.) and 0.6 M NaCl (Fisher Scientific, Bridgewater, NY)) at pH 8.0 with the sample delivery pump turned off after running buffer at a fast flow rate (~160 mL h^{-1}) for 2 min.

Before analysis, the sample pH was adjusted to 8.0 ± 0.1. A fast flow rate (~160 mL h^{-1}) was used to ensure the sample was through the system and that a complete electric circuit was

maintained, after which the flow rate was reduced to the measuring flow rate of 10 mL h^{-1}. The sample was delivered through the system until stabilisation of the potential signal was achieved, which generally took 2 to 5 h. The potential was considered stable when the drift was less than 0.1 mV min^{-1} for at least 5 min. After stabilisation, the final pH was measured. The process was repeated at a pH of 3.3 ± 0.1 for calibration purposes (see below). After the lowest pH measurement, the system was flushed with ASW for 2 min at the fast flow rate, followed by the copper ion buffer for 2 min before the next sample was measured.

A one-point internal calibration was performed using the lower pH measurement to determine free Cu as detailed in Tait et al.[46] The electrode is assumed to have a linear Nerstian response with a slope of 29.6 mV per decade.[46] For the low-pH sample, it is assumed that the total copper equals the free copper, corrected for chloride complexation using the National Institute of Standards and Technology (NIST) formation constant (K) $\log K_{CuCl}$ of 0.3.[47] This allows the free copper to be determined at pH 8.0. Statistical comparisons of the free copper measurements versus DOC were determined using a one-way ANOVA followed by the Student–Newman–Keuls post-hoc test. A limit of $P < 0.05$ was used to indicate significance.

Table 2. Measurements of dissolved organic carbon (DOC) and salinity of the seawater samples used for the toxicity assays

Measurements are shown from both before and after salinity adjustment and filtration; refer to Table 1 for full names of sampling sites

Sample	DOC (mg C L^{-1})		Salinity	
	Before	After	Before	After
BT	4.36	4.83	19.1	30.1
PR	2.13	2.10	23.2	30.2
MKC	7.86	7.57	12.8	29.9
MN	5.50	5.20	4.3	29.9
RB	1.52	1.37	24.4	30.1
HB	1.54	1.28	25.0	30.0
BB	1.45	2.03	8.3	29.9
CB	0.82	0.55	30.3	30.1
JB	1.34	1.13	34.8	30.1

Results and discussion

DOC analysis

DOC concentrations were measured as an approximation of DOM content in each of the nine grab samples before and after salinity adjustment and filtration and are shown in Table 2. DOC concentrations of the nine samples ranged from 0.55 to 7.57 mg C L^{-1} after salinity adjustment and filtration. Some loss of DOC might have been expected in response to increasing the salinity of the samples owing to the salting-out effect. This effect refers to the decrease in solubility of non-electrolytes with an increase in ionic strength.[48,49] This phenomenon has been observed for DOC in marine coastal waters[50] but no 'salting out' of DOC was observed for these samples. A comparison of the DOC measurements before and after salinity adjustment and filtration can be seen in Fig. S1.

Fluorescence measurements

The fluorophores within DOM can be distinguished based on different fluorescent properties. Fluorescence spectroscopy can be used to produce FEEMs. These FEEMs were visualised as contour plots using *MATLAB* to show the fluorescence intensity trends with Ex–Em wavelengths. These plots show a clear qualitative indication of the presence of humic-, fulvic-, tyrosine- and tryptophan-like fractions for each of the samples based on their unique Ex–Em intensity signals. Two examples of contour plots can be found in Fig. 1. Typically, higher intensities at Ex–Em ranges of 340 nm–425 nm and 260 nm–460 nm identify fulvic- and humic-like components. Tryptophan and tyrosine components show higher intensities at 275 nm–350 nm and 225 nm–300 nm respectively.

Fig. 1a represent Major Kollock Creek (MKC) and Fig. 1b corresponds to Chesterman Beach (CB). These two FEEMs illustrate the differences in optical properties between samples. MKC shows a large intensity peak at emissions at 420 and 460 nm, corresponding to fulvic- and humic-like components. In contrast, CB showed large intensity peaks at emissions of

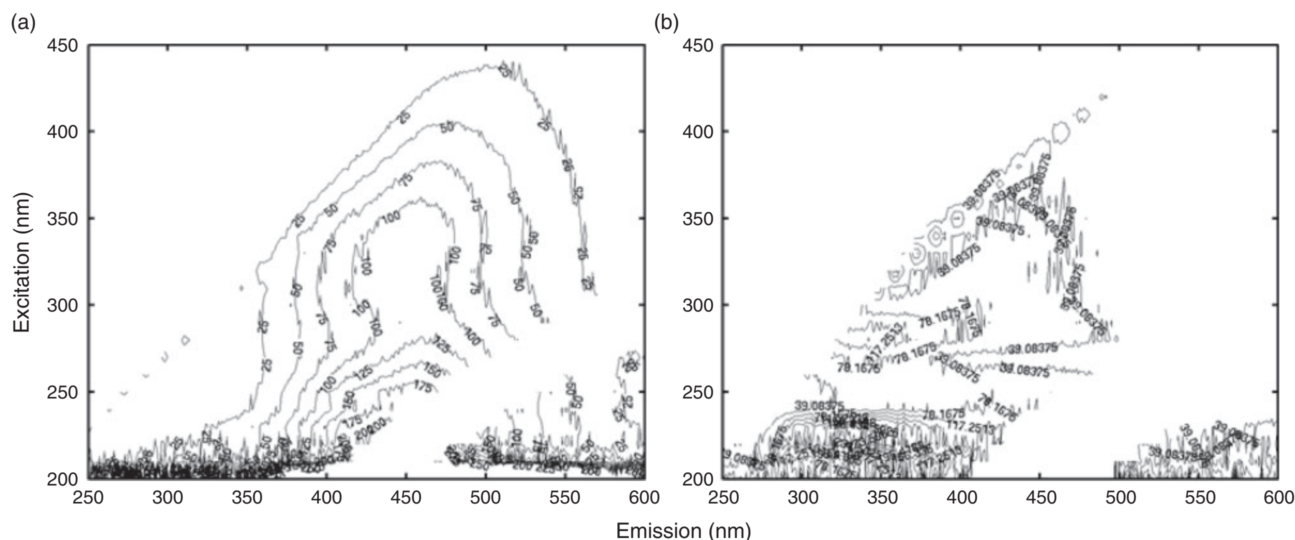

Fig. 1. Fluorescence excitation–emission contour plots for two water samples. Major Kollock Creek (a) has higher fulvic- and humic-like components. Chesterman Beach (b) has higher tyrosine- and tryptophan-like components. Contour labels refer to intensity of emitted fluorescence.

Table 3. Fluorescence measurements of humic- (HA), fulvic- (FA), tryptophan- (Trp) and tyrosine- (Tyr) like components of water used for toxicity assays

Measurements are from before and after salinity adjustments and filtration. Specific absorption coefficient at 340 nm (SAC_{340}) and fluorescence index (FI) of each water sample used for toxicity assay; refer to Table 1 for full names of sampling sites

Sample	HA	FA	Trp	Tyr	SAC	FI
BT	2.12	0.84	0.000	0.10	21.19	1.33
PR	0.53	0.41	0.072	0.15	14.89	1.76
MKC	4.51	1.49	0.000	0.08	39.37	1.25
NH	3.28	1.12	0.000	0.06	28.46	1.26
RB	0.17	0.42	0.079	0.20	12.69	1.28
HB	0.22	0.36	0.036	0.08	20.75	1.27
BB	0.57	0.68	0.066	0.12	26.32	1.43
CB	0.08	0.28	0.121	0.20	31.61	1.77
JB	0.16	0.36	0.044	0.08	10.06	1.64

300 and 350 nm, corresponding to tyrosine- and tryptophan-like components.

Application of the PARAFAC algorithm identified four operationally defined fractions with the FEEMs. These were humic-, fulvic-, tryptophan- and tyrosine-like components as illustrated in Fig. S2, which correspond very closely in shape to previously published components.[22] This analysis described 97.7 % of the variability within the fluorescence data.

The relative concentrations of each component, measured as arbitrary fluorescence units (arb), obtained by PARAFAC are shown in Table 3 along with SAC and FI values. Note that the PARAFAC analysis is quantitative for fluorescence intensity, but qualitative for DOC, because fluorescence intensity per unit C ('fluorescence efficiency') may not be the same for the four components. Indeed, we might expect that fluorescence efficiency would be higher for the two amino acid-like components than for the fulvic and humic-like components, because a lower proportion of structural moities in the latter two categories are expected to fluoresce. The toxicity assays were performed with the samples that had undergone salinity adjustment and filtration; therefore, the tabulated values correspond to measurements in salt-adjusted, filtered samples. SAC_{340} measurements of the samples indicated a range from ~10 to 40. FI ranged from 1.25 to 1.77, suggesting samples ranged from terrestrial to microbial sources.[27] Most sources were terrestrially derived, with FI indexes of 1.25 to 1.45. Jimbo's Bar (JB) had a FI of 1.64, suggesting both terrestrial and microbial input. PR and CB had quite high FI of 1.76 and 1.77 respectively, indicating microbial sources.

Influence of DOC concentration on copper LC_{50}

The Cu LC_{50} ranged from 333 to 980 nM (21 to 62 μg L^{-1}) over a range of DOC from ~0.5 to 8.0 mg C L^{-1} (Fig. 2). Thus, *Branchionus plicatilus* is sensitive to copper in sea water at levels ~10-fold greater than current ambient water quality criteria values (see *Introduction*). Overall DOC was found to be protective against copper toxicity to *Brachionus plicatilis*. This is consistent with literature in which increased DOM concentrations have been shown to be protective to *B. plicatilis*[23] as well as in other marine organisms such as the blue mussel, *Mytilus* sp.[22,26] and the sea urchin (*Parecentrotus lividus*).[24] As well, Arnold et al.[51] found a significant relationship between DOC and EC_{50} values in six different

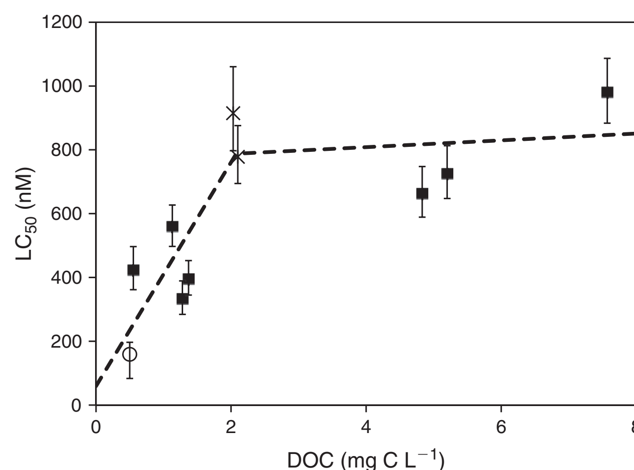

Fig. 2. Total dissolved copper LC_{50} (lethal concentration to half of exposed organisms) as a function of dissolved organic carbon (DOC) suggesting a salt-induced colloid formation trend, as indicated by the two dashed lines. A steep increase in LC_{50} is observed until ~2 mg C L^{-1} DOC ($R^2 = 0.72$, $P = 0.016$), then a plateau effect is observed, suggesting salt-induced colloid formation. Circle (○) data point represents a control artificial sea water (ASW) sample from Arnold et al.[23] Cross (×) data points represent samples from marina locations (see *Discussion*). Error bars correspond to 95 % confidence limits.

species (the blue mussels *Mytilus galloprovincialis* and *M. edulis*, the oyster *Crassostrea virginica*, the sand dollar *Dendraster excentricus*, the sea urchin *Strongylocentrotus purpuratus* and the copepod *Eurytemora affinis*). Where EC_{50} is the concentration resulting in half the embyros not developing normally.

From rotifer LC_{50} values measured here, a plateauing out of the protective effect of DOC is observed (Fig. 2). A low DOC (<0.5 mg C L^{-1}) in an ASW sample from Arnold et al.[23] for *B. plicatilis* using the same ASTM (2004)[44] protocol was added to the data set as a control. The dashed lines represent the trend in the data. A steep increase in LC_{50} was observed when going from zero to ~2 mg C L^{-1} DOC. This resulted in a linear correlation with an R^2 of 0.72 and significant P value of 0.016. At DOC concentrations above this point, further increases in DOC did not significantly affect copper toxicity and a plateauing of the line was observed.

It was hypothesised that the decrease in protectivity as DOC concentrations increased was due to salt-induced colloid formation of DOC particles that could occur at high salinities. This can be described using the Derjanguin–Landau–Verwey–Overbeek (DLVO) theory.[52] The force between two surfaces in liquid is predicted by the continuum theory as the sum of van der Waals force and electrostatic force. Van der Waals forces allow attraction between two similar surfaces whereas electrostatic forces result in repulsion between surfaces of the same charge. However, at high ionic strength, repulsive forces decrease owing to collapse of the electrical double layer. This allows for the possibility of colloidal particles in a liquid medium to form persistent aggregates due to van der Waals force of attraction.[53] At a constant salinity, DOC–DOC interactions increase at higher DOC. The increased DOC–DOC interactions result in fewer binding sites available for copper, and therefore more copper is bioavailable to cause toxicity. As supported by the data in Fig. 2, at low DOC, a linear trend should be seen and then a plateau at high DOC. This trend has been seen in another rotifer toxicity study in which exogenous DOC was added to

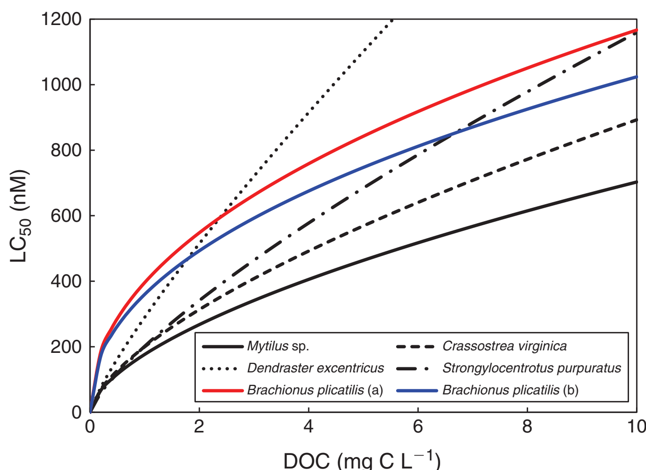

Fig. 3. Comparison of predictive toxicity equations for *Mytilus* sp., *Crassostrea virginica*, *Dendraster excentricus* and *Strongylocentrotis purpuratus*. *Brachionus plicatilis* (a) represents the toxicity equation including all data whereas (b) represents the toxicity equation excluding marina samples.

artificial water.[54] At 30-ppt salinity, a plateau effect was seen at DOC concentrations above ~5 mg C L^{-1}. Brooks et al.[55] found a comparable trend in the Pacific oyster *Crassostrea gigas*. In this case, humic acid concentrations greater than 1.02 mg C L^{-1} did not provide any further protection against copper toxicity. Similarly, copper toxicity in the marine blue mussel (*Mytilus trossolus*) increased in a linear trend when moving from 0 to 10 mg C L^{-1}, with EC_{50} values from 148 to 582 nM respectively. However, when the DOC was doubled to 20 mg C L^{-1}, the EC_{50} plateaued with a value of 614 nM.[26] This relationship has also been observed for lead toxicity. In *M. trossolus* and *M. galloprovincialis*, an increase in DOC from 0 to 2 mg C L^{-1} increased the EC_{50} of lead significantly from 217 and 304 to 564 and 738 nM for *M. trossolus* and *M. galloprovincialis* respectively.[56] However, above 2 up to 12 mg C L^{-1}, there was no significant change in the EC_{50} of *M. trossolus* (521 nM) and *M. galloprovincialis* (758 nM).[56]

Samples Petit Rocher (PR) and Blackberry Bay (BB) were the only samples collected from marina waters and the data from those samples should be considered cautiously. Removing these two sites only moderately changes the best fit model for the data though. The relationship between LC_{50} and DOC can be described by the equation LC_{50} (μg L^{-1}) = $25.15DOC^{0.47}$ including the two marina sites in statistical analysis or LC_{50} (μg L^{-1}) = $22.86DOC^{0.45}$ without including the marina sites. This equation is given in micrograms per litre units to compare with toxicity equations for other species in Arnold et al.[23,57] The relationship has an R^2 of 0.61 and is significant, with a P value of 0.008 including PR and BB or an R^2 of 0.71 and P value of 0.009 excluding those marina samples. These relationships along with the original data are plotted in Fig. S3.

The relationship between LC_{50} and DOC for *B. plicatilis* can be compared with the relationship observed in other species in Fig. 3. This figure shows the relationship between LC_{50} and DOC from the current study as well as for *Mytilus* sp. (EC_{50} (μg L^{-1}) = $11.22DOC^{0.6}$,[57]) the oyster *Crassostrea virginica* (EC_{50} (μg L^{-1}) = $12.7DOC^{0.65}$), the sand dollar *Dendraster excentricus* (EC_{50} (μg L^{-1}) = $18.4DOC^{0.83}$) and the sea urchin *Strongylocentrotus purpuratus* (EC_{50} (μg L^{-1}) = $12.8DOC^{0.76}$).[23] From these comparisons, it can be seen that *B. plicatilis* has a similar relationship to other copper-sensitive aquatic organisms. However, *B. plicatilis* appears to experience greater protectivity at lower DOC concentrations and lower protectivity at higher DOC concentrations compared with the other organisms shown here. This is illustrated by the steeper slope at lower DOC concentrations moving to a shallower slope at increasing DOC concentrations compared with the other model lines.

Influence of DOC quality on copper LC_{50}

SAC_{340} is a measure of colour and has been suggested as a measure of DOM quality.[32] SAC_{340} has shown a good correlation to metal toxicity in freshwater, with a higher SAC_{340} (indicating terrigenous C) shown to decrease copper bioavailability more than organic matter with a lower SAC_{340}.[32,33] Using the SAC_{340} data from Table 3, in the marine samples measured, no correlation was observed between SAC_{340} and LC_{50} normalised to DOC (Fig. S4a).

An approximation of DOM origin was determined using fluorescence indices.[27] FI values of ~1.4 and 1.9 indicate terrestrially derived and microbially derived DOM respectively. Following the idea that terrestrially derived organic matter is more protective,[32] as FI increases, a decrease in protectivity should be observed. However, the opposite was observed when the FI data from Table 3 were plotted with LC_{50} (Fig. S4b). An increase in LC_{50} normalised for DOC is observed as FI increases. This correlation is significant, with a P value of 0.008 and an R^2 of 0.66. This correlation may be due to the fact that samples with higher total DOC were predominantly terrestrially derived sources, and therefore the normalised LC50 values for these samples had been divided by greater DOC concentrations.

It has been found that terrestrial organic material is more protective than microbially derived organic material.[30,32,33,58] Because terrestrial organic matter is mostly composed of fulvic and humic acids,[27,28] it was thought that DOC would show more protectiveness when these components are in higher concentrations. The relationship between the four different fluorescent components of DOC and the LC_{50} is shown in Fig. S5. Both humic- and fulvic-like fractions show a positive correlation with LC_{50}. For fulvic-like (Fig. S5b) fractions, a significant linear increase is observed ($R^2 = 0.53$, $P = 0.027$). Humic-like (Fig. S5a) fractions were just outside the range of significance, with a P value of 0.062 and an R^2 of 0.41. Tryptophan (Fig S5c) and tyrosine (Fig. S5d) showed very weak, slightly negative correlations with LC_{50}, with non-significant R^2 values of 0.18 ($P = 0.261$) and 0.14 ($P = 0.328$) respectively. The very low correlation of tryptophan- and tyrosine-like fluorophores could be due to the low fluorescence intensities, which have larger relative errors that may mask any potential correlations. Similar trends were seen in De Palma et al.,[22] where the total contribution of tyrosine- and tryptophan-like components was found to be a constant fraction of the total fluorescence, independently of DOC.

The protective effect of humic- and fulvic-like fractions agrees with data presented in Lorenzo et al. in which humic acids[59] and fulvic acids[60] proved protective to the sea urchin *Paracentrotus lividus* against copper toxicity. As well, Nadella et al.[26] estimated fulvic-like fraction concentrations from three different DOM sources using fluorescence techniques and found a strong protective effect. The most protective DOM source was found to contain 20 and 40 % higher fulvic substance content than the two less-protective DOM sources.

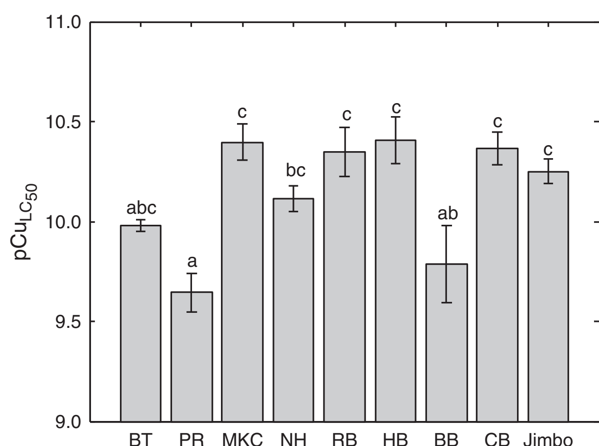

Fig. 4. Free copper measurements, expressed as pCu ($-\log[Cu^{2+}]$), at the LC_{50} (lethal concentration to half of exposed organisms) for each site (refer to Table 1 for full names of sampling sites). Error bars represent 95 % confidence intervals. Letters are based on statistical comparisons of the free copper measurements versus DOC were determined using a one-way ANOVA followed by the Student–Newman–Keuls post-hoc test. A limit of $P < 0.05$ was used to indicate significance.

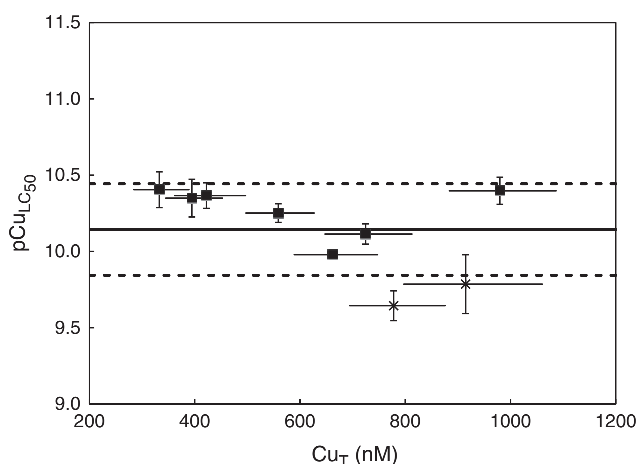

Fig. 5. Free copper at the LC_{50} as a function of total copper at the LC_{50} (lethal concentration to half of exposed organisms). The solid line represents the mean free copper and the dashed lines represent a factor of two of the ion selective electrode data. Error bars represent 95 % confidence intervals. Cross (×) data points represent the marina sites.

Free copper concentration at the LC_{50}

The free copper concentration at the LC_{50} for each sample was measured using the Cu ISE. The comparison of free copper, reported as pCu ($-\log[Cu^{2+}]$), for each site can be found in Fig. 4.

Seven of the nine samples measured were found to be statistically similar. Petit Rocher (PR) was significantly different from all sites excluding Bouctouche (BT) and BB. BB was similar to BT, PR and Naufrage Harbour (NH) but significantly different from the other sites. This supports the suggestion that PR and BB are possible outliers as discussed above. However, overall, these results suggest that although differences in free copper are observed, most of these differences are not significant and free copper is statistically similar in these sites.

Another way to look at these data is by plotting the free copper as a function of LC_{50} (Fig. 5). The solid line represents the mean of the data and the dashed lines represent a factor of two around the data. This factor of two was included because the BLM currently predicts toxicity within a factor of two.[23] In the present study, all samples, save one (PR), have 95 % confidence intervals within the factor of two about the mean. The LC_{50} for total dissolved copper spans a range from 333 to 980 nM (21.1 to 62.3 μg L^{-1}) but as LC_{50} increases, there is no significant increase in free copper, with free copper pCu values ranging from 9.64 to 10.4 (2.5–14.4 ng L^{-1}). As mentioned above, PR and BB measured slightly higher free copper at the LC_{50} than the other samples. Without these measured free copper concentrations, the free copper is very constant, ranging from only 9.98 to 10.40 (2.5–6.7 ng L^{-1}). The slightly higher concentrations of free copper of PR and BB at the LC_{50} may be related to differences in DOC that may affect copper uptake, or alternatively, they could be related to intrinisic properties of these two DOCs, which have positive physiological effects on the organisms.

In ambient clean sea-water samples, a pCu concentration of 11.9 was found in Macquarie Harbour, Tasmania.[17] As well, a pCu of 11.5 was found for clean marine water off the coast of Peru.[61] Toxicity (50 % mortality) was observed for *B. plicatilis* at an average pCu of 10.1, which adds strength to these measurements because toxicity was observed at higher free-copper concentrations than in ambient water samples in which copper toxicity is not observed. In addition, the pCu values found in the present study are consistent with pCu values observed in other copper toxicity assays. In Eriksen et al.,[16] the marine alga *Nitzschia closterium* was found to have decreased growth rates over a pCu range from 11.3 to 8.2. Sunda and Guillard[62] reported reduced growth rates of the marine diatom *Thalassiosira pseudonana* from pCu values of 11.2 to 8.2. As well, seven marine diatom species showed reduced growth rates at pCu values from 10.5 to 11.0.[63]

Free copper is often considered the best indicator of toxicity owing to this species being most available to interact with an organism.[15,16,18] Eriksen et al.[16] reported that free copper measurements directly correlated with growth rate inhibition of the marine diatom *Nitzschia closterium*, whereas anodic stripping voltammetry (ASV) measurements of 'labile' copper (free copper + free copper bound to inorganic ligands) significantly overestimated the toxicity. Stauber et al.[64] found similar results where ASV measurements predicted growth inhibition to *N. closterium* based on the concentrations measured; however, no toxicity was observed. These results lend credence to the theory that free copper is the best estimator of toxicity.

Following this idea, it is proposed that differences in LC_{50} values are due to differences in water chemistry interactions that affect how much total copper is needed to reach the critical free copper concentration required to cause toxicity. For example, in cases where DOC is high, more copper is bound by the DOC and therefore becomes unavailable for uptake; thus, a higher total copper concentration is needed to reach a free copper concentration that causes toxicity. This argument is clearly supported by the measured free copper concentrations accompanying the lowest and highest LC_{50} values obtained in the present study. Hawke's Bay (HB) resulted in the lowest LC_{50} with a value of 333 nM, and MKC had the highest LC_{50} value of 980 nM. However, the free copper measurements for both of these sites were identical, with a pCu of 10.40. This has also been observed for labile copper in the Pacific oyster *Crassostrea gigas*.[55] In this case, the labile copper EC_{50} concentration remained constant at an average concentration of 109 nM, despite total copper EC_{50} ranging from 327 to 638 nM.[33]

The influence of DOC fluorophore components on free copper at the LC_{50}

With respect to the fluorophore components within DOM, there was no significant correlation between the free copper at the LC_{50} and any of the four components (Fig. S7). Overall, the lack of significant trends of DOC characteristics with free copper at the LC_{50} adds strength to the notion that free copper, within variation, is constant regardless of the particular nature of the DOC despite the change in total copper LC_{50}. This reinforces the conclusion that free copper alone is a good predictor of toxicity.

Conclusions

The findings of the present study suggest that DOC is protective against copper toxicity in marine environments, independenlyt of DOC source and quality, which is consistent with De Palma et al.[22] However, the amount of increased protectivity to *Brachionus plicatilis* progressively decreased as DOC increased above 2 mg C L^{-1}. The suggested reason for this plateau effect in LC_{50} as DOC increases is salt-induced colloid formation. The increase in DOC–DOC interactions at high salinity reduces the available binding sites for copper, thereby allowing more copper to be bioavailable to cause toxicity. This is supported by other studies with copper[26,54,55] and lead,[56] which show the same trend. Indeed, qualitatively similar relationships between Cu LC_{50} and DOC have now been observed in several other sensitive marine organisms.[14,22,23,26,51,57] Two marina samples behaved differently than the other samples collected. The slightly higher concentrations of free copper of PR and BB at the LC_{50} may be related to differences in DOC that may affect copper uptake, or alternatively, they could be related to intrinsic properties of these two DOCs that have positive physiological effects on the organisms.[65]

Free copper has been deemed the best predictor of copper toxicity because it is the species most bioavailable. The data here show that regardless of water chemistry, free copper concentrations are fairly constant, within a factor of two, at the LC_{50}. This suggests that a critical free copper concentration is required to cause toxicity; however, the amount of total copper needed to reach this critical concentration changes depending on the water chemistry.

The overall implication of the present study for BLM development is that a source-dependent parameter for DOC is not necessary to include in equilibrium models to predict site-specific LC_{50} values. In addition, the free copper data presented here are consistent with the BLM concept. Future work will consist of applying these methods to a greater variety of natural sea-water samples, ideally in samples with DOC ranges between 2 and 4 mg C L^{-1} or even higher DOC to determine which trend most accurately represents the correlation between LC_{50} and DOC. As well, future studies will further address the influence of DOC source on free copper and toxicity.

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