2013

DOES ECOSYSTEM DISTURBANCE ALTER THE CAPACITY OF DISSOLVED ORGANIC MATTER TO MITIGATE THE IMPACT OF COPPER TO HYALELLA AZTECA?

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Does ecosystem disturbance alter the capacity of dissolved organic matter to mitigate the impact of copper to *Hyalella azteca*?

by

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Honours B.Sc. Biology, Wilfrid Laurier University, 2011

THESIS

Submitted to the Department of Biology

Faculty of Science

in partial fulfilment of the requirements for the

Master of Science in Integrative Biology

Wilfrid Laurier University

2013

Abstract

The potential for aquatic ecosystem recovery as a result of dissolved organic matter (DOM) protecting against metal toxicity has become a significant area of research in environmental toxicology. It is a well-characterized relationship that DOM binds free metal ions in a concentration-dependant manner, making them unavailable for toxic action and a reduction in toxicity is seen. Less understood is source variability and how the upland terrestrial environment influences the protective quality of DOM. The aim of this study was to examine the influence of land disturbance (logging, fire, smelter emissions) on DOM quality by comparing the protective capacity of different sources on Cu toxicity and bioaccumulation in *Hyalella azteca*. Acute (96h) and chronic (28d) toxicity tests were done according to Environment Canada standard methods, and were completed in duplicate (acute) or triplicate (chronic) using 10 *Hyalella* aged 2-9 days old added to solutions of Cu (0-4µM) and DOM sources at a DOC concentration of 5mg C/L (acute) or 7mg C/L (chronic). Test solutions were maintained at pH 7.2±0.1, 21±1ºC, and 13mg/L $CaCO_3$ hardness. Both acute and chronic toxicity tests showed significant variability among sources, with disturbed sites offering less protection than reference sites. The acute results were supported with 6h Cu uptake/binding experiments and optical characterizations (excitation-emission matrix spectroscopy, absorbance at 340nm and fluorescent indices). Chronic toxicity was associated with the dry weight of organisms at Day 28, but not bioaccumulation because it appears that *H. azteca* are capable of regulating Cu. Both acute and chronic toxicity predictions were generated and improved by incorporating SAC_{340} and % humic acid content into the biotic ligand model (BLM). This project contributes toward an improved understanding of DOM quality characteristics and in conjunction with additional studies; it can potentially be applied to large scale ecosystem remediation efforts.

Acknowledgements

I would like to begin by thanking my supervisor Dr. Jim McGeer for providing me with guidance as well as so many invaluable opportunities throughout my M.Sc program. I would also like to thank the members of my committee Dr. Scott Smith and Dr. Kevin Stevens for their time and assistance throughout this project. I want to thank of all my lab-mates (past, present and honorary) for all of the help and friendship that they have given me in the past two years. I can't begin to express my thanks to my family. Andrew and Angela, Mom and Dad – thank you for your continuous support and encouragement not only throughout this program but always. Lastly, Anthony – thank you for your unconditional love and support, I would not have been able to do this without you

Glossary

BLM……..……..biotic ligand model

CETIS……...……comprehensive environmental toxicity information system

DOC…….……..dissolved organic carbon

DOM……..dissolved organic matter

EC20……...the exposure concentration associated with a 20% reduction in growth of exposed organisms compared with unexposed controls

EC50……..……concentration associated with a 50% growth effect

EEMS……....excitation-emission matrix spectroscopy

FA…….fulvic acid

FI……..fluorescence index

HA……...…….humic acid

LA50……...……...……accumulation on the biotic ligand that is associated with a 50% effect level, usually lethality

LC50….....the exposure concentration associated with 50% lethality

PARAFAC…….parallel factor analysis

QF…….…quality factor

SAC…….……specific absoroption coefficient

SEM…….……..standard error of mean

TALER……..……..Terrestrial-Aquatic Linkages for Ecosystem Recovery

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CHAPTER 1
Introduction

New research in ecotoxicology is focusing on the effects of dissolved organic matter (DOM) on metal toxicity. While numerous studies have shown that DOM protects against toxicity by reducing metal bioavailability, the variability among different DOM sources binding metals is not yet fully understood. DOM composition from each ecosystem is likely to have distinct characteristics because every ecosystem is unique. Understanding the ecological role of DOM in aquatic systems negatively impacted by the presence of metals is vital in assessing environmental contamination and refining toxicity predictions to improve remediation strategies.

1.1 Cu in Aquatic Systems

Copper (Cu) is an essential element and is required by most organisms; however when accumulated in excess, vital enzymes and proteins can be damaged, ultimately resulting in acute toxicity (Morris et al., 2003). Cu is known to be a necessary element for the proper functioning of haemocyanin, a respiratory protein (Rainbow, 2002). Cu is a ubiquitous element and is present in aquatic ecosystems as a result of natural geochemistry as well as anthropogenic activities. Naturally, Cu is input to aquatic systems by a multitude of processes including the weathering and erosion of copper-containing minerals which leads to the slow release of the metal into the environment (Georgopoulos et al., 2001). Additionally, Cu may enter aquatic ecosystems via human activities such as waste incineration, coal combustion, plumbing, anti-fouling agents on boats, as well as mining and smelting operations (Georgopoulous et al., 2001). Typically, Cu concentrations in uncontaminated aquatic environments range from 0.5 – 4 µg/L, while in metal-contaminated areas such as Sudbury Ontario, Cu concentrations have been documented to exceed 400 µg/L in certain lakes (Grossel 2011; Keller et al., 1998). Current freshwater quality guidelines, set by the Canadian Council of Ministers of the Environment

(CCME), are 2 – 4 µg/L Cu for waters that have a hardness of less than 120 mg $CaCO_3$ mg/L (CCME, 1999).

Copper can exist in a variety of forms in the water including $CuCO_3$, $CuOH^+$, and Cu^{2+}, among others (Paquin et al., 2000). When Cu^{2+} forms a complex with carbonate ($CuCO_3$) or other anions, it generally becomes unavailable for toxic action to aquatic biota (Paquin et al., 2000). The exception to this is copper-hydroxide which has been associated with toxicity in some aquatic organisms in high pH environments (Grossel et al, 2011). However, it is the free metal ion (Cu^{2+}) that is the associated with being the most toxic form of copper (Di Toro et al., 2001). Cu^{2+} is most detrimental to organisms because it is the most bioavailable form of the metal and once accumulated, it interacts to disrupt organism homeostasis.

1.2 Cu Toxicity

In aquatic organisms (including invertebrates such as *Hyalella azteca*), Cu^{2+} toxicity is best understood through consideration of mechanistic-approaches based on the physiological uptake and internalization of copper, rather than a result of metal adsorption to the surface of the organism (Borgmann, 1998). The mechanisms of Cu^{2+} toxicity in *Hyalella* are not as well established as they are for higher organisms such as rainbow trout. It is well-established that Cu exposure negatively affects ionoregulation in fish by preventing sodium ion (Na^+) uptake as a result of Na^+-K^+-ATP-ase impairment (Laurén and McDonald, 1985). In chronic exposures to Cu it has been documented that *H. azteca*, similar to most other organisms, are capable of internally regulating Cu and this is related to the fact that this is an essential element (Borgmann, 1998).

Cu toxicity may occur across a large range of Cu concentrations, depending on the aquatic medium (e.g speciation influences such as pH and DOC, as well as hardness cations) and

also the ability of the organism to regulate Cu (Sorensen, 1991; Borgmann, 1998). Although the mechanisms of uptake and accumulation are not completely characterized for *H. azteca*, it is generally theorized that toxicity occurs when Cu accumulation exceeds the combined detoxification and elimination capacities. This leads to interactions and sites of toxicity where physiological mechanisms become overwhelmed and disrupted resulting in impaired function (Borgmann, 1998). During acute toxicity, the influx and accumulation occurs rapidly and there is little chance for detoxification responses before the organism is overwhelmed. During a chronic exposure however, accumulated Cu can be detoxified and toxicity is associated with internal thresholds that are extremely localized to specific sites of toxicity. Thus, for chronic exposure, body burden is not a valuable indicator of toxic effects (Rainbow, 2002). Gaining an understanding of the mechanisms of Cu accumulation in *Hyalella azteca* will contribute to the knowledge of Cu toxicity in this amphipod.

1.3 Dissolved Organic Matter (DOM)

DOM is a term used to describe a group of non-anthropogenic complexes and molecules that arise primarily from the breakdown of plant and animal matter. DOMs in aquatic systems are heterogeneous in their composition, vary in complexity and are considered difficult to characterize. Thus, there are a number of approaches used to describe the complexities of DOM. One approach is to classify based on the relative composition of fulvic acid, hydrophilic acid, and humic acid; the three main fractions making up DOMs (Ma et al., 2001). DOMs can also be classified based on their origin, either allochthonous (also known as terrigenous) or autochthonous (McKnight et al., 2001; Lamelas et al., 2005). Terrigenous DOM is comprised mainly of humic acids and fulvic acids and enters into aquatic ecosystems primarily via the terrestrial runoff of decomposed plant and animal matter (Lamelas et al., 2005). Autochthonous

DOM consists mainly of nitrogenous and aliphatic groups as well as hydrophilic acids and originates within the aquatic system by the primary production activity of aquatic photosynthetic organisms such as algae or microbes (McKnight et al., 2001). Often DOM is quantified based on organic carbon content and environmental concentrations of dissolved organic carbon (DOC) typically range from 1 – 15 mg C/L in freshwater ecosystems (Thurman, 1985; David et al., 1997).

1.4 How DOM Affects Metal Bioavailability

DOM has a significant effect on the bioavailability of metal ions in aquatic environments. Organic matter acts as a modifying agent by binding free metal ions, generally considered to be the most toxic form of dissolved metal, reducing their bioavailability and also toxicity (De Schamphelaere et al., 2004a). Copper is a metal that is highly influenced by the presence of organic matter because the negative functional groups (e.g. carboxylics, phenolics, hydroxyls) within DOM have a high binding affinity for Cu (Playle, 1998). For example, the work of Welsh (1996) showed that the LC50 for *H. azteca* increased from 0.13 to 0.94 µM Cu when the DOC increased from 0.4 to 3.4 mg C/L. Other studies with aquatic organisms have shown a similar pattern of the Cu LC50 increases with increasing DOC concentration (Kramer et al., 2004; De Schamphelaere et al., 2004b; Erickson et al., 1996). DOM provides a protective effect against the toxicity of Cu to aquatic organisms in a DOC concentration-dependent manner. This relationship implies that the metal-binding capacity of DOM is correlated to the carbon content and measurements of Cu^{2+} in solution show that complexation by DOM reduces the amount of Cu that is bioavailable for toxic action (De Schamphelaere et al., 2004a).

It has been hypothesized that sources of DOM vary in their potential to ameliorate metal toxicity due to the relative composition of different functional groups (De Schamphelaere et al.,

2004a). A study done by Hicks (2009) examined the influence of DOM quality (ten sources from across the Canadian Shield) on the acute toxicity of Cu to rainbow trout (*Oncorhynchus mykiss*) and found nearly a 3.5-fold difference among DOM sources. It is evident that there are differences among sources of DOM with respect to the ability to bind Cu and reduce metal bioavailability; however it is still unknown why these differences exist and the linkages between source variability and Cu binding capacity. Although it is established that DOM acts to reduce metal toxicity, more research is required in order to understand why the quality (source) of organic matter has such a significant influence on the expression of toxicity in aquatic invertebrates.

1.5 Influence of Upland Vegetation on DOM Quality

The quality of terrigenous DOM is likely defined by upland vegetation characteristics in combination with degradation transfer processes occurring along the land-water interface; however this has never been tested in relation to metal toxicity**.** Hiriart-Baer et al. (2008) documented that the wetlands surrounding aquatic ecosystems play a significant role in the input of terrigenous DOM to the system. Generally, DOC concentrations within an aquatic ecosystem vary over time as a result of several factors that influence the input of decayed organic matter from the surrounding terrestrial environment, including storms, drought, and climate (Snucins and Gunn, 2000). Wetlands are thought to play a key role in DOM sequestration and higher concentrations of DOC are found in lakes that have inputs from these sources. In catchments that are influenced by wetlands DOC concentrations tend to be less variable across seasons because of the slow discharge of water and DOC (Cuss et al., 2010). Schiff et al (1997) conducted a study to determine the process of DOC export from forested catchments into nearby aquatic environments. Radio-labelled carbon was used to determine where the majority of the

DOC originated and it was observed that much of the DOC (55%) came from the breakdown of deciduous leaves; primarily maple leaves (Schiff et al., 1997). Further research is needed to understand how the terrestrial vegetation of surrounding catchments influences the quality of DOC entering aquatic systems.

1.6 Recovery of Damaged Ecosystems

The characteristics of DOM from damaged ecosystems, particularly properties related to toxicity mitigation are the focus of this project. Different types of ecosystem disturbance will be assessed, such as long-term smelter damage, fire, and logging in an attempt to understand how terrestrial damage can influence aquatic DOM quality (Figure 1.1). Because DOM plays a significant role in not only toxicity mitigation, but also in the delivery of carbon and nutrients to aquatic systems, it is crucial to understand how the quality of DOM can differentially protect organisms in contaminated environments (Snucins and Gunn, 2000) and to understand the role that DOM has in the processes that damaged ecosystems undergo to return to reference condition

1.7 *Hyalella azteca*

The short life cycle, reproduction patterns, as well as the amenability to laboratory culturing contribute to why *Hyalella* is a successful organism for toxicity testing. This organism is very sensitive to several metals such as copper, nickel, and cadmium (Borgmann et al., 2005; Borgmann et al., 1989). *Hyalella* can be abundant and are representative of sensitive biota in the aquatic environment. Furthermore, there are established standard test methods for this organism (Environment Canada 1997, 2013). *Hyalella azteca* are good model organisms and highly relevant organism in terms of Canadian environmental protection.

Hyalella azteca is a small, benthic aquatic amphipod from the class Crustacea (Pennak 1978). These organisms are found in most unpolluted freshwater lakes, ponds, and streams across the Canadian Boreal Shield and are commonly abundant in waters that exceed 10ºC in the summer months (Shuhaimi-Othman and Pascoe, 2001; Environment Canada 2013). *Hyalella* are important organisms in aquatic food webs, providing a food source for many animals including birds, fish, and larger invertebrates. *Hyalella* are omnivores, mainly surviving on algae, bacteria, and detritus (Hargrave, 1970). Although this organism is often found swimming freely in the water column, *Hyalella* are known to inhabit sediment-rich areas that provide a source of cover as well as nutrition (Environment Canada, 2013).

Hyalella have an annual life cycle in which at least eight molting periods (instars) occur (Cooper, 1965). Within this life cycle, there are three stages: the immature stage (includes the first 5 molts), the juvenile stage (molting periods 6 and 7) and the adult stage which is the 8th molt and older (Cooper, 1965). Sexual maturation begins within 5-6 weeks of birth and optimal reproduction occurs by approximately 8-12 weeks, however these time points depend on environmental conditions (Borgmann et al., 1989). When eggs are fertilized (in the brood pouch) females can produce between 1 and 50 offspring per brood, with more offspring being produced in summer months with warmer water temperatures (Cooper, 1965). In general, female *Hyalella* produce offspring for 12-14 weeks after sexual maturation (Borgman et al., 1989).

1.8 Biotic Ligand Model (BLM)

The biotic ligand model (BLM) is a recently accepted regulatory tool (e.g. Canada, USA, EU) that provides a means for predicting acute metal toxicity at a specific receptor site (the biotic ligand) on aquatic organisms (Di Toro et al., 2001). The BLM focuses on the free metal ion as the most toxic form of metal to aquatic organisms (Paquin et al., 2000). Disruption of ionic

homeostasis will occur when the free metal ion binds to the biotic ligand, and acute toxicity will occur (Di Toro et al., 2001). Although the site of toxic action is unknown for *Hyalella*, it is suspected that much like higher organisms (e.g. rainbow trout) it is the respiratory surface (i.e. coxal gill) where Cu is accumulated (Borgmann, 1998). The toxicity is estimated based on the site-specific parameters of the surrounding aquatic environment such as: relative concentration of the free metal, complexation with inorganic or organic compounds, competition among cations for uptake at the biotic ligand, and pH (Niyogi and Wood, 2004; Paquin et al., 2000). The previously mentioned water quality parameters can influence metal toxicity by altering the bioavailability of the free metal ion. Figure 1.2 provides a conceptual schematic of the copper BLM and depicts the complexation and competition interactions that influence metal free ion concentrations and uptake (adapted from Santore et al., 2001). Although the BLM is normally used to predict acute Cu toxicity, several studies have successfully developed chronic Cu BLMs for various organisms including *Ceriodaphnia dubia* (Schwartz and Vigneault, 2007), *Daphnia magna* (De Schamphelaere et al., 2004b), and the rotifer *Brachionus calyciflorus* (De Schamphelaere et al., 2006). The BLM is a is the most advanced freshwater toxicological modelling system available and is used in setting regulatory guidelines for metal levels in freshwater systems.

1.8.1 Role of DOM in BLM

In the updated version of the BLM (ver. 2.2.3), DOM is accounted for because it readily binds free metal ions (complexation), but DOM source variability is not traditionally included within the model. Although it is known that sources of DOM differ in their ability to mitigate metal toxicity based on their composition and the relative presence of Cu-binding groups, this variability among sources has not yet been incorporated into the modelling process. In future

models, DOM quality needs to be better understood so that this aspect can be integrated into toxicity prediction BLMs for accurate guidelines to be created in environmental policy-making.

1.9 Objectives

The main long-term objective of this research project was to understand how to assess DOM composition in relation to Cu toxicity mitigation which can potentially help to set better environmental guidelines. Ultimately, this research will potentially provide a basis for better understanding and predicting the recovery and remediation processes of aquatic ecosystems that have been damaged by either metal contamination from mining/smelting emissions, fire, or logging. The short-term objectives of this research project occurred in three phases:

1) To assess how different sources of DOM mitigate Cu toxicity to the chosen model organism (*Hyalella azteca*). This was achieved through a series of acute and chronic toxicity tests with DOM sources from different sources added to Cu solutions. Several sources of DOM from either undisturbed or differently damaged Canadian boreal lakes (e.g. logging, fire, smelter) were tested at a range of Cu concentrations with the purpose of understanding how Cu toxicity mitigation is affected by DOM quality (Fig. 1.3). This work is documented in *Chapter 2 – Chemical, optical, and acute biological characterizations of DOM quality* and *Chapter 3 – Effect of DOM source n the chronic survival, growth, and accumulation of Cu to Hyalella azteca*.
2) To use a suite of optical and chemical methods to characterize the DOM sources in order to gain insight to the reasons why DOM quality differs in protecting against Cu toxicity. The different sources were characterized with techniques such as: ion selective electrode (ISE), excitation emission matrix spectroscopy (EEMS), and absorbance at 340nm (SAC_{340}) to explore the relationships between DOM chemical

characterization and toxicity mitigation. The work focusing on this objective is found in *Chapter 2*.

3) To incorporate the DOM characterizations along with acute and chronic toxicity data into the BLM in order to generate more accurate toxicity predictions that take into account DOM quality as well as ecosystem disturbance and recovery. Results of the modelling can be found in *Chapter 2* and *Chapter 3*.

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1.11 Figures

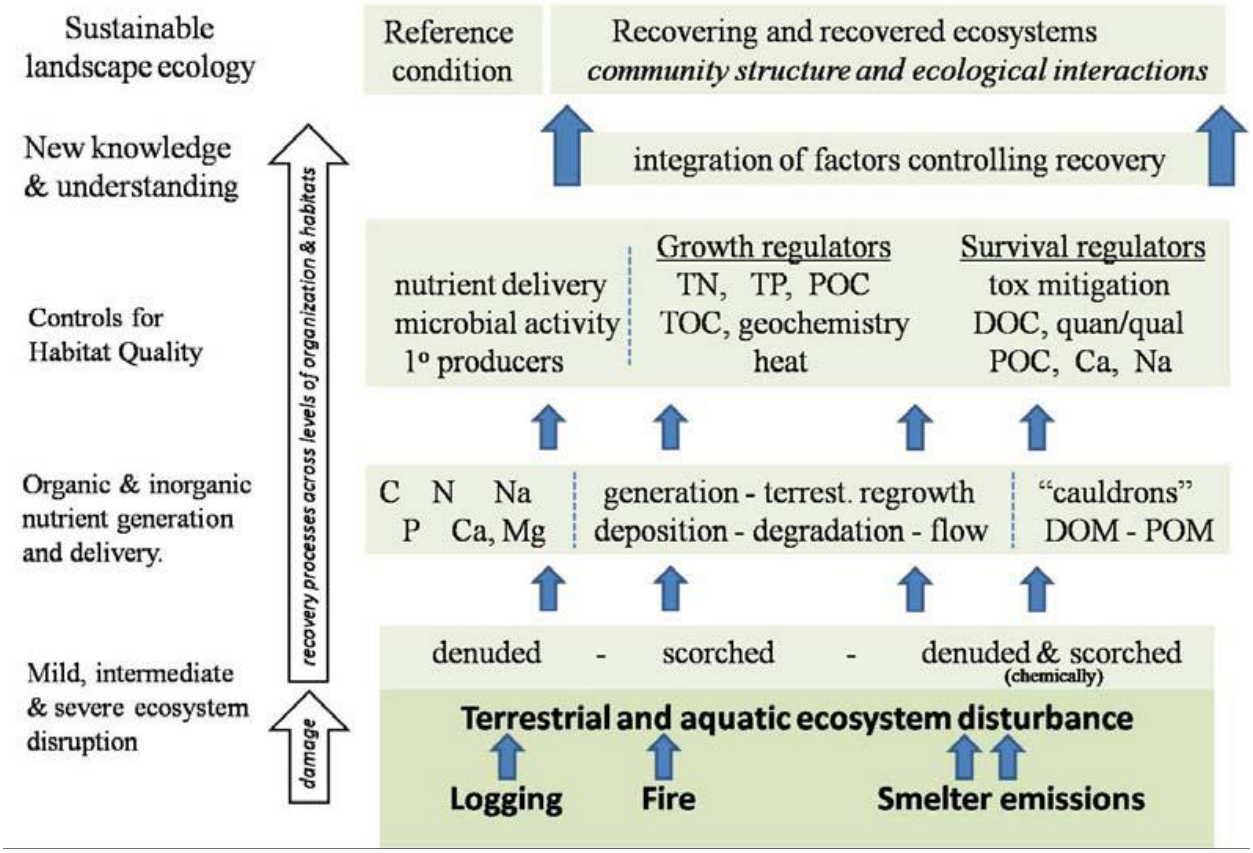

Figure 1.1 – Conceptual framework for the 5 year TALER (Terrestrial-Aquatic Linakages for Ecosystem Recovery) project. This particular aspect of the overall study is focussing on the right hand side of the diagram (survival regulators) and then integrating into the other factors controlling ecosystem recovery.

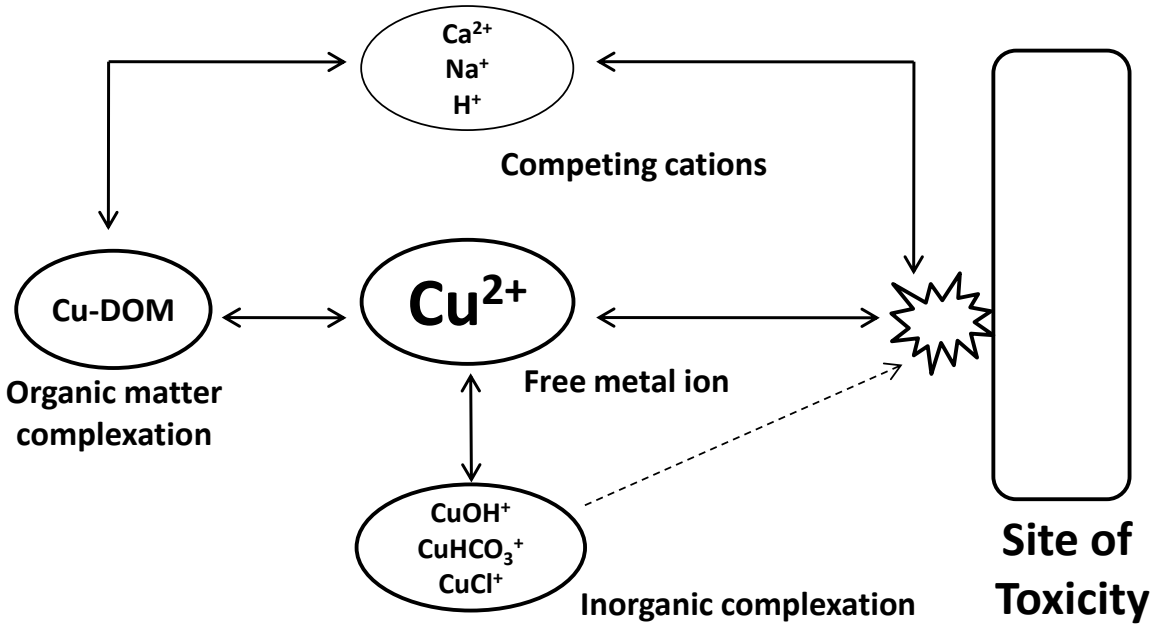

Figure 1.2 – Schematic representation of the Biotic Ligand Model (BLM; Santore et al., 2001) for the speciation and toxicity of Cu^{2+}. Free metal ion (Cu^{2+}) is the toxic form of copper, acting at the respiratory surface (site of toxic action). Cu^{2+} uptake is altered by competing cations or by binding to inorganic or organic compounds. DOM quantity but not quality is taken into account in the BLM.

Dorset, ON ★

Sudbury, ON ★

White River, ON ★

Figure 1.3 – Sources of DOM collected in 2011 and 2012 by portable reverse osmosis. All samples collected are considered to be soft waters from lakes/rivers on the Canadian Shield. Sample sites were White River (red), Subury (purple), and Dorset (black).

CHAPTER 2

Chemical, optical, and acute biological characterizations of DOM quality

2.1 Introduction

Copper (Cu) is an essential element that is required by most organisms; however an excess of this metal can damage vital enzymes and other proteins. This can ultimately lead to acute toxicity (Morris et al., 2003). It is generally believed that the free metal ion (Cu^{2+}) is the most toxic form of copper because it is the most bioavailable form of the metal and once accumulated it can interact to disrupt organism homeostasis (Di Toro et al., 2001). Cu toxicity may occur across a large range of Cu concentrations, depending on the conditions of the aquatic medium (e.g speciation influences such as pH and DOC, as well as hardness cations) and also the ability of the organism to regulate Cu (Sorensen, 1991; Borgmann, 1998).

Hyalella azteca is a small aquatic invertebrate found in most unpolluted freshwater lakes, ponds, and streams across the Canadian Boreal Shield (Shuhaimi-Othman and Pascoe, 2001). It is known to be sensitive to metals such as copper, nickel, and cadmium and has been studied extensively (Borgmann et al., 2005; Norwood et al., 2007). Furthermore, there are established standard test methods for this organism (Environment Canada 1997, 2013). Therefore, *Hyalella azteca* are good model organisms and are considered to be relevant organism in terms of Canadian environmental protection.

Natural dissolved organic matter (DOM) occurs in aquatic systems as a heterogeneous mixture that varies in complexity and is difficult to characterize. DOMs are often classified based on origin, either terrigenous or autochthonous (McKnight and Aiken, 1998). Terrigenous DOM is comprised mainly of humic acids and fulvic acids and enters into aquatic ecosystems primarily via the terrestrial runoff carrying decomposed plant and animal matter (McKnight and Aiken, 1998). Autochthonous DOM consists mainly of nitrogenous and aliphatic groups as well

as hydrophilic acids and originates within the aquatic system by the primary production activity of aquatic photosynthetic organisms (McKnight et al., 2001). DOM is often characterized based on the concentration of dissolved organic carbon (DOC) and the typical range in freshwater ecosystems is from 1 – 15 mg C/L (Thurman, 1985; David et al., 1997). DOM can significantly reduce the bioavailability of metals in aquatic environments, by binding free metal ions and thereby mitigating toxicity (De Schamphelaere et al., 2004). Numerous studies have shown a similar pattern of Cu LC50 increases with increasing DOC concentration (e.g. Al-Reasi et al., 2012; Kramer et al., 2004; De Schamphelaere et al., 2004; Erickson et al., 1996) and Welsh (1996) showed that the LC50 for *H. azteca* increased from 0.13 to 0.94 µM Cu when the DOC concentration increased from 0.4 to 3.4 mg C/L. DOM can provide a protective effect against the toxicity of Cu to aquatic organisms in a DOC concentration-dependent manner.

It is hypothesized that sources of DOM vary in their potential to ameliorate metal toxicity due to the relative composition of different Cu-binding groups (e.g. phenolics, carboxylics, hydroxyls) as a result of unique inputs from the surrounding landscape (De Schamphelaere et al., 2004). Differences (up to four-fold) among DOM sources in mitigating acute Cu toxicity have been observed in studies with *Daphnia magna* (Al-Reasi et al., 2012; Ryan et al., 2009; De Schamphelaere et al., 2004), the fathead minnow (Ryan et al., 2004) and rainbow trout (Gheorghiu et al., 2010; Schwartz et al., 2004; Richards et al., 2001). It is evident that there are differences among sources of DOM with respect to the ability to bind Cu and reduce metal bioavailability and although there are hypotheses, it is still unclear why these differences exist and what the linkages are between DOM source and protection against Cu toxicity.

Numerous chemical and optical methods have been developed for characterization of DOM to quantify the variability among DOM sources. The review by Abbt-Braun et al (2004)

provides a detailed overview of techniques commonly used to characterize DOM. Cu ion selective electrode (ISE) can be used to measure free Cu^{2+} in order to determine the binding capacity and affinity of each DOM source for Cu (Schwartz and Vigneault, 2007; Lu and Allen, 2002). One of the optical characterizations involves measuring the absorbance of the DOM sources at 340 nm to calculate specific absorption coefficient (SAC_{340}) values in order to assess the aromaticity of the samples. Darker, more aromatic DOMs have been generally found to be more protective against acute Cu toxicity (Al-Reasi et al., 2012; Schwartz et al., 2004). Another technique that can be employed to optically distinguish differences among DOM sources is fluorescence excitation emission matrix spectroscopy (EEMS). Through this type of analysis, the fluorescence index (FI) can be used to determine DOM origin (e.g. terrigenous vs. autochthonous; McKnight et al., 2001). Additionally, from EEMS, the relative percent composition of the DOM sources can be determined using a multi-variate analysis known as parallel factor analysis (PARAFAC; De Palma et al., 2011; Stedmon et al., 2003). Briefly, all EEMS data are compiled and PARAFAC identifies as well as quantifies relative amounts of four specific fluorophores (HA-like, FA-like, tyrosine-like, and tryptophan-like) (Al-Reasi et al., 2011; De Palma et al., 2011). This type of analysis elucidates molecular differences among DOM sources and may explain the variability observed among DOM sources in mitigating Cu toxicity. The amount of humic-like substance has been found to generally correlate well with protection against acute Cu toxicity because of the Cu-binding groups (e.g. phenolics) that are found within humic compounds (Al-Reasi et al., 2011). A more detailed explanation of EEMS and PARAFAC analysis can be found in the review by Andersen and Bro (2003).

The biotic ligand model (BLM) is a modelling program used to predict acute metal toxicity at a specific site (the biotic ligand) in aquatic organisms based on site-specific water

quality parameters (Paquin et al., 2000). The parameters that the BLM takes into account are: the relative concentration of the free metal, complexation with organic and/or inorganic compounds, pH, and competition among cations (e.g. Mg^{2+} and Ca^{2+}) for uptake at the biotic ligand (Niyogi and Wood, 2004; Paquin et al., 2000). Within the BLM (ver. 2.2.3), DOC is accounted for because it readily binds free metal ions (complexation), but it is treated as a single homogeneous entity. Although it is known that sources of DOM differ in their ability to mitigate metal toxicity based on their composition, this variability among sources has not yet been incorporated into the modelling process. There are two existing parameters that may be altered within the BLM to account for organic matter: DOC concentration and %HA content (normally set at 10%). In the future, DOM quality should be better understood so that this aspect can be integrated into improved toxicity prediction models.

The characteristics of DOM from damaged ecosystems, particularly properties related to toxicity mitigation were the focus of this project. Different types of ecosystem disturbance were assessed, for example long-term smelter damage, fire, and logging, to determine if/how terrestrial damage influences DOM source variability and quality. Two undisturbed (no human influence) reference sites were also tested. Because DOM plays a significant role in not only toxicity mitigation, but also in the delivery of carbon and nutrients to aquatic systems, it is crucial to understand how the quality of DOM can differentially protect organisms in contaminated environments (Snucins and Gunn, 2000). The goal of this research project was to develop an understanding of DOM composition (using optical and chemical characterization techniques) in relation to acute Cu toxicity mitigation. To achieve this objective, twelve DOM sources were collected from differently damaged watersheds across Ontario (Fig. 1.1, Table 2.1). DOMs were collected and characterized chemically and optically and compared to biologically-

based characterizations using the invertebrate *Hyalella azteca* as a model organism. Additionally, chemical and optical characterizations (ISE, EEMS, SAC_{340}, and FI) were used in conjunction with the modelling software program BLM to determine if the DOM source variability data could be incorporated to improve acute toxicity predictions. Ultimately, this research is a component of a larger study (TALER – Terrestrial-Aquatic Linkages for Ecosystem Recovery) to provide a basis for better understanding and predicting recovery and remediation processes of aquatic ecosystems that have been damaged by either metal contamination from mining/smelting emissions, fire, or logging.

2.2 Methods

2.2.1 DOM Sources and Collection

DOM collections were done between July and November 2011 at ten boreal shield sites representing either lakes (n=4), streams (n=3) or outflow from wetlands (2 sites, one of which was sampled twice) and characterized for optical properties (absorbance and fluorescence), the ability to complex Cu^{2+} in solution and the capacity to influence the bioavailability and toxicity of dissolved Cu. Sites were in the Muskoka region, the Sudbury region and the White River area of Ontario, Canada (Table 2.1). The Sudbury DOM collection sites (Laurentian Lake, Laurentian Wetland, Daisy Lake, Daisy Wetland, Clearwater Lake) were chosen to provide characterization across a gradient of smelter-induced impact and subsequent recovery. All of these lakes were impacted by both acid and metals (Ni, Zn, and Cu for example) and have recovered to varying degrees (Keller et al., 2004). Clearwater Lake has undergone significant natural biological and chemical recovery since the 1970s with numerous biological communities returning to levels of the similar to pristine lakes on the Canadian Shield (Winter et al., 2004).

Two additional sites were collected in August of 2012 from Clearwater Lake at two different depths (surface water and 17m). Clearwater Lake stratifies during the summer and the samples were collected to investigate potential differences between the DOM quality between epilimnion (surface) and hypolimnion (17m depth; Beauclerc and Gunn, 2001). Daisy Lake is classified as a highly contaminated lake due to its previously acidic pH (4.7) and presence of high concentrations of both nickel and copper as a result of waste drainage from nearby mining operations (Dixit et al., 1996). The three White River sites for DOM collection (see Table 2.1) were chosen to gain an understanding of the potential for fire and logging disturbance to influence DOM quality. The White River Site 2 was from a stream draining a watershed that had 80% of the landscape burned by a forest fire 12 years previously (data from Kreutzweiser, Canadian Forest Services) while Site 3 was at the outflow of a catchment that had been logged in 2004 (Kreutzweiser et al., 2010). White River Site 1 and the Muskoka collection site (Harp Lake) represented undisturbed reference sites that had no anthropogenic influence on the DOC or metal concentrations within the water.

All DOM sources were collected and preserved following the methods described by Schwartz et al. (2004) using a custom built portable reverse osmosis unit equipped with 400 Da molecular mass-cutoff membranes (FilmTec FT30, Minneapolis, MN). Typically the collection process involved reducing 250 L of surface water to 8L of concentrate. DOM concentrates were resinated to pH 2 using an H^+ cation-exchange resin (USF C-211 H cation resin, U.S. Filter Corporation, Rockford IL) to remove all residual metals and cations from DOM binding sites, after which it was refrigerated for storage (Schwartz et al., 2004) in polyethylene acid-washed containers.

2.2.2 *Hyalella azteca* Culture

Hyalella azteca used in toxicity testing were collected in August 2010 from Hannah Lake, near Sudbury which is a lake that had been previously damaged but had recovered (as a result of additions of lime in the 1970s; Yan et al., 1996). This liming process has resulted in a stable and neutral pH that supports healthy populations of aquatic invertebrates (Watson, 1992). *Hyalella* were first documented to be in Hannah Lake in 2003 and populations have remained since then (Babin-Fenske et al., 2012). *Hyalella* were identified using taxonomic keys (Pennak, 1978) and the species was confirmed using genetic analysis (Babin-Fenske et al., 2012). The culturing procedures for *Hyalella* followed the Environment Canada Standard Test Methods (Environment Canada 1997). Approximately 50-60 adults were maintained in 1.5 L glass beakers with 1.0 L of artificial soft water made with 0.1 mM $CaCl_2$, 0.1 mM $NaHCO_3$, 0.025 mM $MgSO_4$, and 0.005 mM KCl supplemented with 1 µM NaBr (Borgmann et al., 1996) as well as a 5x5 cm piece of gauze to act as a substrate (Shuhaimi-Othman and Pascoe, 2001). The pH of the culture medium was 7.2 ± 0.1, hardness 13 mg $CaCO_3$/L and the temperature was 21^{o}C. The media was replaced once per week and *Hyalella* were fed 5 mg of ground Tetramin flakes three times per week (Borgmann and Munawar 1989).

2.2.3 Acute Toxicity Tests

Acute (96h) toxicity tests with DOM added at 5 mg DOC/L and Cu concentrations ranging from 0. 5 - 4 µM were done to assess the protective influence of DOM sources and for comparison additional trials were also done with no added DOM (Cu only with concentrations ranging from 0.125 – 4 µM). Tests were carried out in duplicate 400 mL beakers, with 250 mL of test solution made with culture water and containing 10 *H. azteca* of age 2 – 9 days, following standard methods (Environment Canada 1997). Each test series included an unexposed control

that contained only artificial soft water (no Cu or DOM) and in tests with added DOM, a positive control with only DOM (no Cu) was included. Test solutions were prepared 24h prior to test initiation to allow for the equilibration of the solutions (Kim et al., 1999) and during this time a piece of 5x5 cm gauze was also equilibrated with the exposure solution in a separate beaker (Neumann et al., 1999). Food was not added during acute toxicity tests. When conducting toxicity tests with DOM, solutions were prepared by appropriately diluting concentrate and adjusting the pH to 7.2 with 0.1M NaOH (Schwartz et al., 2004).

2.2.4 Short term Cu Bioaccumulation

To assess the capacity of DOM to alter Cu bioavailability, short term (6h) bioaccumulation experiments were done with a subset of selected DOMs. A 6h exposure period was chosen as this was previously shown to be sufficient time to allow for significant accumulation but also before acute toxicological effects became evident (Livingstone undergraduate thesis, 2010). Test solutions were prepared as described for the toxicity testing using the DOM sources that showed elevated protection capacity or weak protective capacity (White River sites 1, 2 and 3, Daisy Lake and Harp Lake) and as with toxicity tests, a concentration of 5 mg DOC/L was used. Short term accumulation was also measured in Cu solutions without added DOM.

2.2.5 Sampling and Characterization

Cu and TOC Analysis

Water sampling during toxicity tests consisted of 10 mL samples for both total and dissolved (0.45 µm filtered; Acrodisc HT tuffryn membranes, Pall Corporation, Ann Arbor, MI) Cu concentrations at test initiation and completion. Cu concentrations were measured via

graphite furnace atomic absorption spectroscopy (GF - AAS: SpectAA-880, with GTA 100, Varian Inc, Palo Alto, CA). Two certified reference materials were used to verify measurements (TMDA 26.3, National Water Research Institute, Burlington, ON and SRM 1643e from National Institute of Standards and Technology, Gaithersburg MD, USA). Samples for GF – AAS were acidified with a 1% volume of 16N HNO_3 (Trace Metal Grade, Fisher Scientific, Nepean, ON). The concentration of ions (Ca^{2+} and $Mg^{2+)}$ in the solutions of a subset of samples from each acute toxicity test was determined via flame - AAS.

Water samples (30 ml) were also collected for subsequent analysis of DOC concentrations in the test media. Samples were filtered (0.45µm as described above) and then kept at 4 ºC until measurement and DOC was measured using a TOC analyzer. DOC concentrations from acute toxicity tests (except for CWL-s and CWL-d) were measured using the Shimadzu TOC-V (with ASI-V autosampler, Mandel Scientific, Guelph, ON) while all other DOC measurements (6h bioaccumulation, ISE, and optical measurements) were completed with Shimadzu TOC-$L_{CPH/CPN}$. Earlier DOC measurements were based on total organic carbon (inorganic carbon was subtracted from the total to obtain the amount of DOC) and were calibrated with inorganic (sodium bicarbonate) and organic (potassium hydrogen phthalate) standards. The later DOC measurements were conducted using non-purgable organic carbon (NPOC) analysis. Standard solutions for total carbon were used as reference when analysis was conducted (5 and 10 mg C/L) and were prepared from potassium hydrogen phthalate (Mandel Scientific, Guelph ON).

Tissue digests

At the end of the short term bioaccumulation tests, individual amphipods were transferred to a paper towel to remove excess moisture and then to put into 0.6 mL sample tubes, dried at 80^{o}C for 48h and then weighed to the to the nearest µg using a Sartorius SE2 Ultra Micro Balance (Sartorius Mechantronics Corp., Bohemia, NY, USA). Next, *Hyalella* were individually digested in 25 µL of 16N HNO_3 for 6 days. Following this, 20 µL of hydrogen peroxide was added and after 24h the final volume was brought to 250 µL per *Hyalella* using de-ionized water (Neumann et al., 1999). Cu content in each organism was measured by GF-AAS as described above.

2.2.6 Characterization of DOM Quality

All quality characterizations were conducted on filtered samples at 5 mg C/L and the pH was adjusted to 7.2 ± 0.1.

DOM Binding Capacity Characterization

Free Cu^{2+} was measured in solutions by ion selective electrode (ISE) using an Orion Ionplus Cu electrode (Orion Ionplus, Thermo Electron Corporation, Beverly, MA**)** to determine the binding capacity and affinity of each DOM source for Cu. The electrode was calibrated before measurements were taken; a two-point calibration was generated with two buffers: glycine (0.001M) and ethylene diamene (0.001M) following the methods outline by Belli and Zirino (1993). Test solutions (50 ml) were prepared by spiking DOM solutions (WR sites 1, 2, 3, DL and HL at 5 mg C/L) with Cu to achieve nominal concentrations of 0.063 – 2µM. Total Cu concentrations were measured by GF-AAS as described above. Free Cu^{2+} was measured in

millivolts (mV) as the solution was passed through the electrode using a flow-through system. The mV response was documented when readings stabilized to $\pm$ 0.1 mV/min.

Optical Characterization

Fluorescence and absorbance measurements were done in a 1 cm quartz cuvette (Hellman Canada Ltd., Concord Canada) that was rinsed with Milli-Q water (18 Mohm, Millipore Corporation, Fisher Scientific, Nepean, ON) and DOM solutions before measurement. EEMS measurements were taken for each DOM source (5 mg C/L) using a fluorometer (Cary Eclipse, Varian, Mississauga, Ontario) following the methodology outlined by Smith and Kramer (1999). Excitation wavelengths started at 200 nm, increasing in 10 nm steps to a maximum of 450 nm while emission was computed across a wavelength range of 250-600 nm. Parallel factor analysis (PARAFAC) was used to analyze the EEMS data (described below). DOM quality was also assessed by measuring the absorbance (against a blank de-ionized water sample) of each source at 340 nm in order to examine the aromaticity of each source. Absorbance measurements were converted into specific absorption coefficients (SAC_{340}; see section 2.1.7; Richards et al., 2001). Fluorescence index (FI) was calculated in order to distinguish between the origins of each DOM source (terrigenous or autochthonous; McKnight et al., 2001).

2.2.7 Calculations and Statistical Analyses

The lethal concentration resulting in 50% mortality (LC50) was calculated with mortality data and measured dissolved copper concentrations using the trimmed Spearman Karber method (Hamilton et al., 1977) within CETIS (Comprehensive Environmental Toxicity Information System, Tidepool Software, 2005). Significant differences between LC50s were deemed to occur when 95% confidence intervals did not overlap (Gillis et al., 2010). However, if any

confidence intervals did overlap, the Litchfield and Wilcoxon method was utilized to determine if they were significantly different (Environment Canada, 2005). Whole organism Cu body burden was normalized for the dry weight of individual *Hyalella* (µg Cu/g dry weight). A one-way ANOVA (Fisher LSD) was used to compare bioaccumulation measurements between exposure concentrations and the control within a test or to compare between toxicity tests at one concentration. The limit for significance was $p<0.05$. Short term bioaccumulation was modelled using body burdens at different exposure concentrations within each tested DOM source using the Michaelis-Menten equation (a Langmuir isotherm):

$$Y = (V_{max} \times X) / (K_m + X) \qquad \text{Equation 1}$$

where Y is the whole body Cu accumulation (µg Cu/g dry weight), V_{max} represents the maximum binding of dissolved Cu to the ligand (µg Cu/g dry weight), X is the concentration in the exposure medium (µM Cu), and K_m is a measure of the binding affinity, the Cu concentration that results in half V_{max}.

Standard curves generated from the ISE procedure were utilized to convert ISE measurements (mV) into free Cu^{2+}. By subtracting free Cu^{2+} from total Cu, the Cu bound to DOC was determined. Free Cu^{2+} and Cu bound to DOC were plotted and binding curves were generated using SigmaPlot. The function used was a hyperbolic rise to saturation as determined by Equation 1, however, where Y is the amount of Cu bound to DOC (mol Cu/mg C), B_{max} is the maximum binding capacity of the DOM source (mol Cu/mg C), K_m is the free Cu^{2+} exposure concentration that produced the half-saturation of the DOM, and X is the free Cu^{2+}. K_m values were expressed by using the negative logarithm (base 10) of the concentration values.

The relative amount of humic acid-like (HA-like) and fulvic acid-like (FA-like) was determined by creating an excitation-emission based characterization of the fluorophores

obtained from the EEMS data (De Palma et al., 2011). The EEMS data was analyzed using the program MatLab™ using the PLS_toolbox version 3.7 (The MathWorks, MA, USA). Two-dimensional contour plots were created, along with PARAFAC analysis, using MatLab™. The resolution of these contour plots was improved with the removal of the Rayleigh-Tyndall scattered light (two high intensity bands diagonal bands of light; De Palma et al., 2011). PARAFAC analysis derived specific components of each DOM source including HA-like and FA-like fluorophores (Stedmon et al., 2003). The HA-like constituents excite at 350 nm and 240 nm and will emit at 400-500 nm, whereas the FA-like component will excite at 320 nm and 240 nm while emitting at 400 nm (Stedmon et al., 2003). The tyrosine-like component is known to excite at 225 and 275 nm while emitting at 360 nm. Finally, the tryptophan-like fluorophore will excite at 220 and 275 nm and emit at 300 nm. With PARAFAC, the relative amount of each component was determined for each source of DOM. The quantity of each component was summed together to allow for the calculation of the relative percent of each component found within each source of DOM. Originally a four component model was utilized to explain the fluorescence trends; however it did not provide a significant explanation for the data set. Therefore, we moved toward a five component model that reduced the trends in the residuals and explained 98% of the data. The fifth component was operationally defined and not associated with a standard component, however it was found to emit at long wavelengths similar to HA-like substances. Furthermore, fluorescence data was used to calculate the fluorescence index (FI) as a method to differentiate between DOM source origins using Equation 2 (McKnight et al., 2001).

$$FI = (EI_{450}) / (EI_{500}) \qquad \text{Equation 2}$$

where EI_{450} and EI_{500} are the emission intensities at 450 and 500 nm following excitation at 370 nm.

Absorption measurements were converted to SAC_{340} values using the following equation from Richards et al (2001):

$$SAC_{340} = [\ 2303 \times (Abs_{340})\ ] / DOC \qquad \text{Equation 3}$$

where Abs_{340} is the absorbance 340 nm and DOC is the measured dissolved organic carbon content (in mg C/L).

The quality factor (QF) used to adjust the BLM was calculated using measured SAC_{340} values according to the equation from Schwartz et al (2004):

$$F = 0.31 \times (\ln SAC340) \qquad \text{Equation 4}$$

where F is multiplied with the measured DOC concentration and input into the BLM as the "improved/surrogate " DOC concentration.

Binding characteristics (B_{max} and K_m from ISE and 6h Cu accumulation studies) were correlated to all of the characterization measurements outlined in Table 5. Correlation coefficients (r) were determined by the Pearson product moment method (n=5) and significance is taken at $p<0.05$.

2.2.8 Modelling

The Biotic Ligand Model (ver. 2.2.3; HydroQual Inc., New Jersey, USA) was used with measured water chemistry concentrations to generate acute toxicity predictions. Because a BLM does not exist for *H. azteca*, the *Daphnia pulex* files were used with adjustments to develop a *Hyalella* specific soft-water BLM. In each modelling scenario the LA50 input value was adjusted to achieve the best overall fit between the measured and predicted LC50s (Clifford and McGeer, 2009). This *Hyalella*-specific BLM was then used to predict the protective effect of different DOM sources. To correct the model for DOM quality, two parameters were altered: %HA and active DOC concentration. The humic acid content was adjusted for each source

based on the results obtained from PARAFAC analysis. The active DOC concentration parameter was adjusted using the quality factor (QF) method developed by Schwartz et al (2004), with SAC_{340} being utilized as a surrogate measure for the DOC concentration. The constant within the QF equation was changed from 0.31 (Schwartz et al., 2004) to 0.339 in order to better fit the model so that organism sensitivity (LA50) did not have to be altered to account for DOM source variability.

2.3 Results

2.3.1 Acute Toxicity Tests

Controls showed good survival during acute toxicity test, always greater than 80% and *Hyalella* also survived well in solutions with added DOM with no added Cu. Measured Cu concentrations were generally with 95% of nominal target concentrations. Measured Cu concentrations for total and dissolved Cu at the beginning of tests were in close agreement; the mean for dissolved concentrations was 96.7% (SEM 0.23, n= 96) with a range from 87 to 101%. This was also true for measurements taken at the end of the tests where the mean for dissolved concentrations was 96.2% (SEM 0.43, n= 96) of total with a range from 85 to 108%). Dissolved Cu concentrations were relatively constant within tests and measured concentrations at the end were 95.5% (SEM of 0.79, n=96) of those at test initiation.

The acute LC50 values varied considerably depending on DOM source, all of which provided at least some degree of protection against the effects of Cu (Fig. 2.1). The average measured LC50 value for six Cu tests with no added DOM was 0.67 µM, while those with added DOM (at 5 mg DOC/L) ranged from 1.1 – 2.5 µM (Fig. 2.1). Five of the DOM sources (LL, LW, CWL, HL, and WR1) offered a statistically significantly higher level of protection against

acute Cu toxicity. The remaining five DOM sources (DL, DW, LW2, WR2, and WR3) provided less protection against Cu toxicity and the LC50s were not significantly different from the Cu-only exposure. DOM samples from CWL were taken in the summer of 2012 at two different depths (surface (CWL-s) and 17m (CWL-d)). The LC50 value for solutions with DOM collected from the hypolimnion (CWL-d) was 1.4 µM while that from the epilimnion (CWL-s) was 1.1 µM. The DOM from either depth did not significantly reduce Cu toxicity (Fig. 2.1) nor did they significantly differ from one another.

2.3.2 Short Term (6h) Accumulation

Short term accumulation was assessed in *Hyalella* exposed to a range of Cu concentrations up to 2 µM. In all tests, Cu accumulation patterns were typical of saturable kinetics, characterized by a rapid increase in whole body burden and then a plateau as saturation occurred at elevated Cu exposures (Fig. 2.2). In tests without DOM, the accumulation was elevated compared to tests with DOM added at 5 mg DOC/L. Curve fitting parameters (binding capacity and binding affinity) were generated using Michaelis-Menten-like kinetics (Table 2.2). *Hyalella* from the Cu only exposure had the highest V_{max} (262 µg Cu/g dry weight) while *Hyalella* sampled from the WR1 (reference site) DOM test exposures had the lowest calculated V_{max} (147 µg Cu/g dry weight; Fig. 2.2; Table 2.2).

2.3.3 Optical Characterizations of DOM

The relative composition (as determined by PARAFAC analysis) was unique for the twelve tested DOM sources (Table 2.3). DL had the highest humic acid content (57%), while CWL-s had the lowest amount of humic acid (6%) but the highest fulvic acid content (50%). The relative amount of proteinaceous compounds (tryptophan and tyrosine) was consistent

among DOM sources, with the exception of the three CWL sites (CWL, CWL-s, and CWL-d) which were found to have the highest amount of protein-like substances (12-28%). The fluorescence index (FI) values ranged from 1.2 (LW-July) to 2.3 (CWL; Table 2.3).

2.3.4 ISE Characterizations of DOM

Results from ISE were used to measure free Cu^{2+} and derive binding capacity coefficients for each source of DOM (Fig. 2.4, Table 2.4). DL, WR2, and WR3 were the least effective sources of DOM at binding Cu, as shown by having the lowest binding capacity coefficients (B_{max}) of 0.6 ± 0.1 µM Cu/mg C, 0.7 ± 0.06 µM Cu/mg C, and 0.7 ± 0.05 µM Cu/mg C respectively (Table 2.4). WR1 DOM had the highest B_{max} (1.0 ± 0.1 µM Cu/mg C), while HL had intermediate B_{max} values (0.8 ± 0.003 and; Table 2.4) indicating an intermediate binding capacity.

2.3.5 Correlations Among Biological, Optical, and Chemical Measures

The HA-content (determined from PARAFAC) was positively correlated to the 96h LC50 values ($r = 0.51$, $p>0.05$) however the relationship was not considered to be significant (Fig. 2.3A, Table 2.5). SAC_{340} values strongly correlated to LC50 values ($r = 0.91$, $p<0.001$) indicating that SAC_{340} measurements provided a strong and reliable indication of how protective each DOM source was when compared to measured LC50 values (Fig 2.3B, Table 2.5).

Correlation analysis (using Pearson Product Moment) among the optical measurements showed that 6 relationships were significant (Table 2.5). Three significant relationships were found between B_{max} (from ISE) and SAC_{340} ($r = 0.87$, $p<0.05$), FI ($r = -0.99$, $p<0.001$), and 96h LC50 ($r = 0.86$, $p<0.05$). Both the SAC_{340} and LC50 increased when the DOM had a higher binding capacity for Cu. Additionally, significant correlations were seen between the LC50 and

FI (r = -0.81, $p<0.05$) and the LC50 and SAC_{340} (r = 0.91, $p<0.0001$). More protective DOM sources (high LC50) had a low FI value but a high SAC_{340}. The highest degree of significance among correlations was found between the LC50 and SAC_{340}, as well as between the B_{max} and FI.

2.3.6 Modelling

BLM predictions for acute Cu toxicity with adjusted quality parameters are plotted in Fig. 2.5. Without any modifications, BLM predictions were constant but measured toxicity was variable. Four DOM samples fell within the 25% boundary of the one-to-one line of prediction (Fig. 2.5A). Using the %HA proportion derived from PARAFAC to adjust from the default %HA value within the BLM (10%), Cu toxicity was fairly well predicted with only four samples that were not predicted within the 25% boundary of the one-to-one line of prediction (Fig. 2.5B). Incorporating the quality factor (QF) to adjust the DOC concentration (based on SAC_{340} values) improved predictions, with all samples being predicted within the 25% boundary (Fig. 2.5C). When both %HA and QF were incorporated into the BLM, there were six samples that were not predicted within the 25% boundary.

2.4 Discussion

This study shows that *Hyalella azteca* are sensitive to Cu and that DOM provides protection against Cu toxicity that varied with source (Fig. 2.1). This protection was associated (not significantly) with a reduction of short term Cu accumulation (Fig. 2.2) and correlated to some of the optical characteristics, particularly SAC_{340} and FI (Fig. 2.3B and Table 2.3). The ameliorative influence of DOM on Cu toxicity was predicted within the BLM by adjusting for

the relative sensitivity of *Hyalella* and predictions were then further improved through modifications to DOC paramaters to account for source quality (Fig. 2.5).

Variations in protective capacity against acute Cu toxicity among DOM sources has also been documented for other species including *Daphnia magna* (De Schamphelaere et al., 2004; Al-Reasi et al., 2012), rainbow trout (Richards et al., 2001; Schwartz et al., 2004), and the fathead minnow (Ryan et al., 2004). Protective differences among our DOM sources (i.e. differences in LC50 among sources when tested at the same DOC concentration) varied by approximately 2.5 fold and this is similar to the DOM source variation observed in previous studies looking at similar parameters such as LT50 (Schwartz et al., 2004) and Cu-gill binding (Luider et al., 2004). Variation in protective capacity among DOM sources has been reported to be as high as 4 fold in other studies where organisms are exposed to Cu or other metals including Hg (Al-Reasi et al., 2012; Ryan et al., 2004; Playle, 1998). In these latter studies, a wide range of DOM source types were compared: autochthonous, sewage-derived, terrigenous, and Aldrich humic acid (coal-derived). In comparison, the DOMs from this study were all of natural origins and primarily of terrigenous origin which may explain the lower degree of variability among the DOM sources in mitigating acute Cu toxicity.

The toxicity mitigation provided by the different DOMs was influenced by both the type and severity of the ecosystem disturbance. The undisturbed sites (WR1 and HL) provided the highest level of protection while the most disturbed sites (WR2 fire site and DL smelter site) were the least effective in protecting against acute Cu toxicity. The landscapes surrounding these watersheds are recovering and it is possible that the DOM is of a lower quality as a result of the ecosystem disruption. The WR3 (logging site) provided an intermediate level of protection, despite being logged seven years prior to sample collection (Kreutzweiser, 2010).

Meyer and Tate (1983) found that clear-cut logging resulted in a reduced concentration of DOC within an aquatic system but the effect on DOM quality was not studied. Although the logging process removes wood material from the terrestrial landscape, debris (e.g leaf litter, brush) are left behind and undoubtedly become important inputs to the recovery process. While this study suggests that the quality of DOM from disrupted environments can vary, the factors that determine DOM characteristics and the terrestrial (and aquatic) process linked to those factors are not understood.

LW (Laurentian Wetland) was found to be very protective, with an LC50 similar to that of the reference sites, although it is a site recovering from long term disturbance. When comparing the protective capacity of Sudbury DOMs, wetland sources were generally more protective than lake sources (Fig. 2.1). DOM from DW provided protection that was 11% higher than that of DL, while LW provided 23% more protection against Cu toxicity compared to LL. This protective effect may be related to the significant role that wetlands play in acting as a long-term repository of terrigenous DOM into aquatic systems (Hiriart-Baer et al., 2008) and quality does not appear to be severely impacted as a result of long term smelter depositions. The LW sampling site was heavily vegetated with deciduous trees and this may have contributed to both the high quantity and quality of organic matter (Schiff et al., 1997).

DOM from the LW site was collected at two different time points (July and November) to determine if toxicity mitigation properties changed seasonally. LW-DOM from the November collection was found to be significantly less protective compared to the sample collected in mid-summer (i.e. the November sample LC50 was only 53% of the summer sample; Fig. 2.1). Cuss et al (2010) noted that catchments that are not influenced by wetlands tend to be more variable across seasons (e.g. carboxylic acid content, modelled Cu speciation), while wetlands are more

stable so it was unexpected to find a significant difference in protectivity between the samples collected in the summer compared to late fall. One possible explanation for the difference between the summer and fall DOM samples is that humic acid-like fluorophores (generally associated with binding free metals) have been found to be at increased levels in the spring and summer months while in the fall and winter, these levels generally decrease (Stedmon and Markager, 2005). This was supported by the fluorescence EEMS measurements which showed differences in humic like fractions (Table 2.3). Another possibility is that the prolonged UV and/or microbial degradation creates smaller molecular weight compounds in the fall samples which are associated with less effective metal binding (Aitkenhead-Peterson et al., 2003). It is worth noting that there was a prolonged period of drought between July and October with little precipitation and no outflow from Laurentian and Daisy Wetlands (personal communication E. Szkokan-Emilson, Laurentian University). This dry period may have led to enhanced UV degradation and oxidation. Sampling in November was following a series of rain events resulting in relative high outflow rates and low DOC concentrations (see Table 2.1). Clearly there are seasonal differences in DOM source quality and these are deserving of further study.

Short term (6h) whole body Cu accumulation, which was measured in the presence of a subset of the DOM sources, generally correlated to 96h LC50 values and provided confirmation of BLM theoretical principles (Di Toro et al., 2001). Cu was rapidly accumulated and saturable uptake kinetics (Michaelis-Menten type) was evident (Fig. 2.2, Table 2.2) as would be expected (McGeer et al 2003; Borgmann, 1998). Cu binding was reduced when LC50 values were increased (Table 2.5) and DOM sources that were protective generally resulted in lower V_{max} values (Fig. 2.2, Table 2.5). Cu accumulation was reduced to the greatest degree when the WR1 (reference site) DOM was present and to a lesser amount when WR2 (fire site) DOM was tested

(Fig. 2.2). An intermediate level of Cu was accumulated when HL (reference site), WR3 (logging site) and DL (smelter site) DOMs were present in solution. The capacity to mitigate against acute toxicity appears to be somewhat correlated with reductions in short term accumulation of Cu (Fig. 2.2 and Table 2.2).

ISE measurements of Cu^{2+} in solution with added DOMs generally supported the toxicity and short term Cu binding results. Sources such as WR1, which was the most protective against Cu toxicity and showed the lowest short term accumulation had the highest affinity as well binding capacity for Cu^{2+} (Table 2.3). DOM sources with a relatively low capacity for toxicity mitigation (a greater short term Cu accumulation), such as Daisy Lake and WR2, had the highest K_m values (lower affinity) and a relatively lower binding capacity (Table 2.3). The binding capacity (B_{max}) of each DOM source was significantly correlated with toxicity mitigation (LC50, Table 2.5). ISE determined that the binding properties of the DOM sources are consistent with the observation that ecosystem level disruption can reduce the quality of DOM. In the Norwegian NOM-Typing Project, a large set of NOM samples were analyzed, however, the variable Cu-binding capacities did not offer an explanation for differences in the toxicity mitigation properties of the NOM sources (Abbt-Braun and Frimmel, 1999). However, based on the binding characteristics of each DOM source and the protective capacity, our data shows that there is an overlap between the "analytical window" and the "toxicological window" and this illustrates that ISE is an effective method to explain the differences observed among DOM sources in mitigating Cu toxicity.

The relative composition of each DOM source, as determined through EEMS and PARAFAC, was somewhat associated with the patterns of ecosystem disturbance observed with the acute toxicity and ISE data (Fig. 2.1, 2.4, Table 2.3). For example, WR1 DOM (one of the

most protective sites) had one of the highest amounts of HA-like content, which is commonly associated with higher levels of protection (Al-Reasi et al., 2012; Schwartz et al., 2004), while WR2 (damaged fire site) was one of the least protective sites and also had one of the lowest amounts of HA-like content. Interestingly, DL (smelter site) was found to have the highest amount of HA-like substance although it was one of the least protective sources of DOM (Fig. 2.3B). There appears to be a component within this DOM that fluoresces similar to humic acid but does not contribute to toxicity mitigation and this may be related to the harsh damage that this site is recovering from (Fig. 2.3B). The three samples from CWL were found to have the highest amounts of protein-like substances (Table 2.3) and this may be a result of human input from the large number of houses surrounding Clearwater Lake (Stokes et al., 1984). It is important to note that the three wetland samples (DW, LW, and LW-N) all had high amounts (>30%) of the undefined fifth component that fluoresces at a longer wavelength than HA (Table 2.3). The remaining DOM sources had less than 22% of this component. It is evident that there is an additional component found within wetlands that may contribute to an improved capacity for toxicity mitigation compared to lake samples. We found no significant correlation between Cu toxicity and the amino acid content of DOM sources which is supported by a study with marine DOM sources and the influence on Cu toxicity to marine mussels (De Palma et al., 2011). There was a positive relationship found between HA and LC50 ($r = 0.5$ $p>0.05$) but the correlation with HA or any of the other components determined by PARAFAC were not considered to be significant.

The SAC_{340} values calculated for the DOMs used in this study varied from a low of 9.7 (CWL-s) to a high of 45 (LW-July; Fig. 2.3B, Table 2.3). A strong correlation was observed between SAC_{340} and the protectivity of each DOM source (Fig. 2.3B, Table 2.3) and this is

similar to other studies that tested the protective effects of Cu on daphnids (Al-Reasi et al. 2012; De Schamphelaere et al 2004), rainbow trout (Schwartz et al., 2004), and the fathead minnow (Ryan et al., 2004). This strong relationship implies that darkly coloured DOMs (i.e. more aromatic) are more effective in mitigating Cu toxicity compared to sources that are lighter in colour. Carbonaro et al (2011) and Al-Reasi et al (2013) discuss why SAC_{340} is considered to be a valuable predictor of DOM quality; the functional groups on the aromatic rings (carboxylics and phenolics) tend to bind Cu strongly as well as allow for multiple binding sites for Cu. This correlation to protection is consistent with the short term accumulation as well as the ISE measurements. Similarly, the fluorescence Index (FI) was found to have a significant (but negative) correlation with both B_{max} and LC50. The DOM sources with lower FI values were found to have increased binding capacities and increased toxicity mitigation potential. Al-Reasi et al (2012) also observed a similar relationship between FI and Cu toxicity mitigation in *Daphnia magna*, however the results were not considered to be statistically significant. In contrast, a number of studies have shown that there is a very weak or no correlation between these two variables (Gheorghiu et al., 2010; Ryan et al., 2004). Although FI has not been an always been effective in describing differences among DOM sources, the relationship observed between the FI and acute Cu toxicity mitigation from these sources allows us to consider FI as a potential indicator for DOM quality of damaged/recovering ecosystems. Additional significant relationships were observed, although they may be less relevant. For example, there were significant positive correlations found between K_m (binding affinity from ISE) and the amino acid content (both tryptophan and tyrosine); there were no similar relationships reported within the literature. However, utilizing the Pearson Product Moment as a method of correlation

analysis allowed us to compare all of the DOM characterization variables to determine which were most significant.

Adjusting the BLM to account for soft waters and *Hyalella* sensitivity to Cu in the presence of different DOM sources allowed us to generate reasonable toxicity predictions (Fig. 2.5). Originally, 5 mg DOC/L was input into the model and an intermediate toxicity prediction was calculated but the goal was to see if we could incorporate any previously discussed quality parameters to account for DOM source variation (Fig. 2.5A). As a result of a strong correlation between %HA-like content and LC50, Al-Reasi et al (2012) were able to indirectly adjust the model to incorporate DOM quality, using SAC_{340} and %HA correlations. However, in our study we did not find observe a significant relationship between the previously mentioned variables so we could not use the same surrogate measure (SAC_{340} to replace %HA) to account for DOM source quality within the BLM.

To incorporate DOM source variability, we first manipulated the %HA content (obtained from PARAFAC analysis) and input this value (adjusted from the original recommended 10% within the BLM) for each source (Fig. 2.5B). When this parameter was adjusted, toxicity predictions were improved which was similar to Al-Reasi et al (2012) who found %HA adjustments to significantly improve acute Cu toxicity predictions. Predictions were not accurate for four of the DOM samples, all of which were from damaged sites (three from Sudbury and the logged site). It is evident that DOM from disturbed sites does not mitigate Cu toxicity as it would be predicted based on the humic acid content. Next, we wanted to use the quality factor described by Schwartz et al (2004) to incorporate DOM quality into the BLM (Fig. 2.5C). The QF uses a specific formula (see "Calculations and Statistical Analyses" in Methods) to incorporate SAC_{340} measurements as a surrogate DOC concentration within the model. This was

considered to be an appropriate method because we observed a strong correlation between the SAC_{340} and LC50. When the QF was calculated and incorporated into the BLM, we obtained the better toxicity predictions. Predictions were consistently close to the one-to-one line and all DOM sources were predicted within the 25% boundary lines. Our next step was to incorporate both adjustments into the model (specific %HA content as well as the QF; Fig 2.5D). Incorporating %HA and QF into the BLM did not improved predictions. With this method, half of the samples were not predicted within the 25% boundary; all of the DOM samples were from disturbed ecosystems (four from Sudbury and the logged and burned sites). The most accurate and consistent toxicity predictions were generated when the SAC_{340} was incorporated into the model.

In general, the predictions for samples collected from damaged areas were not as accurate or consistent as they were for samples from undisturbed reference sites. It appears that although DOM from damaged sites may have a high humic acid content (thus predicted to be protective), it does not protect against Cu toxicity in the same capacity as DOM from the undisturbed sites. However, by using the SAC_{340} to adjust the BLM ecosystem disturbance was taken into account when acute Cu toxicity predictions were generated.

2.5 Conclusions

The results from this study show that there are differences among DOM sources in mitigating acute Cu toxicity to *Hyalella azteca*. DOM source quality is negatively impacted by both the type and severity of ecosystem disturbance, with disturbed sites offering less protection than undisturbed sites. The optical and chemical characteristics (determined by SAC_{340}, FI, EEMS, and ISE) as well as short term (6h) bioaccumulation studies were correlated with the

toxicity mitigation properties of the DOM sources. We found that by incorporating optical characteristics (%HA from EEMS/PARAFAC and using SAC_{340} in the QF) into the BLM, we were able to improve acute Cu toxicity predictions by accounting for DOM source variability as well as ecosystem disturbance. Future research is needed to understand seasonality differences that exist within DOM sources in terms of toxicity mitigation as well as optical/chemical characterizations.

Acknowledgements

This research was supported by NSERC through a CRD grant in partnership with Vale Inc., Xstrata PLC, the City of Sudbury and Natural Resources Canada and with the participation of the Ontario Ministry of the Environment as well as a Discovery Grant to JCM.

2.6 Tables

Table 2.1 – Location, sampling dates, and background information on sites where DOM was collected by portable reverse osmosis.

Sample Site	Legend	Sampling Date	GPS Coordinates	DOC (mg C/L)	Site Characteristic
Daisy Lake	DL	07-Jul-11	N 46º45’ W 80º88’	6.1	Smelter impacted – amphipods not present
Laurentian Lake	LL	08-Jul-11	N 46º44’ W 80º96’	5.2	Smelter impacted – amphipods present
Laurentian Wetland (summer)	LW	08-Jul-11	N 46º45’ W 80º94’	23.5	Smelter impacted – discharges to LL
White River Site 3	WR3	28-Jul-11	N 48º43’ W 85º35’	11.9	Watershed logged in 2002
White River Site 2	WR2	29-Jul-11	N 48º65’ W 85º36’	4.1	Watershed burned in 1999
White River Site 1	WR1	29-Jul-11	N 48º75’ W 85º17’	13.8	Undisturbed site
Clearwater Lake	CWL	30-Sept-11	N 46º37’ W81º05’	2.8	Smelter impacted – amphipods present
Harp Lake	HL	13-Oct-11	N 45º22’ W 81º05’	10.3	Undisturbed site
Laurentian Wetland (Fall)	LW2	12-Nov-11	N 46º45’ W 80º94’	n.d	Smelter impacted – discharges to LL
Daisy Wetland	DW	12-Nov-11	N 46º27’ W80º52’	12.5	Smelter impacted – discharges to DL
Clearwater Lake (surface)	CWL-sur	15-Jul-12	N 46º37’ W81º05’	3.2	Smelter impacted – surface water
Clearwater Lake (depth)	CWL-17m	15-Jul-12	N 46º37’ W81º05’	3.9	Smelter impacted – 17m depth

Table 2.2 – Influence of DOM (5 mg C/L) on short term (6h) Cu bioaccumulation in *Hyalella* (nominal Cu concentrations ranging from 0 – 2 µM). Curve fitting parameters were determined using a Langmuir isotherm kinetics (see equation 1). V_{max} is the binding capacity given ± standard error (n=15 at each concentration). K_m represents the binding affinity of the Cu at the biotic ligand ± standard error (n=15 at each concentration).

NOM Source	V_{max} (binding capacity) (µg Cu/g dry weight)	K_m (binding affinity) (ng/L)	r^2
Cu Only	262 ± 25.8	6.8 ± 3.0	0.89
White River 2	195 ± 8.8	6.9 ± 3.0	0.92
Daisy Lake	166 ± 14.7	5.4 ± 4.0	0.70
White River 3	165 ± 6.9	1.0 ± 4.0	0.88
Harp Lake	162 ± 6.5	2.3 ± 0.9.0	0.89
White River 1	147 ± 4.8	3.6 ± 1.0	0.94

Table 2.3 – Optical characteristics of DOM sources. Relative composition of DOM sources determined by PARAFAC on excitation emission matrix spectroscopy (EEMS) scans. The five components are humic-like (HA), fulvic-like (FA), tryptophan-like (Trp), tyrosine-like (Tyr), and an undefined entity named Component 5. These five components are represented as percent of total fluorescent units, which explain 98% of the variability among the DOM sources.

DOM Source	%HA	%FA	%Trp	%Tyr	%Component 5	FI	SAC_{340}
Daisy Lake	58	6	8	8	20	1.4	13
Harp Lake	47	15	12	6	20	1.4	42
White River Site 1	53	14	9	6	18	1.3	40
White River Site 2	34	28	16	9	13	1.4	14
White River Site 3	55	9	7	5	23	1.4	26
Laurentian Wetland (Jul.)	54	4	5	7	31	1.2	45
Laurentian Wetland (Nov.)	35	14	9	7	34	1.6	12
Laurentian Lake	52	8	13	7	21	1.3	24
Daisy Wetland	16	22	12	8	41	1.7	15
Clearwater Lake	25	26	19	12	18	2.3	14
Clearwater Lake (surface)	6	50	28	14	2	1.9	9
Clearwater Lake (depth)	24	35	17	17	7	1.8	11

Table 2.4 – Maximum binding capacity (B_{max}) and binding affinity (K_m) for 5 DOM sources. Values were determined from Cu binding curves (Fig. 2.4) that were created from Cu^{2+} measurements by Cu ISE. A single site hyperbolic saturation model was utilized from the statistical software program SigmaPlot.

DOM Source	B_{max} (binding capacity) (µM Cu/mg C)	Log K (binding affinity) (nM)	r^2
White River 1	1.0 ± 0.1	8.1 ± 4.8	0.96
White River 3	0.8 ± 0.003	48 ± 5.6	0.99
Harp Lake	0.7 ± 0.05	44 ± 12	0.98
White River 2	0.7 ± 0.06	180 ± 56	0.97
Daisy Lake	0.6 ± 0.1	110 ± 72	0.92

Table 2.5 – Correlation coefficients (r; determined by Pearson Product Moment) for measured DOM variables (Table 2, 3, 4) with ISE binding characteristics along with 6h Cu accumulation characteristics and 96h LC50. A * indicates significance ($p<0.05$), n=12 for optical characteristics; n=5 for ISE and 6h accumulation characteristics.

		Correlation Coefficients						
		SAC_{340}	**FI**	**EEMS + PARAFAC**				**LC50**
				HA	**FA**	**Tyr**	**Trp**	
ISE Binding Characteristics	**B_{max} (µM Cu/mg)**	0.89*	(-)0.99*	0.19	0.03	(-)0.75	(-)0.31	0.86*
	K_m (µM)	(-)0.85	0.76	(-)0.66	(-)0.52	0.86	0.73	(-)0.80
6h Accumulation Characteristics	**V_{max}**	(-)0.71	0.61	(-)0.79	0.65	0.65	0.80	(-)0.67
	K_m	(-)0.67	0.51	(-)0.55	(-)0.52	0.95*	0.66	(-)0.58
	LC50	0.91*	(-)0.81*	0.51	(-)0.36	(-)0.07	(-)0.42	

2.7 Figures

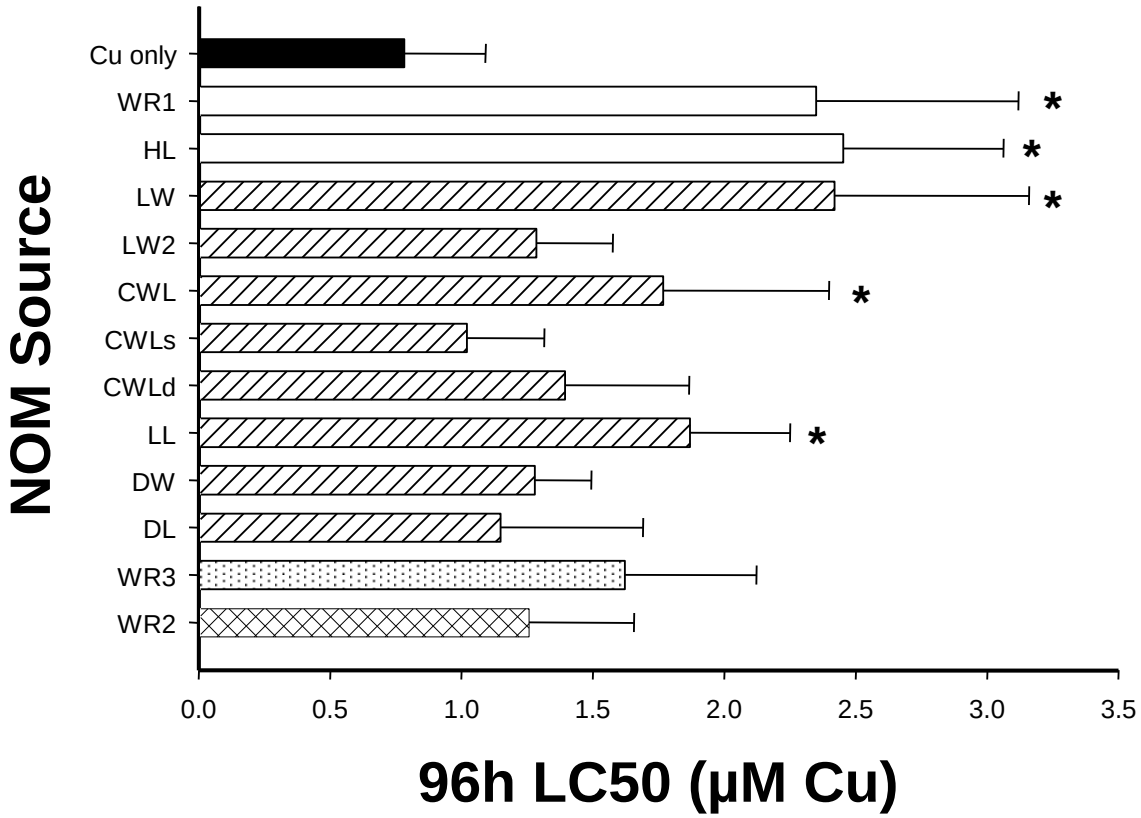

Figure 2.1 – Influence of DOM source on 96h LC50 (+95% CI) for dissolved Cu (µM). The black bar represents the LC50 for a Cu only exposure (no added DOC). DOM source labels are: DL- Daisy Lake, DW- Daisy Lake Wetland, LL- Laurentian Lake, LW- Laurentian Wetland (July 2011), LW2- Laurentian Wetland (Nov. 2011), CWL- Clearwater Lake, CWL-Clearwater Lake Surface, CWLd-Clearwater Lake collected at 17m depth, HL- Harp Lake, WR- White River Sites. Open bars are undisturbed reference sites, side hatch represents Sudbury (smelter-damaged sites), dots mean logging site, and cross-hatched indicates fire site. All DOM sources were added at 5 mg DOC/L and * indicates significant difference from the Cu-only exposure.

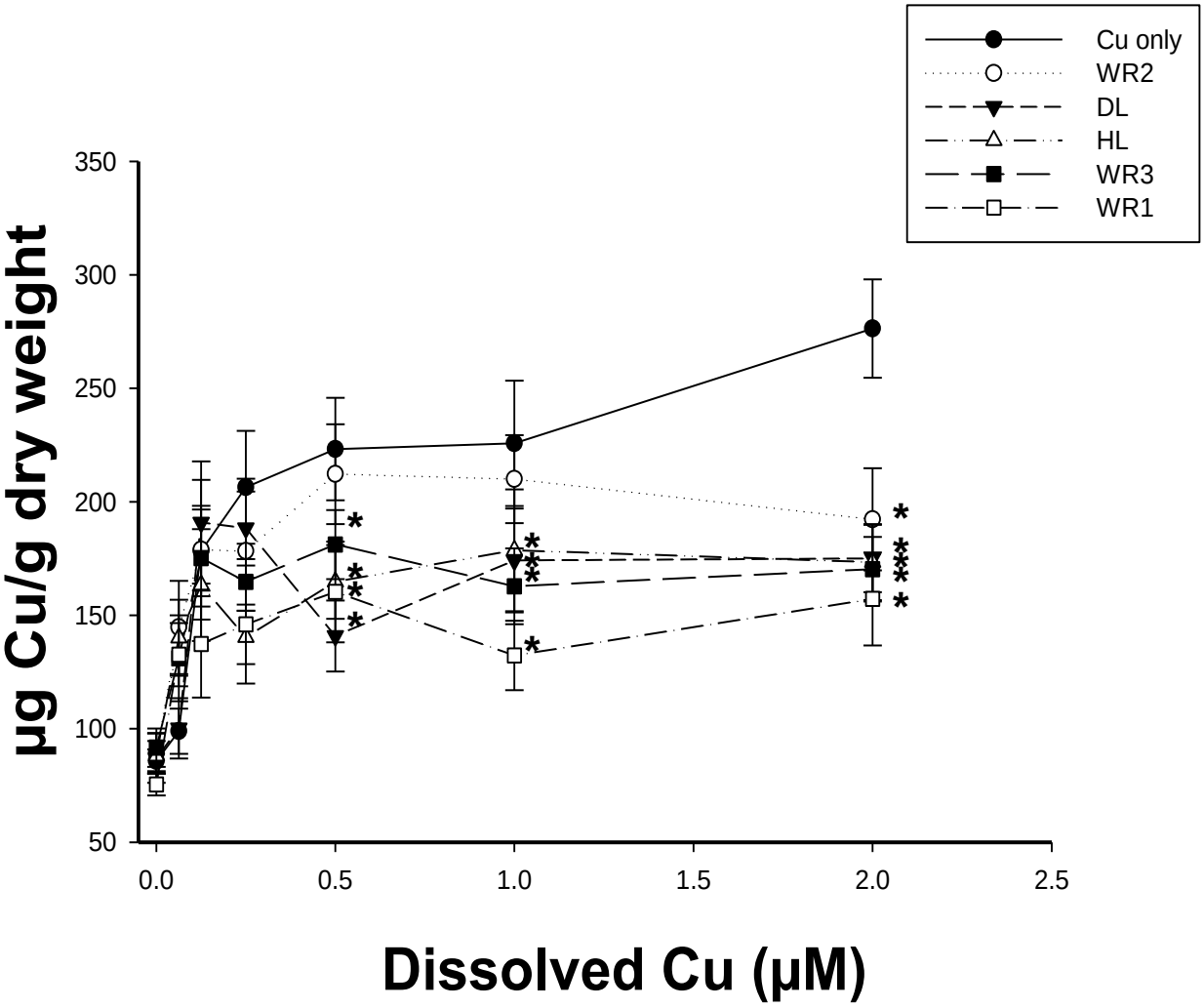

Figure 2.2 – Whole body Cu accumulation (mean ± SEM, n=15 at each exposure concentration) in *Hyalella azteca* over 6h exposures to different concentrations of Cu with no added DOC (filled circles) or DOC at 5 mg C/L. For *Hyalella* exposed to Cu with DOC, five DOM sources were compared: WR1 (open circles), WR2 (filled triangles), WR3 (open triangles), HL (filled squares), and DL (open squares). Cu concentrations reported as nominal (µM). * indicates significant difference ($p<0.05$) from the Cu-only exposure at that specific concentration.

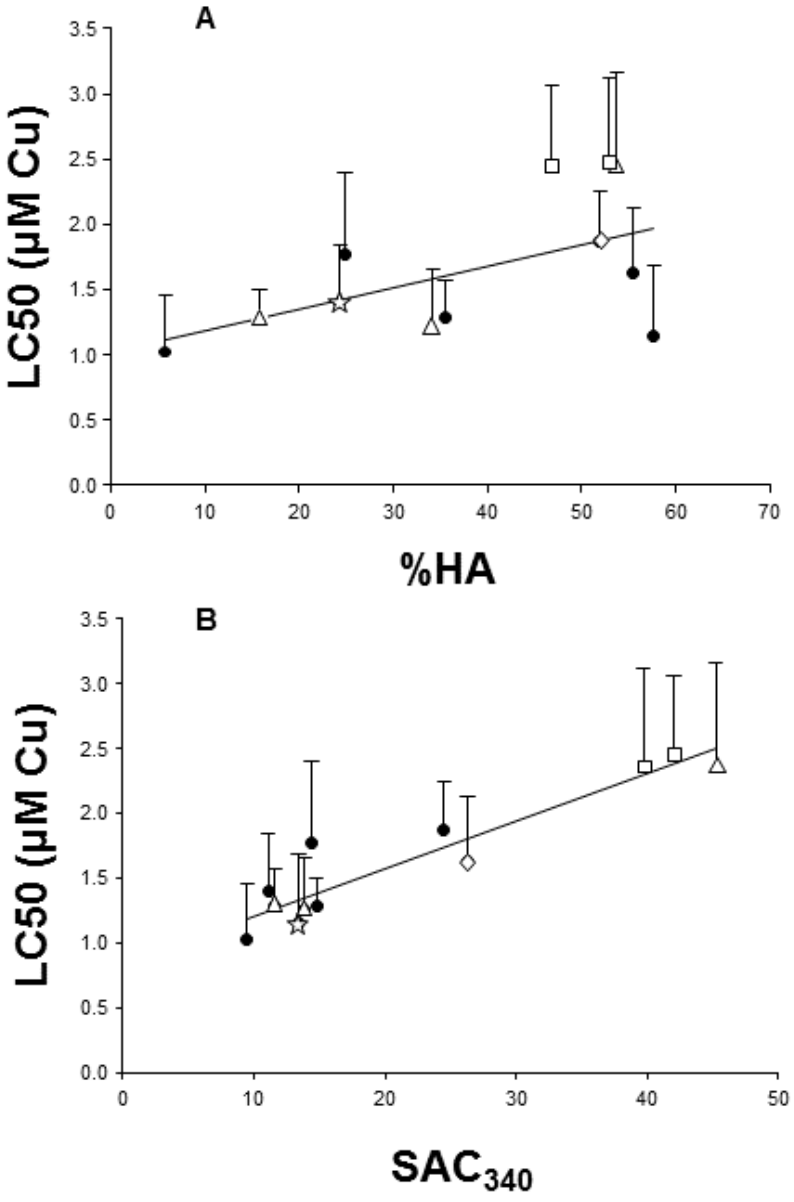
A
LC50 (µM Cu)
%HA
B
LC50 (µM Cu)
SAC340

Figure 2.3 – Correlations between 96h LC50 with 5 mg DOC/L (with 95% c.i.) and measured optical characteristics (humic acid content and SAC_{340}). Symbols show reference sites (open square), Sudbury lakes (filled circle) and wetlands (triangle), as well as burned site (star) or logged (open diamond). Panels show: (**A**) Correlation of 96h LC50s with 5 mg DOC/L (with 95% c.i.) and measured humic acid content from EEMs and PARAFAC analysis. The LC50s were calculated with measured dissolved Cu concentrations (0 – 4 µM). The observed r^2 value was 0.31. (**B**) Correlation of 96h LC50s and measured specific absorption coefficient at 340 nm (SAC_{340}). Absorbance was measured in triplicate and the SAC calculation was based on the average measured value. A significant relationship ($p<0.05$; Table 2.5) was observed with an r^2 value of 0.88.

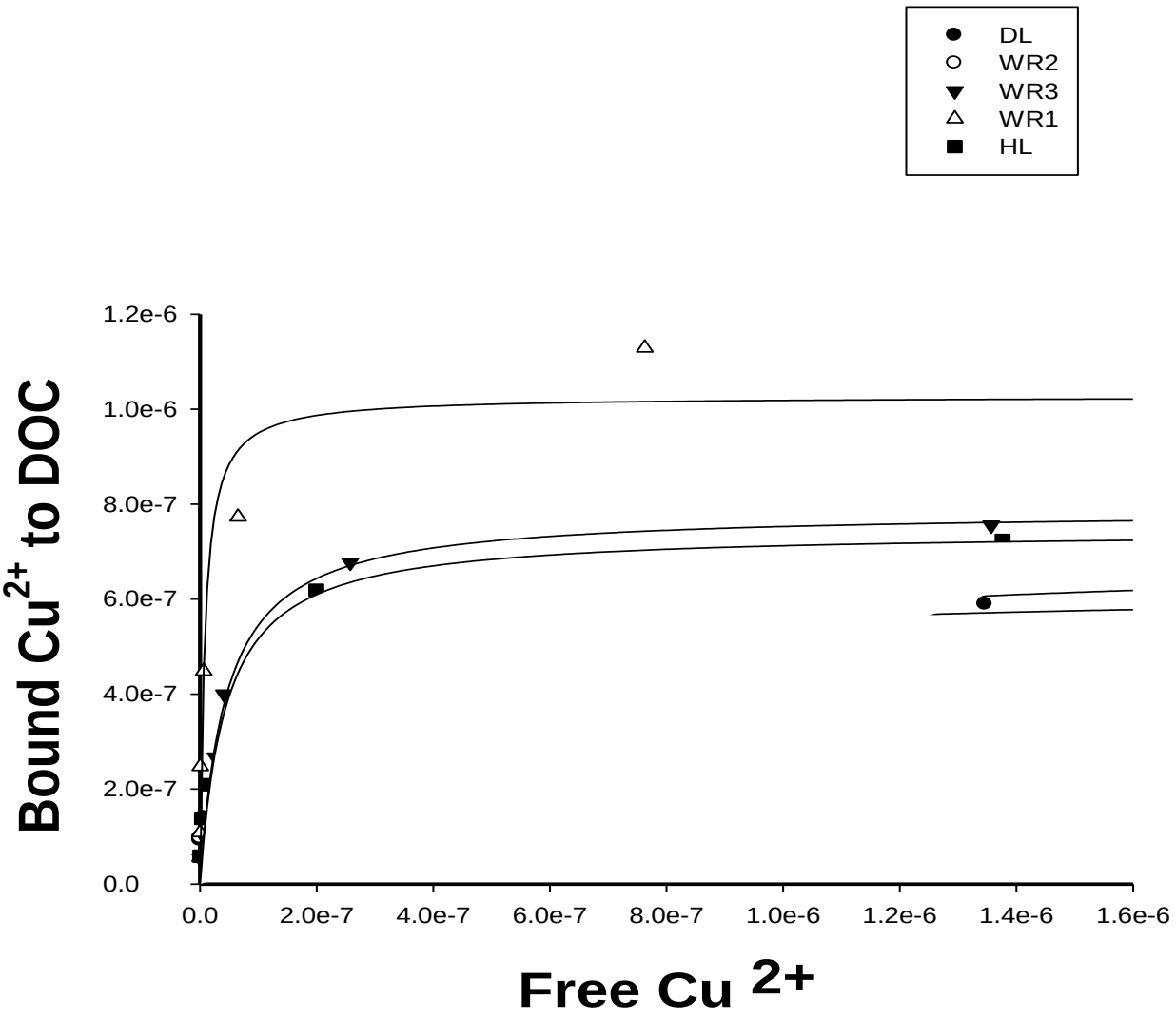

Figure 2.4 – Cu-DOM binding as a function of Cu^{2+} as calculated from ion selective electrode measurements of solution with different concentrations of total Cu (0.063 – 2.0µM) and with DOM added from different sources at a concentration of 5 mg DOC/L. Binding curves were generated using a Langmuir isotherm and these yielded the affinity and maximum binding capacity values for each DOM source (see Table 4). Open triangles represent WR1, filled triangles indicate WR3, squares signify HL, open circles are WR2, and filled circles represent DL. Free Cu^{2+} measured as millivolts and bound Cu^{2+} determined by subtracting free Cu^{2+} from total Cu.

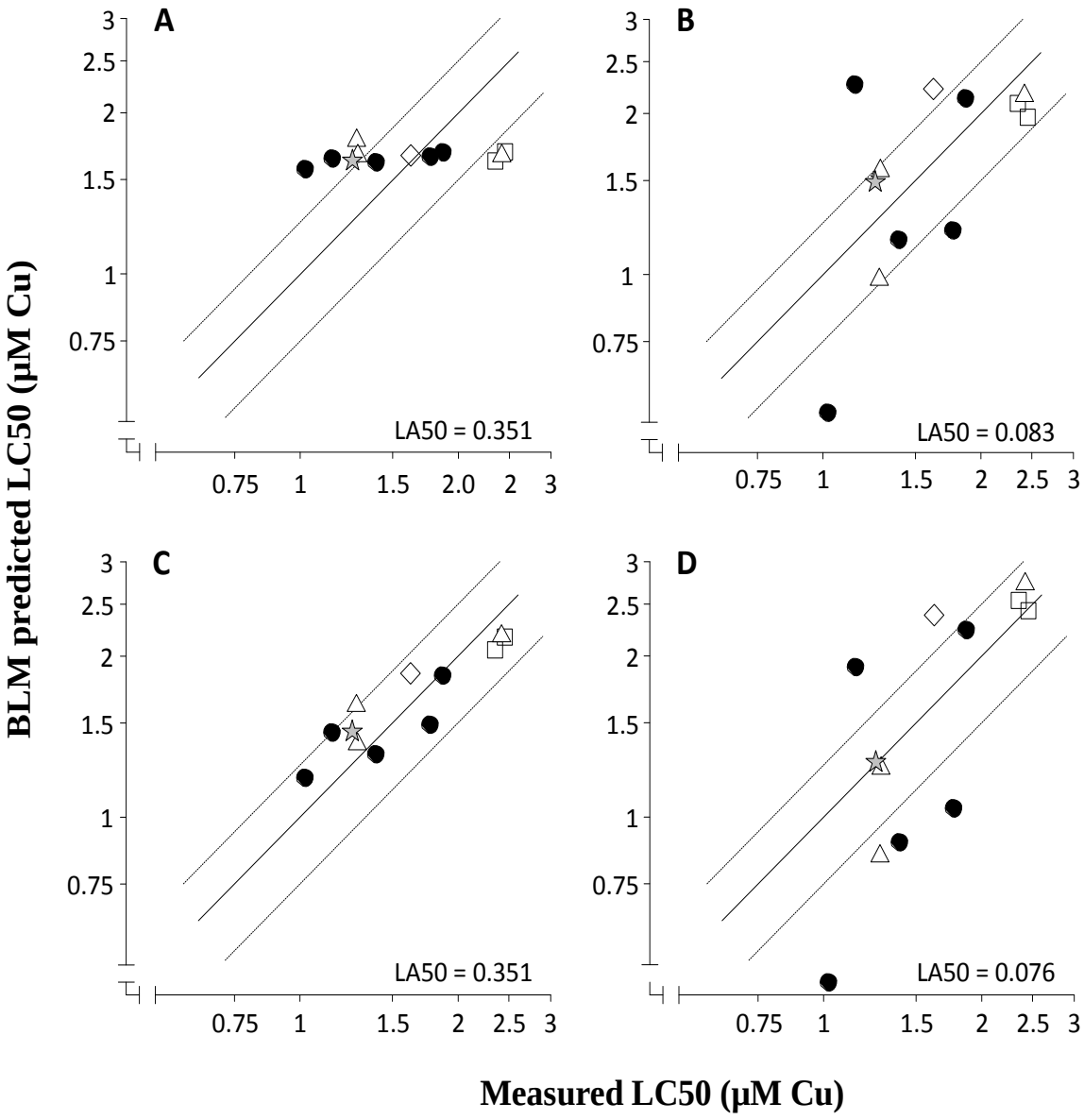
A
B
C
D
LA50 = 0.351
LA50 = 0.083
LA50 = 0.351
LA50 = 0.076
BLM predicted LC50 (µM Cu)
Measured LC50 (µM Cu)

Figure 2.5 – Comparison of measured and BLM predictions of Cu toxicity to *Hyalella azteca* with 5 mg DOC/L from each of 12 different sources. Symbols show reference sites (open square), Sudbury lakes (filled circle) and wetlands (triangle), as well as burned site (star) or logged (open diamond). The solid line shows one-to-one prediction and dotted lines show the ±25% boundary from perfect predictions. Panels show **A**) the unmodified BLM model predictions; **B**) adjustment to %humic acid content derived from PARAFAC analysis of EEMs data; **C**) SAC_{340} adjustments to DOC concentrations using the Quality Factor; and **D**) when the %HA and SAC_{340} based adjustments are combined. In each modelling scenario, the LA50 input is given (nmol Cu/g wet weight) and these were adjusted to achieve the best overall fit between measured and predicted LC50s. Note that the axes are on a Log_{10} scale.

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CHAPTER 3

Effect of DOM source on the chronic survival, growth and accumulation of Cu to *Hyalella azteca*

3.1 Introduction

Copper (Cu) is present in all aquatic environments and concentrations range from less than 0.5 µg/L in uncontaminated areas to >400µg/L in industrially contaminated ecosystems (Grosell, 2011; Keller et al., 1998). Cu is essential to most organisms and this element enters aquatic systems through both natural and anthropogenic routes. Cu toxicity may occur across a wide range of concentrations, depending on an organism's sensitivity as well as water chemistry parameters such as pH, DOC, and hardness cations (Di Toro et al., 2001; Borgmann et al., 1993). In terms of acute toxicity, the free ion form of Cu (Cu^{2+}) is considered to be the most bioavailable form and is associated with toxicity (Di Toro et al., 2001).

Hyalella azteca are small, omnivorous benthic amphipods that are found in most uncontaminated freshwater lakes, ponds, and streams across North America (Shuhaimi-Othman and Pascoe, 2001). *Hyalella* are considered to be important and useful organisms for assessing the toxicity of Cu because they have been shown to be sensitive to a variety of metals including Cu (Borgmann et al., 1998). In addition to being relatively easy to culture in a laboratory setting, they have a relatively short reproductive cycle and there are established guidelines for the culture and testing of this organism (Environment Canada, 2013). The sensitivity of *Hyalella* and their importance in food web dynamics make them a highly relevant organism for studying the potential environmental impacts of Cu contamination.

The chronic effects of Cu, Cd, and Ni on *Hyalella* survival, growth, accumulation, and reproduction have been studied in hard water (e.g. Borgmann et al., 1993; Borgmann and Norwood, 1995; Morris et al., 2003; Schroeder et al., 2010; Borgmann et al., 2010), but the long term effects of Cu in soft water are less studied and it is still unclear what endpoint is the most

sensitive indicator of chronic Cu toxicity. In sediment toxicity tests, growth has been found to be the most sensitive endpoint (Kubitz et al., 1995; Roman et al., 2007) whereas in waterborne exposures, Borgmann et al (1993) have found survival to be more indicative of chronic toxicity because there were no growth effects observed after chronic Cu exposure. *Hyalella* have the ability to regulate internal Cu concentrations during chronic exposure, thus whole body bioaccumulation is not considered to be an effective indicator of long-term toxicity (Borgmann et al., 1993; Borgmann and Norwood, 1995). The chronic effects of Cu exposure on *Hyalella* in the presence of a modifying factor (e.g. dissolved organic matter) have not been documented within the literature.

Dissolved organic matter (DOM) is a term used to describe a group of complexes and molecules that arise primarily from the breakdown of plant and animal matter and enter aquatic ecosystems via runoff from the surrounding landscape (McKnight and Aiken, 1998). DOM can be difficult to characterize because it is heterogeneous and varies in its complexity. In freshwater systems the amount of DOM, quantified as dissolved organic carbon (DOC) concentration, ranges from approximately 1 – 15 mg C/L (Thurman, 1985). DOMs can be classified based on their origin, either autochthonous or terrigenous (allochthonous). Autochthonous DOM is derived from microbial or algal origin from within the aquatic system and this type of DOM is made up mainly of aliphatic and nitrogenous groups. Terrigenous DOM (of terrestrial origin) consists mainly of humic and fulvic acids (McKnight et al., 2001).

It is well-established that DOM acts to mitigate acute metal toxicity in a concentration-dependant manner by binding free metal ions, thus making them unavailable for toxic action (Al-Reasi et al., 2012; Kramer et al., 2004; De Schamphelaere et al., 2004; Erickson et al., 1996). Since each ecosystem is unique, it is hypothesized that all DOMs are distinct because of the

presence of different Cu-binding functional groups as a result of variable inputs from the surrounding landscape (De Schamphelaere et al., 2004). A number of studies with a range of organisms, including *Daphnia magna* (Al-Reasi et al., 2012) and rainbow trout (Richards et al., 2001; Schwartz et al., 2004; Gheorghiu et al., 2010), have shown that DOM source can vary impacts of acute Cu exposure by up to 4-fold. Although DOM source variability has been documented, the reasons behind how DOM sources differ with respect to Cu binding and bioavailability are not as well understood.

A number of techniques exist to chemically and optically characterize and distinguish differences among DOM sources. Cu ion selective electrode (ISE) methods can be used to measure Cu^{2+} in solution in order to determine the binding capacity and affinity of each DOM source for free Cu (Schwartz and Vigneault, 2007; Lu and Allen, 2002). Absorbance measurements (at 340 nm) can be used to calculate SAC_{340} values to assess the aromaticity of the sample (Al-Reasi et al., 2012; Schwartz et al., 2004; Richards et al., 2001). The fluorescence index (FI), based on a ratio of emission intensities at a certain excitation wavelength, is traditionally used to distinguish whether the DOM is derived from microbial or terrestrial origin (i.e. McKnight et al., 2001). Excitation-emission matrix spectroscopy (EEMS), in conjunction with PARAFAC analysis is used to devolve spectral properties into relative composition signatures of each DOM source (De Palma et al., 2011; Holbrook et al., 2006; Stedmon et al., 2003). Components such as those associated with humic-like and fulvic-like substances have been found to generally correlate well with protection against acute Cu toxicity (Al-Reasi et al., 2011; see Chapter 2). There is a lack of knowledge on whether source differences among DOMs influence the response to chronic metal exposure.

The objectives of this study were to extend the understanding of DOM source differences that was developed in the companion work (Chapter 2: optical characterization, binding capacity and acute toxicity mitigation) by comparing sources for the ability to protect against the chronic effects of Cu toxicity. Three DOM sources representing different types of ecosystem disturbance (fire, logging, or smelter-damaged) were compared to a reference source using a standard 28 d chronic test with *Hyalella azteca*. Three endpoints were assessed in these studies in order to determine DOM source variability: survival (d 14 and d 28), dry weight of the organism after 28 days of exposure, and Cu accumulation at day 28 in surviving organisms. This study is contributing to the overall understanding of DOM source quality in mitigating against long term Cu exposure.

3.2 Methods

3.2.1 DOM Sources and *Hyalella azteca* Culture

DOM source collection and characterization as well as the culture methods for *Hyalella azteca* are described in the companion study (Chapter 2: optical characterization, binding capacity and acute toxicity mitigation). Culture and testing was in an artificial medium made with 0.1 mM $CaCl_2$, 0.1 mM $NaHCO_3$, 0.025 mM $MgSO_4$, and 0.005 mM KCl supplemented with 1 µM NaBr (Borgmann et al., 1996). The pH of the culture medium was 7.2 ± 0.1, hardness of 13 mg $CaCO_3$/L and the temperature was 21^{o}C.

3.2.3 Chronic Toxicity Tests

Chronic toxicity tests (28 days) followed the Environment Canada standard static-renewal method for 14-day water only exposures (Environment Canada 2013). Neonates (2-9 days old) were exposed to six Cu concentrations (ranging from 0.0315 – 1.0 µM) for the four

sources of DOM (DL, WR1, WR2, WR3; nominal concentration of 7 mg DOC/L) and a Cu-only test was also completed (no added DOM). All chronic tests had controls (no added Cu or DOM) in triplicate as well as a DOM control (7 mg DOC/L with no added Cu) also done in triplicate (except for the DL chronic, which was done in duplicate due to the limited amount of concentrate available) to verify organism health for the duration of the test.

Static renewal chronic tests were carried out in 400 mL polypropylene beakers (10 *Hyalella* per beaker), with 250 mL of test solution. Test solutions sufficient for the duration of the exposure (10 L) were prepared for each concentration in acid-washed carboys at least 24 h prior to test initiation to allow for Cu-DOM equilibration to occur (Kim et al., 1999). A 5x5 cm piece of gauze was also equilibrated with the test solution in a separate beaker and then added to the exposure beaker upon test initiation (Neumann et al., 1999). Each exposure beaker was fed 5 mg ground Tetramin flakes three times per week (Monday, Wednesday, Friday) following renewal of the exposure medium (100%). At each water renewal, survival was recorded. The pH of test solutions were monitored weekly and were maintained at 7.2 ± 0.1 and temperature was 21ºC ± 2ºC.

3.2.4 Cu and DOC Analysis

Throughout each 28 day test samples (weekly) were collected from the stock test solution carboys and from each of the exposure beakers just prior to test solution renewal. For Cu analysis both unfiltered and 0.45 µm filtered (Acrodisc HT tuffryn membranes, Pall Corporation, Ann Arbor, MI) samples (10 mls) were collected for total and dissolved (respectively). These samples were acidified with a 1% volume of 16N HNO_3 (Trace Metal Grade, Fisher Scientific,

Nepean, ON). For DOC measurement, 30 mls of test solution were filtered (0.45 µm as above) and then stored at 4°C.

Cu concentrations were measured using graphite furnace (GF) atomic absorption spectroscopy (AAS: SpectAA-880 with GTA 100, Varian Inc, Palo Alto, CA). Reference material (SRM 1643e, NIST Gaithersburg MD) was used to verify GF-AAS measurements. DOC was measured as non-purgable organic carbon (NPOC) on carbon-free air purged samples that had not been acidified and this was completed using a total organic carbon (TOC) analyzer (Shimadzu TOC- $L_{CPH/CPN}$, Shimadzu Corporation, Kyoto Japan). A potassium hydrogen phthalate (Mandel Scientific, Guelph ON) reference solution for total carbon (5 and 10 mg C/L made from 1000 mg C/L stock solution) was used to verify measurements. From the 570 samples for Cu analysis per test, a subset (n=85 per test) were selectively chosen for characterization of Ca and Mg and this was done by AAS. Selection of the subset was done to ensure that samples from each concentration within a test were measured for each week of exposure.

3.2.5 Tissue Digestion

After 28 d of exposure, surviving amphipods were removed from exposure solutions and were transferred into a beaker of deionized water (18 mohm, Milli Q A-10, Millipore Corporation, Fisher Scientific, Nepean, ON) for a period of 6 h to allow for gut clearance (Neumann et al., 1999). After the gut clearance, *Hyalella* were transferred onto a Kim wipe and allowed to air dry for five minutes to remove excess moisture. Individual organisms were then transferred to 0.6 ml micro-centrifuge tubes and dried for 48h at 80ºC. Following the drying period *Hyalella* were weighed to the nearest µg (SE2 Ultra Micro Balance Sartorius

Mechantronics Corp., Bohemia, NY, USA). Individual organisms were then digested following the methods described by Neumann et al (1999). In brief, *Hyalella* were digested in 25 µL of HNO_3 for 6 days, followed by 20 µL of 30% H_2O_2 (Sigma-Aldrich Inc., Mississauga ON) for 24h after which the final volume was brought to 250 µL using deionized water (Neumann et al., 1999). Whole body Cu accumulation in each organism was measured by GF-AAS after appropriate dilution of digests.

3.2.6 Calculations and Statistical Analyses

Lethal concentrations at 50% (LC50) were calculated with survival data and measured dissolved Cu concentrations using the trimmed Spearman-Karber method (Hamilton et al., 1977) within the software program CETIS (Comprehensive Environmental Toxicity Information System, Tidepool Software, 2005). Differences across tests between LC50s were considered to be significant when 95% confidence intervals did not overlap (Gillis et al., 2010). If 95% confidence intervals did overlap, the Litchfield and Wilcoxon statistical test was used to determine if there were any significant differences (Environment Canada, 2005). Effect concentrations for growth (dry weight) at 20% and 50% (EC20, EC50) were calculated using Non-linear Regression analysis within CETIS. The dry weight of organisms at day 28 was represented as a % of the DOM-control and the mean was calculated on a per-replicate basis (n=3). The biomass mean (sum of dry weight based on survival) was also calculated on a per-replicate basis (n=3) and was represented ± standard deviation. Growth and accumulation differences at day 28 were determined to be significant ($p<0.05$) within a test compared to the DOM control using a one-way ANOVA followed by the Fisher LSD test within SigmaPlot™ (ver.11). Chronic toxicity data (survival and dry weight) were correlated to the optical and chemical characterization measurements (Chapter 2, see Table 2.2, 2.3, 2.4). Correlation

coefficients (r) were determined by the Pearson product moment method (n=4) and were considered to be significant when $p<0.05$.

3.2.7 Modelling

The Biotic Ligand Model (ver. 2.2.3; HydroQual Inc., New Jersey, USA) was used with measured water chemistry parameters (DOC, Ca, Mg) to generate chronic toxicity predictions. A *Hyalella*-specific model was developed (See Chapter 2; *2.2.8 Modelling*) based on the existing *Daphnia pulex* files within the BLM program. In each modelling scenario the LA50 input was adjusted to achieve the best fit between the predicted and measured LC50s (Clifford and McGeer, 2009). To adjust the model for DOM source variability, two parameters were altered: %humic acid (HA) content (based on results obtained from PARAFAC analysis) and the active DOC concentration. The active DOC concentration parameter was adjusted using the quality factor (QF) method that incorporates SAC_{340} as a surrogate measure of the DOC concentration (see Chapter 2, Schwartz et al., 2004). In the QF equation (See Chapter 2), the 0.31 constant was altered to 0.328 in order to weight different DOM quality without altering the LA50 within the model.

3.3 Results

3.3.1 Water Chemistry

Water chemistry parameters were measured throughout chronic toxicity tests to determine if any changes occurred over the 28 day exposure (Table 3.1, Table 3.2). On average, dissolved Cu concentrations were calculated to be 100 ± 7% of total Cu (n=1260). Furthermore, Cu concentrations at day 28 were calculated to be 96 ± 2% (n=210) of day 0 concentrations. Cu,

DOM, and hardness cation (Ca and Mg) concentrations were consistent among replicates and across exposure series (Table 3.1, 3.2). DOC concentrations were measured at 7 mg C/L and this was expected given the addition of 5 mg C/L against the average background levels of DOC which were measured at 2.1 ± 0.1 mg C/L (n=180, see Table 3.1).

3.3.2 Chronic Mortality

The LC50 values for the Cu-only test (with no added DOM) were 0.089 µM and 0.066 µM for days 14 and 28 respectively (Fig. 3.1). At day 14, all sources of DOM (WR1, WR2, WR3, and DL; added at 7 mg C/L) provided a significant level of protection against chronic Cu toxicity, with LC50 values ranging from 0.44 – 0.83 µM (Fig.3.1). However, at day 28 only WR1 and WR3 DOM offered significant protection (Fig. 3.1). At day 14, WR1 and WR3 DOM were significantly more protective against chronic Cu toxicity compared to day 28, whereas there was no significant difference observed between day 14 and 28 for WR2 and DL DOM.

3.3.3 Growth Effects

In all tests, day 28 dry weight decreased with increased Cu concentration exposure (Fig. 3.2) and there was no significant difference between control solutions (no-DOM and added-DOM) with no added Cu (Table 3.2). The most dramatic reduction in dry weight and biomass occurred in the Cu only exposure (Fig. 3.2). In tests with added DOM from the WR1 and WR3 sites, the effects of Cu on growth were less than it was for the DL and WR2 sources (Fig 3.2). When WR1 and WR3 DOM were present, there was a significant amount of growth compared to the day 0 pre-test controls across all Cu concentrations. However, when WR2 and DL DOM or no DOM (i.e. Cu only exposure) was present, growth did not significantly increase compared to the pre-test dry weight at the highest Cu exposure of 1.0 µM (Table 3.2). Increased growth was

observed in the test series with WR2 where there was a significant increase (150%) compared to the unexposed DOM control dry weight at 0.0315 µM Cu (Fig 3.2A, Table 3.2). A significant trend of increased growth (134% and 128% at exposure concentrations of 0.063 and 0.125 µM, respectively) compared to the DOM control dry weight was also observed with WR3 DOM (Table 3.2, Fig. 3.2A).

Biomass decreased as Cu concentration increased but DOM mitigated the effect (Fig. 3.2B). The largest protective effect of DOM on biomass was observed when WR3 DOM was present. Biomass was significantly reduced at exposure concentrations of 0.25 µM Cu and higher compared to the DOM control (Fig. 3.2B). Only at the highest exposure concentration (1.0 µM) the biomass was not significantly different from the Pre-test Day 0 biomass. On the other hand, when WR2 DOM was present biomass was significantly reduced compared to DOM control at all concentrations from 0.063 µM and beyond (Fig. 3.2B). In this same range of concentrations (0.063-1.0 µM) when WR2 DOM was present, the biomass did not significantly differ from the Pre-test Day 0 biomass. Intermediate mitigation of the impacts of Cu on biomass were observed for WR1 and DL DOM.

Protective growth effects were seen when DOM was added to chronic toxicity tests (Fig. 3.3). With no added DOM, the EC50 and EC20 were 0.0085 ± 0.0071 and 0.0058 ± 0.0018 µM Cu, respectively. The EC50s for these tests with added DOM ranged from 0.061 ± 0.042 µM Cu (WR2) to 0.79 ± 0.2 µM Cu (WR3), with all sources being significantly protective compared to the Cu only exposure and the WR3 EC50 being significantly higher than all other sources of DOM (Fig. 3.3). With DOM present, the EC20s varied between 0.036 ± 0.006 µM Cu (WR2) and 0.25 ± 0.18 µM Cu (WR3), with all sources of DOM providing a significant protective effect compared to the Cu exposure (Fig. 3.3). The EC20 was significantly lower when WR2 DOM

was present compared to the other sources of DOM. It was also observed that there were significant differences between the EC20 and EC50 within the WR3 DOM exposure (EC50 was 3-fold higher than the EC20) and the DL DOM chronic study (EC50 was 4.5-fold higher than the EC20).

3.3.4 Cu Bioaccumulation

Cu bioaccumulation in surviving organisms after 28 d of exposure showed that there were no significant differences between unexposed controls and exposed amphipods within tests. There were no significant differences in whole body Cu burden across all exposures up to and including the highest, 1 µM (Table 3.2).

3.3.5 Correlations with DOM Characteristics

Correlation analysis (using Pearson Product Moment) among the chronic toxicity parameters (survival and growth) and the measured chemical and optical characteristics determined in Chapter 2 showed that there were two significant relationships (Table 3.3). A significant correlation was found between the EC20 and the K_m ($r = -0.98$, $p<0.05$) determined from 6h bioaccumulation studies. There was also a significant correlation found between the Day 28 LC50 and SAC_{340} values ($r = 0.96$, $p<0.05$).

3.3.6 Modelling

Without any adjustments, the BLM generated a single chronic toxicity prediction but variable toxicities were measured (Fig. 3.4A). When the specific %HA determined by PARAFAC was incorporated into the BLM, chronic toxicity predictions were improved for three of the DOM samples (WR1, WR2, WR3; Fig. 3.4B). The predicted and measured LC50 for the

DL sample was not consistent. Chronic toxicity predictions were significantly improved when the SAC_{340} was utilized as an alternate measure of the DOC concentrations (Fig. 3.4C).

3.4 Discussion

This study demonstrates that *Hyalella azteca* are sensitive to chronic Cu exposure and that toxicity was mitigated, to varying degrees, by DOM (Fig. 3.1). The protection afforded by DOM varied with source and was associated with survival and growth (Fig. 3.2, 3.3) but not Cu bioaccumulation (Table 3.2). The optical/chemical characteristics discussed in Chapter 2 did not provide as clear of an explanation for DOM source variability that was found for the acute toxicity data.

Numerous studies assessing chronic Cu toxicity to *Hyalella* have been conducted (Morris et al., 2003; Borgmann and Norwood, 1995; Borgmann et al., 1993). In this study, it was observed that *Hyalella* were sensitive to chronic Cu exposure. The LC50 was approximately 10-fold lower in chronic tests (0.07 µM) compared to acute exposures (0.7 µM). According to the freshwater Cu guidelines set forth by the Canadian Council of Ministers of the Environment (CCME, 1999), *Hyalella* appear to be sensitive since the chronic LC50 (0.07 µM) is just above the existing guidelines of 0.03–0.06 µM Cu. Borgmann et al (1993) found *H. azteca* to be less sensitive to chronic Cu toxicity than we observed, with a 10 week LC50 of approximately 0.5 µM, a 7-fold difference from the chronic Cu exposure LC50 determined in this study. However the Borgmann et al (1993) study was conducted in much harder water (120 mg $CaCO_3$/L) than this study (13 mg $CaCO_3$/L) and this may explain the difference. The Borgmann et al (1993) study also used a different source of *Hyalella* than we did (Valens Conservation Area near Cambridge ON vs Hannah Lake near Sudbury ON) and it is unknown what influence this may

have. This study appears to be the only study within the literature of chronic Cu toxicity to *Hyalella* in such soft water.

The chronic toxicity of Cu, similar to our findings for acute toxicity (Chapter 2), varied with DOM source. Several studies (e.g. De Schamphelaere and Janssen, 2004a; McGeer et al., 2002; Garvey et al., 1991) have illustrated that DOM provides a protective effect against chronic Cu toxicity to a variety of organisms (rainbow trout, *Daphnia magna*, and the green algae *Chlamydomonas reinhardtii*, respectively), however most studies focus on the presence (at different DOC concentrations) or absence of DOM rather than DOM source variability. De Schamphelaere and Janssen (2004a) tested the influence of three different DOM sources on the chronic toxicity of Cu to *D. magna* and found no significant difference between the sources in providing a protective effect. However, that study only compared a few sources within an experimental design that included the influence of DOC concentration, pH and hardness in addition to source (De Schamphelaere and Janssen 2004a). It is also noteworthy that the authors highlight that in a different study with the same sources (De Schamphelaere et al 2003) there were significant differences in both protective capacity (to *Pseudokirchneriella subcapitata*) and complexation capacity among sources. De Schamphelaere and Janssen (2004a) highlighted the need for additional data on how DOM source influences responses to chronic Cu exposure.

This study demonstrates that not all DOM sources offer significant protection against the chronic effects of Cu but the results were equivocal. Based on survival at d 14, all sources provided significant protection however at 28 d the pattern was different. The LC50 values derived from survival over 28 d showed that WR1 and WR3 sites provided significant protection against Cu toxicity while WR2 and DL did not (Fig. 3.1). The DOM from the sites that were protective (WR1 and WR3) also had the highest binding capacity and affinity for Cu^{2+} and the

lowest affinity values (Chapter 2, Table 2.3). The WR1 source (reference) has been shown to offer strong protection against acute Cu toxicity and was highly coloured but the WR3 source (logged site) was intermediate in these measures. A mechanistic rationale for the differences among DOM sources could not be derived but perhaps the protective DOMs were better able to bind free Cu and make it less available for toxic action.

The protective effects of the WR1 and WR3 DOM on survival were also observed for growth over the exposure. Dry weight on d 28 were higher when DOM from the logging site (WR3) was present, followed closely by the undisturbed site (WR1). DOM from the fire (WR2) and smelter damaged (DL) sites were less protective (dry weights were less) and this was consistent with the pattern of finding for acute toxicity. All of the DOM sources provided a significant protection compared to the Cu only (no DOM) exposed organisms.

De Schamphelaere and Janssen (2004b) completed a study assessing chronic dietary Cu exposure on the growth and reproduction of *D. magna* in the presence of 10 mg DOC/L. It was found that in waterborne Cu exposures, dry weight decreased as Cu concentrations increased, although it was not considered to be significant. However, in dietary Cu exposures, dry weight was found to increase with increasing Cu concentrations, up to 75% compared to control organisms (De Schamphelaere and Janssen, 2004b). In sediment toxicity tests, it has been found that growth is a more sensitive indicator than survival in chronic Cu experiments (Kubitz et al., 1995). In contrast, Borgmann et al (1993) observed no significant effect of chronic (10 week) Cu exposure on growth. In our study, waterborne Cu exposure led to significant reductions in dry weight at high Cu concentrations for all exposures but increased growth was observed with the DOM from the WR2 source at low Cu exposure concentrations. It is interesting that this increased growth was seen when WR2 DOM (fire site) was present because although this DOM

provided significant growth protection compared to the Cu only exposure, it was significantly lower than the other three DOMs tested. It is clear that DOM quality is negatively impacted by this type of harsh landscape disturbance (i.e. fire) shown by reduced toxicity mitigation potential but there are also sub-lethal consequences such as reduced growth. From this study, it is evident that there are differences in DOM quality, illustrated by chronic Cu exposures, and there appears to be biological concerns beyond mortality that warrants further investigation.

In accordance with the theory of the BLM, acute toxicity is related to accumulation and this was observed for short term (6 h) bioaccumulation in Chapter 2, however, chronic bioaccumulation was not related to chronic toxicity. There were no significant differences found for Cu body burdens among control and exposed *Hyalella* regardless of the Cu exposure or the presence of DOM (Fig. 3.4). Similar to the results of Borgmann et al (1993) and Borgmann and Norwood (1995), it appears that Cu is regulated during chronic exposure. The Cu regulation observed in *Hyalella* azteca is different from what is seen in other types of amphipods (e.g. *Gammarus zaddachi, Echinogammarus pirloti*) where either no regulation or very little regulation occurs (Amiard et al., 1987; Rainbow and White, 1989). Our measured Cu accumulation levels are supported by the accumulation results from the Borgmann et al (1993) study because we observed comparable chronic Cu bioaccumulation values. For example, in our study, on average, control organisms had whole body concentrations of 89 ± 18 µg Cu/g dry weight and in the Borgmann (1993) study it was noted that control organisms had burdens of 79 ± 20 µg Cu/g dry weight, with no significant differences measured in organisms from any Cu exposure concentrations.

Unlike short-term (e.g. 6h), chronic Cu accumulation is not an effective indicator of toxic effects because of the regulation that occurs during long-term exposure (Borgmann et al., 1993).

Cu accumulation within *H. azteca* has been found to peak after one week of exposure and gradually decrease to approximately similar levels of control organisms because of internal Cu regulating mechanisms (Borgmann and Norwood, 1995). We found that Cu regulation occurred by the end of our chronic tests (4 weeks) which agrees with the 4-6 week timeline outlined by Borgmann and Norwood (1995) for regulation to occur. Importantly, the regulation of Cu is not associated with the weekly molt that *Hyalella* undergo (Borgmann and Norwood, 1995). It is worth noting that the presence of a modifying factor (DOM) did not impact the *Hyalella* Cu regulation mechanisms.

Based on the Pearson Product Moment (see Chapter 2), there were only two significant ($p<0.05$) correlations among chronic toxicity parameters and the optical/chemical characterizations that were completed in Chapter 2 (Table 2.3). The chronic EC20 values were found to negatively correlate with the K_m (binding affinity) calculated from the 6h Cu bioaccumulation studies (see Chapter 2). The relationship between the 6h K_m and the chronic EC20 may act as a useful indicator of growth effects from chronic exposure to Cu and DOM exposures. A positive relationship was found between the Day 28 LC50 and SAC_{340} (see Chapter 2). Similar to the acute toxicity data (Chapter 2), the SAC_{340} value (i.e. the colour of the DOM) is the best indicator of chronic toxicity. This relationship between SAC_{340} and DOM protective ability has been shown for other organisms as well such as rainbow trout (Schwartz et al., 2004), daphnia (Al-Reasi et al., 2012; De Schamphelaere et al., 2004), and fathead minnow (Ryan et al., 2004), however these studies have all focused on acute exposures. This study shows that the functional groups on the aromatic rings, which strongly bind Cu (Carbonaro et al., 2011), appear to be the most important factor in how protective a DOM source is because it is related to the mitigation of both acute and chronic toxicity of Cu to *Hyalella*.

Adjusting our *Hyalella*-specific BLM allowed us to improve toxicity predictions by taking into account DOM source quality (Fig. 3.4). Within the literature there are few chronic Cu BLMs for invertebrates however a chronic BLM has been developed for *Ceriodaphnia dubia* (Schwartz and Vigneault, 2007) as well as a field-validated BLM for *Daphnia magna* (De Schamphelaere and Janssen, 2009). In our study, we utilized the acute Cu BLM to generate chronic toxicity predictions. To adjust the model for DOM source quality we first altered the %HA input by incorporating the specific amount of humic-like substance for each source determined by PARAFAC analysis (Fig. 3.4B). Chronic toxicity predictions were improved when %HA was altered, and this was similar to the study done by Al-Reasi et al (2012) who found that including %HA adjustments within the BLM greatly improved acute Cu toxicity predictions. In general, adjusting the humic acid content was an effective method, but the Daisy Lake DOM sample was not well predicted. As a result of a high measured humic acid content the DL DOM was predicted to be highly protective, however this was not the case. It appears that at this damaged site, there is an entity within the DOM that fluoresces similar to humic acid but does not contribute to toxicity mitigation. The other method to adjust the model for DOM source quality was to utilize the quality factor described by Schwartz et al (2004; Fig. 3.4C). The QF is an adjustment that is based on SAC_{340} values and it becomes an altered input for the DOC concentration within the model. Adjusting the model to include the QF greatly improved chronic toxicity predictions; all samples were accurately predicted. By incorporating optical characteristics into the model, we were able to extend the acute BLM to generate accurate chronic toxicity predictions that accounted for DOM source variability.

3.5 Conclusions

In summary, this study demonstrates that DOM source quality is negatively impacted by ecosystem disturbance. Chronic Cu toxicity to *Hyalella azteca* varied based on DOM source, with the undisturbed and logged sources offering more protection than the more disturbed sites (smelter and fire impacted). The differences in chronic mortality were associated with dry weight patterns at day 28 but not with Cu bioaccumulation at 28 d because during chronic exposure, *Hyalella* appear to regulate Cu. Similar to the companion study (Chapter 2: optical characterization, binding capacity and acute toxicity mitigation), the SAC_{340} values of DOM sources was found to correlate with reductions in chronic Cu toxicity. An improved understanding of Cu-DOM interactions in chronic toxicity studies would contribute towards long term ecosystem remediation efforts.

3.6 Tables

Table 3.1 – Measured water chemistry parameters for 28 day chronic toxicity tests. Parameters for unexposed (control with no Cu or DOM and control with DOM but no Cu) *Hyalella* are also included. Ca, Mg, and pH represented as mean ± SEM (n=85). Measured exposure concentrations are given as total (unfiltered) and dissolved (0.45 µM) Cu and represented as mean ± SEM (n=36). Measured DOC concentrations are given as mean ± SEM (n=36).

Exposure Series	**Nominal Cu** (nM)	**Measured Cu** (nM)		**Measured DOC** (mg C/L)	**Ca** (µM)	**Mg** (µM)	**pH**
		Total	**Dissolved**				
Cu only	0 (Ctrl)	4.0 ± 1.0	4.2 ± 1.0	2.1 ± 0.03	84.8 ± 8.0	26.4 ± 2.1	7.3 ± 0.02
	8	8.0 ± 1.6	9.2 ± 3.5	1.9 ± 0.07			
	16	14 ± 1.8	13.0 ± 1.5	2.1 ± 0.2			
	32	29 ± 2.4	28.0 ± 2.6	2.1 ± 0.08			
	63	60 ± 4.0	55.0 ± 3.4	1.9 ± 0.2			
	125	120 ± 6.2	110.0 ± 6.6	2.1 ± 0.06			
WR1	0 (Ctrl)	3.7 ± 1.5	2.5 ± 1.3	2.2 ± 0.04	82.7 ± 4.1	26.9 ± 1.3	7.3 ± 0.03
	0 (DOM Ctrl)	4.1 ± 1.0	4.5 ± 1.0	7.3 ± 0.09			
	32	39 ± 1.2	38.0 ± 1.1	7.3 ± 0.06			
	63	68 ± 1.5	65 ± 1.6	7.3 ± 0.09			
	125	120 ± 6.1	120.0± 3.6	7.3 ± 0.05			
	250	210 ± 7.1	200.0 ± 6.5	7.2 ± 0.08			
	500	460 ± 15	430.0 ± 13.0	7.3 ± 0.07			
	1000	920 ± 28	910.0 ± 32.0	7.2 ± 0.06			
WR2	0 (Ctrl)	5.1 ± 1.6	4.8 ± 1.6	2.2 ± 0.08	81.9 ± 2.9	28.5 ± 1.9	7.3 ± 0.03
	0 (DOM Ctrl)	7.9 ± 1.9	6.5 ± 1.5	6.6 ± 0.06			
	31.5	24 ± 2.6	23 ± 2.4	7.1 ± 0.09			

	63	63 ± 2.9	62 ± 3.4	7.0 ± 0.1			
	125	120 ± 3.2	120 ± 3.4	7.1 ± 0.09			
	250	260 ± 8.6	250 ± 7.3	7.1 ± 0.2			
	500	490 ± 14	470 ± 14	7.0 ± 0.2			
	1000	890 ± 21	890 ± 24	7.1 ± 0.3			
WR3	0 (Ctrl)	5.1 ± 1.2	4.9 ± 1.3	2.0 ± 0.06	82.6 ± 2.8	27.7 ± 1.4	7.3 ± 0.03
	0 (DOM Ctrl)	6.5 ± 1.5	6.0 ± 1.5	7.2 ± 0.05			
	32	33 ± 2.2	32 ± 2.4	7.2± 0.2			
	63	63 ± 2.3	61 ± 2.4	7.3 ± 0.1			
	125	120 ± 2.7	120 ± 2.5	7.2 ± 0.1			
	250	240 ± 7.3	230 ± 7.0	7.3 ± 0.2			
	500	480 ± 11	460 ± 11	7.2 ± 0.2			
	1000	960 ± 21	920 ± 22	7.3 ± 0.2			
DL	0 (Ctrl)	6.3 ± 1.5	5.9 ± 1.2	1.9 ± 0.2	81.3 ± 3.5	28.8 ± 1.4	7.3 ± 0.03
	0 (DOM Ctrl)	6.4 ± 1.4	6.5 ± 1.2	7.3 ± 0.2			
	32	37 ± 1.4	35 ± 1.6	7.0 ± 0.3			
	63	57 ± 2.8	53 ± 2.3	7.1 ± 0.2			
	125	130 ± 6.7	130 ± 6.4	7.3 ± 0.3			
	250	240 ± 15	230 ± 12	7.2 ± 0.2			
	500	530 ± 22	530 ± 21	7.3 ± 0.1			
	1000	1003 ± 50	950 ± 43	7.3 ± 0.2			

Table 3.2 – Influence of DOM and chronic Cu exposure on survival, mean dry weight, and Cu body burden on Day 28 for *Hyalella azteca*. Dry weight and body burden and dry weight are given as mean ± SEM (n=4-30) for surviving adults. Dry weight and Cu body burden is also shown for 2-9 d old *Hyalella* sampled prior to test initiation (pre-test) and * indicates significant differences from controls with added DOM (DOM Ctrl) while † indicates mean dry weights that are not significantly different from 'pre-test' within a test ($p<0.05$).

Exposure Series	**Nominal Cu** (nM)	**Survival** (%)	**Dry Weight** (mg)	**Cu body burden** (µg/ g dry wt)	**n**
Cu only	Pre-test	n/a	0.016 ± 0.0015	98.07 ± 22.85	24
	0 (Ctrl)	97	0.11 ± 0.0065	87.96 ± 31.45	29
	8	70	0.049 ± 0.0059	104.24 ± 24.27	21
	16	77	0.036 ± 0.0041	111.39 ± 24.19	23
	32	60	0.038 ± 0.0049	110.05 ± 26.75	18
	63.0	30	0.039 ± 0.0041	112.62 ± 19.68	9
	125	30	0.033 ± 0.0051†	146.63 ± 15.6	9
WR1	Pre-test	n/a	0.014 ± 0.0031	94.94 ± 8.94	24
	0 (Ctrl)	100	0.10 ± 0.0059	99.59 ± 14.28	30
	0 (DOM Ctrl)	97	0.097 ± 0.0071	96.99 ± 9.34	29
	32	93	0.068 ± 0.0074*	118.34 ± 13.81	28
	63	77	0.097 ± 0.010	120.83 ± 12.01	23
	125	70	0.085 ± 0.0086	123.39 ± 11.72	21
	250	67	0.055 ± 0.0087*	137.96 ± 18.21	20
	500	63	0.048 ± 0.0042*	134.50 ± 11.23	19
	1000	13	0.040 ± 0.010*	163.15 ± 35.97	4
WR2	Pre-test	n/a	0.012 ± 0.001	86.39 ± 6.56	16
	0 (Ctrl)	100	0.10 ± 0.011	80.42 ± 16.75	30
	0 (DOM Ctrl)	97	0.088 ± .0083	93.78 ± 15.32	29
	32	73	0.14 ± 0.017*	89.52 ± 13.66	22
	63	67	0.059 ± 0.0042*	114.60 ± 15.72	20
	125	57	0.047 ± 0.0055	109.86 ± 11.61	17
	250	53	0.043 ± 0.0035*	200.25 ± 51.76	16
	500	47	0.045 ± 0.0053*	131.66 ± 11.26	14
	1000	13	0.028 ± 0.0064*†	119.83 ± 17.90	3
WR3	Pre-test	n/a	0.013 ± 0.0002	106.85 ± 13.39	15
	0 (Ctrl)	100	0.10 ± 0.0063	93.07 ± 14.52	30
	0 (DOM Ctrl)	100	0.087 ± 0.0081	119.16 ± 16.70	30
	32	87	0.079 ± 0.0069	118.34 ± 17.10	26
	63	77	0.12 ± 0.014*	91.95 ± 16.23	23

	125	70	0.11 ± 0.012*	118.49 ± 14.18	21
	250	63	0.083 ± 0.0086	129.62 ± 17.31	19
	500	53	0.074 ± 0.0093	118.78 ± 18.35	16
	1000	17	0.045 ± 0.0065*	128.45 ± 27.92	5
DL	Pre-test	n/a	0.013 ± 0.0003	108.02 ± 11.14	13
	0 (Ctrl)	100	0.11 ± 0.0057	88.63 ± 14.21	20
	0 (DOM Ctrl)	100	0.11 ± 0.013	109.18 ± 9.30	10
	32	80	0.081 ± 0.0079*	88.01 ± 19.85	16
	63	65	0.093 ± 0.0071	113.31 ± 18.12	13
	125	55	0.072 ± 0.015*	105.83 ± 9.54	11
	250	50	0.058 ± 0.0083*	104.01 ± 12.5	10
	500	45	0.043 ± 0.0055*	110.46 ± 10.12	9
	1000	20	0.020 ± 0.0016*†	152.02 ± 43.46	1

Table 3.3 – Correlation coefficients (r) for measured DOM variables (Table 2.2, 2.3, 2.4) with measured chronic toxicity (survival and growth) values. Correlations calculated with Pearson Product Moment and * indicates significance ($p<0.05$), n=4.

Correlation Coefficients											
	SAC_{340}	**FI**	**HA**	**FA**	**Tyr**	**Trp**	**B_{max} (ISE)**	**K_m (ISE)**	**Vmax (6h)**	**Km (6h)**	**96h LC50**
Day 14 LC50 (µM Cu)	0.65	(-)0.54	0.85	(-)0.76	(-)0.87	(-)0.94	0.58	(-)0.93	(-)0.86	(-)0.92	0.60
Day 28 LC50 (µM Cu)	0.96*	(-)0.92	0.38	(-)0.21	(-)0.66	(-)0.67	0.92	(-)0.89	(-)0.70	(-)0.80	0.90
EC20 (µM Cu)	0.51	(-)0.42	0.54	(-)0.50	(-)0.91	(-)0.69	0.40	(-)0.66	(-)0.44	(-)0.98*	0.36
EC50 (µM Cu)	0.18	(-)0.07	0.61	(-)0.65	(-)0.71	(-)0.70	0.06	(-)0.46	(-)0.30	(-)0.89	0.43

3.7 Figures

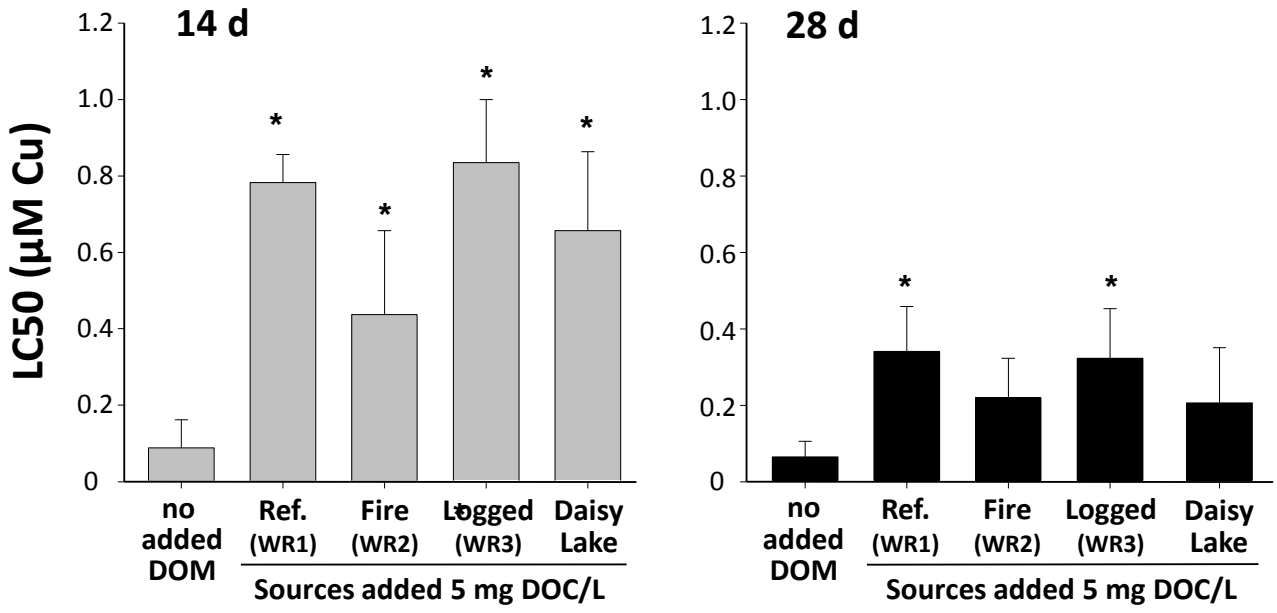

Figure 3.1 – Influence of DOM on chronic Cu toxicity to *Hyalella azteca*. DOM was added at a nominal concentration of 7 mg C/L. LC50 values for Day 14 (grey bars) and Day 28 (black bars) were calculated based on measured dissolved Cu concentrations and are represented with error bar (95% confidence interval). * indicates significant difference from the Cu only exposure at that day.

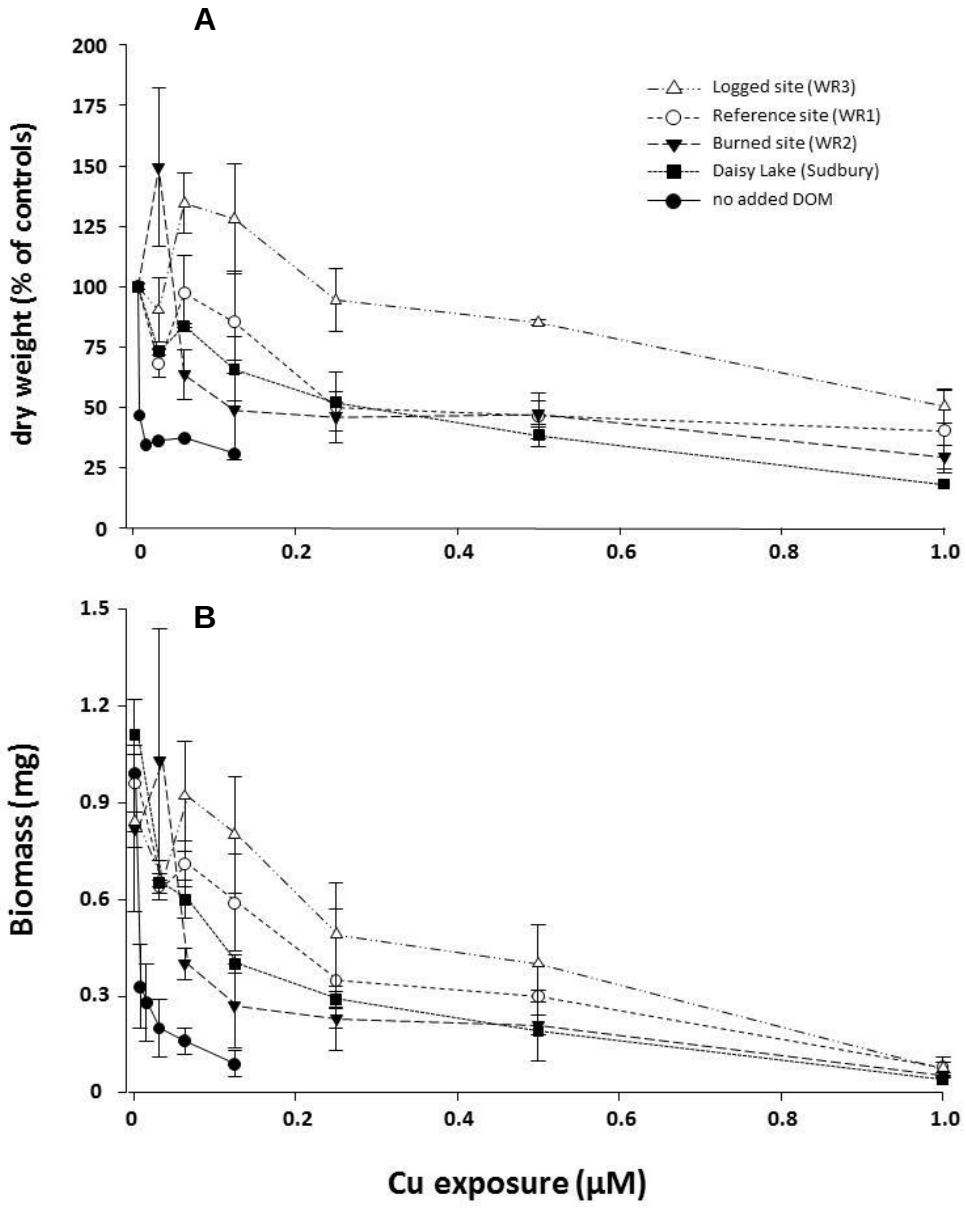

Figure 3.2 – Effect of DOM source on *Hyalella* growth. **A)** Day 28 dry weight (mean % of control ± SD on a per-replicate basis; n=3, except for DL test, n=2). **B)** Day 28 biomass (mean biomass ± SD on a per-replicate basis; n=3 except for DL test, n=2). Cu exposure based on nominal Cu concentrations (0-1µM). DOM sources added at a nominal concentration of 7 mg C/L.

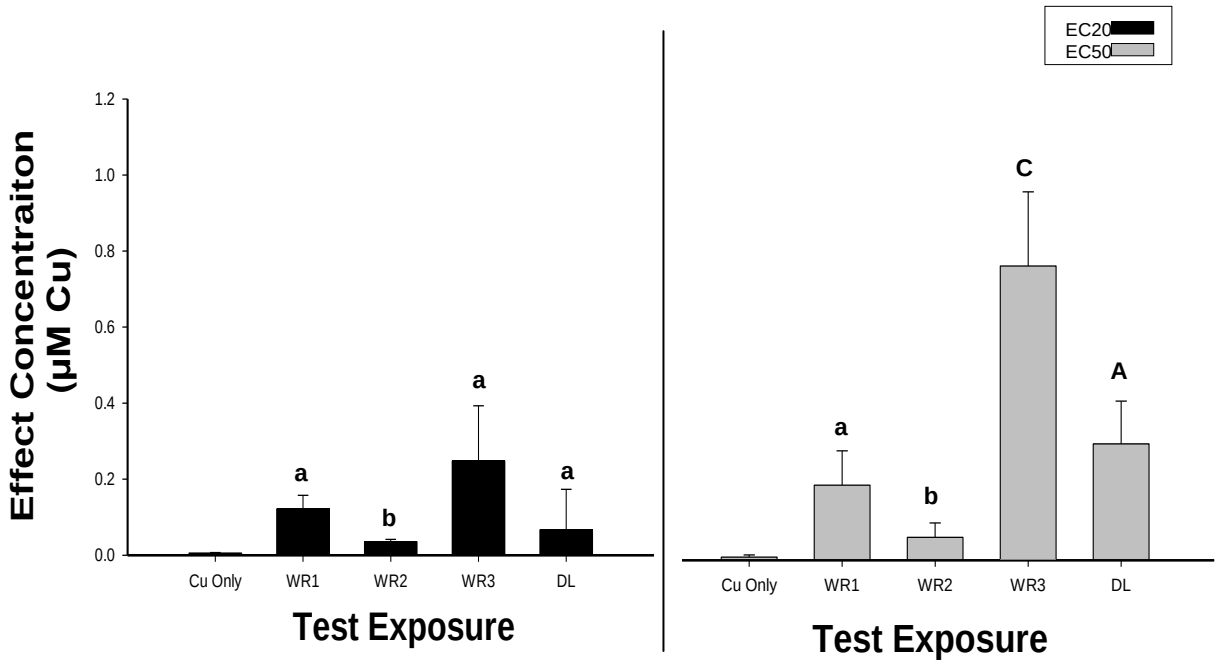

Figure 3.3 – Influence of DOM source variability on growth effect concentration (EC) in *Hyalella*. DOM sources added at nominal concentration of 7 mg C/L. EC20 and EC50 values were calculated based on day 28 survival and dry weight. Black bars represent EC20 and grey bars represent EC50, all bars are represented with 95% confidence intervals. Letters represent significant difference from the Cu only exposure. Capital letters indicate significant difference between EC20 and EC50 for a given exposure type.

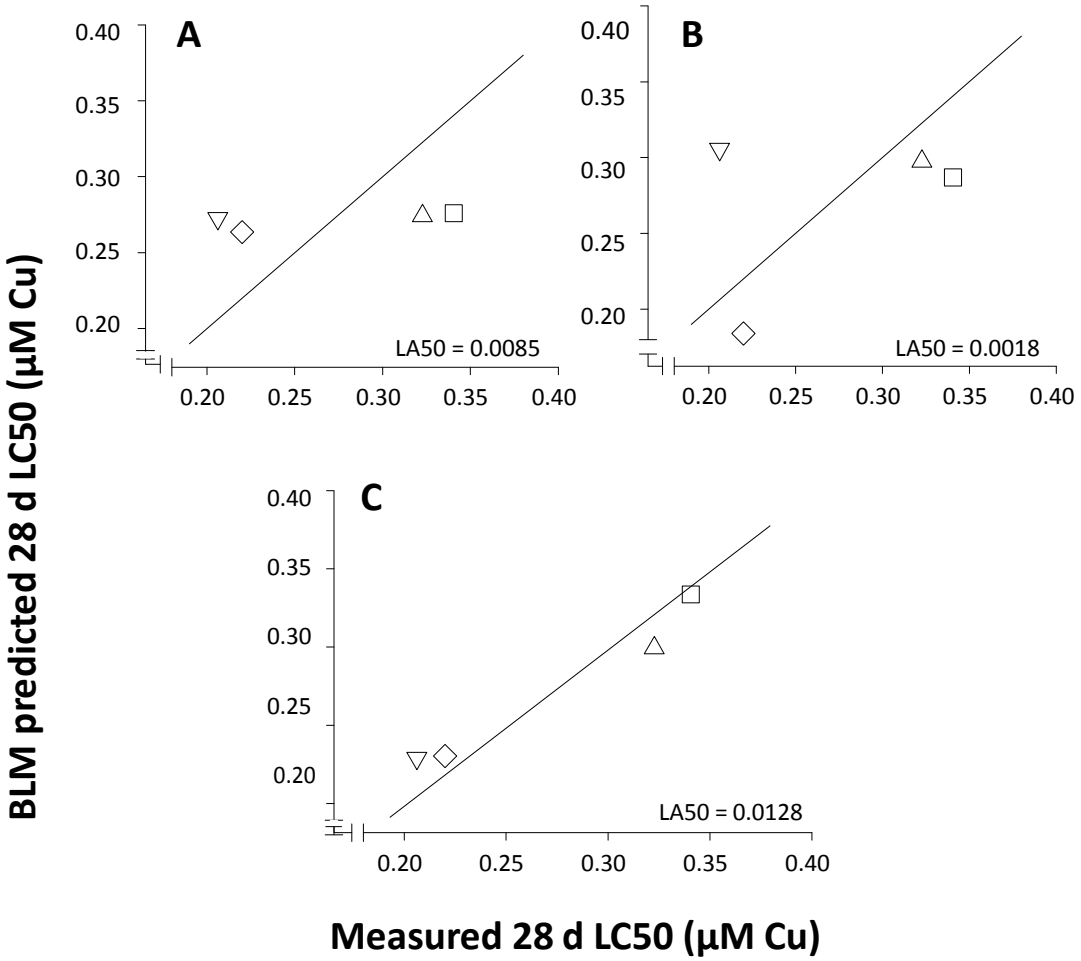

Figure 3.4 – Comparison of measured and BLM predictions of Cu toxicity to *Hyalella azteca* with 7 mg DOC/L from each of 4 different sources. Symbols show the undisturbed site (WR1, squares), Daisy Lake (Sudbury region, inverted triangle) as well as previously burned and logged sites (WR1 and 2, diamond and triangle respectively). The solid line shows one-to-one predictions and panels show: **A**) unmodified BLM predictions; **B**) when the %HA derived from PARAFAC analysis of EEMS data were used to adjust the model; **C**) when SAC_{340} based adjustments to DOC concentrations were applied. In each modelling scenario the LA50 input is given (nmol Cu/g wet weight) and these values were adjusted to obtain the best overall fit between measured and predicted LC50s.

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CHAPTER 4
General Discussion

4.1 Major Findings

The goal of this study was to compare the toxicity mitigating potential of different DOMs impacted by landscape disturbance (e.g. fire, logging, smelting) and how different sources can vary in protecting against acute and chronic Cu toxicity to *Hyalella azteca*. A suite of biological (acute and chronic toxicity testing), chemical (ion selective electrode), and optical (SAC_{340}, EEMS, FI) characterizations were used to explain differences in DOM quality. The optical and biological data were incorporated into the biotic ligand model (BLM) to improve acute and chronic Cu toxicity predictions by accounting for DOM quality. The major findings of this research are as follows:

1. Acute toxicity mitigation varies with DOM source. DOM protective quality is impacted by both the type and severity of disturbance with disturbed sites offering less protection than undisturbed references sites (Chapter 2; see *Acute Toxicity Tests*).

2. Differences in acute toxicity are supported by ion selective electrode and 6h bioaccumulation exposures (Chapter 2; see *ISE Characterization of DOM* & *Short Term (6h) Accumulation*).

3. Optical characterizations are valuable for explaining DOM source quality differences (Chapter 2; see *Correlations Among Biological, Optical, and Chemical Measures*). Specifically, SAC_{340} appears to be the best indicator of DOM protective quality.

4. Incorporating optical characteristics (e.g. %HA from PARAFAC and SAC_{340} as part of the Quality Factor) allowed us to improve both acute and chronic Cu toxicity predictions

that took into account DOM source variability and ecosystem disturbance (Chapter 2; see *Modelling*).

5. Chronic Cu toxicity varies depending on DOM source and there is somewhat of a link between ecosystem disturbance and toxicity mitigation (Chapter 3; see *Chronic Toxicity*).

6. Chronic toxicity is associated with the dry weight of organisms at Day 28 (Chapter 3; see *Chronic Growth Effects*). However, toxicity is not related to Cu bioaccumulation in surviving organisms at Day 28 because regulation appears to occur when *Hyalella* are exposed to Cu in long term studies (Chapter 3; see *Chronic Accumulation*).

7. Chronic toxicity is not explained by most optical and chemical characterizations (Chapter 2; see *Optical Characterizations of DOM* and *ISE Characterization of DOM*). However, similar to the acute toxicity results, the chronic (Day 28) LC50 was significantly correlated to SAC_{340}. In addition to being a rapid and simple measurement to complete, SAC_{340} (i.e. how dark a DOM is) is the best indicator of how protective a DOM source will be against Cu toxicity.

4.2 General Discussion

When comparing acute and chronic studies, there was good agreement between the protective qualities of DOM sources, with similar patterns associated with ecosystem disturbance (Fig. 2.1, 3.1). Although in both acute and chronic studies, Cu toxicity was significantly reduced when DOM was present, the WR3 (logging site) was found to be much more protective in long-term exposures compared to acute tests. In the acute toxicity studies (Fig. 2.1), WR3 DOM provided only an intermediate level of protection against toxicity, whereas in chronic studies

(Fig. 3.1) WR3 DOM offered a protective effect that was comparable to WR1 (undisturbed reference site). This difference may be a result of WR3 DOM having high affinity binding sites that have a low capacity (Table 2.4), thus the DOM provides more protection when lower (chronic) concentrations of Cu compared to higher (acute) exposures. The optical and chemical characteristics of the DOMs did not explain chronic effects as well as the effects seen at the acute level. It is possible that there was not an overlap between the "analytical window" and the "toxicological window" at the lower concentrations tested in chronic exposures, whereas there was overlap seen in the acute toxicity tests (Al-Reasi et al., 2011). However, similar to our acute toxicity results, SAC_{340} was found to be significantly correlated with chronic toxicity. The effectiveness of SAC_{340} as an indicator of DOM quality has been observed in numerous acute studies with both invertebrates and fish species (Richards et al., 2001; Schwartz et al., 2004; De Schamphelaere et al., 2004; Al-Reasi et al., 2012), but at this point in time, there are no studies within the literature that have found a potential indicator of DOM quality with regards to chronic Cu toxicity. Because measuring and calculating the SAC_{340} is such a rapid and uncomplicated technique that most laboratories have access to, it should be considered to be one of the most valuable indicators of DOM quality in terms of both acute and chronic Cu toxicity mitigation. Additionally, incorporating SAC_{340} values (as seen in the study by Schwartz et al (2004)) into the BLM allows for improved toxicity predictions (both acute and chronic) that take into account DOM source variability and ecosystem disturbance (Fig. 2.5, Fig. 3.4)

Whole body Cu bioaccumulation patterns differed between acute and chronic studies. In the acute studies, Cu accumulation decreased when DOM was present (Fig. 2.2) and the protective effect was dependant on DOM source. However, in the chronic exposures, DOM did not reduce Cu bioaccumulation and there was no influence of DOM source variability (Table

3.2). Short term Cu accumulation was related to acute toxicity which supports the theory of the BLM, allowing us to generate acute toxicity predictions using this model (Fig. 2.5). On the other hand, chronic Cu accumulation was not related to chronic toxicity. Based on studies done by Borgmann et al (1993) and Borgmann and Norwood (1995), it was expected that *Hyalella* would regulate Cu during chronic exposure, and that was observed in our study (Table 3.2). Since Cu is regulated when *Hyalella* are chronically exposed, bioaccumulation is not considered to be an effective indicator of toxicity for long-term studies, whereas short term bioaccumulation offers a good indication of acute toxicity (Borgmann, 1998).

Ultimately, this research is contributing to the existing knowledge regarding the role of DOM source quality in the mitigation of metal toxicity. However, there are future research needs in this field to better understand the role that ecosystem disturbance and recovery has in DOM quality. The influence of seasonality needs to be studied more in-depth to determine how and why differences in DOM quality exist in relation to Cu toxicity mitigation. Collecting organic matter from a single sampling site at least once per month between April and November would provide a profile of the DOM that would give insight to if/how seasonality influences DOM quality in terms of toxicity mitigation, as well as optical characteristics. To gain an improved understanding of the link between ecosystem disturbance and DOM quality, an in-depth characterization of the terrestrial landscape features must be completed and studied in conjunction with the DOM quality parameters (biological, optical, and chemical) that were discussed throughout this document. Understanding the linkages between terrestrial and aquatic systems is the goal of the TALER (Terrestrial-Aquatic Linkages for Ecosystem Recovery) project that this research has been a part of. Characterizing these linkages will allow for this research and that of the TALER project to be incorporated into ecosystem remediation efforts

and land usage plans/strategies for regulatory agencies attempting to understand and improve ecosystems that have been impacted by long-term disturbance.

4.3 References

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