2009

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Using Chemical, Optical and Biological Methods to Characterize Cu Complexation by Different Natural Organic Matter Sources Collected from Canadian Shield Waters

by

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THESIS
Submitted to the Department of Biology
in partial fulfillment of the requirements for
Master of Science
in
Integrative Biology
Wilfrid Laurier University 2009

Abstract

The effects of natural organic matter (NOM) source on Cu complexation were characterized chemically, optically and biologically in soft waters. NOM from different Canadian Shield soft water lakes were concentrated using reverse osmosis and for comparison NOM was also sampled from a hard water source. NOM complexation capacity was assessed directly in Cu spiked solutions (with and without additions of NOM) by measuring free Cu activity using an ion selective electrode. Additional chemical and optical characterizations of NOM included specific absorbance coefficient (SAC; at 340 nm), excitation-emission matrix spectroscopy, protein content, and molecular weight fractionation (fraction of DOC<1 kDa). The biological characterization of the ability of various NOMs to complex Cu was measured in rainbow trout (*Oncorhynchus mykiss*) using short term (3 h) gill accumulation, 96h-LC50, and the inhibition of gill Na^+/K^+ ATPase. Results indicate that a more allochthonous NOM having a higher HA content and a higher UV/Vis absorbance complex better with Cu and usually reduce gill Cu accumulation more effectively than autochthonous NOM sources. Those NOM sources which were used for toxicity experiments did not reveal many differences in their chemical or optical properties, yet there was still a 3.5 fold difference in NOM ability to reduce acute Cu toxicity. The geochemical prediction model Windermere Humic Aqueous Model (WHAM), was modified to account for SAC_{340} which increased its predictive capabilities by 18%. This suggests that SAC_{340} could be an important measure when considering NOM quality for better predicting Cu speciation and toxicity in aquatic environments.

Acknowledgements

I would like thank Jim McGeer for his excellent guidance and support throughout my thesis. I would like to thank Bernard Vigneault from the Ecotoxicology Lab at NRCan for his guidance and access to their laboratory facilities. Others at NRCan including Susannah Krack, Yamini Gopalapillai, and Melissa Desforges all provide valuable laboratory assistance for which I am grateful. I would also like to thank Morgan King and Emily-Jane Costa for helping me concentrate NOM. Scott Smith also provided me with great assistance throughout my thesis. Lastly, I would like to thank all those in the McGeer Lab, Wilkie Lab and Smith Lab for their support throughout the completion of this degree.

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Glossary

BLM.....biotic ligand model

B_{max}.....maximum binding capacity

CI.....confidence interval

DOC.....dissolved organic carbon

EEMS.....excitation emission matrix spectroscopy

FA.....fulvic acid

FI.....fluorescence index

HA.....humic acid

IC.....inorganic carbon

ISE.....ion selective electrode

LA50.....median lethal accumulation

LC50.....median lethal concentration

Log K.....conditional stability constant

mV.....millivolt

mw.....molecular weight

NOM.....natural organic matter

PARAFAC.....parallel factor analysis

QF.....quality factor

SAC.....specific absorbance coefficient

SD.....standard deviation

SEM.....standard error of mean

TC.....total carbon

UV.....ultraviolet light

VIS.....visible light

WHAM.....Windermere humic aqueous model

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Introduction

1.1 General overview

Fish and aquatic invertebrates are sensitive to metal inputs to their environments. However, this sensitivity can vary from one water body to the next depending on physicochemical parameters such as pH, hardness, alkalinity, common ions (Na^+, Ca^{2+}, Mg^{2+}, K^+), and natural organic matter (NOM) (Schubauer-Berigan et al., 1993; Erickson et al., 1996; De Schamphelaere and Janssen, 2002). These physiochemical characteristics of water can directly alter metal speciation (i.e. the concentration of free metal ion) and thus metal availability to the aquatic organisms. Toxicity is related to the concentration of free metal ion which binds to biological surfaces (e.g. fish gill), the site of toxic action (Di Toro et al., 2001). The variable influence that water chemistry has on metal toxicity has been incorporated into a toxicity prediction model called the biotic ligand model (BLM). The BLM is based on geochemical equilibrium software and generates water chemistry specific toxicity predictions for metals (Cu, Cd, Pb, Ni, Zn; reviewed Niyogi and Wood, 2004). It predicts toxicity based on bioaccumulation at the site of toxicity and as such, accounts for speciation in the water column, the relative bioavailability of different species of a metal in solution and the mechanism of toxicity.

One of the important toxicity mitigating components in aquatic systems is NOM. NOM complexes with metals, thus reducing the concentration of the free ion forms of metals, usually considered the most bioavailable and toxic species. For example, the work of Richards et al. (1999) showed that complexation of Cu by NOM resulted in reduced Cu accumulation at the gill and prevented most metal-induced physiological changes in rainbow trout.

NOM composition can vary from source to source due to different origins of the organic matters (e.g. aquatically derived or terrestrially derived; McKnight et al., 2001) as well as differing degrees of degradation from either UV exposure or microbial activity (Mann and Wetzel, 1995). Differences in NOM composition may result in different metal complexation capacities thus differentially altering metal speciation and, by extension, toxicity to aquatic organisms. It has been demonstrated with rainbow trout that different sources of NOM differentially protected against the accumulation and toxicity of Hg (Richards et al., 2001), as well as Pb and Cu (Richards et al., 2001; Swartz et al, 2004; Ryan et al., 2004). In all of these studies toxicity and accumulation was correlated to the color of NOM in solution, as measured by absorbance at 340 nm. Although NOM is an important factor that needs to be considered, it is not well understood. The BLM only considers the quantity of the NOM (quantified by dissolved organic carbon; DOC) and other than an adjustment factor for the relative fraction of humic and fulvic acid there are no other toxicity mitigating characteristics incorporated within the model.

The BLM successfully predicts Cu, Ag, Zn, and Cd toxicity to daphnia, rainbow trout, and fathead minnow (Santore et al., 2001; Santore et al., 2002; Di Toro et al., 2001; Heijerick et al., 2002; McGeer et al., 2000). One concern regarding the BLM is that it has primarily been developed and tested in waters of medium to high hardness. The majority of lakes along the Canadian Shield are soft and the ability for the BLM to predict effects accurately in the waters is unknown. Therefore, some of the questions which need to be resolved as the BLM is further developed include, can the BLM be applied to soft waters, and is NOM complexation similar across sources or does it differ

significantly and therefore need to be incorporated within the BLM? These questions are relevant to the application of the BLM to environments that typically occur in the Canadian Shield and need to be resolved in order to validate its use for derivation of aquatic water quality guidelines and for use in risk assessment of metals in aquatic environments.

1.2 NOM composition and characterization

In the soft waters of the Canadian Shield NOM is usually present at concentrations ranging from 2-10 mg C/L (Davidson et al., 1997). NOM can be formed from many different precursors and this affects the structure and composition of NOM making it very complex and heterogeneous (McKnight and Aiken, 1998). Allochthonous types of NOMs represent sources of carbon from outside the aquatic system, derived mainly from terrestrial vegetation and these can differ between wetland and upland sources, depending on the type of vegetation (Schiff et al., 1990). Autochthonous NOM is derived from within the aquatic system and its composition is mostly attributed to algae and macrophytes (Aitkenhead-Peterson et al., 2003). Autochthonous NOM typically has more carbohydrates and nitrogen-containing groups and is lightly coloured, whereas allochthonous NOM is enriched in aromatic humic and fulvic acids, highly coloured and easily absorbs ultraviolet light (Richards et al., 2001). Because lakes typically receive input from terrestrial sources, aquatic NOMs are usually composed of a mixture of allochthonous and autochthonous carbon sources, potentially giving each lake a NOM with unique characteristics. NOM is also subject to degradation by UV radiation and microbial action as well as acidification (Mann and Wetzel, 1995).

The main components of NOM are humic substances making up approximately 50-90% (Thurman, 1985) the remainder being carbohydrates, proteins and lipids. Humic substances are the generic term given to naturally occurring humic acid (HA) and fulvic acid (FA). HA is the portion of NOM that is insoluble below a pH of 2, is darker in colour and has a higher molecular weight (mw) (2-5 kDa, sometimes as large as 100 kDa; Thurman, 1985). HA contain a great proportion of aromatic groups such as methoxyls and phenolics (Aikenhead-Peterson et al., 2003). FA is soluble under all pH conditions, are lightly coloured and are lower in mw (0.5-2 kDa; Thurman, 1985). FA typically contain more carboxyl and hydroxyl groups, and are highly aliphatic (Aikenhead-Peterson et al., 2003). The ratio of HA:FA is usually 10 in low-coloured surface waters, 5 in highly coloured surface waters, and 0.3 in soil solutions (Aikenhead-Peterson et al., 2003).

There are a variety of ways to characterize differences among NOM. Absorbance at wavelengths in the UV and visible range is a common method for distinguishing differences among NOM sources. Absorbance has been measured at 340 nm (Richards et al., 2001; Schwartz et al., 2004) as well as 254 and 436 nm (Abbt-Braun and F.H. Frimmel, 1999). Absorption of light in the UV range is typically caused by aromatic components of humic substances (Abbt-Braun and F.H. Frimmel, 1999) and is a measure of colour (light vs. dark NOMs).

Fluorescence excitation-emission spectroscopy (EEMS) is another method to optically characterize NOM (Coble, 1996). The fluorescent index (FI), calculated as the ratio of emission intensity (450/500 nm) at an excitation wavelength of 370 nm, is one measure which has previously been used to distinguish between NOM sources. A lower

FI indicates an NOM source which is more allocthonous-like where a FI with is higher indicates an NOM source which is more autochthonous-like (McKnight et al., 2001).

Excitation emission data across a matrix of wavelengths is another fluorescence spectroscopy method. This data is transformed into contour plots, which are read like topographical maps, and act like NOM finger prints (Winter et al., 2007). Interpretation of the fluorescent scans can be facilitated by parallel factor analysis (PARAFAC; Stedmon and Markager, 2005). PARAFAC identifies common components within NOM samples that can then be quantified (Stedmon and Markager, 2005). Four common components include HA-like (excitation of 360-390nm; emission of 460-520nm), FA-like (excitation of 320-340nm; emission of 400-450nm), tryptophan-like (excitation 280 and 230nm; emission of 340-350nm) and tyrosine-like (excitation of 280 and 230nm; emission of 300nm) (Winter et al., and references therein). The amino acid tryptophan typically originates from a sewage source or bacteria (Baker, 2001), while tyrosine is indication of an algae source (Determann et al., 1998).

Numerous studies have shown that NOM provides protection against metal toxicity to aquatic animals in a concentration-dependant manner (De Schamphelaere and Janssen 2004; Kramer et al., 2004; Doig and Liber, 2006; Erickson et al., 1996). Given the variability in NOM composition it might be reasonable to assume that different sources (types) of NOM provide different degrees of protection against metal toxicity. However this has not been incorporated into toxicity prediction models like the BLM where NOM complexation considers all sources as similar. As well, geochemically based metal speciation models such as the Windermere Humic Aqueous Model (WHAM: Tipping, 1998) does not distinguish between sources of NOM. Richards et al. (2001)

demonstrated that three different sources of NOM provided varying degrees of protection against the toxicity and accumulation of Cu, Pb, and Hg on the gills of rainbow trout, indicating that metal complexation differed across sources. They optically characterized the NOM samples (absorbance at 350nm) and found that optically darker (more allochthonous) NOM was the most protective; where optically lighter (more autochthonous) NOM was least protective. This same trend with optical absorbance has also been observed with a much larger collection of NOM sources (19 NOM sources) in regards to reducing Cu and Pb accumulation and toxicity in rainbow trout (Schwartz et al., 2004). This was also demonstrated by measuring directly the free Cu^{2+} activity in the presence of different NOM sources (Luider et al., 2004). These studies demonstrated differences among NOM sources in protecting against metal toxicity and gill accumulation in rainbow trout, as well as differences in influencing measured free Cu^{2+} activity. It is therefore important to fully understand the properties of NOM that influence metal toxicity and if those protective effects vary significantly among different NOM sources, to incorporate these properties into the BLM framework.

1.3 The biotic ligand model

The BLM is a more accurate tool for estimating acute metal toxicity compared to conventional methods which consider hardness as the only water chemistry parameter to affect metal toxicity (US EPA, 1986; CCME, 1999). It is now well known that not only hardness, but also alkalinity, pH, as well as various inorganic (Cl^-, HCO_3^-, SO_4^{2-}) and organic ligands (NOM) also influence metal toxicity (Schubauer-Berigan et al., 1993; Erickson et al., 1996; De Schamphelaere and Janssen, 2002). The advantages of using

the BLM approach is that, once it is established, it is cost-effective, quick, and does not require animal testing.

The biotic ligand (BL) is defined as the receptor site on the organism where the metal binds to form a metal-BL complex that results in acute toxicity (Santore et al., 2002). In fish, it is suspected that the sodium and calcium ion channel proteins along the membrane of the gill are the BLs (Niyogi and Wood, 2004). Toxicity occurs because metal binding at the BL results in the disruption of ionoregulatory processes which, if they are severe, results in mortality (Di Toro et al., 2001). A key aspect of the BLM is that a threshold concentration of bound metal can be associated with a toxicity endpoint such as the LC50 (Di Toro et al., 2001).

Typically, it is the free metal ion that is the most bioavailable and the most toxic form (Pagenkopf, 1983). The concentration of free metal ions can be altered by complexation with inorganic ligands (e.g. hydroxides and carbonates) as well as organic ligands like NOM (Paquin et al., 2000). These metal-ligand complexes are less bioavailable than free metal ions and therefore are much less capable of producing toxicity (Paquin et al., 2000). Also, because the metal ions can compete with other cations for the binding sites, metal toxicity can be altered depending on the competing cation concentrations. Examples of competition at the gill surface include those metals that compete with Na^+ for sodium uptake channels (e.g. Cu^{2+} and Ag^+) as well those metals which compete with calcium for Ca^{2+} uptake channels (Cd^{+2}, Co^{+2}, Zn^{+2} and Pb^{+2}; Niyogi and Wood, 2004 and references therein). In addition, at low pH, metals and H^+ compete for proton binding sites on the biotic ligand. A change of pH also affects metal speciation, particularly inorganic complexes, and therefore the relative concentration of

free metal ions is dependent on pH (Campbell and Stokes, 1985). Low pH can also displace metals from NOM to result in an increase in free metal ions (Cabaniss and Shuman, 1988). The complexation of metals with organic and inorganic ligands, together with the competition of free cations, forms the basis of the BLM.

The BLM approach to predicting metal toxicity is based on the assumption that short term accumulation is directly linked with acute toxicity. Accumulation of a metal is predicted mathematically by assigned values that are the binding affinity (Log K) and binding capacity (B_{max}) of the BL. The free ionic form of metal and other competing cations all have specific Log K values. The higher the Log K value is for a particular metal compared to that of other ions the greater is its binding affinity to the BL and consequently the more uptake and the higher its toxicity (Niyogi and Wood, 2004). The binding characteristics of the gill (BL) for a metal are typically measured over 3 hours (Playle et al., 1993a, b) although up to 24 hours has been used (MacRae et al., 1999). The BL also describes the number of binding sites for a metal (binding capacity; B_{max}) which is also used to link accumulation with toxicity. Log K and B_{max} are essential values because the amount of metal binding to the BL will be influenced by the number of binding sites and the affinity of those sites for the metal. Toxicity occurs when metal binding to the BL reaches a critical threshold concentration; this critical concentration, known as the LA (lethal accumulation), defined as the concentration of metal accumulated on the BL that is associated with a toxic endpoint (usually mortality at the 50% level (LC50)). The LA is expressed as the LA_x where x is the effect level associated with the endpoint (e.g. an LA_{50} associated with LC_{50}) and these associations can be determined experimentally with fish by linking short term accumulation with the 96h

LC_{50} (lethal concentration) assays with fish. Therefore, the BLM relates metal binding to the BL with acute toxicity (LC_{50}).

1.4 Copper in the environment

Copper is present in aquatic environments as a result of its natural geochemistry and human activities (Di Toro et al., 2000). Cu originates naturally in the environment from the weathering of copper-bearing minerals of the lithosphere, which over time is incorporated into the soil and eventually enters aquatic systems in particulate and dissolved forms (DiToro et al., 2000). Anthropogenic sources of Cu entering the environment result from metal mining, coal combustion and waste incineration (Georgopoulos et al., 2002). Cu is mainly used for wiring, electronics, plumbing, as a wood preservative, as an anti-fouling coating on boat hulls, and as a pesticide for the control of algae, bacteria and fungi (DiToro et al., 2000; Newman and Unger, 2003). In uncontaminated soft water lakes in Ontario, Cu concentrations range from 0.5-1.5 μg/L (Welsh et al., 1996) and in the Great Lakes, Cu concentrations range from 0.8-1.1 μg/L (Nriagu et al., 1996). Currently, water quality guidelines for Cu set by the Canadian Council of Ministers of the Environment (CCME) are 2-4 μg/L (CCME, 1999). These values are corrected based on hardness only, which generate three possible guidelines based on the hardness of the receiving water. A hardness based correction factor has been proven to be inappropriate since other factors such as NOM have a greater influence on metal speciation and toxicity (Markish et al., 2005). Other jurisdictions have recently adopted the BLM. For example, US EPA now uses the Cu BLM for deriving water

quality criteria in the aquatic environments (US EPA 2007). Within the European Union the BLM approach has been applied in risk assessments (Bodar et al., 2005).

1.5 Mechanism of Cu toxicity

Cu is an essential element to fish and is part of about 30 known enzymatic and glycoprotein complexes (Sorensen, 1991). However, Cu is potentially acutely toxic to freshwater fish in the range of 3μg /L (Mancini et al, 2009 unpublished) to 84,600 μg /L (Hartwell et al., 1989). This large range over which Cu is toxic is largely due to the differences in the physicochemical properties of water (Niyogi and Wood, 2004). For examples, Erickson et al., (1996) demonstrated that increased pH, hardness ions, Na, and dissolved organic matter each decreased Cu toxicity to fathead minnows. In addition, differences in tolerance of various fish species (Matsuo et al., 2004) and fish age (Sorensen, 1991) would contribute to the large range in acute Cu toxicity to fish.

The gill is the first organ to be affected by waterborne exposures to Cu and if concentrations are sufficiently elevated ionoregulatory disruption may result (reviewed in Wood, 2001). In freshwater fish the chloride cell is the primary target cell within the gill. Chloride cells are responsible for the uptake of physiologically essential ions such as Ca^{2+}, Mg^{2+}, Cl^- and Na^+ (Li et al., 1998). When rainbow trout are acutely exposed to Cu, Na^+/K^+ ATPase activity is inhibited resulting in reduced branchial Na^+ and Cl^- uptake (Lauren et al., 1987a, b). At elevated levels, Cu may also stimulate the efflux of NaCl through the tight intercellular junctions due to displacement of Ca (Lauren and McDonald, 1985). Acute exposure of Cu to fish can result in impaired respiration (due to impaired transfer of CO_2 and O_2 across the gill) as well as cardiac failure due to decreases

in Na and Cl concentrations in the plasma (Wilson and Taylor, 1993). Acute Cu exposure has also resulted in elevated plasma ammonia (Wilson and Taylor, 1993) and increased plasma cortisol (Pelgrom et al., 1995).

1.6 Soft waters of the Canadian Shield

In the region of Ontario, along the Canadian Shield, approximately 70% of the lakes are considered soft (Neary et al., 1990). Soft waters are characterized by low alkalinity (<10 mg/L as $CaCO_3$), Ca (<5 mg/L), hardness (<20 mg/L as $CaCO_3$), conductivity (<50 μS/cm), and pH (5.5-7.0) (Neary et al., 1990). Based on this definition, a recent survey of 100 Ontario lakes reveals relatively soft waters due to the following average water chemistry: alkalinity (5.4 mg/L $CaCO_3$), Ca (3.76 mg/L), conductivity (45 μS/cm), DOC (4.7 mg/L), and pH (6.4) (David et al., 1997). Metals (Cu, Pb, Cd, Al, Zn) are suspected to be more toxic in soft waters because of the decrease of competitive cations, thus resulting in high uptake of metal across the gill surface. In addition, the characteristic pH (5.5-6.5) of soft waters increases the concentration of toxic free metals ions. For example, at a higher pH range (6.5-8.0), less toxic Cu species begin to form ($CuCO_3$, $CuOH^+$, $Cu_2(OH)_2^{+2}$; Sorenson, 1991), thus Cu is less toxic at higher pH waters compared to lower pH waters. Due to the prevalence of soft waters along the Canadian Shield, it is important to understand how these soft waters alter metal toxicity to aquatic organisms. The applicability of the BLM to soft waters is unknown because the BLM has mainly been developed and tested in hard waters.

1.7 Research objectives

This research is directed at contributing to improvements in the predictive capabilities of geochemical (such as WHAM) and toxicity (such as BLM) prediction models in soft waters. The proposed studies are focused on NOM, with an overall objective to modify existing predictive models for Cu that takes into account the variability in the quality of NOM. The resulting modified models will better predict Cu speciation, therefore bioavailability and toxicity to aquatic organisms. To accomplish this objective, both biological characterization (Cu accumulation and toxicity) and chemical characterization (NOM-Cu complexation, chemical and optical properties) of NOM from different sources will be performed. There are four sub-objectives:

1. To characterize the chemical, optical and Cu complexing properties of different sources of NOM collected from soft Canadian Shield waters.

2. To evaluate the toxicity reducing capacity of different NOM sources using acute toxicity and short term gill accumulation assays.

3. Determine if chemical and/or optical measures can be used to predict differences among NOM sources in Cu toxicity mitigation

4. To modify the existing geochemical and toxicity prediction models to better simulate Cu speciation as well as Cu accumulation & toxicity to fish in the presence of different NOMs.

Chapter 2

Chemical, Optical and Cu Complexation Properties of Natural Organic Matter

2.1 Introduction

The toxicity of Cu to aquatic organisms is related to the most bioavailable form, free ionic Cu^{2+} (Erickson et al., 1996). The concentration of Cu^{2+} can be reduced by complexation with ligands such as natural organic matter (NOM) and inorganic solutes (e.g. HCO_3^-, CO_3^{2-}, Cl^- and OH^-) thus toxicity is reduced (Erickson et al., 1996). The complexation of Cu^{2+} with NOM is complicated because of the heterogeneous nature of NOM. NOM is derived from terrestrial sources (allochthonous) and sources within (autochthonous) the aquatic system (McKnight and Aiken, 1998). Allochthonous NOM is derived mainly from terrestrial plants, is higher in molecular weight (mw) (2-5 kDa or higher; Thurman, 1985), and is richer in aromatic humic (HA) and fulvic acids (FA) compared to autochthonous sources. Autochthonous NOM is derived mainly from algae and macrophytes, is lower in mw (0.5-2 kDa), and is more aliphatic (Thurman, 1985). In an aquatic system, there will be a mix of allochthonous and autochthonous sources of NOMs making a very complex and heterogeneous mixture. In addition, UV radiation and microbial activity degrades NOM, altering its chemical and optical properties (e.g. Fluorescence, Winter et al., 2007). Therefore NOM may vary in composition depending on source characteristics.

As a result of the heterogeneous nature NOM, the complexation of Cu^{2+} may differ with source and this has been demonstrated previously (McKnight et al., 1983; Cabaniss and Shuman, 1988; Abbt-Braun and Frimmel, 1999; Breault et al., 2006). For example, McKnight et al (1983) examined the Cu binding properties of 18 different water sample sites using an ion selective electrode (ISE). They found two different binding sites with binding affinity (Log K_1) for binding site 1 ranging from 5.4 to 6.6 and binding

capacity (B_{max}) ranging from 0.2-2.0 μmol /mg C. The second binding site had Log K_2 values of 7.0-8.5 and B_{max} ranging from 0.1-0.7 μmol/mg C. Differences among sources of NOM have also been illustrated at the biological level, where by providing varying degrees of protection against Cu toxicity to fish (Ryan et al., 2004; Richards et al., 2001; Schwartz et al., 2004) and invertebrates (De Schamphelaere, 2004).

Geochemical and functional differences among NOMs that result in differential complexation capacities for Cu^{2+} (and other metals) make the development of geochemical speciation models for natural waters challenging. Models such as WHAM (Windermere Humic Aquatic Model; Tipping 1998) predict chemical speciation in natural waters by taking into account inorganic and organic (i.e. NOM) complexation, cation competition and pH (Tipping, 1998). WHAM takes NOM into account as dissolved organic carbon (DOC) and the binding of metal cations is estimated by incorporating multiple metal binding sites with differing affinities. There are two input parameters for DOC, HA and FA, which typically are set to 10% HA and 90% FA and is assumed to be representative of natural waters (Tipping, 2002). However, other than possible differences in the relative amounts of HA and FA, WHAM does account for differences among NOM sources. In some cases the model can predict complexation reasonably well (Dwane and Tipping, 1998; Bryan et al., 2002) while in others it often under-predicts (Guthrie et al., 2005; Unsworth et al., 2006). One reason for these underpredictions may be attributed to variability among humic substances (Guthrie et al. 2005). A better understanding of potential differences among NOMs is required to determine if WHAM can accurately predict Cu speciation and to determine if NOM variability needs to be accounted for.

To better understand NOM in aquatic environments different methods have been developed to characterize it and some of these may be relevant in understanding how it influences metal speciation. The review by Abbt-Braun et al. (2004) discussed a variety of methods used to characterize NOM including chemical/physical analysis (elemental analysis, acid/base titrations), methods in fractionation and degradation (pyrolysis, oxidation, hydrolysis), as well as spectroscopic methods. Spectroscopic techniques including the absorbance at specific wavelength in the UV/VIS spectrum and fluorescence are relatively simple, quick and sensitive techniques for measuring differences among sources of NOM. Measuring absorbance in the UV range, specifically at 254-340 nm, provides a measure of aromatic groups like HA and FA (Abbt-Braun and Frimmel et al., 1999). It is usually accepted that a higher absorbance indicates darkly coloured allochthonous NOM whereas a lower absorbance value indicates a lighter autochthonous sources (Richards et al., 2001).

Excitation emission matrix fluorescence spectroscopy (EEMS) is another optical method used to investigate differences in NOM (Coble, 1996). EEMS is typically measured over multiple excitation and emissions wavelengths (200- 450 nm and 250-600nm, respectively; Smith and Kramer, 1999), results which have been used to show differences in origin (McKnight et al., 2001) including sewage contamination (Baker, 2001), seasonal variations (Vodacek et al., 1997), and patterns of degradation (Brooks et al., 2006; Winter et al., 2007) among NOMs. Due to the large amount of data collected from EEMS, it has been difficult to interpret the results. Recently a multivariate technique called parallel factor analysis (PARAFAC) has been used in conjunction with the EEMS data to analyse excitation emission spectra (Stedmon et al., 2003, Holbrook et

al., 2006). A detailed review of PARAFAC modeling of EEMS data is provided by Andersen and Bro (2003). In brief, PARAFAC combines all EEMS data from multiple samples and identifies and quantifies distinct fluorophore groups. Typically the analysis resolves HA-like, FA-like and protein-like fluorophores (Holbrook et al., 2006). EEMS and PARAFAC may be important characterization methods that could potentially link the ability of NOM to complexation with metals, thus could be incorporated into geochemical speciation modelling like WHAM.

The overall goal of this study was to investigate differences among NOM sources in their ability to complex with Cu and to establish if Cu complexation is related to NOM chemical or optical properties. To accomplish this, nine NOM sources from locations across the Canadian Shield in the provinces of Ontario and Quebec were collected as concentrates and then returned to the lab for characterization. NOM characterization included optical properties such as absorbance and fluorescence as well as total protein content and molecular weight fractions. Additionally, the complexation capacity of NOMs for Cu was measured using an ion selective electrode technique. In addition to exploring linkages between characterization measures and Cu complexation capacity, the ability of the WHAM software to predict NOM effects on speciation was tested.

2.2 Materials and Methods

2.2.1 Sites, collection and preservation of NOM

Samples of NOM from waters throughout south central Ontario and western Quebec were collected and concentrated by reverse osmosis in the period of October 2007 to June 2008. Collections were done at six soft water and one hard water sources

and one of the soft water sources (Brandy Lake) was sampled twice. A previous NOM collection conducted by R. Playle (Wilfrid Laurier University) in 2003 at Lake Nipissing provided a ninth source of NOM. Location and site characteristics are provided in Table 2-1 along with the pH and conductivity which were measured on-site at the time of collection. Water pH was measured using a Mettler Toledo SevenGo^{TM} pH meter (Fisher Scientific) and conductivity using salinity, temperature and conductivity meter (YSI 30, Yellow Springs Instruments, Yellow Springs, OH).

NOM samples were collected as previously described by Schwartz et al. (2004). In brief, near shore surface water (typically 2-3 m from the shore and 0.5 M above the bottom) was pumped (Masterflex peristaltic pump, Cole Palmer Instruments, Vernon Hills IL) through a 1 μm fiber-glass filter (142 mm diameter filters; Geotech Environmental Equipment, Denver, CO) and then concentrated using a stainless-steel portable reverse osmosis unit (Limnological Research, Kelowna, BC). The reverse osmosis filter (FilmTec FT30 U.S. Filters thin composite RO membrane, Minneapolis, MN) had a molecular mass cutoff of 400 Da. Approximately 350 L (range 200 to 500 L depending on source) of water was reduced to approximately 7 L (range 4 to 10 L) of NOM concentrate. The NOM concentrate was collected in 10 L polypropylene containers that had been acid rinsed (0.1% HNO_3) and return to the lab. At each site, 10L samples of surface water were collected and filtered through the 1 μm fiber-glass filter and collected into 10 or 20L polypropylene containers, returned to the lab and stored at 4°C. Upon return to the lab, NOM concentrates were passed through an H^+ cation exchange resin (USF C-211 H cation resin, U.S. Filter Corporation, Rockford IL) to remove residual cationic (metal) contamination. Stabilized NOM concentrates were

stored in the dark at pH 2 and 4°C. Both the concentrates and the collected on-site surface water were measured for dissolved organic carbon (DOC; see below). Subsequent characterizations of NOM from the nine different sources involved the dilution of aliquots of concentrate with MilliQ water (18 mohm, Millipore corporation, Fisher Scientific, Nepean, ON) to give appropriate concentrations of DOC and then solution neutralization to a pH of 6.5 using KOH (Sigma-Aldrich, St Louis, MO).

2.2.2 UV-Vis absorbance and excitation emission matrix spectroscopy

The absorbance of 10 mg C/L DOC solutions at 20 °C was measured for each source in 1nm increments from 200 to 800 nm along a 1cm path length (Ultraspec 4300 pro UV/visible spectrophotometer, Biochrom Ltd., Cambridge, UK). Because pH can have an influence on absorbance (Abbt-Braun and Frimmel, 1999) care was taken to ensure each source solution was adjusted to the same pH of 6.5.

EEMS measurements were done on solutions of 10 mg C/L DOC along a 1 cm pathlength in quartz cuvettes (Hellma Canada Ltd., Concord Ontario) using a fluorometer (Cary Eclipe, Varian, Victoria, Australia) according to the method described by Smith and Kramer (1999). Excitation wavelengths began at 200 nm and increased in 10 nm increments to 450 nm and emission was measured across the range of 250-600 nm. In addition to the nine NOM samples, tryptophan and tyrosine standard solutions (at a concentration of 0.5 and 1 μmol, respectively) were added to facilitate the identification of the amino acid fluorescent peaks during PARAFAC modeling (discussed below).

2.2.3 Ultra-filtration using a 1 kDa membrane

The fraction of DOC less than 1 kDa was determined through ultra-filtration of NOM solutions following methods for characterization of mw of NOM (Thurman et al., 1982). NOM solutions at 10 mg C/L DOC were put in a 250 mL ultra-filtration unit (Stirred Ultrafiltration Cell, Model 8200, Millipore Corporation, Beford, MA, USA) and passed through a new 63.5 mm diameter regenerated cellulose ultra-filtration membrane (Millipore Corporation, Bedford, MA, USA) with a mw cut-off of 1 kDa. Before use, membranes were soaked in ultrapure water for at least 24 h to remove any residual DOC. Prior to filtration 20 mL of solution was collected in 20mL scintillation vials. Solution (10 mL) was passed through the filtration unit and collected in 20 ml scintillation vials. A control was run (MilliQ water) through the ultra-filtration membrane and samples collected, as a control to correct for the possibility of DOC additions by the membrane. Samples were stored at 4°C and measured for DOC within 24 h of collection.

2.2.4 Free Cu^{2+} measurements using an ion selective electrode

Free Cu^{2+} concentrations were measured in solutions using an Orion 5-star Series Meter with an Orion Ionplus Sure Flow Cupric Ion Electrode (Orion 96-29 Ionplus, Thermo Electron Corporation, Burlington, ON) following the method outlined by Schwartz and Vigneault (2007). Test solutions (100 ml) were prepared by spiking soft water with various combinations of Cu (as $CuSO_4 \cdot 5H_20$; Anachemia Canada Inc., Montreal QC) at nominal concentrations of 1, 2, 4, 6 or 8, µmol Cu/L and NOM (nominal DOC at 2.5, 5 or 8 mg C/L). Soft water was prepared by mixing reverse osmosis deionized water with small amounts of local well water (final composition; Ca 76, Mg 36, Na 140; µmol/L) and the pH of solutions was adjusted to 6.5 using KOH. Test

solutions were equilibrated for 24 h (Ma et al., 1999) including overnight refrigeration. The Cu^{2+} response (in millivolts) was measured at 20°C in solutions that were continuously stirred with a magnetic stir bar. Before use, the electrode was washed with soap and rinsed with ultrapure water and filling solution (D Optimum results Thermo) replaced. The of the calibration for Cu was 27.13 mV/decade Cu (n=7). This was less than the theoretical slope of 29.5 mV, however it was consistent. The electrode was calibrated daily using a 6 point standard curve ranging from 0.05 to 10 μmol Cu^{2+} /L, based on dilutions of a 0.1 mol/L cupric ion reference solution (Orion Ionplus, Thermo Electron Corporation, Beverly, MA, USA). Ionic strength adjuster (0.01M $NaNO_3$, Orion Ionplus, Thermo Electron Corporation, Beverly, MA, USA) was added to both standards and solutions to normalize the sample matrix. The mV response of the Cu^{2+} ISE was recorded once readings had remained constant at ± 0.1 mV for 1 min. For each test solution 20 ml water samples were collected in scintillation vials and stored at 4°C for subsequent analysis. Samples for Cu analysis were acidified to 2% with HNO_3 (TraceMetals grade, Fisher Scientific, Nepean ON).

2.2.5 Other analyses

Total protein concentration, calculated on a per mg of DOC basis, was measured directly in the NOM concentrates using the dye binding assay of Bradford (1976). Analysis of total Cu content in ISE test solutions was by done by an inductively coupled plasma atomic emission spectrometer (ICP-AES; Varian Vista RL with Varian SPS-5 autosampler, Varian, Mississauga, ON). Total carbon (TC) and inorganic carbon (IC) concentrations of NOM concentrates and of samples of solutions for UV-Vis absorbance,

ISE, and ultra-filtration were measured with a total carbon analyzer (Shimadzu TOC-V with ASI-V autosampler, Mandel Scientific, Guelph, ON) calibrated with appropriate total carbon and inorganic carbon standards (Pharmaceutical Resource Associates, Arvada, CO). Samples of solutions from fluorescence measurements and some of the NOM concentrates were similarly measured for TC and IC using the same combustion and IR detection method but on a different instrument (Shimadzu TOC-5050A, Tokyo).

2.2.6 Calculations and statistics

DOC was quantified as the difference between TC and IC and ultrafiltration data were corrected for the contributions of DOC from the filtration membrane. The DOC normalized specific absorbance coefficient (SAC) at all wavelengths (200-800 nm) was calculated using the equation provided by Richards et al (2001):

$$SAC_{nm} = [\ 2303 \times (Abs_{nm})\ ] / DOC \qquad \text{Equation 1}$$

where Abs_{nm} is the absorbance at the specified wavelength and DOC is measured in mg C/L. Absorbance at the wavelengths of 254, 340 and 414 nm were selected as source description parameters (Ryan et al., 2004). Fluorescence data was used to calculate the fluorescence index (FI), which has previously been used to distinguish between NOM sources (McKnight et al. 2001). FI was calculated was calculated using the following equation:

FI = (emission intensity @ 450nm / 500nm) @ excitation 370 nm Equation 2

EEMS data was used to develop excitation-emission based characterizations of the relative content of HA-like, FA-like and amino acid-like fluorophores (Smith and Kramer 1999; Stedmon and Markager 2005) in each NOM sample. Data were imported into the software program Matlab™ (The MathWorks, MA, USA) to produce two-dimensional plots with contours for the 3rd dimension (relative intensity) as described in Smith and Kramer (1999). Resolution of the plots was improved by numerically removing the Rayleigh-Tyndall scatter (two high intensity diagonal bands) using Matlab™ as described by Smith and Kramer (1999). Parallel Factor Analysis (PARAFAC) was also carried out in Matlab™ to identify and quantify different NOM components including HA-like, FA-like and amino acid -like fluorophores (Stedmon et al. 2003). Since the amino acid-like fluorophore among the NOM samples had very weak signals, tryptophan and tyrosine standards were added to facilitate PARAFAC modeling in identifying the amino acid fluorophores.

The contour plots of the individual fluorophores identified by PARAFAC are typical of organic fluophores with multiple excitation maxima and single emission maxima (Stedmon et al., 2003). The HA-like component excites at 350 nm & 240 nm and emits at 400-500 nm. The FA-like component excites 320 & 240 nm and emits at 400 nm. The tryptophan-like component excites at 275 & 220 nm and emits at 360 nm. Finally, the tyrosine-like component excites at 275 & 225 nm and emits at 300 nm. The relative concentration of each component for each NOM source is determined by PARAFAC and is given in arbitrary fluorescence units. Actual concentrations cannot be determined because most likely each component is a mix of different compounds fluorescing with similar spectral properties (Stedmon et al., 2005), therefore actual

standards cannot be made. The fluorescence units were normalized for the DOC concentration and then summed together to calculate the relative percent composition of each component within each NOM source. The % HA was divided by the % FA to generate HA:FA ratios.

ISE measurements (mV) were converted to Cu^{2+} using the Cu^{2+} vs. mV standard curve generated for that day. From the measured Cu^{2+}, total Cu and DOC concentrations, the Cu bound to DOC was calculated. Free Cu^{2+} and bound Cu^{2+} were plotted, and binding curves were fitted using functions within the software package SigmaPlot (ver. 11, Systat software, Inc. San Jose, CA, USA). A hyperbolic rise to saturation was defined by:

$$Y = (B_{max} \times X) / (K + X) \qquad \text{Equation 3}$$

where Y is the calculated Cu bound to DOC (moles Cu/mg C), X is the measured free Cu^{2+} (moles/L) in solution, B_{max} is the modelled maximum binding capacity of the NOM (the saturation concentration in (moles Cu/mg C) and K represents the relative affinity of the NOM source (the Cu^{2+} concentration that is associated with at 50% of B_{max}). K values were expressed as conditional equilibrium constants by taking the negative logarithm (base 10) of concentration values.

Correlations among all the variables were assessed using the Pearson product moment method and relationships were considered significant when $p<0.05$.

2.2.7 Modeling free Cu^{2+} activity using WHAM

Solution chemistries from the ISE experiments (see above) were applied within WHAM (version VI, Tipping 1998) to derive predictions of free Cu^{2+} which were compared to the ISE measured actual Cu^{2+}. Measured cation concentrations in solution (which remained consistent across NOM sources) were used as model inputs (see section 2.2.4). In one series of predictions modeling was done with DOC assumed to be 100% active and the HA to FA ratio was 9:1. For comparison, WHAM modeling was also conducted using the relative HA to FA compositions that were determined by EEMS and PARAFAC. To better predict Cu^{2+}, a quality factor (QF) described by Schwartz et al. (2004) was used to take into consideration SAC_{340}, where:

$$QF = 0.31 \ln (SAC_{340}) \qquad \text{Equation 4}$$

This QF value is multiplied by the DOC concentration to yield adjusted DOC concentrations which were then used with the modeling scenarios.

2.3 Results

All of the collected NOMs were from soft and mildly acidic water except for Bannister Lake NOM, which is a hard water lake with neutral pH (Table 2-1). Two collections were made from Brandy Lake and the samples were different even though they were collected only 7 months apart.

2.3.1 UV/Vis absorbance

The SAC values derived from absorbance scans show a decrease as wavelength increases (Fig 2-1). Note that absorbance coefficients are only plotted between 200 nm to 450 nm because absorbance moves increasingly closer to zero. This trend can also be seen in the SAC values at 254, 340 and 436 nm which are shown in Table 2-2. The SAC_{340} values were most variable (up to a 3 fold difference) followed by SAC_{436} and the least variable was SAC_{254} (Table 2-2). Nipissing (collected 2003) and samples collected in 2007 (Echo, Brandy 07 and Bannister; Table 2-2) showed lower SAC values compared to 2008 samples (Brandy 08, Fawn, Dozois, Allard and Rupert). The 2003 and 2007 samples were also more variable among each other, for example SAC_{340} ranges from 11 to 24 while 2008 samples ranged from 26 to 33 (Table 2-2). Overall, Bannister source NOM was lightest represented by the lowest SAC values while Rupert River and Brandy Lake NOMs were darkest represented by the highest SAC values (Figure 2-1, Table 2-2).

2.3.2 Excitation emission matrix spectroscopy

PARAFAC analysis resolved up to four components (HA-, FA-, tyrosine- and tryptophan-like) and overall, this explained 92% of the variation in all the excitation-emission scans. The contour plots of the individual components are given in Figure 2-2 and the contour plots of each NOM source are in Appendix A. The relative percent composition of NOMs, as determined by EEMS and PARAFAC are illustrated in Figure 2-3 and numerically in Table 2-2. HA-like and FA-like components were the most abundant while tryptophan-like and tyrosine-like components were much less abundant. The HA:FA ratios (Table 2-2) were lower for samples collected in 2007 compared to

samples collected in 2008. Evidence of a tyrosine-like fluorophore was present in all NOM sources particularly in Echo and Bannister Lakes. Tryptophan, which was not evident in Lake Nipissing and Brandy Lake 08 samples was highest in Bannister and Echo Lakes. The FIs were similar among the 2008 NOM samples (Table 2-2). Other FI values ranged from 1.28 to 1.46 with Echo Lake and Bannister Lake being the highest (Table 2-2).

2.3.3 Correlations among optical measures

Correlation analysis of the optical variables showed that HA and FA were negatively correlated ($r=-97$, $p<0.0001$, Fig 4A), where an increase in the HA-like fraction was associated with a decrease in the FA-like fraction. HA was positively correlated with SAC and FA was negatively correlated with SAC having the same level of significance (SAC_{254}, $p<0.05$; SAC_{340} and SAC_{436}, $p<0.0001$; data not shown). The FI was positively correlated with the three SAC values where FI and SAC_{254} demonstrated the highest degree of significance ($p<0.0001$; Fig 4B).

2.3.4 Other measures

The fraction of DOC less than 1 kDa and total protein content are also in Table 2-2. The fraction of DOC that was less than 1 kDa was consistently low, less than 17% for all sources except for Bannister Lake and Lake Nipissing which had a much great proportion of the DOC in the <1 kDa fraction (Table 2-2). Total protein content in NOM concentrates varied up to 4 fold among samples (Table 2-2). It was lowest in Bannister Lake and highest in Brandy Lake 07. Tryptophan and tyrosine components resolved

from the EEMS and PARAFAC analysis did not show any relationship with total protein measurements.

2.3.5 Ion selective electrode measurements

The ISE measurements demonstrated differences in Cu complexation among NOM sources. This is graphically illustrated in Figure 2-5 where total Cu is plotted against measured free Cu^{2+}. This is a subset of measurements, all at a DOC concentration of 2.4 ± 0.2 mg C/L. All NOM sources demonstrated some degree to complex with Cu indicated by reduced measured free Cu^{2+} compared to control (no NOM added). Differences in the degree of complexation with Cu are more pronounced at higher total Cu concentrations. Linear regression lines were computed for each source with the slope, y-intercept and r^2 values in Table 2-3. Echo and Bannister had the steepest slopes, and are similar to the slope with no added DOC. Allard River has the smallest slope, demonstrating that it complexes much better to Cu than the other sources.

Calculated stability constants (Log K) and complexation capacity (B_{max}) are in Table 2-4. The Log K values differed up to approximately 0.5 log units (five fold) being lowest in Dozois and highest in Brandy 08. Variability in the B_{max} values was up to a three fold difference. Echo Lake NOM demonstrated the lowest capacity to complex Cu (0.93 μmol Cu /mg C) and Allard River NOM demonstrated the greatest (3.03 μmol Cu /mg C; Table 2.4).

2.3.6 Correlations of NOM-Cu complexation with chemical and optical properties

Log K and B_{max} determined by ISE measurements were correlated with all chemical and optical measures listed in Table 2-2. All correlation coefficients (r) determined by the Pearson product moment correlation method (n=9) are listed in Table 2-5 with an asterisk indicating significance ($p<0.05$). There was no significance in any of the correlations with Log K values. Those NOM variables which correlated well with B_{max} are graphically presented in Figure 2-6. There were significant correlations with B_{max} and the ratio HA:FA and FI. The correlation between SAC_{254} and B_{max} is also illustrated in Figure 2-6 which had demonstrated a good relationship but was not significant ($p=0.054$).

2.3.7 Modeling free Cu^{2+} activity using WHAM

WHAM predicted free Cu^{2+} for ISE measurements are plotted in Figure 2-7 (Fig 2-7A is 1HA: 9FA, Fig 2-7B is measured HA:FA). In both cases, prediction points fall within the factor of ± 2 of measured, 63% of the time. WHAM predicted best at the higher free Cu^{2+} concentrations and for some of the NOM sources WHAM poorly predicted at lower free Cu^{2+} measurements. Changing the ratio of HA:FA from 1:9 to measured values generated from EEMS (ranging from 0.8:1 – 2.7:1) did not alter by much the WHAM prediction except to generally reduce predicted Cu^{2+} concentrations. This either improved the prediction, moving the points closer to the 1:1 line (e.g. Allard, Dozois, Rupert) or worsened the predictions by moving the points further away from the 1:1 line (Brandy 07/08, Fawn, Echo, Bannister and Nipissing). In general, 2007 sources

including Nipissing, were under-predicted whereas, 2008 sources except Brandy 08 and Fawn were over predicted.

WHAM predictions of Cu^{2+} were further modified by incorporating the QF value derived from the SAC_{340} values (Table 2-2). When QF adjusted DOC values were used in the model the ability of WHAM to predict Cu^{2+} increased to 81%, up from 63% (Fig 2-8).

2.4 Discussion

2.4.1 NOM chemical and optical properties

Chemical and optical characterizations revealed differences among NOM sources (Table 2-2). Absorbances at 254nm and 340nm (UV range) are commonly used because at these wavelengths there is absorbance of the aromatic HA and FA compounds (or chromophores; Abbt-Braun et al., 2004). Absorbance in the visible range (436nm) is due to functional groups with quinoide and keto-enol structures (Abbt-Braun and Frimmel, 1999). The NOM sources in this study which demonstrated minimal capacity to absorb UV and VIS light are Echo and Bannister Lake. This indicates that these sources are lighter in colour and therefore contain fewer aromatic groups compared to those sources which absorbed more light. In addition a lower FI value, which is more allochthonous-like (McKnight et la., 2001), was correlated with a higher SAC (Fig 2-4) again illustrating that higher optical absorbance is associated with more aromatic groups (typical of allochthonous NOM). Since Echo and Bannister lake NOMs had lower SAC value and lower FI, this is an indication that these NOM are more autochthonous in

origin. This was not surprising for Bannister, since this was a lake which was heavily colonized by algae, thus receiving lots of NOM derived within the aquatic system.

PARAFAC has been shown to be successful in interpreting the large data sets resulting from EEMS in this study (freshwaters) and others (marine and freshwaters) (Holbrook et al., 2006; Stedmon et al., 2003, Stedmon et al., 2005). Due to the small sample size (9 NOM sources) PARAFAC was limited to only four components which could explain the variability among NOM sources. If the sample size is increased, this increases the variability in fluorophores and more components can be identified. For example, in Stedmon et al. (2003) they had a sample size of 90 and PARAFAC identified 5 components. In Stedmon et al. (2005), they had a sample size of 1,276 and PARAFAC identified 8 components. The four components identified in the NOM samples from this study are similar to components found in other studies. Component 1 and 2 of this study were similar to components 1 and 2 in Holbrook et al. (2006) and component 3 and 4 in Stemond et al. (2003). Component 1 and 2 are identified as HA-like and FA-like (Holbrook et al., 2006). Component 3 in this study is similar to the tryptophan-like (or protein-like) component identified in the literature (Holbrook et al., 2006; Baker et al., 2001; Stedmon et al., 2003). Component 4 in this study is similar to the tyrosine-like component identified in Stedmon et al. (2005). The most abundant components identified in the NOM samples from this study are the HA and FA-like components.

Differences in the ratios of HA:FA were observed between sources. There is a distinct separation between 2007 sources (1:1 ratio) and 2008 sources (2:1 ratio). The reasons for this generalized difference are unknown. Since Brandy lake was collected in both 2007 and 2008, this may indicate that HA:FA differences may be related to seasons

as those 2007 samples were collected in the fall (October and November) and 2008 samples were collected in the late spring/early summer. Higher HA content in the spring/summer 2008 samples may be explained by the increased runoff from soil catchments after the snow melt and spring rains. Lower HA and higher FA in the fall samples may be explained by NOMs longer residency time which would have resulted in more photolysis and microbial degradation. These degradation processes break larger mw compounds into smaller mw compounds (Aitkenhead-Peterson et al., 2003) possibly explaining the decreased HA and increased FA. A seasonal influence on HA:FA was reported by Stedmon et al. (2005) who demonstrated that terrestrial HA-like fluorophores increased in the spring and summer months, and declined in the fall and winter months.

For the protein-like fluorophores, Echo and Bannister Lake were most abundant indicating a possible sewage /microbial origin (tryptophan; Baker et al., 2001) and/or algae origin (tyrosine; Determann et al., 1998). This further indicates that Echo and Bannister Lake are more autochthonous in origin, like previously mentioned. However, amino acid content measured by EEMS did not reflect the total protein content measured, thus EEMS is probably not linked to total protein content.

Additional evidence that suggests that Bannister Lake is autochthonous is the high amount of DOC that is less than 1 kDa (65%). This may indicate that Bannister has a low content of HA (which has a mw higher than 2 kDa; Thurman, 1985), and higher content of algae and bacterial derived NOM which usually have mw smaller than 1 kDa (Thurman, 1985). Echo Lake however, which has demonstrated to be more autochthonous in origin only contains 17% DOC < 1 kDa. Lake Nipissing is intermediate between the autochthonous NOM (Bannister and Echo) and the rest of the

source which are characteristically more allochthonous. This is indicated by intermediate measures of HA:FA ratios, UV/VIS absorbance, and FI. Similar to Bannister, Lake Nipissing also had a high fraction of DOC < 1 kDa, demonstrating more autochthonous-like origins.

Overall, samples collected in the fall 2007 compared to those samples collected in spring of 2008 had a lower ratio of HA:FA, higher FI and lower SAC values at all three wavelengths. These chemical and optical differences among 2007 and 2008 NOM samples are probably a result of the season sampled (e.g. Brandy lake 2007 and 2008) and/or collection from sources which are more autochthonous-like (e.g. Bannister and Echo Lake).

2.4.2 Cu complexation

Ion selective electrode measurements have been shown to be a good method for measuring free Cu (Avdeef et al., 1983). In particular, this method has been used to demonstrate differences among NOM sources in their ability to complex with Cu (McKnight et al., 1983; Luider et al., 2004; Brooks et al., 2007; Breault et al., 1996; Cabaniss and Shuman, 1988). For example, McKnight et al. (1983) who isolated FA from a variety of natural sources measured Cu-FA conditional stability constants (Log K) and binding capacity (B_{max}). They measured both high affinity/low capacity, and low affinity/high capacity binding sites. For the high capacity/low affinity binding sites they had measured similar Log K (5.4-6.6) and B_{max} (0.44-1.9 μmol/mg C) values to this study. Their range of B_{max} values, however, had a smaller range than those measured here (Table 2-4). These differences between the current study and McKnight et al (1983)

could be attributed to the fraction of NOM measured (FA vs. total NOM). By comparing this study to McKnight et al. (1983) it is reasonable to assume that measurements in this study are high capacity and low affinity binding sites.

In 1999, Fremmel and Abbt-Brown measured Cu complexation in isolated FA from different sources and determined B_{max} values ranging from 0.5-2.5 μmol Cu/mg C. Those same authors published another paper which measured Cu complexation in total NOM isolates collected by reverse osmosis and calculated B_{max} values ranging between 1-2.86 μmol/mg C (Abbt-Brown and Frimmel, 1999). These values are comparable to those reported here and in McKnight et al. (1983). Those studies and the one here illustrate that different NOM sources do have varying degrees of B_{max} values with Cu which seem to range anywhere from 0.44 (McKnight et al., 2003) to 3.52 (this study) μmol/mg C for low affinity, high capacity binding sites.

2.4.3 Correlations between Cu complexation and chemical/optical properties

Log K values among NOM sources did not show any significant correlations with the chemical or optical properties measured. The differences in Log K may be attributes to other chemical properties not measured such as functional groups like carboxylic (weaker binding sites) and phenolic groups (stronger binding sites; Perdue, 1998). In contrast to Log K, there were correlations between B_{max} and spectroscopic properties. When more HA-like component was present, NOM had a higher B_{max} (Fig 2-6). Because the HA-like component fluoresces at a higher wavelength, this indicates that it is higher in mw and more aromatic confirming the EEMS data analysis. Increases in aromatic groups, increases the molecular electronegativity of NOMs which results in increased

complexation with Cu^{2+} (Leenheer et al., 1998). Since HA absorbs more light, it was expected that optical absorbance would better correlate with B_{max}, however, this trend was not significant. Additionally, the FI seems to be good a predictor of Cu-NOM complexation (Fig 2-6). These correlations indicate that a more allochthonous NOM which has more aromatic compounds (HA), is optically darker (high SAC values), and that has a lower FI will have a greater ability to complex with Cu than does more autochthonous NOM which has less aromatic compounds, is lighter in colour and has a higher FI.

It has been shown here, and in other studies (Brook et al., 2006; McKnight et al., 1983; Breault et al., 1996) that NOM from different sources can differentially complex with Cu. As a result, it is not surprising to see that NOM from different sources can differentially protect against metal accumulation and toxicity to aquatic organisms (Brooks et al., 2006). For example, Ryan et al. (2004) who exposed larval fathead minnow (*Pimephales promelas*) to Cu and different NOM sources observed up to a six fold difference in 96 h LC50s at the same DOC concentration. In studies conducted by both Schwartz et al. (2004) and Richards et al. (2001) they observed differences in NOM sources in the degree to which metal (Pb and Cu) accumulation and toxicity was reduced in rainbow trout (*Oncorhynchus mykiss*). Pb and Cu toxicity reductions were related to the NOM optical properties, where a more optically dark allochthonous NOM decreased Pb and Cu accumulation and toxicity better than did an NOM that was more optically lighter and autochthonous-like.

2.4.4 WHAM free Cu^{2+} predictions

WHAM predictions for free Cu^{2+} either predicted well (on the 1:1 line), were underestimated by a factor of 1-2, or overestimated by a factor of 1-2, depending on the source of NOM when assuming 100% active DOC and a 1:9 ratio of HA:FA (Figure 2-7). In the literature, it is common that WHAM underestimates free Cu^{2+} (i.e. overestimates NOM-Cu complexation). For example, in metal impacted lakes, WHAM underestimated free Cu^{2+} concentrations by 1 to 2 orders of magnitude (Guthrie et al., 2005). This is comparable to this study, particularly with NOM sources from Echo, Bannister and Nipissing. In other studies, WHAM has underestimated free Cu^{2+} by up to three orders of magnitude in a soft water river and a hard water lake (Unsworth et al., 2006), and up to four orders of magnitude in pore water of contaminated soils (Nolan et al., 2003). Differences in WHAM predicted and measured free Cu^{2+} may be attributed to variability in NOM. WHAM has derived metal humic binding constants from limited studies (Milne et al., 2001) and it's well known that log K and B_{max} values can vary from source to source demonstrated in this study and others (McKnight et al., 1983; Brooks et al., 2007; Breault et al., 1996; Cabaniss and Shuman, 1988).

Initial WHAM modelling applied to the data in this study assumed 100% active sites because this assumption fit best with our data (Fig 2-7). This is not surprising since NOM concentrates were cleaned from metal contamination using an H^+ cation exchange resin, which essentially freed up metal binding sites. Other studies lowered the % active DOC to better fit their data. For example, Guthrie et al. (2005) inputted 65% active DOC into WHAM to better predict metal ions concentrations (Ni, Zn, Cd). Unlike our study, they measured free metals ions in raw water samples from metal contaminated lakes, thus samples were not treated with a cation exchange resin.

To better predict free Cu^{2+}, WHAM was modified using the QF derived from Schwartz et al. (2004). The QF incorporates SAC_{340}, which has been demonstrated in this study and others (Richards et al., 2001; Schwartz et al., 2004) as good measurement for estimating metal binding properties of NOM. Essentially, a NOM which is more autochthonous-like has lower SAC_{340} values which is associated with weak Cu binding properties whereas a NOM which is allochthonous-like has higher SAC_{340} value which is associated with better Cu binding properties. The QF when multiplied by the DOC concentration will either increase (for autochthonous) or decrease (for allochthonous) the DOC concentrations which has the effect of changing the total number of Cu binding sites. When applied to this study, the QF factor did improve the ability of WHAM to predict free Cu^{2+}.

2.5 Conclusion

NOM sources collected across Canadian Shield waters (except for one hard water site, Bannister Lake) varied in their chemical and optical properties. Optical absorbance, HA:FA ratio and FI were good predictors of Cu complexation capacity, where darker more allochthonous NOM containing more HA and lower FI had a greater ability to complex with Cu. Despite differences among NOM sources, particularly in their measured Log K and B_{max} values, WHAM was still able to predict free Cu^{2+} reasonably well without any modification. However, by incorporating the SAC based quality factor (QF), the WHAM predictive capability increases by 18%, suggesting that SAC_{340} may be a good measurement to incorporate into geochemical and toxicity predictions models.

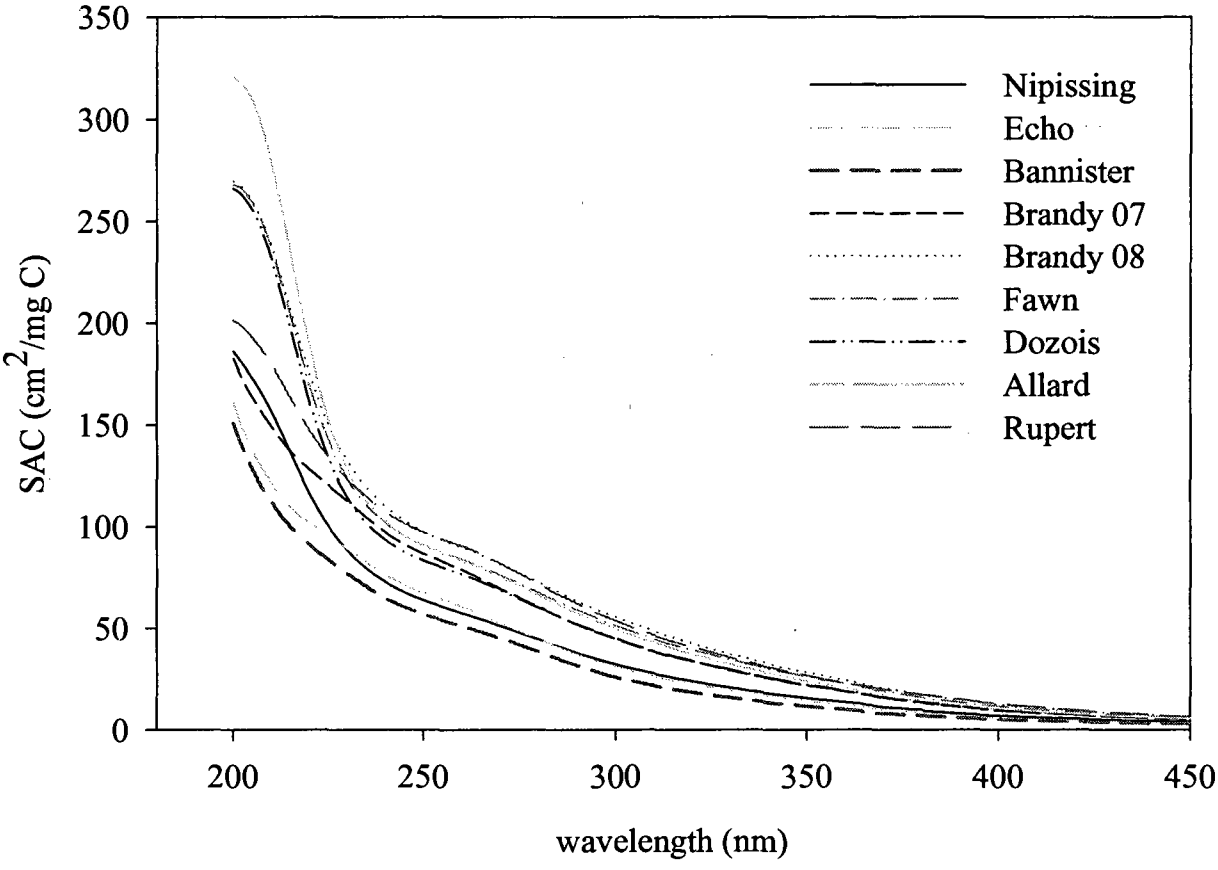


Figure 2-1. Absorbance coefficients for NOM sources at wavelengths ranging from 200 nm to 450 nm.

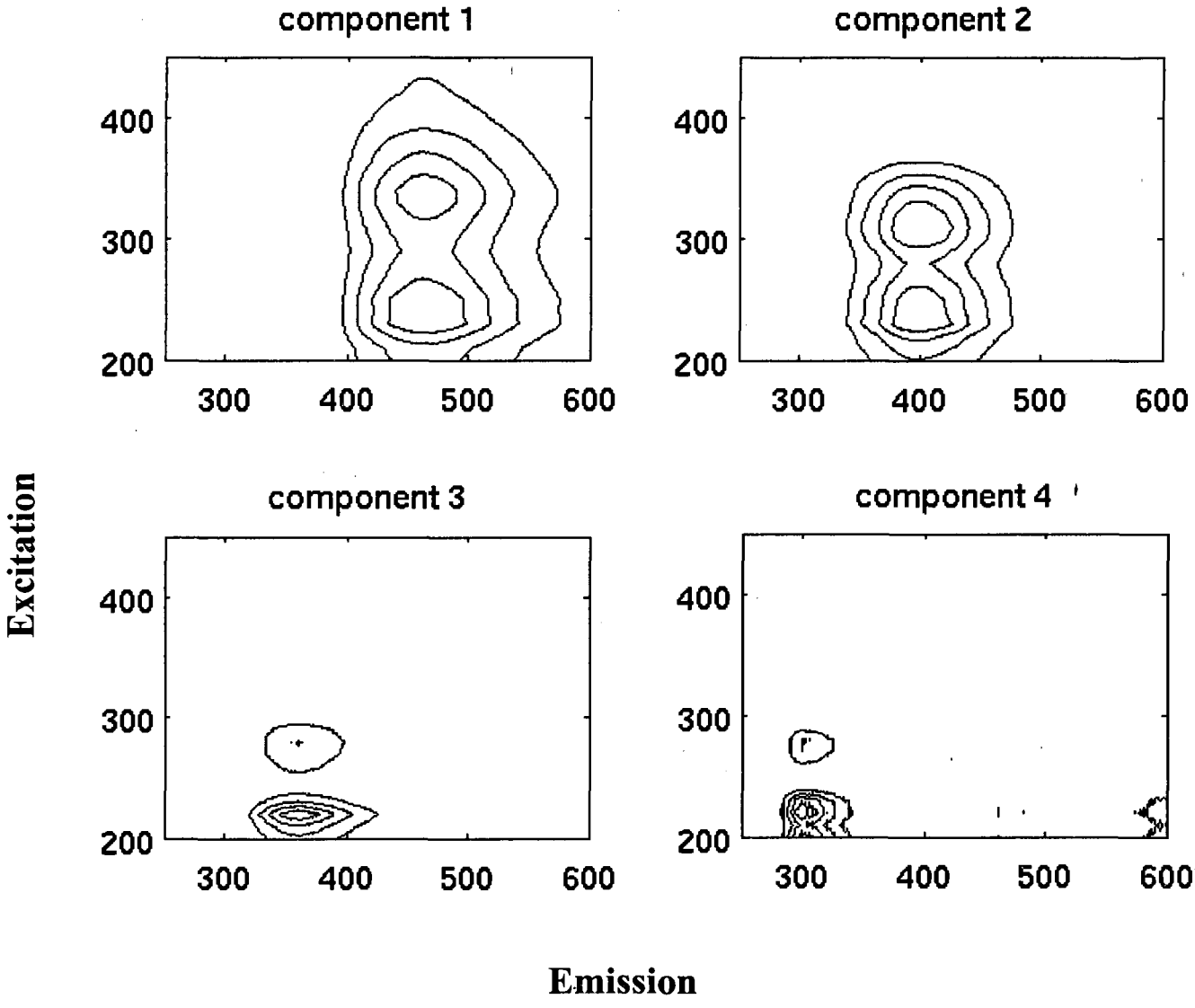


Figure 2-2. Four components identified by PARAFAC from the excitation emission scans of all NOM sources. These four components explain 92% of the variability among NOM sources. The four components identified are: 1- HA like, 2-FA like, 3 tryptophan-like, and 4 tyrosine-like.

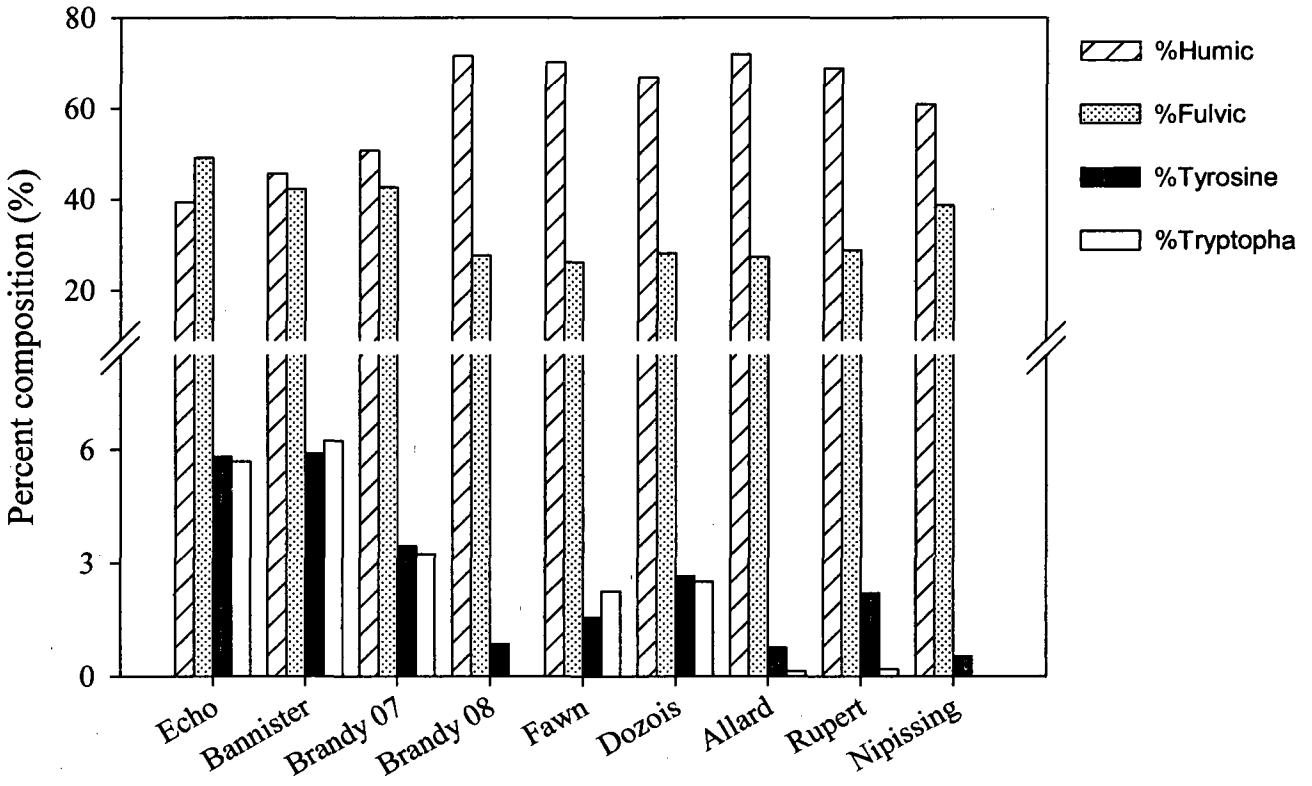


Figure 2-3. Relative composition of the four major fluorescence components as determined by PARAFAC. Measurements of excitation-emission scans were done on 9 NOM samples at 8 mg C/L. The four components identified are humic-like (bars with diagonal lines), fulvic-like (stippled bars), tyrosine-like (solid black bars), and tryptophan-like (open bars).

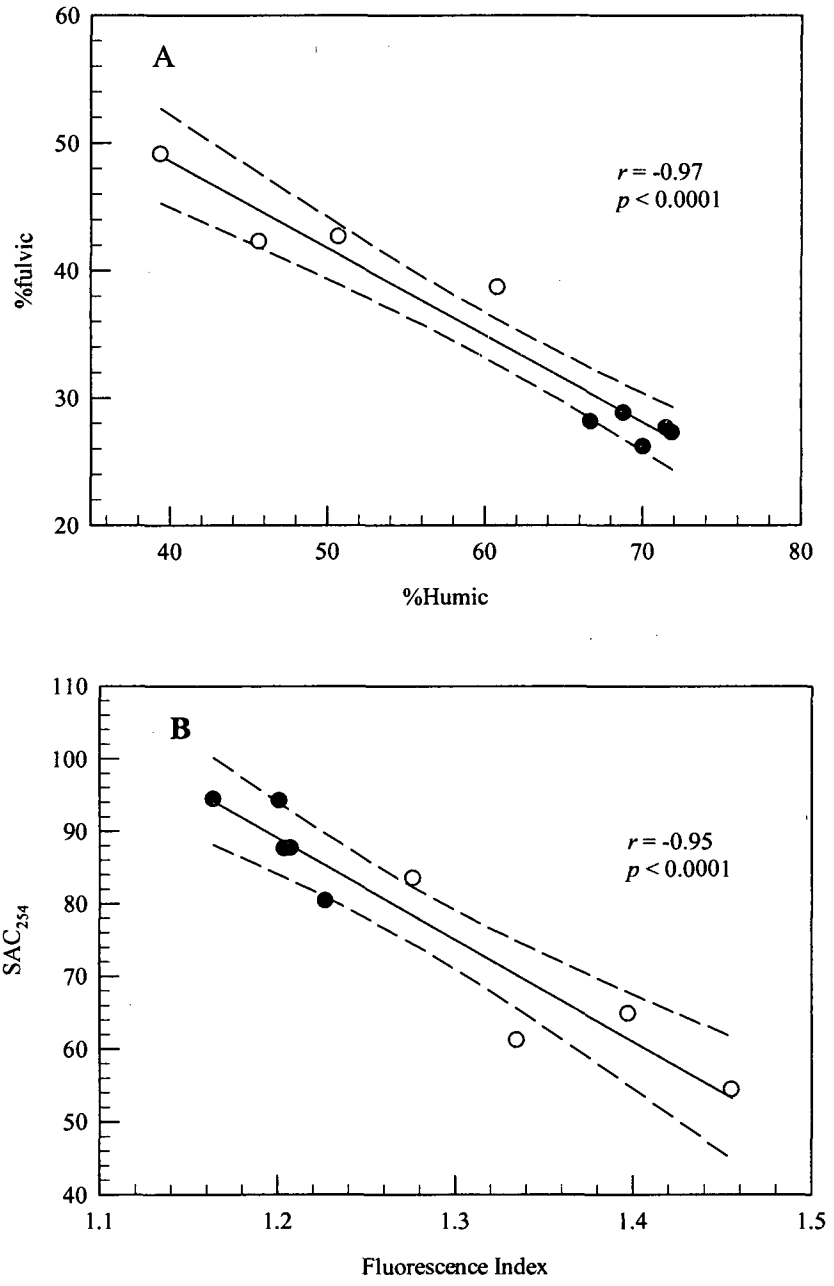


Figure 2-4 Correlations between the spectroscopic properties among the 9 NOM samples. (A) Correlation between humic and fulvic components, (B) correlation between FI and SAC_{254}. Open circles are Nipissing and 2007 sources, while closed circles are 2008 sources. Dashed lines represent the 95% confidence intervals.

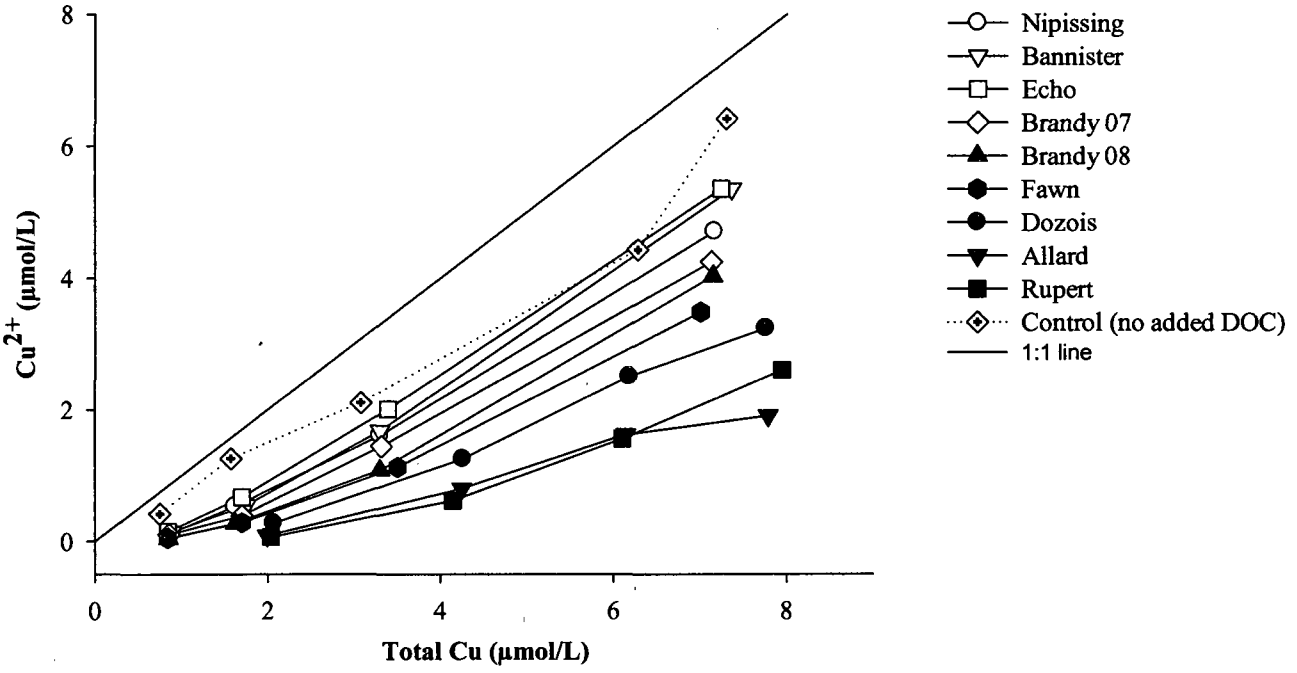


Figure 2-5 Measured Cu^{2+} concentration as a function of total Cu in solutions with 2.4 mg C/L DOC. The 9 NOM sources are shown with additional measurements of solution containing no added DOC (dotted line; diamond symbols with centre dot) and the 1:1 line that would be expected if no complexation occurred is also shown. Open symbols are 2007 sources including Lake Nipissing and closed symbols are 2008 sources.

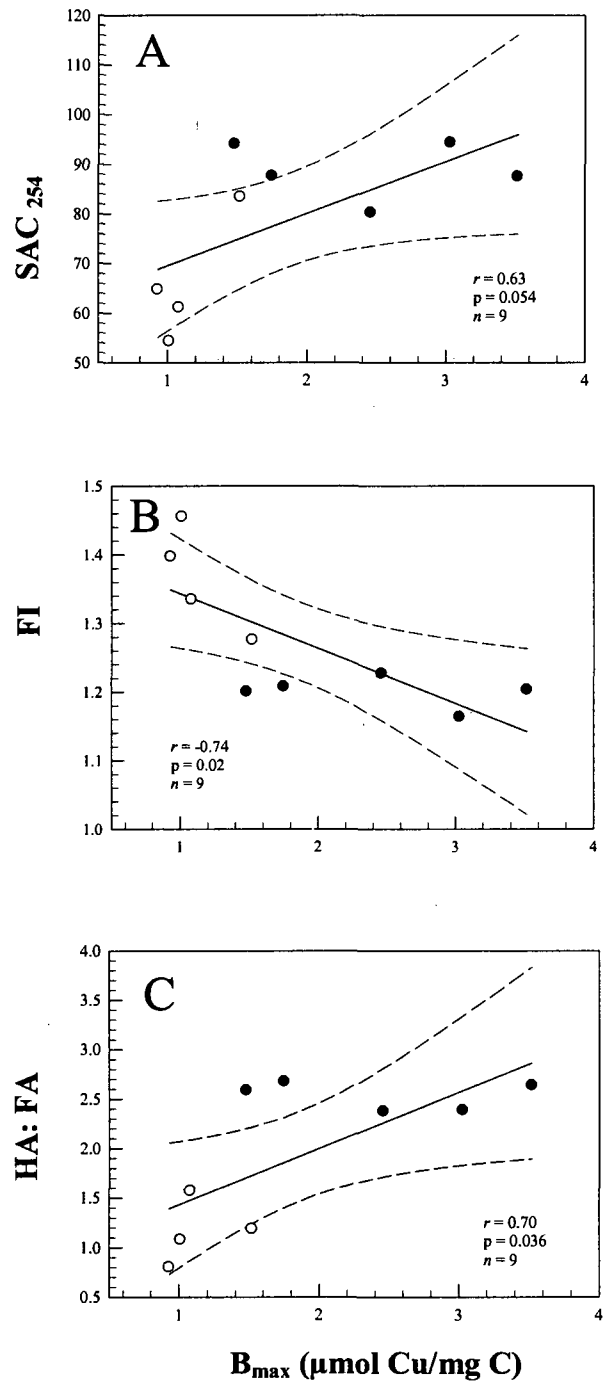


Figure 2-6 Correlations of B_{max} with SAC_{254} (A), FI (B), and HA:FA ratio (C). Correlations were completed with the Pearson product moment method, where n=9 for all correlations. Correlations in B and C were the only two that were significant ($p<0.05$). Open circles are Nipissing and 2007 sources, while closed circles are 2008 sources. Dashed lines represent the 95% confidence intervals.

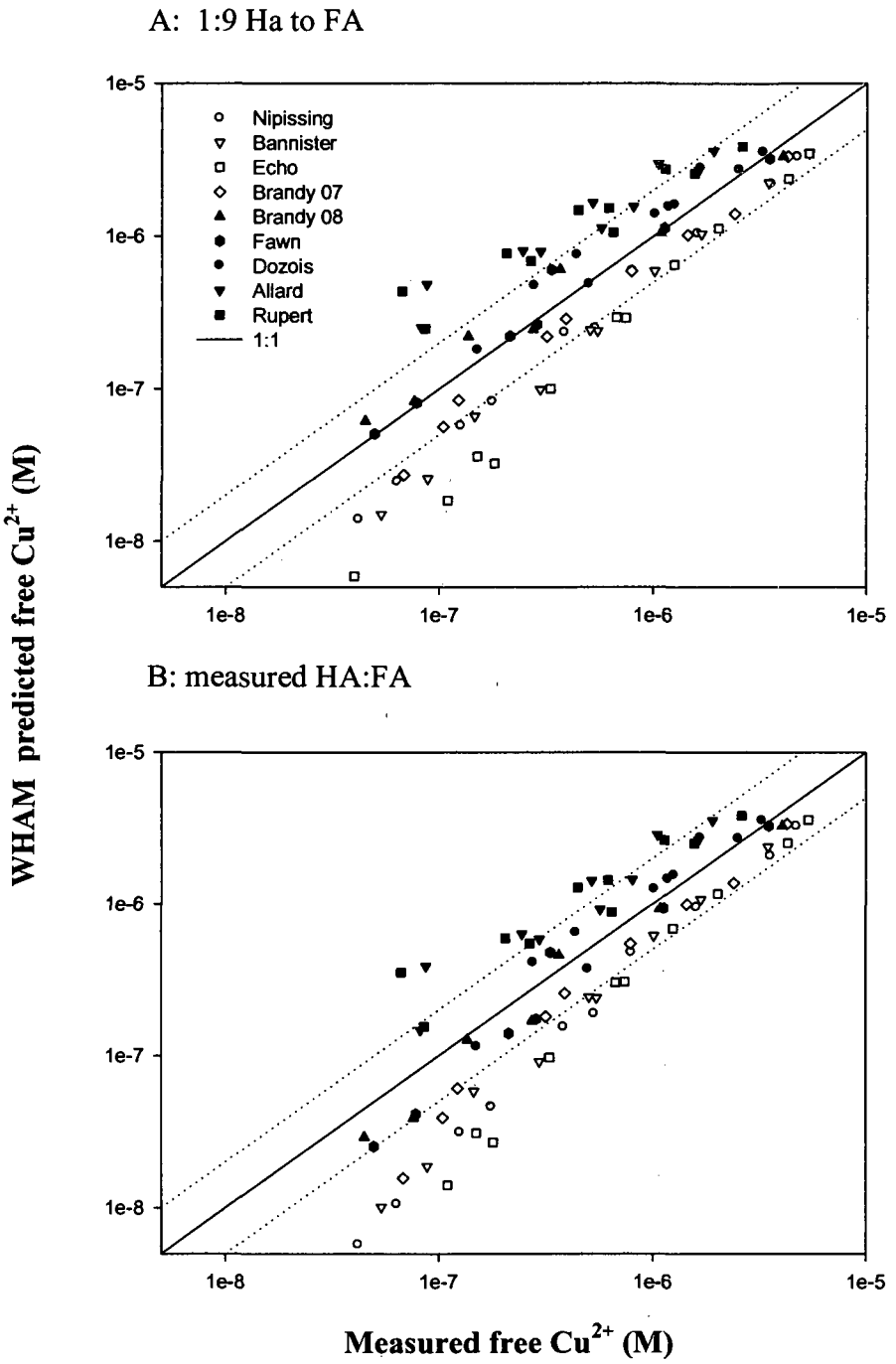


Figure 2-7 WHAM predicted free Cu^{2+} compared to measured free Cu^{2+} from ion selective electrode measurements. WHAM predictions were performed with HA:FA of 1:9 (Fig 7A) and measured HA:FA determined EEMS and PARAFAC (Fig 7B). Open symbols are Nipissing and 2007 sources, while closed symbols are 2008 sources. The solid line represents the line of perfect fit where the dotted lines represent a factor of ± 2. Points between the two dotted lines are accepted as reasonable predictions.

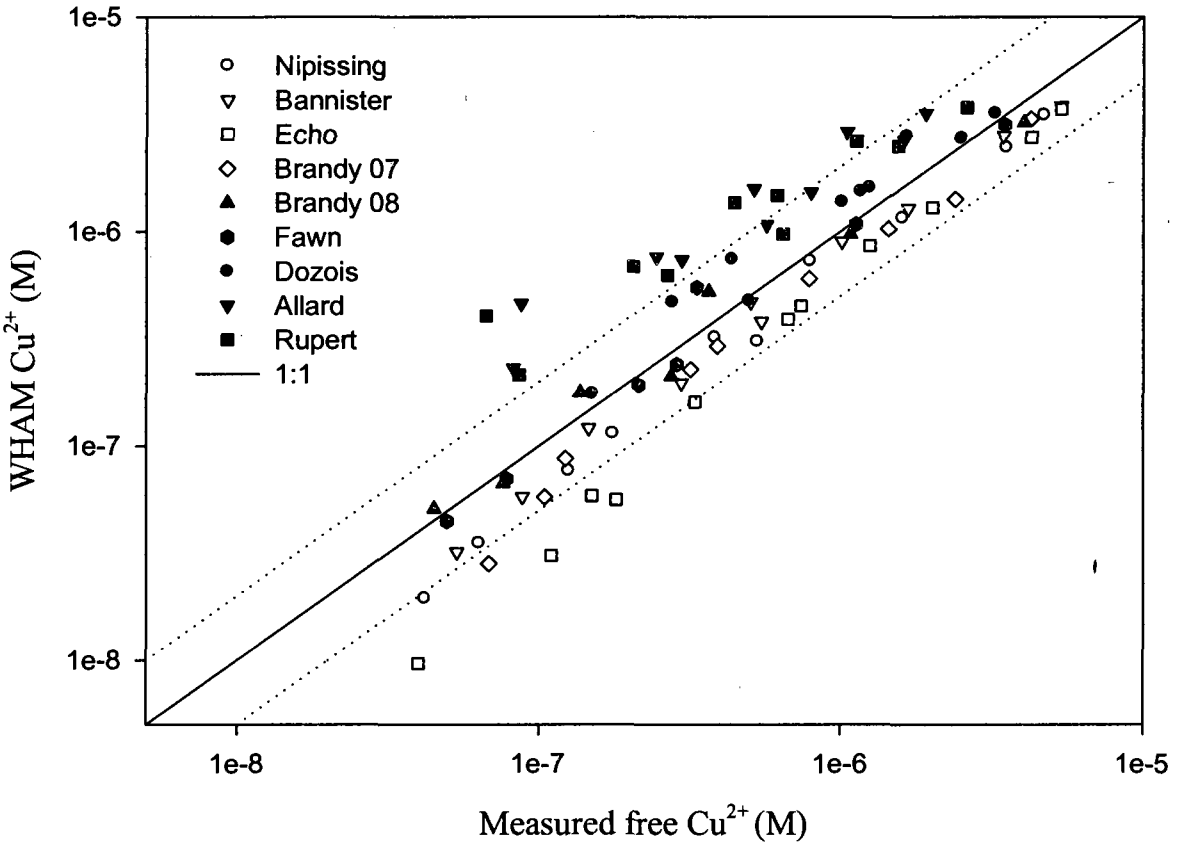


Figure 2-8 Modified WHAM free Cu^{2+} prediction vs. measured free Cu^{2+}. WHAM predictions were still carried out with a 1:9 HA to FA ratio, however with the addition of the quality factor (QF) adopted from Schwartz et al. (2004). The QF = 0.31 ln (SAC_{340}), was multiplied by the DOC concentration to yield a new DOC concentration input. With the addition of the quality factor, WHAM predicted 81% of the time.

Table 2-1 Location, sampling date and background information on the sites from which NOM were collected

Sample Site	Sampling date	GPS Coordinates	Conductivity ($\mu S\ cm^{-1}$)	DOC ($mg\ C\ L^{-1}$)	pH	ecozone
Bannister Lake	06-Oct-07	N 43°18' W 80°23'	275	26.4	7.30	Mixedwood Plains
Echo Lake	10-Nov-07	N 45°16' W 79°06'	27	7.4	6.40	Boreal Shield
Brandy 07 Lake	12-Nov-07	N 45°10' W 79°50'	117	17.5	6.15	Boreal Shield
Brandy 08 Lake	07-Jun-08	N 45°17' W 79°26'	59	13.9	6.29	Boreal Shield
Fawn Lake	08-Jun-08	N 46°17' W 80°00'	28	12.4	5.86	Boreal Shield
Réservoir Dozois	24-Jun-08	N 47°22' W 76°58'	20	4.7	5.78	Boreal Shield
Allard River	25-Jun-08	N 49°34' W 77°48'	78	10.3	6.89	Boreal Shield
Rupert River	26-Jun-08	N 51°21' W 77°24'	16	3.8	6.01	Hudson Plains
Lake Nipissing	01-Jul-03	N 46°17' W 80°00'	nd	nd	nd	Boreal Shield

Notes: nd indicates no data

Table 2-2 The chemical and optical properties of nine NOM sources. The four components humic-like (HA), fulvic-like (FA), tyrosine-like (Tyr) and tryptophan-like (Trypt), as determined by PARAFAC, are given in percent of total fluorescent units. These four components explained 92% of the variability among NOM sources. Total protein content is shown as μg protein per mg DOC and $SAC_{254-436}$ values are in cm^2 per mg DOC. The quality factor (QF) was derived from the SAC_{340} (see text).

NOM Source	%HA	%FA	%Tyr	%Trypt	HA:FA	FI	SAC_{254}	SAC_{340}	SAC_{436}	QF	protein	%DOC < 1 kDa
Echo Lake	39.4	49.1	5.8	5.7	0.8	1.40	65	14	4	0.82	39.6	17.3
Bannister Lake	45.6	42.2	5.9	6.2	1.1	1.46	54	11	3	0.74	14.3	65.3
Brandy Lake (07)	50.7	42.6	3.4	3.2	1.2	1.28	83	24	6	0.99	55.9	12.7
Brandy Lake (08)	71.5	27.6	0.9	0.0	2.6	1.20	94	33	8	1.08	29.8	16.0
Fawn Lake	70.0	26.2	1.6	2.3	2.7	1.21	88	30	7	1.05	30.4	12.7
Réservoir Dozois	66.7	28.1	2.7	2.5	2.4	1.23	80	26	6	1.01	24.9	11.7
Allard River	71.9	27.3	0.8	0.1	2.6	1.20	88	28	6	1.03	39.06	10.04
Rupert River	68.8	28.8	2.2	0.2	2.4	1.16	94	30	8	1.05	40.60	14.22
Lake Nipissing	60.8	38.7	0.5	0.0	1.6	1.33	61	17	5	0.88	35.7	40.6

Table 2-3 Regression line slopes, y-intercepts and r^2 values for the linear relationships of free Cu^{2+} and total Cu at a DOC concentration of 2.4 mg C/L (see Fig 2-5).

NOM sources	Slope	y-intercept	r^2
no added DOC	0.84	-0.26	0.97
Nipissing	0.74	-0.65	1.00
Bannister	0.82	-0.81	0.99
Echo	0.82	-0.67	1.00
Brandy 07	0.68	-0.66	0.99
Brandy 08	0.65	-0.74	0.98
Fawn	0.57	-0.62	0.98
Dozois	0.53	-0.89	0.99
Allard	0.33	-0.55	0.98
Rupert	0.43	-0.98	0.97

Table 2-4 Binding affinity (Log K) and maximum binding capacity (B_{max}) for 9 NOM sources. Values were calculated from the Cu binding curves developed from Cu^{2+} measurements using a single site hyperbolic saturation model (see appendix B).

NOM Source	Log K	B_{max} (μmol Cu/mg C)
Lake Nipissing	6.31	1.08 ± 0.10
Echo Lake	6.19	0.93 ± 0.07
Bannister Lake	6.25	1.01 ± 0.08
Brandy Lake (07)	6.07	1.52 ± 0.17
Brandy Lake (08)	6.45	1.48 ± 0.16
Fawn Lake	6.28	1.75 ± 0.20
Réservoir Dozois	5.97	2.46 ± 0.34
Allard River	6.02	3.52 ± 0.78
Rupert River	6.13	3.03 ± 0.56

Table 2-5 Correlation coefficients (r) for measured NOM variables (Table 2-2) with B_{max} and Log K. A * indicates significance ($p<0.05$), n=9.

	B_{max}	Log K
HA	0.68 *	0.02
FA	(-) 0.70 *	0.04
Tyr	(-) 0.47	(-)0.15
Tryp	(-)0.56	(-)0.10
HA:FA	0.70 *	(-)0.013
FI	(-) 0.74 *	0.18
SAC_{254}	0.66	(-)0.12
SAC_{340}	0.63	(-)0.02
SAC_{436}	0.62	(-)0.01
Protein	0.18	(-)0.30
%DOC< 1 kDa	(-)0.55	0.36

Chapter 3

Short Term Accumulation and Acute Toxicity of Cu to Rainbow Trout in the Presence of Different Natural Organic Matter Sources

3.1 Introduction

The sensitivity of aquatic organisms to metals varies depending on physico-chemical nature of the receiving waters. For example, pH, inorganic (Cl^-, HCO_3^-, SO_4^{2-}) and organic ligands (natural organic matter-NOM) can alter the concentration of the free metal ion, which is usually the form most bioavailable and toxic. As well, cations (Ca^{2+}, Na^+, Mg^{2+}) can compete with the free metal ion for physiological active uptake sites at the biological membrane (Di Toro et al., 2001).

One important toxicity mitigating component in natural waters is natural organic matter (NOM). NOM is divided into two general categories, autochthonous NOM (aquatically derived) and allochthonous NOM (terrestrially derived) (Thurman, 1985). Autochthonous NOM typically has more carbohydrates and nitrogen-containing groups and is lightly coloured, whereas allochthonous NOM is enriched in aromatic humic and fulvic acids, highly coloured and easily absorbs ultraviolet light (Richards et al., 2001). NOM heterogeneous nature makes it difficult to characterize, however, it is known that the humic and fulvic acids have strong binding sites which are capable of complexing metals (Thurman, 1985). Recent studies have focused on determining if NOMs from different sources have different binding capacities for metals, thus differentially affecting metal speciation and toxicity to both fish (Richards et al., 2001; Schwartz et al., 2004; Adam et al., 2004; Luider et al., 2004; VanGenderen et al., 2003) and invertebrates (Kramer et al., 2004; De Schamphelaere et al., 2004; Glover et al., 2005). In addition, these studies attempted to characterize the NOM to determine what NOM property best predicts their ability to reduce metal accumulation and toxicity.

Copper is an example of a metal whose speciation is largely influenced by NOM due to its high binding affinity (Log $K_{NOM\text{-}Cu} = 9.1$; Playle, 1993b). Thus NOM has a large influence on Cu accumulation and toxicity to fish (Erickson et al., 1996). Various studies have examined the effects of NOM sources and Cu accumulation and toxicity to fish, and revealed differences among NOM source protection. For example, Ryan et al. (2004) who exposed larval fathead minnow (*Pimephales promelas*) to Cu in the presence of different NOM sources observed up to a six fold difference in acute Cu toxicity. Studies conducted by both Schwartz et al. (2004) and Richards et al. (2001) observed differences in NOM sources in the degree to which Cu accumulation and toxicity was reduced in rainbow trout (*Oncorhynchus mykiss*). These studies also related NOM characteristics to their ability to reduce Cu accumulation and toxicity, where darker, more aromatic (allochthonous-like) NOM was more protective than lighter, less aromatic (autochthonous-like) NOM.

Measuring Cu accumulation in the gills of fish has been a common method for assessing metal bioavailability because the gills are the first point of contact between waterborne metal and the fish (Wood, 2001). Metal binding to the fish gills, if sufficient enough, typically results in ionoregulatory disruption resulting in death of the fish (Wood, 2001). Therefore, accumulation can be directly linked with toxicity (Di Toro et al., 2001). An understanding of gill accumulation in relation to acute toxicity has led to the development of toxicity prediction models, such as the biotic ligand model (BLM). The BLM predicts toxicity on a site specific water chemistry basis by accounting for competition of the free ionic metal with other cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}), together with complexation by various inorganic (Cl^{-}, HCO_3^{-}, SO_4^{-2}) and organic (NOM) ligands

(Di Toro et al., 2001). Concerns regarding the BLM is that it only considers the quantity of NOM, and other than an adjustment factor for the relative fraction of humic and fulvic acids, there are no other toxicity mitigating characteristics of NOM incorporated within the model.

In a previous study (Chapter 2), Cu binding properties of different NOM sources collected from soft water lakes on the Canadian Shield were examined. NOM sources were biologically characterized by examining their ability to reduce Cu toxicity and accumulation in the gills of rainbow trout (*Oncorhynchus mykiss*). The objective of the present work was to determine if chemical and/or optical characteristics could be used to predict differences among NOM source in Cu toxicity and accumulation mitigation, as well as to evaluate whether the BLM needs to be modified to better predict Cu toxicity in the presence of difference NOM sources.

3.2 Materials and Methods

3.2.1 Fish Husbandry

Juvenile rainbow trout were purchased from Humber Springs Trout Hatchery (Orangeville, Ontario). Fish were held in 180L tanks supplied with well-aerated flowing water. Initially, fish were put in a 1:1 mix of hard (>500 mg/L as $CaCO_3$) well water and soft water produced by reverse osmosis. Fish were acclimatized to soft water by gradually decreasing the flow of the well water over a two-week period. Fish were not used for experimentation prior to acclimation. Final chemistry of the soft water was approximately 67.1 ± 6.5 Ca, 36.3 ± 6.5 Mg, 139.6 ± 11.2 Na (all in μM; n=7), with an

approximate conductivity of 40 μScm^{-1}. Fish were fed daily to satiation; however, they were not fed two days before nor during the experimentation to eliminate the possibility of metal complexation with food or fecal matter. Fish rearing and experimentation was done following CCAC guidelines as administered by the Wilfrid Laurier University Animal Care Committee.

3.2.2 NOM sources

Samples of NOM from waters throughout south central Canada were collected and concentrated by reverse osmosis in the period of October 2007 to June 2008. Collections were done at six soft water sources and one hard water source and one of the soft water sources (Brandy Lake) was sampled twice (2007 and 2008). Previous NOM collections conducted by R. Playle (Wilfrid Laurier University) in 2003 at Lake Nipissing provided a ninth source of NOM. For simplicity, the samples from 2007 and 2003 (Bannister, Echo, Brandy 07, and Nipissing) will be called group A and all 2008 sources (Brandy 08, Fawn, Dozois, Allard, Rupert) will be called group B throughout the paper. More detailed description on locations, site characteristics as well as NOM collection and preservation are in Chapter 2.

3.2.3 Short term gill Cu accumulation

Preliminary experiments

The concentration of waterborne Cu selected for these experiments was based on preliminary gill binding experiments supplemented by modeling to estimate the Cu and DOC mixtures that were likely to produce a gradient of bioavailable Cu for uptake (i.e.

range of concentrations of complexed Cu and Cu^{2+}). Preliminary experiments included different exposure times (3-24 h) and exposure to a variety of Cu concentrations. In the first preliminary experiment, a 6-h exposure to a concentration of 0.25 μM Cu (twice the 96h LC50 for rainbow trout in soft waters determined in our lab water; unpublished results) with the addition of 10 mg C/L DOC from different NOM sources was performed. In the second, a series of Cu titrations were performed with concentrations ranging from 0.5-3 μM Cu with no NOM added to establish short term 3 h gill binding kinetics. For all the experiments with DOC, the concentration was kept in the range of 2-10 mg C/L which is the typical range found in Canadian Shield lakes (David et al., 1997).

3h gill-Cu binding with multiple DOC sources

To assess the effects of different NOM sources on Cu binding to fish gills, fish were exposed to 2 μM total Cu (added as $CuSO_4 \cdot 5H_20$; Sigma-Aldrich, St Louis, MO) with NOM added at nominal concentrations of 0, 2.5, 5, and 8 mg C/L for 3 h. Cu and NOM concentration were chosen based on preliminary results (see below). Separate gill binding experiments were done for group A and group B sources. Cu and NOM exposures were done in acid washed polyethylene tanks filled with 10 L of aerated soft water with the same composure as culture water (see section 3.2.1). For each set of the experiments there was a positive control (Cu alone) and a negative control (no added Cu). One NOM control was run with the Bannister Lake source at 10 mg C/L with no added Cu to determine if NOM altered the background concentration of Cu on the gills. Before exposures, assay water containing both NOM and Cu were equilibrated for 24 h to allow time for Cu to bind to NOM (Ma et al., 1999). Solution temperature was controlled by

placing tanks in a constant temperature water bath and solution pH was maintained at 6.5 using KOH (Sigma-Aldrich, St Louis, MO, USA). Six fish were non-selectively removed from the rearing tank and added to each exposure tank. Water pH, conductivity and temperature were recorded at 0 h and 3 h. During the 3 h experiment, water samples were collected at 0 h (before addition of fish), 1h and 3h, one for DOC analysis, the other for metal and ion analysis. After the 3 h exposures fish were euthanized with 0.2 g/L tricaine methanesulfonate (Aqua Life, Syndel Laboratories Ltd., Vancouver, BC) and gills were excised, rinsed in 100 mL deionized water for 10 s and collected in 1.5 mL centrifuge tubes. Gills were then weighed, digested in a volume of 1N TraceMetal grade HNO_3 (Fisher Scientific, Nepean, ON) equivalent to five times their wet weight, and then baked for 3 h at 80 °C (Richards et al., 2001). After digestion, gill tissues were mixed by vortexing and then centrifuged at 12,000 rpm for 2 min. Supernatant was collected and diluted 10 fold with 1 % acidified (16N TraceMetal grade HNO_3) ultra-pure water to measure for Cu. Short term gill binding tests on group A NOM sources were done as single exposures (unreplicated; due to limited NOM) with an n = 6 fish per concentration per NOM source. Short term gill binding tests on the group B NOM sources followed the same protocol, however, this time in duplicate with n=4 fish in each replicate (n=8).

Cu accumulation and Na^+/K^+ ATPase activity after 24h exposures

Longer-term exposures (24 h) in the presence of Cu and NOM were done to measure gill Cu accumulation and Na^+/K^+ ATPase activity and whether NOMs protected against Cu induced inhibition of enzyme activity. Two NOM sources, Bannister and Brandy Lake 07, were chosen because they revealed the most differences in previous gill

accumulation studies. Exposures were conducted in a similar manner to 3 h gill accumulation (above), except that exposure Cu concentration chosen was 1 μM Cu, since fish could not survive for 24 h at 2 μM Cu. For the exposures there was a control (no Cu or NOM added), Cu only exposure and the Cu with the additions of DOC at nominal concentrations 2.5 and 5 mg C/L from the 2 NOM sources. Six fish were added to 12 L of assay water and gills were sampled at 24 h. The pH tended to increase during the exposure and was adjusted with diluted HNO_3 (TraceMetal grade). Gills were sampled for both Cu accumulation and Na^+/K^+ ATPase activity. Gill sampled for Na^+/K^+ ATPase were immediately frozen in liquid N_2 and stored at -80°C. Gill Na^+/K^+ ATPase activity was measured using microplate method of McCormick (1993) (see appendix C for method details).

3.2.4 Acute toxicity experiments

Acute toxicity experiments (96 h LC_{50}) were conducted with rainbow trout (1-3 g) to determine if different NOM sources provided different degrees of protection against Cu toxicity. Six toxicity tests were conducted, a control (no NOM added) and with the addition of 4 mg C/L for each of five NOM sources from group B. Acute toxicity exposures were not replicated because NOM was limited. Eighty liters of soft water (same composition as culture water) and 4 mg C/L of one of the NOM sources was mixed and pH adjusted to 6.5 with KOH in an acid washed 100 L polyethylene tank. Aliquots of 10 L were divided into eight acid washed polyethylene tanks to which Cu (as $CuSO_4$. $5H_2O$) was added in increasing concentrations. Exposure tanks were placed in a water bath to maintain a constant temperature (14.2 ± 0.7°C) and water was left to equilibrate

for 24 h. Ten fish were non-selectively assigned to each of eight tanks. This was a static renewal system where 50% of the water was renewed after 24 h (which was previously equilibrated for 24 h). During the renewal process, water was siphoned out of each tank removing as much of the fecal matter and debris as possible. The pH was readjusted with KOH after each renewal. The pH, temperature and conductivity were recorded and water samples for metal, ion and DOC analyses were taken at 0, 24, 48, 72, and 96 h. The endpoint of the test was loss of equilibrium or death, in which case they were immediately removed and fish weight was recorded.

3.2.5 Modelling

Acute toxicity predictions were completed using the BLM software version 2.2.3 developed by HydroQual, Inc., Mahwah, New Jersey, USA. (HydroQual 2005). Predictions were carried out by inputting the measured water chemistry variables from each 96-h Cu LC50 into the Cu BLM for rainbow trout. The hardness was calculated based on the measured Ca and Mg concentrations and entered in as alkalinity, since hardness is usually equal to alkalinity. SO_4^{-2} and S were not measured and so were not entered into the BLM. K^+ and Cl^- were estimated since K^+ generally doesn't compete with Cu uptake (Erickson et al., 1996) and CuCl complexes are less likely to form due to its extremely low binding affinity (Log k=0.4; Santore et al., 2001). BLM predictions were employed using different modeling scenarios which included the unmodified BLM, LA50 adjustment for the rainbow trout sensitivity in soft waters, and adjustments to the relative % active DOC and % HA and FA.

3.2.6 Analytical techniques

For metal ion analyses 10 mL samples were collected in 14mL test tubes and acidified to 1% with 16N HNO_3 (TraceMetal grade). Samples for DOC analyses were filtered through a 0.45 μm syringe filter (22 mm Acrodisc HT Tuffryn membranes, Pall Corporation, Ann Arbor, MI, USA) and collected in borosilicate vials (previously combusted for 2 h at 450°C) without any head space and stored at 4°C until analyzed. Water pH was measured using a pH meter (Mettler Toledo SevenGo^{TM} SG2) and conductivity & temperature using a conductivity meter (YSI 30, Yellow Spring, Ohio). Water and gill samples were measured for Cu using a graphite furnace atomic absorption spectrophotometer (SpectraAA 880 GTA100 atomizer, Varian, Mississauga, Ontario). Water Ca, Mg and Na were similarly measured in flame mode (SpectraAA 880). Water samples collected for DOC analyses were measured for total carbon (TC) and inorganic carbon (IC) concentrations using a total carbon analyzer (Schimadzu TOC-5050A, Tokyo, Japan).

3.2.7 Calculations and Statistics

New accumulation of Cu on the gill was calculated by subtracting the background Cu level from the Cu accumulation after 3 h of exposure. DOC was quantified as the difference between TC and IC. The median lethal concentrations (96 h LC50 with 95% confidence intervals (CI)) were calculated within CETIS (Comprehensive Environmental Toxicity Information System; Tidepool Scientific Software, McKinleyville, CA) using the trimmed Spearman-Karber method (Hamilton et. al., 1977). Significant differences between 96 h LC50 values was indicated when 95% CI did not overlap. One-way

analysis of variance (ANOVA) and when appropriate, Tukey's test, was used for multiple comparisons of mean Cu gill accumulation values. Correlations of gill Cu accumulation and of 96 h LC50s with optical, chemical and Cu complexation properties (see results in Chapter 2) were assessed using the Pearson product moment. In all cases, $p<0.05$ was the acceptable level of significance. Graphical presentation, ANOVAs and correlations were performed in the software package SigmaPlot (ver. 11, Systat software, Inc. San Jose, CA, USA). Cu gill accumulation data are presented as means ± SEM and water chemistry variables are presented as means ± 1 SD.

3.3 Results

3.3.1 Short term gill binding

Preliminary experiments

Six hours of exposure to 0.25 μM Cu in the presence of 10 mg C/L DOC from the different sources (group A) resulted in no Cu accumulation on the gills above background levels (Fig 3-1). In the Cu only exposure, gill Cu accumulation increased from approximately 6 nmol/g wet tissue (background) to approximately 10 nmol/g wet tissue. In other preliminary short term gill accumulation experiments there was no difference in the accumulation at 3 h compared to 6 h; therefore, 3 h was chosen. And finally in the third preliminary experiment, a Cu titration exposure demonstrated that Cu accumulation on the gills of rainbow trout at 3 h reached saturation at an exposure concentration of 2 μM total Cu (Fig 3-2). From these preliminary experiments, a concentration of 2 μM Cu

was selected for short term gill Cu accumulation studies on the effect of different NOM sources.

3-h gill accumulation in presence of multiple DOC sources

A control run with NOM only (from Bannister Lake) demonstrated that NOM did not alter gill Cu accumulation compared to background levels. In the presence of group A NOM sources, when the DOC concentration is increased (nominal 2.5, 5 and 8 mg C/L) in the presence of 2 μM Cu, gill Cu accumulation is reduced (Fig 3-3). At 8 mg C/L, new gill Cu is effectively reduced to zero. The reduction of gill Cu accumulation varied between sources. This is particularly clear at the lowest DOC concentration (nominal 2.5 mg C/L). Compared to Cu only exposure, the percent reduction of Cu accumulation on the gills at nominal 2.5 mg C/L was, Bannister Lake (13%), Echo Lake (24%), Lake Nipissing (33%), and Brandy Lake (48%). Bannister Lake was significantly different from Brandy Lake 07. At the higher DOC concentrations, Brandy Lake consistently reduced gill Cu accumulation the most; however, there were no significantly differences among the other sources at the other DOC concentrations. Measured water chemistries for the exposures were similar and are summarized in Table 3-1.

In group B, similar to group A, with increased DOC, there is the common trend of decrease in gill Cu accumulation; however, a couple of the DOC sources (Fawn and Brandy 08) had no effect on gill Cu accumulation going from 5 to 8 mg C/L (Fig 3-4). There were no significant differences among sources in their ability to reduce Cu gill accumulation at DOC concentration of 2.5, 5 or 8 mg C/L. However, at the lower DOC concentration of 2.5 mg C/L, it did appear that Allard NOM reduced gill Cu

accumulation better than Brandy 08. Measured water chemistries for the exposures were similar and are summarized in Table 3-2.

Cu accumulation and Na^+/K^+ ATPase activity after 24 h of exposure

Figure 3-5 illustrates 24 h Cu accumulation and associated Na^+/K^+ ATPase activity. Compared to the Cu only exposure, both NOM sources at each DOC concentration significantly reduced gill Cu accumulation. Among NOM sources, Bannister and Brandy 07 at the lower DOC concentrations (measured at 4.0 and 3.8 mg C/L respectively) did not significantly differ ($p=0.407$). However, there was a significant difference between Bannister and Brandy 07 at the higher DOC concentrations (measured 6.6 and 5.9 mg C/L respectively), where Brandy 07 reduced gill accumulation the most. For Na^+/K^+ ATPase activity measurements, there were no significant differences. In the Cu only exposure, Na^+/K^+ ATPase activity was lowest compared to background levels and with the addition of NOM, Na^+/K^+ ATPase activity seem to rebound to background levels (Fig 3-5). Water chemistry variables measured over the 24 h exposure are summarized in Table 3-3. DOC, Ca, Mg and Cu concentrations remained relatively constant. The pH of test solutions increased over time so the pH at time 0 h and time 24 h are shown in Table 3-3. Similarly Na concentrations also increased over the period of exposure, particularly in the Cu only exposure, therefore values are entered for time 0 h and time 24 h.

3.3.2 Acute toxicity experiments

In all LC50s, there was 100% survival in the Cu-free (control) exposure tests. In the presence of nominal 4 mg C/L, all group B NOM sources significantly increased the 96 h LC50 compared to the no added NOM test (Fig 3-6). Brandy Lake was the least protective and Allard River was significantly the most protective by approximately 3.5-fold. The 96 h LC50 between four of the sources (Brandy, Fawn, Dozois and Rupert) were very similar, but Fawn and Dozois were significantly greater than Brandy. Water ions, pH, and DOC were consistent among the toxicity test (Table 3-4).

3.3.3 Correlations among gill Cu accumulation, toxicity, and NOM characterizations

The chemical, optical and Cu complexation characteristics of the different NOM sources are summarized in Table 2-1 (optical/chemical properties) and Table 2-4 (Log K and B_{max}). Correlation coefficients (r) between NOM measured variables and gill accumulation data (from group A) and acute toxicity (group B) are in Table 3-5. Due to a lack of significant differences between group B sources in the gill Cu accumulation studies (Fig 3-4) and the lack of significant correlation to chemical and optical characteristics, they were not included in Table 3-5. Those NOM variables which demonstrated significant correlations with gill Cu accumulation studies with group A sources were FI and SAC_{340}. FI demonstrated a positive relationship, whereas SAC_{340} demonstrated a negative relationship (Table 3-5).

The relationship between 96h LC50s and short term gill accumulation at 2.5 mg C/L is shown in Figure 3-7. There was a significant negative correlation where more Cu accumulation on the gill was associated with a lower 96 h LC50 value. Among the NOM

variables, %DOC <1 kDa was the only variable which demonstrated a significant relationship with 96 h LC50s where a higher % DOC< 1 kDa resulted in greater toxicity.

3.3.4 BLM modelling

Measured and BLM predicted LC50s are in Table 3-6. The first BLM prediction scenario was the unmodified BLM which consistently underestimated toxicity. The control (no NOM added) LC50 was underestimated by 10 fold. The BLM prediction for Brandy Lake 08 was underestimated by four fold, Fawn, Dozois, Rupert were underestimated by approximately two-three fold and Allard was slightly underestimated, however, it fell within the acceptable factor of 2. The second scenario for predictions was a calibration of the BLM which involved adjusting the LA50 so that the control (no NOM added) 96 h LC50s matched that predicted by the BLM. Predictions with the newly calibrated BLM over estimated the 96 h LC50 when NOM was added. The third scenarios of predictions were carried out with the newly calibrated BLM assuming 50% HA (opposed to the default of 10%). These predictions were more accurate than the other BLM predictions and are all within the acceptable prediction range of a factor of 2. The fourth scenario of predictions was the approach taken by De Schamphelaere et al. (2004) where 50% active DOC (therefore dividing the DOC by 2) and 100% FA are assumed. This increased the predictive capabilities of the BLM. Aside from the control, this BLM prediction scenario was within the acceptable range of a factor of 2.

3.4 Discussion

Gill Cu accumulation, 96 h LC50s, and inhibition of Na^+/K^+ ATPase activity in rainbow trout were used as biological parameters to test whether differences exist in the ability of NOM from different sources to complex with Cu. The range of sources collected across Canadian Shield waters revealed both similarities and differences. Groups A sources demonstrated different degrees in decreasing gill Cu accumulation where more allochthonous-like NOMs were more effective than autochthonous NOMs-like sources (Fig 3-3). Group B sources, which were all allochthonous-like and did not vary much chemically or optically, were similar in the degree with which they reduced gill Cu accumulation (Fig 3-4). However, there was a 3.5 fold difference among NOM ability to reduce acute Cu toxicity among the 5 group B sources (Fig3-6).

Preliminary experimentation was crucial for determining the optimum combination of concentrations of NOM and Cu to reveal any differences among NOM sources in their ability to reduce gill Cu accumulation. Three-hour accumulation studies were chosen because they are commonly used as 3 h allows enough time for Cu to interact at the surface of the gill. Preliminary testing confirmed this (Fig 3-1 and 3-2). Fast binding of Cu to the gill likely represents binding of Cu at physiologically active uptake sites (Campbell, 1995) for example, Na uptake channels. Longer exposures could have resulted in Cu binding to non-specific sites on the gill and thus might not correlate well acute toxicity (Campbell, 1995; Playle, 1998). It is well documented that short term gill Cu accumulation is a good indicator of acute Cu toxicity (Fig 3-7; Playle et al., 1993 a, b).

In both group A and B, greater variability in the NOM sources to reduce gill Cu accumulation were more pronounced at lower DOC concentrations. This is likely because lower concentrations of NOM resulted in lower numbers of Cu binding sites in solution, thus there was more free Cu^{2+} available to bind to the fish gill (Playle et al., 1993a; Hollis et al., 1997). Higher concentration of DOC binds all of the bioavailable Cu resulting in no accumulation (Fig 3-3 and 3-4). A similar trend was observed in Luider et al. (2004) where at higher free Cu^{2+} concentrations they observed greater variability among NOM sources. The variability among NOM sources to reduced gill Cu accumulation is likely a result of a difference in the number of NOM Cu binding sites (B_{max}) and not the relative affinity (Log K) although it did vary. The Log K or B_{max} did not significantly correlate with either gill binding data or acute toxicity. However, a better relationship was established with B_{max} (group A gill binding, $r = -0.879$, $p=0.120$; group B toxicity, $r = 0.755$, $p=0.140$). This suggests that the amount of Cu binding sites among the NOM sources is a lot more influential than the Cu binding affinity. For example, among the group A comparisons, Brandy Lake 07 has the highest B_{max} yet the lowest Log K, and reduced gill Cu accumulation the most. Similarly, among the group B comparisons, Allard River had the higher B_{max} and second lowest Log K, and had the greatest protective effect against Cu toxicity.

Variability among sources could also be attributed to the inorganic chemistry of the waters thus it was important to measure cations in the assay waters, particularly Na, since the primary site of Cu toxicity is the Na transport mechanism in the gill (Lauren and McDonald, 1987). In the present study, the variation that is observed in Cu gill

accumulation cannot be attributed to differences in cationic competition since the concentrations of Na, Mg, and Ca were similar among NOM sources (Table 3-1 and 3-2).

Greater variability among group A sources compared to group B sources in their ability to reduce gill Cu accumulation is likely a result of the greater variability in both NOM chemical and optical properties (Table 3-5). Group A had greater variability in their optical characteristics (SAC_{340}) and in their FI which correlated significantly with the gill Cu accumulation. The protein concentrations among NOM sources in Group A also varied and was suggestive of a relationship, however, it was not statistically significant ($p=0.064$).

SAC_{340} and FI are two characteristics used to distinguish between allochthonous and autochthonous NOMs (McKnight et al., 2001; Richards et al., 2001). SAC_{340} is a measure of colour, where a higher SAC_{340} is indicative of a darker NOM containing more aromatic groups (Richards et al., 2001). Optically darker NOM are characteristic of allochthonous (terrestrial) derived NOM. In group A, SAC_{340} range from 11 (autochthonous-like) to 24 (allochthonous-like). A higher FI is indicative of a more autochthonous NOM where a lower FI is indicative of a more allochthonous NOM (McKnight et al,. 2001). The allochthonous-like NOMs in group A reduced gill Cu accumulation more than the autochthonous-like NOMs demonstrating a possible link between binding of aromatic groups and reduced bioavailability.

These findings suggest that SAC_{340} and/or FI are good measures which could be used to predict differences among NOM sources in the ability to reduce Cu bioavailability and toxicity. This agrees well with other trends in the literature. For example, Ryan et al (2004) demonstrated that higher SAC_{350} values (more allochthonous-

like) among NOMs sources reduced acute Cu toxicity to fathead minnow, more than lower SAC_{350} values. As well, more allochthonous NOM have been demonstrated to better reduce the accumulations of Cu (Luider et al., 2004) and mixtures of Cu and Pb on the gills of rainbow trout (Schwartz et al., 2004; Richards et al., 2001). Optical properties of NOM have also been shown to be good predictors of Ag (Glover et al., 2005) and Cu (De Schamphelaere et al., 2004) toxicity to *Daphnia magna*. The FI has also been shown to improve the prediction of DOC effects on Cu gill accumulation (Luider et al., 2004), however, optical absorbance in the UV range (i.e. SAC_{340}) has been demonstrated to be a better predictor in this study and in Luider et al (2004).

In contrast to group A, group B NOM sources are all characteristically allochthonous-like. They all consistently had a 2:1 HA to FA ratio, low FI and high SAC_{340} values (see Table 2-2). In addition, their protein content range was small (24.9-40.6 μg protein/mg C). Lack of variation in chemical and optical properties was associated with very little variation among the NOM sources and how each reduced gill Cu accumulation. However, toxicity varied by a factor of 3.5 among NOM sources. As well, despite little variation in gill Cu accumulation there was still a correlation among Cu accumulation and toxicity (Fig 3-7). This is not surprising since short term accumulation on the gill is usually linked with acute toxicity (MacRae et al., 1999). In addition to differences in toxicity results, NOM sources from group B also demonstrated variation in their ability to complex with Cu as measured by differences in B_{max} measurements. The only chemical measure which significantly correlated with acute toxicity was %DOC<1 kDa. Though this relationship was significant, the %< 1kDa values only differed by as much as 6%, thus this relationship must be looked at

cautiously. We could still speculate, however, that a larger molecular weight NOM could better complex with Cu.

Measuring Na^+/K^+ ATPase activity did not prove to be a sensitive method to examine the effects of NOM source on short term Cu exposures to rainbow trout (Fig 3-5). This method was carried out as an alternative to acute 96 h LC50s due to limited amount of NOM from group A sources. Accumulation of Cu on the gill after 24 h did result in significant differences compared to background levels (Fig 3-5). As well Brandy significantly reduced gill Cu accumulation better than Bannister at similar DOC concentrations. However, despite those differences, there were no differences among Na^+/K^+ ATPase measurements. There was a small decline in Na^+/K^+ ATPase activity in Cu only exposure. This was expected as Cu^{2+} binds to the SH-groups of Na^+/K^+ ATPase (Kone et al., 1990) inhibiting the action of this enzyme. As well, in the Cu only exposure, total water Na increased dramatically from 57 to 205 μM over the 24 h indicating fish loss of Na which is also an indication of the inhibition of Na^+/K^+ ATPase activity (Lauren and McDonald, 1985). Since there was a significant amount of Cu accumulation on the gill after 24 h compared to control, it was expected this accumulation would result in reduced Na^+/K^+ ATPase activity in the fish gills.

A couple reasons why there is a lack of association between accumulation and Na^+/K^+ ATPase activity is suggested here. First, the Na^+/K^+ APTase assay may not be a sensitive enough method to measure smaller changes in activity which may be associated with toxicity. Secondly, lack of association may also be a result of exposure time. During the 24 h period, Cu may be binding to non-specific sites on the gill. In addition, given enough time, Cu will stimulate mucous secretions (Playle, 1998; Toa et al., 2000)

which will readily bind to Cu. Thus measured gill Cu accumulation after 24 h may be a result of external Cu binding to the surface of the gill and not a reflection of internalized Cu which would be responsible for the inhibition of Na^+/K^+ ATPase activity. Similar 24 h exposures of rainbow trout to Cu have been done which also resulted in the lack correlation between Cu uptake and physiological responses which the authors also attributed to a high degree of surface binding (Lauren and McDonald, 1986).

The Cu BLM for rainbow trout was used to test if the measured 96 h LC50 could be predicted. The BLM can be manipulated in many ways, and for this study we presented four different scenarios. In the first scenario the unmodified BLM underestimated acute Cu toxicity. This underestimation is likely a result of rainbow trout sensitivity to Cu in soft waters which is not only due to lower concentrations of competing cations (which the BLM accounts for), but also due to the physiology of the gill. Soft water acclimated trout, compared to hard water acclimated trout have increased number of chloride cells (Greco et al., 1996) to maintain ionic balance in ion poor waters thus have the ability to take up more Na and Na-agonists like Cu (Lauren and McDonald, 1985). Therefore, the second scenario was to adjust the BLM to better predict Cu toxicity to rainbow trout in soft waters (in the absence of NOM) by lowering the LA50 (from 3.7 to 0.09 nmol Cu/g wet tissue). This produced a new calibrated soft water BLM to account for the relative sensitivity of rainbow trout to Cu in soft waters. This new model overestimated Cu toxicity in the presence of NOM, thus it must be further modified to account for NOM.

Scenario 3 and 4 manipulated the %HA and FA as well as the % active DOC to better predict Cu toxicity in the presence of NOM. Scenario 3 took the soft water BLM

and adjusted the HA to 50%, as opposed to the default of 10% which results in an increase in number of NOM Cu binding sites. This decreases the toxicity predictions to within a factor of 2 of the measured values. The fourth scenario took the unmodified BLM and followed the method of De Schamphelaere et al. (2004). This method assumes only 50% of the DOC to be active in binding Cu all which is in the FA fraction. This decreases the amount of Cu binding sites by half. For example, if the test water contains 10 mg C/L DOC the input into the BLM will be 5 mg C/L DOC the rest considered inert. Applying this approach for BLM modelling increased its ability to predict acute Cu toxicity in the presence of NOM, all within the acceptable factor of 2.

These scenarios illustrate that the BLM must be modified to account for both fish sensitivity to Cu in soft water and NOM fraction (both active and %HA/FA). The BLM was not modified to account for NOM source due to few weak correlations among NOM chemical/optical properties. However, for this study, it was not necessary since the modified BLM (scenario 3 and 4) accurately predicted acute Cu toxicity in the presence of the different NOM sources within a factor of 2.

3.5 Conclusions

This study illustrated that there are differences in NOMs across the Canadian Shield waters. They illustrated differences by their ability to reduce gill Cu accumulation and acute Cu toxicity. Group A sources illustrated that allochthonous-like NOM (high SAC340, low FI) reduced gill Cu accumulation better than autochthonous-like NOM (low SAC340, high FI). Group B sources, however, did not show much variation in their

chemical or optical properties as well as their ability to reduce gill Cu accumulation. However, there were significant differences in their toxicity reducing capacity. Typically, the DOC concentration is the best predictor of acute Cu toxicity (Ryan et al., 2004), however, this study demonstrates that some other factor other than DOC concentration is altering Cu toxicity. The fraction of DOC <1 kDa was the only measure which significantly correlated with acute Cu toxicity therefore mw fractionation of NOM sources may be worthy of study. Although toxicity varied among NOM sources, modification of the BLM to account for both fish sensitivity to Cu in soft waters and NOM fraction accurately predicted acute Cu toxicity. If we were to modify the BLM by accounting for NOM differences it is unknown how we would do that.

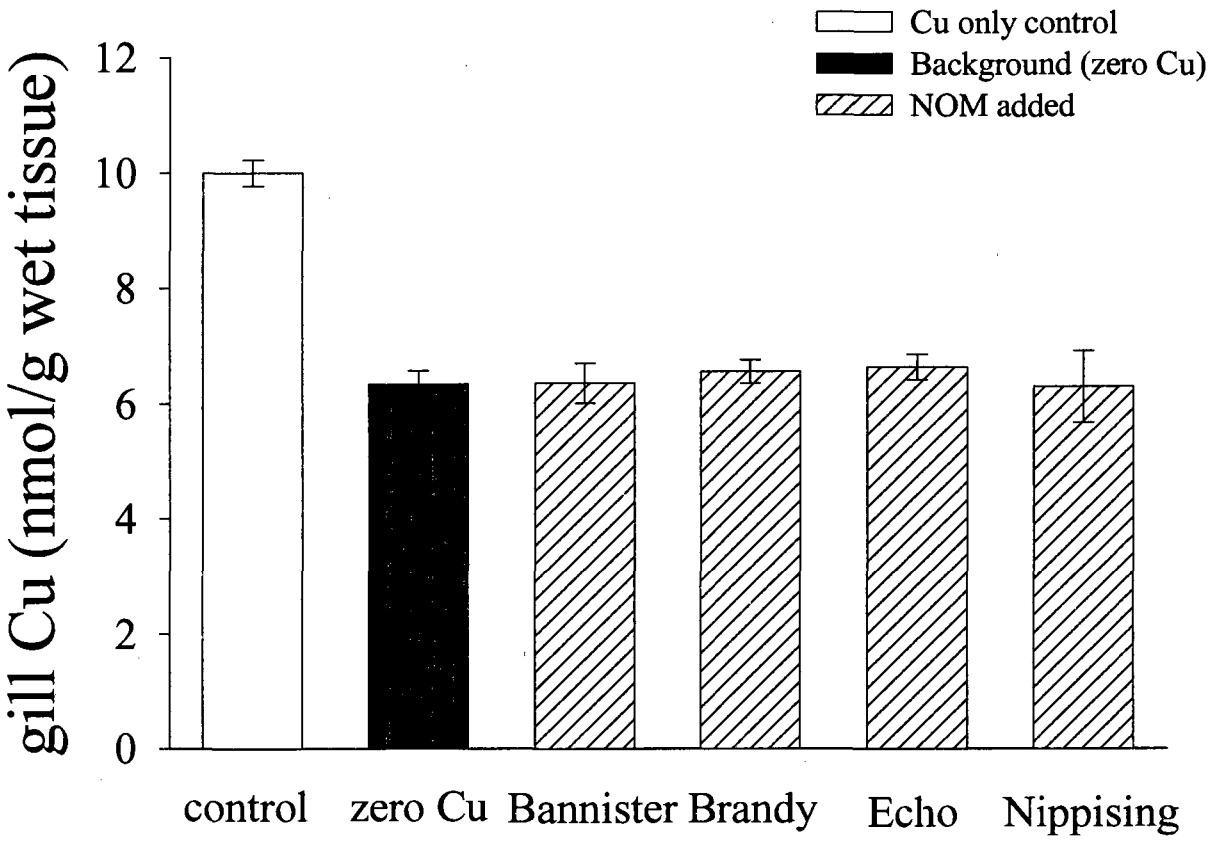


Figure 3-1 Mean gill Cu accumulation after 6 h exposure to 0.25 μM Cu. The open bars shows soft water with no added DOC while the hatched bars show NOM sources at 10 mg C/L. The black bar represents the background gill Cu content. Each bar is the mean of 6 fish (± SEM).

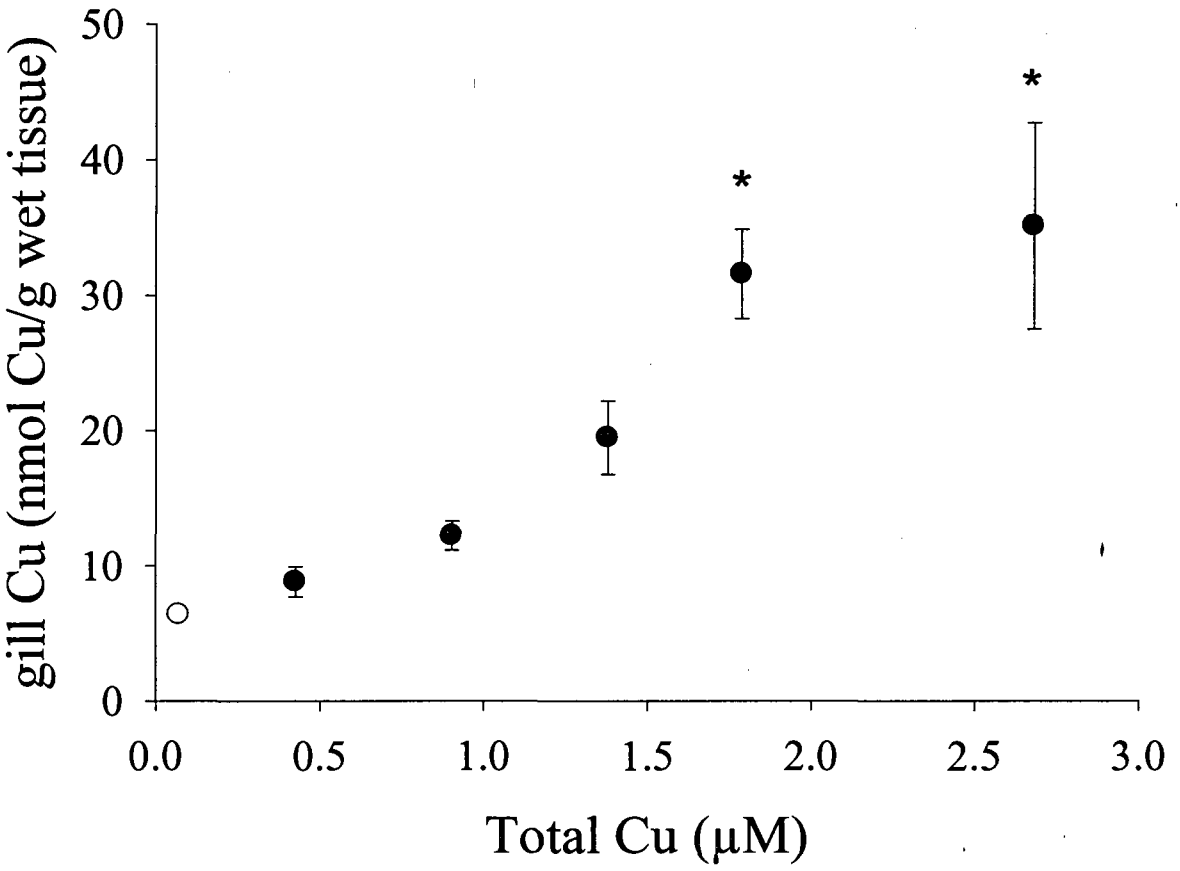


Figure 3-2 Mean short term (3 h) gill Cu accumulation in juvenile rainbow trout exposed to nominal 0.5, 1, 1.5, 2 and 3 μM Cu in soft waters (closed circles). The open circle represents background Cu content. This was a range finder experiment to determine a concentration of Cu which produced significant accumulation. A * indicates significant difference from background Cu content. Each point represents the mean of 6 fish (± SEM).

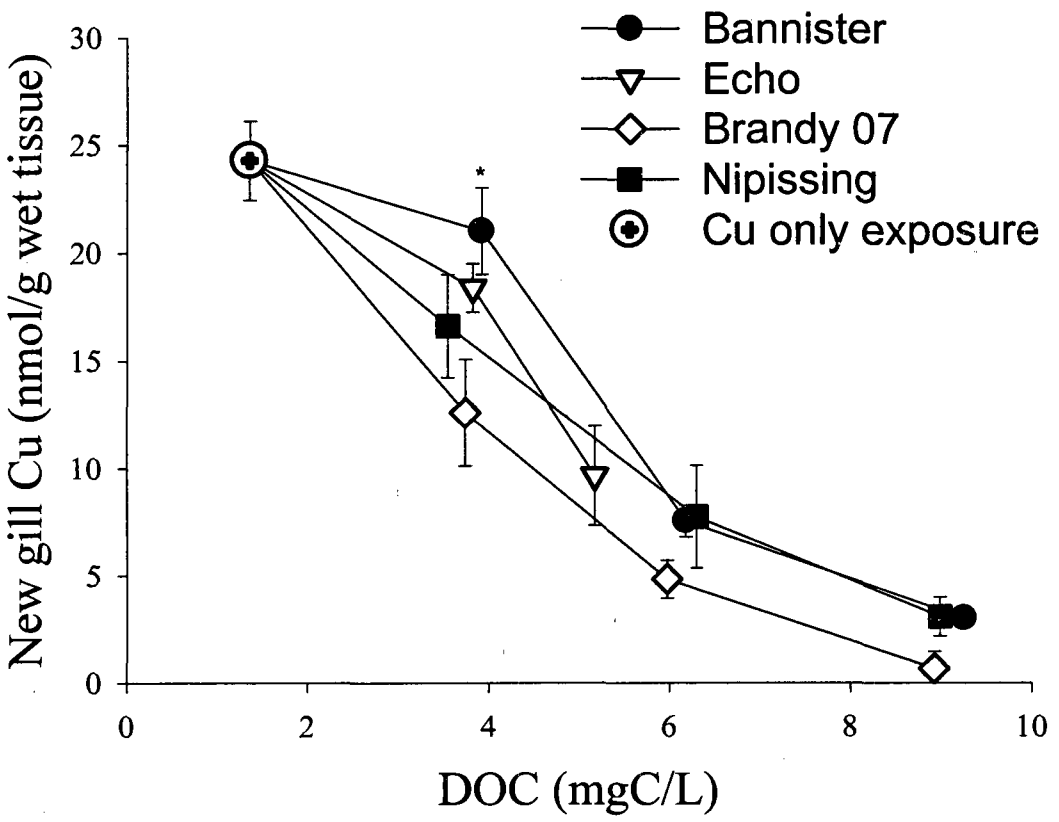


Figure 3-3 Short term (3 h) gill accumulation in juvenile rainbow trout (3-6g) in the presence of Cu and four different sources of NOM (group A). All trout were exposed to 2 μM Cu and varying concentrations of DOC (nominal 2.5, 5, and 5 mg C/L). Measured Cu on gills was subtracted by background levels of Cu (5nmol/g wet tissue) to get new gill Cu. Each point is the mean of 6 fish (± SEM). The * indicates that at 4 mg C/L, Bannister lake is significantly different from Brandy Lake.

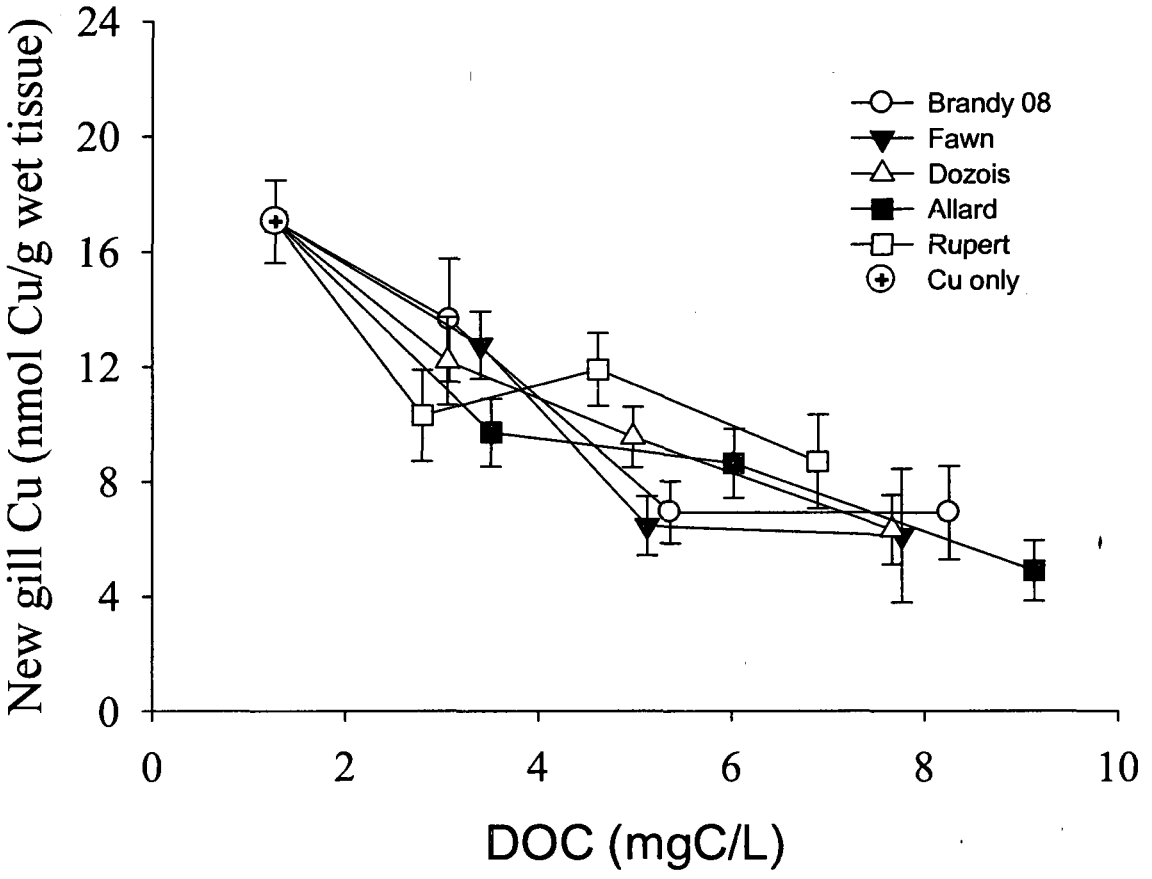


Figure 3-4 Short term (3 h) gill accumulation in juvenile rainbow trout (1-3g) in the presence of Cu and 5 different sources of NOM collected from soft waters (group B). All trout were exposed to 2 μM Cu and varying conc. of NOM (nominal 2.5, 5 and 8 mg C/L). Each point represents the mean of 8 fish (± SEM).

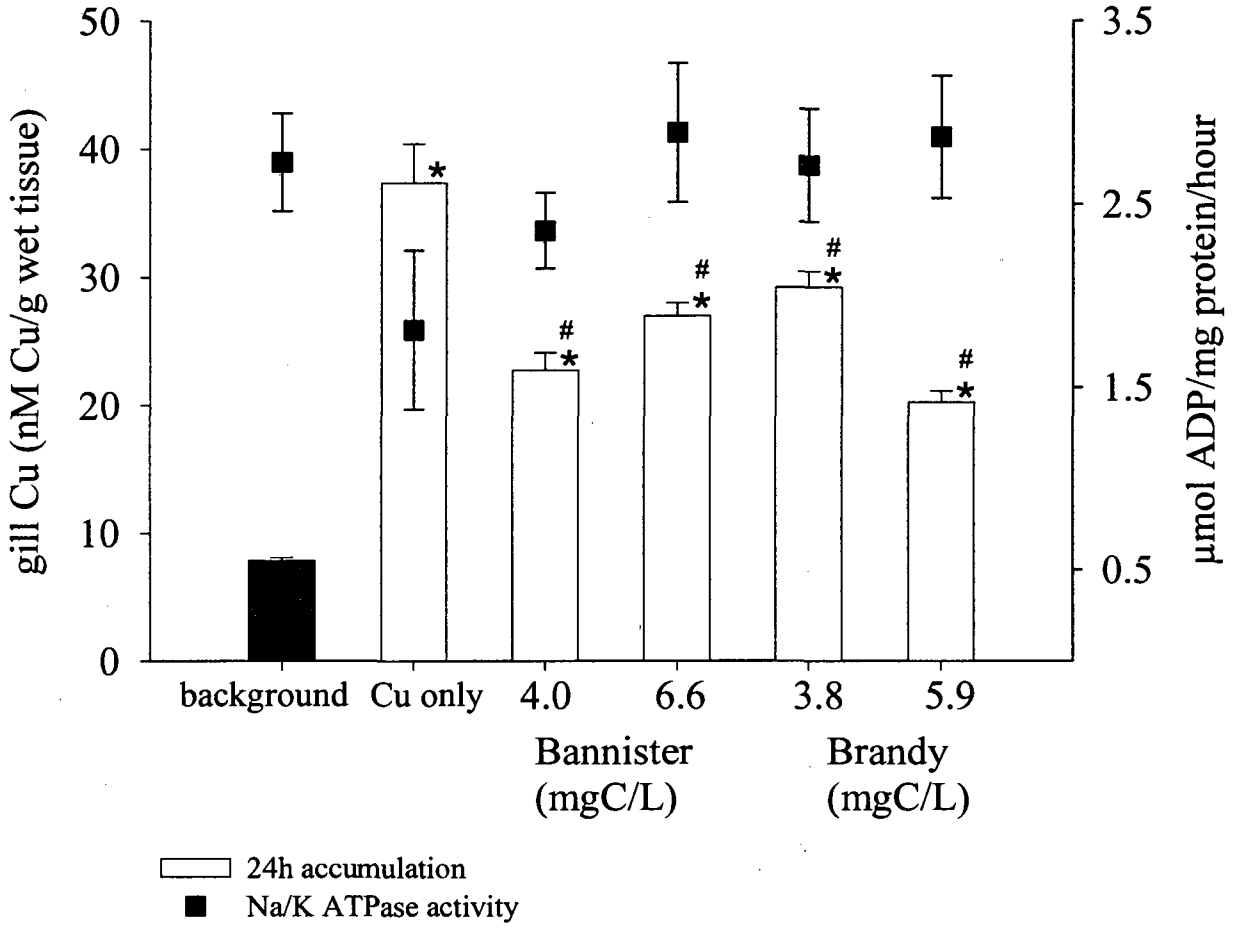


Figure 3-5 Gill Cu accumulation (open bars) and Na^+/K^+ ATPase activity (square symbols) after 24h exposure to 1µM Cu in soft waters. DOC was added from two different NOM sources (Bannister and Brandy 07 Lakes) at nominal 2.5 and 5 mg C/L. The values beneath the bars with added NOM give the actual measured DOC concentrations. The background (black bar) is the Cu content and Na^+/K^+ ATPase activity in unexposed fish. A * indicates accumulation of Cu is significantly different from background Cu content. A # indicates significant reduction of Cu accumulation in NOM exposures compared to Cu only exposure.

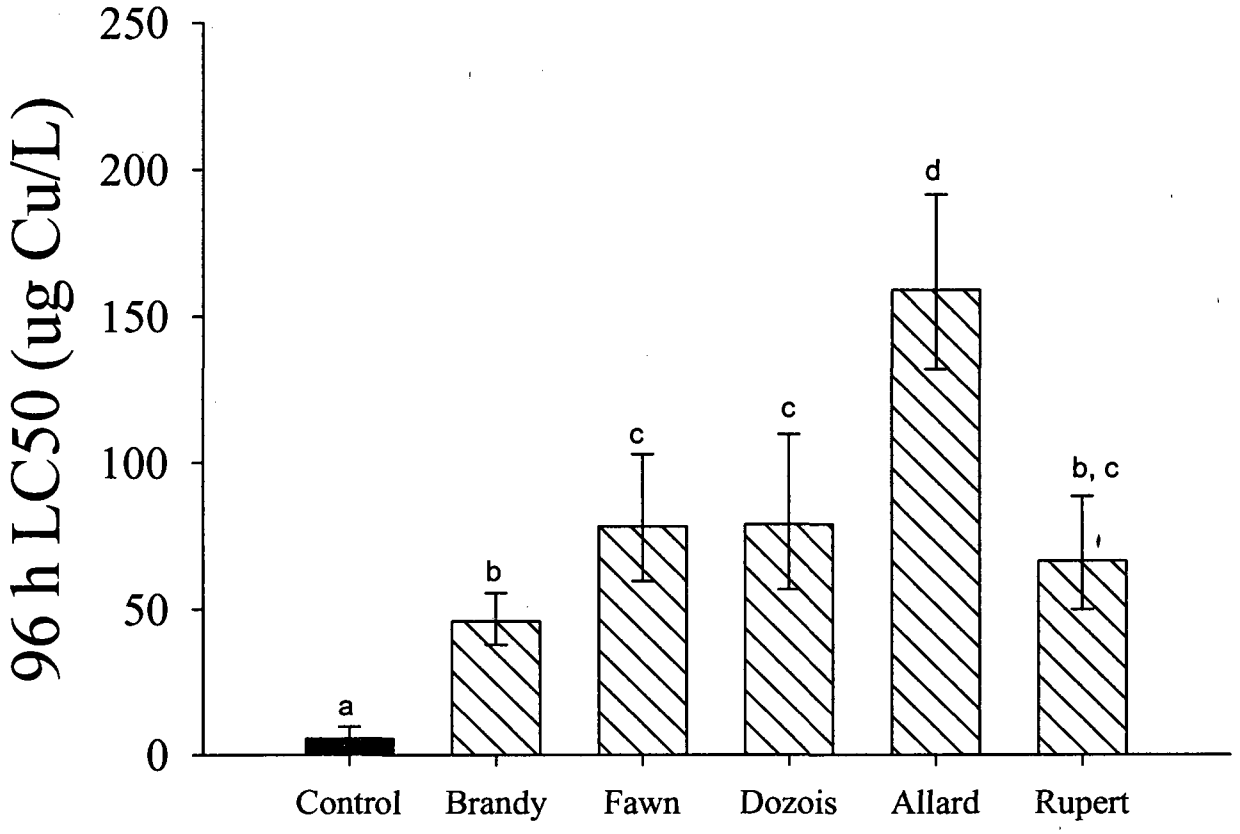


Figure 3-6 Influence of dissolved organic matter from 5 different sources on median lethal concentrations (96 h LC50 ± 95% CI) for total Cu (μg/L). All experiments were conducted in soft waters and nominal 4 mg C/L DOC. The black bar represents the Cu LC50 with no added DOC. Bars with different letter symbols (a-d) indicate significant differences.

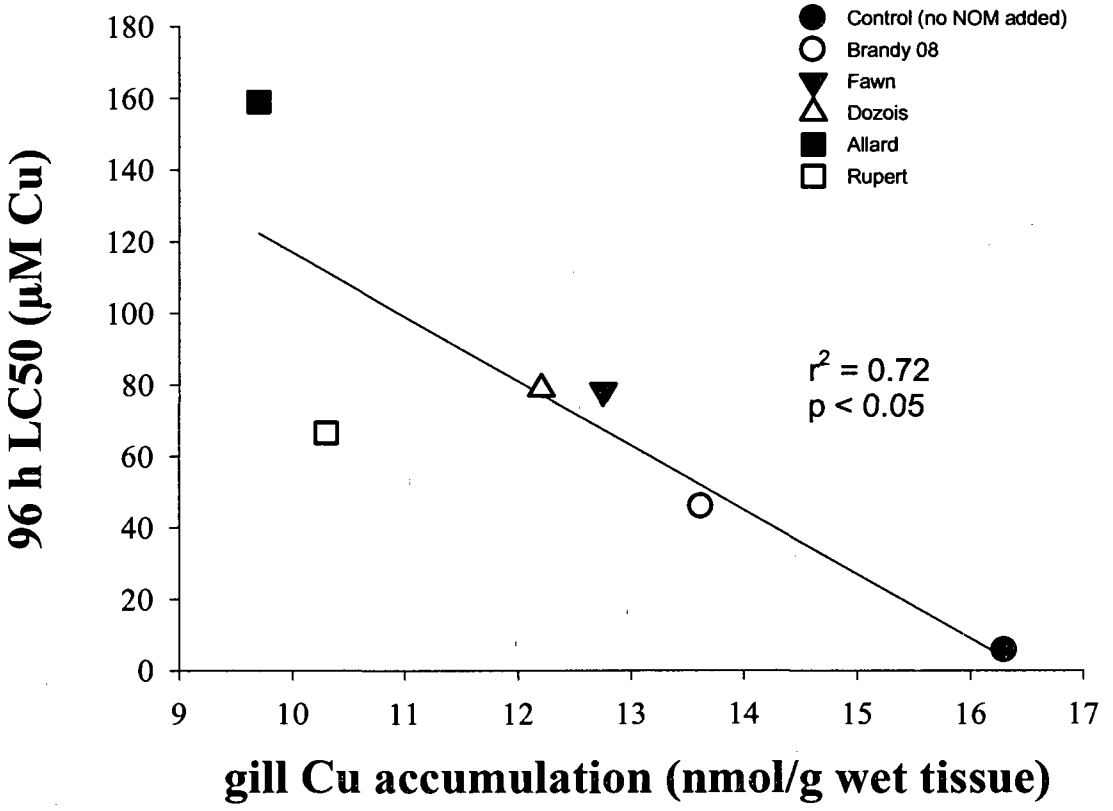


Figure 3-7 Relationship between 96 h LC50 and short term (3 h) gill Cu accumulation in juvenile rainbow trout. Gill accumulation data represents exposure at 2µM Cu and nominal 2.5 mg C/L DOC from each source. (See Fig 3-4)

Table 3-1 Chemistry of exposure water from short term (3 h) gill accumulation experiments using juvenile rainbow trout (4.4 g ± 0.77; n = 96) exposed to Cu in the presence and absence of nominal 2.5, 5 and 8 mg/L DOC from Bannister, Echo, Brandy and Nipissing (group A). All values are expressed as means ± 1 standard deviation with an *n* of 2 or 3. Water temperature during 3 h exposure ranged from 11.0-11.5 °C.

NOM source	Nominal DOC (mg CL^{-1})	DOC (mg C L^{-1})	pH	Conductivity (µS cm^{-1})	Ca µM	Mg µM	Na µM	Total Cu µM
Control	--	0.9 ± 0.1	6.8 ± 0.2	65.4 ± 3.8	137.0 ± 0.0	74.4 ± 0.4	128.1 ± 3.2	0.07 ± 0.07
Cu only	--	0.9 ± 0.2	6.8 ± 0.1	49.2 ± 1.2	136.5 ± 0.7	71.9 ± 0.5	120.8 ± 2.1	1.91 ± 0.07
Bannister	2.5	3.8 ± 0.4	6.8 ± 0.1	64.9 ± 2.8	141.5 ± 0.7	87.8 ± 0.2	126.4 ± 2.3	1.91 ± 0.02
	5	6.0 ± 0.3	6.8 ± 0.2	79.6 ± 1.9	145.5 ± 0.7	100.7 ± 1.5	129.9 ± 2.9	1.98 ± 0.09
	8	8.6 ± 0.1	6.9 ± 0.3	94.8 ± 1.7	151.5 ± 0.7	119.4 ± 2.0	98.7 ± 58.6	2.04 ± 0.01
Echo	2.5	3.8 ± 1.0	6.7 ± 0.1	60.6 ±	152.0 ± 2.1	78.8 ± 2.7	128.2 ± 3.9	2.00 ± 0.01
	5	5.5 ± 1.1	6.6 ± 0.1	74.0 ± 1.6	150.0 ± 0.7	79.6 ± 1.2	134.4 ± 4.4	1.86 ± 0.04
Brandy 07	2.5	3.1 ± 0.3	6.7 ± 0.1	67.3 ± 2.5	153.0 ± 0.0	80.4 ± 0.1	133.7 ± 1.4	1.90 ± 0.05
	5	7.5 ± 2.1	6.6 ± 0.1	70.8 ± 5.2	159.0 ± 0.7	83.2 ± 0.1	113.6 ± 33.7	1.97 ± 0.10
	8	9.7 ± 1.1	6.7 ± 0.0	90.2 ± 3.1	162.0 ± 0.0	87.9 ± 1.6	148.5 ± 2.1	2.07 ± 0.02
Nipissing	2.5	3.3 ± 0.4	6.6 ± 0.1	91.5 ± 2.1	148.0 ± 0.7	81.3 ± 1.4	128.6 ± 3.8	1.98 ± 0.01
	5	5.4 ± 0.2	6.6 ± 0.2	103.9 ± 3.0	159.0 ± 0.7	91.0 ± 0.8	146.0 ± 1.9	2.00 ± 0.01
	8	8.0 ± 0.4	6.6 ± 0.1	129.5 ± 3.5	172.0 ± 0.0	97.6 ± 1.6	170.3 ± 14.9	1.97 ± 0.02

Table 3-2 Chemistry of exposure water from short term (3 h) gill accumulation experiments using juvenile rainbow trout (2.9 g ± 1.0; n = 64) exposed to Cu in the presence and absence of nominal 2.5, 5 and 8 mg/L DOC from Brandy 08, Fawn, Dozois, Allard, Rupert (group B). All values are expressed as means ± 1 standard deviation with an n = 2-6. Water temperature during 3 h exposure ranged from 17.0-17.5 °C.

NOM source	Nominal DOC (mg CL^{-1})	DOC ($mgCL^{-1}$)	pH	Conductivity (µS cm-1)	Ca µM	Mg µM	Na µM	Total Cu µM
Control	--	1.6 ± 0.7	6.7 ± 0.1	46.5 ± 1.2	96.8 ± 12	42.5 ± 8.2	147.3 ± 6.5	<0.01
Cu only	--	1.3 ± 0.4	6.7 ± 0.2	47.9 ± 1.7	104.4 ± 11.8	50 ± 8.5	152.3 ± 6.8	2.1 ± 0.1
Brandy 08	2.5	3.1 ± 0.1	6.7 ± 0.2	59.5 ± 5.9	98.8 ± 14.6	37.2 ± 0.4	145.5 ± 2.6	1.9 ± 0.0
	5	5.4 ± 0.2	6.7 ± 0.1	62.9 ± 1.4	89.7 ± 0.1	39.3 ± 0.3	150.3 ± 2.9	1.8 ± 0.0
	8	8.3 ± 0.1	6.7 ± 0.1	74.8 ± 2.0	96.5 ± 0.1	41.5 ± 0.6	154.4 ± 4.2	2.1 ± 0.1
Fawn	2.5	3.4 ± 0.2	6.8 ± 0.3	52.4 ± 2.2	107.8 ± 10.5	46 ± 0.4	151.6 ± 6.2	1.8 ± 0.0
	5	5.1 ± 0.1	6.8 ± 0.3	55.6 ± 1.0	102.4 ± 1.2	47.2 ± 0.2	152.5 ± 3.6	1.8 ± 0.1
	2.5	7.8 ± 0.1	6.8 ± 0.3	62.5 ± 1.8	109.0 ± 0.8	48.6 ± 0.3	156.0 ± 3.9	2.0 ± 0.0
Dozois	2.5	3.1 ± 0.2	6.9 ± 0.2	49.3 ± 0.9	98.3 ± 0.8	46.0 ± 0.8	151.8 ± 5.4	2.0 ± 0.1
	5	5.0 ± 0.1	6.9 ± 0.2	53.8 ± 0.6	94.6 ± 2.6	46.3 ± 0.5	154.2 ± 3.1	1.8 ± 0.2
	8	7.7 ± 0.4	6.8 ± 0.1	59.8 ± 0.9	90.8 ± 0.2	46.5 ± 0.6	156.7 ± 3.9	1.8 ± 0.1
Allard	2.5	3.5 ± 0.1	6.8 ± 0.2	48.1 ± 0.9	99.8 ± 1.6	47.7 ± 0.5	153.4 ± 2.4	1.9 ± 0.1
	5	6.0 ± 0.1	6.7 ± 0.2	54.9 ± 1.2	105.9 ± 0.2	53.7 ± 0.6	154.5 ± 4.4	2.0 ± 0.1
	8	9.1 ± 0.1	6.7 ± 0.1	56.2 ± 2.5	113.5 ± 0.2	60.7 ± 1.0	158.6 ± 3.8	2.0 ± 0.0
Rupert	2.5	2.8 ± 0.2	6.6 ± 0.1	46.6 ± 1.0	92.1 ± 0.9	45.0 ± 0.9	147.3 ± 5.3	2.0 ± 0.1
	5	4.6 ± 0.1	6.7 ± 0.1	49.6 ± 0.8	92.0 ± 1.6	47.2 ± 0.3	146.8 ± 5.2	1.9 ± 0.1
	8	6.9 ± 0.1	6.7 ± 0.1	52.6 ± 2.1	89.1 ± 1.0	49.3 ± 0.7	146.3 ± 3.6	1.8 ± 0.2

Table 3-3 Chemistry of exposure water from short term (24h) gill accumulation experiments using juvenile rainbow trout (10.1 g ± 4.0; n = 39) exposed to 1 μM Cu in the presence and absence of nominal 2.5 and 5 mg C/L DOC from Bannister and Brandy 07 NOM sources. All values (except pH and Na) are expressed as means ± 1 standard deviation with an n = 3-5. Water temperature during 3 h exposure ranged from 14 - 14.5 °C.

NOM source	Nominal DOC (mg CL^{-1})	DOC (mg CL^{-1})	pH		Conductivity μScm^{-1}	Ca (μM)	Mg (μM)	Na (μM)		Total Cu (μM)
			0h	24h				0h	24h	
Control	--	0.74 ± 0.2	6.62	7.11	49.8 ± 4.9	71.5 ± 1.0	31.7 ± 1.8	72.20	79.80	0.04 ± 0.01
Cu only	--	1.26 ± 0.7	6.62	7.06	50.8 ± 6.7	75.6 ± 1.2	30.8 ± 1.2	57.50	205.10	1.0 ± 0.03
Bannister	2.5	4.08 ± 0.4	6.62	7.07	74.5 ± 4.6	83.2 ± 1.8	46.5 ± 0.9	169.60	184.70	0.9 ± 0.04
	5	6.60 ± 0.4	6.63	7.07	97.5 ± 5.9	111.7 ± 27.3	62.2 ± 1.5	170.30	196.40	1.0 ± 0.02
Brandy	2.5	3.79 ± 0.3	6.58	7.09	62.5 ± 5.4	79.0 ± 0.5	34.8 ± 0.2	92.70	185.20	1.0 ± 0.02
	5	5.85 ± 0.2	6.59	6.94	77.3 ± 4.9	87.1 ± 1.1	39.4 ± 1.1	167.10	173.10	1.1 ± 0.02

Table 3-4 Chemistry of exposure water from six 96 h LC50s for Cu using juvenile rainbow trout (1-3 g) in absence (control) and presence of nominal 4 mg C/L DOC from 5 different NOM sources (group B). Temperature of exposure water ranged from 13-15 °C. All values are expressed as means ± 1 SD (n).

NOM exposure	DOC (mg C/L)	Ca (μM)	Mg (μM)	Na (μM)	pH	Conductivity (μS cm^{-1})	Temp (°C)
Control	1.4 ± 0.2 (10)	95.4 ± 6.1 (7)	46.0 ± 3.3 (8)	145.4 ± 5.4 (8)	6.6 ± 0.1 (30)	50.1 ± 5.8 (30)	15.2 ± 0.2 (30)
Brandy Lake	4.9 ± 0.4 (38)	64.9 ± 2.7 (8)	31.5 ± 0.6 (8)	149.9 ± 8.4 (7)	6.6 ± 0.1 (39)	55.8 ± 3.6 (39)	14.7 ± 0.3 (39)
Fawn Lake	4.5 ± 0.4 (39)	64.8 ± 2.2 (7)	32.9 ± 4.7 (8)	139.0 ± 5.2 (7)	6.7 ± 0.1 (40)	45.4 ± 2.9 (40)	13.9 ± 0.5 (40)
Dozois Lake	4.4 ± 0.5 (36)	59.7 ± 2.3 (8)	29.8 ± 1.1 (8)	144.5 ± 8.1 (6)	6.6 ± 0.1 (37)	55.9 ± 4.8 (37)	13.8 ± 0.3 (37)
Allard River	4.9 ± 0.3 (40)	73.8 ± 4.1 (8)	37.0 ± 1.3 (8)	138.2 ± 8.0 (7)	6.7 ± 0.1 (40)	54.2 ± 2.7 (40)	13.5 ± 0.3 (40)
Rupert River	5.0 ± 0.3 (37)	62.8 ± 7.2 (6)	32.5 ± 3.1 (8)	143.2 ± 5.7 (8)	6.6 ± 0.1 (37)	56.1 ± 2.5 (37)	13.8 ± 0.6 (37)

Table 3-5 Correlations coefficients (r) between biological data (gill accumulation and 96h LC50) and NOM chemical and optical characteristics (see chapter 2). Correlations with 3 h gill accumulation includes 2007 and Lake Nipissing source. Accumulation data is exposure with 2 μM Cu and nominal 2.5 mg C/L DOC. Accumulation data has an n=4, whereas 96h LC50 correlations have an n=5.

variable	3 h gill accumulation (2007)	96h LC50 (2008)
Log K	0.66	(-)0.62
B_{max}	(-)0.88	0.76
HA	(-)0.41	0.34
FA	0.19	(-)0.25
Tyr	0.52	(-)0.36
Tryp	0.54	(-)0.13
HA:FA	(-)0.33	0.34
FI	0.99 *	0.18
SAC_{340}	(-)1.00 **	(-)0.53
Protein	(-)0.94	0.43
%DOC< 1 kDa	0.76	(-)0.89 *

* indicates $p<0.05$

** indicates $p<0.005$

Table 3-6 Measured (95% confidence intervals) and BLM-predicted 96-h Cu LC50s for control (no NOM added) and 5 NOM sources at nominal 4 mg C/L DOC. Presented here are four different scenarios for BLM predictions.

	96-h Cu LC50 (µg / L)				
		BLM predictions (4 scenarios)			
		1	2	3	4
NOM Source	experimental (95% CI)	unmodified	Sensitivity calibration	Calibrated and 50% HA	(unmodified) assume 50% active DOC and 100% FA
Control	5.6 (3.3-9.6)	51.3	5.5	5.5	22.8
Brandy 08	45.8 (37.8-55.5)	180.4	21.0	70.2	72.8
Fawn	78.3 (59.5-102.9)	176.7	22.7	73.7	72.6
Dozois	78.9 (56.7-109.7)	162.4	18.9	63.2	65.6
Allard	158.9 (132.0-191.4)	190.8	24.4	79.3	78.5
Rupert	66.5 (49.9-88.5)	184.2	21.5	71.8	74.2

Chapter 4

General Discussion

NOM sampling strategy to yield different NOM sources

NOM was concentrated from environments which differed in their vegetative type, water system (lake vs. river) and hardness. In addition, NOM was sampled during different seasons. The reverse osmosis technique to concentrate NOM was used because it was highly efficient and has been demonstrated to not significantly alter NOM properties. For example, De Schamphelaere et al. (2005) compared natural water samples to concentrated water samples and saw no significant change in the chemical properties or differences in NOM protection for both Cu and Zn toxicity to aquatic organisms. To prevent microbial alteration after the concentration process, NOM was stored under acidic conditions and at 4°C.

The majority of sampling of NOM took place in the soft Canadian Shield waters. Three different ecozones were sampled from, Boreal Shield, Mixedwood plains and Hudson plains. The majority of the sources are from the Boreal Shield ecozone which are dominated by conifers, black and white spruce, tamarack and balsam fir. Bannister Lake is in the mixedwood plains ecozone, however, only 10% of the mixed forest remains and has been replaced by agriculture, roads, cities and industrial activity. As well Bannister Lake was the only hard water lake and was highly colonized by algae suggesting this lake is mainly autochthonous-like in origin, forming most of its NOM within the aquatic system. The most northern source located in Northwestern Quebec (Rupert River) is located in the Hudson plains ecozone which consists of arctic tundra and boreal forest transition type vegetation.

Environments with differing tree species and vegetation type have been shown to result is different NOM composition (Hongve et al., 2000). For example, molecular size distribution (Suominen et al., 2003) and phenolic acid compounds (Kuiters and Denneman, 1987) in soils have been shown to vary depending on the tree age and type. This suggests that environments differing in their vegetation may potentially be leaching different NOM chemical compounds into the aquatic systems. This may influence the degree to which NOM interacts with metals.

Seasonal variation as opposed to vegetation types, however, has been shown to be a greater influence on NOM chemical composition in soil leachates (Kaiser et al., 2001). This agrees with our study where differences among the NOM samples reflected more the season than the environment. For example, those samples collected in the fall revealed lower SAC_{340} values, higher FI, and a ratio of 1:1 for HA to FA. Samples collected in the spring/summer had higher SAC_{340}, lower FI and a ratio of 2:1 for HA to FA. One exception is Bannister Lake which is highly autochthonous likely related to its environment being located in a semi-rural area and thus receiving agriculture inputs resulting in eutrophication and higher NOM formation within the lake. Further evidence for this is the higher levels of tyrosine present in the Bannister Lake NOM (Fig. 2-3) which is an indication of algae precursor NOM (Determann et al., 1998).

Two samples were collected from the Brandy Lake, once in the fall and once in late spring for seasonal comparison. Chemical differences did arise at the different sampling periods (as mentioned above). When sampled in the fall, Brandy Lake

appeared more autochthonous-like as indicated by the FI, HA content, and higher amounts of tyrosine and tryptophan which are indicative of NOM production within the lake. The spring sample was absent of any trace levels of either tyrosine or tryptophan. Sample characteristics of Brandy Lake in the fall and spring illustrated that one source can be relatively allochthonous-like in one season, and autochthonous-like in another. Interestingly, however, both samples had the same Cu binding capacity (Table 2-4).

Sampling from both lakes and rivers was also a strategy in trying to yield different NOM sources. Differences between NOM in a lake compared to that of a river are mainly attributed to NOM residency time. NOM from a lake would likely have a longer residency time than that of a river. Longer residency time means more exposure to UV light resulting in more photolysis resulting in smaller more labile forms of NOM available for microbial consumption. Thus, lakes will tend to have less allochthonous NOM and more autochthonous-like NOM. Contrary to lakes, fast flowing streams or rivers will have large inputs of allochthonous NOM similar to its precursor organic matter from terrestrial plants and soil catchments (McKnight and Aiken, 1998). The only major chemical difference between the lake and river NOMs from this study is that the two rivers (Allard and Rupert) had the highest ability to complex with Cu (B_{max}; Table 2-4). This may be a result of Allard and Rupert receiving more allochthonous like NOM which generally complexes better with metals (Richards et al., 2001).

Collection of NOM from different sources and in different seasons revealed variation among NOM composition. The large division between those samples collected

in different seasons illustrates that not only NOM source but as well as the season may need to be accounted for when considering differences among NOMs.

Influence of NOM sources on Cu speciation, accumulation and toxicity

It has been well documented that NOM provides protection to aquatic organism against metal accumulation and toxicity (De Schamphelaere et al., 2004; Kramer et al., 2004; Doig and Liber, 2006; Erickson et al., 1996). This was also observed in this study, where in the presence of NOM, free Cu^{2+} measurements, gill Cu accumulation and acute Cu toxicity were always reduced, demonstrating NOM ability to complex with Cu thus reducing free Cu^{2+}, the most bioavailable species. These results are in good agreement with published Cu-gill and Cu-NOM binding constants. Cu binds to NOM 50 times stronger than Cu does to the gill (Log $K_{Cu\text{-}NOM} = 9.1$, log $K_{Cu\text{-}gill} = 8.6$; Playle et al., 1993b) thus explaining why NOM has such a large effect on Cu accumulation and toxicity.

Differences among NOM sources to differentially complex with metals, reduce gill metal accumulation and toxicity have also been documented. This study also illustrated difference among NOM sources in their degree to complexed with Cu and protected against Cu accumulation and toxicity. Free Cu^{2+} measurements using an ion selective electrode illustrated a range in Cu complexation capacity (B_{max}) from 0.9 to 3.5 μmol Cu/mg C. Similar ranges in B_{max} using the same technique with both isolated FA (McKnight et al., 1983) and total NOM concentrates (Abbt-Braun and Frimmel, 1999)

have been found. Variation among NOM sources in 3 h gill Cu accumulation studies was most pronounced at lower DOC exposures. The largest and most significant difference was between Bannister and Brandy Lake 07 NOM (group A sources), where Brandy Lake reduced 35% more Cu accumulation on the gill. In the 96 h toxicity experiments, Allard River NOM reduced acute Cu toxicity 3.5-fold better than Brandy Lake 08.

Compared to this study, other studies have seen greater variability among NOM sources in reducing metal toxicity to aquatic animals. For example, Vangenderen et al. (2003) saw a five fold difference among NOM sources in their ability to reduce Ag toxicity to fathead minnows. Up to a six fold difference was seen among NOM sources in their ability to reduce Cu toxicity to *Daphnia magna* (De Schamphelaere et al., 2004) and to fathead minnow (Ryan et al., 2004). Therefore, different NOM sources have been documented here and in the literature to have different degrees to complex with metals, thus providing different degrees of protection against metal accumulation toxicity to aquatic organisms.

Relating NOM properties to Cu speciation, gill accumulation and toxicity

Chemical and optical characterization revealed NOM sources which were more autochthonous-like and NOMs which were more allochthonous-like. Usually, those sources which were more allochthonous-like better complexed with Cu and reduced accumulation of Cu on the gills. This is likely a result of allochthonous NOM being

more aromatic (darker; Richards et al., 2001) and contains more phenolic groups which strongly bind to metals (Perdue et al., 1998). No optical measure correlated significantly with the acute Cu toxicity data. Therefore, there are factors within NOM that influence bioavailability and toxicity of Cu that are not related to the colour or the (presumed) presence of phenolic/humic substances. The fraction of DOC less than 1 kDa did significantly correlate with toxicity; however, the values were numerically similar to one another differing by only 6%. In the literature, optical measures have been shown to best correlate with the ability of NOM to complex with metals and reduce either accumulation or toxicity.

SAC_{340} has been the most promising measure in predicting NOM ability to protect against Cu toxicity (De Schamphelaere et al., 2004; Ryan et al., 2004; Schwartz et al., 2004) Ag toxicity (Glover et al., 2005) and Pb toxicity (Schwartz et al., 2004; Richards et al., 2001). Other studies have also shown that SAC of different NOM source can predict the accumulation benzo[a]pyrene in the nematode *C.elegans* (Haitzer et al., 1999) and accumulation of other organic contaminants in *Daphnia magna* (Kukkone and Oikari, 1991). Thus this measure may have implication for other contaminants other than metals.

This study agrees with other studies in that SAC_{340} demonstrated to be the property of NOM which had the strongest relationship with gill Cu accumulation ($r = 0.995$; $p<0.005$). Since SAC_{340} is a relatively simple measurement and best predicts

NOM ability to complex with metals, it demonstrates to be the best measure of NOM quality to incorporate into predictions models.

Implications for modeling

The ultimate goal of this thesis was to further improve on the predictive capabilities of existing geochemical and toxicity prediction models by incorporating NOM differences. WHAM proved to predict free Cu^{2+} relatively well, where the majority of predictions were within a factor of 2. Where free Cu^{2+} is being overpredicted this is not much of a concern from a regulatory point of view, since these predictions are only being conservative. The points which are of concern were those points being underpredicted which occurred for some of the autochthonous NOM sources. To improve the WHAM predictions, SAC_{340} was entered in as a quality factor. This increased the predictive capability of WHAM, particularly for those NOM sources which were autochthonous-like. Thus SAC_{340} is potentially an important measure of NOM to incorporate into predictive models for better estimating Cu speciation and toxicity to aquatic organisms.

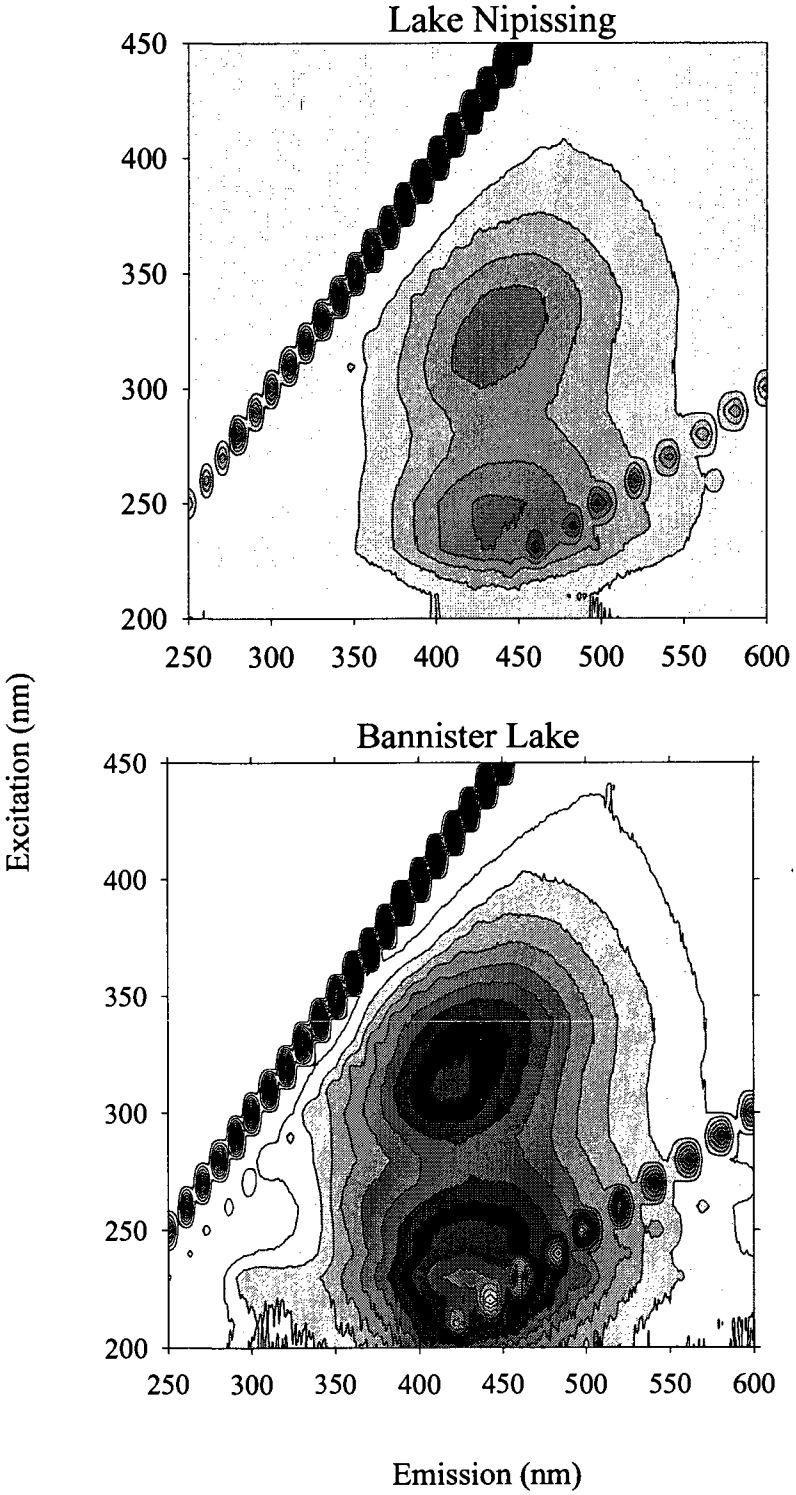
The BLM predicted within a factor of 2 once it was calibrated for fish sensitivity to Cu in soft waters and the relative fraction of active DOC was taken into account. No quality measured was used to improve the ability of the BLM to predict Cu toxicity in the presence of difference NOM sources because no measurement could account for their differences. Unfortunately, only allochthonous-like NOM sources were used for this

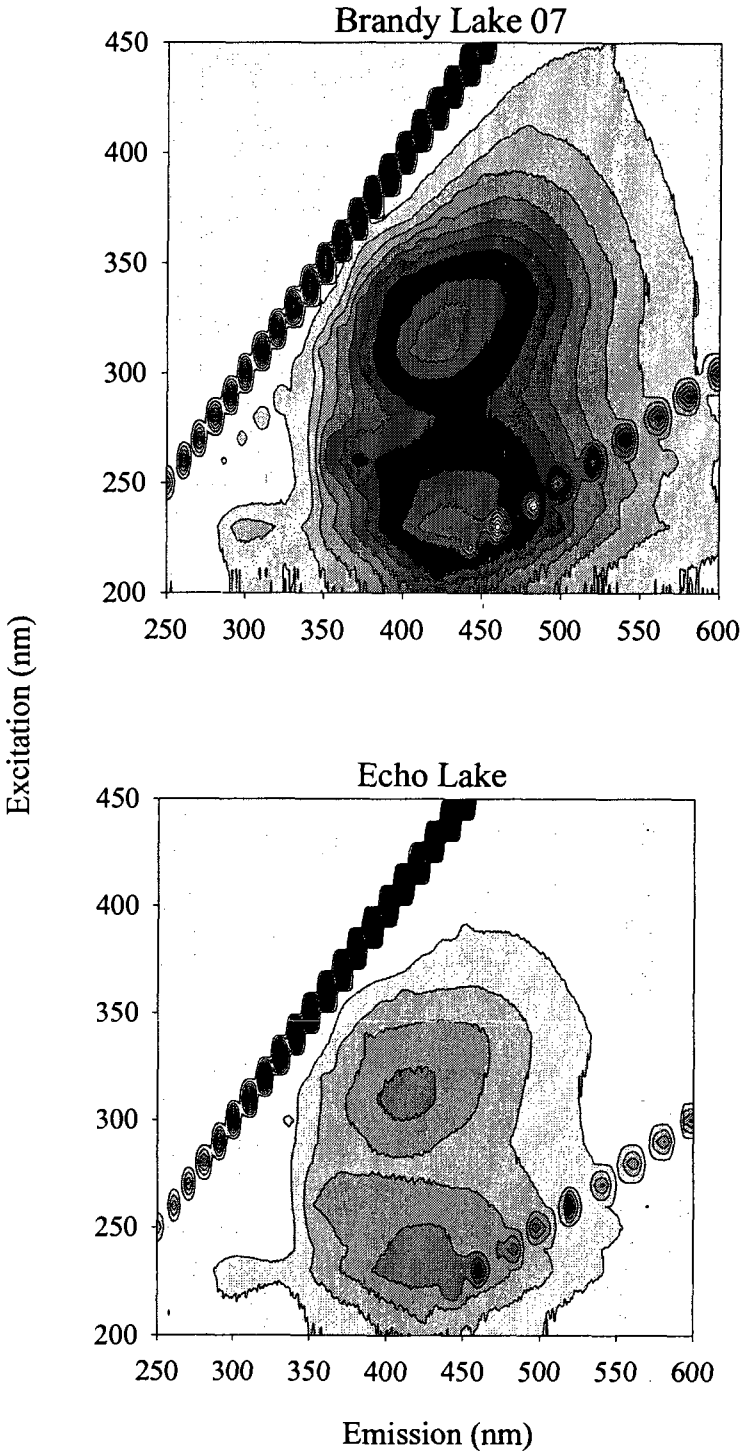
study to conduct acute Cu toxicity tests. The next step would be to test whether the BLM can accurately predict toxicity with autochthonous-like NOM sources. Since WHAM underpredicted Cu^{2+} for autochthonous-like sources, then it is assume the BLM would be the same. Thus, like WHAM, SAC_{340} may also need to be accounted for in the BLM when predicting Cu toxicity to aquatic organisms.

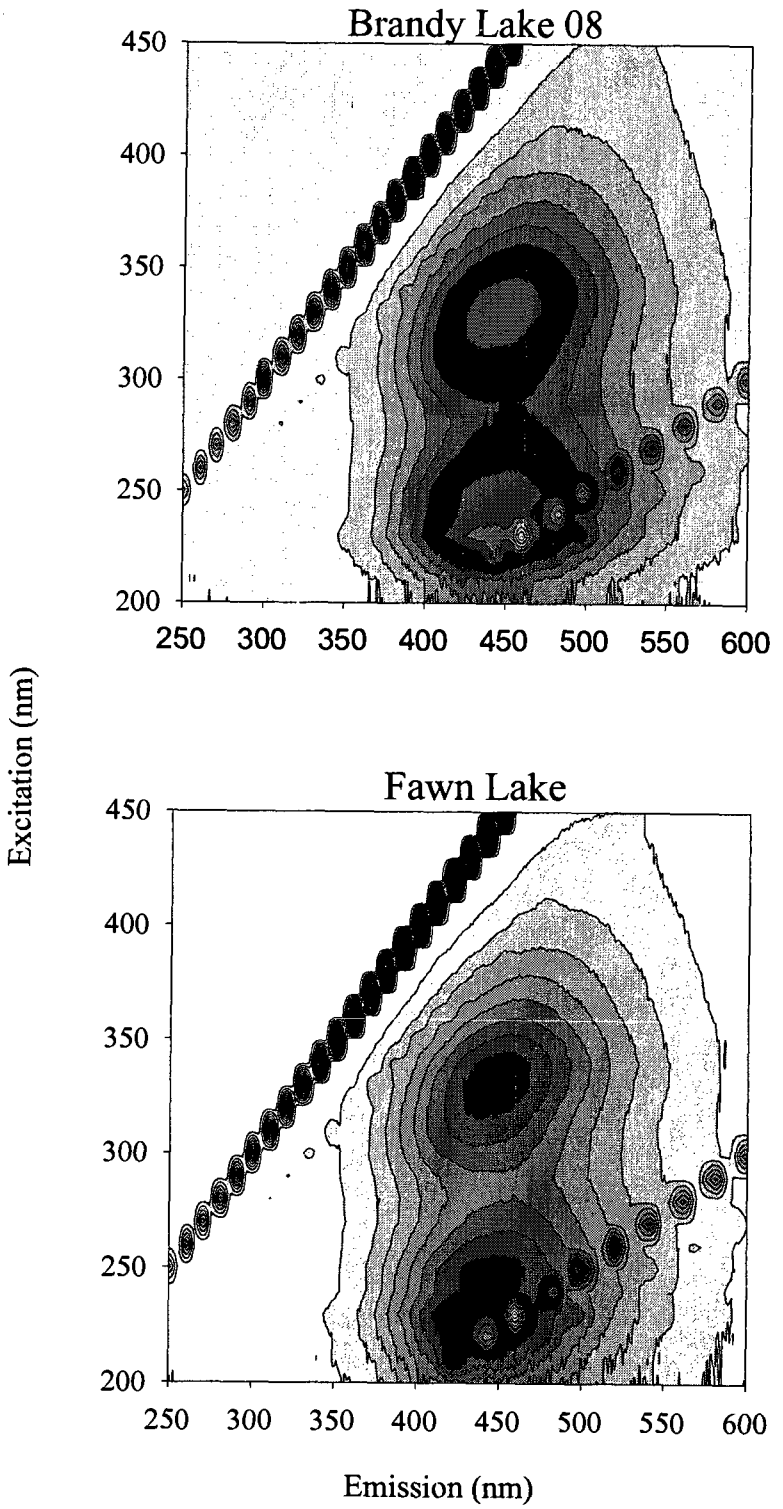
Overall, there are wide differences in NOM quality which is likely dependant on the season as well as the source (lake vs. river). Differences in the ability of various NOMs to reduce Cu accumulation and toxicity are large enough that it should be accounted for in models. Results here show that incorporating NOM characterizations does improve predictions in some cases (e.g. when colour varies; SAC_{340}) but in other cases there are still some unknowns.

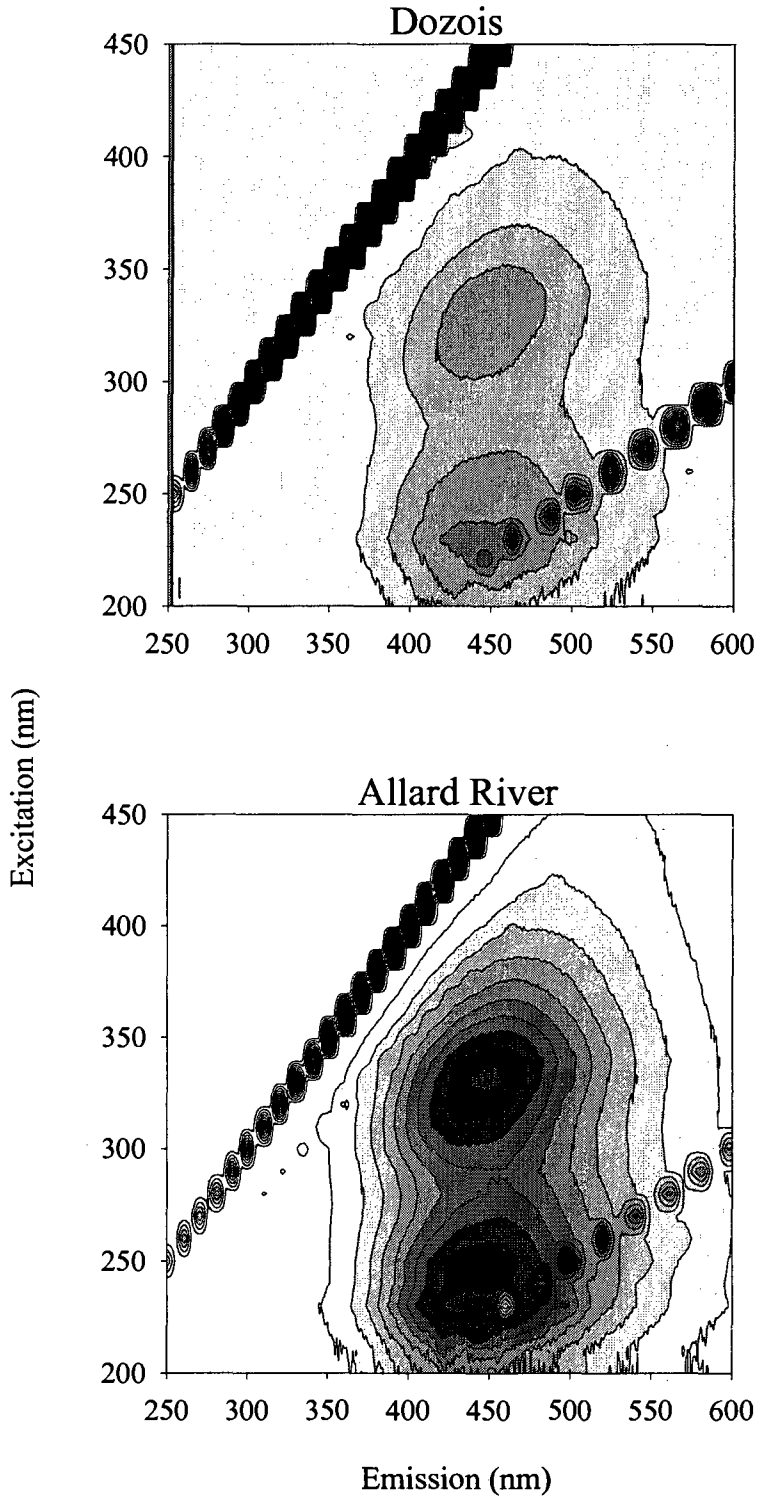
Appendix A
EEMS Contour Plots

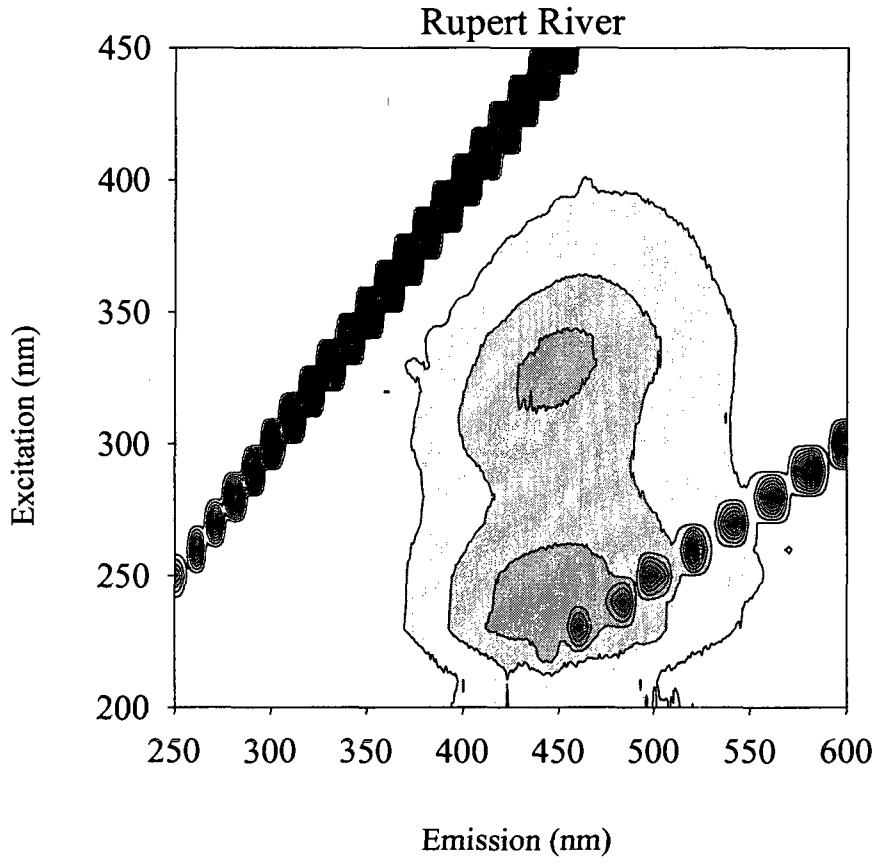
Excitation emission scans of 9 NOM samples at 8 mg C/L DOC. These plots are read like topographical maps. The two dark diagonal lines are the Rayleigh-Tyndall scatter, where the emission wavelengths equal the excitation wavelengths or twice the excitation wavelength. All plots reveal a HA-like peak which excites at 350 and 240 nm and emits at 400-500 nm. The FA-like components excites at 320 and 240 nm and emits at 400 nm. What appears as a shoulder of the humic/fulvic peaks which is more notable in Bannister and Echo lakes are the protein components. Where tryptophan excites at 275 and 220 nm and emits at 360 nm; while tyrosine excites at 275 and 225 nm and emits at 300 nm.


Lake Nipissing
Bannister Lake
Excitation (nm)
Emission (nm)
450
400
350
300
250
200
250
300
350
400
450
500
550
600


Brandy Lake 07
Echo Lake
Excitation (nm)
Emission (nm)
450
400
350
300
250
200
250
300
350
400
450
500
550
600

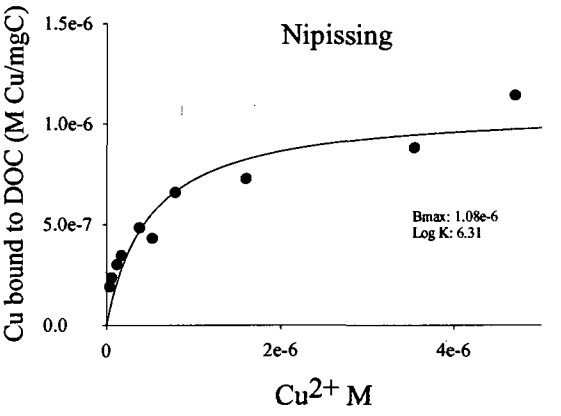

Brandy Lake 08
Fawn Lake
Excitation (nm)
Emission (nm)
450
400
350
300
250
200
250
300
350
400
450
500
550
600

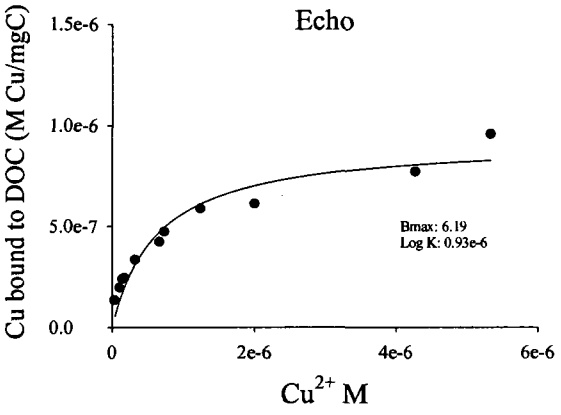

Dozois
Allard River
Excitation (nm)
Emission (nm)
200
250
300
350
400
450
500
550
600

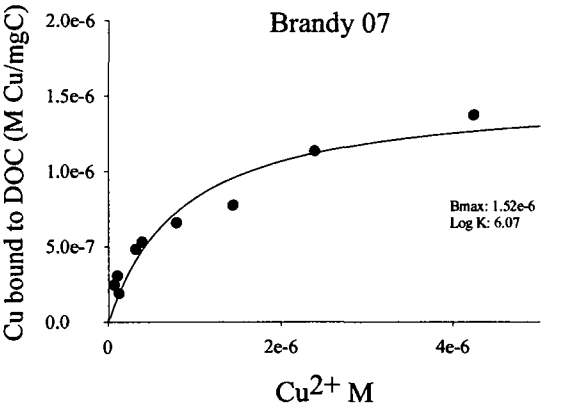

Rupert River
Excitation (nm)
450
400
350
300
250
200
250
300
350
400
450
500
550
600
Emission (nm)

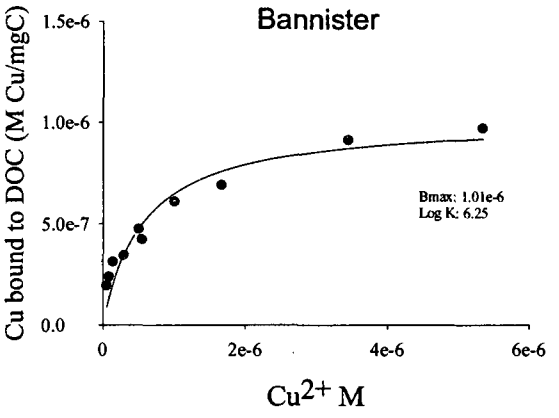
Appendix B
Cu-NOM binding Curves

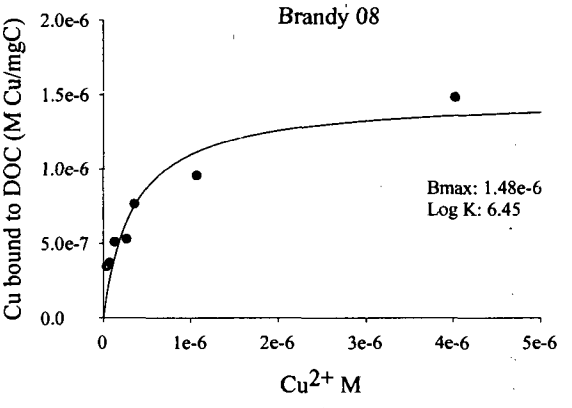
Free Cu^{2+} was measured by ion selective electrode in solutions with varying combinations of total Cu and DOC from 9 NOM sources. Some plots have fewer points than others as a result of the electrode not being able to read Cu within the standard curve due to too much complexation. As well, an additional Cu concentration was added to the NOM sources Dozois, Allard and Rupert. Cu bound to DOC (y-axis) was calculated as the difference between total Cu and Cu^{2+} divided by the DOC concentration. NOM binding curve for Cu were modelled to give binding capacity (B_{max}) and affinity (Log K) constants using a hyperbolic function $y = (B_{max} + x) / (\text{Log } K \cdot x)$.

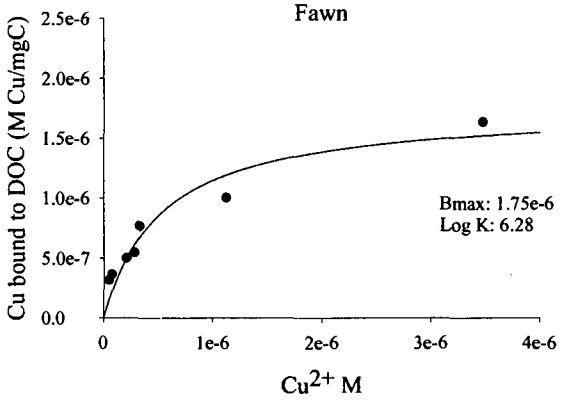

Nipissing
Cu bound to DOC (M Cu/mgC)
1.5e-6
1.0e-6
5.0e-7
0.0
0
2e-6
4e-6
Cu2+ M
Bmax: 1.08e-6
Log K: 6.31

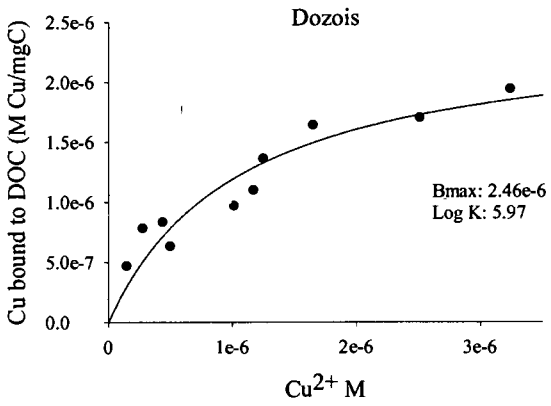

Echo
Cu bound to DOC (M Cu/mgC)
1.5e-6
1.0e-6
5.0e-7
0.0
0
2e-6
4e-6
6e-6
Cu2+ M
Bmax: 6.19
Log K: 0.93e-6

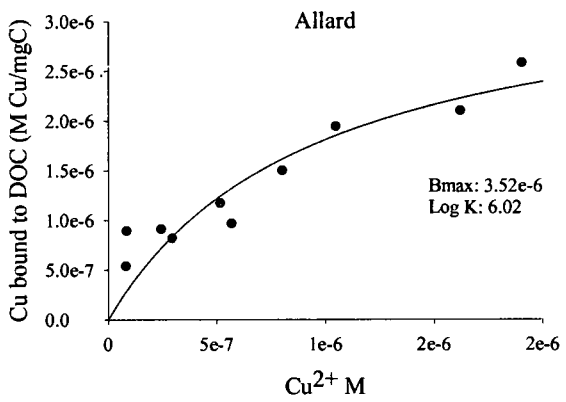

Brandy 07
Cu bound to DOC (M Cu/mgC)
2.0e-6
1.5e-6
1.0e-6
5.0e-7
0.0
0
2e-6
4e-6
Cu2+ M
Bmax: 1.52e-6
Log K: 6.07

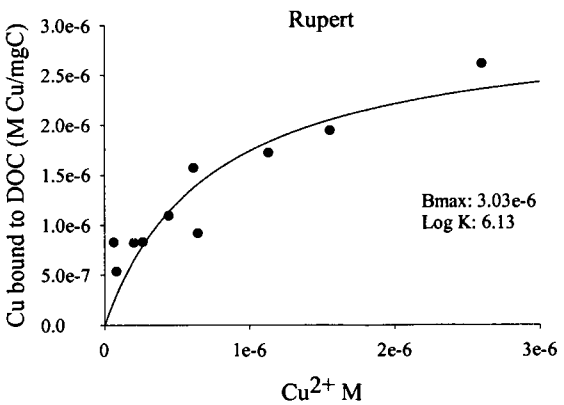

Bannister
Cu bound to DOC (M Cu/mgC)
1.5e-6
1.0e-6
5.0e-7
0.0
Bmax: 1.01e-6
Log K: 6.25
0
2e-6
4e-6
6e-6
Cu2+ M


Brandy 08
Cu bound to DOC (M Cu/mgC)
2.0e-6
1.5e-6
1.0e-6
5.0e-7
0.0
Bmax: 1.48e-6
Log K: 6.45
0
1e-6
2e-6
3e-6
4e-6
5e-6
Cu2+ M


Fawn
Cu bound to DOC (M Cu/mgC)
2.5e-6
2.0e-6
1.5e-6
1.0e-6
5.0e-7
0.0
Bmax: 1.75e-6
Log K: 6.28
0
1e-6
2e-6
3e-6
4e-6
Cu2+ M


Dozois
Cu bound to DOC (M Cu/mgC)
2.5e-6
2.0e-6
1.5e-6
1.0e-6
5.0e-7
0.0
0
1e-6
2e-6
3e-6
Cu2+ M
Bmax: 2.46e-6
Log K: 5.97


Allard
Cu bound to DOC (M Cu/mgC)
3.0e-6
2.5e-6
2.0e-6
1.5e-6
1.0e-6
5.0e-7
0.0
0
5e-7
1e-6
2e-6
2e-6
Cu2+ M
Bmax: 3.52e-6
Log K: 6.02


Rupert
Cu bound to DOC (M Cu/mgC)
3.0e-6
2.5e-6
2.0e-6
1.5e-6
1.0e-6
5.0e-7
0.0
0
1e-6
2e-6
3e-6
Cu2+ M
Bmax: 3.03e-6
Log K: 6.13

Appendix C

Na^+/K^+ ATPase Assay Protocol

All reagents for the assay were purchased from Sigma Aldrich. First, approximately 25 mg of frozen gill tissue is added to a 1.5 ml bullet tube containing 500 µL of medium (400 µl SEI: 150 mM Sucrose, 10 mM EDTA, and 50 mM imidazole; and 100 µL of SEID: 100 µl SEI with 0.5 mg Na Dexoycholate). Tissue was homogenized for 10-15 s and centrifuged at 5,000 g for 30 s to remove insoluble material. Next, 25 µL of supernatant was added to either 200 µL of solution A or Solution B in triplicate to 96-well microtitre plates. Solution A contains 4 u/ml lactate dehydrogenase, 5 u/ml pyruvate kinase, 2.8 mM phophoenolpyruvate, 3.5 mM ATP, 0.44 mM NADH and 50 mM Imidazole. Solution B is the same composition as solution A but with the addition of 0.5 mM ouabain (Na^+/K^+APTase inhibitor). Both solution A and B are prepared in a 3:1 solution of 3 parts Soln A or B and 1 part salt solution (189 mM NaCl, 10.5 mM MgCl*6 H_2O and 50 mM imidazole). Once assay solutions are in the wells, the plate was placed in a temperature controlled (25°C) spectrophotometer and the linear rate of NADH disappearance is measured at the wavelength 340 nm for 10 minutes. The linear rate from 0-10 minutes is determined and Na^+/K^+APTase activity is calculated as the difference in ATP hydrolysis in the absence and presence of ouabain. Na^+/K^+APTase is expressed as µmol ADP /mg protein/hour. Protein was measured in the tissue homogenates using the Bradford assay (Bradford, 1977). Before each day, a standard

curve was run with ADP standards containing 0 to 20 nmol with the addition of solution A.

References

Abbt-Braun, G., Frimmel, F.H. 1999. Basic characterization of Norwegian NOM samples-similarities and differences. Environ. Int. 25: 161-180.

Abbt-Braun, G., Lankes, U., Frimmel, F.H. 2004. Structural characterization of aquatic humic substances-the need for a multiple method approach. Aquat. Sci. 66: 151-170.

Andersen, C.M., Bro, R. 2003. Practical aspects of PARAFAC modeling of fluorescence excitation-emission data. J. Chemometrics. 17: 200-215.

Avdeepf, A., Zabronsky, J., Stuting, H.H. 1983. Calibration of copper ion selective electrode response to pCu 19. Anal. Chem. 55: 298-304.

Balm, P.H.M., Pepels, P., Helfrich, S., Hovens, M.L.M., and Wendelaar, B.S.E. 1994 Adrenocorticotropic hormone in relation to interregnal function during stress in tilapia (*Oreochromis mossambicus*). Gen. Comp. Endocrinol. 96: 347-360.

Baker, A., 2001. Fluorescense excitation-emission matrix characterization of some sewage-impacted rivers. Environ. Sci. Technol. 35: 948-953.

Bodar, C.W.M., Pronk, M.E.J., Sijm, D.T.H. 2005. The European Union risk assessment on zinc and zinc compounds: the process and the facts. Integrated Environmental Assessment and management. 1(4): 301-319.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Breault, R.F., Colman, J.A., Aiken, G.R., McKnight, D. 1996. Copper speciation and

binding by organic matter in copper-contaminated streamwater. Environ. Sci. Technol. 30 (12): 3477-3486.

Brooks, M.K., Meyer, J.S., Boese, C.J. 2007 (A). Toxicity of copper to larval *Pimephales promelas* in the presence of photodegraded natural dissolved organic matter. Can. J. Fish. Aquat. Sci. 64: 391-401

Brooks, M.J., Joseph, S.M., McKnight, D.M. 2007. Photooxidation of wetland and riverine dissolved organic matter: altered copper complexation and organic composition. Hydrobiologia. 579: 95-113.

Bryan, S.E., Tipping, E., Hamilton-Taylor, J. 2002. Comparison of measured and modeled copper binding by natural organic matter in freshwaters. Comp. Biochem. Physiol. Part C. 133: 37-49.

Cabaniss, S.E., Shuman, M.S. 1988. Copper binding by dissolved organic matter: I. Suwannee river fulvic acid equilibria. Geochemica Cosomochimica Acta. 52: 185-193.

Campbell, P.G.C., and Stokes, P.M. 1985. Acidification and toxicity of metal to aquatic biota. Can. J. Fish. Aquat. Sci. 42: 2034-2049.

Campbell, P.G.C. 1995. Interactions between trace metals and aquatic organisms: A critique of the free-ion activity model. In: Metal speciation and bioavailability in aquatic systems. Tessier, A., and Turner D.R. (eds), John Wiley & Sons.

CCME (Canadian Council of Ministers of the Environment). 1999. Canadian water quality guidelines. Environment Canada, Ottawa.

Coble, P.G., 1996. Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. Mar. Chem. 51: 325-346.

Curtis, P.J., Schindler, D.W. 1997. Hydrologic control of dissolved organic matter in low-order Precambrian Shield lakes. Biogeochemistry. 36: 125-138.

David, S.M., Somers, K.M., Reid, R.A., Ingram, R. 1997. Crayfish species assemblages in soft water lakes in seven tertiary watersheds in south-central Ontario. Ontario Ministry of Environment and Energy Report, ISBN 0-7778-6504-1. Pp 100.

De Schamphelaere K.A.C., Janssen C.R. 2002. A biotic ligand model predicting acute copper toxicity for daphnia magna: the effects of calcium, magnesium, sodium, potassium and pH. Environ. Sci. Technol. 36: 48-54.

De Schamphelaere K.A.C., Janssen C.R. 2004. Effects of dissolved organic carbon concentration and source, pH, and water hardness on chromic toxicity of copper to *Daphnia magna*. Environ. Toxicol. Chem. 23(5): 1115-1122.

De Schamphelaere, K.A.C., Unamuno, V.I.R., Tack, F.M.G., Vanderdeelen, J., Janssen, C.R. 2005. Reverse osmosis sampling does not affect the protective effect of dissolved organic matter on copper and zinc toxicity to freshwater organisms. Chemosphere. 58: 653-658.

Determann, S., Lobbes, J.M., Reuter, R., Rullkotter, J., 1998. Ultraviolet fluorescence excitation and emission spectroscopy of marine algae and bacteria. Mar. Chem. 62: 137-156.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C.

ecological effect of metals in aquatic system. In: Copper in the environment and Health. International Copper Association, Ltd, NY NY

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20: 2383-2396.

Doig, L.E., Liber, K. 2006. Influence of dissolved organic matter on nickel bioavailability and toxicity to *Hyalella azteca* in water-only exposures. Aquat. Toxicol. 76: 203-216.

Dwane, G.C., Tipping, E. 1998. Testing a humic speciation model by titration of copper-amended natural waters. Environ. Int. 24: 609-616.

Erickson, R.J., Benoit, D.A., Mattson, V.R., Nelson, H.P., Leonard, E.N. 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. Environ. Toxicol. Chem. 15: 181-193.

Frimmel, F.H., Abbt-Braun, G. 1999. Basic characterization of reference NOM from central Europe-similarities and differences. Environ. Int. 25(2/3): 191-207.

Georgopoulos, P.G., Roy, A., Opiekun, R.E., Lioy, P.J. 2002. Environmental dynamics and human exposure to copper: Environmental dynamics and human exposure issues (Volume 1). International Copper Association, Ltd. New York, NY.

Gerchakov, S.M., Hatcher, P.G. 1972. Improved technique for analysis of carbohydrates in sediments. Limnol. Oceanogr. 72: 938-943.

Glover, C.N., Sharma, S.K., Wood, C.M. 2005. Heterogeneity in physicochemical

properties explains differences in silver toxicity amelioration by natural organic matter to *Daphnia magna*. Environ. Toxicol. Chem. 74(11): 2941-2947.

Greco, A.M., Fenwick, J.C., Perry, S.F. 1996. The effects of soft water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*. Cell Tissue Res. 285: 75-82.

Guthrie, J.W., Hassan, N.M., Salam, M.S.A., Fasfous, I.I., Murimboh, C.A., Murimboh, J.,Chakrabarti, C.L., Grégoire, D.C. 2005. Complexation of Ni, Cu, Zn, and Cd by DOC in some metal-impacted freshwater lakes: a comparison of approaches using electrochemical determination of free-metal-ion and labile complexes and a computer speciation model, WHAM V and VI. Anal. Chem. Act. 528: 205-218.

Kaiser, K., Guggenberger, G., Haumaier, L., Zech, W. 2001. Seasonal variation in the chemical composition of dissolved organic matter in organic forest floor layer leachates of old-growth Scots pine (*Pinus sylvestris* L.) and European beech (*fagus sylvatica* L.) stands in northeastern Bavaria, Germany. Biogeochemistry. 55: 103-143.

Kone, B.C., Brenner, R.M., Gullams, S.R. 1990. Sulfhydryl-reactive heavy metals increase cell membrane K^+ and Ca^{2+} transport in renal proximal tubule. J. Membr. Biol. 113:1-12.

Kramer, K.J.M., Jak, R.G., Hanttum, B.V., Hooftman, R.N., Zwolsman, J.J.G. 2004. Copper toxicity in relation to surface water-dissolved organic matter: biological effects to *Daphnia magna*. Environ. Toxicol. Chem. 23: 2971-2980.

Kuiters, A.T., Denneman, A.J. 1987. Water-soluble phenolic substances in soils under several coniferous and deciduous tree species. Soil Biol. Biochem. 19(6): 765-769.

Kukkonen, J., Oikari, A. 1991. Bioavailability of organic pollutants in boreal waters with varying levels of dissolved organic material. Water Res. 25: 455-463.

Haitzer, M., Abbt-Braun G., Traunspurger, W., Steinberg, C.E.W. 1999. Effects of humic substances on the bioconcentration of polycyclic aromatic hydrocarbons: Correlations with spectroscopic and chemical properties of humic substances. Environ. Toxicol. Chem. 18: 2782-2788.

Hartwell, S.I., Jin, J.H., Cherry, D.S., Cairns, J. 1989. Toxicity versus avoidance response of golden shiner, *Notemigonus crysoleucas,* to five metals. J. Fish. Biol. 35: 447-456.

Heijerick, D.G., De Schamphelaere, K.A.C, Janssen, C.R. 2002. Predicting acute zinc toxicity for Daphnia magna as a function of key water chemistry characteristics: development and validation of a biotic ligand model. Environ. Toxicol. Chem. 21: 1309-1315.

Honge, D., Van Hees, P.A.W., Lundstrom, U.S. 2000. Dissolved components in precipitation water percolated though forest litter. European Journal of Soil Science. 51: 667-677.

Holbrook, D.R., Yen, J.H., Grizzard, T.J. 2006. Characterizing natural organic material from the Occoquan watershed (Nothern Virgina, US) using fluorescence

spectroscopy and PARAFAC. Sci. Tot. Environ. 361: 249-266.

Hollis, L., Muench, L., Playle, R.C. 1997. Influence of dissolved organic matter on copper binding and calcium on cadmium binding by gills of rainbow trout. J. Fish. Biol. 50: 703-720.

Hudson, N., Baker, A., Reynolds, D. 2007. Fluorescence analysis of dissolved organic matter in natural, waste and polluted waters: A review. River. Res. Applic. 23: 631-649.

Lauren, D.J., MacDonald, D.G. 1985. Effects of copper on branchial ionoregulation in rainbow trout, *Salmo gairdneri*: J. Comp. Physiol. B. 155: 635-644.

Lauren, D.J., Macdonald, D.G. 1986. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salma gairdneri*. Can. J. Fish. Aquat. Sci. 43: 1488-1497

Lauren, D.J., MacDonald, D.G. 1987a. Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. Can. J. Fish. Aquat. Sci. 44: 99-104.

Lauren, D.J., MacDonald, D.G. 1987b. Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. Can. J. Fish. Aquat. Sci. 44: 105-111.

Leenheer, J.A., Brown, P.M., Cabaniss, S.E. 1998. Models of metal binding structures in fulvic acids from the Suwannee river, Geogia. Environ. Sci. Technol.

Li, J., Quabius, E.S., Bonga, S.E.W., Flik, G., Lock, R.A.C. 1998. Effects of water-borne copper on branchial chloride cells and Na^+/K^+ ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*) Aquat. Toxicol. 43: 1-11.

Luider, C.D., Crusius, J., Playle, R.C., Curtis, P.J. 2004. Influence of natural organic matter source on copper speciation as demonstrated by Cu binding to fish gill, by ion selective electrode and by DGT get sample. Environ. Sci. Technol. 32: 2865-2872.

MacRae, R.K., Smith, D.E., Swoboda-Colberg, N., Meyer, J.S., Bergman, H.L. 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) gills: implications for assessing bioavailable metal. Environ. Toxicol. Chem. 18: 1180-1189.

Mann, C.J., Wetzel, R.G. 1995. Dissolved organic carbon and its utilization in a riverine wetland ecosystem. Biogeochemistry. 31: 99-120.

Markich, S.J., Batley, G.E., Stauber, J.L., Rogers N.J., Apte, S.C., Hyne R.V., Bowles, K.C., Wilde, K.L., Creighton, N.M. 2005. Hardness corrections for coper are inappropriate for protecting sensitive biota. Chemosphere. 60: 1-8.

Matsuo, A.Y., Playle, R.C., Val, A.L., Wood, C.M. 2004. Physiological action of dissolved organic matter in rainbow trout in the presence and absence of copper: \
Sodium uptake kinetics and unidirectional flux rates in hard and soft water. Aquat. Toxicol. 70: 63-81.

McCormick, S.D. 1993. Method for non-lethal gill biopsy and measurement of Na+, K+ ATPase activity. Can. J. Fish. Aquat. Sci. 50: 656-658.

McGeer, J.C., Playle, R.C., Wood, C.M., and Galvez, F. 2000. A physiologically based biotic ligand model for predicting the acute toxicity of waterborne silver to

rainbow trout in fresh waters. Environ. Sci. Technol. 24: 4199-4207.

McKnight, D.M., Feder, G.L., Thurman, M.E., Wershaw, R.L. 1983. Complexation of copper by aquatic humic substance from different environments. Sci. Tot. Environ. 28: 65-76.

McKnight D.M., Aiken, G. 1998. Sources and age of aquatic humus: In Hessen, D.O., Travic L.J. (Eds) Aquatic humic substances: ecology and biochemistry Vol. 133. Springer Verlag Berlin Heidelberg, Germany.

McKnight, D.M., Boyer, E.W., Westhoff, P.K., Doran, P.T., Kulbe, T., Andersen, D.T. 2001. Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic matter and aromaticity. Limnol. Oceanogr. 46: 38-48.

Milne, C.J., Kinniburgh, D.G., Tipping, E. 2001. Generic NICA-Donnan model parameters far proton binding by humic substances. Environ. Sci. Technol. 25: 2049-2059.

Neary, B.P., Dillon, P.J., Munro J.R., Clark, B.J. 1990. The acidification of Ontario lakes: an assessment of their sensitivity and current status with respect to biological damage. Ontario Ministry of the Environment. Toronto, Ont. p171.

Newman, M.C., Unger, M.C. 2003. Fundamental of ecotoxicology, 2nd edition. CRC, Boca Raton, FL, USA. pp 28-29.

Niyogi, S., Wood, C.M. 2003. Effects of chronic waterborne and dietary metal exposure on gill metal-binding: implications for the biotic ligand model. Hum. Ecol. Risk

Assess. 9: 813-846.

Niyogi, S., Wood, C.M. 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Tech. 38: 6177-6192.

Nolan, A.L., Mclaughlin, M.J., Mason, S.D. 2003. Chemical speciation of Zn, Cd, Cu and Pb in pore waters of agricultural and contaminated soils using donna dialysis. Environ. Sci. Technol. 37: 90-98.

Nriagu, J.O, G, Lawson, H.K.T. Wong, Cheam, V. 1996. Dissolved trace metals in lakes Superior, Erie, and Ontario. Environ. Sci. Techno. 30: 178-187.

Pagenkopf, G.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: Role of complexation, pH, and water hardness. Environ. Sci. Technol. 17 (6): 342-347.

Paquin, P.R., Santore, R.C., Wu, K.B., Kavvadas, C.D., Di Toro, D.M. 2000. The biotic ligand model: a model of the acute toxicity of metals to aquatic life. Environ. Sci. and Pol. 3: S175-S182.

Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M., and Wendelaar Bonga, S.E. 1995. Effects of combined waterborne Cd and Cu exposures on ionic composition and plasma cortisol in tilapia, Oreochromis mossambicus. Comp. Biochem. Physiol. C. Comp. Pharmacol. 111: 227-235.

Perdue, E.M. 1998. Chemical composition, structure and metal binding properties: In Aquatic humic substances. Edited by D.O. Hessen and L.J. Tranvik. Spring, pp 41-61.

Playle, R.C., Dixon, D.G., and Burnison K. 1993a. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. Can. J. Fish. Aquat. Sci. 50: 2678-2687.

Playle, R.C., Dixon, D.G., and Burnison K. 1993b. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modelling of metal accumulation. Can. J. Fish. Aquat. Sci. 50: 2678-2687.

Playle, R.C. 1998. Modelling metal interactions at fish gills. Sci. Tot. Environ.. 219: 147-163.

Richards, J.G., Burnison, B.K., Playle, R.C. 1999. Natural and commercial DOM protects against the physiological effects of combined cadmium and copper exposure on rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 56: 407-418.

Richards, J.G., Curtis, J.P., Burnison, K.B., Playle, R.C. 2001. Effects of natural organic matter source on reducing metal toxicity to rainbow trout (*Oncorhynchus mykiss*) and on metal binding to their gills. Environ. Toxicol. Chem. 20: 1159-1166

Ryan, A.C., Van Genderen, E.J., Tomasso, J.R., Klaine, S.J. 2004. Influence of natural Organic matter source on copper toxicity to larval fathead minnows (*Pimephales promelas*): implications for the Biotic ligand model. Environ. Toxicol. Chem. 23: 1567-1574.

Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E., Meyer, J.S. 2001. Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in

freshwater fish and daphnia. Environ. Toxicol. Chem. 20: 2397-2402.

Santore, R.C., Mathew, R., Paquin, P.R., Di Toro, D. 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*. Comp. Biochem. Physiol. 133: 271-285.

Schubauer-Berigan, M.K., Diekes, J.R., Monson P.D., Ankley G.T. 1993. pH-dependent toxicity of Cd, Cu Ni, Pb and Zn to *Ceriodaphnia dubia*, *Pimephales promelas*, *Hyalella azteca* and *Lumbriculus variegatus*. Environ. Toxicol. Chem. 12: 1261-1266.

Schwartz, M.L., Curtis, P.J., Playle, R.C. 2004. Influence of natural organic matter source on acute copper, lead, and cadmium toxicity to rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 23: 2889-2899.

Schiff, S.L., Aravena, R., Trumbore, S.E., Dillon P.J. 1990. Dissolved organic carbon cycling in forested watersheds: A carbon isotope approach. Water Resour. Res. 26: 2949–2957

Smith, D.S., Kramer, J.R., 1999. Fluorescence analysis for multi-site aluminum binding to natural organic matter. Environ. Int. 25: 295-306

Sorensen, E.M.B. 1991. Metal poisoning in fish. CRC, Boca Raton, FL, USA. pp 235-276.

Stedmon, C.A., Markager, S., Bro, R. 2003. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. Mar. Chem. 82, 239-254.

Stedmon, C.A., Markager, S., 2005. Resolving the variability in dissolved organic matter fluorescence in a temperate estuary and its catchments using PARAFAC analysis. Limnol. Oceanogr. 50: 686-697.

Suominen, K., Kitunen, V., Smolander, A. 2003. Characteristics of dissolved organic matter and phenolic compounds in forest soils under silver birch (*Betula pendula*), Norway spruce (*Picea abies*) and Scots pine (*Pinus Sylvestris*). Euro. J. Soil. Sci. 54: 287-293.

Sweileh, J.A., Lucyk, D., Kratochvil., B., Cantwell, F.F. 1987. Specificity of the ion exchange/atomic absorption method for free copper (II) species determination in natural waters. Anal. Chem. 59(4): 586-592.

Thacker, S.A., Tipping, E., Baker, A., Gondar, D. 2005. Development and application of functional assays for freshwater dissolved organic matter. Wat. Res. 39: 4559-4573.

Thurman, E.M, Wershaw, R.L., Malcolm, R.L., Pinckney, D.J. 1982. Molecular size of aquatic humic substances. Org. Geochem. 4: 27-35.

Thurman., E.M. 1985. Organic geochemistry of natural waters. Kluwe Academic, Bonston, MA, USA.

Tipping, E. 1998. Humic ion-binding model VI: An improved description of the interations of protons and metal ions with humic substances. Aquatic Geochemistry. 4: 3-48.

Tipping, E. 2002. Cation binding by humic substances. Cambridge University Press.

Toa, S., Long, A., Liu, C., Dawson, R. 2000. The influence of mucus on copper speciation in the gill microenvironment of carp (*Cyrpinus carpio*). Ecotoxicol. Environ. S. 47: 59-64.

Unsworth, E.R. et al. 2006. Model predictions of metal speciation in freshwaters compared to measurements by in situ techniques. Environ. Sci. Technol. 40: 1942-1949.

US EPA (United States Environmental Protection Agency). 1986. Quality criteria for water. US EPA Office of Water Regulations and standards, Washington, DC. EPA 440/5-85-001.

US EPA (United States Environmental Protection Agency). 2007. . Aquatic life ambient freshwater quality criteria – copper 2007 revision. Washington, DC. EPA-822-R-07-001.

VanGenderen, E.J., Ryan, A.C., Tomasso, J.R., Klaine, S.J. 2003. Influence of dissolved organic matter source on silver toxicitgy to *Pimephales promelas*. Environ. Toxicol. Chem. 22 (11), 2746-2751.

Vodacek, A., Blough, N.V., Degrandpre, M.D., Peltzer, E.T., Nelson, R.K. Seasonal variation of CDOM and DOC in the middle Atlantic might: terrestrial input and photooxidation. Limnol. Oceanogr. 42(4): 647-686.

Welsh, P.G., Parrott, J.L., Dixon, D.G., Hodson, P.V., Spry, D.J., Mierle, G. 1996. Estimating acute copper toxicity to larval fathead minnow (*Pimephales promelas*) in soft water from measurements of dissolved organic carbon, calcium, and pH.

Can. J. Fish. Aquat. Sci. 53: 1263-1271.

Welsh, P.G., Skimore, J.F., Spry, D.J., Dixon, D.G., Hodson, P.V., Hutchinson, N.J., Hickie, B.E. 1993. Effect of pH and dissolved organic carbon on the toxicity of copper to larval fathead minoow ((*Pimephales promelas*) in natural lake waters of low alkalinity. Can. J. Fish. Aquat. Sci. 50: 1356-1362.

Wilson, R.W., and Taylor, E.W. 1993. The Physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. J. Comp. Physiol. B. 163: 38-47.

Winter, A.R., Fish, T.A.E., Playle, R.C., Smith, C.D., Curtis, J.P. 2007. Photodegradation of natural organic matter from diverse freshwater sources. Aquat. Toxicol. 84: 215-222.

Wood, C.M. 2001. Toxic responses of the gill. In: Schlenk D and Benson WH (eds), Target Organ Toxicity in Marine and Freshwater Teleosts, vol 1, Organs, pp 1-89. Taylor and Francis, London, UK.