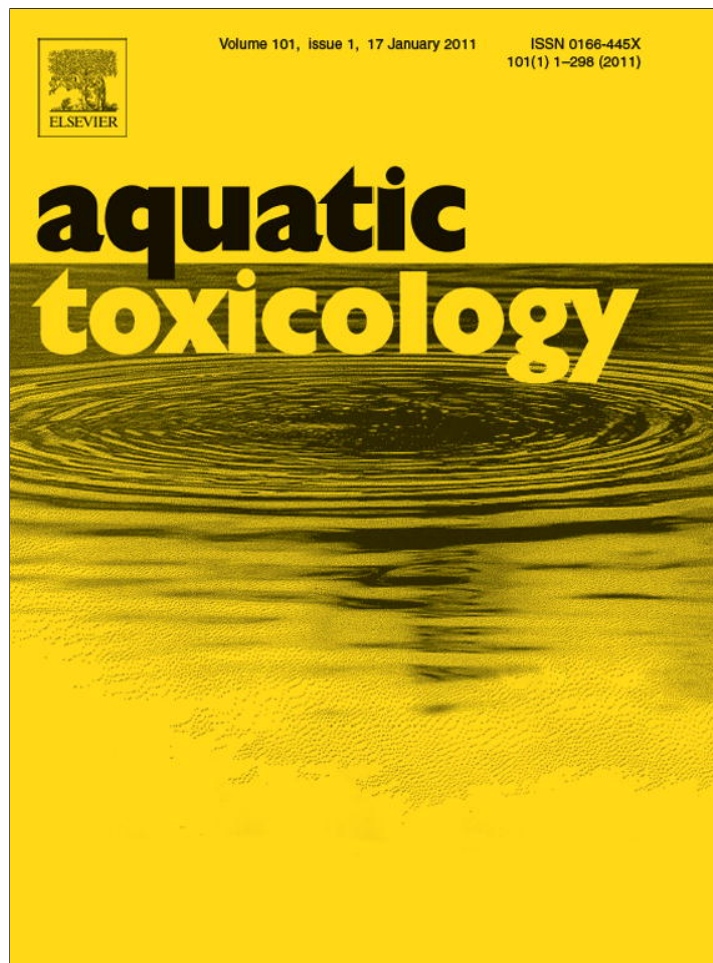


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Review

Physicochemical and spectroscopic properties of natural organic matter (NOM) from various sources and implications for ameliorative effects on metal toxicity to aquatic biota

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ABSTRACT

Natural organic matter (NOM), expressed as dissolved organic carbon (DOC in mg CL^{-1}), is an ubiquitous complexing agent in natural waters, and is now recognized as an important factor mitigating waterborne metal toxicity. However, the magnitude of the protective effect, judged by toxicity measures (e.g. LC50), varies substantially among different NOM sources even for similar DOC concentrations, implying a potential role of NOM physicochemical properties or quality of NOM. This review summarizes some key quality parameters for NOM samples, obtained by reverse osmosis, and by using correlation analyses, investigates their contribution to ameliorating metal toxicity towards aquatic biota. At comparable and environmentally realistic DOC levels, molecular spectroscopic characteristics (specific absorbance coefficient, SAC, and fluorescence index, FI) as well as concentrations of fluorescent fractions obtained from mathematical mixture resolution techniques (PARAFAC), explain considerable variability in the protective effects. NOM quality clearly influences the toxicity of copper (Cu) and lead (Pb). NOM quality may also influence the toxicity of silver (Ag), cadmium (Cd) and inorganic mercury (Hg), but as yet insufficient data are available to unequivocally support the latter correlations between toxicity reduction and NOM quality predictors. Cu binding capacities, protein-to-carbohydrate ratio, and lipophilicity, show insignificant correlation to the amelioration offered by NOMs, but these conclusions are based on data for Norwegian NOMs with very narrow ranges for the latter two parameters. Certainly, various NOMs alleviate metal toxicity differentially and therefore their quality measures should be considered in addition to their quantity.

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1. Introduction

Natural organic matter (NOM) is a complex mixture of poorly defined organic molecules which occur in natural waters. In simple mass concentration terms, NOM exceeds in abundance most other inorganic and organic components in natural waters (Thurman, 1985; Tipping, 2002). The common concentration range reported is 1–15 mg CL⁻¹ in freshwater systems (Thurman, 1985). These molecules arise biologically from the decomposition of lignin-rich plant materials (Ertel et al., 1984) and the decay of dead organic remains of animals and microbes in a poorly understood process known as humification (Hatcher and Spiker, 1988). Unlike other organic compounds, NOM molecules are not described in term of a unique chemical structure (Gaffney et al., 1996; Filella et al., 2004). Instead, they are operationally defined based on two criteria. The first is water sample filtration through a 0.45- μ m membrane. The filtrate is considered “dissolved” organic matter (DOM), and since approximately 50% by mass of DOM is carbon, which can be easily measured by oxidative or combustion techniques, DOC is often used as a surrogate concentration measure (Tipping, 2002). For the purposes of this review, only “dissolved” NOM will be considered, because it is this fraction which affects the toxicity and bioaccumulation of “dissolved” metals, operationally defined by the same filtration technique (Playle et al., 1993; Erickson et al., 1996; Ryan et al., 2004; Schwartz et al., 2004). The second criterion is acid–base solubility (Gaffney et al., 1996). At low pH, high molecular weight molecules (“humic” acids) of an NOM sample precipitate, whereas the low molecular weight molecules (“fulvic” acids) remain in the solution (e.g. Ma et al., 2001; Ryan et al., 2004). For NOM of terrigenous origin, the major fraction (~50–90%) of the aquatic NOM is represented by so-called “humic substances”, which are a heterogeneous combination of fulvic and humic acids (Thurman, 1985; Tipping, 2002). The non-humic bio-macromolecules (e.g. carbohydrates, proteins, and amino acids) account for lower proportions of the NOM sample (Thurman, 1985).

Organic matter of an aquatic system can also be characterized by source or origin, and classified as terrigenous (NOM produced on land and then washed into the water body; the term “allochthonous” is also sometimes used) or autochthonous (NOM generated within the water column by microorganisms such as algae and bacteria) (McKnight et al., 2001). Due to their absorbance in the visible region, water samples containing high concentrations of NOM are usually yellow to brown in color (Tipping, 2002). In general terrigenous NOM tends to be dark in color, while autochthonous NOM is much lighter.

Aquatic NOM is now recognized as a global regulator of many processes, both biotic and abiotic, in freshwater ecosystems (Petersen, 1991; Kullberg et al., 1993; Williamson et al., 1999; Steinberg et al., 2006). Three areas of recent interest are the direct physiological impacts of NOM on aquatic organisms (e.g. Campbell et al., 1997; Wood et al., 2003; Matsuo et al., 2004; Glover et al., 2005a; Galvez et al., 2009), the toxic effects of NOM itself on them (e.g. Matsuo et al., 2006; Meinelt et al., 2007), and the ability of NOM to alter the uptake and toxic effects of organic chemicals (e.g. Haitzer et al., 1998; Qiao and Farrell, 2002). However, these areas will not be explored further in the current review; rather, the focus of our analysis will be on the ability of NOM to complex metals, and thereby reduce their bioaccumulation and/or toxicity. This is particularly topical because regulatory authorities have now started to

realize that NOM is an important water quality variable affecting the acute toxicity of metals. Indeed, there is now a trend to incorporate NOM (as DOC concentration) into predictive algorithms used to establish ambient water quality criteria. A prime example is the Biotic Ligand Model (BLM) for Cu, now approved in the USA for the establishment of site-specific criteria for Cu (USEPA, 2007). Di Toro et al. (2001), Santore et al. (2001), and Niyogi and Wood (2004) provide the theoretical background for the BLM approach. At present, this and related approaches generally use total DOC concentration as the input variable.

In general, NOM reduces metal toxicity by chelating and sequestering metal cations and consequently making them less bioavailable (e.g. Playle et al., 1993; Hollis et al., 1997). The phenomenon is concentration-dependent (Playle et al., 1993; Erickson et al., 1996; Ryan et al., 2004). To date, the influence of NOM quality or physicochemical properties on protective ability remains poorly understood. This is because of the structural irregularity, heterogeneity and complexity of NOM (McDonald et al., 2004). However, there is abundant evidence that NOM quality matters, at least for some metals and some endpoints. The source-dependent protection offered by different NOMs was originally demonstrated by Playle (1998). Later, his work and that of colleagues, confirmed the importance of NOM quality (i.e. physicochemical properties) with terrigenous NOM having stronger protective effects than autochthonous NOM against metal toxicity (Richards et al., 2001; Schwartz et al., 2004; Luider et al., 2004).

NOM samples from various aquatic environments have been obtained, characterized and utilized for metal toxicity tests, but investigations are inconsistent in term of isolation procedures, natural sources sampled, chemical characterizations and experimental conditions of toxicity tests employed. The data generated are scattered and it is generally hard to find complete description of the NOM's used. The objectives of this review are to summarize available data on NOM physicochemical characteristics, which have been reported to play role in alleviating metal toxicity, and to assess whether the quality of the NOM, in addition to its simple mass concentration, could be a useful input parameter to improve the precision of toxicity-prediction models, such as the BLM.

2. Data compilation and treatment

Several publications of the NOM-Typing Project have indicated that chemical characteristics potentially vary considerably according to the methods used to obtain NOM samples (low-pressure low-temperature evaporation versus reverse osmosis) (e.g. Gjessing et al., 1999; Abbt-Braun and Frimmel, 1999). In the present review, only data for NOMs originally isolated by reverse osmosis (RO, Sun et al., 1995) have been included, for several reasons. The restriction to chemical characteristics and toxicity information obtained with RO-NOM was intended to avoid differences due to isolation methods. Moreover, RO-NOM samples have been reported to exhibit similar protective ability to those of natural waters from which they were isolated (De Schampelaere et al., 2005), suggesting the usefulness of RO in providing representative natural NOM samples. In addition, most investigations on metal toxicity tests examining qualitative aspects of NOM have employed RO-NOMs (e.g. Richards et al., 2001; VanGenderen et al., 2003; Schwartz et al., 2004; De Schampelaere et al., 2004; Ryan et al., 2004; Glover et al., 2005b). Indeed, there are only very limited tox-

ecological data available for NOMs obtained using other isolations procedures.

In our present analyses, we have exploited an excellent data source on the various physicochemical techniques used to characterize NOMs, the publications of the NOM-Typing Project of the Norwegian NOM samples (special issue of *Environment International* 25:143–388). Data on metal toxicity (e.g. effective concentration (EC50), lethal concentrations (LC50), and lethal time (LT50, time to reach 50% mortality in test organisms), in the presence of these Norwegian NOM samples (Pempkowiak et al., 1999; VanGenderen et al., 2003; Ryan et al., 2004) and others from Europe and North America (e.g. Schwartz et al., 2004; De Schampelaere et al., 2004; Glover et al., 2005b) have been collated, together with additional physicochemical characterizations. In addition, we have included very recent toxicological data on RO-NOM's from Canadian freshwater environments (Hicks, 2009; Gheorghiu et al., 2010). All studies included in this review are summarized in Table 1.

The isolation and characterization of individual NOM molecules is impractical (Leenheer and Croué, 2003). However, absorbance and fluorescence spectroscopy have frequently been successfully employed to distinguish the molecular variability among natural samples from various sources, as well as between fulvic and humic acids from the same source (Senesi et al., 1991). Optical properties such as the specific absorbance coefficient, SAC (estimated as $2.303 \times$ absorbance at a specific wavelength often 340 nm and normalized to TOC) (Curtis and Schindler, 1997) and the fluorescence index (FI, determined as fluorescence intensity_{450nm}/fluorescence intensity_{500nm}; both taken at excitation wavelength of 370 nm) (McKnight et al., 2001) have been reported to distinguish NOM sources and composition. Operationally, "humic substances" can be defined based on the low energy wavelengths of light emitted when an NOM sample is excited by higher energy light. The humic fraction of the sample tends to produce emission at longer wavelengths relative to that of the fulvic component (Wu et al., 2007). Recently, a more advanced spectral resolution and multivariate statistical approach (PARAFAC) for excitation–emission fluorescence spectroscopy has been developed which allows for greater molecular discrimination (Stedmon et al., 2003; Stedmon and Bro, 2008). Using complete excitation–emission matrices for a particular NOM, PARAFAC resolves the underlying moieties or fluorophores into their peaks, each identified by its corresponding excitation–emission wavelength pair and relative concentrations. Therefore, SAC, FI and relative concentrations of PARAFAC-identified fluorophores (fulvic-like and humic-like) of NOM from Canada and Norway have been collected here. These spectroscopic characteristics and additional measurements of potential diagnostic significance (proton-binding capacities and copper-complexation capacities, protein-to-carbohydrate ratio, lipophilicity) have been compiled and included in our data analysis (Table 2).

It is important to note that most of the experimental conditions were relatively comparable at environmentally relevant levels of DOC (Table 1). The coefficient of variation (CV, equals the standard deviation divided by the mean) was utilized as a statistical measure of the dispersion for DOC levels used within each study (Table 1). Another important concern is that sodium and hardness ions, usually concentrated during NOM isolation may ameliorate metal toxicity (Winner, 1985; Erickson et al., 1996). However, the concentrations of Na and Ca were checked and found to be relatively consistent among toxicity tests within each study included in the analysis.

Graphical representation and data exploration have been performed using SigmaPlot (Version 10.0 for Windows, 2006, Systat Software, Inc., Chicago, IL). The coefficient of determination (R^2) of the regression analysis was employed to test correlations between the physicochemical properties or quality parameters (i.e. predic-

tor variables on the x -axis) and the measured toxic responses (EC50 LC50, LT50, metal gill binding and accumulation rates which are outcome variables on the y -axis). The degree of significance for statistical analysis was established at the 0.05 probability level.

3. Do quality predictors explain the protective effects of NOM?

3.1. Aromaticity

The near UV absorptivity has been utilized as a standard measure of color in natural waters (Cuthbert and Giorgio, 1992) and to quantify the concentrations of light-absorbing moieties (i.e. chromophores) of NOM. As stated above, the specific absorption coefficient (SAC), the absorbance of NOM at 300–350 nm normalized to DOC, can serve as an index of the aromatic composition (Curtis and Schindler, 1997; Richards et al., 2001). The influence of aromaticity, as represented by SAC, on the protective effect of different NOM sources against metal toxicity is presented in Fig. 1.

For Cu, significant positive relationships between the SAC's of different NOM sources and the measured toxic responses were noted for three freshwater organisms (fathead minnows, rainbow trout, *Daphnia magna*) (Fig. 1). In samples collected from Europe and the US, SAC₃₅₀ could explain most of variation ($R^2 = 0.70$) in ameliorative behaviour of NOM against Cu EC50 to *D. magna* (De Schampelaere et al., 2004) (Fig. 1A). Highly significant correlations were also found between the aromatic composition of both Norway-NOMs and Canada-NOMs-1 versus Cu LC50 in fathead minnow, *Pimephales promelas* (Ryan et al., 2004) (Fig. 1A) and Cu LT50 for rainbow trout, *Oncorhynchus mykiss* (Schwartz et al., 2004) (Fig. 1B), respectively.

For Pb, the aromatic index of Canada-NOMs-1 accounted for 66% of the protection against Pb toxicity to *O. mykiss* (Schwartz et al., 2004) (Fig. 1C). However, in a separate study, no correlation ($R^2 = 0.002$, $p = 0.923$, $n = 7$) was found between SAC₃₄₀ of Canada-NOMs-2 versus Pb LT50 in the same species (MacDonald et al., 2002) (Fig. 1C). The SAC₃₄₀ values of Canada-2 samples spanned a more limited range (~ 5 – $17 \text{ cm}^2 \text{ mg}^{-1}$) relative to that (~ 5 – $60 \text{ cm}^2 \text{ mg}^{-1}$) of Canada-1 samples, which likely explains the lack of significant relationship seen with Canada-2 NOMs. For Cd, a weak, but marginally significant relationship ($R^2 = 0.28$, $p = 0.04$, $n = 13$) was recorded between aromaticity, as a predictor of mitigation of toxicity by NOM, and 96-h Cd LT50 to *O. mykiss* (Schwartz et al., 2004) (Fig. 1B). No correlation was detected between SAC₃₄₀ and Ag toxicity to *P. promelas* in the presence of Norway-NOMs (VanGenderen et al., 2003) (Fig. 1D).

There was also weakly significant (for Hg) and non significant correlations (for Cu) between SAC₃₄₀, as a quality parameter of Canada-NOMs-3 and Canada-NOMs-4 samples, and gill burden of inorganic Hg ($R^2 = 0.40$, $p = 0.05$, $n = 10$) (Klinck et al., 2005) or Cu ($R^2 = 0.00$, $p = 0.96$, $n = 8$) to *O. mykiss* (Hicks, 2009; Gheorghiu et al., 2010). In a 21-day chronic exposure using Norway-NOMs, a lack of relationship was also seen when accumulation rates of Cd and Cu ($\mu\text{g g}^{-1}$ dry weight day⁻¹) in the gills of the blue mussel (Pempkowiak et al., 1999) were plotted versus the SAC₃₅₀ ($R^2 = 0.04$, $p = 0.62$, $n = 8$ and $R^2 = 0.24$, $p = 0.22$, $n = 8$, respectively). For Ag toxicity to *D. magna*, the relative protective units of Canada-NOMs-5 showed no significant relationship with their aromatic composition estimated by SAC₃₀₀ ($R^2 = 0.14$, $p = 0.36$, $n = 8$). However, when additional non-RO NOMs were tested and Aldrich humic acid was excluded, the relationship became significant (Glover et al., 2005c).

For the very soft metals (Ag and Hg), the correlations were not strong. Soft metals (metals with a polarizable valence shell) tend to interact most strongly with soft ligands such as reduced sulphur, whereas harder metals (with more tightly held valence electrons)

Table 1
Summary of the toxicity data obtained by conducting metal exposures in the presence of natural organic matter (NOM) samples isolated by reverse osmosis (RO) from many freshwater systems in Europe and North America.

NOM ID ^a	Test organism	Toxicity data in presence of NOMs ^b					DOC levels (mg CL ⁻¹)		Reference
		Toxicity parameters				Range	CV ⁱ		
		Endpoint ^g (unit)	Metal	Range ^h	Variation				
Norway-NOMs ^b	Blue mussel ^e (<i>Mytilus trossulus</i>)	Accumulation rate ($\mu\text{g g}^{-1}$ dry wt. day ⁻¹)	Cu	3.20–9.67	3.0	6.0	NA	Pempkowiak et al. (1999)	
			Cd	10.70–22.09	1.3				
Norway-NOMs ^b	Fathead minnow (<i>Pimephales promelas</i>)	96-h LC50 ($\mu\text{g L}^{-1}$)	Ag	6.20–32.9	5.3	3.3–5.1	0.14	VanGenderen et al. (2003)	
		96-h LC50 ($\mu\text{g L}^{-1}$)	Cu	137.0–671.2	4.9	2.1–3.0	0.11	Ryan et al. (2004)	
Canada-NOMs-1 ^c	Rainbow trout (<i>Oncorhynchus mykiss</i>)	96-h LT50 (h)	Cu	24.0–200.0	8.3	5.7–6.5	0.04	Schwartz et al. (2004)	
			Pb	48.0–105.0	2.2	3.0–4.4	0.12		
			Cd	42.0–78.0	1.9	5.6–6.9	0.05		
Canada-NOMs-2	Rainbow trout (<i>Oncorhynchus mykiss</i>)	96-h LT50 (h)	Pb	90.0–168.0	1.4	2.8–4.0	0.14	MacDonald et al. (2002)	
Canada-NOMs-3	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Gill burden (nmol g^{-1} wet wt.)	Hg	5.6–39.7	7.1	7.5–10.5	0.10	Klinck et al. (2005)	
Canada-NOMs-4	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Gill binding (nmol g^{-1} wet wt.)	Cu	3.7–13.6	3.7	4.0	NA	Hicks (2009), Gheorghiu et al. (2010)	
Canada-NOMs-5 ^d	Water flea (<i>Daphnia magna</i>)	Relative protective unit	Ag	0.57–3.66	6.4	NA	NA	Glover et al. (2005b,c)	
Europe and US-NOMs	Water flea (<i>Daphnia magna</i>)	48-h EC50 ($\mu\text{g L}^{-1}$)	Cu	50.6–129.0	2.5	2.0–3.0	0.20	De Schampelaere et al. (2004)	

^a Each group of NOMs was assigned an identity label for easier comparison in the text. For complete details of sampling sites, refer to the specific reference.

^b Because Norway-NOMs were exchanged and shipped between different laboratories around the globe, RO isolates had been freeze-dried (Pempkowiak et al., 1999; VanGenderen et al., 2003; Ryan et al., 2004).

^c Commercially available Aldrich humic acid and Suwannee River-NOM are included as NOM samples. These are freeze-dried organic matter.

^d Two reverse-osmosis NOM isolates from USA and two commercially available NOMs (Nordic Reservoir and Aldrich humic acid) are included as samples in the analysis.

^e Exposures were conducted in Baltic seawater.

^f For toxicity data in control (NOM-free exposure), consult the specific reference.

^g LC50: lethal concentration needed to result in 50% mortality of test organisms; LT50: time to reach 50% mortality of test organisms; EC50: effective concentration required to immobilize 50% of *D. magna* neonates (De Schampelaere et al., 2004). Accumulation rates were measured in the gills of *M. trossulus* over 21-day chronic exposure tests (Pempkowiak et al., 1999). Relative protective units for different NOMs against Ag toxicity to *D. magna* were calculated by first determining the linear regression relationship for Ag LC50 against Aldrich humic acid concentration, and then computing the ratio of the LC50 for the particular NOM to the LC50 for Aldrich humic acid at the same DOC concentration (Glover et al., 2005b,c).

^h The range has the same unit as the endpoint.

ⁱ The coefficient of variation.

Table 2

Summary of the physicochemical characteristics of natural organic matter (NOM) samples isolated by reverse osmosis (RO) from freshwater systems in Europe and North America and used for metal toxicity tests (see Table 1).

NOM ID ^a	Quality parameters (unit) ^b	Range	Range factor	Reference
Norway-NOMs	Aromaticity: SAC ₃₅₀ (cm ² mg ⁻¹ C)	18.73–35.63	1.90	Ryan et al. (2004)
	Fluorescence index (FI)	1.13–1.33	1.17	This study
	Humic-like fraction concentration (%) estimated by PARAFAC	63.13–100	1.58	
	Fulvic-like fraction concentration (%) estimated by PARAFAC	0.00–33.98		
	Proton-binding capacities (μmol mg ⁻¹ TOC)	4.30–27.10	6.30	Smith and Kramer (1999)
	Copper-binding capacities (μmol mg ⁻¹ TOC)	0.14–1.41	10.07	Takács et al. (1999)
	Protein-to-carbohydrate ratio ^c	0.14–0.37	2.64	Christy et al. (1999)
	Lipophilicity ^e	0.09–3.06	34.00	Gjessing et al. (1999)
	Octanol solubility log K _{ow}	0.53–0.96	1.81	Egeberg and Alberts (2002)
Canada-NOMs-1	Aromaticity: SAC ₃₄₀ (cm ² mg ⁻¹ C)	5.50–53.20	9.67	Schwartz et al. (2004) ^f
	Fluorescence index (FI)	0.89–1.73	1.94	This study
	Humic-like fraction concentration (%) estimated by PARAFAC	10.34–84.74	8.20	This study
	Fulvic-like fraction concentration (%) estimated by PARAFAC	9.68–66.07	6.83	
Canada-NOMs-2	Aromaticity: SAC ₃₄₀ (cm ² mg ⁻¹ C)	6.20–12.03	1.94	MacDonald et al. (2002)
Canada-NOMs-3	Aromaticity: SAC ₃₄₀ (cm ² mg ⁻¹ C)	2.70–32.2	11.93	Klinck et al. (2005)
	Fluorescence index (FI)	1.20–2.02	1.68	This study
	Humic-like fraction concentration (%) estimated by PARAFAC	0.00–84.74		
Canada-NOMs-4	Fulvic-like fraction concentration (%) estimated by PARAFAC	15.26–66.07	4.33	
	Aromaticity: SAC ₃₄₀ (cm ² mg ⁻¹ C)	4.70–16.90	3.60	Hicks (2009), Gheorghiu et al. (2010), This study
	Fluorescence index (FI)	1.18–1.87	1.58	
	Humic-like fraction concentration (%) estimated by PARAFAC	36.32–70.70	1.95	
Canada-NOMs-5	Fulvic-like fraction concentration (%) estimated by PARAFAC	15.26–48.32	3.17	
	Aromaticity: SAC ₃₀₀ (cm ² mg ⁻¹ C)	32.26–123.65	3.83	Glover et al. (2005c)
Europe and US-NOMs	Fluorescence index (FI)	1.32–2.31	1.75	
	Aromaticity: SAC ₃₄₀ (cm ² mg ⁻¹ C)	13.20–49.10	3.72	De Schampelaere et al. (2004)

^a Each group of NOMs was assigned an identity label for easier comparison in the text. For complete details of sampling sites, refer to the specific reference.

^b The units of each parameter are indicated, unless it is unitless. SAC is the specific absorbance coefficient normalized to DOC; FI is the fluorescence index. See text for description of each quality parameter.

^c Protein-to-carbohydrate ratios were calculated using concentrations of proteins and carbohydrates determined by Christy et al. (1999).

^e Gjessing et al. (1999) estimated octanol solubility as absorbance ratio: (A₂₅₄)_{octanol}/(A₂₅₄)_{water}. Egeberg and Alberts (2002) approximated log K_{ow} values using reverse-phase high pressure liquid chromatography (RP-HPLC).

^f For each metal exposure, Schwartz et al. (2004) performed SAC measurements of NOMs used. Here, SAC values from all exposures were averaged and means were used in data analysis. Commercially available Aldrich humic acid and Suwannee River-NOM were included as NOM samples.

tend to interact most strongly with oxygen (Smith et al., 2002). It could be that softer binding sites within NOM macromolecular structure are not associated with aromatic groups, whereas oxygen sites (such as phenolic and carboxylic groups) likely are associated with aromatic moieties. However, it should be noted that Glover et al. (2005c) reported that was no relationship between the sulphide content of various NOMs and their protective ability against Ag toxicity to *D. magna*.

3.2. Fluorescence index

The FI, another optical quality parameter, is a simple ratio (emission intensity at 450 nm/emission intensity at 500 nm; both taken at excitation at 370 nm) which is thought to differentiate organic matter from different sources (McKnight et al., 2001). Within-water column microbially derived NOM (i.e. autochthonous) is assigned values very close to 1.9, whereas terrestrially derived NOM from lignin-degradation (i.e. terrigenous) has values of approximately 1.4 (McKnight et al., 2001). The FI of Norway-NOMs ranged between about 1.1 and 1.3, implying an exclusively terrestrial origin.

As illustrated in Fig. 2, this quality predictor could not explain the variability in protection offered by these samples against Cu

(Ryan et al., 2004) (Fig. 2A) and Ag (VanGenderen et al., 2003) (Fig. 2A) to fathead minnow. However, in contrast, Cu (Fig. 2A) and Pb LT50 (Fig. 2B) in rainbow trout (Schwartz et al., 2004) were found to correlate extremely well with the FI of the Canada-NOMs-1 samples ($R^2 = 0.58$, $p < 0.05$, $n = 8$, $R^2 = 0.55$, $p < 0.05$, $n = 8$, respectively). Regarding gill burden or accumulation rates of metals, a moderate relationship ($R^2 = 0.42$, $p < 0.05$, $n = 10$) occurred between Hg accumulated in the gill of *O. mykiss* (Klinck et al., 2005) (Fig. 2C) and the FI of Canada-NOMs-3. In the same species exposed to Cu in the presence of Canada-NOMs-4 samples, gill Cu-burden showed no association with the FI (Hicks, 2009; Gheorghiu et al., 2010) (Fig. 2C). Cu or Cd accumulation rates in gills of blue mussel were only weakly associated with FI (Pempkowiak et al., 1999) (Fig. 2D) which may again indicate that this toxic endpoint is not sensitive enough to detect the influence of an NOM quality predictor, or simply that the range of FI in these tests was insufficient to detect a significant relationship. Glover et al. (2005c) did find a significant correlation between relative protective units of NOMs (RO and non-RO samples) against Ag toxicity. However, upon excluding non-RO NOMs from the analysis, the influence of FI turned out to be insignificant ($R^2 = 0.33$, $p = 0.14$, $n = 8$).

Unlike the SAC₃₅₀ of Norway-NOMs, the FI of the same samples could not explain variation in Cu toxicity to *P. promelas* (Ryan

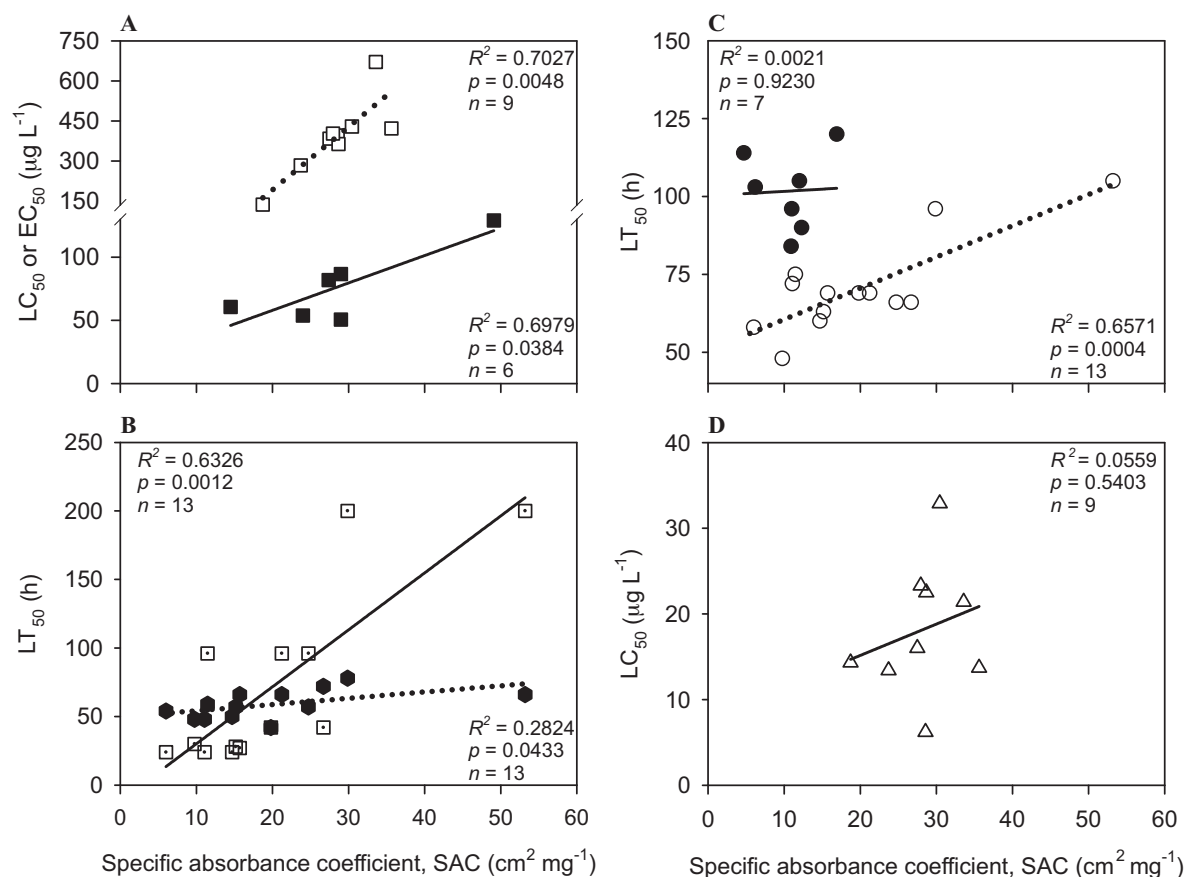


Fig. 1. The relationships between specific absorbance coefficient (SAC, in cm² mg⁻¹, an index of aromaticity) of various NOMs and lethal toxicity measures of several metals against different aquatic organisms. (A) Cu-LC₅₀ (□) of fathead minnow in Norway-NOMs and Cu-EC₅₀ (■) of *Daphnia magna* in US and Europe-NOMs. (B) Cu-LT₅₀ (□) and Cd-LT₅₀ (●) of rainbow trout in Canada-NOMs-1. (C) Pb-LT₅₀ (○, ●) of rainbow trout in Canada-NOMs-1 and Canada-NOMs-2, respectively. (D) Ag-LC₅₀ (△) of fathead minnow in Norway-NOMs. See text for details on the toxicity measures and refer to Table 1 for DOC levels in each study.

et al., 2004) (Fig. 2A). This observation emphasizes the heterogeneous nature of NOM which could be differentiated by one quality parameter (e.g. aromaticity index), but not by the other (e.g. fluorescence index). This inconsistency may be closely related to characteristics of NOM molecules of the system during their formation which varies spatially and temporally (Hatcher and Spiker, 1988). Both SAC and FI measurements initially require an absorption of light but only specific molecular structures allow for subsequent fluorescence emission. This specificity of fluorescence makes it a selective measurement technique. The types of moieties that fluoresce tend to have aromatic structure but are also influenced by the molecular environment. If a molecule is too “floppy” the added energy from the excitation light will be deactivated via non-fluorescent pathways including molecular movements and collisions. However, the FI of more representative Canada-NOMs-1 ranging widely from autochthonous to highly terrigenous (Schwartz et al., 2004) resulted in relationships with Cu and Pb toxicities (Fig. 2B) which were of comparable strength to those reported with SAC. Possibly, the observation of more Hg binding to the gill of *O. mykiss* with NOMs of high FI (i.e. autochthonous; Klinck et al., 2005) (Fig. 2C) could be attributed to the chemical nature of the organic matter. Usually, autochthonous NOMs are composed of photosynthetic products of primary producers (e.g. algae) and microbial decomposition of terrestrially derived NOM (Curtis and Schindler, 1997; McKnight et al., 2001). In general these tend to be rich in simple molecules of carbohydrates and amino acids, with low metal-chelating abilities, and therefore offer little protection against gill metal binding.

3.3. Fluorescent components by PARAFAC analysis

Parallel factor analysis (PARAFAC) is a relatively recent technique which was not available during most of the characterization studies summarized here. We were able to obtain the original excitation versus emission fluorescence data for several of the studies summarized here and re-analyze these archived data using PARAFAC fluorescence resolution techniques summarized in DePalma et al. (2011a). Data analysis was performed using the PLS Toolbox from Eigenvector Research Inc. (Wenatchee, WA, USA) as implemented on the Matlab™ platform (The Mathworks Inc. Natick, MA, USA).

The PARAFAC resolution method utilized here starts with an *a priori* assumption of 4 representative underlying fluorophores (two humic-like materials and two proteinaceous substances) present in each NOM sample. High relative humic and fulvic content should correspond to terrigenous input, and high amino acid-like fluorescence should correspond to autochthonous origin. Because of the pronounced heterogeneous nature of NOM, PARAFAC-resolved component spectra and their contents may not reflect the actual concentrations of humic and fulvic acids in the samples. However, the operational definition of fluorescent composition based on wavelengths of light emitted is useful for tracking molecular variability in samples from different environments and their influence on protective ability against metal toxicity. For example, Nadella et al. (2009) noticed a trend of increasing protective effect (against Cu toxicity to mussel larvae) with NOM enriched in fulvic-like fluorophore relative to those enriched in humic-like fluorophore in sea water. These relative concentrations enabled us to distinguish

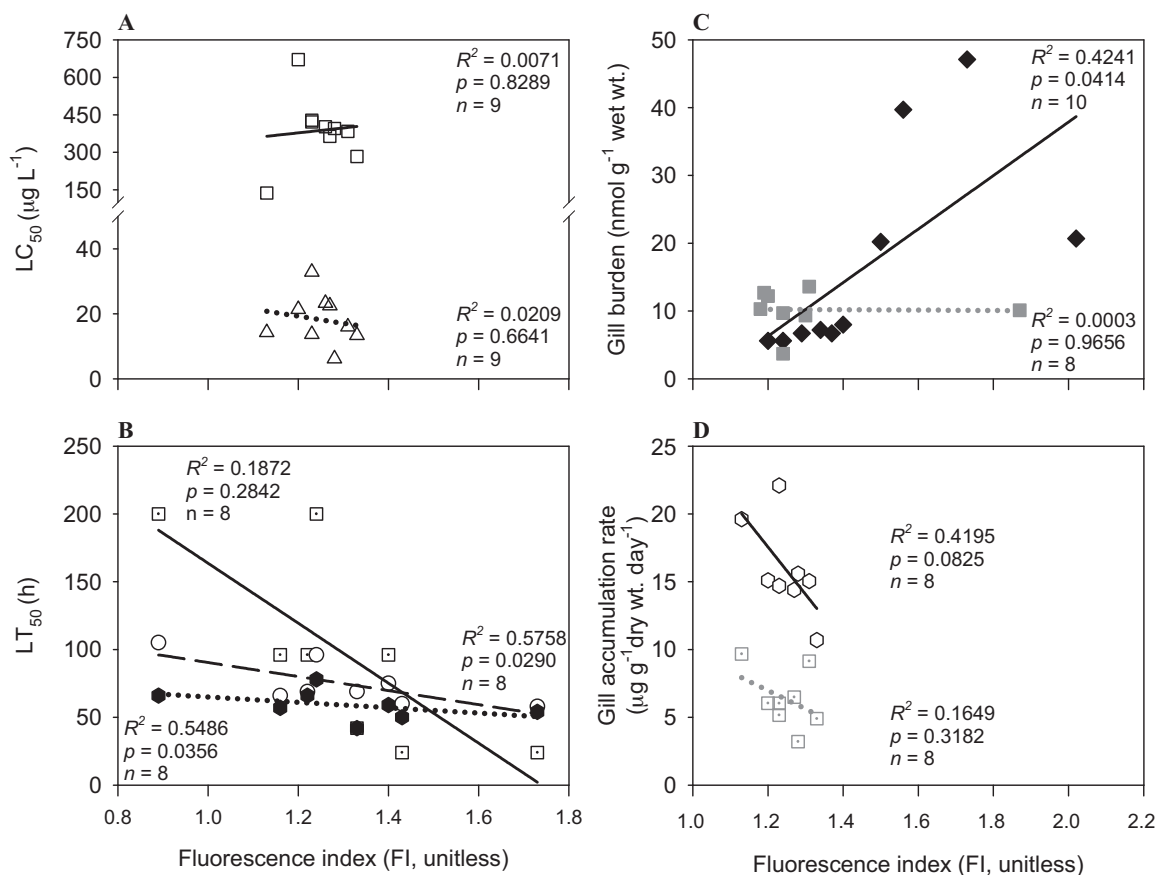


Fig. 2. The relationships between fluorescence index (FI, an indicator of origin) of various NOMs and toxicity measures of metals in different aquatic organisms. (A) Lethal concentrations (LC₅₀; $\mu\text{g L}^{-1}$) of Cu (□) and Ag (△) of fathead minnow in Norway-NOMs. (B) Lethal time (LT₅₀, hours) of Cu (□), Pb (○) and Cd (●) of rainbow trout in the presence of Canada-NOMs-1. (C) Gill binding (nmol g^{-1} wet weight) of Hg (◆) and Cu (■) of rainbow trout in Canada-NOMs-3 and Canada-NOMs-4, respectively. (D) Accumulation rates ($\mu\text{g g}^{-1}$ dry weight day^{-1}) of Cu (□) and Cd (○) in gill of blue mussel in Norway-NOMs. See text for details on the toxicity measures and refer to Table 1 for DOC levels in each study.

a wide range of samples according to their fluorescent composition and compare their protective effects against metal toxicity. The PARAFAC method has an advantage over FI or SAC in that greater information is obtained about the molecular nature of the NOM. The PARAFAC method utilized here takes multidimensional fluorescence surfaces and reduces the information to four summary numbers representing organic matter quality.

3.3.1. Humic-like component

Our PARAFAC analysis indicates that Cu toxicity to larval fathead minnow (Ryan et al., 2004) (Fig. 3A) and juvenile rainbow trout (Schwartz et al., 2004) (Fig. 3B) was inversely related to concentrations of humic-like fluorophore of Norway-NOMs and Canada-NOMs-1, respectively. An increase in the humic-like fractions of the samples augmented Cu LC₅₀ and LT₅₀ values. On the other hand, the ameliorative effects of Norway-NOMs on Ag toxicity to fathead minnow (VanGenderen et al., 2003) were not significantly related to their proportions of humic composition (Fig. 3D). The same was true of Pb and Cd toxicity to *O. mykiss* (Schwartz et al., 2004) in the presence of Canada-NOMs-3 (Fig. 3B). Likewise, neither gill burdens of Hg (Klinck et al., 2005) (Fig. 3C) and Cu to *O. mykiss* (Hicks, 2009; Gheorghiu et al., 2010) (Fig. 3C), nor gill accumulation rates of Cu and Cd in blue mussel (Pempkowiak et al., 1999) (Fig. 3D) were significantly related to the relative concentrations of humic-like component. Nevertheless, in most cases the slopes of the relationships were in the direction to be expected if greater relative concentrations of the humic-like fluorophore were to be protective, and some of these were close to significant.

Operational quantification of humic content by PARAFAC supported conclusions based on quantification by classical acid fractionation. Ryan et al. (2004) determined humic acid concentration and found it to be an effective parameter in explaining variation of Cu LC₅₀ values (Ryan et al., 2004). Since humic fractions of aquatic NOM tend to comprise larger molecules (McDonald et al., 2004), it would be generally accepted that bioavailability of larger metal complexes for uptake by organisms may be restricted, with a resulting greater ameliorative effect.

3.3.2. Fulvic-like component

Opposite to what was observed with humic-like fractions, a significant negative relationship between Cu toxicity to larval fathead minnow (Ryan et al., 2004) and relative concentrations of the fulvic-like component of Norway-NOMs was detected ($R^2 = 0.53$, $p < 0.05$, $n = 9$) (Fig. 4A). Similarly, a significant negative correlation ($R^2 = 0.68$, $p < 0.05$, $n = 8$) was seen for Cu LT₅₀ of juvenile *O. mykiss* (Schwartz et al., 2004) (Fig. 4B) in the presence of Canada-NOMs-1 while the relationship with Pb LT₅₀ was just below significance (Fig. 4B). Interestingly, a very strong ($R^2 = 0.72$), highly significant ($p < 0.01$) negative correlation was noticed between Cd LT₅₀ and fulvic-like contents of Canada-NOMs-1 (Fig. 4B). NOM samples with high amounts of fulvic acid have less protective effects against the toxicity of Cu and Cd to aquatic animals.

Ostensibly, De Schampelaere et al. (2004) reached exactly the opposite conclusion by use of their BLM optimization procedure: the greater the “percentage active fulvic acid” (% AFA), the greater was the protective ability of NOM against Cu toxicity to *D. magna*.

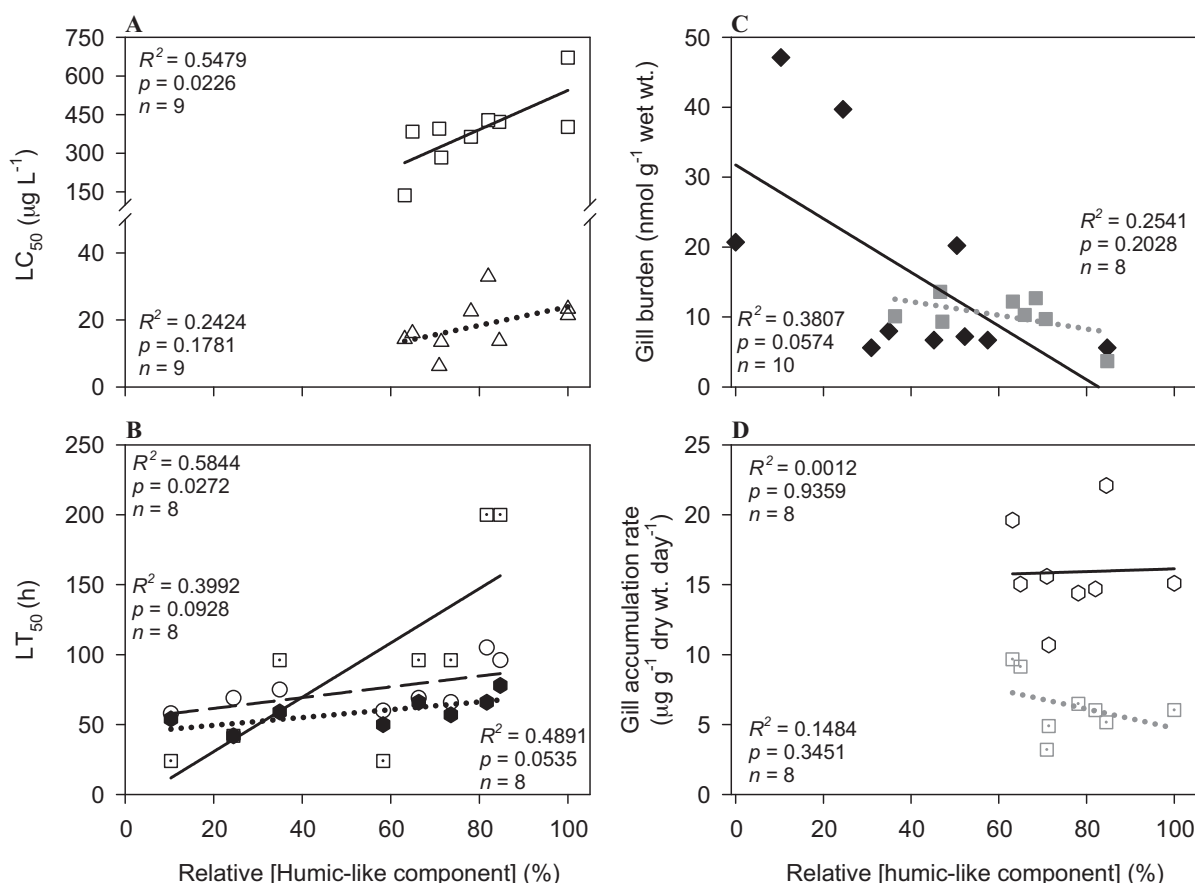


Fig. 3. The influence of relative concentrations of the humic fraction (%) of various NOMs, as revealed by PARAFAC analysis, on toxicity measures of metals in different aquatic organisms. (A) Lethal concentrations (LC₅₀; µg L⁻¹) of Cu (□) and Ag (△) of fathead minnow in Norway-NOMs. (B) Lethal time (LT₅₀, hours) of Cu (□), Pb (○) and Cd (●) of rainbow trout in the presence of Canada-NOMs-1. (C) Gill binding (nmol g⁻¹ wet weight) of Hg (◆) and Cu (■) of rainbow trout in Canada-NOMs-3 and Canada-NOMs-4, respectively. (D) Accumulation rates (µg g⁻¹ dry weight day⁻¹) of Cu (□) and Cd (○) in gill of blue mussel in Norway-NOMs. See text for details on the toxicity measures and refer to Table 1 for DOC levels in each study.

At first glance, this is confusing. However, *a priori*, the BLM used by these researchers assumed that Cu binding capacity was a positive function of “% AFA”, and indeed they found that the optimized “% AFA” was strongly correlated with the UV absorption coefficient at 350 nm – in other words, with the humic-like fluorophore! Thus there is in fact no conflict with the conclusions based on PARAFAC or those based on classical acid fractionation, but in hindsight, the choice of terminology for their modelled parameter is unfortunate.

Interestingly, the positive correlation of the relative fulvic-like composition (derived by PARAFAC) with gill burdens of Hg in *O. mykiss* was significant (Klinck et al., 2005) (Fig. 4C). However, there were no significant relationships with gill accumulation rates of Cu in *O. mykiss* or of Cu and Cd in blue mussels (Pempkowiak et al., 1999) (Fig. 4D).

Overall, there are two possible explanations for the generally lower protective ability of fulvic-like fluorophores. Firstly, fulvic acids are generally recognized to have weaker metal association constants than humic acids (Gondar et al., 2006). Secondly, given their solubility at all pHs (Gaffney et al., 1996), smaller molecular weights (McDonald et al., 2004) and higher surface activity on biological membranes (Visser, 1985), all these factors may allow the passage of some of this fraction and possibly its metal-complexes across biological membranes and accumulation in tissues.

3.3.3. Amino acid-like components

The NOM samples of autochthonous origin are usually enriched in proteinaceous matter such as amino acids. Fluorescence signa-

tures of tryptophan- and tyrosine-like components are commonly reported in aquatic NOMs. For Canadian and Norwegian NOMs, neither the content of tryptophan nor that of tyrosine were correlated to the toxicity measures. Our analysis is supported by recent observations of lack of influence of fluorescent composition of tryptophan and tyrosine in many marine NOM samples on the toxicity of Cu to marine mussels (DePalma et al., 2011b).

3.4. Other characteristics

3.4.1. Binding capacities

Complexation of metals is governed by presence of many different binding sites on NOM. The total concentration of proton-binding sites was fairly variable among Norwegian NOMs, with carboxylates and phenolic alcohols as the dominant ligands (Smith and Kramer, 1999; Takács et al., 1999). Nonetheless, Cu LC₅₀ for fathead minnow ($R^2 = 0.09$, $p = 0.45$, $n = 9$) and accumulation rates of Cd and Cu in gill of blue mussel ($R^2 = 0.12$, $p = 0.40$, $n = 8$; $R^2 = 0.37$, $p = 0.11$, $n = 8$, respectively) appeared not to be influenced by total proton-binding sites. Similarly, copper-binding capacities did not explain the protective effect offered by Norway-NOMs. It is important to note that capacity is not necessarily related to protective effects. The “analytical window” and “toxicological window” have to overlap for a binding site to exhibit an observable protective effect. Consider the equilibrium constant (K) for a one to one complex stoichiometry for metal (M) binding to ligand (L) to form

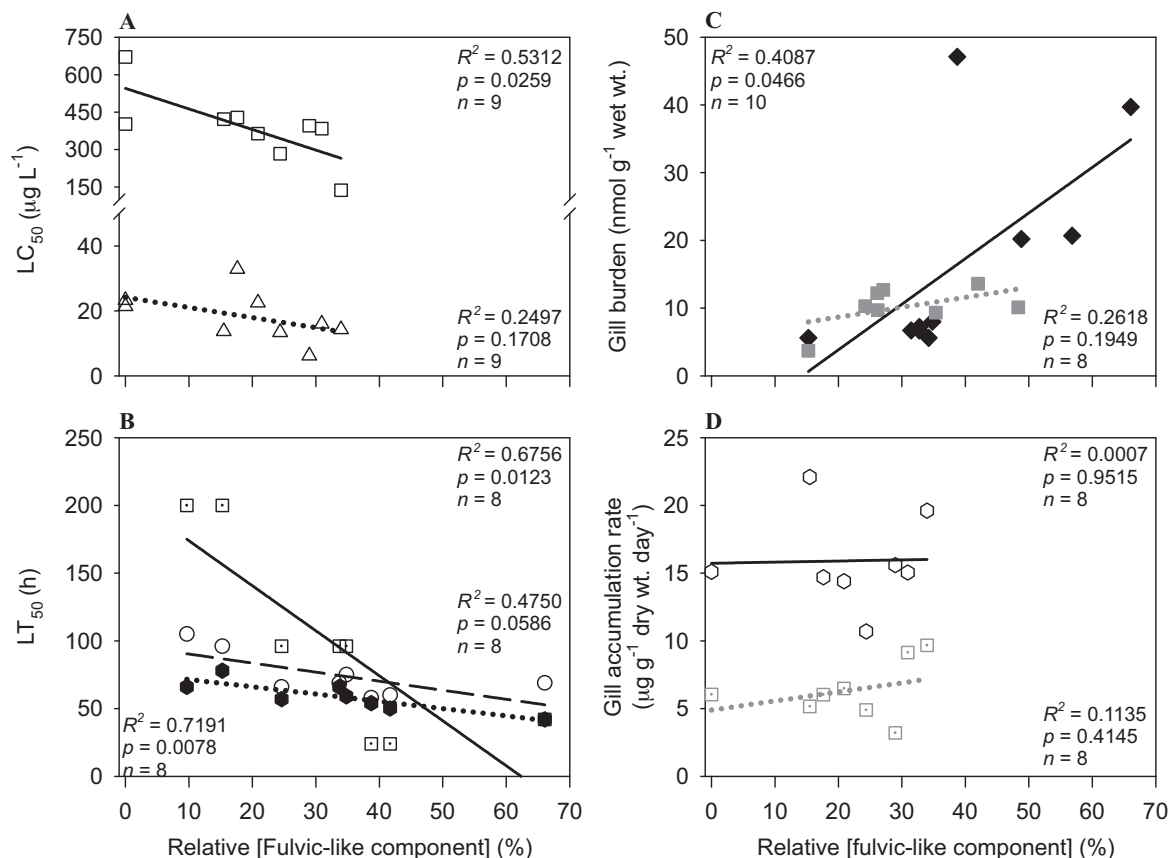


Fig. 4. The influence of relative concentrations of the fulvic fraction (%) of various NOMs, as revealed by PARAFAC analysis, on toxicity measures of metals in different aquatic organisms. (A) Lethal concentrations (LC50; $\mu\text{g L}^{-1}$) of Cu (□) and Ag (△) of fathead minnow in Norway-NOMs. (B) Lethal time (LT50, hours) of Cu (□), Pb (○) and Cd (●) of rainbow trout in the presence of Canada-NOMs-1. (C) Gill binding (nmol g^{-1} wet weight) of Hg (◆) and Cu (■) of rainbow trout in Canada-NOMs-3 and Canada-NOMs-4, respectively. (D) Accumulation rates ($\mu\text{g g}^{-1}$ dry weight day^{-1}) of Cu (□) and Cd (○) in gill of blue mussel in Norway-NOMs. See text for details on the toxicity measures and refer to Table 1 for DOC levels in each study.

complex (ML):

$$K = \frac{[ML]}{[M][L]}, \text{ rearranged to } K[M] = \frac{[ML]}{[L]} \quad (1)$$

It can be seen that when the free metal concentration is equal to $1/K$ the bound ligand and free ligand are equal. For much higher free metal concentrations, the binding site will be completely saturated. The concentration of free metal in solution at the toxicity endpoint determines which binding sites (K values) are active in complexation when toxicity is observed. Thus, a high capacity of sites with a weak binding constant will not be relevant to determining toxic endpoints. Likewise, a low concentration of strong binding sites will probably be completely saturated before toxicity is observed and again will not be correlated with toxicity.

3.4.2. Protein-to-carbohydrate ratio

Surveying 3 RO-NOM isolates, Richards et al. (2001) proposed that protein-to-carbohydrate ratios (0.5 to 1.1 in their samples) can differentiate NOMs and reflect their protective action towards metal toxicity. In the presence of Norway-NOMs, neither Cu and Ag LC50 values for fathead minnow (Ryan et al., 2004; VanGenderen et al., 2003) nor Cu and Cd accumulation rates in blue mussel gills (Pempkowiak et al., 1999) were related to the protein-to-carbohydrate ratio with a more restricted range (0.14–0.40) (Christy et al., 1999) Once more, this makes obvious that Norwegian NOMs included in the international NOM-typing project might not be representative of the full natural ranges in all freshwater systems. There is a need to re-visit this index in the future with toxicity testing using

samples that span a wider range of protein-to-carbohydrate ratios.

3.4.3. Lipophilicity

Direct interaction of NOM with biological surfaces is known (Visser, 1985; Campbell et al., 1997; Galvez et al., 2009) and availability of NOM-metal complexes for uptake has been reported by some workers (Marr et al., 1999; Boulemant et al., 2007) though not by others (Playle et al., 1993). Lipophilicity, estimated by the octanol–water partition coefficient (K_{ow}), is a fundamental property of many organic compounds. Using absorbance as an estimate of NOM partitioning between octanol and water as $(A_{254})_{\text{octanol}}/(A_{254})_{\text{water}}$, Gjessing et al. (1999) calculated octanol solubility for NOMs from 8 Norwegian reservoirs and reported a range of 0.09–3.06. For the same samples, Egeberg and Alberts (2002) estimated lipophilicity by reverse-phase high pressure liquid chromatography (RP-HPLC) and reported results on the logarithmic scale ($\log K_{ow}$) with values less than 2.0 for all samples. Investigations have suggested that aquatic NOM molecules are highly water-soluble with their lipophilic nature showing dependence on pH (Gjessing et al., 1999; Egeberg and Alberts, 2002; Namjesnik-Dejanovic and Cabaniss, 2004) and isolation procedure (Namjesnik-Dejanovic and Cabaniss, 2004). None of the toxic responses to metals (Cu, Ag, and Cd) were related to the lipophilicity of the isolates tested.

4. Are the spectroscopic measures related?

In the above sections, spectroscopic properties (SAC, FI, fulvic- and humic-like components of fluorescence as quantified by

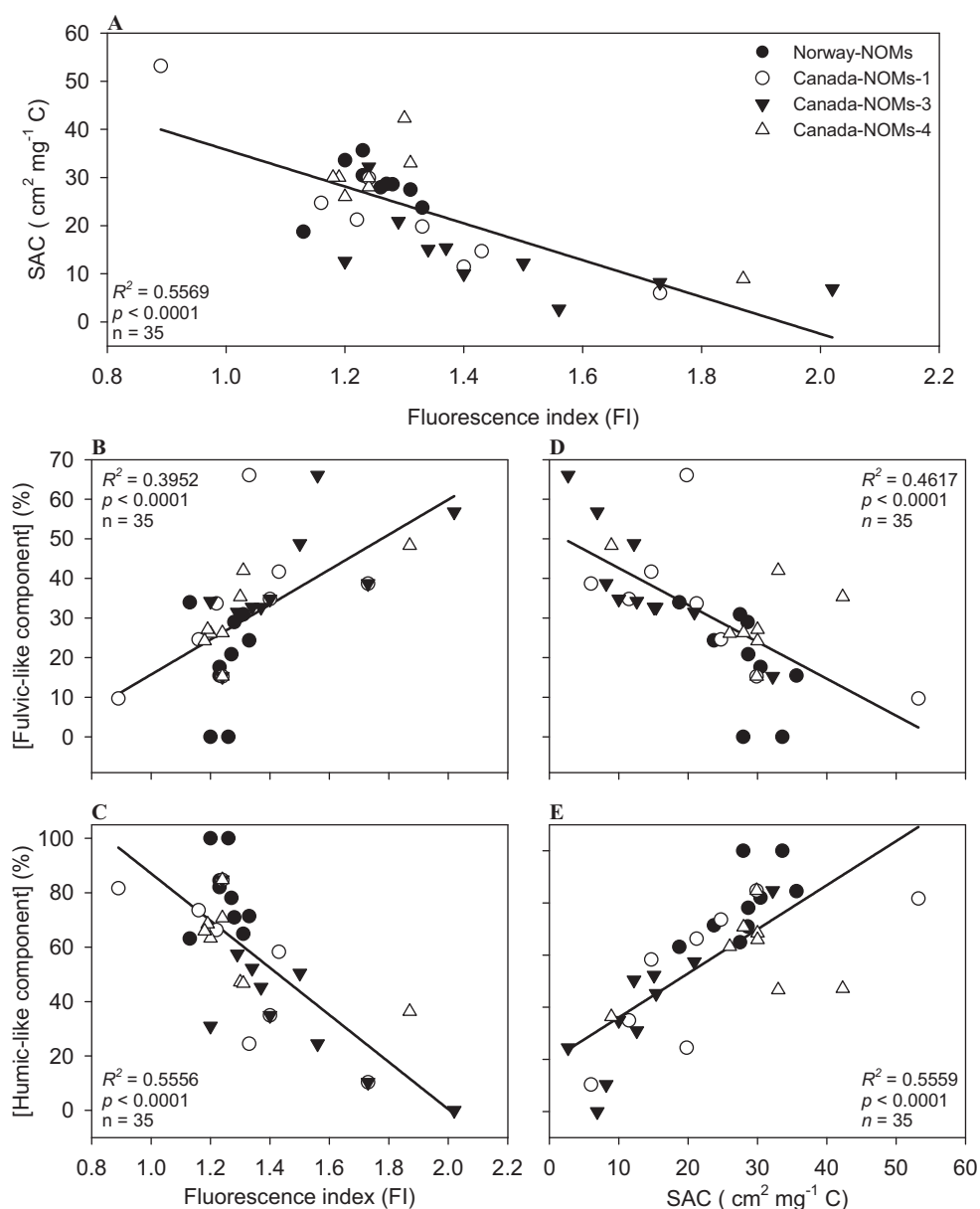


Fig. 5. For Canadian and Norwegian NOM samples, the correlations between (A) aromaticity index (SAC) and fluorescence index (FI), (B and C) the FI and the operationally defined fulvic- and humic-like composition of the samples as revealed by PARAFAC analysis and (D and E) the SAC index and the operationally defined fulvic- and humic-like composition of the samples as revealed by PARAFAC analysis. The symbols for the various NOMs are given in panel (A).

PARAFAC) as molecular probes for aquatic NOM quality were related to measures of toxicity for several metals. Fig. 5 shows relationships between spectroscopic measures themselves. SAC and FI were quality predictors strongly correlated to the protective effect of NOM. The two properties were inversely correlated (Fig. 5A) and were related to the operationally defined (i.e. PARAFAC-derived) humic-like and fulvic-like contents of different NOM samples (Fig. 5B–E). Optically light autochthonous NOM (having relatively lower SAC values and high FI values) tend to be relatively enriched in fulvic-like fluorophores compared to optically dark terrigenous NOM (having relatively higher SAC values and lower FI values) with higher proportions of humic-like components. The major contribution to color intensity (i.e. SAC, aromaticity index) appears to come from the humic-like fraction of NOM samples. On the other hand, an increase in fulvic-like fluorophores of NOM samples is more likely to decrease the color intensity and thus reflect lower aromatic composition. As described here, the trend of operationally defined fulvic-like and humic-like concentrations reinforces the

earlier observations of autochthonous NOM being less protective against metal toxicity than terrigenous NOM (Richards et al., 2001; Schwartz et al., 2004).

5. Conclusion

The concentration-dependent ability to alleviate metal toxicity is well-established for aquatic NOM, however the influence of its quality is complicated because of structural and compositional heterogeneity. Compared to simple DOC measurement, the assessment of quality parameters seems to depend on the isolation method, the analytical technique used to estimate them, as well as the sources from which they were isolated. In the presence of RO-NOMs, physicochemical characteristics, in particular aromaticity and fluorescence indices (SAC and FI, respectively), were found to be useful in differentiating between protection efficiencies of different NOMs. As resolved by PARAFAC, it is likely that NOMs enriched in humic-like fractions (i.e. with longer emis-

sion wavelength) offer better ameliorative effects than those with higher fulvic-like composition. Although insignificant correlations were noted between toxicity responses and other properties such as proton- and copper-binding capacities, protein-to-carbohydrate ratio and hydrophobicity, they have been systematically tested only for Norwegian NOMs. These samples might not be well representative of wide ranges of aquatic environments based on the ranges reported for their quality predictors.

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