Evaluating the ameliorative effect of natural dissolved organic matter (DOM) quality on copper toxicity to *Daphnia magna*: improving the BLM

Hassan A. Al-Reasi · D. Scott Smith · Chris M. Wood

Abstract Various quality predictors of seven different natural dissolved organic matter (DOM) and humic substances were evaluated for their influence on protection of *Daphnia magna* neonates against copper (Cu) toxicity. Protection was examined at 3 and 6 mg l^{-1} of dissolved organic carbon (DOC) of each DOM isolate added to moderately hard, dechlorinated water. Other water chemistry parameters (pH, concentrations of DOC, calcium, magnesium and sodium) were kept relatively constant. Predictors included absorbance ratios $Abs_{254/365}$ (index of molecular weight) and $Abs\text{-}octanol_{254}/Abs\text{-}water_{254}$ (index of lipophilicity), specific absorption coefficient (SAC_{340}; index of aromaticity), and fluorescence index (FI; index of source). In addition, the fluorescent components (humic-like, fulvic-like, tryptophan-like, and tyrosine-like) of the isolates were quantified by parallel factor analysis (PARAFAC). Up to 4-fold source-dependent differences in protection were observed amongst the different DOMs. Significant correlations in toxicity amelioration were found with $Abs_{254/365}$, $Abs\text{-}octanol_{254}/Abs\text{-}water_{254}$, SAC_{340}, and with the humic-like fluorescent component. The relationships with FI were not significant and there were no relationships with the tryptophan-like or tyrosine-like fluorescent components at 3 mg C l^{-1}, whereas a negative correlation was seen with the fulvic-like component. In general, the results indicate that larger, optically dark, more lipophilic, more aromatic DOMs of terrigenous origin, with higher humic-like content, are more protective against Cu toxicity. A method for incorporating SAC_{340} as a DOM quality indicator into the Biotic Ligand Model is presented; this may increase the accuracy for predicting Cu toxicity in natural waters.

Keywords *Daphnia magna* · Copper · Dissolved organic matter · Absorbance and fluorescence quality parameters · Biotic ligand model

Introduction

Metal toxicity is a global environmental concern in natural waters. Copper (Cu), for example, is one of the essential micronutrients required for various metabolic processes in living organisms, nevertheless it becomes toxic to many freshwater organisms at higher concentrations (Flemming and Trevors [1989]). According to USEPA's Water Quality Criteria for Copper (U.S. Environmental Protection Agency [2007]), the natural background Cu concentrations may range widely from 0.2 to 30 µg l^{-1} in pristine freshwater and up to 100 µg l^{-1} or more in systems moderately contaminated by anthropogenic inputs, while in mining areas Cu levels may approach 200,000 µg l^{-1}. Toxicological manifestations of Cu have been linked to the interference with sodium (Na^+) regulation and metabolism in freshwater animals (Grosell et al. [2002]; Grosell and Wood [2002]). In natural waters, or the exposure water of laboratory based experiments, the concentration of Na^+ and the presence of Cu-detoxifying agents such as natural dissolved organic matter (DOM) play an important role in determination of the toxic outcome. The influence of water chemistry on Cu toxicity has resulted in the development of models such as the Biotic Ligand Model (BLM), a computer program that uses water chemistry parameters (model inputs) to predict Cu toxicity to

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organisms, and to calculate Cu criteria for freshwater (the model output) (Di Toro et al. 2001; Santore et al. 2001; Niyogi and Wood 2004). Input parameters of water chemistry include pH, concentrations of hardness ions (Ca^{2+} and Mg^{2+}), salinity ions (Na^+ and Cl^- ions), alkalinity, and dissolved organic carbon (DOC) concentrations. The BLM considers cations (e.g. Na^+, Ca^{2+} and Mg^{2+}) as species which compete with Cu ions for binding sites on the biological surface or biotic ligand (Paquin et al. 2002). On the other hand, anions (e.g. Cl^- and SO_4^{2-}) and DOC tend to form Cu-complexes, resulting in reduction of Cu bioavailability.

With dissolved organic carbon (DOC) representing roughly 50% of its elemental composition, DOM is probably the most abundant copper-binding moiety in many freshwaters, exceeding many inorganic components (Thurman 1985). DOM results from the decomposition of lignin rich plant materials (Ertel et al. 1984) and the decay of dead organic remains of animals and microbes (Hatcher and Spiker 1988). The major composition (50–90%) of aquatic DOM is a heterogeneous mixture of fulvic and humic acids (Thurman 1985). The DOC concentrations vary widely, but the typical concentrations in freshwater systems range from 1 to 15 mg C l^{-1} (Thurman 1985). It is well established that the ameliorative effect of DOM against Cu toxicity is concentration-dependent (Erickson et al. 1996; Kramer et al. 2004). Recent studies have indicated that protection may also be source-dependent (e.g. Richards et al. 2001; Ryan et al. 2004; Schwartz et al. 2004), highlighting the possible influences of chemical structure and composition of DOM source on its protective ability.

Typically, aquatic DOMs are classified by origin as terrigenous (DOM produced on land and then washed into the water body, also referred to as allochthonous DOM) or autochthonous (DOM synthesized within the water column) (McKnight et al. 2001) or a mixture of both types. Because of their heterogeneity and structural irregularity, DOM molecules cannot be described in term of unique chemical structures because characterization of individual molecules is unattainable (McDonald et al. 2004). As an alternative, use of spectroscopic measurements has been successful (Senesi et al. 1991; Chin et al. 1994) and has provided several optical indices to distinguish DOM from various freshwater sources. For example, absorbance ratios such as $Abs_{254/365}$ serves as a proxy for molecular weight (Dahlén et al. 1996), specific absorbance coefficient (SAC_{340} = Absorbance at 340 nm × 2.303/DOC) as an aromaticity index (Curtis and Schindler 1997), and fluorescence index (FI_{370} = emission intensity at 450 nm/emission intensity at 500 nm) as an origin indicator (McKnight et al. 2001) have all been used extensively in the characterization literature and can be employed as quality predictors. Parallel factor analysis (PARAFAC) is a new advance in handling three-dimensional fluorescence spectra of DOM which has emerged as powerful approach to probe molecular differences in various DOM sources (Stedmon and Bro 2008; DePalma et al. 2011a). Using PARAFAC, the complex excitation-emission spectra can be decomposed into individual fluorescent signals or fluorophores enabling their qualitative and quantitative description (Stedmon and Bro 2008).

In general, toxicological studies to date indicate that terrigenous DOM is a better ameliorative agent against Cu than autochthonous DOM. The former tends to be optically darker, composed of larger molecules with a higher aromatic content, while the latter is optically lighter, and composed of smaller molecules with lower aromatic content (Richards et al. 2001; Ryan et al. 2004; De Schamphelaere et al. 2004; Schwartz et al. 2004). In addition, characterization and toxicological data recently compiled by Al-Reasi et al. (2011) revealed that fluorophore concentrations obtained by PARAFAC, as another potential DOM quality parameter, explain considerable variability in the protective effects against Cu toxicity.

As a potential detoxifying agent, the role of DOM can be described quantitatively (i.e. DOC concentrations) and qualitatively (i.e. optical characteristics). Although the role of DOM quality towards protection against Cu toxicity has been proposed based on optical properties (e.g. De Schamphelaere et al. 2004), little is known on how DOMs of different qualities determine the protective effect, particularly at similar DOC concentrations. In the currently used predictive toxicity models (e.g. BLM), one quality aspect of DOM which can be changed is the percentage humic acid. However, incorporation and manipulation of the quality factor does not discriminate between DOM from various sources, rather than the default assumption of 10% of DOC being humic acid. Taking water chemistry factors (e.g. pH, hardness ions and DOC concentrations) into consideration, the present study uses a range of natural DOMs plus two commercial DOM preparations to quantitatively explore the influence of several DOM quality parameters on the aquatic toxicity of Cu under environmentally relevant conditions. The three main objectives of the present study were: (i) to show that qualitatively different aquatic DOM sources vary greatly in their ability to ameliorate Cu toxicity; and (ii) to ascertain the physicochemical characteristics (i.e. optical or other indices) that can be used to predict their differential protective abilities and (iii) to test the ability of these physiochemical parameters to improve BLM predictions.

Materials and methods

DOM collection and treatment

To obtain DOM isolates spanning a wide range between autochthonous and terrigenous nature, DOMs were

concentrated from various aquatic environments using a portable reverse osmosis unit. Details about methodology and its applicability to provide representative DOMs of the aquatic systems are discussed in Sun et al. (1995) and De Schamphelaere et al. (2005), respectively. On the sites (Table 1), water was pumped from the source, pre-filtered through 1 μm wound string filters to remove large debris and particulates and collected in pre-washed food-grade plastic buckets. The filtered water was then pumped into the reverse osmosis unit which concentrated organic matter gradually (Sun et al. 1995). In order to remove most metal cations built up during this process, DOM concentrates were treated with cation exchange resin (Amberlite IR-118 (H), Sigma-Aldrich). Briefly, 3.6 l of resin was first pre-washed three times with 6 l of deionized water (18.2 MΩ cm, Millipore Corporation, Billerica, MA, USA), followed by soaking in 0.6 l of 4 N trace metal grade HCl and finally rinsed five times with 2 l of deionized water each time. About 1.5 l of the clean resin was used to rinse 4 l of each DOM isolate. Because of the acidic nature of washed resin, DOM isolates were acidified (pH ≤ 2), and then kept refrigerated in a dark cold room at 4°C, in polyethylene containers until their use for chemical analyses and exposure tests. Table 1 provides information about freshwater aquatic sources and some physicochemical parameters of DOM isolates. Aldrich humic acid, AHA (Sigma-Aldrich Chemical, St. Louis, MO, USA) and Nordic Reservoir DOM, NR (International Humic Substances Society, St. Paul, MN, USA) were the two commercially

available humic substances included along with the aquatic DOM isolates. In contrast, they were obtained as freeze-dried powder and therefore the stock solutions were prepared in deionized water ($\geq$17.5 MΩ cm; Barnstead Nanopure II, Thermo Scientific Barnstead, USA).

DOM and humic substances characterization

Absorbance and fluorescence measurements were carried out to describe the quality parameters of DOMs. Triplicate DOM solutions (10 mg C l^{-1}) were prepared by diluting the DOM concentrates with the appropriate volume of Milli-Q water. Each DOM solution was then adjusted to $\sim$pH 7.0 by using 0.1 M NaOH or 5.0 M KOH. For octanol solubility determination, 75 ml of each adjusted DOM solution was mixed with 25 ml of octanol (Sigma Aldrich, USA) and shaken for 3 h (Gjessing et al. 1999). Then, the aqueous and octanol fractions were separated and left to stand for another 3 h, after which absorption of both fractions were measured at 254 nm. All absorbance and fluorescence scans of the samples were performed in a 1-cm quartz cuvette (Helma Canada Ltd., Concord, ON, Canada). The cuvette was pre-rinsed thoroughly with Milli-Q water, followed by a few washes with DOM sample being measured. The cuvette was always filled using a 10-ml syringe (Becton, Dickinson and Company, BD, Fraklin Lakes, NJ, USA) fitted with a 0.45-μm acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI, USA). For each sample, absorbance and fluorescence scans were

Table 1 Characteristics of DOM isolates and humic substances used in the analysis

DOM source[a]	Coordinates	Type	SAC$_{340}$ (cm^2 mg^{-1})[d]	Abs$^e_{254/365}$	Octanol solubility[f]	Fluorescence index (FI)[g]
Dechlorinated Hamilton water (DC)[b]	–	Tap water isolate	3.72	15.72	0.088	1.75
Lake Ontario (LO)	43°29′N 79°79′W	Autochthonous	4.85	9.75	0.106	2.54
Bannister Lake (BL)	43°30′N 80°38′W	Autochthonous	14.16	6.31	0.024	1.51
Preston Effluent (PE)	43°39′N 80°35′W	Sewage-derived	14.77	5.40	0.058	1.94
Nordic Reservoir (NR)[c]	–	Terrigenous	28.76	4.50	0.012	1.21
Luther Marsh (LM)	43°37′N 80°26′W	Terrigenous	39.30	3.72	0.016	1.19
Aldrich humic acid (AHA)[c]	–	Coal-derived	79.98	2.53	0.030	0.83

[a] Sorted based on increasing specific absorbance coefficient (SAC$_{340}$)

[b] The source for dechlorinated Hamilton tap water is Lake Ontario. The tap water isolate was different from the Lake Ontario isolate due to removal of most of organic matter during purification processes and addition of organic matter from activated charcoal during dechlorination

[c] Commercially-available humic substances obtained by freeze-drying

[d] Proxy for aromaticity of DOM molecules, the specific absorbance coefficient (SAC$_{340}$) = (2.303 × absorbance at 340 nm)/DOC (Curtis and Schindler 1997)

[e] Proxy for molecular weight of DOM molecules, calculated as ratio of absorbance at 254 nm to that at 365 nm (Dahlén et al. 1996)

[f] Proxy for lipophilicity of DOM molecules, the ratio of absorbance at 254 nm in octanol phase to water phase (Abs-octanol$_{254}$/Abs-water$_{254}$) (Gjessing et al. 1999)

[g] An index of DOM source, the fluorescence index (FI) = emission intensity of 450 nm/emission intensity of 500 nm, both taken at excitation at 370 nm (McKnight et al. 2001)

conducted using a Varian Cary 50 UV/visible spectrophotometer and Varian ECLIPSE Cary fluorescence spectrophotometer (Varian Incorporation, Old Oak, NJ, USA), respectively. For absorption of the samples, measurements were determined for the wavelength range of 200–800 nm, while for their fluorescence, the excitation wavelengths were between 200 and 450 nm with 10-nm increments and emission intensities were collected for wavelengths of 250–600 nm every 1-nm increment. For excitation–emission fluorescence measurements, Milli-Q water, standard solutions of tryptophan (0.5 μM) and tyrosine (1.0 μM) (Sigma Aldrich, St. Louis, MO, USA) and a mixed solution (10 mg C l^{-1} of a well-characterized DOM isolate plus 0.5 μM tryptophan and 1.0 μM tyrosine) were scanned along with the samples as a control procedure to check for instrument drift.

As quality predictors, absorption ratios ($Abs_{254/365}$ and Abs-octanol$_{254}$/Abs-water$_{254}$) were utilized as indicators of the molecular weight and the lipophilic nature of DOMs as described by Dahlén et al. (1996) and Gjessing et al. (1999), respectively. The aromatic composition of DOMs was estimated by SAC_{340} according to Curtis and Schindler (1997). The fluorescence index (FI) was used as an indicator of DOM origin as suggested by McKnight et al. (2001). Excitation–emission matrices (EEMs) obtained from fluorescence measurements were contour-plotted to identify the fluorescent components or peaks present in each samples. Visual interpretation of contour surfaces revealed the presence of four prominent component peaks, two at shorter emission wavelengths ($\sim$300 and 350 nm) and two at longer wavelengths ($\sim$410 and 460 nm). DOM fluorescence is characterized by broad intensities when excited between 200 and 450 nm (Senesi et al. 1991). The longer emission wavelengths are usually labelled as fulvic-like and humic-like components, whereas the emission spectra of shorter wavelengths are labelled tryptophan-like and tyrosine-like fluorophores (DePalma et al. 2011a). To quantify relative abundance of each component for every sample, the EEMs were modeled using PARAFAC as implemented in the PLS toolbox (Eigenvectors Research Inc, WA, USA) which resulted in score vectors as estimates of relative concentrations.

Test organisms

The original *Daphnia magna* adults were purchased from Aquatic Research Organisms (ARO, Hampton, NH, USA). Neonates (< 24 h old) were obtained from adults cultured for several generations in the laboratory under the same constant conditions used in the tests: dechlorinated Hamilton city tap water ([Na$^+$] = $\sim$0.7 mM, [Ca^{2+}] = $\sim$1.0 mM, [Mg^{2+}] = $\sim$0.3 mM, [DOC] = 2.5 ± 0.4 mg l^{-1}, pH 7.5–8.0, at 23°C with a 12 h light: 12 h dark photoperiod. Daphnia were fed unicellular green algae (*Selenastrum capricornutum*) and YCT (Yeast, CERO-PHYLL®, and Trout chow) in a 2:1 ratio. Neonates were used in all tests and not fed during the duration of the experiment (48 h).

Experimental exposures

Standard 48-h acute toxicity tests were conducted in the dechlorinated water alone (no DOM added) or in dechlorinated water with added DOMs or commercial humic substances. Prior to the start of experimentation, neonates (<24 h old) were collected in fresh dechlorinated water and 10 of them were transferred individually into 100-ml glass exposure chambers with a minimum amount of water to avoid changing the target Cu and DOC concentrations. At the end of exposure (48 h), dead neonates were counted and recorded for each test chamber.

Extracts of the five aquatic DOMs and commercial humic acid and Nordic Reservoir DOM were added to dechlorinated Hamilton tap water to obtain two nominal DOC concentrations (3 and 6 mg C l^{-1}). For each treatment, a control and five nominal concentrations (3.2, 10, 18, 32, 100 and/or 320 μg l^{-1}) of Cu stock solutions (40 and 160 mg Cu l^{-1} solutions prepared from CuSO$_4$·5H$_2$O; J.T. Baker, Phillipsburg, NJ, USA) were prepared for the dechlorinated water alone and dechlorinated water with DOM. Eight replicates per concentration were conducted for tests in dechlorinated tap water alone and three to four replicates for dechlorinated water with DOM added. As recommended by Glover et al. (2005a), solutions were prepared at least 24 h prior to start of experimentation and stored at 4°C in the dark. pH was checked (Accumet® Basic AB15 pH meter, Fisher Scientific) and adjusted, if necessary back to 7.5–8.0, using 5.0 or 1.0 *M* KOH. Exposure solutions were sampled for total and dissolved Cu, as well as DOC, Ca^{2+}, Mg^{2+} and Na$^+$ concentrations before the start and at the end of exposure. Water samples for total Cu were acidified immediately with trace metal HNO$_3$ and those for dissolved Cu, DOC, Ca^{2+}, Mg^{2+} and Na$^+$ were filtered through the 0.45-μm Acrodisc® syringe filter and then acidified right away. All samples were then kept refrigerated in a cold room (4°C) until analyses.

Chemical analyses

For each analytical instrument, blank reagents and calibration standards were prepared according to specifications stated in the instrument user manual, and the validity of the calibration curve and reproducibility were assessed every 12 samples using a standard solution of the analyte being measured. Total and dissolved Cu concentrations were measured using graphite furnace atomic absorption spectrometry (SpectroAA220, Varian, Mulgrave, Australia).

During determinations, the nominal and measured values for the certified analytical reference materials (fortified waters for trace metals known as TM-15, TM-24.3 and TM-25.3, National Research Council Canada, Ottawa, ON, Canada, made with diluted, filtered Lake Ontario water as a natural water matrix with 0.2% nitric acid) were 18.20 ± 1.99 and 17.57 ± 2.95 µg Cu l^{-1} ($n = 41$, recovery confidence interval, CI 92.5–101.6%), 6.79 ± 0.64 and 5.91 ± 0.62 µg Cu l^{-1} ($n = 45$, CI 84.3–89.7%), 27.60 ± 2.84 and 25.09 ± 1.58 µg Cu l^{-1} ($n = 47$, CI 89.3–92.6%), respectively. Cupric ion activity was measured using an Orion cupric ion selective electrode with a separate double junction Ag/AgCl reference electrode, and the method of Rachou et al. (2007) modified using a flow through cell as per Eriksen et al. (1999). Ethylene diamine buffers at variable pH and fixed copper activity were utilized to calibrate the electrode. The total DOC concentration was measured directly using a Shimadzu TOC-V$_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). The reproducibility of the TOC analyzer using standard total carbon solutions of 5 and 10 mg l^{-1} (prepared from potassium hydrogen phthalate) yielded 5.26 ± 0.66 mg C l^{-1} ($n = 44$) and 10.24 ± 0.43 mg C l^{-1} ($n = 77$). The concentrations of Ca^{2+}, Mg^{2+} and Na^+ were determined by flame atomic absorption spectrometry (SpectroAA220FS, Varian, Mulgrave, Australia). The 50 µM Ca standard solution yielded 51.00 ± 2.57 µM ($n = 22$), the 25 µM Mg standard solution gave 25.80 ± 2.13 µM ($n = 25$) and the 50 µM Na standard solution yielded 50.10 ± 0.94 µM ($n = 40$). A major focus of this study was evaluating and improving the BLM, so water chemistry (apart from DOC) has been reported using molar units rather than g l^{-1} units. Regardless, the units can be easily converted and expressed in µg or mg l^{-1} through knowledge of molecular weight of the particular ion.

LC_{50} calculation and statistical analyses

For each nominal Cu concentration, the averaged values of total and dissolved Cu at the beginning and the end of exposure were used for LC_{50} determination. The 50% lethal concentrations (LC_{50}, Cu concentrations required to kill five neonates in each concentration chamber) were calculated using probit analysis with the trimmed Spearman–Karber method (Hamilton et al. 1977). An LC_{50} point estimate was determined for each replicate and then LC_{50} values were averaged for determinations in presence of each specific DOM. All values have been reported as mean ± standard deviation.

Data were statistically analyzed using SPSS Statistics 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Cu LC_{50} data were first checked for normality of distribution and homogeneity of variance using the Shapiro–Wilk test and Levene's test, respectively. Data points were log-transformed when they violated these assumptions. Student's t tests were applied to check for the difference in LC_{50} at 3 and 6 mg C l^{-1} of the same DOM and one-way analysis of variance (ANOVA) was employed to detect variation in LC_{50} in various DOM. The ANOVA was followed by the post hoc Tukey test. The relationship between each DOM quality predictor and Cu toxicity measure (i.e. LC_{50} values) was analyzed by linear regression.

Principal component analysis (PCA) was used to explore the DOM quality predictors, summarize the pattern of intercorrelations among them, and identify those accounting for most variance in data. Quality predictors (SAC_{340}, $Abs_{254/365}$, humic-like fluorophore concentration and FI) were identified and retained by the PCA and therefore they were included in the multiple regression models. From a statistical point of view, both approaches (PCA and multiple regression) work best when the variables are neither highly correlated (i.e. $r \geq 0.9$) nor not correlated at all. For multiple regression models, data for AHA were excluded from the analysis based on statistical criteria as outliers, especially for SAC_{340} data, and the fact that AHA as a humic substance is not a real aquatic DOM (Malcolm and MacCarthy 1986). Since SAC_{340} is supported by previous research for its strong influence on Cu toxicity (De Schamphelaere et al. 2004; Richards et al. 2001; Ryan et al. 2004; Schwartz et al. 2004), the method for placing SAC_{340} in models was the forced entry, while backward stepwise entry was used for the other variables, according to the guidance of Field (2005). Although octanol solubility showed no correlation with LC_{50} in the presence of 3 mg C l^{-1}, this quality parameter was also added to the aforementioned predictors based on its significant relationship with LC_{50} at 6 mg C l^{-1}. Little is actually known about the role of $Abs_{254/365}$, humic-like fluorophore concentration, FI and octanol solubility. Accordingly, the backward method places all predictor variables in the model and calculates the contribution of each one by looking at the significance value of the t test for each predictor (Field 2005). If the predictor is not making a statistically significant contribution to how well the model predicts the outcome (i.e. LC_{50}), it is removed from the model and the model is re-estimated for the remaining predictors (Field 2005). The degree of significance for all statistical analyses was established at the 0.05 level.

LC_{50} prediction using the BLM

One of the DOM quality parameters which is already included in the current BLM (Windows version 2.2.3, HydroQual Inc.) and which can be modified in the BLM interface is the humic acid percentage (%HA; the default recommendation is 10% HA). As explained in detail in the "Results and discussion" section, we optimized the BLM

for our data set, and inserted estimates of % HA derived directly from PARAFAC, or indirectly through SAC versus PARAFAC-estimated %HA relationships into the BLM with the goal of improving its predictive ability. Measured inputs included pH, concentrations of Na^+, Ca^{2+}, Mg^{2+} and DOC of the exposure waters of the present study. The other inputs (Cl^-, SO_4^{2-}, K^+, alkalinity and total sulphate) were taken from Mann et al. (2004) for dechlorinated Hamilton tap water.

Results and discussion

Cu toxicity as a function of DOC and other water chemistry parameters

Figure 1a presents the 48-h dissolved LC_{50} values of Cu for *D. magna* in the presence of the various DOM sources listed in Table 1. The toxicity varied greatly (approximately 4-fold) from one source to another at the same nominal DOC concentrations. At background DOC concentrations (2.52 ± 0.44 mg l^{-1}) in dechlorinated water, the control LC_{50} averaged 19.1 ± 4.9 and 18.2 ± 5.2 µg l^{-1} for total and dissolved Cu, respectively. Addition of 3 mg C l^{-1} of various exogenous DOMs to the dechlorinated water raised total Cu LC_{50} to values ranging from 26.4 µg l^{-1} (DOM isolate from the dechlorinated tap water, DC) to 160.4 µg l^{-1} (Luther Marsh, LM). Similar trends were observed for the dissolved Cu LC_{50} values (range: 23.4–160.1 µg l^{-1}) which accounted on average for 95% of the total Cu LC_{50} values. Overall, doubling the nominal amount of DOC added to the dechlorinated water from each isolate resulted in a strong trend for increased dissolved LC_{50} (Fig. 1a); the elevation in LC_{50} at the higher DOC concentration was significant for 5 of the 7 different DOMs tested, the only exceptions being those from Lake Ontario (LO) and Luther Marsh (LM) (Fig. 1a). Figure 1b shows Cu ion activity measurements obtained at the total Cu LC_{50} concentrations for each DOM isolate at nominal DOC concentration of 3 mg C l^{-1}. The Cu ion activity at the LC_{50} in presence of all DOMs was similar (ANOVA, $F = 1.92$, $P = 0.13$) to that of the control (Fig. 1b), suggesting that the unifying feature of the different DOM types and concentrations was their ability to bind sufficient copper such that a relatively constant Cu ion activity prevailed so as to cause 50% toxicity at the LC_{50} in the different tests. This observation is consistent with the assumptions of the BLM protection by complexation.

The addition of exogenous DOM isolates has the potential to change other water chemistry parameters which may alter aquatic Cu toxicity, including the ionic composition and pH (Erickson et al. 1996). However, both at 3 or 6 mg C L^{-1} of added DOM, the mean measured

values for pH and concentrations of Na^+, Ca^{2+}, Mg^{2+} and DOC did not vary substantially among toxicity tests in dechlorinated water with added DOM from each source (Table 2). The single exception was Preston Effluent (PE) where the DOM was isolated from a sewage treatment outflow, and Na^+ concentrations were elevated approximately 8 fold (probably a reflection of road salting). However in subsequent analyses (see below), this treatment was never an outlier, so the influence of this Na^+ elevation appears to have been negligible. Given the observed similarity in water chemistry of the exposure water in all other treatments, variability in Cu LC_{50} to *D. magna* supports the source-dependent phenomenon reported earlier by Richards et al. (2001), Ryan et al. (2004), Schwartz et al. (2004) and De Schamphelaere et al. (2004). In the following sections, the absorbance and fluorescence indices as quality predictors of DOMs are explored and discussed for their influence on the protective effect of DOMs against Cu toxicity (Cu LC_{50}) to *D. magna*.
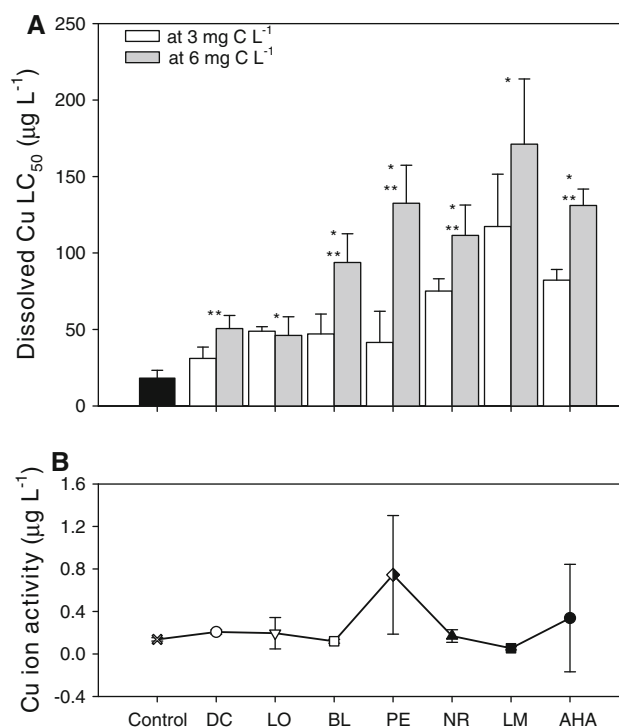
Fig. 1 a The 48-h 50% lethal concentrations (LC_{50}) of dissolved copper to *D. magna* in the presence of DOMs from various aquatic environments. **b** Copper ion activity measurements obtained at the total Cu LC_{50} concentrations for each DOM isolate at a nominal DOC concentration of 3 mg l^{-1}. The measured DOC concentrations are reported in column 3 of Table 2. The *bars* represent mean ± standard deviation. Refer to Tables 1 for abbreviations of DOMs. * Specifies significant difference from control while ** indicates significant difference between 3 and 6 mg C l^{-1} concentrations of the same DOM

Table 2 pH, dissolved organic carbon (DOC) concentration, sodium, calcium and magnesium concentrations of control (dechlorinated tap water alone) and dechlorinated water with added dissolved organic matter (DOM) on copper toxicity to *Daphnia magna* added at two nominal concentrations of 3 and 6 mg C l⁻¹ of each DOM isolate

Treatment	N^a	pH	DOC (mg l⁻¹)	Na⁺ (µM)	Ca²⁺ (µM)	Mg²⁺ (µM)
Control (dechlorinated water alone)[b]	8	7.75 ± 0.30	2.52 ± 0.44	686 ± 64	947 ± 25	345 ± 8

Dechlorinated water with added DOM isolate	At nominal DOC concentration of 3 mg C l⁻¹						At nominal DOC concentration of 6 mg C l⁻¹					
	n	pH	DOC (mg l⁻¹)	Na⁺ (µM)	Ca²⁺ (µM)	Mg²⁺ (µM)	n	pH	DOC (mg l⁻¹)	Na⁺ (µM)	Ca²⁺ (µM)	Mg²⁺ (µM)
Dechlorinated water (DC)	3	7.73	5.63	753	954	528	3	7.93	8.15	846	1009	619
Lake Ontario (LO)	3	7.69	4.91	832	1067	451	3	7.59	7.13	931	1260	557
Bannister Lake (BL)	4	7.51	4.94	613	934	349	3	7.48	9.36	819	978	355
Preston Effluent (PE)	3	7.50	4.74	5442	1262	522	4	7.47	7.33	4463	1536	669
Nordic Reservoir (NR)	3	7.71	4.72	745	941	348	3	7.76	7.57	747	829	316
Luther Marsh (LM)	4	7.86	5.48	669	931	346	3	7.73	8.57	810	925	358
Aldrich humic acid (AHA)	4	7.58	4.36	805	949	352	3	7.83	6.99	802	939	346

[a] Number of LC50 determinations

[b] Values are mean ± standard deviation

Absorbance indices

The ratio of the absorbances at 254 and 365 nm (Abs$_{254/365}$; *index of molecular weight*) is independent of DOC concentration and is considered to be inversely related to molecular weights of DOMs (Dahlén et al. 1996). Accordingly, the wide range of this absorbance ratio seen for DOM samples included in the analysis (Table 1) may suggest lower molecular weights for the autochthonous DOMs and higher molecular weights for terrigenous DOMs. The relationships between this quality parameter and total (Table 3) or dissolved Cu LC$_{50}$ (Fig. 3) were not significant in the presence of 3 mg C l⁻¹ of added DOMs. On the other hand, the relationships became significant at 6 mg C l⁻¹ (Table 3; Fig. 2). A high ratio of Abs$_{254/365}$ (i.e. smaller DOM molecules) was linked to high Cu toxicity while a low ratio (i.e. larger DOM molecules) corresponded with low Cu toxicity in the presence of 6 mg C l⁻¹ of DOMs. Overall, these results indicate that DOMs of higher molecular weights may have more capacity to decrease Cu toxicity to *D. magna* than those of lower molecular weights.

The absorbance ratio of DOM in octanol versus water at 254 nm (Abs-octanol$_{254}$/Abs-water$_{254}$; *octanol solubility*) provides an operational definition of the lipophilicity of a particular DOM (Gjessing et al. 1999). This physicochemical property determines the tendency of DOM molecules to be associated with other organic materials including biological membranes. The 9-fold range of octanol solubilities of DOMs (0.012–0.106) included in the present study (Table 1) is lower than the range (0.09–3.06) recorded by Gjessing et al. (1999) for DOM samples from 8 Norwegian reservoirs. This index was significantly correlated with toxicity when DOMs were added at 6 mg C l⁻¹ (Table 3; Fig. 3). Higher total and dissolved Cu LC$_{50}$ values were found in the presence of relatively less lipophilic DOMs (AHA, LM, and NR) compared to those in relatively more lipophilic ones (DC, and LO). However, as with Abs$_{254/365}$, the relationships were not significant at 3 mg C l⁻¹ (Table 3; Fig. 3). Campbell et al. (1997) demonstrated direct binding of DOMs to the surface membranes of living cells (algae and isolated fish gill cells), an effect with more pronounced magnitude at lower water pH and higher DOC concentration. There is some evidence for uptake of small Cu–organic complexes into aquatic animals and diffusion through their membranes has been observed (Daly et al. 1990; Playle et al. 1993; Marr et al. 1999). Overall, these results suggest possible uptake of Cu-DOM complexes and probably more available Cu ions in the interface boundary microenvironment between the organism and its surrounding water, especially at higher DOC levels, when octanol solubility is high.

Specific absorbance coefficient (SAC$_{340}$) of the DOMs, considered an index of aromaticity (Curtis and Schindler

Table 3 Linear regression parameters for the influence of some physicochemical parameters on copper toxicity (LC_{50}) to *Daphnia magna* in the presence of dissolved organic matter (DOM) added at nominal concentrations of 3 and 6 mg C l^{-1} to dechlorinated Hamilton tap water
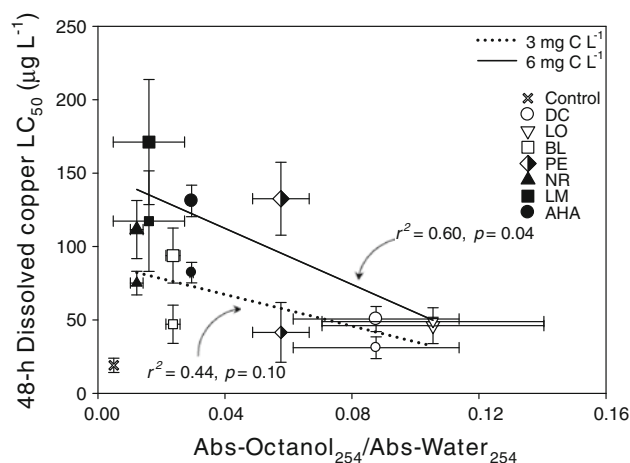
Quality predictors	DOC added at	Total Cu LC_{50}				Dissolved Cu LC_{50}			
		Slope	Intercept	r^2	P	Slope	Intercept	r^2	P
Molecular weight index ($Abs_{254/365}$ ratio)	3 mg l^{-1}	−5.0	101.0	0.52	0.07	−4.6	94.7	0.48	0.08
	6 mg l^{-1}	−9.1	171.3	0.73	0.01*	−8.3	162.0	0.68	0.02*
Octanol solubility ($Abs\text{-}254_{octanol}/Abs\text{-}254_{water}$)	3 mg l^{-1}	−537.6	92.4	0.40	0.12	−538.4	88.9	0.44	0.10
	6 mg l^{-1}	−1012.9	157.3	0.61	0.04*	−955.1	150.6	0.60	0.04*
Specific absorption coefficient, SAC_{340nm}	3 mg l^{-1}	2.0	26.0	0.85	<0.01*	2.1	23.0	0.87	<0.01*
	6 mg l^{-1}	3.1	46.8	0.83	0.01*	3.1	47.0	0.78	0.02*
Relative humic-like fluorophore concentration	3 mg l^{-1}	0.7	25.0	0.79	<0.01*	0.7	24.5	0.74	0.01*
	6 mg l^{-1}	1.2	42.8	0.85	<0.01*	1.0	46.1	0.75	0.01*
Relative fulvic-like fluorophore concentration	3 mg l^{-1}	−1.0	99.3	0.83	<0.01*	−0.9	93.4	0.77	<0.01*
	6 mg l^{-1}	−1.4	153.9	0.67	0.02*	−1.2	143.3	0.54	0.06
Fluorescence index (FI)	3 mg l^{-1}	−38.4	127.0	0.48	0.08	−33.9	116.5	0.41	0.12
	6 mg l^{-1}	−63.3	208.4	0.56	0.05	−53.0	188.4	0.43	0.11
Relative tryptophan-like fluorophore concentration	3 mg l^{-1}	−2.2	82.9	0.32	0.19	−2.1	78.1	0.29	0.21
	6 mg l^{-1}	−5.0	145.4	0.61	0.02*	−4.9	140.4	0.72	0.02*
Relative tyrosine-like fluorophore concentration	3 mg l^{-1}	−2.7	78.2	0.28	0.22	−2.5	74.1	0.28	0.22
	6 mg l^{-1}	−6.2	135.7	0.66	0.03*	−5.9	130.5	0.66	0.03*

* Indicates significant ($P < 0.05$) relationship
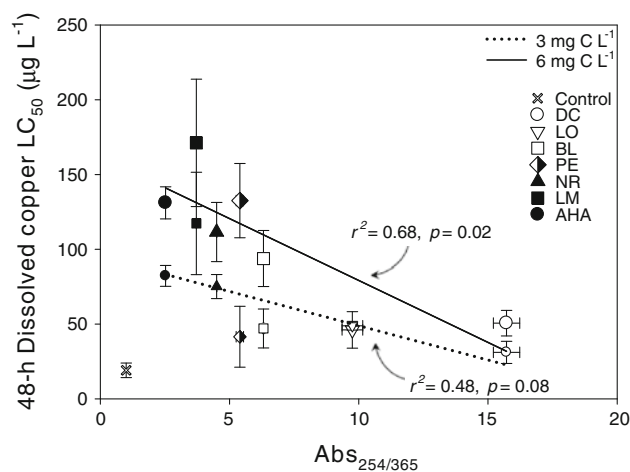
Fig. 2 Influence of molecular weight of DOM molecules, as estimated by $Abs_{254/365}$ ratio, on copper toxicity to *D. magna* in the presence of DOMs from various aquatic environments added at 3 and 6 mg l^{-1}. The values represent mean ± standard deviation. Refer to Tables 1 and 3 for abbreviations of DOMs and regression parameters, respectively. The control data were not included in the regression analysis. The larger symbols are for data obtained in the presence of 6 mg C l^{-1}


Fig. 3 Influence of octanol solubility of DOM molecules, an index of lipophilicity, calculated as $Abs\text{-}octanol_{254}/Abs\text{-}water_{254}$ ratio, on copper toxicity to *D. magna* in the presence of DOMs from various aquatic environments added at 3 and 6 mg l^{-1}. The values represent mean ± standard deviation. Refer to Tables 1 and 3 for abbreviations of DOMs and regression parameters, respectively. The control data were not included in the regression analysis. The larger symbols are for data obtained in the presence of 6 mg C l^{-1}

1997), varied widely between 3.72 (dechlorinated Hamilton tap water isolate, DC) to 79.98 cm^2 mg^{-1}(Aldrich humic acid, AHA) (Table 1), indicating highly variable contents of ringed chromophores. Lower SAC_{340} values were found for autochthonous DOMs while higher values were seen for

terrigenous DOMs (Table 1). Strong and significant correlations were observed between SAC_{340} as the quality predictor and dissolved Cu LC_{50} in the presence of both 3 and 6 mg C l^{-1} (Fig. 4). Similar observations were noted for the relationship of the total Cu LC_{50} versus the aromatic
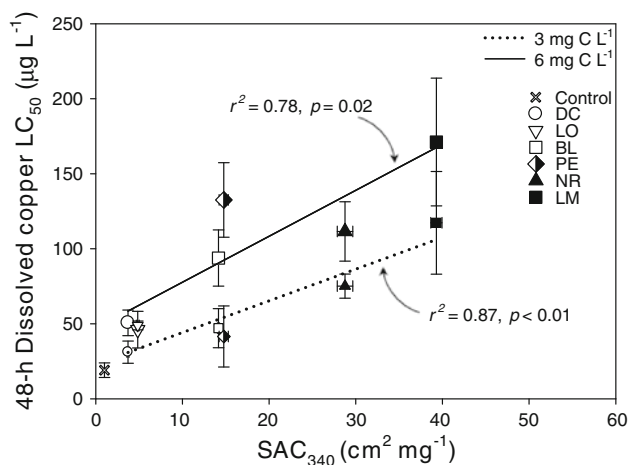
Fig. 4 Influence of aromaticity of DOM molecules, estimated by SAC_{340}, on copper toxicity to *D. magna* in the presence of DOMs from various aquatic environments added at 3 and 6 mg l^{-1}. The values represent mean ± standard deviation. Refer to Tables 1 and 3 for abbreviations of DOMs and regression parameters, respectively. The control data were not included in the regression analysis. The larger symbols are for data obtained in the presence of 6 mg C l^{-1}

composition index at both DOC levels (Table 3). This implies high effectiveness of darkly colored DOMs in alleviation of Cu toxicity compared to the lightly colored DOMs. Previous research has recognized this property as a fundamental characteristic not only in distinguishing aquatic DOMs from various sources but also in affecting their ameliorative effects toward Cu toxicity to several aquatic animals (De Schamphelaere et al. 2004; Richards et al. 2001; Ryan et al. 2004; Schwartz et al. 2004).

Fluorescence indices

Fluorescence index (*FI*) is a simple characteristic providing information about the source or origin of DOM isolate. It has a value of ~1.9 for DOM which is microbially derived within the water column (i.e. autochthonous) and approximately ~1.4 for DOM which is terrestrially derived principally from lignin degradation (i.e. terrigenous) (McKnight et al. 2001). The DOMs from Lake Ontario (LO) and Preston effluent (PE) had FI magnitudes close to or exceeding 1.9, implying an exclusive microbial origin of the organic matter in these isolates. Values of 1.19 and 1.21 for isolates from Luther Marsh (LM) and Nordic Reservoir (NR), respectively, designated their terrestrial origin according to FI. However, the relationships between this quality predictor and acute Cu toxicity to *D. magna* were not statistically significant for both total and dissolved LC$_{50}$ at either 3 mg C l^{-1} or at 6 mg C l^{-1} (Table 3). Nevertheless, autochthonous DOMs (BL and LO) appeared less protective, as judged by LC$_{50}$ values (Fig. 1a), than terrigenous DOMs (LM and NR).

Fluorescent components refer to DOM molecules that produce a specific intensity characterized by a function of excitation wavelength on one axis and emission wavelength on the other in excitation-emission spectra. Besides classical fractionation based on pH, this is another operational characterization to overcome the heterogeneity and molecular irregularity of DOM molecules. Here, four fluorescent components or fluorophores were identified and characterized qualitatively and quantitatively by PARA-FAC (Stedmon and Bro 2008) using the methods presented in DePalma et al. (2011a). As resolved by this statistical technique, contour plots of humic-like, fulvic-like, tryptophan-like and tyrosine-like fluorophores and their relative concentrations in the DOMs and AHA (normalized to DOC concentration) are shown in Fig. 5. For excitation wavelengths of 200–275 nm, fluorescence of proteinaceous materials occurred at emission wavelengths of ~350 nm for tryptophan-like and ~300 nm for tyrosine-like. For humic substances, molecules labelled fulvic-like produced emission intensity at ~410 nm emission and those labelled humic-like produced emission intensity at ~475 nm for excitation wavelengths of 250–350 nm. These excitation-emission wavelength pairs are similar to those reported by Baker (2001) for riverine DOM samples.

The relative content of these fluorescent molecules differed greatly among DOMs included in the analysis (Fig. 5). For humic substances, terrigenous DOMs (NR and LM) were composed mainly of fluorescent organic molecules of moderately longer wavelengths (humic-like) compared to autochthonous DOMs (DC, LO), chiefly made of fulvic-like fluorophores (Fig. 5a). Another characteristic feature of autochthonous DOMs (DC, LO, BL), revealed by quantification using PARAFAC, was their higher proportions of proteinaceous materials (Fig. 5b), which is considered a distinctive feature of microbially generated organic matter.

At 3 and 6 mg C l^{-1}, a higher protective effect against Cu toxicity (i.e. higher dissolved Cu LC$_{50}$) in the presence of terrigenous DOMs was strongly correlated to higher relative amounts of humic-like fluorophores (Fig. 6). Humic substances are higher molecular weight than fulvics, thus this observation is consistent with the observed trend that higher molecular weight DOMs seems to be more protective (Fig. 2). Equally, relative humic-like fluorophore concentrations were positively and strongly correlated with total Cu LC$_{50}$ when DOMs were added at 3 and 6 mg C l^{-1} (Table 3). Conversely, the negative relationship between Cu LC$_{50}$ and fulvic-like fluorophore concentration highlighted the lower protective actions of DOMs relatively enriched with this fluorescent component (Table 3) at the same DOC concentrations. The exception was the relationship for the dissolved Cu LC$_{50}$ that was not significant at 6 mg C l^{-1} (Table 3). Ryan et al. (2004) fractionated DOMs into humic acid (insoluble below pH 2.0) and fulvic acid portions
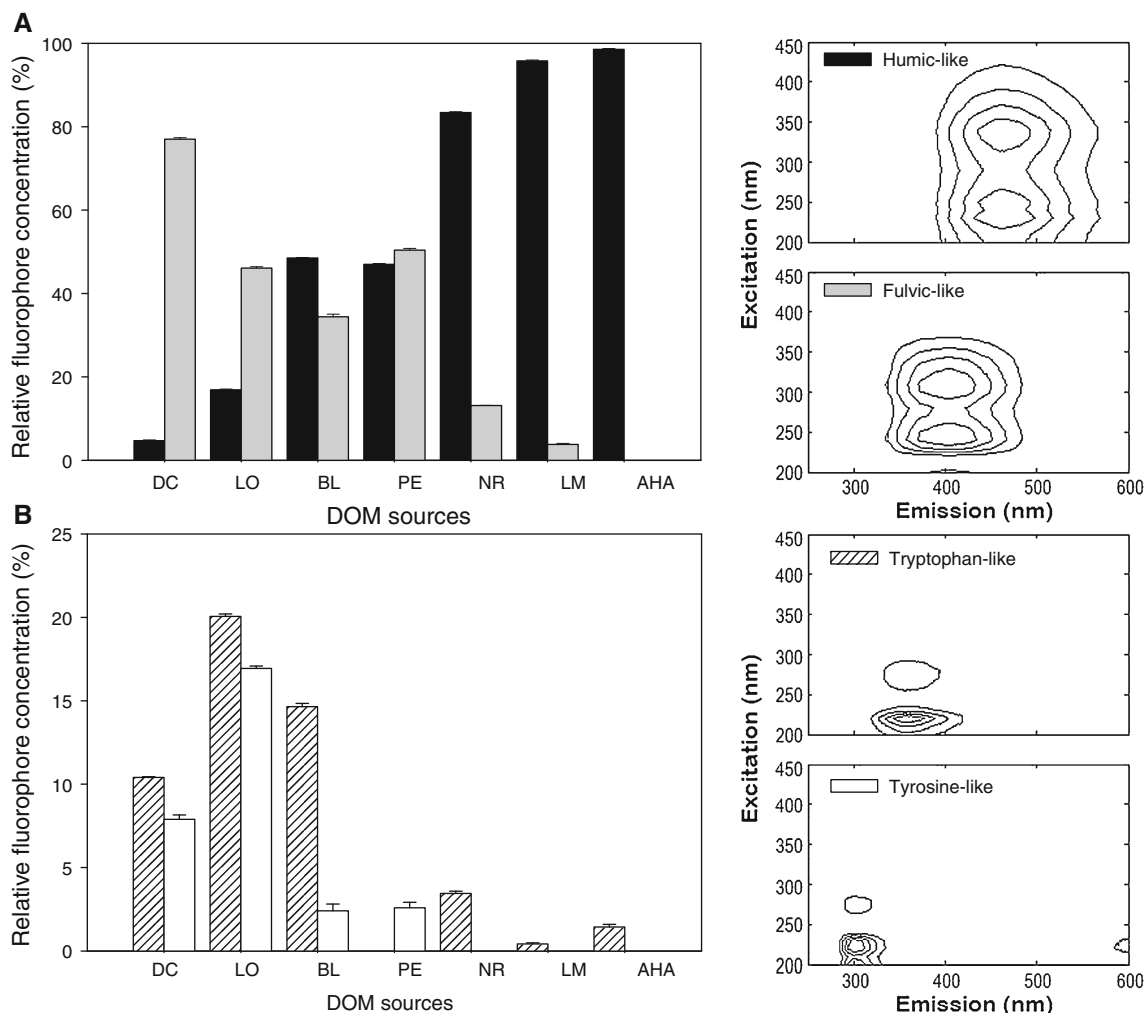
Fig. 5 The relative concentrations of humic-like, fulvic-like, tyrosine-like, and tryptophan-like components in each DOM isolate resolved by PARAFAC. Refer to *right hand panels* for bar codes. The *right-hand panel* of **a** shows the two fluorescence components (fulvic-like and humic-like) with longer emission wavelengths ($\sim$410 and 475 nm) and that of **b** illustrates the two fluorescence components (tryptophan-like and tyrosine-like) at shorter emission wavelengths ($\sim$300 and 350 nm). Refer to Table 1 for abbreviations

(soluble below pH 2.0) and observed that DOMs with higher humic acid concentrations provided greater protective effects against Cu toxicity to larval fathead minnows than did samples with relatively lower humic acid concentrations. Quite the opposite, when dissolved in seawater, freshwater DOMs with higher content of PARAFAC quantified fulvic acid were found to offer more protection against Cu toxicity to marine mussel larvae (Nadella et al. 2009). The reason for this difference is unclear, but may relate to either the effect of high ionic strength on the conformation of DOM molecules, or the effect of high cation concentrations on their Cu binding sites.

No significant relationships were observed between the relative tryptophan and tyrosine-like components of DOMs and Cu LC_{50} values at 3 mg C l^{-1}, but they were significant in the presence of 6 mg C l^{-1} (Table 3). This may suggest that these components do not participate in the protective action against Cu toxicity at lower concentrations but probably become involved as DOC concentration increases. In contrast, DePalma et al. (2011b) observed no effect of these components of marine organic matters of diverse origin against Cu toxicity for marine mussel larvae.

Principal component analysis (PCA) and regression models to explore quality predictors of DOM protective ability

As noted above, SAC_{340}, $Abs_{254/365}$, humic-like fluorophore concentration, FI and octanol solubility (Abs-octanol$_{254}$/Abs-water$_{254}$) were the quality parameters identified and retained by the PCA. Exclusion of other quality variables (fluorophore concentrations of fulvic-like, tryptophan-like and tyrosine-like) was a necessary step, not only to get a valid and reliable number of components accounting for
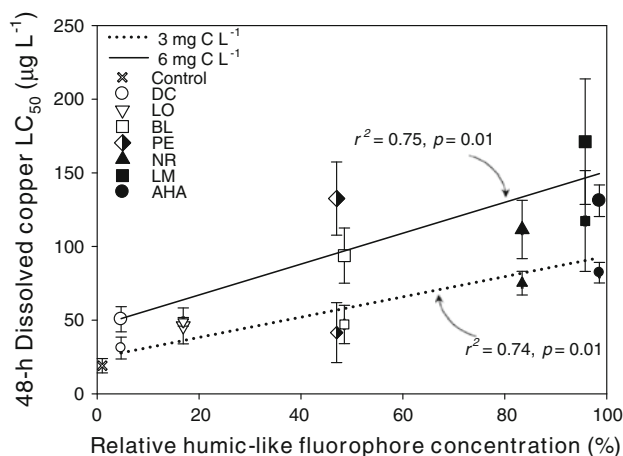
Fig. 6 The influence of relative humic-like fluorophore concentration, resolved by PARAFAC, of DOMs from various aquatic environments added at 3 and 6 mg l^{-1}, on copper toxicity to *D. magna*. The values represent mean ± standard deviation. Refer to Tables 1 and 3 for abbreviations of DOMs and regression parameters, respectively. The control data were not included in the regression analysis. The larger symbols are for data obtained in the presence of 6 mg C l^{-1}

most variance in DOM qualities, but also to overcome high multicolinearity, a fundamental assumption for the PCA. However, only one component factor resulted from this analysis and explained 82.2% of the variance, suggesting that they all are probably influenced by a common underlying dimension (i.e. the presence of aromatic moieties). The multiple regression models revealed that most variation in Cu toxicity to *D. magna* could be explained by SAC_{340} for the dissolved LC_{50} in the presence of 3 mg C l^{-1} ($r^2 = 0.87$, $P = 0.006$) and at 6 mg C l^{-1} ($r^2 = 0.79$, $P = 0.02$). Similarly, SAC_{340} accounted for most proportions (86 and 83%) of variance in the total LC_{50} at 3 and 6 mg C l^{-1}, respectively. The inclusion of the other variables could not improve the prediction of Cu toxicity beyond the point accounted for by SAC_{340}. Nevertheless, toxicity measures (i.e. total and dissolved Cu LC_{50}) were significantly correlated with several DOM quality predictors (i.e. absorbance and fluorescence indices) (Table 3; Figs. 2, 3, 4 and 6), when the impacts of predictors were assessed individually. Large molecular structures with an enriched aromatic composition of optically dark components exhibited more effective ameliorative action than smaller DOM molecules. In contrast, lower alleviation of Cu toxicity, likely due to weaker complexation of Cu ions by smaller straight carbon molecules, was noted for the optically lighter DOMs. These data re-inforce previous observations that aromaticity may be the key characteristic determining the effectiveness of DOMs in protecting aquatic organisms against Cu toxicity (De Schamphelaere et al. 2004; Richards et al. 2001; Ryan et al. 2004; Schwartz et al. 2004).

**Potential mechanisms by which DOM protects against Cu toxicity**

The mechanisms by which DOM molecules complex and chelate metals, including Cu, are not fully understood. The higher SAC_{340} of optically darker DOMs, which were more protective, was attributed to the presence of more phenolic functional groups (Schwartz et al. 2004). The aromatic structures may not be involved directly in chelating Cu ions, but they can influence the reactivity of the functional groups directly attached to them. From a copper-binding perspective, aromatic carboxylic and phenolic sites on aromatic rings tend to bind stronger than aliphatic carboxylic and hydroxyl acids (Carbonaro et al. 2011). In addition, ring structures allow for multiple chelation sites when functional groups are orthogonal to each other on the aromatic ring and chelation dramatically increases binding strength (Carbonaro et al. 2011). Certainly the binding, however it occurred, resulted in relatively constant Cu activity at the various LC_{50} values (Fig. 1b). In addition, direct DOM interactions with aquatic organisms should be considered. These have recently emerged as an active research area (Wood et al. 2011) and could serve as additional mitigation mechanisms against Cu toxicity. Several relevant direct effects on aquatic animals have been reported, including the sorption of DOMs to their cell surfaces by hydrophobic interaction or hydrogen bonding (Campbell et al. 1997), stimulation of active Na$^+$ uptake (Matsuo et al. 2004; Glover et al. 2005b), hyperpolarization of gill transepithelial potential (Galvez et al. 2009) and reduction of paracellular permeability (Wood et al. 2003). Most of these interactions are not only considered beneficial for aquatic organisms for their physiology but also regarded as possible defensive mechanisms against the toxic effects of metals. Notably SAC_{340} was the optical property that correlated strongly with the magnitude of the hyperpolarization, a direct influence of DOMs on membrane physiology with possible consequences on ion transport (Galvez et al. 2009).

Using the fluorescence-derived humic acid (%HA$_{PARAFAC}$) and SAC_{340} to improve the predictive capacity of the BLM

Initially, we used the BLM (version 2.2.3, HydroQual Inc) to predict the 48-h LC_{50} for the exposure water data at 3 mg C l^{-1}, employing the default lethal accumulation (LA_{50}) of 0.119 nmol g^{-1} wet weight with the default assumption that 10% of the DOM was HA (i.e. 10% HA). We then compared the output when the same model was run with the PARAFAC-derived %HA (%HA$_{PARAFAC}$) at the same default LA_{50}. At this LA_{50}, the predicted LC_{50} values were overestimated (on average 2.7 and 3.9 times higher than the measured LC_{50} in this study for the default

%HA and %HA$_{PARAFAC}$, respectively, Fig. 7a). Even though the predicted and observed LC$_{50}$ were not realistically matched when using the %HA$_{PARAFAC}$, the relationship was much stronger ($r^2 = 0.80$; $P < 0.0001$) than when using the default 10% HA ($r^2 = 0.32$, $P < 0.001$. Similar results were seen when these predictions were conducted using the exposure water data at 6 mg C l^{-1}. This suggests that capturing the variability in HA contents of DOMs could improve the predictive capacity of the BLM.

The next step was to optimize the LA$_{50}$ so as to achieve a slope of 1.00 for the regression equation between predicted and observed LC$_{50}$. The optimized LA$_{50}$ (0.0120 nmol g^{-1} wet weight) yielded the best correlation between the predicted and observed LC$_{50}$ using
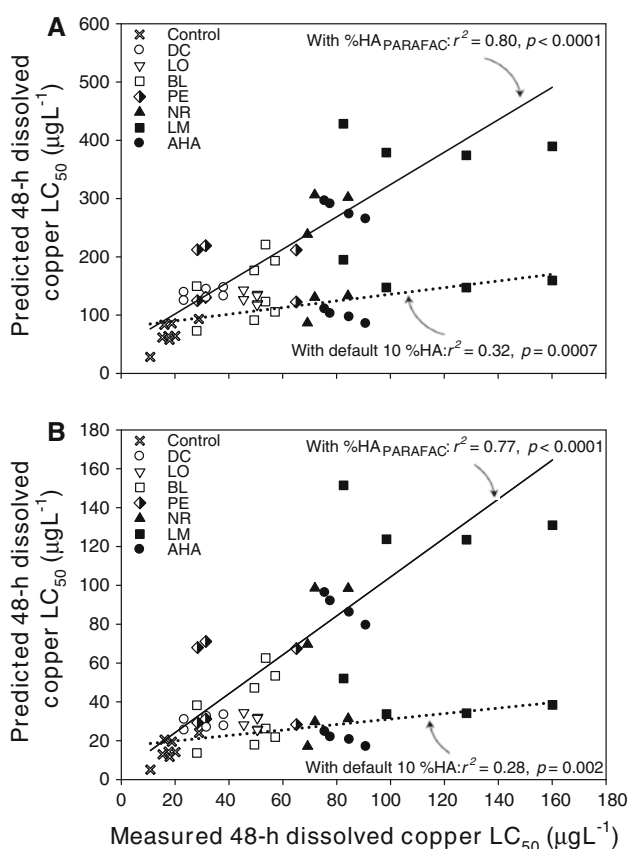
Fig. 7 The relationships between the values of predicted and observed copper LC$_{50}$ at the **a** default lethal accumulation (LA$_{50}$) of 0.119 nmol g^{-1} wet weight and **b** the optimal LA$_{50}$ of 0.0120 nmol g^{-1} wet weight in the BLM (Windows version 2.2.3, HydroQual Inc.). In **a**, for a default LA$_{50}$ of 0.119 nmol g^{-1} wet weight, the *solid line* represents the relationship using the %HA$_{PARAFAC}$ (Predicted LC$_{50}$ = 2.78 Measured LC$_{50}$ + 46.30), and the *dotted line* represents the relationship using the default 10%HA (Predicted LC$_{50}$ = 0.57 Measured LC$_{50}$ + 78.60). In **b**, for an optimized LA$_{50}$ of 0.0120 nmol g^{-1} wet weight, the *solid line* represents the relationship using the %HA$_{PARAFAC}$ (Predicted LC$_{50}$ = 1.00 Measured LC$_{50}$ + 4.16) and the *dotted line* represents the relationship using the default 10% HA (Predicted LC$_{50}$ = 0.14 Measured LC$_{50}$ + 17.04). Refer to Table 1 for abbreviations of DOMs

%HA$_{PARAFAC}$ ($r^2 = 0.77$; $P < 0.0001$) while the predicted LC$_{50}$ were still greatly underestimated using the default 10%HA ($r^2 = 0.28$; $P = 0.002$) (Fig. 7b). While this relatively good agreement between observed and predicted LC$_{50}$ using %HA$_{PARAFAC}$ is encouraging, the relationship may not be very useful in the regulatory arena, as few labs have access to excitation-emission fluorescence and PARAFACs capability.

However, specific absorbance is easily measured, and SAC$_{340}$ in the present study was highly correlated ($r^2 = 0.95$) with %HA$_{PARAFAC}$ of the DOMs (excluding data of AHA) (Fig. 8a), as well as with Cu toxicity amelioration (Fig. 4; Table 3). Similar relationships between SAC and %HA$_{PARAFAC}$, and between SAC and toxicity amelioration have been seen for various other freshwater DOMs (Al-Reasi et al. 2011). We therefore explored the potential
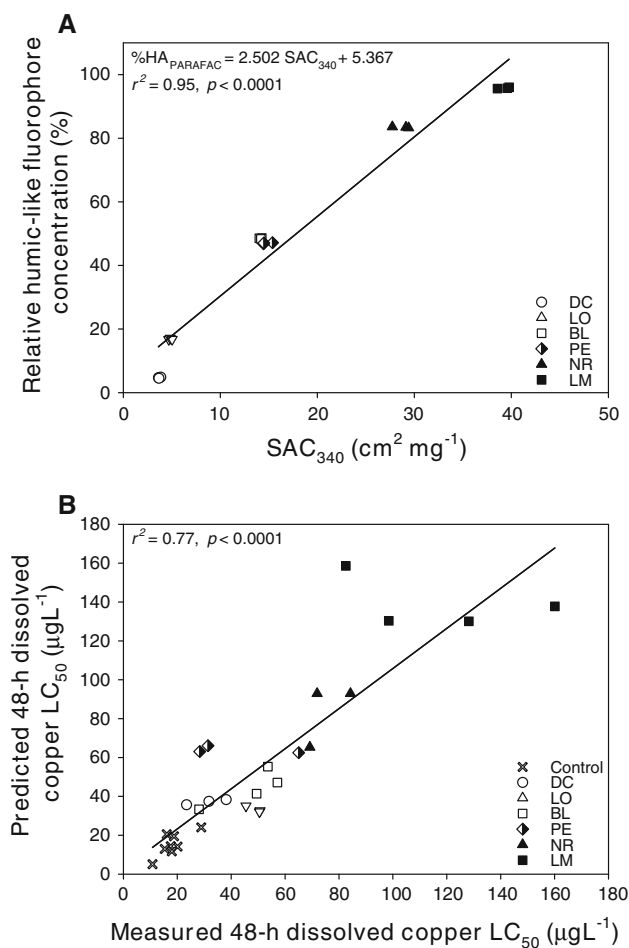
Fig. 8 a The relationship between the relative concentrations of humic-like fluorophore resolved by PARAFAC (%HA$_{PARAFAC}$) and specific absorbance coefficient (SAC$_{340}$) of the DOMs (excluding data of AHA) used in the exposure. **b** The relationships between the values of predicted and observed copper LC$_{50}$ obtained using the estimated %HA$_{PARAFAC}$ from SAC$_{340}$, as explained in the text, and the optimized LA$_{50}$ of 0.0120 nmol g^{-1} wet weight. Refer to Table 1 for abbreviations of DOMs

for using the SAC_{340} as an easily measured surrogate for %$HA_{PARAFAC}$ so as to appropriately adjust the BLM output. We simply used the unadjusted relationship between SAC_{340} and %$HA_{PARAFAC}$ ($r^2 = 0.95$). In the other words, we estimated the %$HA_{PARAFAC}$ by using the regression equation (Fig. 8a) and then inserted estimated %$HA_{PARAFAC}$ values as % HA into the optimized BLM (i.e. using $LA_{50} = 0.0120$ nmol g^{-1} wet weight) in order to predict LC_{50}. The relationship between the predicted and measured LC_{50} was still strongly linked ($r^2 = 0.77$, Fig. 8b), supporting the use of the easily measured aromaticity index (i.e. SAC_{340}) for approximation of %HA content of DOMs. These results support the inclusion of SAC_{340} as a quality factor, through %HA, into the BLM to improve Cu toxicity prediction. Similarly, De Schamphelaere et al. (2004) incorporated a quality factor for DOMs by adjusting the DOC input into the program based on absorbance correction. The present work showed that predictive capacity of the BLM can be improved with the adjustment of %HA through the use of a fluorescence quality measure (i.e. humic-like fluorophore concentration) or with estimation of %HA from a simple absorbance measurement (i.e. SAC_{340}).

In conclusion, various physicochemical measurements can be applied to overcome the heterogeneous nature of aquatic DOMs including standard UV/Visible absorbance and fluorescence measurements. Several indices were determined, providing qualitative information about the nature and molecular composition of DOM from various natural freshwater sources. Applying several optical characteristics, physicochemically distinguished DOMs exhibited differential protection against Cu toxicity to *D. magna*. Of these characteristics, the present observations support the incorporation of the aromaticity index (i.e. SAC_{340}) as the DOM quality input in the BLM to increase the accuracy of predictions of Cu toxicity in natural waters.

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