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Quantifying the Direct and Indirect Effects of Dissolved Organic Matter (DOM) on Aquatic Organisms: Interaction with pH and Quality Measures

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THE DIRECT AND INDIRECT EFFECTS OF DOM ON AQUATIC ORGANISMS

QUANTIFYING THE DIRECT AND INDIRECT EFFECTS OF DISSOLVED ORGANIC MATTER (DOM) ON AQUATIC ORGANISMS: INTERACTION WITH PH AND QUALITY MEASURES

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ABSTRACT

Dissolved organic matter (DOM) in natural waters is a heterogeneous mixture of organic molecules with direct and indirect influences on aquatic organisms. Although the influences are usually attributed to DOM quantity (quantified as Dissolved Organic Carbon, DOC), the role of quality (optical and binding characteristics obtained by absorbance and fluorescence spectroscopy and potentiometric titration, respectively) is not well-understood. Through an initial critical review of the literature, followed by experimental geochemical, toxicological, and physiological investigations, a number of conclusions were reached that improve our knowledge in this area. Freshwater DOM sources exhibit source-dependent protection against metal toxicity, in particular copper (Cu). Generally, for this indirect effect, optically-dark terrestrially-derived or allochthonous DOMs offer better protection than microbially-derived or autochthonous sources. Linear regressions revealed that the better ameliorative effect is principally related to a higher aromatic composition (specific absorption coefficient, SAC_{340}) and a greater humic-like fluorescent component as quantified by parallel factor analysis (PARAFAC). In addition, the allochthonous DOMs were shown to have relatively higher magnitudes of titration index (TI), a new summary of chemical reactivity of DOM molecules obtained by titration analysis, and closely related to optical properties. TI was strongly correlated with SAC_{340}, suggesting greater binding capacities for DOM molecules with higher SAC_{340}. Consequently, a method for incorporation of SAC_{340} as a DOM quality measure into the Biotic Ligand Model (BLM) was developed which

improved Cu toxicity predictions in experimental tests with natural DOMs. For direct effects, two basic physiological functions (Na^+ metabolism and nitrogen excretion) of the adult water flea (*Daphnia magna*, a cladoceran crustacean) and the zebrafish (*Danio rerio*, a teleost fish) were investigated at circumneutral and acidic pH (≥ 7 and ~ 5, respectively). Three previously characterized, chemically-distinct natural DOM sources as well as a commercial humic acid (AHA) were examined. Regardless of the pH conditions, while Na^+ regulation of *D. magna* remained unaffected by the presence of all DOMs, the passive diffusive efflux of Na^+ in zebrafish was attenuated, indicating ameliorative action against unidirectional Na^+ loss. In addition, only a distinct allochthonous-autochthonous DOM source stimulated the Na^+ uptake rate of zebrafish at low pH. Ammonia excretion rates of *D. magna* were reduced at circumneutral pH by the most highly coloured, allochthonous DOM, and at low pH by all three natural DOMs. Both in *D. magna* and in *D. rerio*, urea excretion rates at both pH conditions were not influenced by the presence of the various DOMs, and the same was true for ammonia excretion in the zebrafish. A commercially prepared humic acid (Aldrich humic acid, AHA) exerted anomalous actions relative to those of natural DOMs, and does not appear to be representative of their normal effects. In contrast to the actions of DOM in detoxifying metals, these direct effects of DOMs on freshwater organisms appeared highly unpredictable with variable dependencies on the source, pH and species. This thesis has advanced our understanding of the relationships between DOM quality and its indirect and direct effects on aquatic organisms, and points to new directions for future work.

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PREFACE

This thesis is organized in the "sandwich thesis" format approved by McMaster University. Chapter 1 provides a general introduction and objectives of the study. Chapters 2–6 were written as separate manuscripts of which two (Chapters 2 and 3) were published before completion of thesis work. Chapters 4 and 5 were submitted for publication prior to submission of this thesis. The planned date for submission of Chapter 6 is indicated. Chapter 7 summarizes and discusses the main findings of the preceding chapters and puts them in context of current knowledge.

Thesis organization and format:

CHAPTER 1: GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

CHAPTER 2: PHYSICOCHEMICAL AND SPECTROSCOPIC PROPERTIES OF NATURAL ORGANIC MATTER (NOM) FROM VARIOUS SOURCES AND IMPLICATIONS FOR AMELIORATIVE EFFECTS ON METAL TOXCITY TO AQUATIC BIOTA

Authors: Al-Reasi H.A., Wood C.M. and Smith, D.S.

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Comments: H.A. Al-Reasi compiled the literature data and wrote the manuscript under the supervision of D.S. Smith and C.M. Wood.

CHAPTER 3: **EVALUATING THE AMELIORATIVE EFFECTS OF NATURAL DISSOLVED ORGANIC MATTER (DOM) QUALITY ON COPPER TOXCITY TO *Daphnia magna*: IMPROVING THE BLM**

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CHAPTER 4: **CHARACTERIZATION OF FRESHWATER NATURAL DISSOLVED ORGANIC MATTER (DOM): EXPLANATIONS FOR PROTECTIVE EFFECTS AGAINST METAL TOXICITY AND DIRECT EFFECTS ON ORGANISMS**

Authors: Al-Reasi H.A., Wood C.M. and Smith D.S.

Submitted on: August 2, 2012

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CHAPTER 5: **THE EFFECT OF DISSOLVED ORGANIC MATTER (DOM) ON SODIUM TRANSPORT AND NITROGENOUS WASTE EXCRETION OF THE FRESHWATER CLADOCERAN (*Daphnia magna*) AT CIRCUMNEUTRAL AND LOW pH**

Authors: Al-Reasi H.A., Yousuf U., Smith D.S. and Wood C.M.

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CHAPTER 6: **THE INFLUENCE OF DISSOLVED ORGANIC MATTER (DOM) ON GILL FUNCTION IN THE FRESHWATER TELEOST, ZEBRAFISH (*Danio rerio*): SODIUM HOMEOSTASIS, PERMEABILITY, AND NITROGENOUS WASTE EXCRETION AT CIRCUMNEUTRAL AND LOW pH**

Authors: Al-Reasi H.A., Smith D.S. and Wood C.M.

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CHAPTER 7: **GENERAL CONCLUSIONS AND DISCUSSION**

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LIST OF ABBREVIATIONS

$(Abs_{254})_{octanol}$	Absorbance at 254 in the octanol phase
$(Abs_{254})_{water}$	Absorbance at 254 in the aqueous phase
$Abs_{254/365}$	Absorbance ratio of 254 to 365 nm
$Abs_{254/436}$	Absorbance ratio of 254 to 436 nm
AHA	Aldrich humic acid
AFA	Active fulvic acid
BL	Bannister lake
BLM	Biotic ligand model
β	Buffer capacity
CON	Control
Cpm	Counts per minute
DC	Dechlorinated water isolate
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EC_{50}	Effective concentration impacting 50% of test organisms
EEMs	Excitation-emission matrices
FI	Fluoresence index
FOCUS	Fully optimized continuous model
HA	Humic acid
J_{in}	Na^+ influx rate
J_{max}	The maximal Na^+ influx rate
J_{out}	Na^+ efflux rate
K	Equilibrium constant
K_m	Uptake affinity
K_{ow}	Octanol-water partition coefficient
L	Ligand
LA_{50}	Lethal accumulation needed to cause 50% mortality of test organisms

LC_{50}	Lethal concentration killing 50% of test organisms
LM	Luther Marsh
LO	Lake Ontario
L_T	Proton site density or binding capacity
LT_{50}	Lethal time required for 50% mortality of test organisms
M	Metal
MRCs	Mitochondria-rich cells
ML	Metal-ligand complex
^{22}Na	Radiolabeled sodium
NOM	Natural organic matter
NR	Nordic Reservoir
PARAFAC	Parallel factor analysis
PCA	Principal component analysis
PE	Preston Effluent
pK_a	Acidity or proton binding constant
POC	Particulate organic matter
Rh	Rhesus
RO	Reverse osmosis
SA	Specific activity
SAC	Specific absorbance coefficient
$SCOA_{436}$	Normalized absorbance at visible light wavelength of 436 nm
SRN	Suwanee River natural organic matter
$SUVA_{254}$	Normalized absorbance at ultraviolet wavelength of 254 nm
TI	Titration index
[^{3}H] PEG-4000	Tritium-labelled polyethylene glycol
TOC	Total organic carbon
UT	Urea transporters
WHAM	Windermere humic aqueous model

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

1.1 Dissolved organic matter (DOM)

Aquatic natural organic matter (NOM) is composed of extremely heterogeneous mixtures of organic compounds. The major constituents of NOM are humic substances generated from dead organic biomass through humification, a process which is a poorly-understood yet one of the most important ecosystem functions on the earth (Buffle, 1984; Ertel *et al.*, 1984; Hatcher and Spiker, 1988). Although the elemental composition is well documented (45–60% carbon, 4–5 % hydrogen, 25–45 % oxygen, 1–2% nitrogen, 1% sulfur and phosphorus) (Filella *et al.,* 2004; Thurman, 1985), molecules of humic substances are very irregular in terms of chemical structure (Gaffney *et al.*, 1996; Filella *et al.*, 2004; Stevenson, 1994) with a wide range of molecular weights (between ~ 500 up to 300000 daltons) and diverse functional groups (Shin *et al.*, 1999; Thurman, 1985). Hypothetical chemical structural models (e.g. Fig. 1.1) for NOM molecules have been proposed based on several analytical techniques. Aquatic NOM can be categorized physically into particulate organic matter (POM) and dissolved organic matter (DOM) (Buffle, 1984). The latter fraction passes through through a 0.45-μm membrane and is chemically made of humic and fulvic fractions or acids (Buffle, 1984; Thurman, 1985). At pH $\leq$ 2.0, humic acid is the fraction that precipitates, while the fulvic acid is the one that remains in solution (Thurman, 1985). In addition, non-humic constituents including carbohydrates, lipids, fatty acids and proteinaceous materials (e.g. amino acids, peptides and proteins) account for about 20% of DOM (Buffle, 1984; Thurman, 1985).

In freshwater systems, DOM (also referred to as gilvin from Latin word '*gilvus*': pale yellow or gelbstoff: yellow matter, in German) is responsible for the yellow to brown

colour of surface water in the lakes and streams (Tipping, 2002). The DOM of an aquatic system (e.g. lake) can be either autochthonous (organic matter released or excreted by bacteria, macrophytes and phytoplankton) or allochthonous (also known as terrigenous, organic matter imported from the surrounding watershed) (McKnight *et al.*, 2001; Williamson *et al.*, 1999). Biochemically, DOM of terrigenous origin is composed mainly of highly coloured humic organic matter (i.e. humic and fulvic acids), while that of autochthonous source is made of humic substances with higher percentages of carbohydrates and proteinaceous materials (Buffle, 1984; McKnight *et al.*, 2001). Dissolved carbon makes ≥ 50% of the aquatic DOM, thus dissolved organic carbon (DOC) concentrations (in mg C L^{-1}) are often used as estimates of DOM concentrations in natural waters (Tipping, 2002). Surface natural freshwaters exhibit large variations in DOC, but the commonly reported range is 1–15 mg C L^{-1}(Thurman, 1985).

1.2 Molecular nature and quality measures of DOM

The protection offered by DOM against metal toxicity appears to be source-dependent (Richards *et al.*, 2001; De Schamphelaere *et al.*, 2004; Ryan *et al.*, 2004; Schwartz *et al.*, 2004) with terrigenous DOMs being better ameliorative agents than autochthonous DOMs. Likewise, some source-dependent direct impacts (Table 1.1) have suggested the involvement of distinct chemical properties in modulating the differences in effects from one DOM source to another. However, chemical properties responsible for such dependence are largely unknown. Spectroscopic measurements have been successfully employed to provide qualitative informantion about some of the chemical

characteristics of freshwater DOM molecules (Senesi *et al.*, 1991; Chen *et al.*, 2002; Her *et al.*, 2003). These absorbance and fluorescence methodologies are selective and sensitive, can be applied to bulk water samples without any chemical pretreatments, and do not alter compositional and structural features (Chen *et al.*, 2002). They have been employed to detect the presence of chromophores (i.e. light-absorbing moieties) and major fluorophores of DOMs (i.e. longer wavelength light-emitting moieties when exposed to shorter wavelength light). Despite the fact that the absorption spectra of DOMs are usually featureless (Dobbs *et al.*, 1972), several measures have been proposed at specific wavelengths to gain qualitative and quantitative information on aliphatic and aromatic character. For example, the specific absorbance coefficient (SAC) estimates aromaticity (Curtis and Schindler, 1997), the absorbance ratio of 254 to 436 nm ($Abs_{254/436}$) determines the relative intensity of UV-absorbing absorbance groups to yellow-brown ones (Abbt-Braun and Frimmel, 1999) and the absorbance ratio of 254 to 365 nm ($Abs_{254/365}$) approximates molecular weights (Dahlén *et al*., 1996). Such indices are considered quality measures to reflect the distinct molecular and structural nature of various DOM sources.

The excitation-emission matrix (EEM) is a simultaneous collection of numerous emission wavelengths over a range of excitation wavelengths (DePalma *et al*., 2011 McKnight *et al.*, 2001; Smith and Kramer, 1999a). The EEM can be depicted as a three dimensional counter plot on which the fluorophores or fluorescent components can be visualized as a function of the excitation-emission pair of wavelengths. Each EEM is considered as a unique fingerprint of the DOM source, providing qualitative information

on the presence of the fluorescent groups (e.g. humic fractions, fulvic fractions, protein-like materials). With the recent advances in handling of florescence spectra by parallel factor analysis (PARAFAC) (e.g. Stedmon and Bro, 2008; Mueller *et al*., 2012), now it is possible to estimate the abundance of each fluorophore in the DOM sample. A better resolution of the heterogenous nature of DOM is a necessary step to distinguish DOM sources and comprehend their direct and indirect biological activity.

Another important molecular feature of DOM is the presence of various functional groups dominated by carboxylates and phenolic groups (Smith and Kramer, 1999a). They are involved in the interactions of DOM with solutes in the water environment (e.g. metal binding) (e.g. Tipping, 2002). In addition, DOMs, as large and multi-charged polyelectrolytes, can alter the charge on biological surfaces (Kullberg *et al*., 1993). Properties of metal detoxification and interaction with organisms have been related to optical properties (e.g. SAC) of DOM (e.g. Galvez *et al*., 2009; Schamphelaere *et al.*, 2004; Ryan *et al.,* 2004; Schwartz *et al.,* 2004), but the link between the chemical reactivity (presence of various functional groups) and spectroscopic characteristics is still lacking.

1.3 Direct and indirect effects of DOM

Many abiotic processes in freshwater ecosystems are affected directly and indirectly by the presence of DOM. As a global regulator, DOM is involved in the global cycling of carbon, attenuation of solar radiation, alteration of nutrient availability, interference with drinking water purification, and modulating acidity of surface water

(Williamson *et al.*, 1999). In the literature, these processes and others have been extensively explored (e.g. Kullberg *et al.*, 1993; Thomas, 1997; Tipping, 2002; Williamson *et al.*, 1999). Although the investigation of the biological influences of DOM on aquatic organisms is not a new research theme (e.g. Hargeby and Petersen, 1988; Petersen and Persson, 1987), there have been limited advances in this subject area largely because of the heterogeneous nature of DOM molecules.

Several direct and indirect effects have been identified, emphasizing the significance of freshwater DOM, not only for abiotic roles towards nonliving components, but also for interactions with organisms. Classical examples of indirect effects include the protection against toxicity and bioaccumulation of metals (e.g. De Schamphelaere *et al.*, 2004; Erickson *et al.*, 1996; Playle *et al.*, 1993; Ryan *et al.*, 2004; Schwartz *et al.*, 2004) and modification of uptake and toxic effects of organic pollutants (e.g. Haitzer *et al.*, 1998; Haitzer *et al.*, 1999; Qiao and Farrell, 2002). Fundamentally, these are considered indirect actions since DOM molecules are not interacting directly with organisms, rather they complex and sequester metals, or other pollutants, in water (Winter *et al.*, 2005). For metals, the ameliorative action varies from one metal to another, but shows dependency on DOC concentrations as well as DOM source (e.g. De Schamphelaere *et al.*, 2004; Playle, 1998; Ryan *et al.*, 2004). While most of the studies show decreases in bioconcentration of organic toxicants in the presence of DOM, some examples of enhancement of the bioconcentration has been reported at low DOM levels, up to 10 mg C L^{-1}(Haitzer *et al.*, 1998).

Several direct biological influences of DOM have been reported for different

freshwater species, ranging from the molecular level to the whole organism (Table 1.1). It is important to note that such effects have rarely been attributed to the uptake of DOM, although experiments based on uptake of ^{14}C-labelled organic molecules (e.g. produced by oxidation of caffeic acid or organic matter released from cultures of diatoms) support the direct uptake of dissolved carbon by different species (Roditi *et al.,* 2000; Speas and Duffy, 1998; Steinberg *et al.,* 2003). However, the oxidative products may not exhibit the inherited heterogeneity of precursor materials and the complexity of real-world DOM molecules (Hatcher and Spiker, 1988; Stevenson, 1994). Alternatively, the work of Campbell and his colleagues (Parent *et al.*, 1996; Campbell *et al.*, 1997; Vigneault *et al.*, 2000, Table 1.1) has provided evidence that DOM molecules may directly interact with biological surfaces. This interaction appears to be pH-dependent and is associated with increased membrane permeability, suggesting hydrogen and hydrophobic binding, and is in accord with earlier thoughts of a surfactant character for DOM (Petersen, 1991). Interestingly, observations from both *in vitro* (e.g. Campbell *et al.*, 1997) and *in vivo* experiments (e.g. Petersen and Persson, 1987; Wood *et al*., 2003) reveal that the impact of DOM is most pronounced at an environmentally low pH range (4–5). However, the effects are not exclusively limited to the low pH range (e.g. Barth and Wilson, 2010; Galvez *et al.*, 2009). The direct actions are DOC concentration-dependent and DOM source-dependent in many cases (Table 1.1). Roy and Campbell (1997) and Wood *et al*. (2011) have speculated that some of the so-called indirect protective effects of DOM against metal toxicity (usually attributed to metal complexation) may actually be due in part to the ability of surface-bound DOM to promote some of the physiological processes

described in subsequent sections.

1.4 Metal toxicity to freshwater organisms and the Biotic Ligand Model (BLM)

Acute metal toxicity to freshwater animals occurs by interference with ionoregulatory processes on the gills and body surface (Wood, 2001). For example, copper (Cu) and silver (Ag) interfere with active sodium ion (Na^+) uptake, while zinc (Zn), cadmium (Cd), and lead (Pb) interfere with active calcium ion (Ca^{2+}) uptake, and virtually all metals may also increase passive ion loss rates (Wood, 2001; Niyogi and Wood, 2004). Metal toxicity to freshwater organisms is affected by water chemistry with DOM acting as a protective factor as described above. For development of site-specific water quality criteria, the use of the Biotic Ligand Model (BLM) has gained widespread interest among the scientific and regulatory authorities in particular for Cu toxicity (Di Toro *et al.*, 2001; Niyogi and Wood, 2004; Paquin *et al*., 2002; Santore *et al.*, 2001). The BLM a conceptual model which can be represented within a chemical equilibrium framework in a computer program that uses water chemistry parameters (model inputs) to predict Cu toxicity to organisms, and to calculate Cu criteria for freshwater (the model output) (Di Toro *et al.*, 2001; Santore *et al.*, 2001; Niyogi and Wood, 2004). The BLM considers competition for binding of Cu ions between the biotic ligand (e.g. Na^+ channel or transporter proteins in the gill surface) and the other aqueous anionic ligands, particularly DOM, in a geochemical modeling framework (Di Toro *et al.*, 2001). Simultaneously, the competition of water cations (e.g. Na^+, Ca^{2+} and Mg^{2+}) with Cu ions for binding sites on the biotic ligand (Di Toro *et al.*, 2001; Paquin *et al.*, 2002) is taken into account. The BLM is

implemented using the Windermere humic aqueous model (WHAM) of metal-DOM complexation (Tipping, 1998) and relates predicted toxicity to the concentration of the Cu (Di Toro *et al.*, 2001). While the source-dependent protection against Cu toxicity by DOM has been widely observed (e.g. De Schamphelaere *et al.*, 2004; Erickson *et al.*, 1996; Playle *et al.*, 1993; Ryan *et al.*, 2004; Schwartz *et al.*, 2004), the currently used BLM (Windows version 2.2.3, HydroQual Inc.) employs total DOC concentration as the input variable, and does not discriminate between DOM from various sources. Rather a default assumption that 10% of the DOC is humic acid is written into the program, and only this aspect of DOM chemistry can be manipulated by the user.

1.5 Sodium metabolism and nitrogenous waste excretion of freshwater organisms

Freshwater organisms must maintain Na^+ homeostasis (Marshall, 2002) and excrete toxic by-products arising from deamination of amino acids and catabolism of proteins, so as to minimize build up of nitrogenous wastes in their tissues (Evans *et al.*, 2005). These by-products are mainly ammonia and urea; ammonia refers to sum of gaseous ammonia, NH_3 and ammonium ion, NH_4^+, unless otherwise specified. The situation is exacerbated when they are stressed by metal exposure (e.g. Bianchini and Wood, 2003; Grosell and Wood, 2002; Zimmer *et al*., 2012) or low pH (4) water (e.g.Wood *et al.*, 1998; Wood *et al.*, 2003), causing disturbances in ionoregulation and ammonia excretion. Overall, freshwater fish and crustaceans share similar apical epithelial entities for Na^+ uptake mechanisms and nitrogenous waste excretion. However, the actual ion-transporting cells (i.e. ionocytes or mitochondria-rich cells, MRCs) may

differ in distribution pattern and, transporter localization within epithelial cells may differ among species (Kirschner, 2004; Evans *et al.,* 2005). Compared to hardness, pH and salinity, DOM has been neglected for the investigation of the basic physiology of sodium metabolism and nitrogen excretion in aquatic organisms until recently (e.g. Wood *et al.*, 2003; Glover and Wood, 2005).

1.6 Sodium metabolism and nitrogenous waste excretion of *Daphnia magna* (water flea) and *Danio rerio* (zebrafish)

As well-known models for environmental and toxicological studies, the water flea (*Daphnia magna*), which is a cladoceran crustacean, and the zebrafish (*Danio rerio*), which is a teleost fish, were utilized in this research project. Respective functional models of ion transport for both organisms are presented in Figure 1.2. Adults of *D. magna* are able to absorb ions with ion-transporting cells on epipodites located on thoracic appendages, which may be also involved in osmoregulation and excretion (Goldmann *et al.,* 1999; Kikuchi, 1983). Apical uptake of Na^+ ions from the external water into the epithelial cells takes place via the electrogenic $2Na^+/1H^+$ exchanger (Glover and Wood, 2005b; Bianchini and Wood, 2008) similar to other freshwater crustaceans such as crayfish (Kirschner, 2004). Two apical pathways are widely accepted for Na^+ uptake in freshwater fish: (1) the absorption of Na^+ ions by an epithelial Na^+ channel (often referred to as ENaC) which is energetically powered by the H^+-ATPase; and (2) the electroneutral exchange of Na^+ for H^+ ions via a Na^+/H^+ exchanger (NHE) (Evans *et al.*, 2005; Hwang, 2009). Both pathways have been debated for long time, given the lack of molecular

evidence for existence of a true ENaC in teleost genomes, and the unknown mechanism to drive NHE's in the face of apparent thermodynamic constraints (Hwang, 2009; Hwang *et al*., 2011). In zebrafish, the NHE-3 protein in H^+-ATPase rich cells, a subset of MRCs, appears to be responsible for active Na^+ uptake (Hwang, 2009; Yan *et al.*, 2007). Evidence is also available for the presence of the other Na^+ uptake mechanism mediated by H^+-ATPase through ENaC-like channels (Boisen *et al*., 2003, Horng *et al*., 2007; Kumai and Perry, 2011). The basolateral Na^+/K^+-ATPase powers the apical uptake of Na^+ ions indirectly by shuttling ions from absorbing cells to the extracellular fluids, and therefore creating an electrochemical gradient to drive more Na^+ ions into the absorbing cells from external water (Evans *et al*., 2005; Krischner, 2004).

While little is known about the mechanisms controlling nitrogenous waste excretion in *D. magna*, there is a general understanding of ammonia and urea excretion from fish studies. Although both ammonia and urea were traditionally thought to diffuse passively across gill epithelia along partial pressure or concentration gradients, transporters for ammonia (Rhesus glycoproteins, Rh) and urea (UT) have been recently described in the gills of freshwater teleosts (Braun *et al.*, 2009; Nakada *et al.*, 2007; Nawata *et al.*, 2007). Wright and Wood (2009) proposed a new general model for ammonia excretion in freshwater fish, incorporating the recent discovery of ammonia transporters and addressing the several mechanisms proposed and debated for a long time. The model provides variable linkages of ammonia efflux to both Na^+ uptake as well as acid (H^+) excretion (Wright and Wood, 2009). According to Figure 1.2B, the gaseous NH_3 diffuses across the apical gill membrane *via* an Rh protein (Rhcg1 in zebrafish),

down the partial pressure gradient. Once in the water on the apical side, ammonium ion (NH_4^+) is formed by combining NH_3 with H^+ ions which are pumped by H^+-ATPase and/or NHE-3 (Hwang *et al.*, 2011; Wright and Wood, 2009). Wright and Wood (2009) suggested that the acid excretion may provide the needed electrochemical force to drive apical Na^+ uptake through the Na^+ channel, implying an indirect "Na^+/NH_4^+ exchange system" proposed earlier (e.g. Wilkie, 2002). Simple transcellular diffusion of NH_3 has also been proposed for ammonia extrusion (Wilkie, 2002). Catalytic conversion of expired metabolic CO_2 by carbonic anhydrase (CA) provides additional acid (i.e. H^+ ions) in the unstirred water next to the gill epithelium (i.e. boundary layer) for trapping more NH_3 molecules as NH_4^+ ions (Wright *et al.*, 1986; Wright *et al.*, 1989).

1.7 Objectives of the study

This thesis addressed two main themes; (1) the direct and indirect influences of DOM on two freshwater organisms (the water flea and the zebrafish) and (2) the DOM quality measures needed to account for the influences. The following major objectives were set to be accomplished by the end of the research project:

1. To investigate whether qualitatively different aquatic DOM sources vary greatly in their ameliorative ability against metal toxicity, as indirect effects, according to their physicochemical characteristics (Chapter 2 and 3).
2. To test the applicability of using the physicochemical characteristic(s), as a DOM quality input parameter, to improve metal toxicity prediction by a widely used metal toxicity prediction tool, the Biotic Ligand Model (BLM) (Chapter 3).

3. To find simple-to-measure quality measures to account for source-dependent phenomena observed with respect to the direct and indirect effects of different DOMs on aquatic organisms (Chapter 4).
4. To develop a summary parameter linking the chemical reactivity (i.e. functional groups from potentiometric titration) of DOM sources with their spectroscopic characteristics (Chapter 4).
5. To elucidate the influences of natural DOM sources on the basic physiology (Na^+ regulation and ammonia excretion) of freshwater organisms (Chapters 5 and 6).
6. To ascertain the pH-dependent influences of natural DOM sources facilitating direct interactions with freshwater organisms (Chapter 5 and 6).
7. To compare the influences of the natural DOM sources on the basic physiology to that of commercially-prepared humic substances (Chapters 5 and 6).

1.8 Hypotheses and overview of the chapters

The focus of Chapter 2 was to review and summarize available data on DOM physicochemical characteristics which have been reported to play a role in alleviating metal toxicity toward aquatic biota. Data from chemically-characterized DOM isolates, obtained by reverse osmosis, were compiled from published literature. Quality measures obtained by absorbance (specific absorption coefficient, SAC) and fluorescence spectroscopy (fluorescence index, FI, and fluorescent composition) were collected. For limited DOM isolates, binding capacities, protein-to-carbohydrate ratio and lipophilicity were also included. Toxicity measures (effective concentration- EC_{50}, lethal

concentration- LC_{50}, lethal time- LT_{50} and gill binding and accumulation rates) of several metals for different aquatic organisms in the presence of these isolates were collected. Taking into account water chemistry, regression analyses were employed to explore correlations between the quality measures and the toxicity measures. Overall, SAC and FI as well as concentrations of fluorescent fractions (humic-like and fulvic-like components) obtained by parallel factor analysis (PARAFAC), explain considerable variability in the protective effects against toxicity of copper (Cu) and lead (Pb), in particular.

Chapter 3 tested the hypothesis that since the protective effects of DOM are source-dependent, lethal Cu toxicity (LC_{50}) in various DOM sources will vary with their quality measures. Different quality measures of various DOM sources were evaluated with respect to the protection of *Daphnia magna* neonates against lethal Cu toxicity (LC_{50}). Significant correlations in toxicity amelioration were found with absorbance ratios $Abs_{254/365}$ (an index of molecular weight), Abs-octanol $_{254}$/Abs-water $_{254}$ (an index of lipophilicity), SAC_{340} (an index of aromaticity), and with the relative concentration of the humic-like fluorescent component quantified by PARAFAC. The results demonstrated that optically dark, more aromatic DOMs of terrigenous or allochthonous nature are more protective against Cu toxicity. In addition, the data suggested that additional ameliorative effect can be attributed to larger molecules and the lower lipophilic nature of allochthonous relative to autochthonous DOM sources. Therefore, a method for incorporation of SAC_{340}, as a DOM quality input, into the Biotic Ligand Model was developed to increase the accuracy for predicting Cu toxicity in natural waters.

Chapter 4 summarized and explored the correlations between the quality measures obtained by absorbance and fluorescence spectroscopy and potentiometric titrations. The titration data were utilized to calculate a summary parameter (the titration index or TI). The TI was calculated from the measurement of acid, base and intermediate proton binding capacities. This index and the optical characteristic (i.e. SAC_{340}) exhibited a very high positive correlation for a number of natural DOM sources. Thus, the chemical reactivity, as summarized by acid-base titration, of each source was linked to a fundamental optical property (SAC_{340} or aromaticity index) of DOM. This is a significant step toward understanding the mechanisms involved in the higher protection against Cu toxicity observed in the presence of darker organic matter (Chapter 3).

In Chapters 5 and 6, the hypotheses that DOMs would impact the processes of Na^+ regulation and nitrogenous waste excretion, that the effects would vary depending on the type of DOM (autochthonous versus allochthonous, natural versus commercially-prepared sources), and that responses would be more pronounced at lower environmental pH, were tested. Tests were conducted with adult *D. magna* and zebrafish in the presence of three natural DOM sources ranging from allochthonous to autochthonous, as well as a commercial humic acid (AHA), under two pH conditions (circumneutral pH $\geq$ 7 and acidic pH $\sim$ 5). For *D. magna*, results showed that the various natural DOMs had negligible impact on Na^+ homeostasis, and that differences attributable to pH occurred only with the "unnatural" commercial (Aldrich) humic acid preparation (AHA). Nevertheless, low pH alone greatly increased ammonia and urea excretion in *D. magna*, and the natural DOMs, especially the most highly coloured allochthonous DOM from a

marsh, exerted some marked effects on ammonia excretion, particularly at low pH (Chapter 5). For zebrafish, very different results were obtained. A reduction of passive Na^+ efflux was observed in the presence of all DOM sources regardless of pH condition and stimulation of Na^+ uptake was restricted to a mixed autochthonous-allochthonous DOM source only at low pH. In addition, a kinetic parameter of active Na^+ uptake (the maximal Na^+ uptake rate, J_{max}) was stimulated only in the presence of a commercially prepared humic acid source. Neither ammonia excretion nor urea excretion were affected by the presence of DOMs (Chapter 6). Overall, the results of these two chapters demonstrate that direct DOM effects are not only source-dependent, but also pH-dependent and organism-specific, and that the effects of commercially prepared humic acid differ from those of natural DOMs.

1.9 Tables and figures

Table 1.1: Summary of direct effects of DOM on freshwater organisms

Organism		Measured effect
Algae	-	Increased membrane permeability (Knauer and Buffle, 2001; Parent *et al.,* 1996; Vigneault *et al.,* 2000)*
	-	Binding to cell surfaces (Campbell *et al.,* 1997; Knauer and Buffle, 2001; Parent *et al.,* 1996; Vigneault *et al.,* 2000)*
Fungi	-	Source-dependent growth inhibition or stimulation (Meinelt *et al.,* 2007)
Nematode	-	Source-dependent effects on reproduction (Hoss *et al.,* 2001; Menzel *et al.,* 2005)
	-	Behavioral attraction (Menzel *et al.,* 2005)
Crustacean	-	Interference with Na^+ uptake (Glover *et al.,* 2005; Glover and Wood, 2005)
	-	Higher survival in acidic water (Hargeby and Petersen, 1988; Petersen and Persson, 1987)*
	-	DOC-dependent increase of glutathione *S*-transferase (Meems *et al.,* 2004)
	-	Mortality at higher DOC concentrations (Petersen and Persson, 1987)*
	-	Induction of heat shock protein (Timofeyev *et al.,* 2004)
	-	Activity changes of peroxidase (Timofeyev *et al.,* 2004)
Amphibian	-	Stimulation of thyroid hormone (Lutz *et al.,* 2005)
	-	Growth and swimming improvement (Barth and Wilson, 2010)
Fish	-	*In vitro* binding to gill cells (Campbell *et al.,* 1997)*
	-	Interference with Na^+ regulation (Gonzalez *et al*., 1998 and 2002; Wood *et al.,* 2003; Matsuo *et al.,* 2004)
	-	Source-dependent amplification of gill transepithelial potential (Galvez *et al.,* 2009)
	-	Source-dependent induction of cytochrome P450 (Matsuo *et al.,* 2006)
	-	Communication disturbance (Heidi *et al.,* 2006)

* The effect is either enhanced or seen at low pH only.

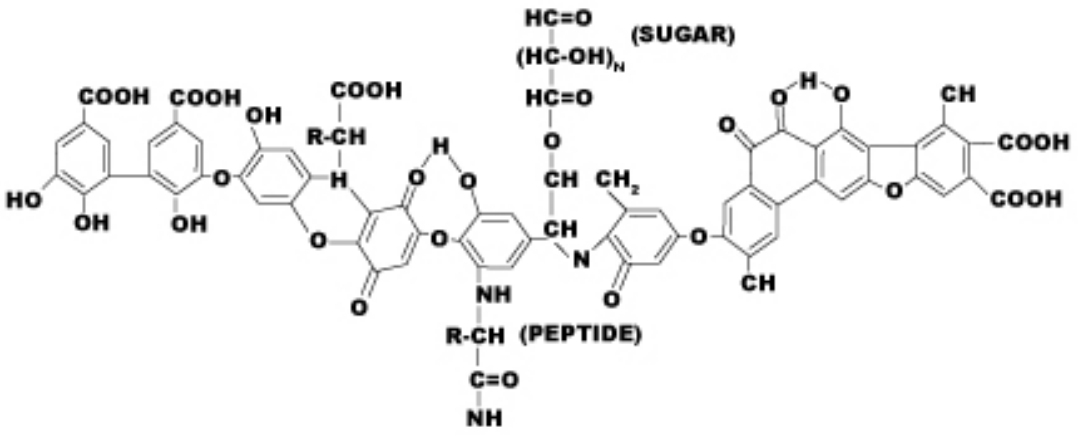

Figure 1.1 A proposed model structure for humic acid (Stevenson, 1982). Unlike other organic compounds, DOM molecules are not described in term of a unique chemical structure (Gaffney *et al.*, 1996; Filella *et al.*, 2004).

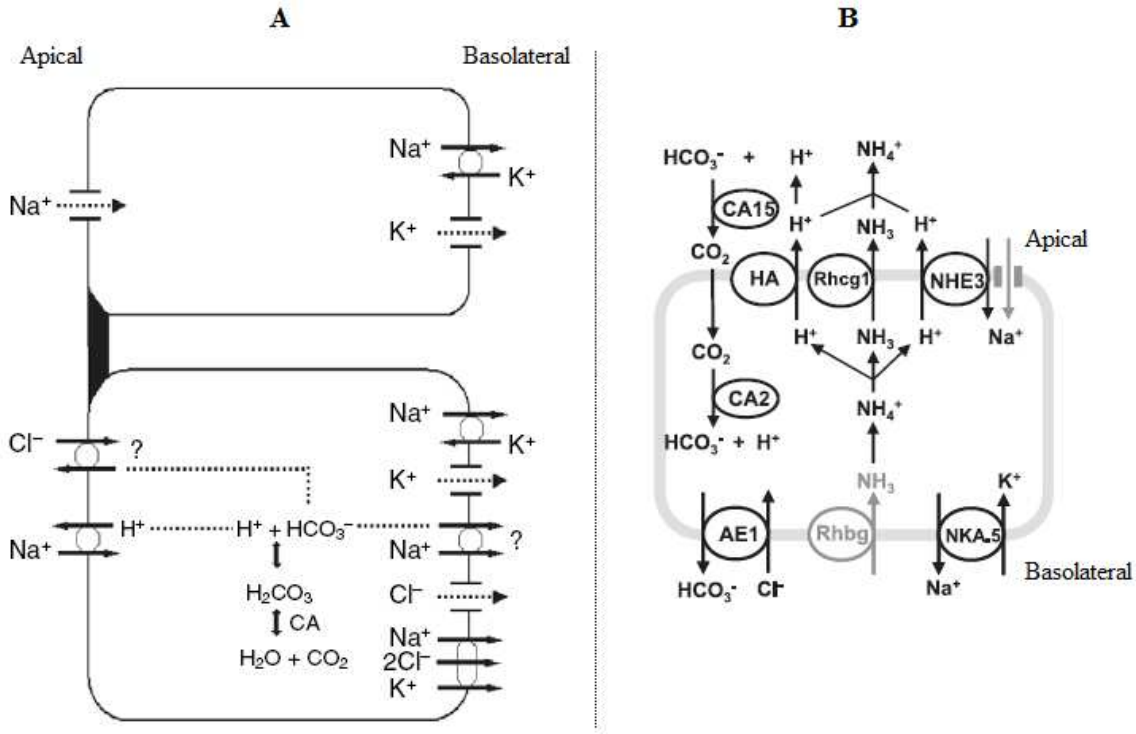

Figure 1.2 Functional models of ionoregulation and ammonia excretion of the freshwater (**A**) water flea (*D. magna*) and (**B**) zebrafish (*D. rerio*). For (**A**), the two kinds of cells, dark and light types, of the ion-transporting epithelia of *D. magna* (Kikuchi, 1983) are depicted where solid and broken lines indicate transport and diffusion, respectively (Bianchini and Wood, 2008). For (**B**), only an H^+-ATPase-rich cell is depicted, a type of MRCs found in zebrafish gill epithelium, responsible for apical Na^+ uptake and ammonia excretion (Hwang *et al*., 2011). Abbreviations: AE: anion exchanger, CA: carbonic anhydrase, HA: H^+-ATPase, NHE3: Na^+/H^+ exchanger, Rhcg: Rh transporter for ammonia and NKA: Na^+/K^+-ATPase (Hwang *et al*., 2011). The (?) indicates the need for more data and/or apparently not present.

1.10 References

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CHAPTER 2

PHYSICOCHEMICAL AND SPECTROSCOPIC PROPERTIES OF NATURAL ORGANIC MATTER (NOM) FROM VARIOUS SOURCES AND IMPLICATIONS FOR AMELIORATIVE EFFECTS ON METAL TOXICITY TO AQUATIC BIOTA

2.1 Abstract

Natural organic matter (NOM), expressed as dissolved organic carbon (DOC in mg C L^{-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. LC_{50}), 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.

2.2 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 C L^{-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-µm 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 microrganisms 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 colour (Tipping, 2002). In general terrigenous NOM tends to be dark in colour, 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 (Peterson, 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 U.S.A. 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 an 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.

2.3 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 Schamphelaere *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 Schamphelaere *et al.*, 2004; Ryan *et al.*, 2004; Glover *et al.*, 2005b). Indeed, there are only very limited toxicological data available for NOMs obtained using other isolation procedures.

In our present analyses, we have exploited an excellent data source on the various physico-chemical 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 (EC_{50}), lethal concentrations (LC_{50}), and lethal time (LT_{50}, 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 Schamphelaere *et al.*, 2004; Glover *et al.*, 2005b) have been collated, together with additional physico-chemical 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 2.1.

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 × absorbance at a specific wavelength often 340nm and normalized to TOC) (Curtis and Schindler, 1997) and the fluorescence index (FI, determined as fluorescence intensity$_{450\ nm}$/fluorescence intensity$_{500\ nm}$; 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 2.2).

It is important to note that most of the experimental conditions were relatively comparable at environmentally-relevant levels of DOC (Table 2.1). 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 2.1). 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. predictor variables on the x-axis) and the measured toxic responses (EC_{50} LC_{50}, LT_{50}, 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.

2.4 Do quality predictors explain the protective effects of NOM?

2.4.1 Aromaticity

The near UV absorptivity has been utilized as a standard measure of colour in

natural waters (Cuthbert and del 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 Figure 2.1.

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. 2.1). In samples collected from Europe and the U.S, SAC_{350} could explain most of variation (R^2= 0.70) in ameliorative behaviour of NOM against Cu EC_{50} to *D. magna* (De Schamphelaere *et al.*, 2004) (Fig. 2.1A). Highly significant correlations were also found between the aromatic composition of both Norway-NOMs and Canada-NOMs-1 versus Cu LC_{50} in fathead minnow, *P. promelas* (Ryan *et al.*, 2004) (Fig. 2.1A) and Cu LT_{50} for rainbow trout, *O. mykiss* (Schwartz *et al.*, 2004) (Fig. 2.1B), 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. 2.1C). However, in a separate study, no correlation (R^2= 0.002, p = 0.923, n = 7) was found between SAC_{340} of Canada-NOMs-2 versus Pb LT_{50} in the same species (MacDonald *et al.*, 2002) (Fig. 2.1C). The SAC_{340} values of Canada-2 samples spanned a more limited range (~ 5–17 cm^2 mg^{-1}) relative to that (~ 5–60 cm^2 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 LT_{50} to *O. mykiss* (Schwartz *et al.*, 2004) (Fig. 2.1B). No correlation was detected between SAC_{340} and Ag toxicity to *P. promelas* in the presence of Norway-NOMs (VanGenderen *et al.*, 2003) (Fig. 2.1D).

There were also weakly significant (for Hg) and non-signifcant correlations (for Cu) between SAC_{340}, 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 (μg g^{-1} dry weight day^{-1}) in the gills of the blue mussel *(*Pempkowiak *et al.*, 1999) were plotted versus the SAC_{350} (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 SAC_{300} (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) 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 reduced sulphur content of various NOMs and their protective ability against Ag toxicity to *D. magna.*

2.4.2 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 to 1.3, implying an exclusively terrestrial origin.

As illustrated in Fig. 2.2, this quality predictor could not explain the variability in protection offered by these samples against Cu (Ryan *et al.*, 2004) (Fig. 2.2A) and Ag (VanGenderen *et al.*, 2003) (Fig. 2.2A) to fathead minnow. However, in contrast, Cu (Fig. 2.2A) and Pb LT_{50} (Fig. 2.2B) 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. 2.2C) 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. 2.2C). Cu or Cd accumulation rates in gills of blue mussel were only weakly associated with FI (Pempkowiak *et al.*, 1999) (Fig. 2.2D) 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 SAC_{350} of Norway-NOMs, the FI of the same samples could not explain variation in Cu toxicity to *P. promelas* (Ryan *et al.*, 2004) (Fig. 2.2A). 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. 2.2B) 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. 2.2C) 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.

2.4.3 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^{TM} 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 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.

2.4.3.1 Humic-like component

Our PARAFAC analysis indicates that Cu toxicity to larval fathead minnow

(Ryan *et al.*, 2004) (Fig. 2.3A) and juvenile rainbow trout (Schwartz *et al.*, 2004) (Fig. 2.3B) 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 LC_{50} and LT_{50} 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. 2.3D). The same was true of Pb and Cd toxicity to *O. mykiss* (Schwartz *et al.*, 2004) in the presence of Canada-NOMs-3 (Fig. 2.3B). Likewise, neither gill burdens of Hg (Klinck *et al.*, 2005) (Fig. 2.3C) and Cu to *O. mykiss* (Hicks, 2009; Gheorghiu *et al.*, 2010) (Fig. 2.3C), nor gill accumulation rates of Cu and Cd in blue mussel (Pempkowiak *et al.*, 1999) (Fig. 2.3D) 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 LC_{50} 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. Diffusion of DOM-metal complexes through the biological membrane has been reported for small model molecules (Marr *et al*., 1999),

though it is unclear whether this occurs with natural DOM.

2.4.3.2 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. 2.4A). Similarly, a significant negative correlation (R^2= 0.68, p < 0.05, n = 8) was seen for Cu LT_{50} of juvenile *O. mykiss* (Schwartz *et al.*, 2004) (Fig. 2.4B) in the presence of Canada-NOMs-1 while the relationship with Pb LT_{50} was just below significance (Fig. 2.4B). Interestingly, a very strong (R^2= 0.72), highly significant (p < 0.01) negative correlation was noticed between Cd LT_{50} and fulvic-like contents of Canada-NOMs-1(Fig. 2.4B). NOM samples with high amounts of fulvic acid have less protective effects against the toxicity of Cu and Cd to aquatic animals.

Ostensibly, De Schamphelaere *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*. 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. 2.4C). 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. 2.4D).

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. However, these observations may only be true for small model molecules.

2.4.3.3 Amino acid-like components

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

2.4.4 Other characteristics

2.4.4.1 Binding capacities

Complexation of metals is governed by the 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 LC_{50} 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 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.

2.4.4.2 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 LC_{50} 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 to 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.

2.4.4.3 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 based on model molecules (Marr *et al.*, 1999; Boullemant *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 $(Abs_{254})_{octanol}/(Abs_{254})_{water}$, 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.

2.5 Are the spectroscopic measures related?

In the above sections, spectroscopic properties (SAC, FI, fulvic- and humic-like components of fluorescence as quantified by PARAFAC) as molecular probes for aquatic NOM quality were related to measures of toxicity for several metals. Figure 2.5 shows relationships among spectroscopic measures themselves. SAC and FI were quality predictors strongly correlated to the protective effect of NOM. The two properties were

inversely correlated (Fig. 2.5A) and were related to the operationally-defined (i.e. PARAFAC-derived) humic-like and fulvic-like contents of different NOM samples (Fig. 2.5B-E). Optically-light autochthonous NOM (having relatively lower SAC values and high FI values) tends 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 colour 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 colour 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).

2.6 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 among protection efficiencies of different NOMs. As resolved by PARAFAC, it is likely that NOMs enriched in humic-like fractions (i.e. with longer emission 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.

2.7 Tables and figures

Table 2.1 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 [f]						Reference
		Toxicity parameters				DOC levels (mg C L^{-1})		
		Endpoint [g] (unit)	Metal	Range [h]	Variation	Range	CV [i]	
Norway-NOMs [b]	Blue mussel [e] (*Mytilus trossulus*)	accumulation rate (µg g^{-1} dry wt. day^{-1})	Cu Cd	3.20 – 9.67 10.70 – 22.09	3.0 1.3	6.0	NA	Pempkowiak *et al.*, 1999
	Fathead minnow (*Pimephales promelas*)	96-h LC_{50} (µg L^{-1})	Ag	6.20 – 32.9	5.3	3.3 – 5.1	0.14	VanGenderen *et al.*, 2003
	Fathead minnow (*Pimephales promelas*)	96-h LC_{50} (µg 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 (*Oncorhynchus mykiss*)	96-h LT_{50} (h)	Cu Pb Cd	24.0 – 200.0 48.0 – 105.0 42.0 – 78.0	8.3 2.2 1.9	5.7 – 6.5 3.0 – 4.4 5.6 – 6.9	0.04 0.12 0.05	Schwartz *et al.*, 2004
Canada-NOMs-2	Rainbow trout (*Oncorhynchus mykiss*)	96-h LT_{50} (h)	Pb	90.0 – 168.0	1.4	2.8 – 4.0	0.14	MacDonald *et al.*, 2002
Canada-NOMs-3	Rainbow trout (*Oncorhynchus mykiss*)	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 (*Oncorhynchus mykiss*)	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	Relative protective	Ag	0.57– 3.66	6.4	NA	NA	Glover *et al.*, 2005b,c

	(*Daphnia magna*)	unit						
Europe and US-NOMs	Water flea (*Daphnia magna*)	48-h EC_{50} ($\mu g\ L^{-1}$)	Cu	50.6 – 129.0	2.5	2.0 – 3.0	0.20	De Schamphelaere *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] LC_{50}: lethal concentration needed to result in 50% mortality of test organisms; LT_{50}: time to reach 50% mortality of test organisms; EC_{50}: effective concentration required to immobilize 50% of *D. magna* neonates (De Schamphelaere *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 LC_{50} against Aldrich humic acid concentration, and then computing the ratio of the LC_{50} for the particular NOM to the LC_{50} 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 2.2 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 2.1**).

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	Tackás *et al.*, 1999
	Protein-to-carbohydrate ratio [c]		0.14 – 0.37	2.64	Christy *et al.*, 1999
	Lipophilicity [e]	Octanol solubility	0.09 – 3.06	34.00	Gjessing *et al.*, 1999
		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		
	Fulvic-like fraction concentration (%)estimated by PARAFAC		15.26 – 66.07	4.33	
Canada-NOMs-4	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	
	Fulvic-like fraction concentration (%)		15.26 – 48.32	3.17	

	estimated by PARAFAC			
Canada-NOMs-5	Aromaticity: SAC_{300} ($cm^2\ mg^{-1}\ C$)	32.26 – 123.65	3.83	Glover *et al.*, 2005c
	Fluorescence index (FI)	1.32 – 2.31	1.75	
Europe and US-NOMs	Aromaticity: SAC_{340} ($cm^2\ mg^{-1}\ C$)	13.20 – 49.10	3.72	De Schamphelaere *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: $(Abs_{254})_{octanol}/(Abs_{254})_{water}$. 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

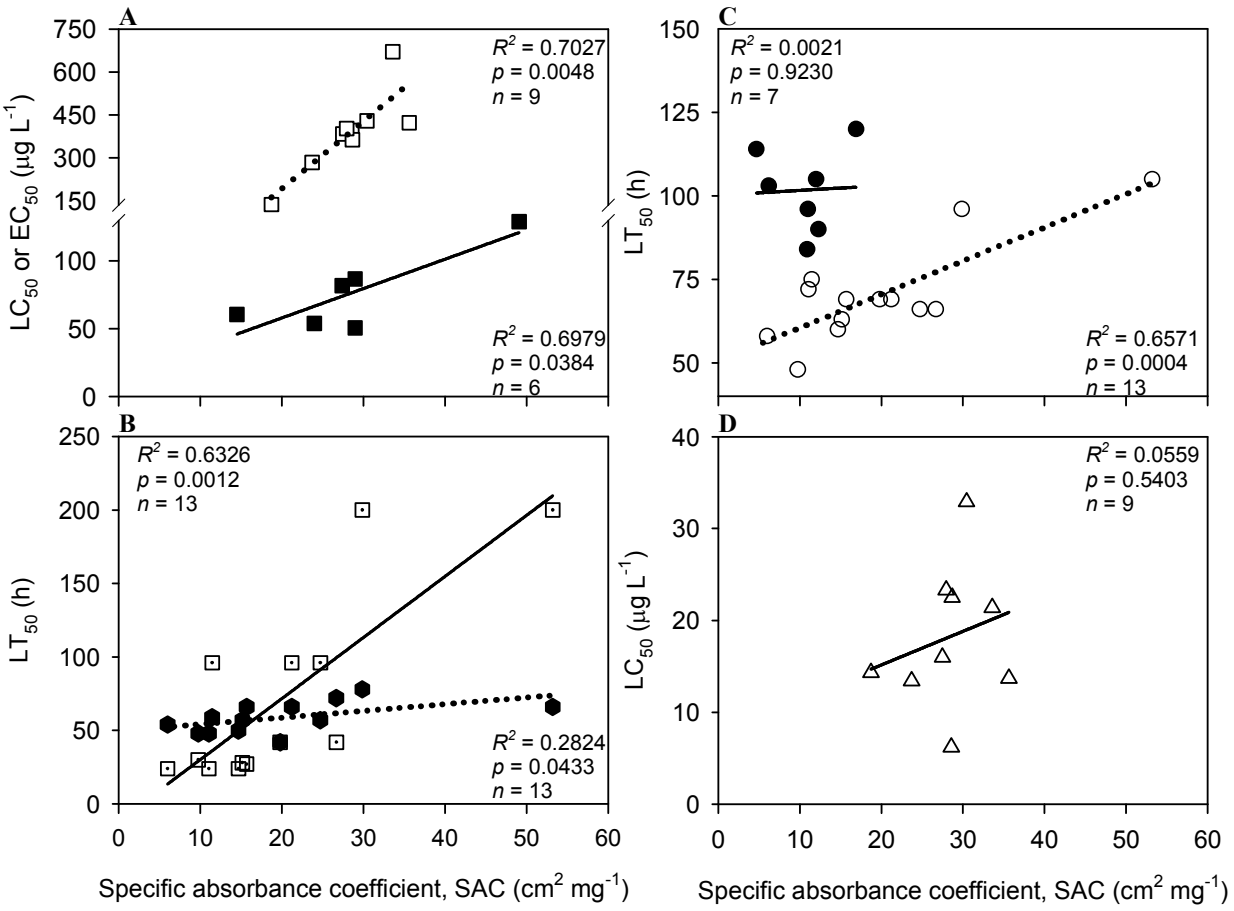


Figure 2.1 The relationships between specific absorbance coefficient (SAC, in cm^2/mg C, an index of aromaticity) of various NOMs and lethal toxicity measures of several metals against different aquatic organisms. (**A**) Cu-LC_{50} (□) of fathead minnow in Norway-NOMs and Cu-EC_{50} (■) of *Daphnia magna* in US and Europe-NOMs. (**B**) Cu-LT_{50} (⊡) and Cd-LT_{50} (⬢) of rainbow trout in Canada-NOMs-1. (**C**) Pb-LT_{50} (●,○) of rainbow trout in Canada-NOMs-1 and Canada-NOMs-2, respectively. (**D**) Ag-LC_{50} (△) of fathead minnow in Norway-NOMs. See text for details on the toxicity measures and refer to Table 2.1 for DOC levels in each study.

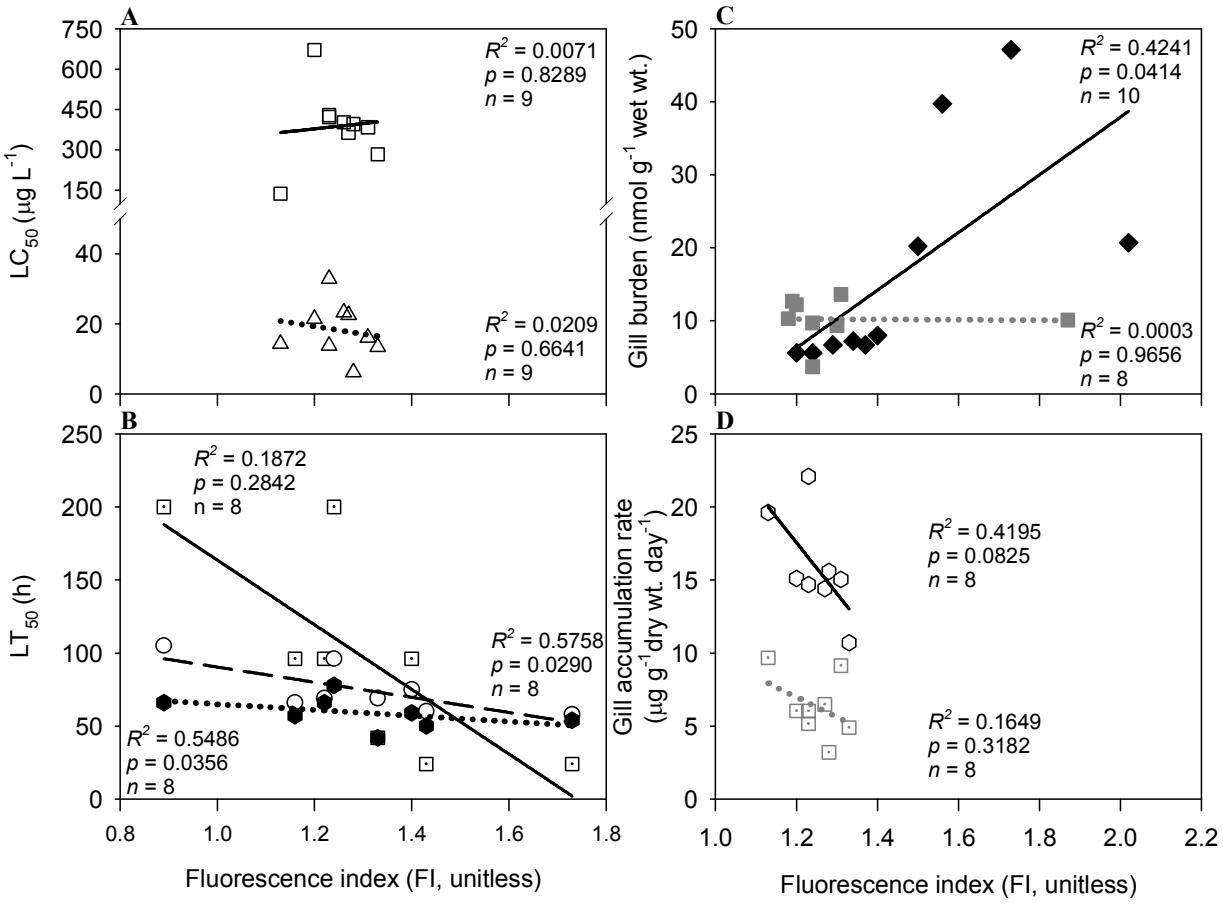


Figure 2.2 The relationships between fluorescence index (FI, an indicator of origin) of various NOMs and toxicity measures of metals in different aquatic organisms. (**A**) Lethal concentrations (LC_{50}; µg L^{-1}) of Cu (□) and Ag (△) of fathead minnow in Norway-NOMs. (**B**) Lethal time (LT_{50}, 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 2.1 for DOC levels in each study.

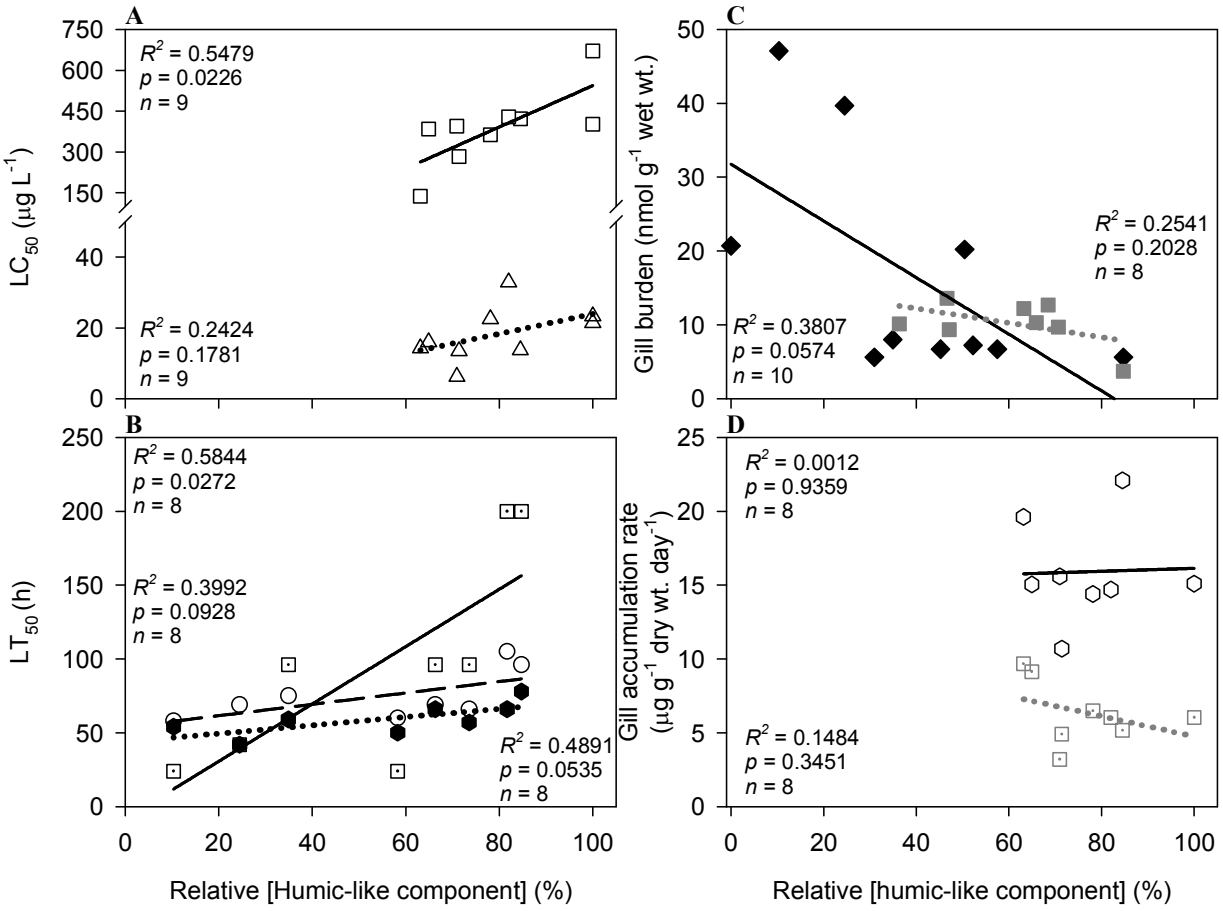


Figure 2.3 The influence of relative concentrations of the humic fraction (%) of various NOMs, as revealed by PARAFAC analysis, on toxicity measures of metals in different aquatic organisms. (**A**) Lethal concentrations (LC_{50}; µg L^{-1}) of Cu (□) and Ag (△) of fathead minnow in Norway-NOMs. (**B**) Lethal time (LT_{50}, hours) of Cu (⊡), Pb (○) and Cd (⬢) of rainbow trout in the presence of Canada-NOMs-3. (**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 2.1 for DOC levels in each study.

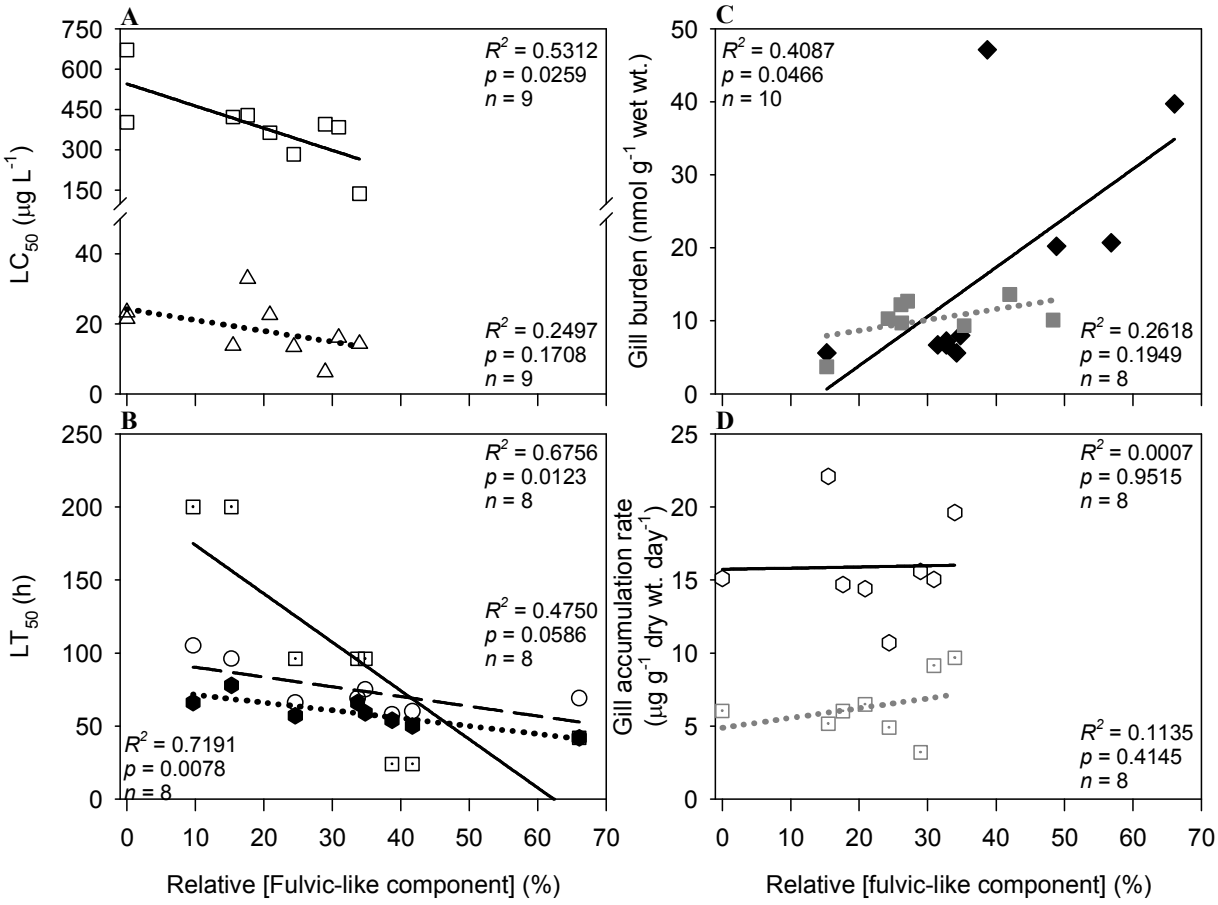
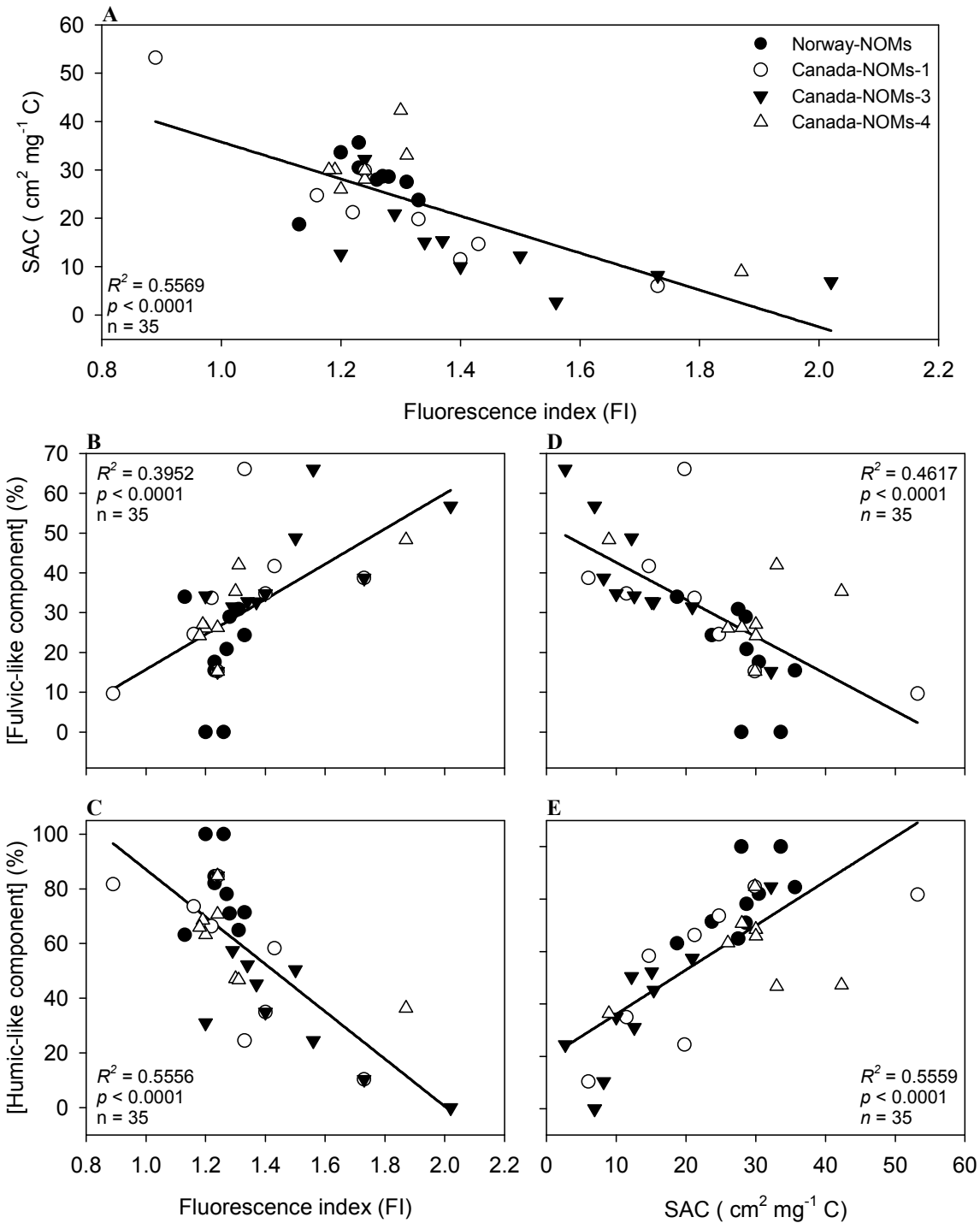


Figure 2.4 The influence of relative concentrations of the fulvic fraction (%) of various NOMs, as revealed by PARAFAC analysis, on toxicity measures of metals in different aquatic organisms. (**A**) Lethal concentrations (LC_{50}; µg L^{-1}) of Cu (□) and Ag (△) of fathead minnow in Norway-NOMs. (**B**) Lethal time (LT_{50}, hours) of Cu (⊡), Pb (○) and Cd (⬢) of rainbow trout in the presence of Canada-NOMs-3. (**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 2.1 for DOC levels in each study.

Figure 2.5 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**).


A
Norway-NOMs
Canada-NOMs-1
Canada-NOMs-3
Canada-NOMs-4
SAC (cm2 mg-1 C)
R2 = 0.5569
p < 0.0001
n = 35
Fluorescence index (FI)
B
R2 = 0.3952
p < 0.0001
n = 35
[Fulvic-like component] (%)
D
R2 = 0.4617
p < 0.0001
n = 35
C
R2 = 0.5556
p < 0.0001
n = 35
[Humic-like component] (%)
Fluorescence index (FI)
E
R2 = 0.5559
p < 0.0001
n = 35
SAC (cm2 mg-1 C)

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CHAPTER 3

EVALUATING THE AMELIORATIVE EFFECTS OF NATURAL DISSOLVED ORGANIC MATTER (DOM) QUALITY ON COPPER TOXCITY TO *Daphnia magna*: IMPROVING THE BLM

3.1 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-octanol $_{254}$/Abs-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-octanol $_{254}$/Abs-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 of 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.

3.2 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 (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. In mining areas, Cu levels as high as 200,000 μg L^{-1} have been reported (USEPA's Water Quality Criteria for Copper, 2007), though it is unclear whether all of this remains in the solution. 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 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, carbonate anion (CO_3^{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 x 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 that 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 among 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.

3.3 Materials and methods

3.3.1 DOM collection and treatment

To obtain DOM isolates spanning a wide range between autochthonous and terrigenous natures, 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 3.1), 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 ($pH \leq 2$), and then kept refrigerated in dark cold room at 4°C, in polyethylene containers until their use for chemical analyses and exposure tests. Table 3.1 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 (≥ 17.5 MΩ cm; Barnstead Nanopure II, Thermo Scientific Barnstead, USA).

3.3.2 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 ~ 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, Franklin 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 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 – 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 (~ 300 and 350 nm) and two at longer wavelengths (~ 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 (Eigen-vectors Research Inc, WA, USA) which resulted in score vectors as estimates of relative concentrations.

3.3.3 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 ($[Na^+]$ = ~ 0.7 m*M*, $[Ca^{2+}]$ = ~ 1.0 m*M*, $[Mg^{2+}]$ = ~ 0.3 m*M*, [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, CEROPHYLL®, 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).

3.3.4 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 $CuSO_4 \cdot 5H_2O$; 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 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.

3.3.5 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) 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 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.

3.3.6 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. On average, the total and dissolved Cu concentrations had a reduction range of 0 to 15 % between the beginning and end of the exposure. The 50% lethal concentrations (LC_{50}, Cu concentrations required to kill 5 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. Before applying t-test 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) to detect variation in LC_{50} in various DOMs, data points were log-transformed when they violated these assumptions. 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 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 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.

3.3.7 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.

3.4 Results and discussion

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

Figure 3.1A presents the 48-h dissolved LC_{50} values of Cu for *D. magna* in the presence of the various DOM sources listed in Table 3.1. 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 DOC's 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 an increase trend of the dissolved LC_{50} (Fig. 3.1A). The further increases at 6 mg C L^{-1} (relative to 3 mg C L^{-1}) were statistically significant for all DOMs except those from Lake Ontario (LO), Nordic Reservoir (NR) and Luther Marsh (LM) (Fig. 3.1A). The variability in Cu toxicity in presence of each nominal DOC level is obvious as indicated by the standard deviation (Fig. 3.1A). In fact, determination of LC_{50} replicates within the same water source has been shown to be similarly variable in other studies (e.g. Santore *et al.*, 2001), presumably reflecting biological variation. Figure 3.1B 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. 3.1B), suggesting that the unifying feature of the different DOC 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 DOC 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 DOC, the mean measured values for pH and concentrations of Na^{+}, Ca^{2+}, Mg^{2+} and DOC in test water did not vary substantially among toxicity tests in dechlorinated water with added DOM from each source (Table 3.2). The single exception was Preston Effluent (PE) where the DOC 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*.

3.4.2 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 3.1) 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 3.3) or dissolved Cu LC_{50} (Fig. 3.3) were just below the significance level (p = 0.07 and 0.08, respectively) 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 3.3, Fig. 3.2). 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 be more effective in binding Cu 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. Nevertheless, this parameter based on the absorbance spectrocopy has never been validated. The 9-fold range of octanol solubilities of DOMs (0.012–0.106) included in the present study (Table 3.1) 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 3.3; Fig. 3.3). 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 3.3; Fig. 3.3). 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 to 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. It should be noted that Cu activity alone (Fig. 3.1B) is sufficient to explain the observed toxicity results. Each DOM source protects by reducing free Cu to a free Cu LC_{50} value. However, the possibility of small DOM complex bioavailability should be considered in future work as it might represent a mechanism of Cu uptake.

Specific absorbance coefficient (SAC_{340}) of the DOMs, considered an index of aromaticity (Curtis and Schindler, 1997), varied widely between 3.72 (dechlorinated Hamilton tap water isolate, DC) to 79.98 cm^2 mg^{-1}(Aldrich humic acid, AHA) (Table 3.1), 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 3.1). 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. 3.4). Similar observations were noted for the relationship of the total Cu LC_{50} versus the aromatic composition index at both DOC levels (Table 3.3). This implies high effectiveness of darkly coloured DOMs in alleviation of Cu toxicity compared to the

lightly coloured 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).

3.4.3 Fluorescence indices

Fluorescence index (FI) is 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 3.3). Nevertheless, allochthonous DOMs (BL and LO) appeared less protective, as judged by LC_{50} values (Fig. 3.1A), 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 PARAFAC (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. 3.5. 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 to 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. 3.5). 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. 3.5A). Another characteristic feature of autochthonous DOMs (DC, LO, BL), revealed by quantification using PARAFAC, was their higher proportions of proteinaceous materials (Fig. 3.5B), 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. 3.6). 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. 3.2). 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 3.3). 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 3.3) 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 3.3). Ryan *et al.* (2004) fractionated DOMs into humic acid (insoluble below pH 2.0) and fulvic acid portions (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 3.3). 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.

3.4.4 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 ($Abs\text{-}octanol_{254}/Abs\text{-}water_{254}$) 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 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 3.3, Fig. 3.2, 3.3, 3.4 and 3.6), 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). From a chemical perspective, the relationship between the aromaticity of DOM sources and their effectiveness in protecting against Cu toxicity does not solely reflect the involvement of aromatic moieties but also functional groups (especially oxygen-containing sites) associated with aromatic parts of DOM molecules.

3.4.5 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 if occurred, resulted in relatively constant Cu activity at the various LC_{50} (Fig. 3.1B). 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).

3.4.6 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. 3.7A). 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}. An LA_{50} of 0.0120 nmol g^{-1} wet weight was recovered from the optimization. This is value is much lower than the default LA_{50} (0.119 nmol g^{-1} wet). It is important to note that the optimized value could not be compared to a "true" or measured LA_{50} due to lack of experimental data. In fact, LA_{50} is BLM parameter that is adjusted for different organisms as a way to implement the change in sensitivity of various organisms, especially invertebrates (e.g. *D. magna*) to

copper toxicity (U.S. EPA, 2003). For example, optimization of BLM for a closely related species, *D. pulex* resulted in a LA_{50} value of 0.035 nmol g^{-1} wet weight (U.S. EPA, 2003), which is not too far from the value found here for *D. magna*. From a practical perspective, the adjustment of LA_{50} affects only the sensitivity of the model but the response of the model to changes in chemistry is determined by log K values and is not affected by changes in the LA_{50} (U.S. EPA, 2003). The optimized LA_{50} yielded the best correlation between the predicted and observed LC_{50} using $\%HA_{PARAFAC}$ (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. 3.7B). 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. 3.8A), as well as with Cu toxicity amelioration (Fig. 3.4, Table 3.3). 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 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. 3.8A) 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. 3.8B), 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 toxicity predictions toxicity for Cu in natural waters.

3.5 Tables and figures

Table 3.1 Characteristics of DOM isolates and humic substances used in the analysis

DOM Source [a]	Coordinates	Type	SAC_{340} (cm^2 mg^{-1}) [d]	$Abs_{254/365}$ [e]	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).

Table 3.2 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} DOC of each DOM isolate.

Treatment	*n* [a]	pH	DOC (mg L^{-1})	Na^+ (µM)	Ca^{2+} (µM)	Mg^{2+}(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)	Ca^{2+} (µM)	Mg^{2+} (µM)	*n*	pH	DOC (mg L^{-1})	Na^+ (µM)	Ca^{2+} (µM)	Mg^{2+} (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 LC_{50} determinations.
[b] Values are mean ± standard deviation.

Table 3.3 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 L^{-1} DOC to dechlorinated Hamilton tap water.

Quality predictors		Total Cu LC_{50}				Dissolved Cu LC_{50}			
	DOC added at	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-$254_{octanol}$/ Abs-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.

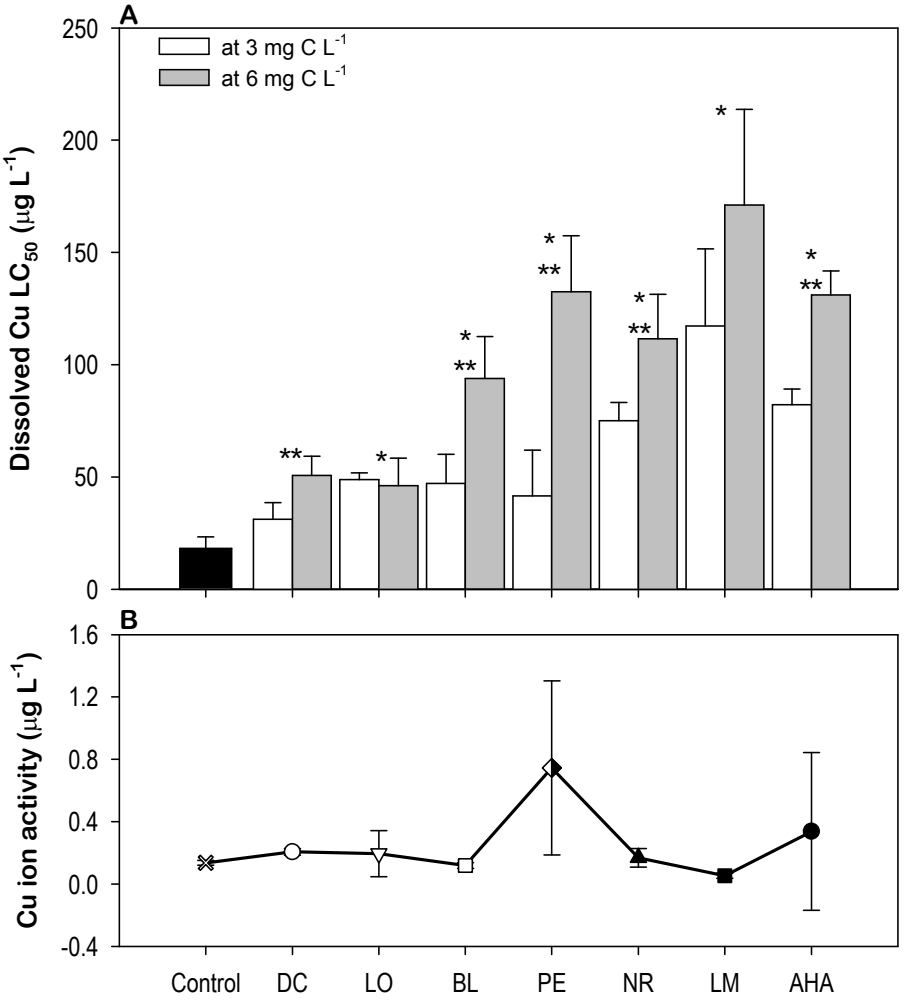

Figure 3.1 (**A**) The 48-h 50% lethal concentrations (LC_{50}) of dissolved copper to *D. magna* in the presence of DOMs from various aquatic environments.(**B)** Copper ion activity measurements obtained at the total Cu LC_{50} concentrations for each DOM isolate at a nominal DOC concentration of 3 mg C L^{-1}. The measured DOC concentrations reported in column 3 of Table 3.2.The bars represent mean ± standard deviation. Refer to Tables 3.1 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.

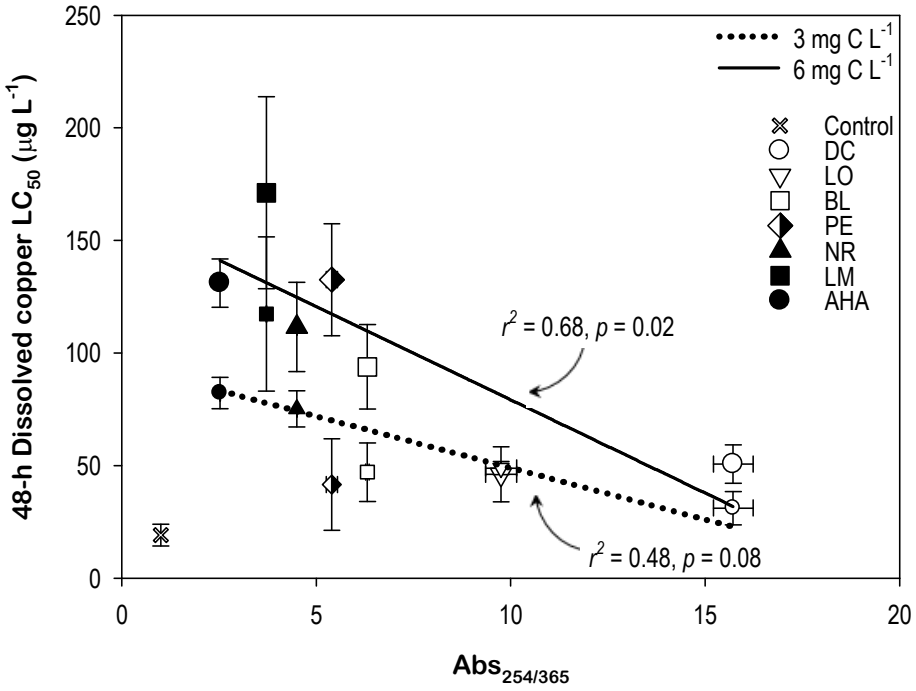

Figure 3.2 Influence of molecular weight of DOM molecules, as estimated by $Abs_{254/365}$ ratio, on copper toxicity to *D. magna* in the presence of DOMs from various aquatic environments added at 3 and 6 mg L^{-1}. The values represent mean ± standard deviation. Refer to Tables 3.1 and 3.3 for abbreviations of DOMs and regression parameters, respectively. The control data were not included in the regression analysis. The larger symbols are for data obtained in presence of 6 mg C L^{-1}.

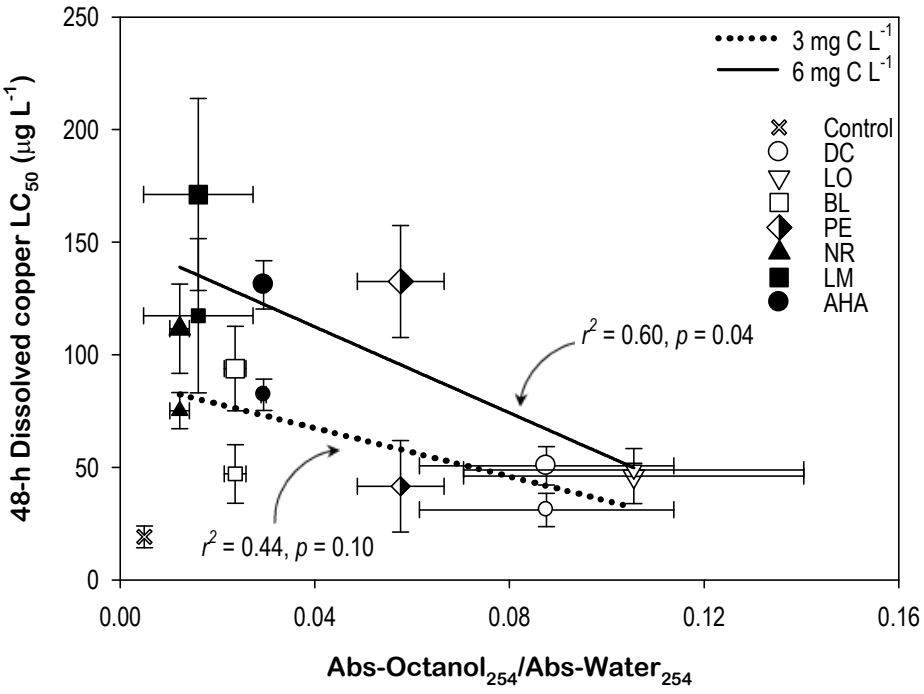

Figure 3.3 Influence of octanol solubility of DOM molecules, an index of lipophilicity, calculated as Abs-octanol $_{254}$/Abs-water $_{254}$ ratio, on copper toxicity to *D. magna* in the presence of DOMs from various aquatic environments added at 3 and 6 mg L^{-1}. The values represent mean ± standard deviation. Refer to Tables 3.1 and 3.3 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 presence of 6 mg C L^{-1}.

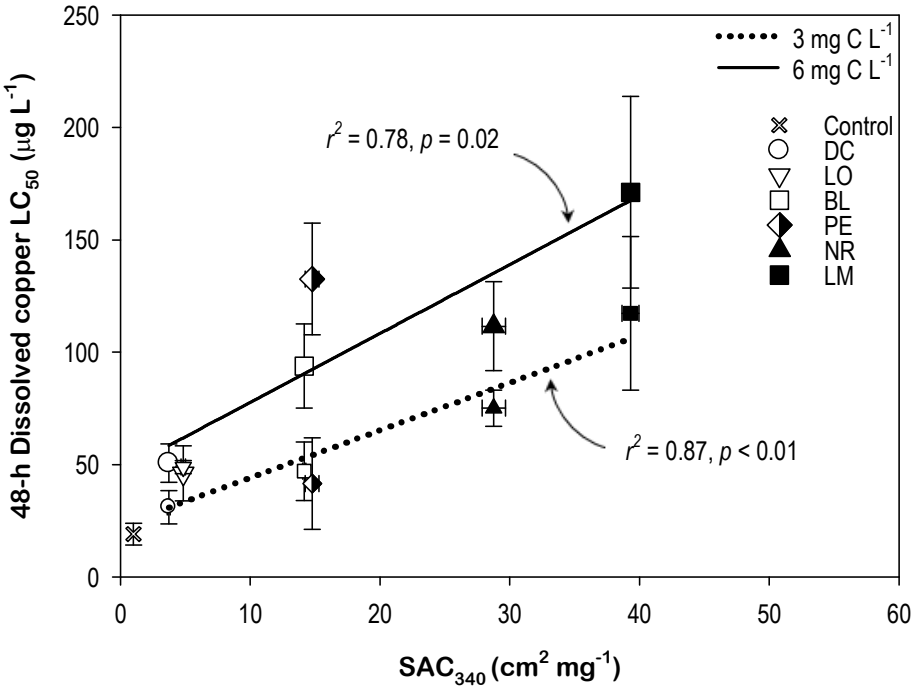

Figure 3.4 Influence of aromaticity of DOM molecules, estimated by SAC_{340}, on copper toxicity to *D. magna* in the presence of DOMs from various aquatic environments added at 3 and 6 mg L^{-1}. The values represent mean ± standard deviation. Refer to Tables 3.1 and 3.3 for abbreviations of DOMs and 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}.

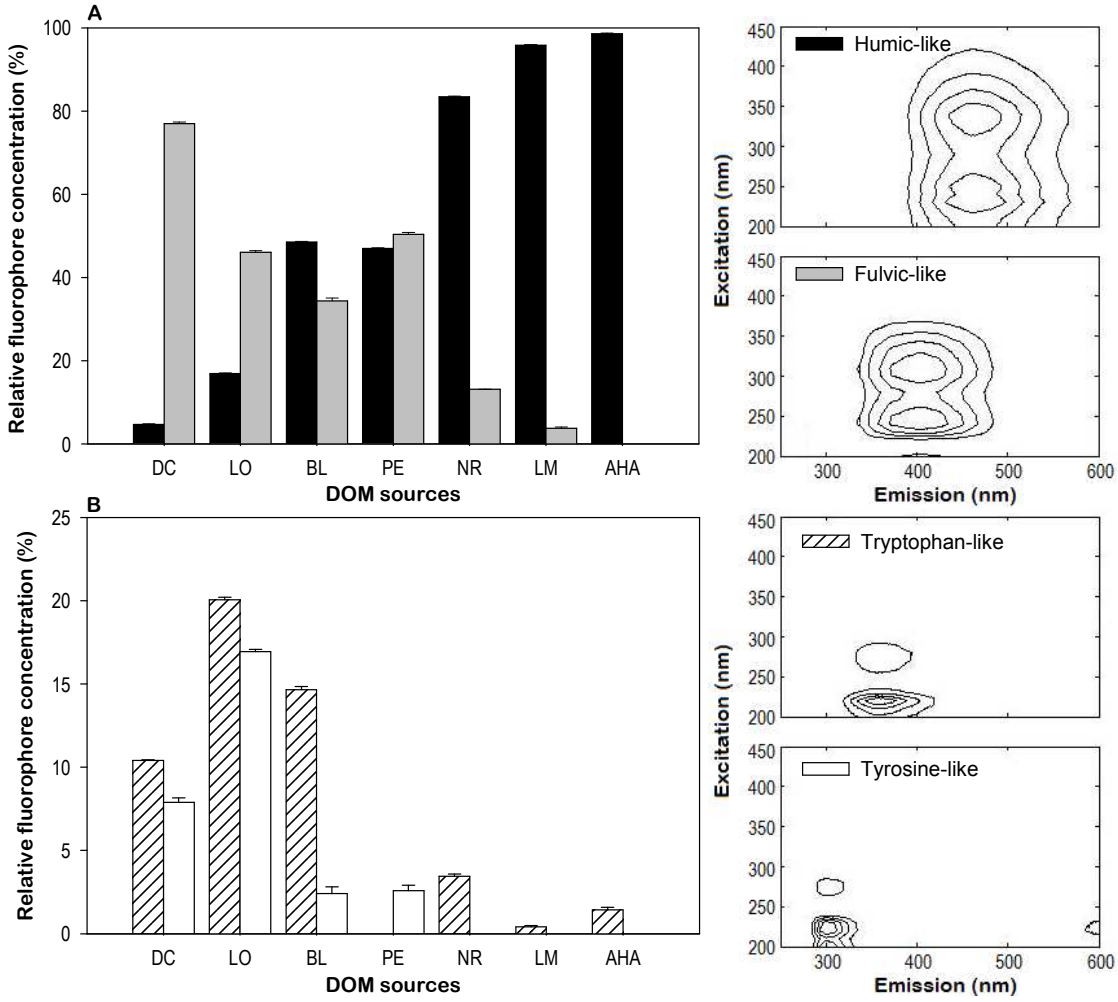

Figure 3.5 The relative concentrations of humic-like, fulvic-like, tyrosine-like, and tryptophan-like in each DOM isolate resolved by PARAFAC. Refer to right hand panels for bar codes. The right-hand panel of (**A)** shows the two fluorescence components (fulvic-like and humic-like) with longer emission wavelengths (~ 410 and 460 nm) and that of (**B**) illustrates the two fluorescence components (tryptophan-like and tyrosine-like) at shorter emission wavelengths (~ 300 and 350 nm). Refer to Table 3.1 for abbreviations.

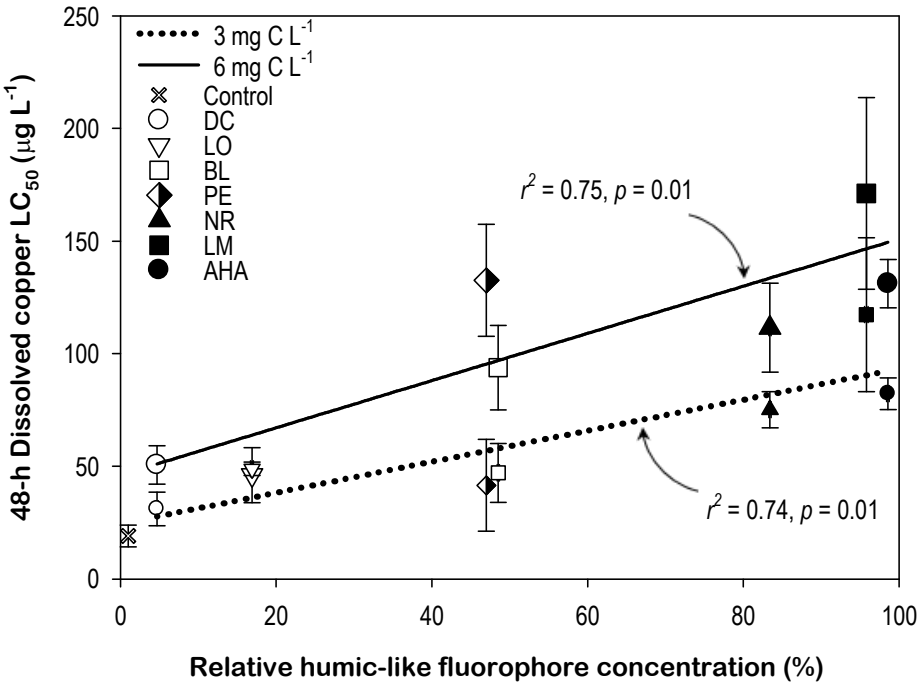

Figure 3.6 The influence of relative humic-like fluorophore concentration, resolved by PARAFAC, of DOMs from various aquatic environments added at 3 and 6 mg L^{-1}, on copper toxicity to *D. magna*. The values represent mean ± standard deviation. Refer to Tables 3.1 and 3.3 for abbreviations of DOMs 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}.

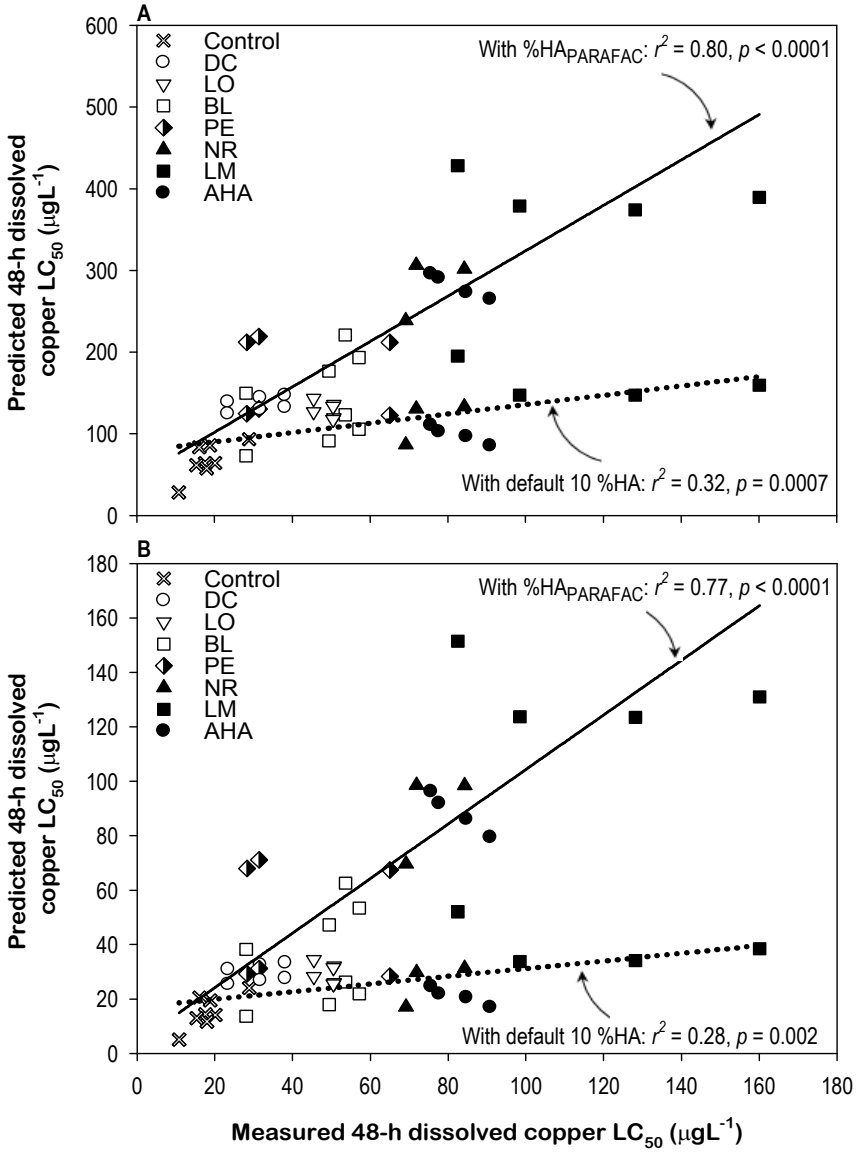

Figure 3.7 The relationships between the values of predicted and observed copper LC_{50} at the (**A**) default lethal accumulation (LA_{50}) of 0.119 nmol g^{-1} wet weight and (**B**) the optimal LA_{50} of 0.0120 nmol g^{-1} wet weight in the BLM (Windows version 2.2.3, HydroQual Inc.). In (**A**), 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 3.1 for abbreviations of DOMs.

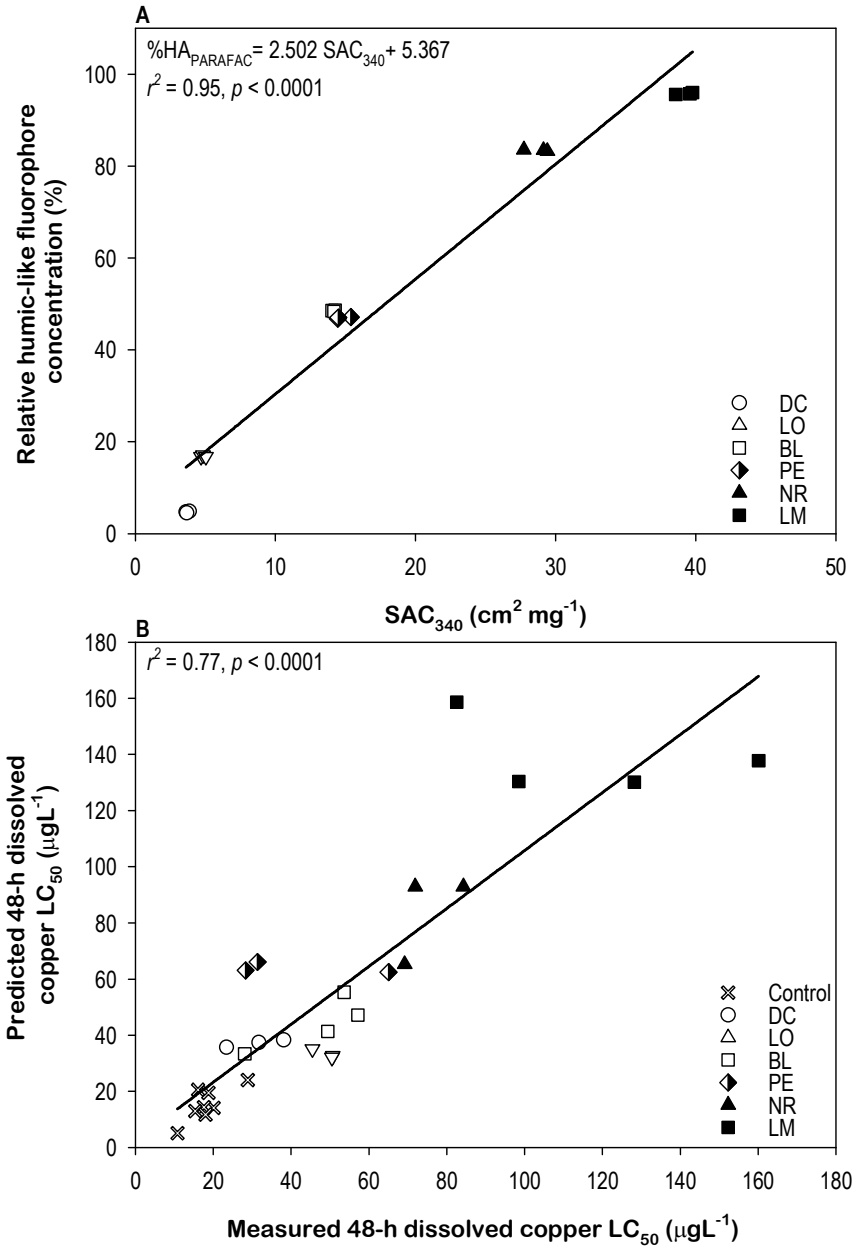

Figure 3.8 (**A**) The relationship between the relative concentrations of humic-like fluorophore resolved by PARAFAC (%$HA_{PARAFAC}$) and specific absorbance coefficient (SAC_{340}) of the DOMs (excluding data of AHA) used in the exposure. (**B**) The relationships between the values of predicted and 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 3.1 for abbreviations of DOMs.

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CHAPTER 4

CHARACTERIZATION OF NATURAL DISSOLVED ORGANIC MATTER (DOM): EXPLANATIONS FOR PROTECTIVE EFFECTS AGAINST METAL TOXICITY AND DIRECT EFFECTS ON ORGANISMS

4.1 Abstract

Dissolved organic matter (DOM) exerts direct and indirect influences on aquatic organisms. In order to better understand how DOM causes these effects, a wide range of autochthonous and terrigenous freshwater DOMs have been characterized by potentiometric titration, absorbance spectroscopy (specific absorptivities at 254, 340 and 436 nm), and fluorescence spectroscopy (excitation-emission, with resolution by parallel factor analysis, PARAFAC). PARAFAC resolved the spectra into four fluorescent components: humic-like, fulvic-like, tryptophan-like, and tyrosine-like. Substantial differences in the compositional and molecular nature among the DOMs were revealed, corroborating the use of absorbance and fluorescence measurements for addressing their quality and yielding easy-to-measure indices for aromaticity, molecular weight, octanol solubility, content of coloured moieties and the relative concentration of fluorophores. Acid-base titrations revealed the dominance of carboxylic and phenolic ligands with a trend for more autochthonous sources to have higher total proton binding capacity. With the introduction of a summary parameter, referred to as the titration index (TI), it is possible to directly relate optical characteristics (i.e. SAC_{340}) and chemical reactivity as summarized by acid-base titration. Thus, the tendencies observed in the literature that darker organic matter is more protective against metal toxicity and more effective in altering physiological processes in aquatic organisms can now be rationalized on a basis of chemical reactivity to protons.

4.2 Introduction

In freshwater environments, many abiotic and biotic processes are affected by water chemistry including natural organic matter (NOM). Aquatic NOM is a ubiquitous, naturally-occurring, heterogeneous mixture of organic compounds formed from the degradation of lignin-rich plant materials and the decay of dead organic biomass (Thurman, 1985). Based on filtration, the NOM fraction passing through a 0.45-µm membrane is known as dissolved organic matter (DOM) of which ≥ 50% by mass is carbon (Thurman, 1985). The concentration of DOM is widely variable in freshwater and commonly reported in mg C L^{-1} as dissolved organic carbon (DOC) (Thurman, 1985). The major chemical components (~ 50–90%) of DOM are humic substances which are operationally divided into humic and fulvic acids (MacCarthy, 1989; Thurman, 1985). In addition, carbohydrates, proteins, and amino acids make up lower proportions of most DOM samples (Thurman, 1985). DOM of allochthonous or terrigenous origin is composed mainly of humic substances, while that of autochthonous source (synthesized by biological activity within the water column) is made of humic substances with higher percentages of proteinaceous materials (McKnight *et al*., 2001).The heterogeneous nature of aquatic DOM is also reflected in the presence of diverse functional ligands expressing a wide range of acidity constants (pK_a) (Ritchie and Perdue, 2003; Smith and Kramer, 1999; Thurman, 1985).

As a global regulator in freshwater ecosystems, DOM has been investigated intensively for several indirect actions on organisms; these abiotic functions including attenuation of solar radiation, influences on carbon cycling and nutrient availability, and

alteration of contaminant toxicity (Williamson *et al*., 1999). For example, protection against copper toxicity in the presence of aquatic DOM was correlated to the aromatic carbon content of DOM, estimated as specific absorbance at 254 or 340 nm (Al-Reasi *et al*., 2012; De Schamphelaere *et al*., 2004; Schwartz *et al*., 2004). In addition, humic fractions of 9 Norwegian DOMs showed strong correlation with the protective effect against copper toxicity (Ryan *et al*., 2004). Most recently, several absorbance and fluorescence characteristics were reviewed as quality indices of aquatic DOMs, and found to account for considerable variability in the protective effects against metal toxicity (Al-Reasi *et al*., 2011). Many direct interactions of DOM with aquatic organisms have also been demonstrated. For example, DOM molecules may accumulate on biological surfaces, influence membrane permeability, affect basic physiological functions, and induce toxic actions (Campbell *et al*., 1997; Galvez *et al*., 2009; Glover *et al*., 2005; Matsuo *et al*., 2006; Meinelt *et al*., 2007; Vigneault *et al*., 2002; Wood *et al*., 2003). These responses are widely variable, again possibly reflecting different DOM qualities.

Addressing the issue of DOM quality is a key step to understand how these ubiquitous substances act both directly and indirectly on organisms. Spectroscopic measurements have been successfully employed to tackle the quality of freshwater DOMs (Chen *et al*., 2002; Senesi *et al*., 1991). The UV-Visible absorption properties have been utilized to detect the presence of chromophores (i.e. light absorbing moieties) of the heterogeneous DOMs. Despite the fact that the absorption spectra of DOMs are usually featureless, several measures have been proposed at specific wavelengths to approximate aromaticity (specific absorbance coefficient at 340 nm, SAC_{340}, Curtis and Schindler,

1997), the intensity of UV-absorbing absorbance groups to yellow-brown ones (absorbance ratios of 254 to 436 nm, Abs $_{254/436}$, Abbt-Braun and Frimmel, 1999), molecular weights (absorbance ratios of 254 to 365 nm, Abs $_{254/365}$, Dahlén *et al*., 1999) and octanol solubility (Abs-octanol $_{254}$/Abs-water $_{254}$, Gjessing *et al*., 1999). Similarly, fluorescence spectroscopy provides useful measures such as fluorescence index (FI = the ratio of the emission intensity at a wavelength of 450 nm to that at 500 nm, both obtained at excitation wavelength of 370 nm) which can distinguish source or origin of various DOMs (McKnight *et al*., 2001). It also offers detailed qualitative molecular information about the major fluorophores of DOMs (i.e. light emitting moieties). For instance, excitation-emission matrices (EEMs) comprise simultaneous collections of numerous emission wavelengths over a range of excitation wavelengths (DePalma *et al*., 2011; McKnight *et al*., 2001). The EEMs can be depicted as three dimensional contour plots and as a result, the fluorophores or fluorescent components can be visualized as a function of excitation-emission pairs of wavelengths. Recent advances in handling of the EEMs by parallel factor analysis (PARAFAC) have improved identification of the components, and estimation of their abundance and the contribution of each one to the total fluorescence (Stedmon and Bro, 2008).

The long term objective of our research is to find simple-to-measure quality parameters to facilitate source-dependence corrections to DOC inputs for metal bioavailability and toxicity modeling, and to predict direct physiological effects on organisms. To this end, many spectroscopic techniques have been tested here, and acid-base titrations have been utilized as an integrated measure of overall chemical reactivity

for the organic matters studied.

4.3 Materials and methods

4.3.1 Collection, absorbance and fluorescence measurements of DOMs

Terrigenous and autochthonous DOM samples were collected by a portable reverse-osmosis unit from various natural freshwater bodies ranging widely in colour and DOC concentrations (the clear water of Lake Ontario with ambient DOC of only 2 mg C L^{-1} to the brownish water of Luther Marsh of approximately 50 mg C L^{-1}). In addition, two commercially available humic substances, namely Aldrich humic acid (AHA, Sigma-Aldrich Chemical, St. Louis, MO, USA) and Nordic Reservoir NOM (NR, International Humic Substances Society, St. Paul, MN, USA) were included in chemical characterization. The sources and their abbreviations are listed in Table 4.1. Al-Reasi *et al*. (2012) provide coordinates of the natural water bodies where the DOMs were isolated. The absorbance and fluorescence measurements of the different DOM sources have been detailed elsewhere (Al-Reasi *et al*., 2012). Briefly, the aromatic composition of DOM was estimated by SAC_{340} according to the equation: $SAC_{340} = (2.303 \times absorbance_{340\ nm})/DOC$ (Curtis and Schindler, 1997). The absorption ratio ($Abs_{254/365}$) was utilized as an indicator of the molecular weight as described by Dahlén *et al*. (1999) and the absorption ratio at 254 nm in octanol to water ($Abs\text{-}octanol_{254}/Abs\text{-}water_{254}$) was employed as an index of the lipophilic nature of our DOM isolates according to Gjessing *et al*. (1999). In addition, normalized absorptions at the ultraviolet wavelength of 254 nm ($SUVA_{254}$ =

Abs_{254}/ DOC) and at 436 nm ($SCOA_{436}$ = Abs_{436}/DOC) were determined to estimate the presence of UV-absorbing and coloured moieties of the samples, respectively (Abbt-Braun and Frimmel, 1999).

The fluorescence index (FI) was used as an indicator of DOM origin (McKnight *et al*., 2001) to evaluate its ability to distinguish sources of our samples. For fluorescence scans, EEMs of the DOMs were obtained after re-zeroing the fluorescence spectrophotometer (i.e. subtracting EEMs of the blank, ultrapure water) to minimize the influence of Raman scattering. The absorbance at 254 nm of the terrigenous isolates exceeded 0.3 absorbance units and therefore the EEMs of these samples were corrected using the absorbance in 200-600 nm as suggested by Ohno (2002). For each sample, the three-dimensional EEMs were processed to remove Rayleigh scattering, and then the processed EEMs were utilized to construct contour plots, a detailed fingerprint of the fluorescent components of each DOM source.

For PARAFAC analysis, the spectral EEMs were modeled using the PLS Toolbox from Eigenvector Research Inc. (Wenatchee, WA, USA) as implemented on the Matlab™ platform (The Mathworks Inc. Natick, MA, USA) as described elsewhere (DePalma *et al*., 2011; Al-Reasi *et al*., 2012). The PARAFAC modeling was carried out based on an a *priori* assumption of the presence of four fluorophores or components contained in the underlying fluorescence signal. Mathematically, the choice of the right number of the components to describe the fluorescence signal is difficult but it should be sufficient to describe the variation within the data set (Stedmon and Bro, 2008). In the present study, the selection of the four components was justified according to the fact that humic

substances of aquatic DOMs are operationally defined into fulvic and humic acids (Thurman, 1985), representing the two humic materials to be resolved by PARAFAC as humic-like and fulvic-like fluorophores. The heterogeneous DOM molecules can be separated into two chromatographic peaks; one with smaller molecular sizes and shorter fluorescence wavelengths (i.e. fulvic acids) and the other with bigger molecular sizes and longer fluorescence wavelengths (i.e. humic acids) (Wu *et al*., 2003; Wu *et al*., 2007). The other two fluorophores were proteinous materials and labeled as tryptophan-like and tyrosine-like. The model decomposed and quantified the underlying fluorophores mathematically (93.9% of the variability was explained), resulting in scores that were relative estimates of the abundances for each DOM sample.

4.3.2 Potentiometric acid-base titrations

Acid-base titrations were performed on diluted solutions (30 mg C L^{-1} of BL, PE, NR, LM and AHA) and the concentrated DOM isolates of LO and DC (Table 4.1). Eight to 16 titration replicates were carried out for each DOM sample. The ionic strength of each DOM solution was adjusted to 0.01M with addition of 5 M potassium nitrate (KNO_3, Sigma Aldrich) and then the sample was transferred to the titration vessel. The sample was initially acidified with concentrated hydrochloric acid (HCl, Sigma Aldrich) to bring the pH down to ~ 2.0 and titrated at ~ 0.1 pH intervals by addition of 0.1N sodium hydroxide (NaOH, made from standardized 1.005*N* NaOH, Sigma Aldrich) to pH 12. At room temperature, all titrations were conducted in a CO_2-free atmosphere (i.e. under purge of ultrapure N_2 gas) using an automated titrator (848 Titrino Plus attached to

801 magnetic stirrer with support rod, Metrohm Canada) with a pH electrode (Orion 8101BNWP ROSS Half-Cell Electrode, Thermo Scientific) and a double junction Ag/AgCl reference electrode (Orion 900200 Sure-Flow Reference Half Cell Electrode, Thermo Scientific). To estimate proton binding constants (pK_a) and their site densities (L_T, μmol mg^{-1}), the experimental titration data were fitted to a fully optimized continuous (FOCUS) model using in-house Matlab™ programs as described by Smith and Ferris (2001). Binding site densities within a specified pK_a range were determined by integration of the area under the curve in the pK_a spectrum.

4.3.3 Statistical analyses

Data have been presented as means ± 1 standard error (n) throughout. Normal distribution of the data for each quality measure was checked by Kolmogorov-Smirnov test. If not normally distributed, they were $\log_{10}$ transformed. The correlation between different DOM quality measures was then performed by the Pearson product moment correlation coefficient using SigmaStat for Windows (Version 3.5, Systat Software, Inc., Point Richmond, CA, USA). Significant correlation was established when $p < 0.05$.

4.4 Results and discussion

4.4.1 Absorbance and fluorescence indices

Absorbance and fluorescence indices have been summarized in Al-Reasi *et al*. (2012). Data assessing the aromatic composition (SAC_{340}) and the presence of the UV-absorbing molecules ($SUVA_{254}$) and yellow-brown coloured moieties ($SCOA_{436}$) are

presented in Table 4.1. The wide range of the aromaticity indices ($SUVA_{254}$ and SAC_{340}) indicated highly variable aromatic content of organic matter of the samples. Autochthonous DOMs (LO and BL) and PE (from a sewage treatment plant) had lower SAC_{340} values than those of terrigenous DOMs (NR and LM) and AHA. Usually, the terrigenous organic matter is composed of higher amounts of aromatic carbon and phenols than the microbially derived or autochthonous DOMs and that is why the former is optically darker than the latter. Both the $SUVA_{254}$ and $SCOA_{436}$ increased from the colourless DC and LO to golden-brown AHA (Table 4.1). The ratio ($Abs_{254/436}$) provided an approximation of the ratio of UV-absorbing functional groups to the coloured ones (Abbt-Braun and Frimmel, 1999). The $SUVA_{254}$ has been frequently employed to estimate aromatic content of organic compounds because the absorbance of energy at 254 nm corresponds to π- π^* transitions typical of aromatic rings (Abbt-Braun and Frimmel, 1999). On the other hand, absorption of DOM in the visible range ($SCOA_{436}$) has been attributed to organic compounds with quinoide and ketoenol functional groups (Abbt-Braun and Frimmel, 1999). SAC_{340}, $SUVA_{254}$, and $SCOA_{436}$ emphasized the higher aromatic coloured organic entities for the terrestrially derived DOMs compared to autochthonous DOMs. These quality indices were strongly correlated with one another (r = 0.87–0.99), implying the consistency among all these measures to reflect the presence of aromatic and coloured moieties in organic matter.

As indirect estimates of molecular weight, relatively higher values of $Abs_{254/365}$ were recorded for the autochthonous DOMs (LO, BL) and PE and lower values for the terrigenous ones (NR, LM) and AHA (Al-Reasi *et al*., 2012). Previous studies have

demonstrated that the ratio increases as the average molecular weight of DOMs decreases (Dahlén *et al*., 1999; Chin *et al*., 1994). Therefore, $Abs_{254/365}$ may rank the examined DOMs in this decreasing order of molecular weights, AHA > LM > NR > PE > BL > LO > DC. This index ($Abs_{254/365}$) and that of the yellow-brown coloured moieties ($SCOA_{436}$) were negatively correlated (r = - 0.97), suggesting that smaller DOM molecules (i.e. higher values of $Abs_{254/365}$) may not contribute substantially to the light absorbance by the isolates, particularly autochthonous sources.

The UV-Vis absorbance-based octanol solubilities (Abs-octanol $_{254}$/Abs-water $_{254}$) of our DOMs have been published elsewhere (Al-Reasi *et al*., 2012). This approach was earlier employed to approximate the liphophilic nature of aquatic DOM samples from the Norwegian Lakes (Gjessing *et al*., 1999). With exception of BL, DOMs of those considered autochthonous (i.e. DC, LO and PE) had higher octanol solubility than the terrigenous ones (LM, NR) and AHA (Al-Reasi *et al*., 2012). The positive correlation between this physicochemical property and the molecular weight index (r = 0.78) may imply that the smaller DOM molecules are more octanol soluble (i.e. more lipophilic). The liphophilic nature index was not related to aromatic carbon composition (SAC_{340} and $SUVA_{254}$) or to the presence of coloured moieties of DOM ($SCOA_{436}$). Thus, aromatic and coloured DOM molecules may not partition into the octanol phase. This is consistent with non-polar and colourless aliphatic carbon representing the types of moieties soluble in the octanol.

According to source classification based on FI (McKnight *et al*., 2001), values of 2.5 and 1.9 for LO and PE, respectively, labeled their organic matter as autochthonous

(Al-Reasi *et al*., 2012). On the other hand, NR and LM exhibited FI values of 1.2, indicating that organic matter in these freshwater sources is exclusively terrestrially-derived (Al-Reasi *et al*., 2012). The organic matter of BL, when originally sampled, was thought to be autochthonous based on visual examination on site, but it turned out to be of mixed origins (i.e. autochthonous and terrigenous) with an FI value of 1.5. An anomaly to this classification (where FI of ~ 1.9 represents autochthonous origin and ~ 1.4 represents terrigenous origin, McKnight *et al*., 2001) was the commercial AHA with a much lower FI of 0.83. Although it has been extensively used as a DOM model, AHA is not a real aquatic DOM substitute (Malcolm and MacCarthy, 1986). Interestingly, DC had a lower FI (1.8) than LO (2.5), shifting towards a terrigenous nature. This is most likely explained by changes in the molecular and structural composition of DC due to partial removal of the original organic matter from the source (LO) by filtration and addition of new organic matter probably by leaching of the activated charcoal during dechlorination. While the origin index (FI) was inversely correlated with the aromatic composition of the isolates (SAC_{340}, r = - 0.81, and $SUVA_{254}$, r = - 0.86), it was positively associated with the octanol solubility index (Abs-octanol $_{254}$/Abs-water $_{254}$, r = 0.84). Consequently and in contrast to terrigenous isolates, autochthonous DOMs would have higher amounts of dissolved organic molecules characterized by smaller sizes and higher octanol solubility.

The excitation-emission spectral contour plots for the 4 fluorophores (humic-like, fulvic-like, tryptophan-like and tyrosine-like) and their concentrations (normalized to DOC) resolved by PARAFAC were earlier presented in Al-Reasi *et al*. (2012). Other studies have reported similar fluorophores with characteristic fluorescence wavelengths

for DOMs in different natural waters (Baker, 2001; DePalma *et al*., 2011; Fellman *et al*., 2008; Stedmon and Bro, 2008). The humic-like component was the dominant fluorescent component in LM, NR and AHA accounting for > 80% of their underlying fluorescence signal. In LO, BL and PE, between 16–50% of the fluorescence was humic-like fluorophore (Al-Reasi *et al*., 2012). On the contrary, the abundance of the fulvic-like component was prominent for the autochthonous isolates (LO and PE) and DC (Al-Reasi *et al*., 2012). The contribution of tryptophan- and tyrosine-like fluorophores to the total fluorescence was much lower than that of the humic- and fulvic-like ones. In terrigenous isolates (LM and NR) and AHA, the fluorescence of proteinaceous materials was either negligible (tryptophan-like accounted for < 5% of the total fluorescence) or completely absent (tyrosine-like fluorophore) (Al-Reasi *et al*., 2012). On the other hand, up to 20% of fluorescent organic molecules of DC, LO and BL were composed of tryptophan-like fluorophores, and there were variable contents of tyrosine-like fluorophores in autochthonous isolates (LO and PE), DC and PE (Al-Reasi *et al*., 2012).

Highly significant relationships were revealed for fluorescent components, in particular for the humic-like constituents. For example, the humic-like component appeared to govern the aromaticity of DOMs as this component was strongly and positively ($r \geq 0.90$) associated with SAC_{340}, $SUVA_{254}$ and $SCOA_{436}$. Opposite significant correlations were found between the fulvic-like fluorophore and SAC_{340} (r = -0.78), $SUVA_{254}$ (r = -0.82) and $SCOA_{436}$ (r = -0.80). Similarly, negative relationships were recorded for the humic-like component with octanol solubility index (r = -0.84), $Abs_{254/365}$ (r = -0.89) and FI (r = -0.86), implying that the molecules making up this

fluorophore tend be of a less lipophilic nature with higher molecular sizes. No significant correlations were observed for the fulvic-like fluorophore with octanol solubility index, $Abs_{254/365}$ and FI. For protein-like materials, the tryptophan-like component did not show any significant association with the other spectroscopic quality measures. On the other hand, the tyrosine-like component was positively related to octanol solubility (r = 0.90) and FI (r = 0.89) and negatively correlated to humic-like fluorophore (r = -0.81). This is an indication of the tendency of the autochthonous DOMs to have a relatively higher proportion of protein-like substances.

4.4.2 Proton binding site densities and pK_a

The acid–base properties (acidity constants (pK_a) and their densities (L_T, μmol mg^{-1})) of the aquatic DOM samples are summarized in Figure 4.1 as pK_a spectra, where site concentrations were assigned to each pK_a value in the range 2.6 to 11.2. The FOCUS method was based on the *a priori* assumption that the pK_a spectra will vary smoothly. Table 4.2 summarizes proton binding capacities in the range $pK_a \leq 5$ (acidic), $5 < pK_a \leq 8.5$ (intermediate) and $pK_a > 8.5$ (basic). In general, the proton affinity spectra showed peaks at the acidic end with pK_a maximum values around 3.5 and similar sized peaks at the basic end centered around a pK_a of 10. More variable positioned peaks occurred in the intermediate pK_a range. The most acidic peaks were generally interpreted as carboxylic sites, and the highest pK_a peaks as hydroxyl and more specifically phenolic sites (Smith and Kramer, 1999). Basic proton binding capacities (and $pK_a > 8.5$) demonstrated significant correlations with octanol solubility index (r = 0.82), FI (r =

0.88) and humic-like fluorophore (r = 0.77). The sum of binding capacities was significantly related with fulvic-like fluorophore (r = 0.79). Inspection of Figure 4.1 illustrated that the darker organic matters tend towards less total capacity; the maximum L_T values tend towards smaller values as the SAC_{340} values increase. To better visualize the relationship between colour and proton reactivity, a contour plot is presented in Figure 4.2 with the scans presented in the same order as in Figure 4.1. There seemed to be little trend in the acidic pK_a values, but overall the intensity (i.e. L_T) decreased as the DOM became darker in colour. The most basic peaks were more variable in location but with the same overall trend of decreasing intensity as the samples became darker in colour. Based on $SAC_{340–350}$, overall darker organic matter has been observed to be more protective towards metal toxicity (e.g. Al-Reasi *et al*., 2012; De Schamphelaere *et al*., 2004; Ryan *et al*., 2004; Schwartz *et al*., 2004). Protons react at the same types of functional groups as metals; thus, at first appearance the decreased capacity with darker colour appeared counterintuitive. However, carboxylic and phenolic sites are not strong metal binding centers in and of themselves, and capacity alone is not sufficient to predict potential impacts on metal bioavailability. For example, high capacity weak sites would not tend to be very protective if binding sites on the organism have higher affinity (log$K$). However, what determines the competition between complexation in solution and binding to the organism surface is not the affinity constant alone (Log$K$), but rather the product ([L] x K) (SETAC, 2009).

4.4.3 Relationship between spectroscopic properties and acidic functional group analysis

Several different approaches were tested to link chemical reactivity, as defined by proton pK_a and L_T to optical properties. Individual acid, base and intermediate binding sites did not link to spectroscopy in any clear way, statistically or conceptually. Thus, a mathematical combination of proton binding sites is required to link reactivity and spectroscopy. The logic of the new proposed proton binding metric is as follows. In metal speciation modeling, tridentate sites represent very strong metal binding sites. This concept is typified by the representation of the strongest binding sites as tridentate sites in the geochemical complexation model, Windermere Humic Aqueous Model (WHAM) (Tipping, 1998). There are three main proton binding classes of ligands identified here and a model tridentate ligand would have three pK_a values (for example, consider citric acid, phosphoric acid or diethylenetriamine). If these three sites are in 1:1:1 proportions, DOM could be prepresented as a single triprotic ligand. Here a Titration Index (TI) is used to measure the difference between actual measurements and the model tridentate ligand hypothesis. To estimate the potential of the organic matters in this study to show strong tridentate binding, a Titration Index (TI) was calculated as:

$$TI = \frac{int}{((acid + base)/2)}$$

where, *TI* is a function of the measured *acid*, *base* and intermediate (*int*) proton binding capacities. The idea of this calculation was that the numerator represents the average capacity for bidentate complex formation (i.e. salicylic acid-like with a phenolic group

ortho to a carboxylic group). The average value was selected because both acid and base sites have error associated with them and there is no way to know which would be stoichiometrically limiting. The third proton binding site that could be involved in a tridendate complex was represented by the sum of intermediate proton binding capacity. A value of TI = 1 would correspond to a stoichiometric amount of intermediate proton binding sites to match the hypothetical bidentate site. Higher values of TI would represent stronger potential for binding. Results of TI calculations are presented in Table 4.2 and plotted versus SAC_{340} in Figure 4.3.

Overall, darker organic matter had a higher TI value and is expected to be more protective against metal toxicity. The seven samples used in this study were plotted (Fig. 4.3), as well as DOM samples from the NOM-Typing project as titrated in Smith and Kramer (1999). The NOM-Typings samples were not used in determination of the regression line in Figure 4.3 and without calibration these samples fall within the 95% confidence interval of the new data presented here. For comparison, the TI value calculated from the data of Smith and Kramer (1999) for titration of Suwannee River Fulvic Acid has also been included. Thus, except for the "unnatural" AHA, all the samples considered follow the general trend of darker organic matter having higher TI. This interpretation is consistent with the observation that darker organic matter is more protective (Al-Reasi *et al*., 2011; De Schamphelaere *et al*., 2004; Ryan *et al*., 2004; Schwartz *et al*., 2004). From a detailed consideration of acid-base titration data, it seems that darker organic matter has a greater potential to form strong binding with metals as demonstrated by the TI calculation. The TI calculation involves a large assumption; that

three ionisable sites are involved in tridentate metal complexation nevertheless does produce excellent correlations with colour and thus tendencies for metal reactivity. Therefore, it can be speculated that DOM sources with higher TI should interact more strongly with biological surfaces such as fish gills.

Addressing the quality issue of DOM is of environmental significance not just for the direct and indirect effects on aquatic organisms but also for other processes in natural waters. For example, simple optical properties of DOM can help to improve predictions of nitrate removal (Barnes *et al*., 2012) and can be employed to allocate the seasonal and spatial variations in the formation of the harmful disinfection byproducts associated with the chlorination of raw water (Herzsprung *et al*., 2012). In future, it will be of interest to evaluate the TI approach in this regard.

The quality indices, explored above, provide easy-to-measure parameters obtained using simple absorbance, fluorescence, and titration measurements to probe molecular and structural chemistry of distinct aquatic DOM sources. These indices, as quality measures, will help researchers to evaluate the abiotic and biotic roles of DOM in the natural freshwaters. Concerning the protective effect against metal toxicity in particular, the spectroscopic parameters can be related to chemistry of metal binding through the TI parameter. Our overall conclusion is that darker organic matter is more protective against metal toxicity, and more effective at interacting with physiological processes, because darker organic matter has a greater proton binding ratio as determined by TI.

4.5 Tables and figures

Table 4.1 Spectroscopic indices of aromatic composition (SAC_{340}), UV-absorbing molecules ($SUVA_{254}$) and yellow-brown coloured moieties ($SCOA_{436}$)*. Data (mean ± standard errors of $n = 3$).

DOM source**	Code	SAC_{340}	$SUVA_{254}$	$SCOA_{436}$
Dechlorinated Hamilton water	DC	3.72 ± 0.07	1.51 ± 0.01	0.01 ± 0.00
Lake Ontario	LO	4.85 ± 0.10	1.40 ± 0.01	0.05 ± 0.00
Bannister Lake	BL	14.16 ± 0.07	2.49 ± 0.01	0.12 ± 0.00
Preston Effluent	PE	14.77 ± 0.30	2.32 ± 0.03	0.16 ± 0.01
Nordic Reservoir	NR	28.76 ± 0.52	3.78 ± 0.07	0.28 ± 0.01
Luther Marsh	LM	39.30 ± 0.37	4.42 ± 0.04	0.39 ± 0.00
Aldrich humic acid	AHA	79.98 ± 0.96	6.78 ± 0.08	1.34 ± 0.02

* SAC_{340}, $SUVA_{254}$ and $SCOA_{436}$ are the absorbances at 340 nm, 254 and 436 nm, respectively divided by DOC concentrations. See Al-Reasi *et al*. (2012) for other spectroscopic quality indices on these same samples.
** Sorted based on increasing colour intensity according to SAC_{340}

Table 4.2 Binding capacities for acidic sites ($pK_a < 5$), intermediate sites (pK_a 5 to 8.5) and basic sites ($pK_a > 8.5$) as determined by integration of the area under the curve within the specified pK_a value ranges. Bidentate capacity is determined as the average of acidic and basic site capacities. Titration Index (TI) is the ratio between intermediate pK_a and bidentate capacity. Data (mean ± standard errors for *n* observations).

Code	Binding capacities (L_T, μmol mg^{-1})				
	Acidic	Intermediate	Basic	TI	*n*
DC	2.56 ± 0.99	0.36 ± 0.13	2.86 ± 0.96	0.13 ± 0.05	10
LO	1.32 ± 0.32	0.50 ± 0.09	3.75 ± 0.66	0.20 ± 0.04	10
BL	4.26 ± 0.68	0.89 ± 0.24	1.79 ± 0.55	0.30 ± 0.08	9
PE	2.67 ± 0.86	0.38 ± 0.09	4.08 ± 0.60	0.11 ± 0.03	8
NR	1.58 ± 0.27	0.31 ± 0.04	0.79 ± 0.20	0.26 ± 0.03	11
LM	1.74 ± 0.53	0.70 ± 0.08	1.45 ± 0.29	0.44 ± 0.05	16
AHA	1.89 ± 0.23	0.49 ± 0.05	1.17 ± 0.31	0.32 ± 0.04	10

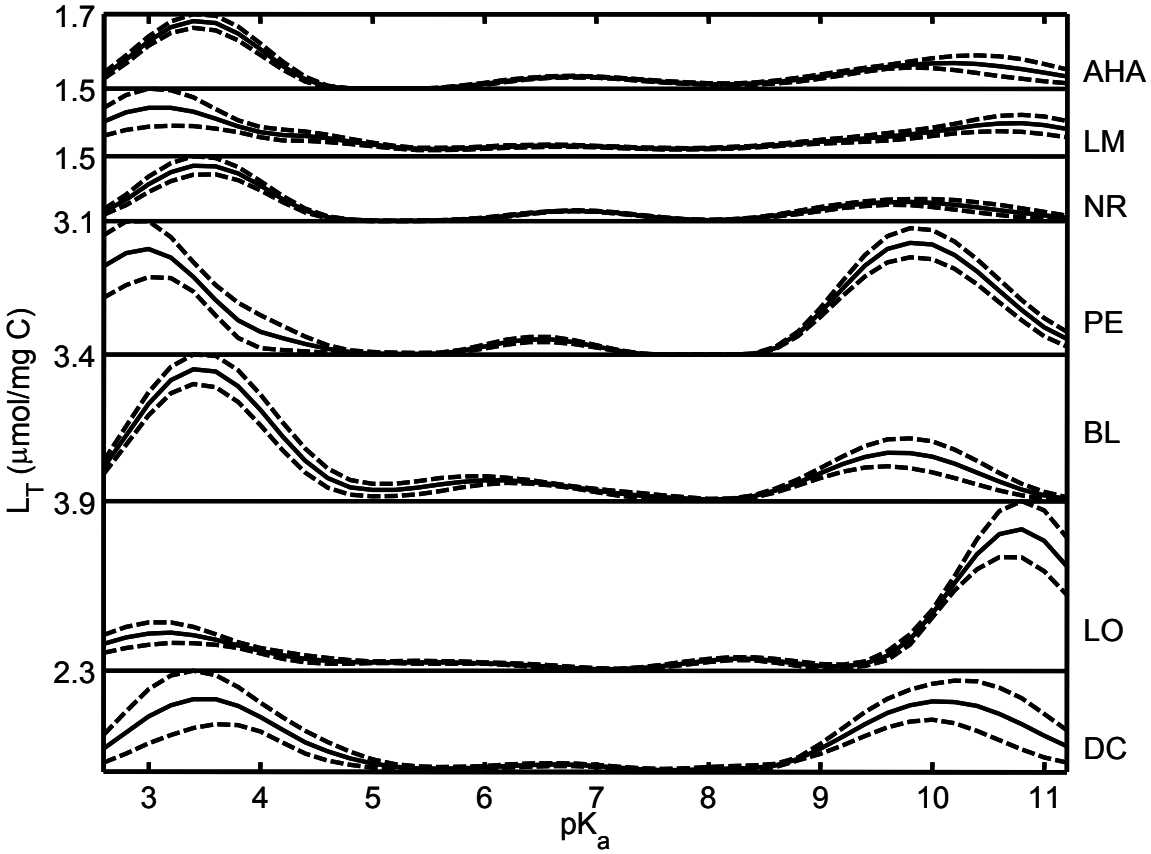

Figure 4.1 pK_a spectra for each organic matter sample. The specific sample is indicated to the right of each plot. The y-axis labels for each solid line correspond to the maximum binding capacity of the pK_a spectrum below the line. Solid lines correspond to the mean spectrum (n in the range 5 to 15) and the dashed lines correspond to standard errors. The samples are stacked from highest SAC_{340} for the top spectra to the lowest SAC_{340} value at the bottom.

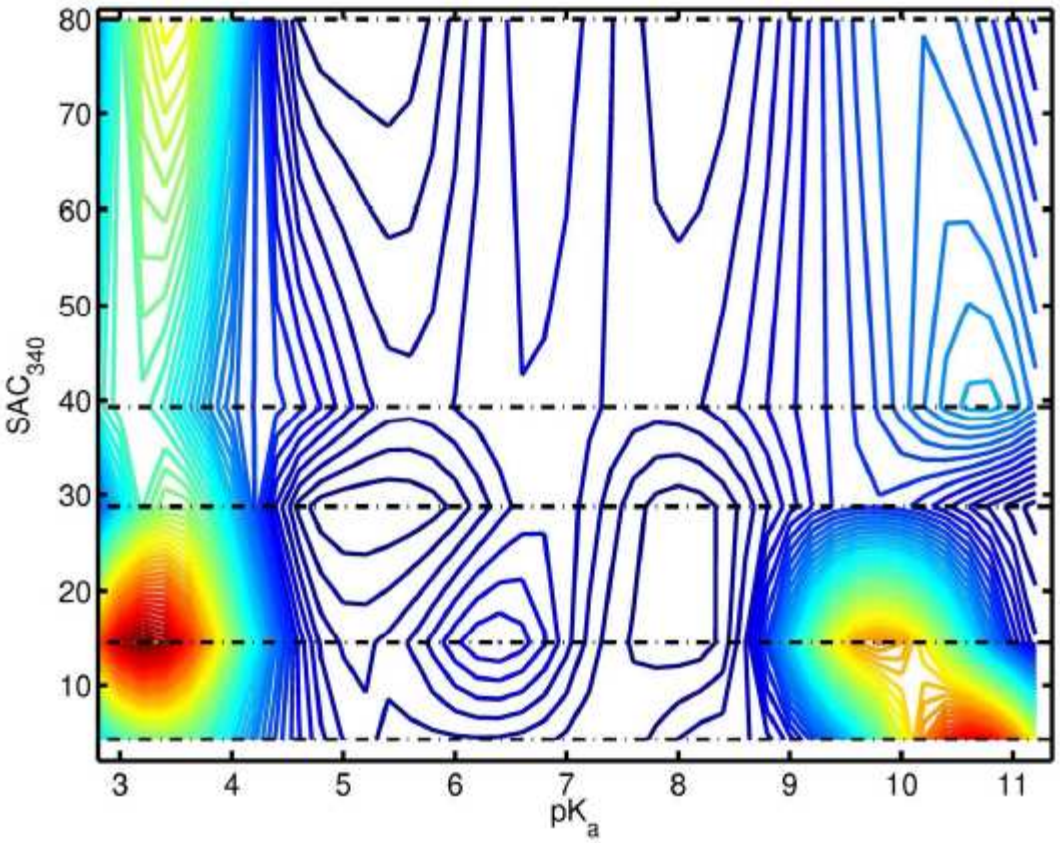

Figure 4.2 Contour plot of the binding capacity (L_T) versus pK_a and SAC_{340}. The horizontal dashed lines correspond to each of 5 different SAC_{340} values where the values near 4 and 15 are the average of DC and LO, and BL and PE samples, respectively.

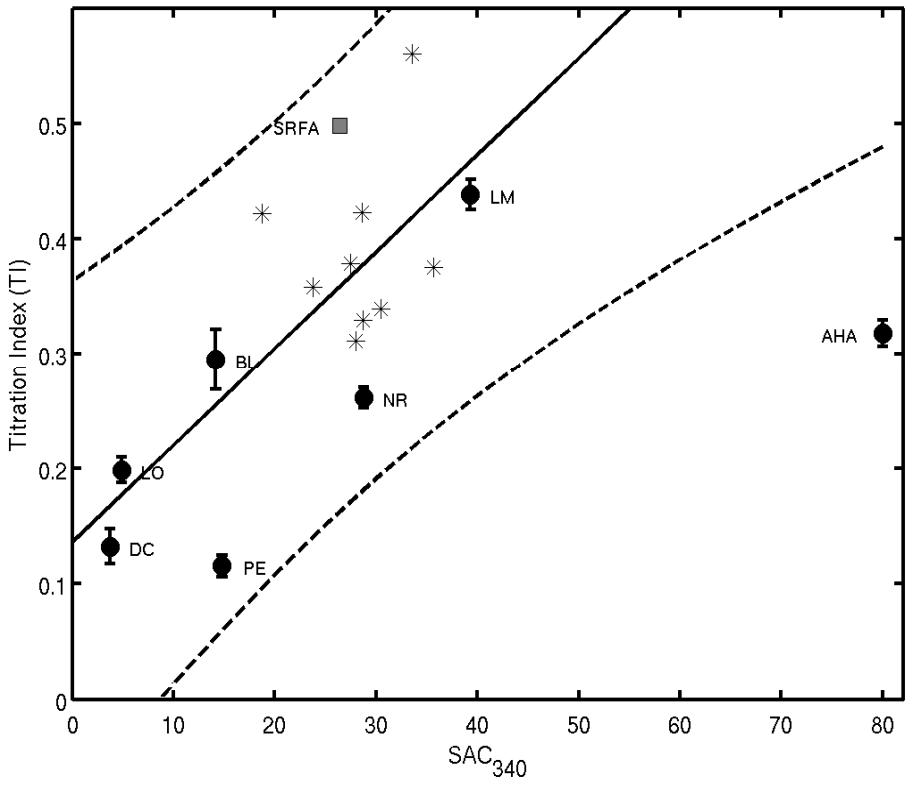

Figure 4.3 Titration index (IT) versus SAC_{340}, where the description of how to calculate titration index is given in the text. The error bars correspond to standard errors about the measured data. Each data point is labeled with the sample identity including SRFA for Suwannee River Fulvic Acid. ✳ symbols correspond to DOM samples from the NOM-Typing project (e.g. Abbt-Braun and Frimmel, 1999; Gjessing *et al*., 1999). Dashed lines indicate 95% confidence interval about the regression line.

4.6 References

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CHAPTER 5

THE EFFECTS OF DISSOLVED ORGANIC MATTER (DOM) ON SODIUM TRANSPORT AND NITROGENOUS WASTE EXCRETION OF THE FRESHWATER CLADOCERAN (*Daphnia magna*) AT CIRCUMNEUTRAL AND LOW pH

5.1 Abstract

Dissolved organic matter (DOM) is a heterogeneous mixture of fulvic and humic acids found in all natural waters with many abiotic roles. Recently, several direct biological influences of DOM on physiological functions have emerged including possible impacts on sodium (Na^+) regulation. However, little is known about the transport of essential solutes such as Na^+ and ammonia of freshwater animals in the presence of natural DOM. The whole-body Na^+ content, unidirectional influx and efflux rates of Na^+(measured with radiolabeled $^{22}Na^+$), and nitrogen (ammonia and urea) excretion rates of the water flea (*Daphnia magna*) were measured in the presence of three previously characterized, chemically-distinct natural DOM sources, as well as commercially available Aldrich humic acid (AHA), at circumneutral and acidic pH (≥ 7 and ~ 5, respectively). Regardless of the pH conditions, neither Na^+ influx nor efflux rates were affected, suggesting no influence of the DOM sources on active uptake and passive diffusion of Na^+ ions, respectively. This was supported by the unaffected whole-body Na^+ content of *D. magna* in the presence of the added DOMs. Differences associated with pH were restricted to AHA. Ammonia and urea excretion rates were both increased at low pH in most treatments. Ammonia excretion rates were reduced at circumneutral pH by the most highly coloured, allochthonous DOM, and at low pH by all three natural DOMs, but not by AHA. The reductions may be attributed to the higher buffering capacities of natural DOM sources, as well as their ability to interact with biological membranes. Urea excretion rates at both pH conditions were not influenced by the presence of the various DOMs.

5.2 Introduction

Dissolved organic matter (DOM) is a complex group of molecules produced during the decomposition of lignin-rich plant materials and the decay of dead organic biomass in a poorly-understood process known as humification (Ertel *et al*., 1984; Hatcher and Spiker, 1988). In freshwater ecosystems, DOM molecules are ubiquitous and their mass (usually ≥ 50% dissolved organic carbon or DOC as a heterogeneous mixture of humic and fulvic acids) exceeds that of living organisms (Thurman, 1985; Thomas, 1997). The source of DOM in the ecosystem can be allochthonous (i.e. terrigenous - organic matter produced on land and then washed into the water body), autochthonous (organic matter generated within the water column by microrganisms such as algae and bacteria), or of mixed autochthonous and allochthonous origin (McKnight *et al*., 2001). Depending on their concentrations and origins, DOM molecules are responsible for the yellow to brown colour of surface water; allochthonous DOMs tend to be darker in colour. Although DOM has been considered as a global regulator of freshwater ecosystems with several abiotic roles (Williamson *et al*., 1999), the direct interaction of DOM with freshwater organisms has been largely overlooked until recently (Steinberg *et al*., 2006).

Nevertheless, several direct influences of DOM on aquatic animals have been reported in the literature. For example, DOM molecules have been shown to accumulate on cell membranes (Campbell *et al*., 1997) with impact on their permeability (Vigneault *et al*., 2000), especially under conditions of low water pH coupled with higher DOC concentrations. Even at circumneutral pH, the presence of added DOM induced a more

negative transepithelial potential in trout gills, and the effects were greater with darker, more allocththonous DOMs (Galvez *et al*., 2009). Some studies have indicated that DOM molecules have the potential to induce toxicity (e.g. Matsuo *et al*., 2006; Meems *et al*., 2004; Steinberg *et al*., 2006; Timofeyev *et al*., 2004). However, other investigations have found that organisms in soft acidic waters experience higher survival (Hargeby and Petersen, 1988) and improved growth (Barth and Wilson, 2010) in the presence of DOMs, and are protected against negative changes in ionoregulation (Gonzalez *et al*., 1998, 2002; Matsuo *et al*., 2004; Wood *et al*., 2003). DOMs may also facilitate increased ammonia excretion at low pH, resulting in alkalinisation of the gill surface (Wood *et al*., 2003). Similar to low pH conditions (e.g. Wood *et al*., 1998; Havas *et al*., 1984), metals such as copper (e.g. Grosell and Wood, 2002; Alsop and Wood, 2011; Zimmer *et al.*, 2012) have been reported to disrupt Na^+ regulation and ammonia excretion in freshwater organisms. From metal toxicity studies, DOM molecules are well-known to offer protection by sequestering metal ions as complexes in the water, thereby reducing bioavailability and uptake. However, Wood *et al*. (2011) have postulated that part of the protective effect of DOM may also be a direct interaction with the processes of Na^+ transport. Indeed, Matsuo *et al*. (2004), Glover *et al*. (2005), and Glover and Wood (2005a) reported that the presence of DOM may stimulate active Na^+ uptake in the rainbow trout (*Oncorhynchus mykiss*) and the water flea (*Daphnia magna*), respectively. Overall, it is apparent that DOMs may interact in a positive or negative fashion with the functioning of gills or other transporting epithelia of freshwater animals (Galvez *et al*., 2009; Glover *et al*., 2005; Matsuo *et al*., 2004; Wood *et al*., 2003). Furthermore, there is some evidence that

commercially prepared DOMs may have different direct actions than natural DOMs (e.g. Wood *et al*., 2003; Glover *et al*., 2005, Glover and Wood, 2005a) but the mechanism(s) of all such effects are poorly understood.

The present study investigated the influence of DOMs on two fundamental physiological functions, Na^+ regulation and nitrogenous waste excretion, of the freshwater cladoceran, *D. magna*. We hypothesized that DOMs would impact the processes of ionoregulation and nitrogenous waste excretion, that the effects would vary depending on the type of DOM (autochthonous versus allochthonous, natural versus commercially prepared sources), and that responses would be more pronounced at lower environmental pH. Tests were conducted with adult daphnids (5 – 6 days old) using three natural DOM sources ranging from allochthonous to autochthonous, as well as commercially available Aldrich humic acid (AHA), under two pH conditions (circumneutral pH $\geq$ 7 and acidic pH $\sim$ 5). Whole body Na^+ content, unidirectional Na^+ influx and efflux rates (determined with radiolabeled ^{22}Na), and net ammonia and urea excretion rates were recorded. Overall, the results do not support our hypotheses with respect to the effects of natural DOM on Na^+ homeostasis, but revealed some differences with AHA, and marked effects of both low pH and various DOMs on nitrogenous waste excretion in *D. magna*.

5.3 Materials and methods

5.3.1 Test organisms

Daphnia magna, a widely used freshwater crustacean in ecotoxicological and

physiological research, was employed as a model organism in the present study. The original *D. magna* adults were acquired from Aquatic Research Organisms (ARO, Hampton, NH, USA) and cultured for several generations under laboratory conditions (at ~23 ºC with a 12 h light: 12 h dark photoperiod) in dechlorinated Lake Ontario water (city of Hamilton tap water). The water has the following chemistries; $[Na^+]$ = ~ 0.7 mM, $[Ca^{2+}]$ = ~ 1.0 mM, $[Mg^{2+}]$ = ~ 0.3 mM, [DOC] = 2.5 ± 0.4 mg L^{-1} and pH~ 7.5–8.0 (Al-Reasi *et al*., 2012). Thirty two to 35 organisms were reared in 650 ml of dechlorinated water which was renewed by 500 ml replacement with fresh water twice per week. The daphnids were fed once per day with unicellular green algae (*Selenastrum capricornutum*) and YCT (Yeast, CEROPHYLL®, and Trout chow). The organisms were starved for 24 h prior to experimentation and were not fed during exposures. Adult daphnids (5 to 6 days old, mean wet weight ± standard error = 0.77 ± 0.01 mg, *n* = 574) were utilized for all experiments.

5.3.2 Dissolved organic matter (DOM) solutions

Four different DOM sources were tested, 3 of which were natural isolates collected by reverse-osmosis from Lake Ontario (LO), Bannister Lake (BL) and Luther Marsh (LM). Details of collection and treatment of these DOMs are provided in Al-Reasi *et al*. (2012). A commercially available humic acid (AHA, Sigma-Aldrich Chemical, St. Louis, MO, USA), which has been extensively used as a DOM analogue in earlier studies (e.g. Glover *et al*., 2005; Glover and Wood, 2005a) was included for comparison. Absorbance and fluorescence properties of DOMs are provided in detail elsewhere (Al-

Reasi *et al*., 2012). In brief, of the three natural DOMs, LO is the most lightly coloured and autocththonous, whereas LM is the most highly coloured and allochthonous. All DOM solutions (at DOC concentrations of 6 and 12 mg L^{-1}) were prepared using dechlorinated city of Hamilton tap water which was employed as a control (no added DOM). Since the addition of reverse-osmosis collected DOM isolate has the potential to change concentrations of ions, especially sodium (Na^+) and calcium (Ca^{2+}), all exposure solutions including control were checked and balanced for Ca^{2+} and Na^+ levels. Maintaining similar ion levels for all treatments was essential, as for example Ca^{2+} is known to play a role in regulating membrane permeability (McDonald and Rogano, 1986) and affecting Na^+ uptake in a concentration-dependent manner (Glover and Wood, 2005b). Therefore, appropriate amounts of calcium carbonate ($CaCO_3$ powder, Sigma-Aldrich Chemical, St. Louis, MO, USA) and sodium chloride (NaCl salt, Caledon Laboratories LTD, Georgetown, ON, Canada) were added to each solution. Because of the low solubility of $CaCO_3$, all solutions were bubbled overnight with pure carbon dioxide (CO_2, Air Liquide Canada Inc., Burlington, ON, Canada). The next day, the solutions were vigorously bubbled with air for 24 h to remove excess CO_2. About 16–20 h before exposure, each solution was initially adjusted to the desired pH (≥ 7 or ~ 5) by addition of diluted H_2SO_4 solution (made from 95–98% H_2SO_4, ACS specification, Caledon Laboratories LTD, Georgetown, ON, Canada) or/and KOH solution (made from KOH crystal, ACS specification, Caledon Laboratories LTD, Georgetown, ON, Canada). An SP70 portable pH meter with Ag/AgCl pH electrode (VWR sympHony, VWR International, Beverly, MA, USA) was employed throughout. In all these steps, solutions

were stored in foil-wrapped plastic bottles to minimize the degradation of DOM due to light exposure. The Na^+ transport and whole body Na^+ content were evaluated at both DOC concentrations (6 and 12 mg C L^{-1}), but nitrogenous waste excretion rates were examined only at 6 mg C L^{-1}.

5.3.3 Whole body sodium content

Ten *D. magna* adults were transferred individually into 100 ml of each exposure solution. The duration of the exposure was 24 h. The pH was checked and adjusted before the introduction of the organisms. Two pH readings for each solution were taken at the start and the end of the exposure at pH ≥7 and approximately every 2–3 hours for experiments at pH~ 5 during the light period. The pH was not adjusted in the dark period but the change in pH was within 0.2–0.3 units. No mortality was recorded over the 24 h exposure in all tested DOM sources. At the end of the exposure, individuals were counted, removed and rinsed in deionized water (≥17.5 MΩ cm; Barnstead Nanopure II, Thermo Scientific Barnstead, NH, USA) for 30 seconds. Then, organisms were blotted dry on Whatman® No. 1 filter paper. In addition, water samples for each exposure solution were obtained at the end of the exposures for measurements of Na^+, Ca^{2+}, and DOC concentrations. All daphnids were then placed individually into pre-weighed micro-centrifuge tubes, which were weighed again using an UMT2 electronic microbalance (Mettler-Toledo AG, Laboratory and Weighing Technologies, Greifensee, Switzerland). Each individual was then digested by the addition of 15 µl of concentrated trace metal

grade HNO_3 (67–70% HNO_3, Fisher Scientific, Fairlawn, NJ, USA) and placed in an oven for 4 h at ~ 65 ºC in a sealed micro-centrifuge tube. Then, 450 µl of deionized water was added to the digested individual and the micro-centrifuge tube was mixed on a Vortex Genie 2 Shaker (Scientific Industries, Bohemia, NY, USA). The solution was then transferred to a pre-weighed 2.0 ml centrifuge tube where it was diluted to a total volume of ~ 1.5 ml using the deionized water. All tubes were weighed again in order to determine the exact volume of the solution, and then assayed for Na^+ concentration (see below). The factor 1.25 was used to correct the final whole body concentration for water trapped by the carapace as suggested by Stobbart *et al*. (1977). The whole-body Na^+ content of *D. magna* was expressed as µmol mg^{-1} wet weight.

5.3.4 Sodium influx rate

In 10 ml of each solution, 10 organisms were exposed to 1.25 µCi of radioactive $^{22}Na^+$ as NaCl (Eckert and Ziegler isotope products, Valencia, CA, USA) for 1.0 h. The pH of the exposure solutions was checked and adjusted as necessary according to the target pH (≥ 7 or ~ 5). After 1 h, individuals were rinsed individually in a high Na^+ "cold displacement" solution (1.0 M NaCl) for 10 seconds to get rid of any $^{22}Na^+$ ions adsorbed to the surface of the organism, followed by a rinse in deionised water for 30 seconds as suggested by Stobbart *et al*. (1977) and Glover *et al*. (2005). The daphnids were then processed as described above for whole-body Na^+ experiments. The solutions were sampled for analysis of $^{22}Na^+$ radioactivity and the concentrations of Na^+ and Ca^{2+}. Organisms and water radioactivity, as counts per minute (cpm), were measured using a

"Wizard 3" 1480 automatic gamma counter (Perkin-Elmer, Woodbridge, ON, Canada). The unidirectional Na^+ influx rate (J_{in}) was calculated based on the amount of radioactivity incorporated into the organism (Glover *et al*., 2005):

$$J_{in} = \frac{cpm}{SA \times m \times t}$$

where cpm is the counts per minute of each individual, SA is the specific radioactivity of the exposure water (cpm/μmol), m is the mass of the organism (g) and t is the time of exposure (h). In this calculation, the mass of each individual was divided by 1.25 to correct for water trapped by the carapace (Stobbart *et al.*, 1977).

5.3.5 Sodium efflux rate

Daphnids were incubated in 1.0 L of dechlorinated water inoculated with 50–100 μCi of radioactive $^{22}Na^+$ for 24 h before experimentation. Thereafter, each individual was first rinsed in fresh dechlorinated water and immediately in deionized water for 1 min. This ensured the removal of any excess ^{22}Na adsorbed to the surface of the organism or in the water trapped by the carapace. Then each daphnid was individually placed in 1.5 ml of each exposure solution and allowed to undergo efflux for 2 h. In preliminary experiments, it was found that this time period ensured a relatively abundant amount of radioactivity (i.e. cpm) appeared in the exposure water. At the end of the exposure, 1 ml of the water was sampled and *D. magna* individuals were processed as above for whole-body Na^+ content and their weights were corrected as described above for the influx experiments. Radioactivity of individuals and exposure water samples was measured and

the unidirectional Na^+ efflux rate (J_{out}) was calculated based on the appearance of $^{22}Na^+$ radioactivity in the exposure water (Glover *et al*., 2005):

$$J_{out} = \frac{\text{cpm}}{\text{SA} \times m \times t}$$

where cpm is the total counts per minute of the 1.5 ml exposure water for each individual, SA is the specific radioactivity of the organism (cpm/μmol), *m* is the corrected mass of the organism (g) and *t* is the time of exposure (h).

5.3.6 Nitrogenous waste excretion rates

Five *D. magna* individuals were transferred into 12 ml of each exposure solution. As detailed above for whole body Na^+ experiments, pH was maintained at the desired levels (≥ 7 or ~ 5) during the course of the exposure. Two-ml water samples were obtained 10 min after introduction of the organisms and after 24 h. The samples were frozen immediately after collection and kept at –20 ºC until chemical analysis. The excretion rates (J_x) of ammonia or urea were calculated as follow:

$$J_x = \frac{(Tx_f - Tx_i) \times V}{m \times t}$$

where Tx_f and Tx_i are final and initial concentrations of ammonia or urea, respectively, *V* is the volume of the exposure chamber, *m* is the corrected mass of the organism (g) and *t* is the time of exposure (h).

5.3.7 Chemical analyses

The total DOC concentration in water samples was measured directly using a Shimadzu TOC-$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). Whole body Na^+ content of *D. magna* and Na^+ and Ca^{2+} concentrations of the water samples were determined using flame atomic absorption spectrometry (SpectroAA220FS, Varian, Mulgrave, Australia). The accuracy and reproducibility of the flame spectrometer and TOC analyzer was assured using certified standards diluted according to the manufacturers' manuals. Total ammonia and urea concentrations in water were determined in triplicate via spectrophotometry according to methods of Verdouw *et al*. (1978) and Rahmatullah and Boyde (1980), respectively, using a SpectraMAX 340pc microplate reader (Molecular Devices, Sunnyvale, CA, USA). For these assays, blanks and standards were prepared in 6 mg L^{-1} solutions of each DOM isolate to account for any colour change due to the presence of DOM.

5.3.8 Buffer capacity determination

Isolates of these DOM samples were titrated previously (Al-Reasi *et al.*, submitted). From these acid-base titrations, proton binding capacities at specified pK_a values were determined (so-called pK_a spectroscopy). The buffer capacity (β) of the organic acid was determined at specific pH (and corresponding $[H^+]$) values using the following equation for monoprotic acids (Stumm and Morgan, 1996):

$$\beta = 2.303 \sum_{i=1}^{n} \left(\frac{L_{Ti} K_{ai} [H^+]}{(K_{ai} + [H^+])^2} \right)$$

using the summation across all dissociation constants, nK_{ai} values with associated proton site densities, L_{Ti} values. The calculated buffer capacity is in units of μmol pH^{-1} mg C^{-1}. To determine β (μmol $pH^{-1}L^{-1}$) for specific samples, this value was multiplied by the measured DOC concentration (mg C L^{-1}).

5.3.9 Statistical analyses

Statistical analyses were conducted using SigmaStat for Windows (Version 3.5, Systat Software, Inc., Point Richmond, CA, USA). Before applying the appropriate statistical techniques, normality and homogeneity of variance of data were checked by the Kolmogorov-Smirnov test and Levene median test, respectively. Differences among various DOM treatments were detected using one-way analysis of variance (ANOVA). Student's two-tailed *t*-test was used to evaluate the difference between the two pH conditions of the same DOM treatment and between 6 and 12 mg C L^{-1} at each pH condition for Na^+ data. When the assumptions of the normal distribution and homogeneity of variance were violated, data were first transformed on base 10 logarithmic scale (log_{10}) and then the ANOVA and/or *t*-test were performed. In a few cases where the transformation did not work, nonparametric equivalents to ANOVA and *t*-test (Kruskal-Wallis Analysis of Variance on ranks, *H*, and Mann-Whitney Rank Sum test, *U*, respectively) were employed. When significant differences were detected, the ANOVA and/or the nonparametric equivalent were followed by multiple *post hoc* comparisons (Tukey test and Dunn's method, respectively). Values have been reported as means ± 1 standard error and the degree of significance for all statistical analyses was established at

the 0.05 level.

5.4 Results

5.4.1 The influence of DOM on whole-body Na^+ content

The whole body Na^+ content for control daphnids averaged 0.036 ± 0.004 and 0.034 ± 0.005 μmol mg^{-1} wet weight for circumneutral pH (≥ 7) and acidic pH (~5), respectively. For exposures at DOM concentrations of 6 mg C L^{-1} (Fig. 5.1A), the range was between 0.031 ± 0.003 (BL) to 0.041 ± 0.002 (AHA) μmol mg^{-1} wet weight for circumneutral pH and 0.029 ± 0.004 (AHA) to 0.038 ± 0.004 μmol mg^{-1} wet weight (LM) for low pH. Similar ranges were found for exposure at 12 mg C L^{-1} (Fig. 5.1B). There were no significant differences among treatments (including the control, with no added DOM) under circumneutral conditions (ANOVA, $F_{4, 43} = 1.205$, $p = 0.322$) or acidic water (ANOVA, $F_{4, 43} = 1.198$, $p = 0.326$) in the presence of 6 mg C L^{-1}. Similarly, treatments with added DOM at 12 mg C L^{-1} did not differ from control (no added DOM) at pH ≥ 7 (ANOVA, $F_{4, 54} = 0.521$, $p = 0.720$) or pH ~ 5 (ANOVA, $F_{4, 44} = 1.003$, $p = 0.416$). For both DOC concentrations (6 and 12 mg C L^{-1}), daphnids had similar whole-body Na^+ content in the same treatment regardless of pH conditions. The single exception was AHA at 6 mg C L^{-1}. In the presence of AHA, daphnids in the acidic medium experienced a significant reduction in their Na^+ content (*t*-test, $t = -2.714$, $df = 16$, $p < 0.05$) relative to those at cirumneutral pH (Fig. 5.1A). This was not seen at the higher AHA concentration (12 mg C L^{-1}), but at pH ≥ 7 daphnids had lower Na^+ content at 12 mg C L^{-1} relative to 6 mg C L^{-1} (*t*-test, $t = -3.400$, $df = 15$, $p < 0.01$).

5.4.2 The influence of DOM on Na^+ influx

For controls, the averages of Na^+ influx rates for pH ≥ 7 and ~ 5 were 5.31 ± 0.62 and 4.53 ± 0.38 μmol g^{-1} h^{-1}, correspondingly. In DOC concentrations of 6 mg C L^{-1} (Fig. 5.2A), values ranged from 3.32 ± 0.73 (AHA) to 5.84 ± 0.88 μmol g^{-1} h^{-1} (LM) and 4.51 ± 0.44 (LM) to 6.61 ± 1.07 μmol g^{-1} h^{-1} (BL) for pH ≥ 7 and ~ 5, respectively. Slightly higher influx rates were determined for the organisms when exposed to DOM at 12 mg C L^{-1} at both pH's (Fig. 5.2B). In the latter circumstance at the circumneutral pH, Na^+ influx rates ranged from 6.17 ± 0.61 in the presence of BL to 7.59 ± 1.37 μmol g^{-1} h^{-1} in the presence of AHA. Under acidic conditions, daphnids tended to increase their Na^+ influx rates (6.39 ± 0.40 in LM to 8.88 ± 0.82 μmol g^{-1} h^{-1} in the presence of AHA). Notably, *D. magna* in the acidic control treatment for the 12 mg C L^{-1} series exhibited a significantly higher Na^+ influx rate (*t*-test, $t = 3.900$, $df = 27$, $p < 0.001$) than that of the individuals in the circumneutral control treatment (Fig. 5.2B). Furthermore, the organisms exposed to 6 mg C L^{-1} AHA at pH ~ 5 had significantly higher influx rates compared to those at pH ≥ 7 (*t*-test, $t = 2.535$, $df = 18$, $p < 0.05$, Fig. 5.2A). In the presence of 6 mg C L^{-1} (Fig. 5.2A), significant differences were revealed among treatments at pH ≥ 7 (ANOVA, $F_{4, 52} = 3.013$, $p < 0.05$), but not at pH~ 5 (Kruskal-Wallis ANOVA on ranks, $H = 1.965$, $df = 4$, $p = 0.742$). The *post hoc* Tukey test showed that daphnids in the presence of AHA had a significantly lower Na^+ influx rate than those exposed to LM (Fig. 5.2A). All other comparisons were not significant. In contrast, at DOC concentrations of 12 mg C L^{-1}, no differences were seen among treatments at pH ≥ 7 (ANOVA, $F_{4, 51} = 2.003$, $p = 0.108$) or pH~ 5 (ANOVA, $F_{4, 41} = 1.367$, $p = 0.262$) (Fig. 5.2B). When the two DOM

concentrations (6 versus 12 mg C L^{-1}) were compared, higher uptake rates were found for *D. magna* individuals at 12 mg C L^{-1} in LM (Mann-Whitney test, $U = 73$, $p < 0.05$) and AHA (*t*-test, $t = -3.943$, $df = 18$, $p < 0.01$) at low pH and in LO (*t*-test, $t = -2.293$, $df = 16$, $p < 0.05$) and AHA (*t*-test, $t = -3.414$, $df = 16$, $p < 0.01$) at circumneutral pH.

5.4.3 The influence of DOM on Na^+ efflux

For exposures at DOM treatments at 6 mg C L^{-1}, control organisms had average Na^+ efflux rates of 3.80 ± 0.71 at circumenutral pH and 6.43 ± 1.45 μmol g^{-1} h^{-1} at low pH (Fig. 5.3A). In water containing the added DOMs, Na^+ loss rates displayed a range from 4.05 ± 0.99 for AHA to 5.78 ± 1.48 μmol g^{-1} h^{-1} for LM at pH ≥ 7 and from 3.26 ± 0.54 for LO to 6.94 ± 1.92 μmol g^{-1} h^{-1} for AHA at pH ~ 5. Although considerable variability was observed, there were no significant differences in Na^+ efflux rates among different DOM treatments either at circumneutral pH (ANOVA, $F_{4, 45} = 1.331$, $p = 0.273$) or at low pH (ANOVA, $F_{4, 31} = 1.312$, $p = 0.287$) when 6 mg C L^{-1} was present in the exposure water. There were also no differences attributable to pH. In the 12 mg C L^{-1} treatments, Na^+ efflux rates tended to be higher, especially in acidic waters, but this was also true for the controls, so the differences could not be attributed to the effects of DOM (Fig. 5.3B). As at 6 mg C L^{-1}, no significant differences among the different treatments were observed in the presence of 12 mg C L^{-1} (ANOVA, $F_{4, 50} = 0.625$, $p = 0.647$ and ANOVA, $F_{4, 28} = 0.400$, $p = 0.807$ at pH ≥ 7 and pH~ 5, respectively). Generally, there was a trend of increasing Na^+ efflux rates in the acidic pH condition. However, *t*-tests revealed no significant differences within the same DOM treatment at the two pH's for most

treatments (Fig. 5.3). The one exception was when LO was present at 12 mg C L^{-1} where the adults at low pH had significantly higher Na^+ efflux rates than those at circumneutral pH (*t*-test, $t = 2.295$, $df = 12$, $p < 0.05$) (Fig. 5.3B). At pH ~5, organisms in presence of LO (*t*-test, $t = -5.153$, $df = 12$, $p < 0.01$) and LM (*t*-test, $t = -2.376$, $df = 13$, $p < 0.05$) experienced higher Na^+ efflux rates in 12 relative to 6 mg C L^{-1}. Similarly, at pH ≥ 7, higher loss was observed in the presence of AHA at 12 relative to 6 mg C L^{-1}(*t*-test, $t = -2.485$, $df = 15$, $p < 0.01$).

5.4.4 The influence of DOM on ammonia and urea excretion

Ammonia excretion rates of *D. magna* in control (i.e. no added DOM) for circumneutral pH (≥ 7) and low pH (~ 5) were 2.76± 0.20 and 5.27± 0.30 μmol g^{-1} h^{-1}, while urea excretion rates were 0.30 ± 0.01 and 0.64 ± 0.06 μmol g^{-1} h^{-1} , respectively (Fig. 5.4). Overall, these order of magnitude differences between ammonia and urea excretion rates were consistent across treatments. Therefore, on a unit N basis (i.e. 2 N per urea molecule), urea-N excretion rates were about 20% of ammonia-N excretion rates. When DOM was present in water at 6 mg C L^{-1}, the ranges of ammonia excretion rates at pH ≥ 7 were between 0.54 ± 0.04 in LM to 2.57 ± 0.22 μmol g^{-1} h^{-1} in BL, and at pH ~5 from 2.35 ± 0.29 in LM to 4.04 ± 0.43 μmol g^{-1} h^{-1} in AHA. At pH ≥ 7, organisms demonstrated similar excretion rates in all treatments except those in LM where the rate was substantially lower (19–23% of rates in other treatments; ANOVA, $F_{4, 20} = 46.675$, $p < 0.001$) (Fig. 5.4A). At pH~ 5, ammonia excretion rates were substantially increased relative to pH ≥ 7 in all treatments, including the controls; the differences were significant

in all groups except BL (*t*-test, $t = 2.220$, $df = 7$, $p = 0.062$, Fig. 5.4A). Furthermore at pH~ 5, the ANOVA ($F_{4, 20} = 46.675$, $p < 0.001$) followed by the multiple *post hoc* comparisons revealed significantly lower ammonia excretion rates in the presence of the 3 natural DOM sources (LO, BL and LM) relative to the control treatment. Interestingly, similar excretion rates were recorded at low pH for daphnids in the control and in AHA treatments.

Urea excretion rates ranged from 0.28 ± 0.02 (BL) to 0.43 ± 0.09 μmol g^{-1} h^{-1} (LM) at circumneutral pH and 0.42 ± 0.03 (BL) to 0.61 ± 0.05 μmol g^{-1} h^{-1} (LO) at low pH (Fig. 5.4B). No significant differences were revealed for urea excretion rates among treatments at circumneutal pH (Kruskal-Wallis ANOVA on ranks, $H = 5.265$, $df = 4$, $p = 0.261$) or acidic pH ($F_{4, 37} = 68.142$, $p = 0.051$). However, as with ammonia excretion rates, *D. magna* individuals exhibited substantially higher urea excretion rates at low pH compared to those at circumneutral pH, differences which were significant in all treatments except LM (*t*-test, $t = 0.107$, $df = 8$, $p = 0.918$, Fig. 5.4B).

5.4.5 The influence of DOM on water buffering capacities

Figure 5.5 shows the buffer capacities for the exposure solutions at both pH's. At circumneutral pH, the control had an average β of 1.45 ± 0.10 μmol pH^{-1} L^{-1} while a higher range of 3.86 ± 0.35 (LO) to 8.90 ± 1.69 (BL) μmol pH^{-1} L^{-1} was recorded with addition of various DOM sources. In acidic pH, an average β of 4.62 ± 0.63 μmol pH^{-1} L^{-1} was calculated for control and a range of 5.21 ± 0.40 (AHA) to 22.85 ± 6.16 (BL) μmol pH^{-1} L^{-1} was estimated when added DOMs present in solution. Overall, pH ~5 samples

demonstrated consistently higher buffer capacity than pH ~7 samples. Significant differences were observed for β between treatments at pH ~7 ($F_{4, 18} = 2.900$, $p = 0.051$) and pH 5 (Kruskal-Wallis ANOVA on ranks, $H = 29.965$, $df = 4$, $p < 0.01$). *Post hoc* comparisons illustrated significant differences among the treatments at both pH's (Fig. 5.5).

5.5 Discussion

5.5.1 Overview

Based on previous studies with several different freshwater organisms, we had predicted that DOMs would impact the processes of Na^+ metabolism and nitrogenous waste excretion, that the effects would vary depending on the type of DOM (autochthonous versus allochthonous, natural versus commercial origin), and that responses would be more pronounced at lower environmental pH (see Introduction). Overall, our results show that in contrast to prediction, the three natural DOMs had negligible impact on Na^+ homeostasis, but might offer a subtle protective effect against the effects of low pH as explained below. Clear differences attributable to pH occurred only with the "unnatural" AHA. Nevertheless, low pH alone greatly increased ammonia and urea excretion rates in *D. magna*, and the natural DOMs, especially the most highly coloured allochthonous LM, exerted some marked effects on ammonia excretion, particularly at low pH.

5.5.2 Sodium metabolism

The whole-body Na^+ contents, as well as the unidirectional Na^+ influx and efflux rates reported here either in control treatments (no added DOM) or in the presence of the DOMs were comparable to those found by Glover *et al*. (2005) and Glover and Wood (2005a, b) for the same species. In our study, *D. magna* in both circumneutral and low pH's had very similar whole-body Na^+ contents (Fig. 5.1), and generally similar unidirectional influx (Fig. 5.2) and efflux rates (Fig. 5.3), indicating no significant whole-body Na^+ loss at this low pH. Nevertheless, there was a tendency for higher rates of both influx and efflux at the lower pH (i.e. an increase in Na^+ turnover rates), though this was not significant in most treatments. The explanation may be slightly greater permeability for passive loss and compensating slightly greater active inward transport at pH ~5. This cladoceran species has been generally reported to experience reduction in whole body Na^+ concentrations only under extreme low pH's of $\leq$ 4.5 and/or in very soft water (Potts and Fryer, 1979; Havas *et al*., 1984; Havas, 1985; Havas and Likens, 1985). For example, severe inhibition of Na^+ uptake was observed at pH 4.0 in water of comparable Ca^{2+} concentration (1 mmol L^{-1}) to that of the present study (Glover and Wood, 2005b). In laboratory tests, *D. magna* appear to successfully regulate Na^+ metabolism over a pH range of 4.6 – 9.0, similar to the range reported for the abundance of several *Daphnia* species in northern hemisphere lakes (Salonen and Hammer, 1986).

A key finding, and a notable contrast to one of our initial hypotheses, was that Na^+ metabolism of *D. magna* adults remained generally unaffected in the presence of any of the three added natural DOMs, regardless of their very different sources and

chemistries (Al-Reasi *et al*., 2012). The few effects seen in the present study were restricted to the "unnatural" AHA, which is consistent with several previous studies. For example in the studies of Glover and colleagues, Na^+ content exhibited an increasing trend when *D. magna* adults were exposed to increasing concentrations of AHA, but another commercially purchased DOM (Suwanee River, SRN) did not have this effect (Glover *et al*., 2005; Glover and Wood, 2005a). Although both AHA and SRN increased unidirectional Na^+ uptake of *D. magna*, these two different commercial sources resulted in contrasting effects on Na^+ efflux rates, explaining the different effects on whole body Na^+ content (Glover *et al*., 2005; Glover and Wood, 2005a). In agreement with the present study, no effect was seen in the presence of a natural DOM (another isolate from Luther Marsh, one of the sources tested in the present study; Glover *et al*., 2005). Our results with AHA showing reduced whole body Na^+ content (Fig. 5.1A) and increased Na^+ influx (Fig. 5.2A) only at low pH were also consistent with those of Glover and Wood (2005a). Interestingly, no influence was seen on Na^+ efflux in the presence of AHA in this study or in Glover *et al*. (2005). Furthermore, at low pH, the presence of AHA was observed to cause severe exacerbation of the passive Na^+ loss of stenohaline freshwater stingrays (*Potamotrygon* spp.), while natural DOM protected against this loss (Wood *et al*., 2003). We conclude that the effects of commercial humic acids (e.g. AHA) on Na^+ metabolism may often be contradictory to those of natural DOMs, which appear to be inert with respect to active Na^+ uptake and passive diffusive efflux, at least at circumneutral pH in *D. magna*.

In the present study, our natural DOMs were obtained by reverse-osmosis, a

method which isolates the organic matter directly from the natural source water (i.e. Lake Ontario, Bannister Lake or Luther Marsh) by differential membrane filtration. This method has been proven to reproduce representative organic matter of the natural waters (De Schamphelaere *et al*., 2005). In contrast, commercial AHA and similar humic acids are lyophilized, and they have distinct aliphatic and aromatic molecular composition but similar elemental composition to natural water DOMs (Malcolm and MacCarthy, 1986). Absorbance and fluorescence spectroscopy has demonstrated that AHA deviates substantially from the natural DOMs utilized in this study in term of aromatic composition and pure humic nature compared to natural DOMs which have mixed humic and fulvic molecular compositions (Al-Reasi *et al*., 2012).

At least under the present water chemistry conditions (Table 5.1), the unaltered Na^+ uptake (Fig. 5.2A) may imply no interference by DOM with the mechanisms responsible for the active Na^+ uptake in *D. magna* such as the electrogenic $2Na^+/1H^+$ exchanger (Glover and Wood, 2005b; Bianchini and Wood, 2008). The higher Na^+ influx in the presence of 6 mg L^{-1} of AHA at pH ~ 5 (Fig. 5.2A) may be regarded as a consequence to compensate for whole-body Na^+ reduction under this acidic condition (Fig. 5.1A). Acidic pH simulated Na^+ influx of *D. magna* only in the absence of added DOMs (control, Fig. 5.2B), implying possible subtle beneficial effects of DOMs for *D. magna* in low pH environments since organisms would not need to accelerate Na^+ uptake.

5.5.3 Ammonia and urea excretion

The ranges of ammonia and urea excretion rates in this study (Fig. 5.4) were

higher than the ranges of 0.00–2.94 and 0.10–0.17 μmol g^{-1} h^{-1}, respectively, reported for starved *D. magna* (Wiltshire and Lampert, 1999). This zooplankter (*D. magna*) liberates ammonia as the dominant excretory nitrogenous waste followed by urea (Wiltshire and Lampert, 1999) similar to other crustaceans and fish (Weihrauch *et al*., 2009). While Na^+ homeostasis was not affected by the acidic condition, lower environmental pH resulted in higher ammonia and urea excretion rates by daphnids relative to circumneutral pH (Fig. 5.4). One of the proposed mechanisms of ammonia excretion is passive diffusion of gaseous NH_3 facilitated by acid trapping (i.e. combining H^+ with NH_3 to from ammonium ion, NH_4^+) in the water next to ion-transporting epithelia (i.e. boundary layer) (Wilkie, 2002). The pH of this layer is usually lower than that of the bulk surrounding water (Wright *et al*., 1988) and further acidification would result in more ammonia being excreted as an adaptive response by daphnids to raise boundary layer pH. Alternatively, acidic water may be a stressful situation where protein catabolism of *D. magna* is accelerated, resulting in higher ammonia and urea production and excretion rates.

No significant effects of DOMs were observed on urea excretion rates at either circumneutral pH or acidic pH, but the most allochthonous DOM (LM) markedly depressed ammonia excretion in both conditions (Fig. 5.4). Indeed, at the lower water pH, ammonia excretion rates were significantly attenuated by the presence of all three natural DOMs, but not by AHA (Fig. 5.4A). At least in part, the reduction in ammonia excretion in the presence of the natural DOMs may be attributable to their ability to raise the buffering capacity of the water, and effect which is greater at pH ~ 5 than at circumneutral pH (Fig. 5.5). Published humic acid titrations demonstrate consistent buffer

capacity values similar to those calculated here. For various humic acids, pH 5 buffer capacity calculated for 6 mg C L^{-1} are in the range of 15 to 30 and for pH 7 in the range 7.5 to 15 µmol pH^{-1} L^{-1} (Boguta and Sokolowska, 2012). The trend of higher pH samples having lower buffering makes sense because buffer capacity maxima are when the pH= pK_a. DOM tends to have more acidic (pK_a 4-5) values than neutral pK_a values.

The lack of effect of AHA may be explained by its similar buffering capacity to control (no added DOM), as well as by its distinct chemical nature (Al-Reasi *et al*., 2012; Al-Reasi *et al*., submitted). However, the most highly coloured allochthonous source (LM) did not have the greatest effect in raising the buffering capacity of the water (Fig. 5.5), yet was effective in reducing ammonia excretion at both pH's (Fig. 5.4A), and also in preventing the increase in urea excretion at low pH observed with other DOMs (Fig. 5.4B). Among the DOM sources tested, this source has the highest Titration Index (TI) of 0.44 ± 0.05. The TI range in other sources (LO, BL and AHA) is between 0.20–0.32. This index is derived as a measure of how closely the DOM approximates a hypothetical tridentate ligand and thus can be thought of as a measure of the ability to form strong complexes (Al-Reasi *et al*., submitted). Indeed, we have predicted that DOMs with higher TI should interact more strongly with functional groups on the external physiological membranes of aquatic animals (Al-Reasi *et al*., submitted). The potency of LM in attenuating nitrogenous waste excretion in daphnids supports this prediction. One possibility is the direct interaction of DOM molecules with ammonia transporters (e.g. Rhesus proteins as transporters for NH_3/NH_4^+) proposed for ammonia excretion in aquatic organisms including crustaceans (Weihrauch *et al*., 2009).

5.5.4 Future perspectives

It is noteworthy that the effects of natural DOMs in depressing ammonia excretion at low pH in *D. magna* are exactly opposite those reported in one study on fish where natural, highly allochthonous DOM greatly raised ammonia excretion rate at low pH (Wood *et al*., 2003). Furthermore, natural DOMs had marked positive effects on ionoregulation in several species of fish at low pH (Wood *et al*., 2003), while such effects were not generally seen in daphnids. At present it is unclear why these responses differ, and whether the different response patterns in *D. magna* are adaptive, as they are thought to be in fish. Clearly, there is a need for more work in this area on both crustaceans and fish, using natural DOMs with detailed physico-chemical characterization, rather than commercially prepared surrogates.

5.6 Tables and figures

Table 5.1 Water chemistry of the exposure water used for examining the influence of dissolved organic matter (DOM) on sodium transport and nitrogenous wastes excretion by the water flea (*D. magna*). Data (mean ± standard errors).

Treatment	Exposure in the presence of ~ 6 mg C L^{-1}				Exposure in the presence of ~ 12 mg C L^{-1}			
	pH	Na^+ (mM)	Ca^{2+} (mM)	DOC (mg C L^{-1})	pH	Na^+ (mM)	Ca^{2+} (mM)	DOC (mg C L^{-1})
CON	7.75 ± 0.03 / 5.19 ± 0.07	0.93 ± 0.01	1.45 ± 0.02	2.26 ± 0.03	7.69 ± 0.11 / 5.16 ± 0.05	1.23 ± 0.02	1.85 ± 0.05	2.12 ± 0.11
LO	7.63 ± 0.05 / 5.16 ± 0.07	0.94 ± 0.02	1.40 ± 0.01	6.83 ± 0.08	7.36 ± 0.10 / 5.06 ± 0.03	1.18 ± 0.03	1.81 ± 0.03	12.58 ± 0.16
BL	7.67 ± 0.04 / 5.09 ± 0.05	0.91 ± 0.01	1.44 ± 0.10	6.61 ± 0.15	7.40 ± 0.11 / 5.08 ± 0.03	1.20 ± 0.02	1.90 ± 0.04	12.77 ± 0.30
LM	7.67 ± 0.04 / 5.12 ± 0.05	0.93 ± 0.00	1.43 ± 0.01	5.73 ± 0.06	7.39 ± 0.11 / 5.08 ± 0.03	1.24 ± 0.02	1.90 ± 0.04	11.18 ± 0.09
AHA	7.71 ± 0.04 / 5.13 ± 0.05	0.94 ± 0.01	1.54 ±0.05	5.11 ± 0.71	7.43 ± 0.11 / 5.07 ± 0.03	1.23 ± 0.02	1.87 ± 0.04	12.38 ± 0.25

CON-control (dechlorinated tap water with no added DOM from exogenous source); DOM isolates from Lake Ontario (LO), Bannister Lake (BL), Luther Marsh (LM) and Aldrich humic acid (AHA) were added to dechlorinated water for the other treatments. Data of n = 4–8 for concentrations of Na^+, Ca^{2+} and DOC and n = 6–30 for pH.

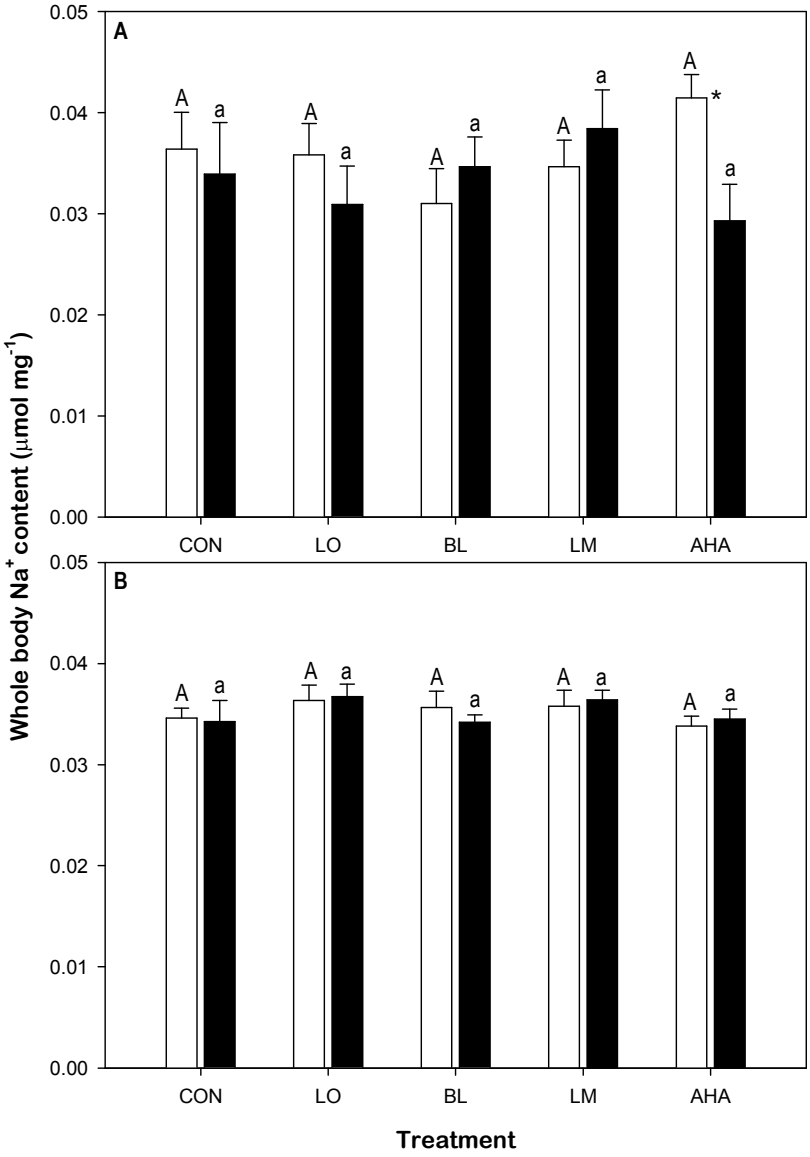

Figure 5.1 Whole body Na^+ content (μmol mg^{-1} wet weight) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at (**A**) 6 mg C L^{-1} and (**B**) 12 mg C L^{-1}dissolved organic carbon (DOC) at circumneutral pH ≥ 7 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors for n = 7 – 14 of 5-6 days old adults. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

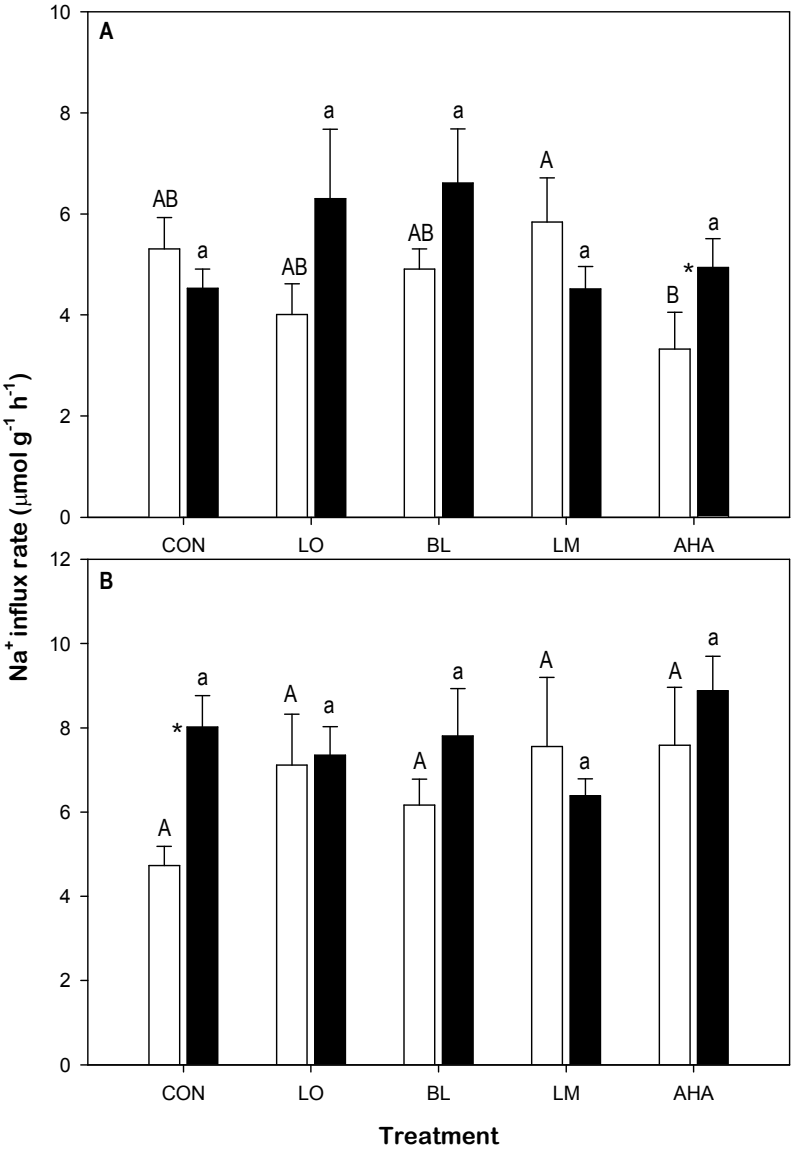

Figure 5.2 Na^+ influx rates (μmol g^{-1} h^{-1}) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at (**A**) 6 mg C L^{-1} and (**B**) 12 mg C L^{-1} dissolved organic carbon (DOC) at circumneutral pH ≥ 7 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors for Na^+ influx rates over 1 h of $n = 9 – 20$ of 5-6 days old adults. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

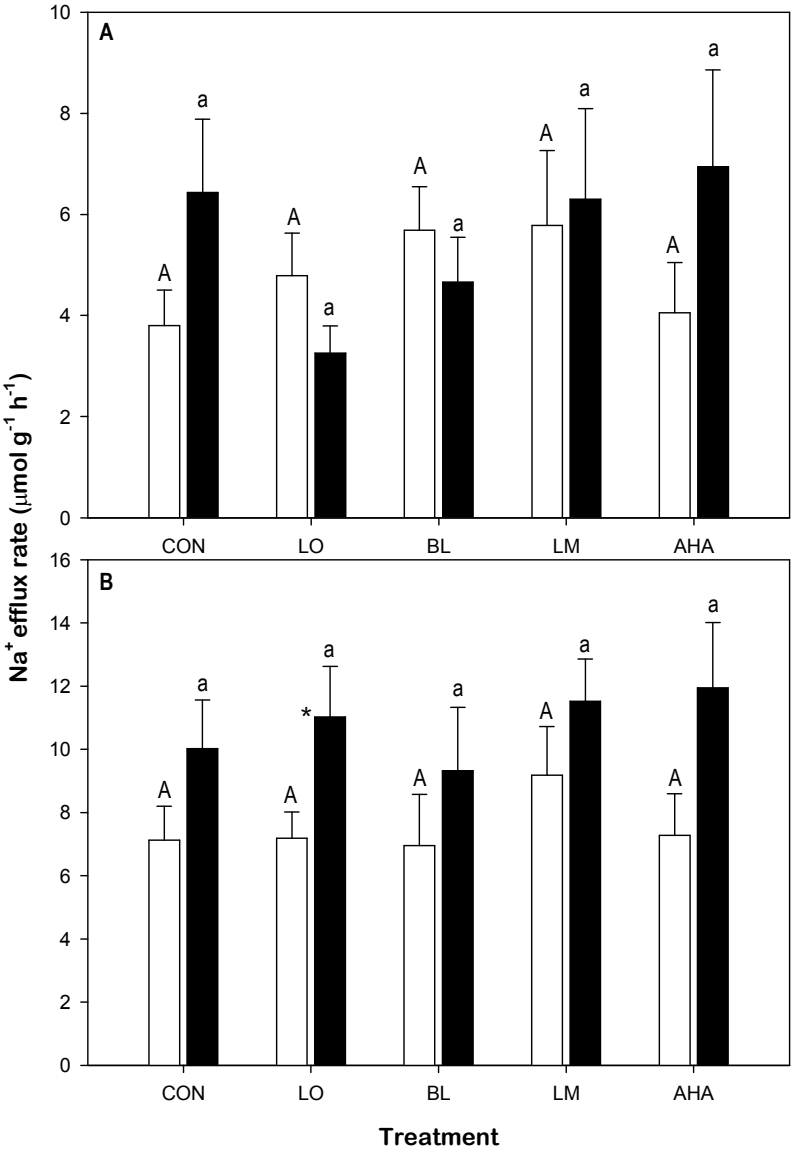

Figure 5.3 Na^+ efflux rates (μmol g^{-1} h^{-1}) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at (**A**) 6 mg C $L^{-1}$and (**B**) 12 C mg L^{-1}dissolved organic carbon (DOC) at circumneutral pH ≥ 7 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors for Na^+ efflux rates over 2 h of $n = 6 – 19$ of 5-6 days old adults. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

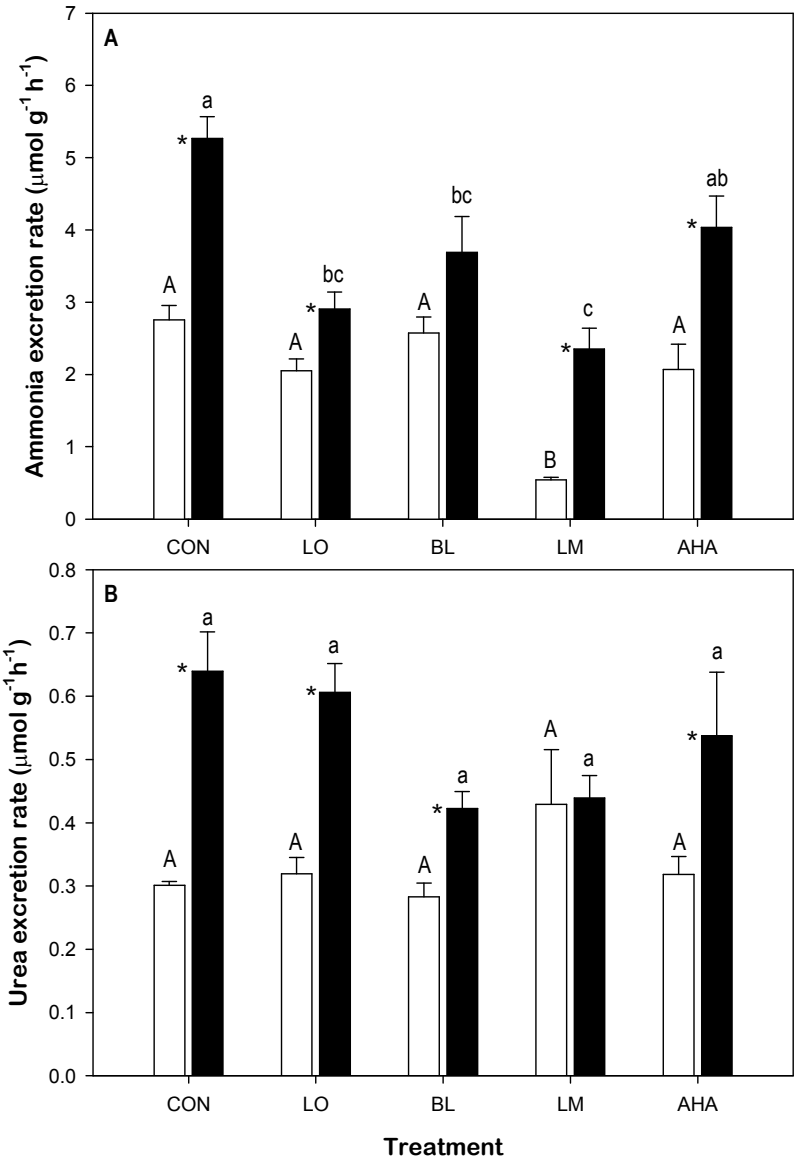

Figure 5.4 Ammonia excretion rates (**A**) and urea excretion rates (**B**) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at 6 mg C L^{-1} DOC at circumneutral pH (≥ 7, white bars) and low pH (~ 5, black bars). Plotted values represent the mean ± standard errors for rates over 24 h of n = 4 – 5 determinations with 5 individuals for each determination, using 5-6 days old adults. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

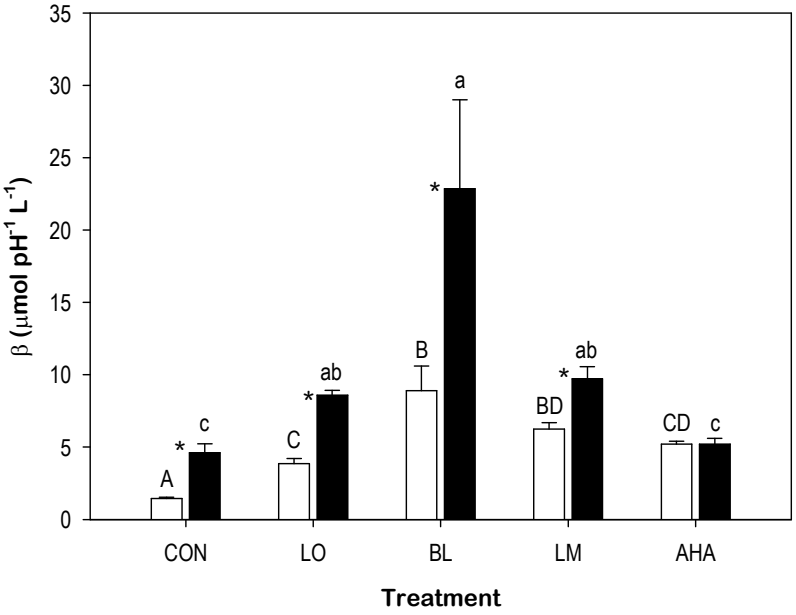

Figure 5.5 Buffer capacities (β) of control (CON, no added DOM) and treatments with DOMs added at 6 mg C L^{-1} DOC at circumneutral pH (≥ 7, white bars) and low pH (~ 5, black bars). Plotted values represent the mean ± standard errors of the capacities calculated from $n = 4 – 12$ titrations. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment. Details of titrations are provided in Al-Reasi *et al*. (submitted).

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CHAPTER 6

THE INFLUENCE OF DISSOLVED ORGANIC MATTER (DOM) ON GILL FUNCTION IN THE FRESHWATER TELEOST, ZEBRAFISH (*Danio rerio*): SODIUM HOMEOSTASIS, PERMEABILITY, AND NITROGENOUS WASTE EXCRETION AT CIRCUMNEUTRAL AND LOW pH

6.1 Abstract

In prior studies, the potential influences of dissolved organic matter (DOM, as a water chemistry factor) on basic features of gill function (Na^+ homeostasis, nitrogenous waste excretion, transcellular and paracellular permeability) have been largely overlooked. In the present study, unidirectional influx and efflux rates of Na^+ and the concentration-dependent kinetics of Na^+ influx have been evaluated in the zebrafish (*Danio rerio*) in the presence of different freshwater DOM sources and a commercial humic acid (AHA) added at 6 mg C L^{-1}, at both circumneutral and low pH ($\geq$ 7 and ~ 5). Additionally, ammonia and urea excretion rate, potassium ion (K^+) net loss rate (an indicator of branchial transcellular permeability) and [^{3}H] PEG-4000 uptake rate through non-gut routes (an indicator of branchial paracellular permeability) and through the gut (a measure of drinking rate) were also determined. In accord with several previous studies, a commercially obtained preparation, Aldrich humic acid (AHA) stimulated unidirectional Na^+ uptake by increasing the maximal Na^+ uptake rate (J_{max}). However, this effect was not significant in the presence of natural DOMs. A natural DOM source characterized by its distinctive autochthonous-allochthonous nature promoted Na^+ uptake rate at low pH, likely due to its higher acidic binding and buffer capacities. At both pH's, Na^+ uptake in the presence of other DOMs was not affected. In contrast, unidirectional Na^+ efflux rates were consistently reduced in the presence of all DOMs, indicating ameliorative action against passive Na^+ diffusive loss. The Na^+ efflux reductions could not be exclusively explained by indicators of either transcellular permeability or paracellular permeability. In contrast to Na^+ homeostasis, neither ammonia nor urea excretion rates, nor drinking

rates were substantially influenced by the presence of DOMs at both pH's.

6.2 Introduction

Ionoregulation and ammonia excretion are essential physiological functions for teleosts. The gill of freshwater fish is an organ equipped with specialized epithelial ion-transporting cells (i.e. ionocytes, mainly mitochondria-rich cells, MRCs) for ion uptake and nitrogenous waste excretion (Evans *et al*., 2005). To survive in hypo-ionic media, fish have to maintain a constant ionic homeostasis, especially for Na^+ and Cl^-, counterbalancing the continual diffusive loss of ions by actively concentrating them from the surrounding dilute water (Marshall, 2002). Fish excrete nitrogenous waste, mostly ammonia (the term referring to $NH_3 + NH_4^+$, unless specified in the text) directly into the surrounding water, predominantly through the gills (Wood, 1993). Our understanding of the physiology of aquatic organisms has come largely from laboratory-based trials whereas some actual environmental situations have been overlooked. Transport of essential solutes (Na^+ and ammonia) and gill permeability regulation represent classic examples of such a situation: the influences of water chemistry factors such as pH, hardness and salinity on these processes have been studied extensively (Marshall, 2002; Evans *et al*., 2005), but another ubiquitous water chemistry factor, dissolved organic matter (DOM), has received very little attention until recently.

Freshwater DOM molecules are a heterogeneous mixture of humic and fulvic acids with ≥ 50% of their mass as carbon (Thurman, 1985). The DOM origin can be allochthonous or autochthonous. Allochthonous DOMs are terrestrially-derived or

terrigenous organic matter characterized by high aromatic carbon content, whereas autochthonous DOMs are formed in water bodies due to photosynthetic activity or bacterial degradation of allochthonous organic matter. Autochthonous DOMs are distinguished by their lower aromaticity and higher proportion of nitrogenous materials (McKnight *et al.*, 2001). In natural waters, the amount of DOM is usually estimated as dissolved organic carbon (DOC) concentration (Thurman, 1985).

Evidence is now available for several direct influences of DOM on aquatic organisms including accumulation on biological surfaces (Campbell *et al.*, 1997), increasing membrane permeability (Vigneault *et al.,* 2000) and interference with the electrical properties of fish gills (Galvez *et al.,* 2009). In the presence of DOMs, higher survival and improved growth were observed for organisms in acidic soft water (Hargeby and Petersen, 1988; Barth and Wilson, 2010). Gonzalez *et al.* (1998, 2002), Matsuo *et al.* (2004), and Wood *et al.* (2003) documented ameliorative actions of DOM in protecting against disruptions of ionoregulation caused by metal or low pH exposure, and in enhancing ammonia excretion under acidic conditions. Even in the absence of these disturbances, studies suggested that these substances may stimulate active Na^+ uptake in several different freshwater animals (Matsuo *et al.*, 2004; Glover *et al.*, 2005). While some direct effects of DOM appeared to be pronounced at low pH (Campbell *et al.*, 1997; Vigneault *et al.*, 2000; Hargeby and Petersen, 1988), interactions with aquatic organisms also occurred at normal pH ranges (Barth and Wilson, 2010). Indeed, source-dependent effects of different DOMs on trout gill transepithelial potential were clearly seen at circumneutral pH (Galvez *et al.*, 2009). On the other hand, commercial humic substances

exerted contrasting effects on Na^+ regulation of aquatic organisms (e.g. Glover *et al.*, 2005; Matsuo *et al.*, 2004; Wood *et al.*, 2003). It is possible that DOM molecules interact with the epithelial component(s) (e.g. transporters, channels, junctional proteins) responsible for Na^+, ammonia, and permeability regulation, yet it is not clear how DOM mediates the actions mechanistically.

With the overall objective of gaining a better understanding of DOM influences on the basic physiology of aquatic organisms, the present study examined the effects of various DOMs on Na^+ homeostasis, nitrogenous waste excretion, and indices of permeability regulation in the gills of the freshwater teleost, zebrafish (*Danio rerio*) at two pH conditions (circumneutral pH of ≥ 7 and acidic pH of ~ 5). Our hypotheses stated that the presence of DOM in water would provoke positive influences on Na^+ transport and ammonia excretion, and would generally reduce passive gill permeability. Given the surface activity of DOM at low pH (Campbell *et al*., 1997; Vigneault *et al.,* 2000) and the observations by Matsuo *et al.* (2004) and Wood *et al.* (2003), we predicted higher unidirectional Na^+ uptake, lower diffusive Na^+ loss, enhanced ammonia excretion, and lower transcellular and/or paracellular permeability when DOM was present in water, especially at low pH. In addition, it was expected that commercial humic substances would behave differently compared to the natural DOM isolates. The technique used for assessment of paracellular permeability also yielded measures of drinking rate, which proved to be surprisingly high in freshwater zebrafish. Although our hypotheses were not supported completely, our results reveal reduction of passive Na^+ loss in the presence of all DOM sources, implying interaction with epithelial permeability. In addition, one

source of mixed nature (allochthonous-autochthonous) enhanced the active uptake of Na^+ at low pH. The effect of commercial AHA was restricted to stimulating maximal Na^+ uptake in kinetic studies.

6.3 Materials and methods

6.3.1 Test organisms

The adult zebrafish (*D. rerio*) were obtained from a local supplier (AQuality Tropical Fish Wholesale Inc., Mississauga, ON, Canada) and kept in 40-L aquaria in aerated, dechlorinated Lake Ontario water (City of Hamilton tap water, moderately hard water, Table 6.1) at 26–27 ºC. Fish were subjected to a 12 h light: 12 h dark photoperiod and were fed Nutrafin® Max tropical fish flakes (Rolf C. Hagen Inc., Montreal, QC, Canada) twice per day. The water of the aquaria was filtered with Aqua Clear 300 aquarium filters with activated carbon, foam and bio-max inserts (Rolf C. Hagen Inc., Baie d'Urfe, QC, Canada). Fish were allowed to acclimate to the dechlorinated water for at least 14 days before acclimation to low pH or use in experiments. In all experiments, fish were starved for 48 h prior to experimentation and all exposures were conducted during daytime with no feeding. All procedures for animal use were carried out according to the McMaster University Animal Research Ethics Board and conform to the guidelines of the Canadian Council on Animal Care.

6.3.2 Acclimation to low pH

For all experiments at acidic pH (~5), fish were acclimated to low pH for one

week before exposure. Thirty fish were held in a 20-L aquarium where water pH was kept within 4.90–5.10 using a Radiometer-Copenhagen pH meter-titrator system (PHM82 Standard pH meter and TTT80 titrator, Bach-Simpson Ltd., London, ON, Canada). The system was connected to a valve which controlled dripping of 0.25 N H_2SO_4 (prepared from 95–98% H_2SO_4, ACS specification, Caledon Laboratories LTD, Georgetown, ON, Canada) into the aquarium to keep the water at or just slightly below the pH endpoint set by the titrator. On a daily basis, the pH of the aquarium water was checked using a SP70 portable pH meter with Ag/AgCl pH electrode (VWR sympHony, VWR International, Beverly, MA, USA), the pH endpoint was adjusted, if necessary, and about 1/4 of the water was replaced with fresh dechlorinated water adjusted to pH 5.

6.3.3 Dissolved organic matter (DOM) solutions

Three different DOM isolates were collected by reverse-osmosis from Lake Ontario (LO), Bannister Lake (BL) and Luther Marsh (LM). Details of collection and treatment of these DOM sources are provided in Al-Reasi *et al.* (2012). A commercially available humic acid (AHA, Sigma-Aldrich Chemical, St. Louis, MO, USA) was included for comparison. The various chemical characteristics obtained by absorbance and fluorescence spectroscopy, and by potentiometric titration of these four different DOMs, are discussed elsewhere (Al-Reasi *et al.*, 2012; Al-Reasi *et al.*, submitted a and b). All DOM solutions for experiments measuring unidirectional Na^+ fluxes and uptake of polyethylene glycol ([^{3}H] PEG-4000) were prepared at a nominal DOC concentration of 6 mg C L^{-1} using dechlorinated water which was employed as a control (no added DOM).

For Na^+ influx kinetic experiments, Na^+-free artificial freshwater was prepared (see below). Since the addition of DOC from LO isolate in particular increased the concentrations of Na^+ and Ca^{2+} ions, all exposure solutions including the control were checked before exposure for Ca^{2+} and Na^+ levels. Appropriate amounts of calcium carbonate ($CaCO_3$ powder, Sigma-Aldrich Chemical, St. Louis, MO, USA) and sodium chloride (NaCl salt, Caledon Laboratories LTD, Georgetown, ON, Canada) were added so as to remove differences, because calcium (Ca^{2+}) is known to play a role in regulating membrane permeability (McDonald and Rogano, 1986) and affecting Na^+ uptake in a concentration-dependent manner (Glover and Wood, 2005b). Because of the low solubility of $CaCO_3$, all solutions were bubbled overnight with pure carbon dioxide (CO_2, Air Liquide Canada Inc., Burlington, ON, Canada). The next day, the solutions were aerated for 24 h to remove excess CO_2. About 16–20 h before exposure, each solution was initially adjusted to the desired pH ($\geq$ 7 or ~ 5) using the portable pH meter by addition of diluted H_2SO_4 solution or/and KOH solution (made from KOH crystal, ACS specification, Caledon Laboratories LTD, Georgetown, ON, Canada). In all these steps, solutions were stored in foil-wrapped plastic bottles to minimize the degradation of DOM due to photolysis.

6.3.4 Na^+ influx kinetics

Experiments measuring the concentration-dependence of Na^+ influx (i.e. "Na^+ influx kinetics") were performed in artificial Na^+-free water with average ionic composition similar to the dechlorinated tap water used to house the fish (Table 6.1). The

Na^+-free water was prepared using $CaCO_3$ and magnesium carbonate ($4MgCO_3 \cdot Mg(OH)_2 \cdot 4H_2O$, Mallinckrodt® analytical grade) added to deionized water (≥17.5 MΩ cm; Barnstead Nanopure II, Thermo Scientific Barnstead, NH, USA) based on the recipe formulated by Goss and Wood (1990). As described above for dissolution of $CaCO_3$, the solution was bubbled using first CO_2 and then air. Water chemistry of the dechlorinated tap water and the Na^+-free artificial water is listed in Table 6.1. Organic matter was added to the artificial water from BL and LM isolates and AHA at DOC concentrations of ~ 6 mg C L^{-1}. Because of high Na^+ content of the LO isolate, it was not included in the Na^+ influx kinetics experiments.

All kinetic exposures were run in shielded aerated polyethylene chambers containing 60 ml of each exposure solution. Six *D. rerio* adults (mean weights in Table 6.2) were transferred individually into each of the nominal Na^+ concentrations of 75, 150, 300, 600, 1,200 and 2,400 mM (as NaCl). The pH was checked and adjusted before the introduction of the organisms, and again at the end of exposure. Fish were allowed to recover from handling for 30 min before addition of ^{22}NaCl (Eckert and Ziegler isotope products, Valencia, CA, USA). The radiotracer $^{22}Na^+$ was added proportionally to nominal [Na^+] in order to keep relatively similar specific activities (SA) for all solutions. In a 3 h-period flux, Na^+ uptake was determined based on $^{22}Na^+$ incorporation by the fish. One ml of water was sampled at the beginning and at the end of the experiment for determination of [Na^+] and counts per minute (cpm). At the end, fish were rinsed in a high concentration "cold displacement Na^+ solution" (250 mM NaCl) for 1 min to get rid

of any $^{22}Na^{+}$ ions adsorbed to the surface, humanely sacrificed with an overdose of neutralized TMS (~ 1.0 g L^{-1} tricaine methanesulfonate, Syndel Laboratories Ltd., Qualicum Beach, BC, Canada) followed by a quick rinse in deionised water, and blotted dry on Whatman® No. 1 filter paper. Fish were then transferred into tared plastic vials and weighed. In addition, water samples were obtained for ions and DOC concentrations.

6.3.5 Unidirectional Na^{+} influx rate

Unidirectional Na^{+} influx measurements were conducted in 60 ml of each solution with 6 or 3 fish for 3-h or 6-h exposure periods, respectively. The pH of solutions was checked and adjusted before and during the exposure to the desired pH condition (≥ 7 or ~ 5). After 30 min recovery time from handling, ~1.0 μCi of $^{22}Na^{+}$ was added to each solution and a 10-min equilibration period was allowed before collecting the first 1 ml sample. The second 1 ml sample was taken at the end of the exposure after which the fish were handled as described above for Na^{+} influx kinetic experiments. The radioactivities of fish and water samples, as counts per minute (cpm), were determined using a Wizard 3" 1480 automatic gamma counter (PerkinElmer, Woodbridge, ON, Canada). Additional water samples were obtained for measurements of Na^{+} and DOC concentrations.

6.3.6 Unidirectional Na^{+} efflux and net K^{+}, ammonia, and urea excretion rates

Overnight, 15 fish were held in 1.0 L aerated, dechlorinated water inoculated with 50–100 μCi of radioactive $^{22}Na^{+}$ so as to load their internal Na^{+} pool with radiolabel. Prior to efflux measurements, each fish was rinsed first in fresh dechlorinated water for 2

min and then quickly washed in deionized water to remove any $^{22}Na^{+}$ adsorbed to the surface of the organism. Efflux chambers contained 30 ml of control water (no added DOM) or waters with added DOMs, adjusted to the appropriate pH. Fish were then transferred individually into these solutions and allowed to undergo efflux for 6 h. For the entire course of the exposure, pH was checked and adjusted using the diluted H_2SO_4 solution for exposure at both pH conditions for all solutions except that of solutions containing LO isolate. Diluted KOH solution was used to correct pH readings of the LO solutions for the circumneutral pH experiments only. At 10 min after fish introduction, 3 water samples of 1 ml were obtained at the beginning of the exposure for measurements of $^{22}Na^{+}$counts, ammonia and urea. Ammonia and urea samples were collected on ice and immediately after collection were kept in a freezer at –20 ºC until chemical analysis. Similar water samples were taken after 3 and 6 h (end of the exposure). At the end, additional samples were taken for the measurement of DOC and ion concentrations. Fish were then taken out of the chambers, rinsed in fresh dechlorinated water to remove $^{22}Na^{+}$ traces on the surface, and killed by overdose of neutralized TMS. The whole bodies were placed individually into pre-weighed tubes, which were re-weighed. Radioactivity of the samples was measured as described above for the unidirectional Na^{+} influx experiments.

Each fish was then digested by the addition of 1.8 ml of 1.0 N trace metal grade HNO_3 (prepared from 67–70% HNO_3, Fisher Scientific, Fairlawn, NJ, USA) and placed in the oven for 48 hours at ~ 65 ºC. During this period, the samples were mixed 3–4 times with a Vortex Genie 2 Shaker (Scientific Industries, Bohemia, NY, USA) to homogenize the digested fish solutions. The solutions were then transferred to micro-centrifuge tubes

and centrifuged at 4000 revolutions per min (rpm) for 10 min. Known amounts of the supernatant were diluted 50 times with 1% HNO_3 to determine the whole body Na^+ content of each fish, in order to calculate the internal SA of Na^+.

As a marker for transcellular permeability of freshwater gills, external water potassium (K^+) concentrations were measured to determine net K^+ efflux rate. Because K^+ ions are highly concentrated inside cells with 100-fold higher concentration than that of blood plasma, Lauren and McDonald (1985) considered the K^+ loss rates to largely reflect transcellular permeability. The K^+ fluxes could not be quantified in the LO tests at circumneutral pH because KOH was used for pH adjustment in these trials.

6.3.7 Uptake of [^{3}H] polyethylene glycol ([^{3}H] PEG-4000)

Whole body uptake of [1, 2] tritium-labelled polyethylene glycol ([^{3}H] PEG-4000), after correction for accumulation in the gut through drinking, yields extra-gut uptake, which provides an index of gill paracellular permeability (Kumai *et al*., 2011). Extra-gut uptake rates and drinking rates were determined by exposing fish to [^{3}H] PEG-4000 (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) for 4.5 h, in waters of similar chemistry to those of the unidirectional Na^+ flux experiments. Fish were introduced into 60 ml of each solution in groups of 3, and allowed to settle for 30 min prior to addition of [^{3}H] PEG-4000. The pH of solutions was kept at the desired values throughout the experiments. About 10 μCi of the radiolabeled [^{3}H] PEG-4000 was added to each solution and a water sample (1 ml) was taken 10 min later and another sample when the experiment was terminated. After that, the fish were rinsed in fresh

dechlorinated water and killed using the lethal dose of neutralized TMS. Then, each fish was rinsed quickly in deionised water, and dissected carefully to remove the entire gastrointestinal tract. The tracts and fish were placed in separate pre-weighed micro-centrifuge tubes and plastic vials, respectively and their weights were recorded.

Each fish was digested as described above for the unidirectional efflux measurements. The gastrointestinal tract was digested in 1 ml of 2 N HNO_3 for 48 hours at ~ 65 ºC with 2–3 times mixing on the vortex. The samples were centrifuged and supernatants (1.5 ml of fish digest and ~0.9 ml of tract digest) were poured into glass scintillation vials to which 5 times volume of Ultima Gold^{TM} scintillation cocktail (PerkinElmer, Inc., Waltham, MA, USA) was added and the mixture was shaken. For water samples, 2 ml of Optiphase 'Hisafe' 3 scintillation cocktail (PerkinElmer, Inc., Waltham, MA, USA) was added to 1 ml of the sample and mixed. After 1 hour of incubation in the dark to minimize chemiluminescence, the radioactivities of tissue and water samples were quantified using a liquid scintillation analyzer (Tri–Carb 2900TR, PerkinElmer Life and Analytical Services, Downers Grove, IL, USA). Readings of all samples were corrected for the background cpm and each tissue sample (fish and gastrointestinal tract) was corrected for quenching effect by internal standardization, so as to have the same counting efficiency as water samples.

6.3.8 Chemical analyses

The whole body Na^+ concentration of zebrafish and concentrations of water Na^+, Ca^{2+}, Mg^{2+} and K^+ were analyzed using flame atomic absorption spectrometry

(SpectroAA220FS, Varian, Mulgrave, Australia). The total DOC concentration was measured directly using a Shimadzu TOC-$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). Assays for ammonia and urea concentrations in water were performed in triplicate in microplates according to methods of Verdouw *et al.* (1978) and Rahmatullah and Boyde (1980), respectively. To minimize any possible interference of DOM molecules with colour development in these assays, blanks and standards for ammonia and urea were prepared in 6 mg L^{-1} solutions of each DOM isolate. Microplates were scanned at the appropriate wavelengths using a SpectraMAX 340pc microplate reader (Molecular Devices, Sunnyvale, CA, USA).

6.3.9 Calculations and statistical analyses

The Na^+ influx kinetic parameters were determined graphically from the plots of unidirectional Na^+ influx rate versus water Na^+ concentration produced by SigmaPlot for Windows (Version 10.0, Systat Software, Inc., Point Richmond, CA, USA) according to the Michaelis-Menten equation (Wood, 1992):

$$J_{in} = \frac{J_{max} \times [Na^+]}{K_m + [Na^+]}$$

where J_{in} is Na^+ influx rate, J_{max} is maximum influx rate (i.e. an index of the number of sites available for Na^+ uptake) and K_m is an index of the binding affinity of the sites for Na^+ transport and is equal to water $[Na^+]$ when J_{in} is 50% of J_{max}. As K_m magnitude increases, the affinity of sites or transporters decreases (Wood, 1992). The unidirectional Na^+ influx (J_{in}) and efflux (J_{out}) rates were calculated based on the amount of

radioisotopic $^{22}Na^{+}$ incorporated into the fish (for influx) and the appearance of $^{22}Na^{+}$ in the exposure water (for efflux), respectively, according to the following equations:

$$J_{\text{in}} = \frac{\text{cpm}}{\text{SA} \times m \times t}$$

where cpm is the counts per minute of each fish, SA is the external specific radioactivity of the exposure water (cpm/μmol), m is the fish mass (kg) and t is the time of exposure (h), and

$$J_{out} = \frac{(\text{cpm}_i - \text{cpm}_f) \times V}{\text{SA} \times m \times t}$$

where cpm_i and cpm_f are initial and final counts per minute lost by fish into the external water, V is volume (L) of exposure water, SA is the internal specific activity of the fish, m is the fish mass (kg) and t is the time of exposure (h). The net flux rates of K^{+}, total ammonia and urea were calculated from the differences between the initial and final concentrations ($[\text{solute}]_i - [\text{solute}]_f$, in μM), divided by the fish mass, m (kg), taking into account volume, V (L) and time, t (h) as follow:

$$J_{\text{net}} = \frac{([\text{solute}]_i - [\text{solute}]_f) \times V}{m \times t}$$

All fluxes were expressed in μmol kg^{-1} h^{-1}. Extra-gut uptake of [^{3}H] PEG-4000 was estimated as clearance from the external water, calculated from the cpm in the whole body digest (excluding the gastrointestinal tract) factored by water cpm of [^{3}H] PEG-4000 (cpm ml^{-1}), the total fish mass (kg, the whole body mass including the gastrointestinal tract) and time (h). Wood and Grosell (2012) demonstrated that *in vitro* absorption of [^{3}H] PEG-4000 by the fish gastrointestinal tract is very low, suggesting that this route would

make a negligible contribution to the extra-gut uptake. Similarly, the drinking rate was estimated as cpm of the total tract digest, again divided by the total fish mass and time. The units for both the extra-gut uptake and drinking rate were ml kg^{-1} h^{-1}.

All statistical analyses were performed using SigmaStat for Windows (Version 3.5, Systat Software, Inc., Point Richmond, CA, USA). Normality and homogeneity of variance of data were checked by the Kolmogorov-Smirnov test and Levene median test, respectively, before applying the appropriate statistical techniques. One-way analysis of variance (ANOVA) was employed to test whether significant differences occurred among multiple treatments, and Student's two tailed *t*-test was used to check for significant differences between the two pH conditions of the same DOM treatment. Before performing ANOVA and/or *t*-tests, data were first $\log_{10}$-transformed in the case of data sets which violated assumptions of normal distribution and homogeneity of variance. The negative values for unidirectional efflux rates were multiplied by -1 before the logarithmic transformation. Nonparametric equivalents to ANOVA and *t*-test (Kruskal-Wallis Analysis of Variance on ranks, *H*, and Mann-Whitney Rank Sum test, *U*, respectively) were employed rarely where the transformation did not work. Multiple *post hoc* comparisons (Tukey's test or Dunn's method) were employed whenever significant differences were detected. Spearman's correlation coefficient (r_s) was utilized to examine relationships between two data variables. All values have been reported as means ± standard errors, and a significance level of $p < 0.05$ was set for all statistical tests.

6.4 Results

6.4.1 The influence of DOM on the whole body Na^+ content

The whole body Na^+ contents of zebrafish in all treatments, measured in the unidirectional Na^+ efflux trials, are listed in the right hand column of Table 6.3. No differences in the mean values of Na^+ content were found among the treatments at circumneutral pH (Kruskal-Wallis ANOVA, $H = 0.649$, $df = 4$, $p = 0.957$) or low pH (ANOVA, $F_{4,\ 38} = 1.433$, $p = 0.242$). For each treatment, fish had similar Na^+ concentrations at both pH's (Table 6.3).

6.4.2 The influence of DOM on Na^+ influx kinetics

Under the specified water chemistries (Table 6.2), unidirectional Na^+ uptake rates by zebrafish were concentration-dependent, exhibiting Michaelis-Menten saturation kinetics. The kinetic parameters (maximal Na^+ transport rate - J_{max} in μmol kg^{-1} h^{-1} and uptake affinity - K_m in μM) were estimated for all treatments and detailed in Fig. 6.1. Relative to control (DOM-free water), an increasing trend of J_{max} and higher K_m values were observed in the presence of DOMs. Statistically, a significant difference was revealed by ANOVA ($F_{4,\ 20} = 4.775$, $p < 0.05$) and the *post hoc* multiple comparisons showed that fish had higher J_{max} in the presence of AHA relative to those in controls and in the presence of BL (Fig. 6.1). However, similar K_m values were observed for all treatments (ANOVA, $F_{4,\ 20} = 0.782$, $p = 0.518$).

6.4.3 The influence of DOM on unidirectional Na^+ influx rate

Over the 3-h exposure period, zebrafish had similar Na^+ unidirectional influx rates (average control: 780 ± 122 μmol kg^{-1} h^{-1}; range in the presence of DOM: 692 ± 85 μmol kg^{-1} h^{-1} in BL to 840 ± 218 μmol kg^{-1} h^{-1} in LO) in all treatments (ANOVA, $F_{4, 25}$ = 0.180, p = 0.947) at circumneutral pH (Fig. 6.2A). At pH ~5, while control fish had an average rate of 621 ± 50 μmol kg^{-1} h^{-1}, a broad range (between 597 ± 98 μmol kg^{-1} h^{-1} in AHA to 1011± 98 μmol kg^{-1} h^{-1} in BL) was noticed when DOM was present in water. Significant differences were revealed among the treatments at pH ~5 (ANOVA, $F_{4, 34}$ = 3.068, $p < 0.05$). Fish in the presence of BL at acidic pH demonstrated higher Na^+ uptake rates than those in control conditions or in the presence of AHA (Fig. 6.2A). However, rates were not significantly different within the same treatment at the two pH conditions (Fig. 6.2A). For exposures over the 6-h period, somewhat greater rates of Na^+ influx were seen (ranging between 705 ± 95 μmol kg^{-1} h^{-1} in AHA to 1090 ± 138 μmol kg^{-1} h^{-1} in BL) in the presence of various DOM sources at pH ≥ 7 relative to the control (average: 595 ± 63 μmol kg^{-1} h^{-1}) (Fig. 6.2B; ANOVA, $F_{4, 25}$ = 2.829, p = 0.046), but the *post hoc* test detected no significant individual differences. Compared to control and also to the presence of other DOMs, fish doubled their Na^+ uptake in the presence of BL at pH ~5 (Fig. 6.2B). In addition, fish exposed to LM at pH ~ 5 had significantly lower influx rates compared to those at pH ≥ 7 (*t*-test, t = 2.402, df = 10, $p < 0.05$, Fig. 2A).

6.4.4 The influence of DOM on unidirectional Na^+ efflux rate

Unidirectional Na^+ efflux rates tended to decline with time. Over the 0–3 and 3–6

h phases, control fish had average Na^+ efflux rates of -1752 ± 301 and -1126 ± 375 μmol kg^{-1} h^{-1} for circumneutral pH, and -3009 ± 633 and -925 ± 308 μmol kg^{-1} h^{-1} for acidic pH, respectively. Compared to control, fish exposed to DOMs had lower mean ranges of -855 ± 115 (LM) to -1398 ± 162 (LO) at 0–3 h, and -354 ± 58 (LO) to -447 ± 62 (BL) μmol kg^{-1} h^{-1} at 3-6 h at pH ≥ 7, whereas at pH ~5, the corresponding ranges were -643 ± 88 (BL) to -1192 ± 130 (LO) at 0-3 h and -292 ± 47 (AHA) to -691 ± 66 (LO) μmol kg^{-1} h^{-1} at 3-6 h. For the first 3 h of exposure, significant reductions in Na^+ efflux rates were noticed for BL and LM at circumneutral pH, whereas in the acidic water, all DOMs tested caused significant reductions (Fig. 6.3A). For the 3–6 h phase, the situation was somewhat different. Significant reductions in Na^+ efflux rates occurred for all DOMs tested, except BL where the difference was just below significance ($p < 0.08$). Therefore, at the circumneutral pH, the decreased rates persisted in the presence of LO, LM and AHA until the end of exposure (Fig. 6.3B). At pH ~ 5, the average values for Na^+ efflux rates of fish in the presence of various DOMs tended to be lower than those of control fish (Fig. 6.3B), but no significant difference could be detected among treatments by the *post hoc* comparisons, despite the significant ANOVA ($F_{4, 24} = 4.190$, $p < 0.05$), likely because of considerable variability observed within the control fish. Student's *t*-tests revealed no difference in Na^+ loss rates between the two pH's within the same treatment, except for fish in the presence of LO (Fig. 6.3). Fish experienced higher Na^+ ion losses when exposed to LO in the acidic environment in comparison to fish at the circumneutral water pH (*t*-test, $t = -3.839$, $df = 10$, $p < 0.01$).

6.4.5 Ammonia and urea excretion in the presence of DOM

During the 0–3 h period of exposure (Fig. 6.4A), the range of ammonia excretion rates were -1848 ± 643 in control to -1018 ± 106 μmol kg^{-1} L^{-1} in LM for pH ≥ 7 and -1634 ± 86 in control to -1073 ± 90 μmol kg^{-1} l^{-1} in AHA for pH ~5. Correspondingly, the excretion rates dropped by 9–47% and 14–27% for all treatments in the second time phase of the exposure (3–6 h, Fig. 6.4B). The exception was in presence of AHA at low pH where the fish displayed increased rates by 5% overall (Fig. 6.4B). While fish had comparable ammonia excretion rates for all the treatments during the 0–3 h period at circumneutral pH range, at low pH they dropped their excretion rates in the presence of AHA relative to those in control and in the presence LO (Kruskal-Wallis ANOVA, H = 12.224, df = 4, $p < 0.05$; Fig. 6.4A). The other comparisons were not significant. Additionally, fish exhibited different ammonia excretion rates between the pH conditions when AHA present in the exposure water (t-test, t = 3.009, df = 12, $p < 0.05$). For the second 3 h of exposure, no significant differences were revealed for ammonia excretion rates between treatments at circumneutral pH (ANOVA, $F_{4, 25} = 0.307$, $p = 0.871$) or acidic pH ($F_{4, 39} = 0.935$, $p = 0.454$).

Urea excretion rates were very low (5-15%) relative to those of ammonia and were approximately consistent over the entire exposure duration (Fig. 6.5). Thus, urea-N excretion (2 N's per urea molecule) was only 10–30% of ammonia-N excretion. For the 0–3 h time period (Fig. 6.5A), no significant differences were revealed for urea excretion rates between treatments at circumneutral pH (Kruskal-Wallis ANOVA, H = 2.022, df = 4, $p = 0.732$) or acidic pH ($F_{4, 39} = 1.006$, $p = 0.416$). Likewise, rates in all treatments at

both pH's were similar to the control and to each other during the 3–6 h phase. One exception was for *D. rerio* individuals in the presence of LO at acidic pH where they demonstrated higher urea excretion rates than those in the presence of BL and LM (Fig. 6.5B). Similar urea excretion rates were observed for both pH conditions of each treatment throughout the exposure.

6.4.6 The influence of DOM on net K^+ efflux rate

Net K^+ loss rates (indicative of transcellular permeability), were fairly stable over time at circumneutral pH, but tended to increase greatly during the first 3h of low pH exposure with attenuation in the second 3h (Fig. 6.6). Overall, there was negligible effect of DOMs on K^+ loss rates. Over the 0–3 and 3–6 h phases, control fish had average net K^+ loss rates of -190 ± 44 and -156 ± 35 μmol kg^{-1} h^{-1} for circumneutral pH; note that K^+ fluxes could not be quantified in the LO tests at circumneutral pH because KOH was used for pH adjustment in these trials. At acidic pH, control K^+ loss rates were -1392 ± 270 and -420 ± 85 μmol kg^{-1} h^{-1} at 0-3 and 3-6 h respectively. Compared to control, fish exposed to DOMs exhibited mean rates ranging from -268 ± 32 (BL) to -513 ± 112 (AHA) at 0-3 h and -186 ± 51 (AHA) to -327 ± 47 (LM) μmol kg^{-1} h^{-1} at 3-6 h at pH ≥ 7. At the lower pH ~5, rates were again correspondingly higher at -658 ± 107 (BL) to -967 ± 133 (LO) and -380 ± 60 (AHA) to -1207 ± 289 (LO) μmol kg^{-1} h^{-1}. At circumneutral pH, only the fish in the presence of AHA had significantly higher net K^+ loss rate than those of control for the first 3 h of exposure (Fig. 6.6A), and by the second 3 h, similar K^+ losses were noticed for all treatments (Fig. 6.6B). At acidic pH, no significant differences were

observed between treatments for the 0–3 h time period (Fig. 6.6A), while the increase in K^+ efflux rate in the presence of LO for the 3–6 h time phase was significant only relative to BL (Fig. 6.6B). Relative to exposure at pH ≥ 7, significantly higher K^+ loss rates were observed for control, BL and LM at pH ~ 5 for the first 3 h (Fig. 6.6A). The exception was for fish in the presence of AHA which exhibited similar rates at both pH's (*t*-test, t = -1.352, df = 9, p = 0.209, Fig. 6.6A). For the second 3 h period, the higher net K^+ loss rates persisted in control (*t*-test, t = -2.727, df = 10, $p < 0.05$, Fig. 6.6B), but not in the presence of BL or LM (*t*-test, t = -0.454, df = 10, p = 0.659 and *t*-test, t = -0.643, df = 10, p = 0.534, respectively, Fig. 6.6B). By the end of the exposure, fish in the presence of AHA experienced higher K^+ ion losses in the acidic environment in comparison to the circumneutral water pH (*t*-test, t = -2.404, df = 9, $p < 0.05$, Fig. 6.6B).

6.4.7 The influence of DOM on the extra-gut uptake of [^{3}H] PEG-4000 and on drinking rates

A surprisingly high fraction (40-50%) of whole body [^{3}H] PEG-4000 uptake occurred via drinking, with 50-60% entering via extra-gut routes, presumably mainly the gills. Overall, effects of DOM on both parameters were negligible. The average extra-gut uptake rates (indicative of paracellular permeability) of [^{3}H] PEG-4000 uptake in control were 3.3 ± 0.4 and 5.4 ± 1.0 ml kg^{-1} h^{-1} at circumneutral and acidic pH (Fig. 6.7A), respectively. In the presence of DOM, the rates spanned between 2.4 ± 0.1 ml kg^{-1} h^{-1} in LO to 4.1 ± 0.5 ml kg^{-1} h^{-1} in BL at circumneutral pH, and 3.3 ± 0.2 ml kg^{-1} h^{-1} in LM to 3.7 ± 0.4 ml kg^{-1} h^{-1} in BL and AHA at low pH (Fig. 6.7A). While no significant

difference was found among the treatments at pH ~5 (Kruskal-Wallis ANOVA, H = 3.604, df = 4, p = 0.462), at pH ≥ 7 fish in the presence of BL had a higher extra-gut [^{3}H] PEG-4000 uptake rate than those in the presence of LO and AHA (Fig. 6.7A). In each treatment, fish showed similar uptake rates under both pH conditions, except in the presence of LO where fish in acidic water had higher [^{3}H] PEG-4000 uptake rate than fish in normal pH (t-test, t = 3.156, df = 10, $p < 0.05$, Fig. 6.7A).

Over the 4.5-h period, control fish had drinking rates of 2.3 ± 0.5 and 3.0 ± 0.7 ml kg^{-1} h^{-1} for circumneutral and acidic conditions, respectively. When DOM was present in water, the drinking rates were between 2.0 ± 0.4 in LO and 2.9 ± 0.5 ml kg^{-1} h^{-1} in AHA at pH ≥ 7, and between 2.6 ± 0.9 in AHA to 3.8 ± 0.8 ml kg^{-1} h^{-1} in BL at pH ~ 5. The rates were statistically similar at the circumneutral pH (ANOVA, $F_{4, 24}$ = 0.648, p = 0.634, Fig. 6.7B) and low pH (ANOVA, $F_{4, 23}$ = 0.383, p = 0.818, Fig. 6.7B). Although the average drinking rates at pH ~ 5 were higher than those at pH ≥ 7 for most treatments (Fig. 6.7B), no significant differences were observed.

6.5 Discussion

6.5.1 Overview

Originally, we hypothesized (see Introduction) that the presence of DOMs in the exposure water would result in higher unidirectional Na^+ influx rates, lower passive Na^+ efflux rates and lower transcellular and/or paracellular permeability, especially at low pH. Similarly, we had predicted reduction of ammonia excretion rates for zebrafish in the presence of natural DOMs, as seen for *D. magna* (Al-Reasi *et al.*, submitted b). Source-

dependent effects were anticipated such that allochthonous DOM sources would cause greater effects compared to the autochthonous ones. Furthermore, we hypothesized that the influence of the commercial AHA would differ from that of the natural DOM isolates. All tests were run in the presence of 6 mg C L^{-1}, well within the reported DOC concentrations of the natural habitat of *D. rerio* (the River Ganges, which has DOC concentration range of 2–9 mg C L^{-1}, Ittekkot *et al*., 1985) while keeping other exposure conditions relatively alike. Only one of these hypotheses was unequivocally confirmed, two were partially supported, and some of the results conflicted with our predictions. The one major positive finding was that there was a reduction of unidirectional Na^+ efflux in the presence of all DOM sources regardless of pH condition, indicating ameliorative action against passive Na^+ loss. Interestingly, these same DOM sources had negligible impact on Na^+ homeostasis of *D. magna* (Al-Reasi *et al.*, submitted b), indicating organism-specific differences. With respect to the prediction of higher Na^+ influx rates in the presence of DOMs, there seemed to be a general trend for this phenomenon with various DOMs, but significant effect was restricted to a mixed autochthonous-allochthonous source at acidic condition. Furthermore, the kinetics analyses indicated a trend for generally higher J_{max} and higher K_m values in the presence of DOMs, but this was significant only for AHA. AHA also exerted several other effects which were different from those of natural DOMs, providing some support for our hypothesis that its actions are not consistent with those of natural DOM isolates. This is in agreement with our findings on *D. magna* (Al-Reasi *et al.*, submitted b) and a growing literature on the unusual properties of commercial DOM preparations (Terekhova *et al*., 2010; Molnar *et*

al., 2012). There was no clear evidence for effects of DOMs on transcellular or paracellular permeabilities, or on ammonia and urea excretion rates in the zebrafish. For *D*. *magna*, reduction of ammonia excretion was observed in the presence of the natural DOMs particularly at low pH (Al-Reasi *et al.*, submitted b), so again, there are organism-specific mechanisms of ammonia excretion.

6.5.2 The Influence of DOM on Na^+ influx kinetics

For zebrafish (*D*. *rerio*), J_{max} values were similar to those found by Kumai *et al.* (2011) at circumneutral pH, but higher in magnitude than those reported by Boisen *et al.* (2003) for hard water acclimated fish. Lower values of K_m (112–160 μM) for this species were found in both studies (Boisen *et al.*, 2003; Kumai *et al.*, 2011) relative to the present values (246–476 μM). The reasons for these differences are unclear, as they were not related to consistent differences in the chemistry (e.g. Na^+ and/or Ca^{2+} concentrations) of the acclimation waters in the various studies.

The general trend of higher maximal uptake rate (J_{max}) associated with decreased uptake affinity (i.e. higher K_m) in the presence of DOMs suggested uncompetitive stimulation of Na^+ uptake of zebrafish. While K_m values did not vary significantly across treatments, a significant increase in J_{max} was noticed for AHA (Fig. 6.1). Similarly, Matsuo *et al.* (2004) found a significant increase in J_{max}, but unchanged K_m for rainbow trout (*O*. *mykiss*) in the presence of AHA. Enhanced Na^+ transport by the water flea (*D*. *magna*) was observed with the augmentation of J_{max} by both AHA and another commercial preparation, Suwannee River natural organic matter, SRN (Glover *et al.*,

2005). In the same study, the SRN also resulted in significantly higher K_m. In contrast, Amazonian black water (8.6 mg C L^{-1}) had no effects on both kinetic parameters (J_{max} and K_m) of freshwater stingrays relative to reference water with low DOC concentration of 0.6 mg C L^{-1} (Wood *et al.*, 2003). Matsuo *et al.* (2004) and Glover *et al.* (2005) tentatively attributed the enhanced Na^+ uptake in the presence of AHA to increased membrane permeability because of the surfactant character of humic substances especially at low pH (Campbell *et al.*, 1997; Vigneault *et al.*, 2000), and a reduction in external Ca^{2+} ions (Matthews, 1986) due to complexation by DOM molecules. However, it should be noted that Vigneault *et al.* (2000) demonstrated the effect of DOM using lipophilic substrates rather than cations in algal cell membranes. This explanation may work for commercial humic substances, but not for the natural DOM samples tested in the present study and others (Matsuo *et al.*, 2004; Wood *et al.*, 2003) in which Na^+ influx kinetics (J_{max} and K_m) did not change significantly. In future studies (currently under way), it will be of interest to determine Na^+ influx kinetics at low pH in these same treatments.

6.5.3 Unidirectional Na^+ influx rates in the presence of DOM

The reported unidirectional Na^+ influx rates were within the range found for zebrafish in other studies (Boisen *et al.*, 2003; Kumai *et al.*, 2011). The influx rates obtained from the unidirectional Na^+ influx trials agreed well with the rates obtained by the kinetic analysis in control water (no added DOM) and in the presence of natural DOM sources tested at a Na^+ concentration of 0.6 μM. The exposure water for the unidirectional

influxes contained a Na^+ level of ~ 0.9 μM (Table 6.3). For AHA, the kinetics analysis resulted in higher influx rates compared to the influx rates from the unidirectional Na^+ influx trials. The reason for this difference is unknown, but the result again highlights the anomolous actions of AHA.

Two features of the Na^+ influx results are of particular interest. Firstly, unidirectional Na^+ uptake rates by zebrafish remained generally unchanged in acidic conditions (pH ~ 5) relative to circumneutral conditions (pH $\geq$ 7.0; Fig. 6.2) (but see below for one exception). This result contrasts with the greatly increased Na^+ influx reported recently by Kumai *et al.* (2011) in zebrafish exposed to much more acidic water (pH 3.8–4.0). This difference was likely because of higher pH (by ~1.0–1.2 units) in our experiments. These observations were also supported by the constant whole body Na^+ contents of zebrafish at both circumneutral and acidic pH (Table 6.3). In addition, the rates of unidirectional Na^+ uptake (Fig. 6.2) and Na^+ efflux (Fig. 6.3) of each treatment under both pH conditions were essentially similar, implying that Na^+ homeostasis was not severely impacted by pH ~ 5 exposure. In another freshwater organism (*D. magna*), low pH (~ 5) had no negative effects on the whole body Na^+ content or influx rates (Al-Reasi *et al.*, submitted b).

Secondly, the 6 h data indicated a tendency for most DOMs to increase Na^+ influx rates at circumneutral pH, in accord with the trends seen in the influx kinetic relationships (Fig. 6.2), though the changes were not significant. However at low pH, there was a substantial significant stimulation of Na^+ uptake in the presence of BL but not in the presence of the other DOM sources. Unlike other sources of either autochthonous or

terrigenous origin, the organic matter of BL is of mixed origins (i.e. autochthonous and terrigenous) (Al-Reasi *et al.*, 2012). It is the DOM source with the highest acidic binding capacity (Al-Reasi *et al.*, submitted a) and buffer capacity, especially at low pH (Al-Reasi *et al.*, submitted b). From a chemical perspective, this isolate also has a high Titration Index (TI) of 0.30 ± 0.08 (the range recorded for the tested DOM sources: 0.20–0.44). This value can loosely be interpreted as a closer spacing of functional groups to allow for greater coordination of proton binding sites (Al-Reasi *et al.*, submitted a). Thus, the latter characteristics imply that BL molecules may have greater potential to interact more effectively with ionizable sites on epithelial membrane surfaces, including entities responsible for apical Na^+ uptake in adult zebrafish (e.g. Na^+/H^+ exchanger in H^+-ATPase rich cells, Hwang, 2009; Yan *et al.*, 2007). Alternatively, BL molecules may behave differentially under acidic conditions, because there was a time-dependent enhancement of Na^+ uptake not seen with other DOM sources, including the commercial AHA (Fig. 6.2). Influx kinetic experiments are currently being conducted at pH ~ 5, and may provide insights about the behaviour of this particular DOM isolate toward maximal Na^+ uptake (J_{max}) and uptake affinity (K_m).

6.5.4 Unidirectional Na^+ efflux rates in the presence of DOM

While the same sources had no influence on the passive diffusive efflux of Na^+ in *D. magna* (Al-Reasi *et al.*, submitted b), the presence of DOM exerted positive actions by minimizing unidirectional Na^+ efflux rates at both pH conditions (Fig. 6.3). Although it usually assumed that Na^+ ions move passively through paracellular pathways down the

concentration gradient (blood to external water) in freshwater fish, the regulation of Na^+ diffusion is not a well-understood process. Indeed, some recent studies have suggested that significant Na^+ efflux may also occur through the transcellular pathway, modulated by the covering/uncovering of the ionocytes by pavement cells (Wood *et al*., 2009; Iftikar *et al*., 2010; Matey *et al*., 2011). Diffusion across the cell membrane (i.e. the transcellular pathway) has been proposed to increase as a consequence of humic substances binding (Campbell *et al.,* 1997; Vigneault *et al.,* 2000). The present observations indicated exactly the opposite; the unidirectional Na^+ efflux rates were significantly reduced in the presence of DOMs (Fig. 6.3), reinforcing our hypothesis that the presence of DOM would reduce passive permeability for Na^+ ions (see Introduction). This is discussed subsequently.

6.5.4.1 Is the reduction of Na^+ efflux in the presence of DOM due to transcellular permeability?

Potassium (K^+) is thought to move primarily through the transcellular pathways of the gills (Lauren and McDonald, 1985; Wood *et al*., 2009; Iftikar *et al*., 2010), and therefore serves as a marker for transcellular permeability. The observed responses in K^+ net loss rates (Fig. 6.6) with respect to DOM treatments were very different from those of unidirectional Na^+ efflux rates (Fig. 6.3). In particular, K^+ loss rates increased markedly at low pH, and the various DOMs offered no protection against this loss. Furthermore, the net K^+ flux rates were not correlated with the Na^+ efflux rates (r_s = 0.18, $p > 0.05$, respectively, Fig. 6.8A). These results argue against DOM effects on transcellular permeability.

Similarly, trends in ammonia excretion rates (Fig. 6.4) with respect to DOMs did not correspond to those in unidirectional Na^+ efflux rates (Fig. 6.3). Nevertheless, the total ammonia flux rates and Na^+ efflux rates were significantly related, albeit with considerable variability ($r_s = 0.36$, $p < 0.001$, Fig. 6.8B). Recent evidence suggests that a substantial portion of ammonia efflux in fish occurs through the transcellular pathway mediated by specific facilitated diffusion channels called Rhesus (Rh) glycoproteins, and that this process is loosely coupled to Na^+ uptake (Wright and Wood, 2009; Weihrauch *et al*., 2009; Kumai and Perry, 2011). While the reduction of transcellular permeability due to DOMs may not be exclusively ruled out, the results also point to a possible action on the paracellular pathway.

6.5.5 Paracellular permeability and drinking rate in the presence of DOM

Revisiting the conventional assumption of Na^+ ion efflux through paracellular pathways, [^{3}H] PEG-4000 uptake was utilized as a gill paracellular permeability marker (Curtis and Wood, 1991; Scott *et al.*, 2006; Wood *et al*., 2009; Kumai *et al*., 2011). Since we placed this marker in the external environment and measured its appearance in the fish, it was important to correct for uptake through drinking into the gut. An added benefit of this approach is that it provided measurements of drinking rate, which proved to be surprisingly high in freshwater zebrafish. Usually, freshwater fish drink very limited quantities of water because of the hypotonic nature of surrounding dilute water. The drinking rates reported here (ranges: 2.0–2.9 and 2.6–3.8 ml $kg^{-1}h^{-1}$ at circumneutral and acidic pH, respectively) are much higher than the average drinking rates of 0.3–2.0 ml kg^{-}

$^{1}h^{-1}$ reported for the freshwater goldfish (*Carassius auratus*), common shiner (*Natropis cornutus*) and mottled sculpin (*Cottus bairdi*) (Beasley *et al*., 1986). Lower drinking ranges of 0.5–1.5 and ~0.5 to < 1.0 ml $kg^{-1}h^{-1}$ were documented for the freshwater rainbow trout (Pyle *et al.*, 2003) and the euryhaline killifish (*Fundulus heteroclitus*) when transferred to freshwater (Scott *et al.*, 2006), respectively. Nevertheless, at these drinking rates, simple calculations indicate that the acquisition of Na^+ ions via absorption from the gut of zebrafish (*D. rerio*) is negligible (< 1%) relative to Na^+ uptake by the gill. Gill paracellular permeability as well as drinking rates were generally not affected by the presence of the various DOMs at both pH's (Fig. 6.7). Such observations may mean that the reduction of Na^+ loss by the presence of DOMs may not be attributed to effects on the paracellular permeability of the branchial epithelium.

In adult *D. rerio*, a recent study (Kumai *et al.*, 2011) demonstrated upregulation of tight junction proteins (e.g. claudins, which are thought to be involved in regulating paracellular efflux) when fish were exposed to acidic water. However, both passive Na^+ efflux rates and the extra-gut uptake of [^{3}H] PEG-400 were significantly elevated at a pH range of 3.80–4.00 (Kumai *et al.*, 2011). Here, we showed that paracellular permeability, estimated by extra-gut uptake of [^{3}H] PEG-4000, is not affected by the presence of DOM at both pH conditions (≥ 7 and ~ 5) (Fig. 6.7), while the unidirectional Na^+ efflux rates were significantly attenuated (Fig. 6.3), suggesting that the decrease in passive Na^+ loss may not be necessarily coupled with paracellular permeability as estimated by [^{3}H] PEG-4000. However, the net K^+ flux results also suggested that this effect was not coupled with transcellular permeability either (Figs. 6.6, 6.8). One possible explanation is that the

regulation of the paracellular permeability by tight junction proteins is selective, such that effects on paracellular Na^+ permeability (a small charged ion) may be different from those on PEG-4000 permeability (a large uncharged molecule). In fact, the regulation of paracellular permeability by the tight junction proteins is driven by discrimination based on size and charge of solutes (Reuss, 2001). Specifically, claudins are directly responsible for creating charge-selective channels for movement of ions (e.g. Na^+) in the paracellular space (Colegio *et al*., 2002; Van Itallie *et al*., 2001). Therefore, it is possible that the presence of DOM may act directly on stabilization of the paracellular junctions, resulting in tighter epithelia and reducing passive Na^+ effluxes but not influencing the [^{3}H] PEG-4000 uptake.

6.5.6 The influence of DOM on nitrogenous waste excretion

The excretion rates of ammonia and urea were not affected either by pH conditions or by the presence of natural DOMs (Fig. 6.4 and 6.5), favouring the alternative hypothesis that DOM molecules do not interact with transporting mechanisms of both excretory solutes. The results contrast with the reduction of ammonia excretion by *D. magna* observed in the presence of the same natural DOMs at low pH (Al-Reasi *et al.*, submitted b). At present, most evidence indicates that substantial proportions of both ammonia excretion and urea excretion through the gills occur by specific facilitated diffusion transport proteins, the Rh proteins for ammonia, and the UT proteins for urea (Weihrauch *et al.*, 2009; Wright and Wood, 2009). Nakada *et al.* (2007) reported that acclimating adult zebrafish to acidic water (3 days in pH 5.0) did not change the

expression of the Rhesus protein (Rhcg1) in the gill and kidney. In *D. rerio*, the ammonia Rh transporter (Rhcg 1) is also localized on the apical membrane of the H^+-ATPase rich cells, one of several subsets of the MRC cells (Braun *et al.*, 2009; Hwang *et al.*, 2011; Nakada *et al.*, 2007). In Amazonian stingrays, relative to reference water at pH 4.0, the ammonia excretion rate was increased by 60% by the natural DOM of the black water of Rio Negro, but this response did not occur when AHA was added to reference water at the same DOC level as that of the black water (Wood *et al.*, 2003). Little is known about the response of urea transporters to changing water chemistry, but any influence on ammonia excretion would be expected to impact urea excretion since a recent molecular study suggested that ammonia and urea excretion may be tightly linked in adult zebrafish (Braun *et al.*, 2009).

6.5.7 Future perspectives

Our results emphasize a significant positive impact of natural DOMs in attenuating the passive Na^+ loss of a "model" freshwater fish at low pH, in accord with earlier observations on Amazonian fishes endemic to low pH, high DOM waters (Gonzalez *et al*., 1998, 2002; Wood *et al*., 2003). However, the exact mechanism of DOM interaction with gill epithelia is still lacking. Given the selectivity of claudins in regulation of paracellular traffic of ions such as Na^+ (Colegio *et al*., 2002; Van Itallie *et al*., 2001), the proposed interaction of DOM molecules with these tight junction proteins needs to be confirmed. Here, the influence of DOM was generally restricted to reduction of Na^+ loss. Wood *et al*. (2003) reported remarkable stimulation of Na^+ active uptake and

attenution of passive diffusive efflux by natural DOM in the freshwater stingray at low pH. Then again, Na^+ regulation of *D. magna* was not affected by DOMs (Al-Reasi *et al.*, submitted b). While we did not observe any influence of DOM on ammonia excretion of zebrafish, the reduction of ammonia excretion at low pH in *D. magna* (Al-Reasi *et al.*, submitted b) is exactly the opposite to the significant enhancement of ammonia excretion in Amazonian fish species in the presence of a natural DOM at low pH (Wood *et al.*, 2003). Certainly, these conflicting data should be addressed in the future studies for a better understanding of the direct effects of DOM on aquatic organisms. In addition, the high drinking rates reported here for freshwater zebrafish, compared to other freshwater fish, is unusual and should be revisited in the future investigations of osmoregulation and water balance in this species.

6.6 Tables and figures

Table 6.1 Water chemistry of the dechlorinated tap water and artificial Na^+-free water. The Na^+-free water was used for making DOM solutions for Na^+ influx kinetics of zebrafish (*D. rerio*). Data (mean ± standard errors).

Parameter	Dechlorinated tap water [a]	Na^+-free water [b]
Na^+ (mM)	0.67 ± 0.01	0.00 ± 0.00
Ca^{2+} (mM)	0.95 ± 0.00	1.08 ± 0.00
Mg^{2+} (mM)	0.35 ± 0.00	0.29 ± 0.00
DOC (mg C L^{-1})	2.52 ± 0.06	0.66 ± 0.02
pH	7.75 ± 0.04	7.94 ± 0.03
Titratable alkalinity (μM) [c]	1.71 ± 0.01	1.72 ± 0.02

[a] Data of n = 46–48 for concentrations of ions, DOC and pH, taken from Al-Reasi *et al.* (2012).
[b] Data of n = 3
[c] Titratable alkalinity (to fixed endpoint pH= 4.00) was determined according to McDonald and Wood (1981).

Table 6.2 Water chemistry of the artificial Na^+-free water without added DOM (control) and with DOM added at 6 mg C L^{-1} from isolates of Bannister Lake (BL), Luther Marsh (LM) and Aldrich humic acid (AHA) used for Na^+ influx kinetics of zebrafish (*D. rerio*). Fish weights are listed in the righthand column. Data (mean ± standard errors).

Treatment	pH	Ca^{2+} (mM)	Mg^{2+} (mM)	DOC (mg l^{-1})	Fish weight (g)
CON (DOM-free)	7.77 ± 0.13	1.04 ± 0.00	0.31 ± 0.01	2.24 ± 0.17*	0.34 ± 0.01
BL	7.82 ± 0.10	1.19 ± 0.04	0.47 ± 0.02	7.50 ± 0.33	0.34 ± 0.02
LM	7.77 ± 0.13	1.02 ± 0.01	0.29 ± 0.00	6.21 ± 0.12	0.36 ± 0.02
AHA	7.71 ± 0.11	1.04 ± 0.01	0.29 ± 0.00	5.86 ± 0.06	0.32 ± 0.01

Data of n = 12 for pH, n = 18 for Ca^{2+} and Mg^{2+} concentrations, n = 6 for DOC level and n = 35–36 for fish weights.

* Higher DOC concentrations (relative to Na^+-free water in Table 6.1) for CON because of fish mucus produced during exposure.

Table 6.3 Summary of water chemistry of the exposure water used for examining the influence of dissolved organic matter (DOM) on Na^+ unidirectional fluxes, [^{3}H]PEG uptake, and nitrogen excretion by zebrafish (*D. rerio*) at circumneutral and low pH. Fish weights from all experiments and whole body Na^+ concentrations of zebrafish from the unidirectional efflux trials are listed in the two righthand column. Data (mean ± standard errors).

Treatment	pH	Na^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)	DOC (mg L^{-1})	Fish weight (g)	Whole body Na^+ content (mmol kg^{-1})
CON	7.85 ± 0.02	0.97 ± 0.02	1.50 ± 0.05	0.37 ± 0.00	3.37 ± 0.07	0.35 ± 0.05	30.78 ± 1.59
	5.00 ± 0.02					0.32 ± 0.04	34.82 ± 1.30
LO	7.76 ± 0.02	0.95 ± 0.02	1.55 ± 0.02	0.65 ± 0.01	8.92 ± 0.11	0.40 ± 0.06	30.34 ± 0.62
	5.02 ± 0.02					0.35 ± 0.04	32.32 ± 1.69
BL	7.86 ± 0.02	0.98 ± 0.02	1.50 ± 0.05	0.51 ± 0.01	8.19 ± 0.18	0.35 ± 0.04	29.45 ± 0.96
	5.02 ± 0.02					0.35 ± 0.03	31.38 ± 0.79
LM	7.84 ± 0.02	0.91 ± 0.07	1.46 ± 0.05	0.36 ± 0.01	6.95 ± 0.07	0.35 ± 0.05	30.50 ± 1.81
	5.03 ± 0.03					0.34 ± 0.04	31.31 ± 1.24
AHA	7.87 ± 0.02	0.95 ± 0.02	1.47 ± 0.06	0.36 ± 0.01	5.93 ± 0.09	0.35 ± 0.05	30.07 ± 0.65
	5.03 ± 0.02					0.36 ± 0.05	30.68 ± 1.43

Data of n = 71–148 readings for pH, n = 18 for ions concentrations, n = 28–32 determinations for DOC concentrations, n = 24–31 for fish weights and n = 6–9 for whole body Na^+ content.

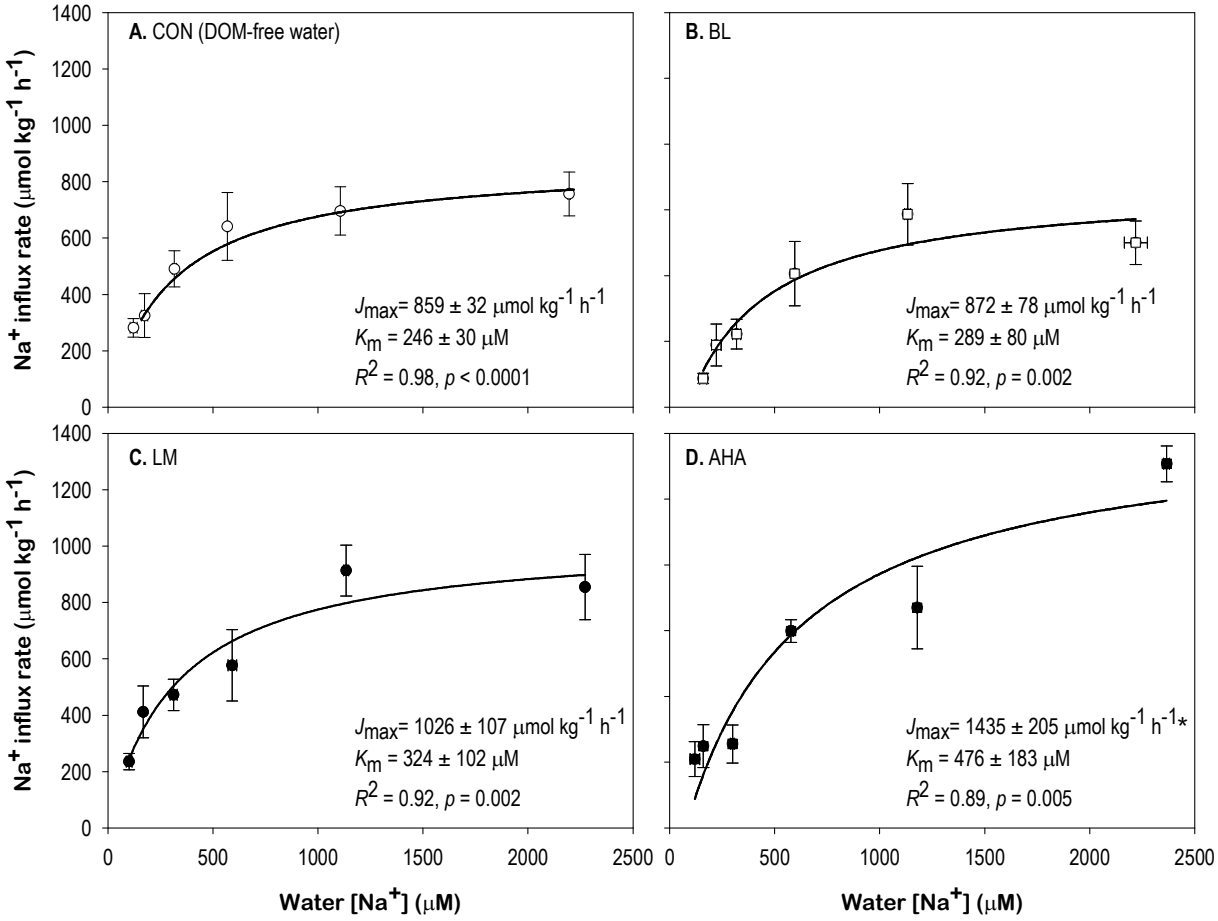

Figure 6.1 The effect of 3 DOM sources added at 6 mg l^{-1} DOC on the Michaelis-Menten transport kinetic parameters (maximal Na^+ transport rate or J_{max} in μmol kg^{-1} h^{-1} and uptake affinity or K_m in μM) of Na^+ influx in zebrafish (*D. rerio*). Each point represents the mean ± standard errors of n = 5–9. Kinetic parameters were calculated directly from plots and presented as mean ± standard errors. While J_{max} in the presence of AHA was significantly higher than those of control (DOM-free water) and BL, similar uptake affinities (K_m) were found in all treatments (see text).

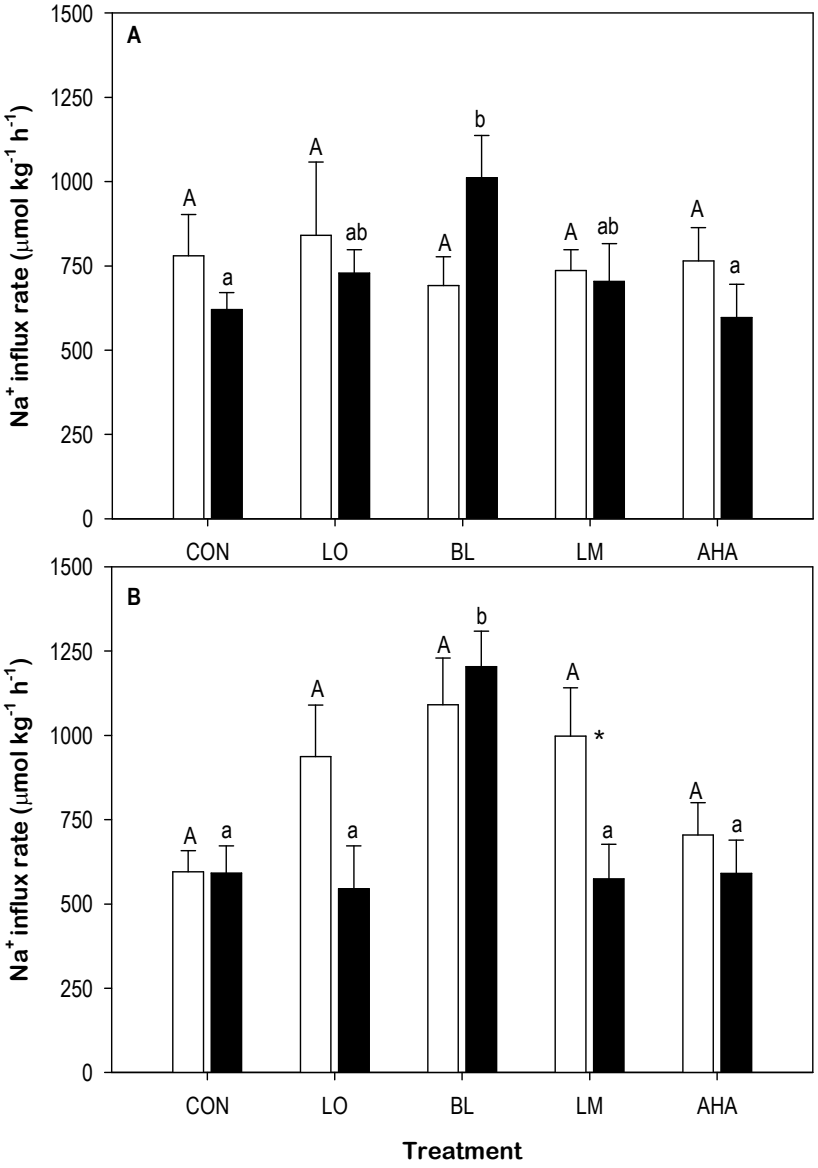

Figure 6.2 The unidirectional Na^+ influx rate (μmol kg^{-1} h^{-1}) of zebrafish in the absence (CON, no added DOM) or presence of DOMs added at 6 mg C L^{-1} at circumneutral pH ≥ 7 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors (n = 5 – 9) for Na^+ influx rates over (**A**) 3-h and (**B**) 6-h exposure periods. In the presence of BL at acidic pH, fish exhibited higher Na^+ uptake rate over both exposure periods. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

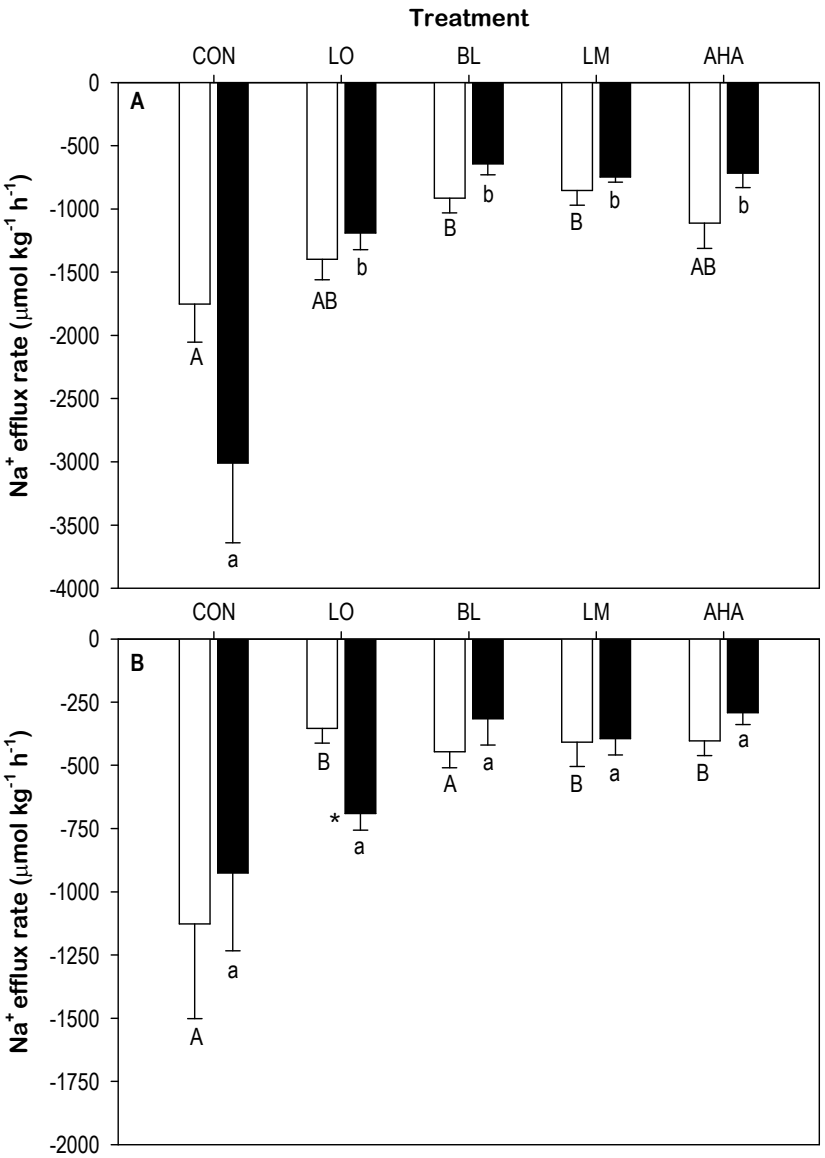

Figure 6.3 Effect of DOM on the unidirectional Na^+ efflux rate (μmol kg^{-1} h^{-1}) of zebrafish at circumneutral pH ≥ 7 (white bars) and low pH ~5 (black bars) over (**A**) 0–3 h and (**B**) 3–6 h exposure periods. Efflux rates demonstrated declines over time in all treatments. Plotted values represent the mean ± standard errors of n = 5 – 6. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

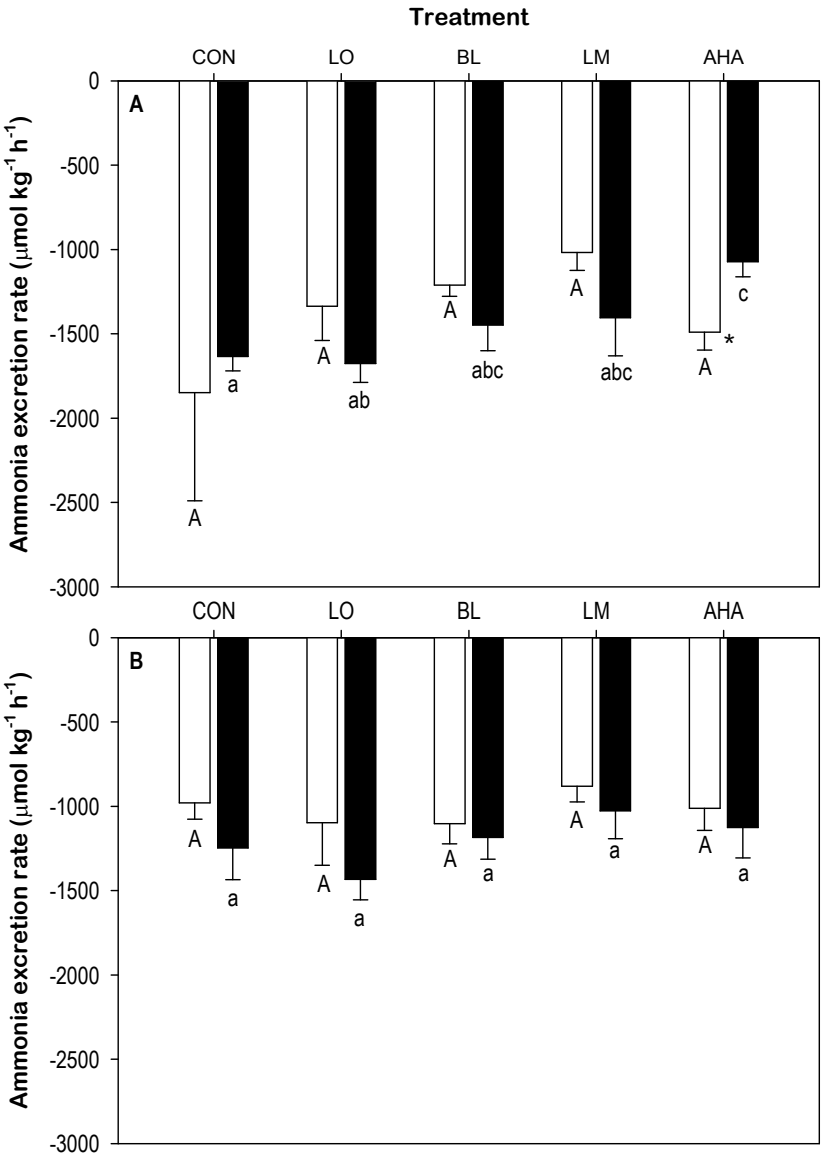

Figure 6.4 Total ammonia excretion rate of zebrafish in the absence (CON, no added DOM) or presence of DOMs added at 6 mg L^{-1} DOC at circumneutral pH ≥ 7 (white bars) and low pH ~ 5 (black bars) over (**A**) 0–3 h and (**B**) 3–6 h exposure periods. Plotted values represent the mean ± standard errors of n = 6. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different.

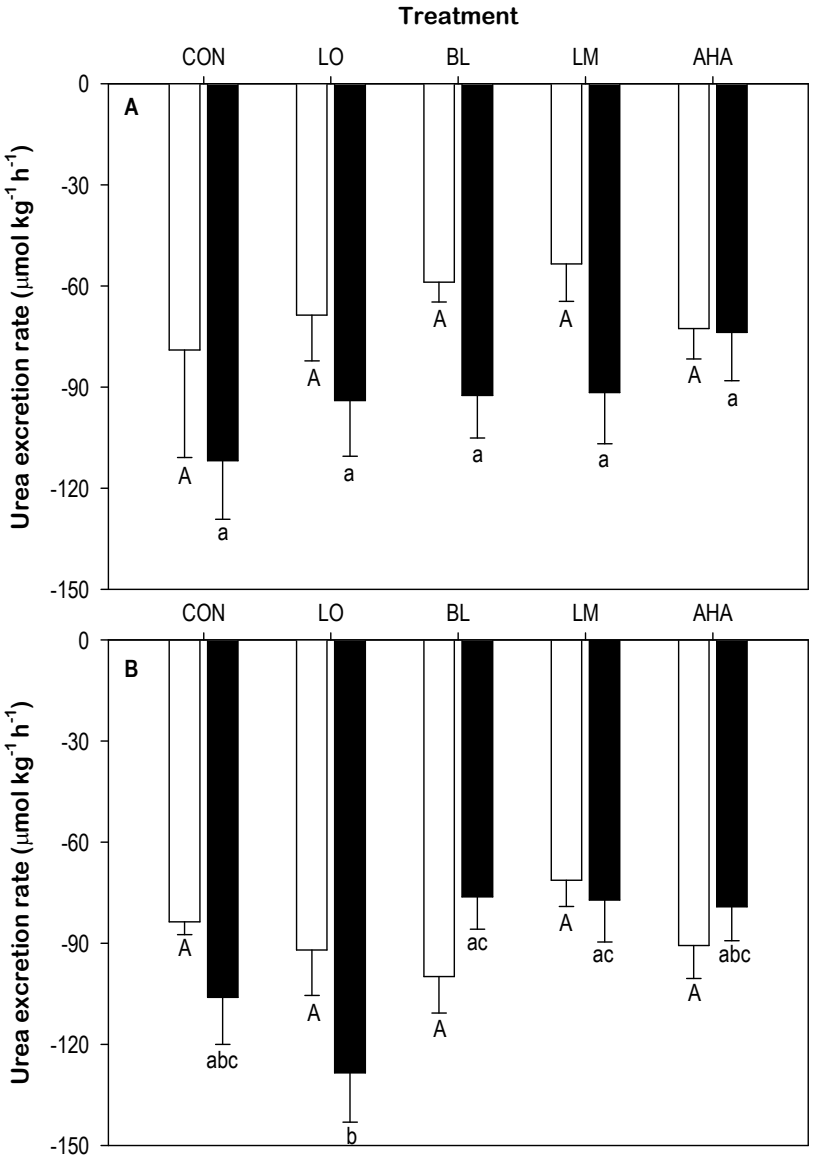

Figure 6.5 Urea excretion rate of zebrafish in the absence (CON, no added DOM) or presence of DOMs added at 6 mg L^{-1} DOC at circumneutral pH ≥ 7 (white bars) and low pH ~ 5 (black bars) over (**A**) 0–3 h and (**B**) 3–6 h exposure periods. Plotted values represent the mean ± standard errors of n = 6. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

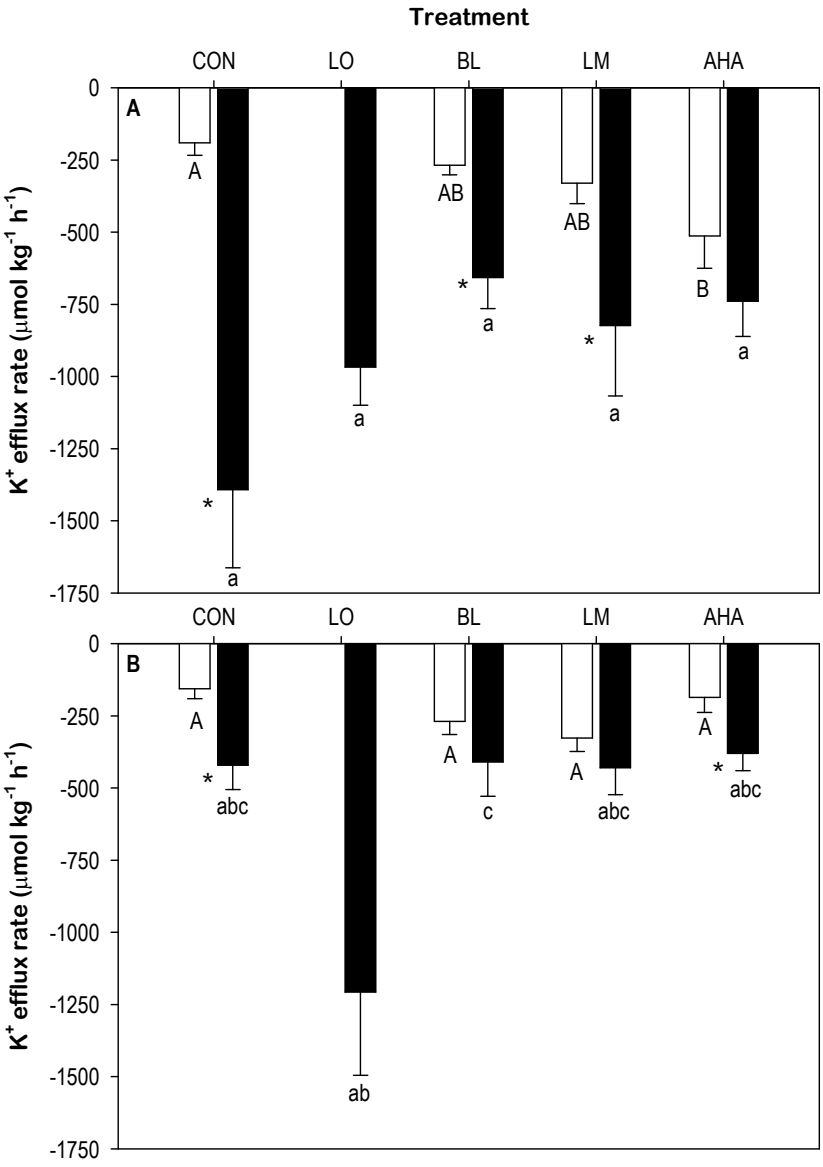

Figure 6.6 The net K^+ loss rates (μmol g^{-1} h^{-1}) of zebrafish at circumneutral pH ≥ 7 (white bars) and low pH ~5 (black bars) over (**A**) 0–3 h and (**B**) 3–6 h exposure periods. The K^+ efflux was measured as a marker for transcellular permeability of freshwater gills (see text). Plotted values represent the mean ± standard errors of n = 5–6. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment. Note that K^+ fluxes could not be quantified in the LO tests at circumneutral pH because KOH was used for pH adjustment in these trials.

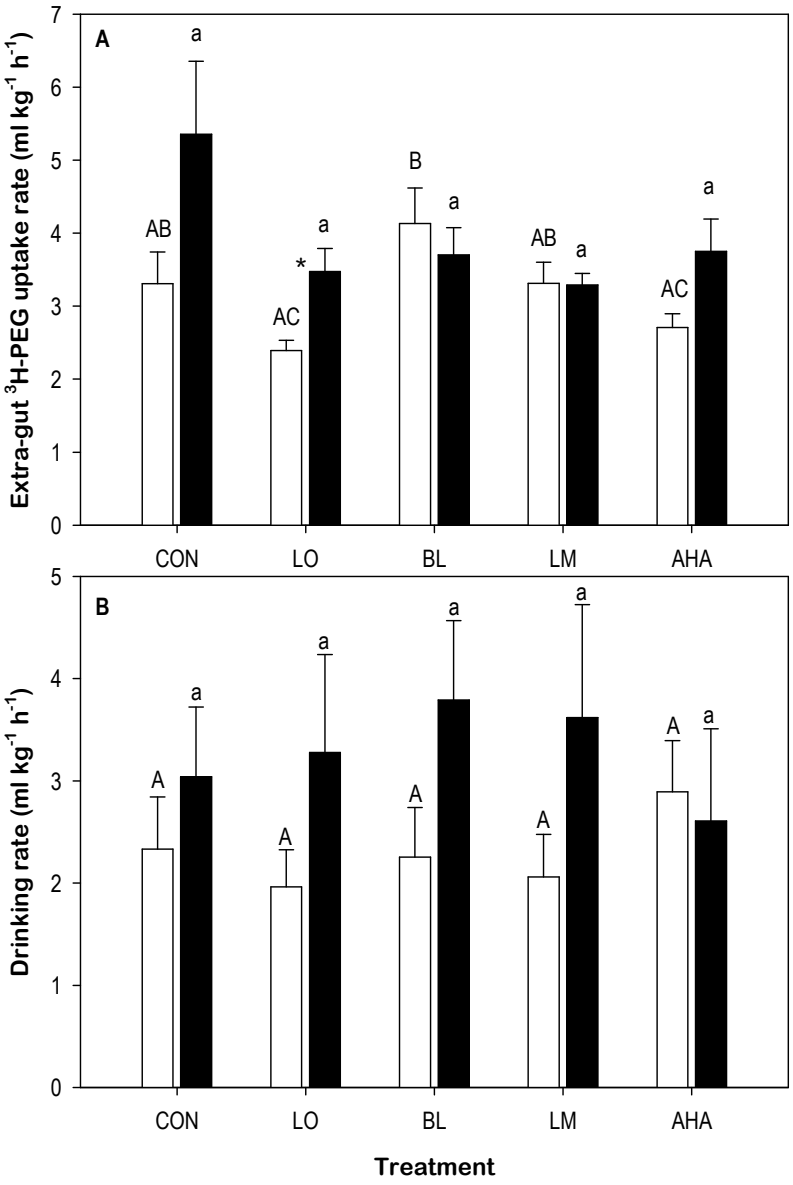

Figure 6.7 The influence of DOMs on (**A**) the extra-gut ^{3}H-PEG uptake rates (as a paracellular permeability marker) and (**B**) the drinking rates of zebrafish at circumneutral pH ≥ 7 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors of n = 5–6. Within a pH, bars sharing the same letter (upper case for pH ≥ 7, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

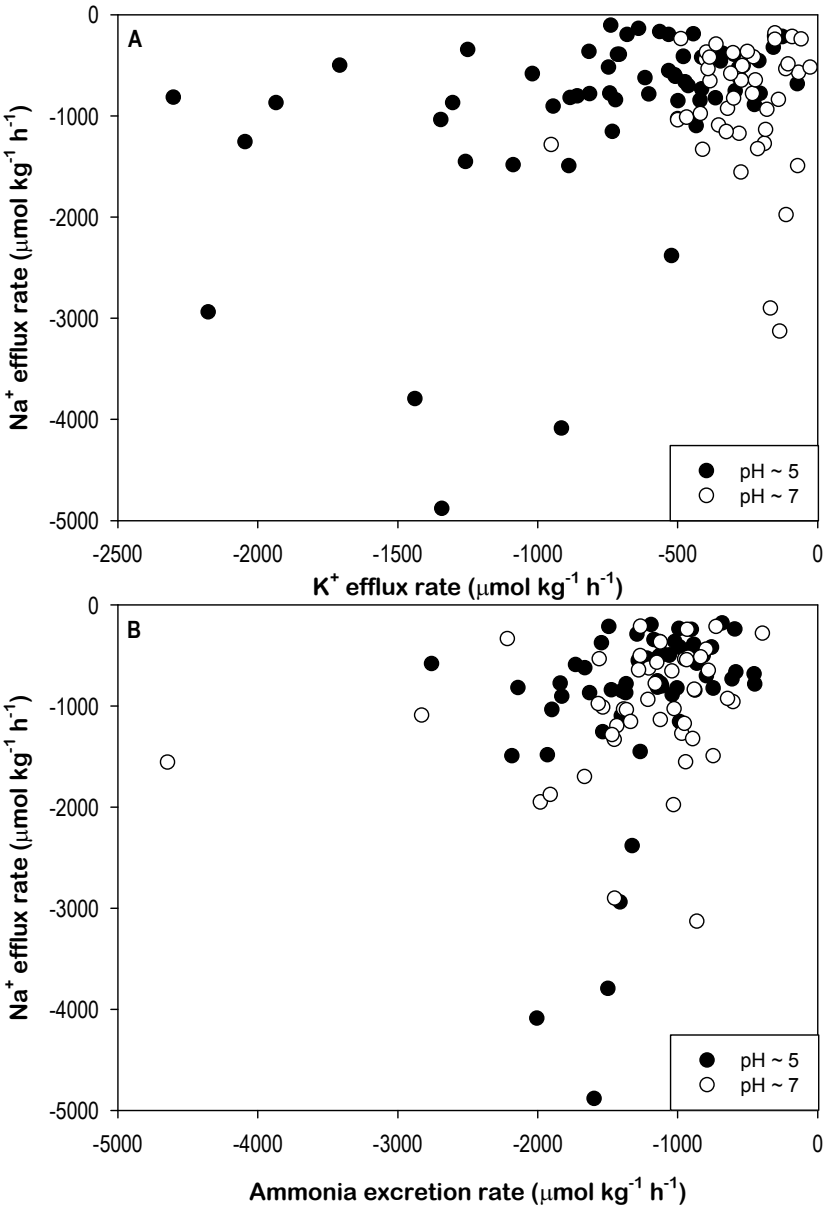

Figure 6.8 The relationships between unidirectional Na^+ efflux rate and (**A**) the net K^+ efflux rate and (**B**) total ammonia excretion rate of zebrafish in the absence (No added DOM) or presence of DOMs added at 6 mg L^{-1} DOC over 3 and 6 h at circumneutral pH $\geq$ 7 and low pH ~5 conditions.

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CHAPTER 7

GENERAL CONCLUSIONS AND DISCUSSION

7.1 Overall conclusions of this research

Dissolved organic matter (DOM) is a heterogeneous mixture of organic acids and macromolecules found in all natural waters. This thesis has shown that it exhibits indirect and direct influences on aquatic organisms including source-dependent protection against metal toxicity and organism-dependent interactions with basic physiological processes of ionoregulation and nitrogenous waste excretion. The following Discussion addresses these findings with respect to the original objectives (*in italics*) and hypotheses (***in bold italics***).

The first objective was *to investigate whether qualitatively different aquatic DOM sources vary greatly in their ameliorative ability against metal toxicity, as indirect effects, according to their physicochemical characteristics (quality measures)*. Indeed, the literature survey of Chapter 2 (Al-Reasi *et al.*, 2011) revealed significant contributions of the molecular and structural nature (quality) of DOM molecules towards their protective effects against metals, comparable in influence to the quantity of DOM (estimated as dissolved organic carbon, DOC, concentration). As quality measures, the specific absorbance coefficient (SAC) and the fluorescence index (FI) as well as the concentrations of fluorescent fractions (humic-like and fulvic-like components) obtained by parallel factor analysis (PARAFAC), explained considerable variability in the protective effects against toxicity of copper (Cu) and lead (Pb), in particular (Chapter 2, Al-Reasi *et al.*, 2011). As a follow-up study, Chapter 3 (Al-Reasi *et al.*, 2012) evaluated the different quality measures of various natural DOM sources and humic substances on the protection of *Daphnia magna* neonates against lethal Cu toxicity (LC_{50}). The

experimental data of Chapter 3 demonstrated that the effect of DOM sources on Cu toxicity could be explained solely in terms of their ability to complex Cu and decrease the concentrations of free Cu ions. Furthermore, the data supported the conclusions of the literature review of Chapter 2, and suggested that in addition to their aromatic composition (SAC_{340}), allochthonous or terrigenous DOM sources, because of larger molecules with higher percentages of humic-like fluorescent components and lower lipophilic nature, may be more protective than autochthonous sources.

The second objective of this work was *to test the applicability of using the physicochemical characteristic(s), as a DOM quality input parameter, to improve metal toxicity prediction, by a widely used metal toxicity prediction tool, the Biotic Ligand Model (BLM).* Chapter 3 (Al-Reasi *et al.*, 2012) developed a method for incorporation of SAC_{340} as a DOM quality measure into the BLM, through manipulating the default 10% humic acid (HA) assumption for DOM input in the model. The HA% for DOM sources was estimated based on the easily measured aromaticity index (SAC_{340}), taking into account the strong relationship between SAC_{340} and the relative humic-like fluorescence component from PARAFAC analysis. BLM optimization using the adjusted HA% resulted in a strong relationship between the predicted and measured LC_{50}, providing more precision in the metal toxicity prediction of the currently used BLM.

The third objective of the present research project was *to find simple-to-measure quality measures to account for source-dependent phenomena observed with respect to the direct and indirect effects of different DOMs on aquatic organisms.* Chapter 3 (Al-Reasi *et al.*, 2012) tested the hypothesis that ***since the protective effects of DOM are***

source-dependent, lethal Cu toxicity (LC_{50}) in various DOM sources will vary with their quality measures. The Cu toxicity results demonstrated that the magnitude of LC_{50} was substantially related to the simple absorbance ratios (e.g. $Abs_{254/365}$ as an index of molecular weight, Abs-octanol $_{254}$/Abs-water $_{254}$ as an index of lipophilicity and SAC_{340} as an index of aromaticity) and the fluorescence property (e.g. the relative concentration of humic-like fluorescent component by PARAFAC) of the examined DOM sources (Chapter 3, Al-Reasi *et al*., 2012), reinforcing the hypothesis. However, the role of DOM quality in influencing sodium transport and ammonia excretion by *Daphnia magna* and zebrafish was inconsistent (see below). Chapter 4 (Al-Reasi *et al.* submitted a) aimed *to develop a summary parameter linking the chemical reactivity (i.e. functional groups from potentiometric titration) of DOM sources with their spectroscopic characteristics*. A summary parameter, referred to as the titration index (TI) was introduced which summarized the chemical reactivity of various DOMs based on acid-base titration. The TI is defined as the sum of intermediate proton binding capacity divided by the average capacity for bindentate complex formation (i.e. average binding capacities of acid and base). The basis for this calculation is testing to see how closely the actual DOM titrations resemble titrations of a theoretical tridentate ligand. Given the logic assumptions of its derivation, high TI can be interpreted as closer spacing of functional groups for stronger metal binding (Chapter 4, Al-Reasi *et al.*, submitted a). TI was positively related to the SAC_{340}, emphasizing why the optically-dark DOM sources were more protective against Cu toxicity than the optically-light ones (Al-Reasi *et al.*, 2012).

Chapter 5 (Al-Reasi *et al.*, submitted b) and Chapter 6 tested the hypotheses that

DOMs would impact the processes of Na^+ regulation and nitrogenous waste excretion, that the effects would vary depending on the type of DOM (autochthonous versus allochthonous, natural versus commercially prepared sources), and that responses would be more pronounced at lower environmental pH. Na^+ metabolism and nitrogenous waste excretion of the water flea (*D. magna*) and zebrafish (*Danio rerio*) were investigated in the presence of three previously characterized, chemically-distinct natural DOM sources, as well as a commercial humic acid (AHA), at circumneutral and acidic pH (≥ 7 and ~ 5, respectively). Regardless of the pH conditions, neither Na^+ uptake nor passive loss rates of *D. magna* were affected by the presence of the various DOMs (Chapter 5, Al-Reasi *et al.*, submitted b). For zebrafish, a natural DOM source characterized by its distinctive autochthonous-allochthonous nature promoted Na^+ uptake rate at low pH, likely due to its higher acidic binding and buffer capacities (Chapters 4 and 5, Al-Reasi *et al.*, submitted a and b). At both pH's, Na^+ uptake in the presence of other DOMs was not significantly influenced. Only commercial AHA stimulated unidirectional Na^+ uptake by increasing the maximal Na^+ uptake rate (J_{max}). For ammonia and urea excretion of *D. magna*, excretion rates of both solutes were generally increased at low pH. In contrast, in zebrafish, the unidirectional Na^+ efflux rates were consistently reduced by all DOMs, indicating ameliorative action against the passive Na^+ diffusive loss (Chapter 6). The Na^+ efflux reductions could not be exclusively explained by indicators of either transcellular permeability (net fluxes of ammonia and potassium, K^+) or paracellular permeability (Tritium-labelled polyethylene glycol, [^{3}H] PEG-4000). Ammonia excretion rates of *D. magna* were reduced at circumneutral pH by the most

highly coloured, allochthonous DOM, and at low pH by all three natural DOMs, but not by AHA. The reduction was attributed to higher buffer capacities of natural DOM sources as well as their ability to interact with biological membranes (Chapter 5, Al-Reasi *et al.*, submitted b). Urea excretion rates at both pH conditions were not influenced by the presence of the various DOMs. In zebrafish, neither ammonia nor urea excretion rates, (nor drinking rates) were substantially influenced by the presence of DOMs at both pH's, in contrast to effects on Na^+ homeostasis (Chapter 6).

Re-visiting the main objectives for Chapters 5 (Al-Reasi *et al.*, submitted b) and Chapter 6, the results revealed both similarities and difference for the responses of the two test organisms (*D. magna* and *D. rerio*) in the presence of various DOMs. For example, one objective was *to elucidate the influences of natural DOM sources on the basic physiology (Na^+ regulation and ammonia excretion) of freshwater organisms*. While Na^+ regulation of *D. magna* remained unaffected by the presence of DOM (Chapter 5, Al-Reasi *et al.*, submitted b), the passive Na^+ diffusive efflux of zebrafish was reduced (Chapter 6). Another objective was *to ascertain the pH-dependent influences of natural DOM sources facilitating direct interactions with freshwater organisms*. Most effects of DOM observed occurred independent of pH for both species. However, the attenuation of ammonia excretion of *D. magna* by all the natural DOM sources and the stimulation of Na^+ uptake of zebrafish by one natural source were pronounced at low pH (Chapters 5 and 6). The final objective was *to compare the influences of the natural DOM sources on the basic physiology to that of commercially-prepared humic substances*. Some notable differences were seen. For example, the commercial AHA did not reduce

the ammonia excretion of *D. magna* compared to the natural DOMs at low pH, but it caused disturbance of Na^+ regulation at low DOC concentration (Chapter 5, Al-Reasi *et al.*, submitted b). For zebrafish, the significant increase of J_{max} in presence of AHA was not seen when the natural DOMs were present in the exposure water.

7.2 Effects of DOM on aquatic organisms

7.2.1 Role of quality measures

Physicochemical characteristics, as quality measures, have been described largely by absorbance and fluorescence spectroscopy (e.g. Chapters 2, 3, and 4; McKnight *et al.*, 2001; Mueller *et al*., 2012) and to some extent by acid-base titrations (e.g. Chapter 4; Scott and Kramer, 1999; Brooks *et al*., 2007). Strong evidence is now available to support the importance of the molecular composition for alleviating metal toxicity to diverse aquatic animals (Chapters 2 and 3; De Schamphelaere *et al.*, 2004; Ryan *et al.*, 2004; Schwartz *et al.*, 2004). The aromatic composition estimated by specific absorbance coefficient, SAC (Chapters 2 and 3; De Schamphelaere *et al.*, 2004; Ryan *et al.*, 2004; Schwartz *et al.*, 2004) and HA% by acid fractionation (Ryan *et al.*, 2004) or PARAFAC-based humic-like fluorescent component (Chapters 2 and 3) appeared to be the fundamental property in distinguishing the effectiveness of the protection against toxicity of metals, especially Cu.

Chapter 3 confirmed the earlier observations of darkly-coloured, more aromatic allochthonous material being more protective than lightly-coloured, less aromatic autochthonous DOM sources (Schamphelaere *et al.*, 2004; Ryan *et al.*, 2004; Schwartz *et*

al., 2004) and suggested that ameliorative effect can be related to larger molecules with higher percentages of humic-like fluorescent components and a lower lipophilic nature. The protection may now be rationalized based on the higher titration indices (TI) of allochthonous or terrigenous DOMs (Chapter 4). TI is a summary of proton reactivity profiles of DOM and a high value of TI corresponds to a high potential for metal binding. The conclusions of Chapters 2, 3 and 4 are in accord with those of several previous studies (De Schamphelaere *et al.*, 2004; Ryan *et al.*, 2004; Schwartz *et al.*, 2004) as to the importance of inclusion of a DOM quality measure in the BLM. Notably, Chapter 3 has demonstrated how an easy-to-measure indicator of aromatic composition may be incorporated into the BLM so as to improve toxicity predictions for Cu.

Earlier investigations indicated that DOM may exert source-dependent direct effects on freshwater organisms (e.g. Galvez *et al.*, 2009; Matsuo *et al.,* 2006; Menzel *et al.*, 2005). For example, the darker, more allocththonous DOMs induced a more negative transepithelial potential in trout gills (Galvez *et al.*, 2009) and were more efficient in reducing fungal growth (Meinelt *et al.*, 2007). However, in the present study (Chapters 5 and 6), as well as in several previous investigations (Hoss *et al.*, 2001; Meems *et al.*, 2004), other responses did not exhibit a clear trend with the aromatic content of DOMs. For example, the most highly coloured, allochthonous DOM did reduce the ammonia excretion of *D. magna* at both circumneutral and low pH conditions (Chapter 5). However, the same DOM source did not show any influence on ammonia excretion by zebrafish (Chapter 6), suggesting species-dependent responses. In general, there was not a clear influence of DOM quality on either Na^+ regulation of *D. magna* (Chapter 5) or

ammonia excretion of zebrafish (Chapter 6). It can be argued that the influence of the DOM quality on direct effects may depend on both the response being measured as well as on the organism.

Addressing the quality issue of DOM is an issue of environmental significance not just for the direct and indirect effects on aquatic organisms but also for other processes in natural waters. For example, a recent paper (Barnes *et at.*, 2012) has reported that the chemical composition of DOM, as revealed by simple spectroscopic methods similar to those used in Chapter 3, can explain variability in denitrification under elevated and ambient nitrate conditions, suggesting that simple DOM optical properties could help to improve predictions of nitrate removal in the environment. Another recent study (Herzsprung *et al.*, 2012) has shown that the simple ultraviolet absorption at 254 nm and humic-like fluorescence can be employed as DOM quality measures, to allocate the seasonal and spatial variations in the formation of disinfection byproducts associated with the chlorination of raw water.

7.2.2 Direct effects of DOM on aquatic organisms: contradiction versus inertness

For long time, DOM has been considered inert, yet its heterogeneous nature has been blamed for contradictory positive and negative biological influences in aquatic environments (Petersen, 1991). DOM exerted negligible effects on Na^+ regulation in *D. magna* (Chapter 5), but generally reduced Na^+ passive loss in zebrafish (Chapter 6). Similarly, while DOMs did not impact urea transport in either *D. magna* or zebrafish, or ammonia excretion in zebrafish (Chapters 5 and 6), the natural DOM sources reduced

ammonia excretion in *D. magna* especially at low pH (Chapter 5). It would appear that the biological behavior of DOMs in freshwater oscillates between exerting effects which are highly variable or behaving as inert substances causing no effect. For instance, Hoss *et al.* (2001) used different isolates and reported stimulatory, inhibitory and no effect on the reproduction of the nematode (*Caenorhabditis elegans*). Glover *et al.* (2005) found that the commercial humic substances stimulated Na^+ uptake in *D. magna*, but not the natural sources (Glover and Wood, 2005). It may be speculated that the variable effects observed in these studies vary with DOM sources. However, in the present study, an examination which included well-characterized DOM sources ranging between highly autochthonous to highly allochthonous in nature did not support the source-dependent effect on Na^+ regulation and ammonia excretion (Chapters 5 and 6). The conclusion that can be drawn here is that the direct effects of DOM on freshwater organisms at present remain highly unpredictable, more so than the indirect effects such as metal toxicity reduction. Clearly, there is a need for more work in this area.

7.2.3 Direct effects of DOM on aquatic organisms: are they pH-dependent?

Under typical environmental conditions (pH ≥ 7), DOM molecules and biological surfaces are negatively charged and therefore their interaction may be unlikely. This may explain the lack of direct effects of DOM at circumneutral pH reported in some previous studies (e.g. Knauer and Buffle, 2001; Petersen and Persson, 1987; Vigneault *et al.*, 2000). It is hypothesized that DOM acts as surfactant on the biological surfaces at lower pH's because the greater availability of H^+ ions neutralizes the negative charges on the

biological surfaces to facilitate the binding of DOM molecules (Campbell *et al.*, 1997; Vigneault *et al.* 2000). This in turn may account for the more pronounced effects seen in acidic waters in some investigations (e.g. Chapter 5; Petersen and Persson, 1987; Campbell *et al.*, 1997; Parent *et al.*, 1996; Vigneault *et al.*, 2000; Wood *et al*., 2003). However, there are also studies showing lack of pH-dependence. For example, at circumneutral pH, the presence of added DOM induced a more negative transepithelial potential in trout gills (Galvez *et al.*, 2009) and reduced the passive Na^+ efflux of zebrafish (Chapter 6). Moreover, Chapters 5 and 6 documented no effect on Na^+ regulation of *D. magna* and ammonia and urea excretion of zebrafish at the acidic pH. These observations suggest that the interaction between water pH and DOM is intricate and may be related to the ambient DOM of water to which the organisms are acclimated, as well as to the particular physiolohgical processes under study.

7.2.4 Anomalous effects of commercial humic substances: AHA as an example

Commercially-available humic substances such as Aldrich humic acid (AHA) has been extensively utilized as an experimental substitute for aquatic DOM sources (e.g. Glover et al., 2005; Glover and Wood, 2005; Matsuo *et al*., 2004; Matsuo *et al*., 2006; McGeer *et al*., 2002; Petersen and Persson, 1987). Nonetheless, AHA exhibited marked differences in term of effects on organisms compared to the natural DOMs. While *D. magna* experienced a significant reduction in their Na^+ content when exposed to AHA (Chapter 4), Glover *et al*. (2005) reported increase of Na^+ content when the organism was exposed to increasing concentrations of AHA. The commercial AHA increased Na^+

influx at low pH for the same species (Chapter 4; Glover and Wood, 2005), but this effect was not seen in zebrafish (Chapter 6). The stimulation of the Na^+ kinetic parameter (the maximal Na^+ uptake rate, J_{max}) of *D. magna* (Glover and Wood, 2005), zebrafish (Chapter 6) and rainbow trout (Matsuo *et al*., 2004) appears to be limited to the "unnatural" AHA. Wood *et al*. (2003) documented severe exacerbation of the passive Na^+ loss of freshwater stingrays (*Potamotrygon* spp.) in the presence of AHA, however no influence was seen on the passive Na^+ efflux in daphnids (Chapter 5; Glover *et al*., 2005). While the ammonia excretion rates of *D. magna* were not attenuated by AHA (Chapter 5), the presence of AHA in the exposure water was found to facilitate ammonia excretion of freshwater stingrays (Wood *et al.*, 2003). These observations highlight the anomalous actions of AHA which are likely explained by its molecular nature. AHA has a lower fluorescence index (Chapters 3) which does not fit the classification of origin developed by McKnight *et al*. (2001) for the natural DOMs. Absorbance and fluorescence spectroscopy has demonstrated that AHA deviates substantially from the natural DOMs in term of aromatic composition and pure humic nature compared to natural DOMs which have mixed humic and fulvic molecular compositions (Chapter 3). In addition, the commercial AHA and similar humic acids are usually prepared by freeze-drying, while the natural sources used in this study were obtained by reverse-osmosis, a method proven to produce representative DOMs of the natural waters (De Schamphelaere *et al.*, 2005). These findings support the distinct aliphatic and aromatic molecular composition observed earlier (Malcolm and MacCarthy, 1986) and therefore caution should be taken with the use of AHA as surrogate for aquatic DOM.

7.2.5 Future directions

This thesis has contributed to a better understanding of the environmental significance of freshwater DOM. The present data highlight the role of DOM quality in influencing metal-detoxifying ability and the basic physiology of freshwater organisms. Concerning protection against metal toxicity, there is need for data to be generated for metals other than Cu. There is also a need to test whether or not the method developed in the present study for the incorporation of DOM quality into the BLM works well for other metals, as well as for chronic as opposed to acute BLMs. For direct biological effects, the conflicting physiological responses between different organisms should be resolved and the mechanism(s) of DOM interaction with the epithelial transporting cells, where influences have been observed, should be investigated at cellular and molecular levels. It would be very useful to understand how DOMs react with moieties (e.g. channels, transporters, tight junctions) on epithelial cell membranes, and whether DOM molecules are incorporated into the membranes or actually penetrate through them. It would also be helpful to investigate whether the presence of DOMs alters boundary layer conditions (e.g. pH, ionic and nitrogenous waste concentrations) at the respiratory surfaces. In addition, while most of the investigations have been conducted over relatively short time periods, there is need for chronic and long term exposures to examine the direct and indirect effects of DOM.

7.3 References

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