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Effects of Salinity and Dissolved Organic Matter on Cu Toxicity to Americamysis bahia in Estuarine Environments

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Effects of Salinity and Dissolved Organic Matter on Cu Toxicity to *Americamysis bahia* in Estuarine Environments

By

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Abstract

As salinity increases the geochemical speciation of Cu is altered as a result of organic/inorganic complexation/competition. Such salinity changes may further challenge the osmoregulatory capabilities of euryhaline organisms. This chemical-biological interaction complicates the understanding of the impacts of Cu in estuarine waters. Dissolved organic matter (DOM) has been widely established to be an important modifier of Cu toxicity in freshwaters however its effectiveness in modulating Cu toxicity across the range of salinities that occur in estuarine conditions has not been studied in a systematic manner. Site to site differences in DOM quality with respect to the potential for toxicity mitigation are also not well understood. The purpose of this study was to examine the mitigating effects of salinity and DOM on acute/chronic Cu toxicity to mysids (*Americamysis bahia)* using EPA-standardized 96h and 7d toxicity tests. An array of Cu concentrations (0 – 800 µg/L) were tested in duplicate (acute) and quadruplicate (7d) exposure over a wide range of salinities (5 – 40 ppt) with DOM from 4 different sources (at 0-4 mg C/L). A protective effect of salinity on acute Cu toxicity was observed however the organism was found to be more sensitive to Cu at salinity extremes. A protective effect of salinity was observed only for biomass and minimal effect was observed for other chronic end-points. The presence of DOC resulted in a protective effect to *A. bahia* against Cu toxicity at both 15 and 25 ppt. This protection was variable among sources, with some sources imparting greater protective effects than others and this difference could not be explained by optical characteristics of DOM. There was little variation among sources and resultant toxicity suggesting that DOM quality may not be as important in predicting Cu toxicity in estuarine environments. Overall, the results of

this study suggest that toxicity prediction in estuarine environments may not only be dependent on Cu geochemistry but the physiological capabilities of the organisms. Future estuarine toxicity prediction models for estuarine systems therefore need to account for the variability in physiology of estuarine organisms to develop models that accurately predict Cu toxicity. This project helps towards improving the understanding of Cu toxicity in estuarine systems and contributes data towards development of toxicity prediction models which may contribute to guidelines/criteria development for protection of aquatic biota.

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Glossary

BLM……..……...Biotic Ligand Model

ppt..…….……..Parts Per Thousand (measure of salinity)

DOM……...…..Dissolved Organic Matter

DOC…….…….....Dissolved Organic Carbon (measure of DOM)

EEMS…….…....Excitation-Emission Matrix Spectroscopy

FI……Fluorescence Index

SAC…….……Specific Absorption Coefficient

HA……...…...…Humic Acid

FA……...……Fulvic Acid

TRP…….….Tryptophan

TYR…….....Tyrosine

PARAFAC……..…….Parallel Factor Analysis

MYSID……..*Americamysis bahia*

CETIS……...…Comprehensive Environmental Toxicity Information System

LC_{50}…….…...Exposure concentration resulting in 50% lethality

EC_{20}……...Exposure concentration associated with a 20% effect on toxicity end-points

EC_{50}…..……..…Exposure Concentration responsible for a 50% effect on end-points

SEM…...…….…….Standard Error of Mean

1.0 Introduction

1.1 Copper

Copper (Cu) is an essential trace element found in a variety of tissues. It acts as a cofactor for enzymes, such as cytochrome *c* oxidase and tyrosinase and assists in biological processes involved in growth, development, maintenance and survival (Uauy et al., 1998; Gaetke et al., 2003). It is also a component of hemocyanin thereby helping in oxygen transport and it has a role in the formation of myelin for the central nervous system (Gaetke et al., 2003; Flemming et al., 1989). Being an essential metal, Cu is actively taken up and excreted by all organisms, however, increased ambient levels in aquatic systems can result in increased Cu accumulation in the organisms resulting in toxicity (Bambang et al., 1995; Pinho et al., 2010; Martins et al., 2011).

Copper is present in all aquatic systems as a result of both natural and anthropogenic activities (Canadian Council of Ministers of the Environment, 1999; United States Environmental Protection agency, 2007). As a component of earth's crust Cu is generally present in all waters and natural processes such as weathering contribute to further input into surface water (Georgopoulous et al., 2001). Natural background concentrations of Cu range from 0.2 to 30 µg/L in fresh water systems and 0.06 to 17 µg/L in coastal regions (USEPA, 2007). Elevated Cu concentration in aquatic systems can also be attributed to anthropogenic activities. Sources of contamination include fossil fuel burning, anti-fouling paints, mining processes, manure, fertilizers, the leather industry (tanneries), electrical equipment and municipal waste, but are not limited to such (Georgopoulous et al., 2001). Increased Cu concentrations, of up to 200,000 µg/L have

been reported in surface waters surrounding mining areas (USEPA, 2007). Despite the essentiality of Cu, elevated levels due to both natural and anthropogenic activities in surface waters have been known to result in toxicity of aquatic biota (Lauer et al., 2010; Bianchini et al., 2004).

1.2 Water Quality Guidelines and Criteria

Water quality criteria and guidelines for environmental contaminants are used to ensure protection of fresh water and marine organisms. The Canadian Council of Ministers of Environment (CCME) as well as Unites States Environmental Protection Agency (USEPA) have developed guidelines and criteria (respectively) that can be used for many purposes, including as benchmarks in assessing risk. CCME guidelines are intended to provide protection from anthropogenic stressors and toxicity of the contaminant is determined to a variety of species to produce a numerical value that will allow for protection of all forms of aquatic life. The numerical values provide a consistent nationally accepted guideline for aquatic life in Canada (CCME, 2003). For fresh water the Canadian water quality guideline values for many metals are adjusted based on water hardness and therefore are site specific. The CCME water quality guidelines for Cu input into surface waters are based on water hardness and can be calculated using the following equation:

$$Copper\ concentration = e^{0.8545[ln(\underline{hardness})]-1.465} * 0.2\ \mu g/L \qquad \text{Equation 1}$$

and this provides a value of 2 µg/L at a hardness of 0-120 mg/L $CaCO_3$ (CCME,1999). No values are currently in place for estuarine or marine waters.

USEPA publishes ambient water quality criteria both fresh and marine conditions. They include both acute and chronic criteria. The acute criteria is given as the criterion maximum concentrations (CMC), a concentration to which the organism was briefly exposed not resulting in a toxic lethal effect. The chronic criteria are given as criterion continuous concentrations (CCC), a concentration to which organisms can be exposed long term without a toxic effect. The fresh water CMC is based on application of the biotic ligand model (BLM) (USEPA, 2007). The BLM is a tool developed to predict acute metal toxicity based on the effects of water chemistry parameters (Santore et al., 2001) and it discussed in detail in section 1.4. A marine BLM has been proposed but at this time single criterion concentration values are still used. The marine CCC for dissolved Cu is 3.1 µg/L while the CMC is 4.8 µg/L (USEPA, 2007).

Current water quality guidelines/criteria for estuarine conditions are generated using both fresh water and marine values as the CCME freshwater guidelines are for water of 1ppt salinity or less while USEPA criteria is set for marine systems. The use of both fresh and marine models to formulate guidelines/criteria for estuarine conditions introduces uncertainty in setting values. Increasing salinity and other changes in water chemistry as fresh water flows into salt water will alter the toxicity of metals and therefore neither fresh water models calibrated for very low salinity nor ones for full strength sea water may accurately predict toxicity thresholds in estuarine conditions. The lack of an estuarine specific toxicity prediction models that account for a wide range of salinities represents a gap in the understanding of the impacts of metals.

1.3 Cu toxicity

1.3.1 Cu toxicity in marine environments

Copper toxicity in marine environments has been found to vary considerably. Seawater usually ranges from 30ppt to 37ppt and the increased Cl^- content of seawater has been found to provide a protective effect for toxicity through complexation with Cu ions. A study looking at effects of salinity on marine invertebrate species showed an LC_{50} of 6.3, 11.2, 18.9 and 14.8 µg Cu/L for *Mytilus galloprovincialis (mussel)*, *Crassostrea virginica (Atlantic oyster)*, *Dendraster excentricus (sand dollar)*, and *Strongylocentrotus purpuratus (sea urchin)* respectively at salinities ranging from 29-32ppt (Arnold et al., 2010). An LC_{50} of 181 µg/L for Cu was observed for *Americamysis bahia (mysid)* at a salinity of 30ppt (Lussier et al., 1985). In another test at pH of 8.0 the LC_{50} was calculated to be 250 µg/L for *A. bahia* at 30ppt (Ho et al., 1998). In the same study by Ho et al. (1998) *Ampelisca abdit*, a marine amphipod was found to be more sensitive to Cu with an LC_{50} of 90 µg/L. In toy shrimp, *Heptacarpus futilirostris*a an LC_{50} was determined to be 131 µg/L in seawater in comparison to an LC_{50} of 84.4 µg/L found in red sea bream (Mochida et al., 2006). At the extreme end, *Penaeus japonicas*, a prawn, exhibits a very high tolerance for Cu as at full strength sea water a 96h LC_{50} is observed at 2050 µg/L of Cu (Bambang et al., 1995).

1.3.2 Cu toxicity in estuarine environments

Estuarine environments range in salinity from 1ppt up to full strength sea water and this changing salinity can have a dramatic effect on Cu toxicity. Several studies have demonstrated the protective effects of salinity in estuarine systems over a wide range of salinities. Protective effects of salinity on acute Cu toxicity have been observed in the

estuarine copepod *Acartia tonsa* as a 1.8 fold increase in 48h EC_{50} values as salinities increased from 5 and 15ppt (Pinho et al., 2010). For sheepshead minnow (*Cyprinodon variegates*) a 4 fold increase in percent survival was observed as the salinity was increased from 2.5ppt to 18.5ppt and this was associated with a decrease in whole body Cu from 200 µg/g at 2.5ppt to 75 µg/g (Adeyemi et al., 2012). Pinho et al. (2010) showed a threefold increase in toxicity of Cu to *Acartia tonsa* as the 48h LC_{50} decreased from 110 ug/L at 30ppt to 30 µg/L at 5ppt. *Brachionus plicatilis*, a euryhaline rotifer, showed a salinity dependent increase in its 24h Cu LC50 (from 38.2 µg/L to 78.4 µg/L) moving across a salinity gradient from 6-29ppt (Arnold et al., 2010). Similarly acute dissolved Cu toxicity (96h LC_{50}) to *Callinectes sapidus (blue crab)* was higher at a salinity of 2ppt (5.3 µM Cu) than at 30ppt (53 µM) of Cu (Martins et al., 2011). EC_{50} values for egg production in *A. tonsa* after Cu exposure were 9.9, 36.8, and 48.8 mg/L (dissolved Cu) at salinities of 5, 15, and 30ppt, respectively (Lauer et al., 2010). A protective effect on Cu toxicity was also observed for mysid (*Neomysis integer)* as a decrease in Cu toxicity was observed at 25ppt as the LC_{50} doubled from 41 µg/L at 5ppt to 83 µg/L at 25ppt (Verslycke et al., 2003).

This protective effect of salinity on Cu toxicity, however, is not consistent across the salinity gradient that occurs in estuarine environments. While increased salinity reduces toxicity (see above) this only occurs up to a certain threshold after which protective effects can be reduced. Killifish for example were found to be very tolerant of Cu at intermediate salinities, with the highest EC_{50} value exhibited at 10ppt (EC_{50} of 1000 µg/L, Blanchard et al., 2006) and were most sensitive in both FW (EC_{50} of 18 µg/L) and full strength SW (EC_{50} of 294) (Grosell et al., 2007). Although Pinho et al. (2010)

found a significant change in the 48h EC_{50} of Cu to *A. tonsa* in salinities from 5- 15ppt (see above), EC_{50} was reduced when it was further increased to 30ppt. Acute Cu toxicity to *E. affinis* was reduced at 5ppt (104 µg/L) in comparison to 15ppt (67.6 µg/L) and 25ppt (58.1 µg/L; Hall et al., 2008). At a low range of salinity there appears to be a direct relation between EC_{50} and salinity however this protective effect of salinity plateaus as the organism reaches its iso-osmotic point (a point at which the external salinity of medium/water matches the internal salinity of the organism) and at salinities past the osmoregulatory threshold an increased in Cu sensitivity is observed (Blanchard et al. 2006; Grosell et al., 2007; Adeyemi et al., 2012). Cu toxicity in estuarine waters therefore may be dependent on the reduced uptake of metal associated with Na^+ and Cl^- effects and on the physiological capabilities of the organisms at different salinities.

1.3.3 Physiological Stresses vs Cu Stress

In estuarine waters, osmo-regulatory capabilities of euryhaline organisms may determine the extent of Cu toxicity. The mechanism of toxicity for Cu has now been widely established to be disruption of ion (Na^+) regulation, which leads to disruption of osmoregulation resulting in mortality (Grosell et al., 2007). At iso-osmotic, a salinity disruption of osmoregulatory capabilities may have minimal effects because internal and external Na^+ concentrations are similar and this was demonstrated as at an approximate iso-osmotic point of 10ppt, a decrease in Cu toxicity in killifish was observed (see above) (Grosell et al., 2007). In full strength sea water (37ppt), *Penaeus japonicas*, completely lost their ability to maintain internal Na^+ concentrations at Cu concentrations of 1000 and 1500 µg Cu/L while a 73% reduction in internal Na^+ was observed at low concentrations (500 µg Cu/L) (Bambang et al., 1995). When salinity was lower (17ppt) the same Cu

exposure concentration had a much smaller disruptive effect on Na^+ balance (Bambang et al., 1995). Reduced Cu toxicity was observed for sheepshead minnow at 10.5ppt, their iso-osmotic point and no net increase or decrease in whole body Na^+ levels was found (Adeyemi et al., 2012). Osmo-regulation stress was similarly measured for *A. bahia* without addition of Cu and increased survival was observed at salinities 23-25ppt as their iso-osmotic point is understood to be 25ppt as seen in Figure 1 (De Lisle et al., 1986, 1987).

Whereas a disturbance in ionoregulation is generally believed to occur as a result of Cu exposure in marine and euryhaline organisms, as confirmed by experiments conducted with rainbow trout (Wilson et al., 1993), gulf toadfish (Grosell et al., 2004) as well as seawater-adapted flounder (Stagg et al., 1982), it is not always found to be the main cause of mortality. An expected increase in plasma Na^+ levels in killifish was not observed, but instead a disturbance in ammonia excretion was found to be the primary cause of toxicity for Cu at low (120 µg/L Cu) concentrations in salt water (Blanchard et al., 2006). A general increase in the expression of Na^+/K^+-ATPase was observed in rainbow trout which counteracted the inhibition of the enzyme (Stagg et al., 1982). A lack of ionic disruption due to Cu toxicity has been shown to be a result of an increased expression of Na^+/K^+-ATPase as well as production of different isoforms for the enzyme that might not be inhibited through the same mechanism (Richards et al., 2003, Grosell et al., 2004). For cod, *Gadus morhua*, disturbances in acid-base balance as well as problems with ammonia excretion resulted in mortality (Larsen et al., 1997). Ammonia has been found to the main component of nitrogenous waste excretion for mysids and an increase

in ammonia elimination in mysids was observed as the Cu concentration and exposure time were increased (Garnacho et al., 2001).

1.3.4 Cu toxicity in relation to Dissolved Organic Matter (DOM)

In marine waters complexation of Cu with dissolved organic matter can further reduce Cu toxicity (Arnold, 2005). Arnold (2005) found that at 30ppt, DOM (measured as dissolved organic carbon ;DOC); provided a strong protective effect as an 8 fold increase was observed in EC_{50}s for *Mytilus galloprovincialis* (6 µg C/L at 0.6 mg C/L to 50.5 µg/L at 9 mg C/L). A decrease in toxicity for *M. galloprovincialis* was also observed by De Palma et al. (2011) when the DOC concentrations were increased from 0.8 mg C/L to 8.7 mg C/L. A 4.8 fold increase in measured EC_{50}s for *Mytilus sp.* was observed when DOC was increased from 2.9 mg/L to 8.6 mg/L (Arnold et al., 2006). Nadella et al. (2009) found a 4 fold increase in EC_{50} for Cu in seawater when 20 mg/L of DOC was added, changing the EC_{50} from 9.6 µg/L to 39 µg/L for *M. galloprovincialis*. While protective effects of DOM have been studied in marine conditions, a limited number of studies are available that test the potential changes in the protective effect of DOC over a range of salinities. In *Eurytemora affinis* Cu became less toxic as DOC concentrations increased from 2 mgC/l (EC_{50} of 76.2 µg/L) to 8 mgC/L (EC_{50} of 166 µg/L) at 10 ppt (Hall et al., 2008). For *Brachionus plicatilis* at 7ppt, an 8 fold increase was observed in LC_{50} at 4 mg C/L (393 µg Cu/L) in comparison to an LC_{50} of 43 µg Cu/L in 0.6 mg C/L at 6ppt (Arnold et al., 2010). Further research is required to build an understanding of the interactive effects of salinity and DOC in estuarine waters.

Dissolved organic matter concentrations can vary across the salinity gradient. An overall decrease in DOC concentrations is observed as salinity is increased. McCallister

et al. (2006) found that an increase in salinity from 0 to 20ppt decreased DOC concentration from 424 µM to 263 µM. Using carbon and nitrogen as characterizing parameters of DOM a net decrease was observed in C:N ratio as the salinity was increased going from the mouth of the estuary to the river (McCallister et al., 2006). Gerringa et al. (1998) found a net decrease in both dissolved Cu (13.8 nM to 7.6 nM) as well as DOC concentrations (378 µM to 155 µM) as salinity increased from 11ppt to 30ppt. In a study conducted on Cape Fear (North Carolina), estuarine transects can increase in salinity from 3.3ppt to a downstream salinity of 33ppt exhibited a 6 fold decrease in DOC concentration, 1286 µM to 214 µM (Shank et al., 2004). Samples collected at San Jose station in San Francisco Bay estuary at a 5ppt exhibited a DOC content of 5.5 mg C/L in comparison to Red Rock station for the same estuary where a DOC concentration of 1.5 mg C/L was measured at 25ppt (Ndung'u et al., 2003*).*

1.4 The Biotic Ligand Model

The Biotic Ligand Model (BLM) is a model that predicts the toxicity of a metal based on its bioavailability and uptake as determined via estimations of geochemical speciation (Di Toro et al., 2001). The BLM incorporates the biology (the biotic ligand) with the water chemistry (both inorganic and organic (i.e. DOM)) to determine the toxicity of metals in a variety of organisms (see Figure 2; Arnold et al., 2005; Paquin et al., 2000). It takes into account the competition for uptake among metal ions and cations, the complexation with anions as well as DOM to predict site-specific metal accumulation at the biotic ligand (Di Toro et al., 2001; Santore et al., 2001; Niyogi et al., 2004 Arnold

et al., 2010). The BLM has successfully been implemented for Cu and other metals in fresh water but marine and estuarine BLM models have yet to be established.

1.4.1 Cu speciation

The environmental and chemical factors influencing Cu speciation define its bioavailability and therefore its toxicity, at the site of toxic action (biotic ligand) in the organism (Georgopoulus et al., 2001; Arnold et al., 2005). Copper in water is not always available in its most toxic form, the free cupric ion (Cu^{2+}, Blanchard et al., 2006; Paquin et al., 2002; USEPA, 2007; Hall et al., 2008), because it interacts with inorganic anions as well as DOM (Arnold et al., 2010; USGS, 1997; CCME, 1997). Species such as OH^-, Cl^- , CO_3^{2-} as well as S^{2-} represent species that form complexes with Cu (Figure 3) and work as ligands rendering Cu less bio-available resulting in lowered toxicity (Flemming et al., 1989; Arnold et al., 2005; Arnold et al., 2010). Ions such as Mg^{2+}, Ca^{2+} and K^+ also interact with Cu but here acting as competitors to replace Cu as the ion being absorbed at the site of uptake and/or the site of toxic action, resulting in a lowered toxicity (Figure 2) (Allen et al., 1996; Arnold et al., 2005; Blanchard et al., 2006; Arnold et al., 2010: Pinho et al., 2010; Martins et al., 2011). Furthermore, Cu can also bind to DOM resulting in a limited concentration of available free copper ion (Cu^{2+}) in water and hence increased Cu concentrations are required to result in toxicity (Kramer et al., 2004; De Schamphelaere et al., 2004; Hall et al., 2008; Arnold et al., 2010; Erickson et al., 1996).

1.4.2 Dissolved Organic Matter

DOM can be produced through decomposition of plant and animal matter in the terrestrial system and is then transported and deposited into the surface waters or within

the water column. It consists of dissolved form of carbons, nitrates as well as acids (humic and fulvic) (Hansell et al., 2009). There are two types of organic matter (Figure 4), allochthonous (i.e., terrigenous) and autochthonous (i.e., plankton, photosynthetic organisms in water) (McCallister et al., 2006). The allochthonous component of DOM consists (50-90%) of humic and fulvic acids (Al-Reasi et al., 2011) and is the DOM produced in terrestrial environments, which is then transported to surface waters (Lozovick et al., 2005; Wood et al., 2011). Autochthonous DOM is composed of nitrogenous compounds as well as carbohydrates and is comprised of detritus from degradation of organism i.e. phytoplankton, bacteria, algae (Lozovick et al., 2005; Wood et al., 2011).

DOM in aquatic systems presents itself as the ligand to which free metal ions readily bind resulting in their decreased bioavailability and therefore reduced toxicity (Kramer et al., 2004; Arnold et al., 2005; Al-Reasi et al., 2011). DOM entering estuaries can be modified in several ways such as photolytic reactions, homogenization between different sources of DOM, production of autochthonous material as well as further degradation through UV (McCallister et al., 2006) and this may alter the quality of DOM and its capacity to protect against metal toxicity. Given the large variability in DOM sources and composition, it might be reasonable to assume that different sources of DOM might provide different degrees of protections. This variability in DOM composition can be analyzed using various optical techniques which will allow for better understanding of metal binding to DOM and therefore its availability and toxicity.

Optical Characterization of DOM can be accomplished to understand the components that have previously been shown to provide a protective effect (Hicks, 2009; De Shamphelaere et al., 2004). Absorbance at varying UV lengths can be used to characterize components of DOM and distinguish among DOMs from different sources (Murphy et al., 2008). Optical techniques including specific absorbance at 340 nm (aromaticity index) as well as a fluorescence excitation at 370 nm wavelengths have been successful in distinguishing DOM sources and composition (Al-Reasi et al., 2011). Relationships between fluroesence index (FI) and toxicity have also been demonstrated (McKnight et al., 2001; Brooks et al., 2007; Hicks, 2009) but this relationship is not considered to be a universal measure of DOM composition. Fluorescence excitation emission matrix spectroscopy (EEM) can also help distinguish DOM of a variety of composition and from a range of sources (Murphy et al., 2008). DOM fractions have the ability to fluoresce under UV and blue light and these EEMs can then be interpreted using multivariate analysis techniques such as parallel factor analysis (PARAFAC) to distinguish the components of a DOM source in contour plots (Stedmon et al., 2003, 2005).

1.5 *Americamysis bahia*

Invertebrates are commonly used as indicator organisms in toxicology. *A. Bahia* is a euryhaline organism from the order of mysida and has been established as an indicator organism by the USEPA for standardized acute and chronic toxicity testing (De Lisle et al., 1986; USEPA, 1990). When investigating contaminant impacts in estuarine conditions, organisms that tolerate a wide array of salinities and have the ability to iono-

regulate successfully are required. The optimal salinity for *A. bahia* is at 25ppt. The study of De Lisle et al. (1987) found that mysids have well defined osmoregulatory abilities and can rapidly adjust their haemolymph to hyper or hypo-osmotic condition within 95 minutes.

Mysids are crustaceans most commonly found in estuaries and marine waters all over the world (Verslycke, 2003). The size of *Americamysis bahia* is approximately 5 to 10 mm and they have a transparent body with a slight yellow tint (Lussier et al., 1988). Mysids do not have gills and obtain oxygen through the cuticle (Garnacho et al., 2001). They are omnivorous and in culture will feed on brine shrimp *Artemia* sp. Ongoing cultures require a sufficient dark period are required in culturing as reproduction occurs during this time (Lussier et al., 1988). The reason for selecting *A. bahia* in these studies is because they are easy to culture, have a short life span, abundant in estuaries around the world and they are intermediate in their sensitivity to metals (Figure 5) and they tolerate a wide range of salinities. These characteristics in addition to salinity tolerance makes them an ideal species to conduct both acute and chronic species for biological monitoring studies (Lussier et al., 1988; De Lisle et al., 1987).

1.6 Objectives

The overall aim of this project was to develop an understanding of how DOM and salinity influence the toxicity of Cu to *Americamysis bahia*. This project focused on 96h and 7-day toxicity tests to determine Cu toxicity. The results of this study will contribute to the development of an estuarine BLM for estimating Cu toxicity. The objectives were met through four studies, each with a specific goal:

1. Determine the effect of salinity on Cu toxicity (acute and chronic). This was achieved by conducting acute toxicity tests across a salinity gradient, ranging from 5ppt to 40ppt (Chapter 2) and 7-day short term chronic toxicity tests (Chapter 3).
2. Identify the physiological effects of salinity change in mysids in relation to Cu toxicity. This was carried out by culturing organisms at different salinities (e.g. 15 and 25ppt) and then conducting acute toxicity tests as in objective 1 for each of the cultures (Chapter 2).
3. Understand the influence of DOM on the toxicity of Cu (acute and chronic). Possible protective effects were investigated across different salinities.
4. Assess and characterize various sources of DOM to determine if there are site specific differences in DOM quality in relation to Cu toxicity. This characterization was carried out through optical and chemical analyses.

Hypotheses linked to each of the short term goals were as follows:

1. Salinity will have a protective effect on acute as well as chronic Cu toxicity however this protective effect will be lost at very low and high salinities (5 or 40ppt).
2. Acclimation to test salinities will lower Cu toxicity and prevent stress during salinity changes (Figure 1).
3. DOM will exhibit a protective effect on Cu toxicity and higher protective effect will be observed at lower salinities.
4. There will exist both optical as well as chemical differences in DOM from different collection sites and these will be reflected in differences in toxicity mitigation capacity.

1.7 Figures

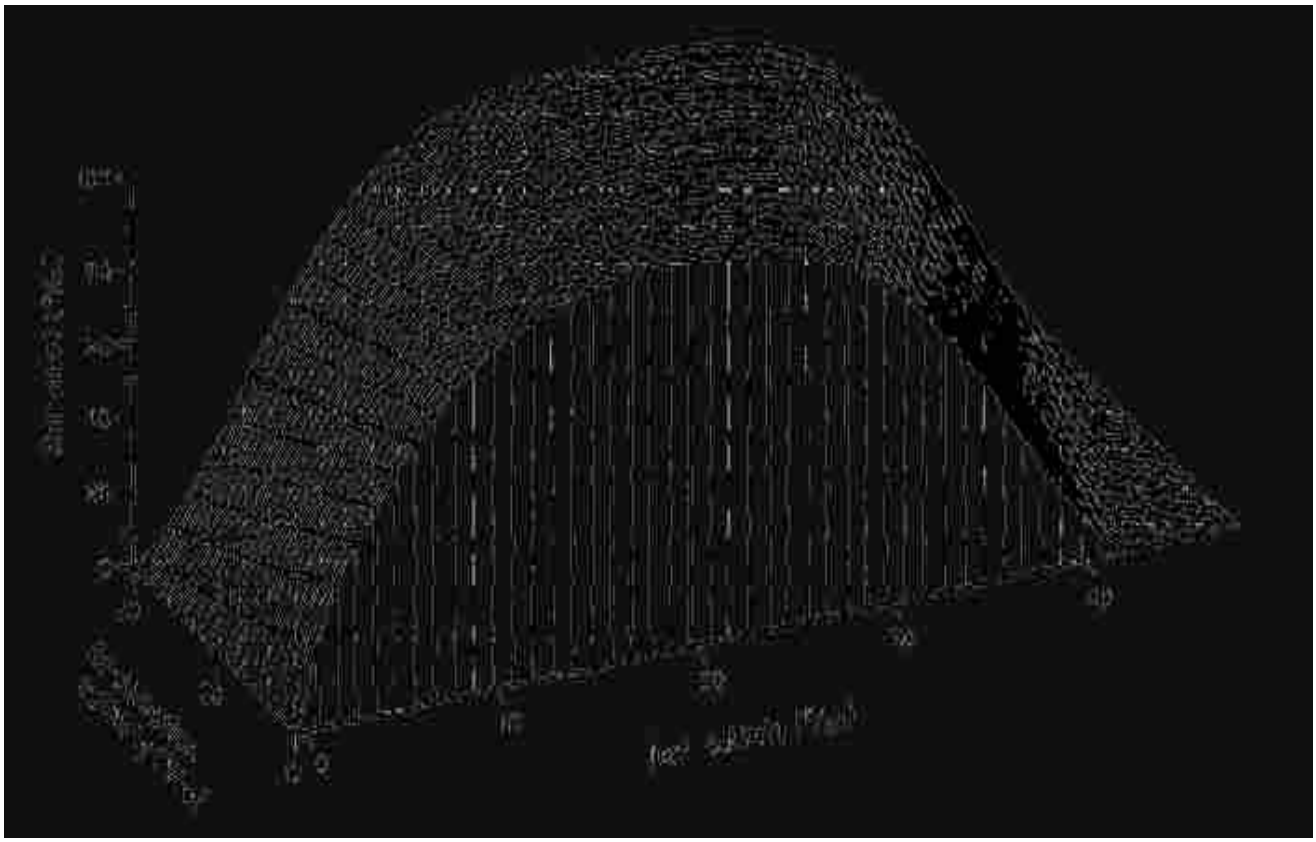

Figure 1: Fitted response of estimated percent survival at different salinities for *Americamysis bahia* (Figure 2; De Lisle et al., 1986). The survival increases as the salinity increases to peak around 22-25ppt as 25ppt is the iso-osmotic point for *A. bahia* and then decreases as the salinity threshold is crossed.

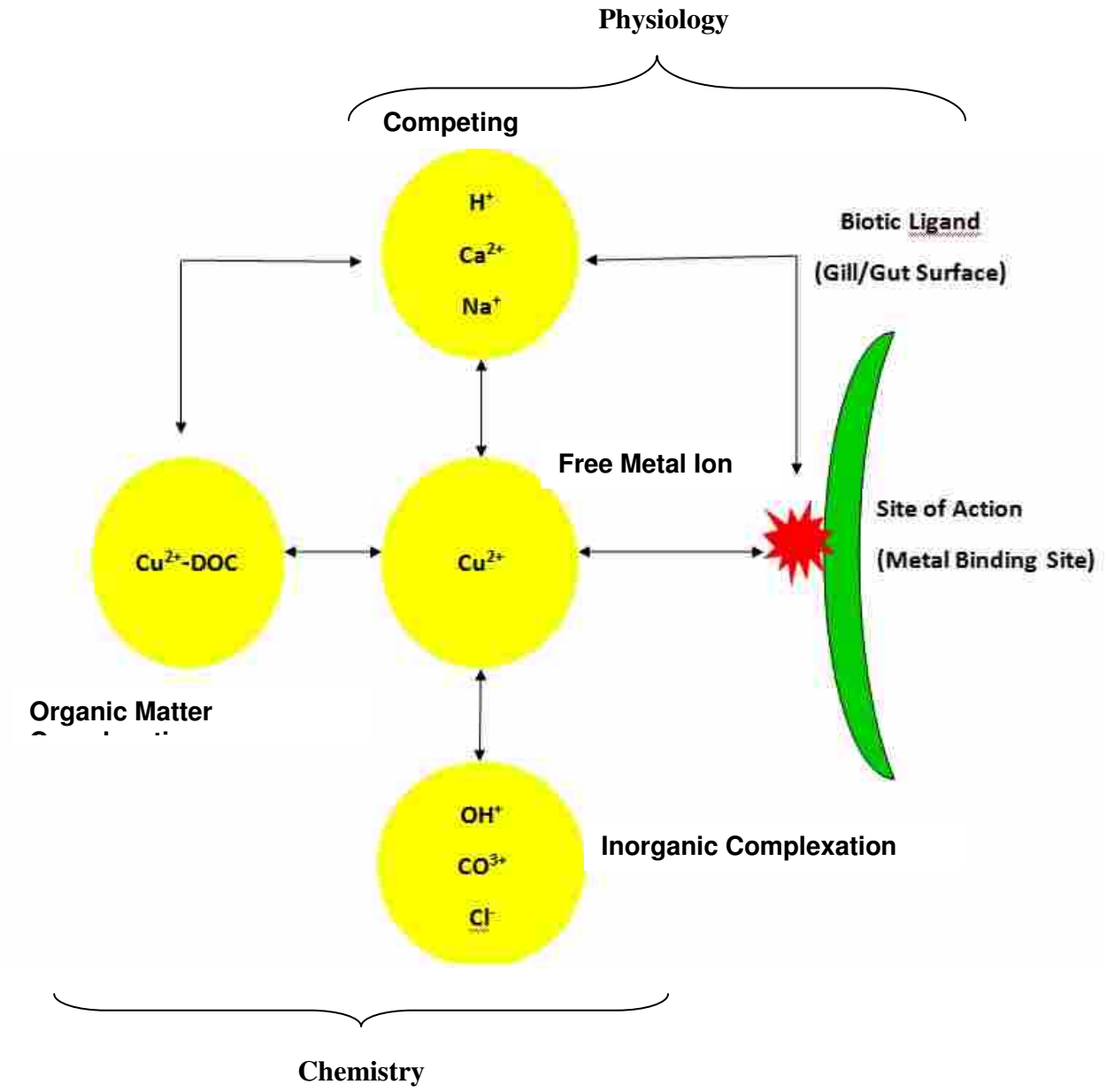

Figure 2: Schematic representation of the BLM framework for Cu speciation (Di Toro et al., 2001). Competing cations, organic and inorganic complexations reduce Cu^{2+} uptake and availability (respectively) therefore altering its interaction at the biotic ligand.

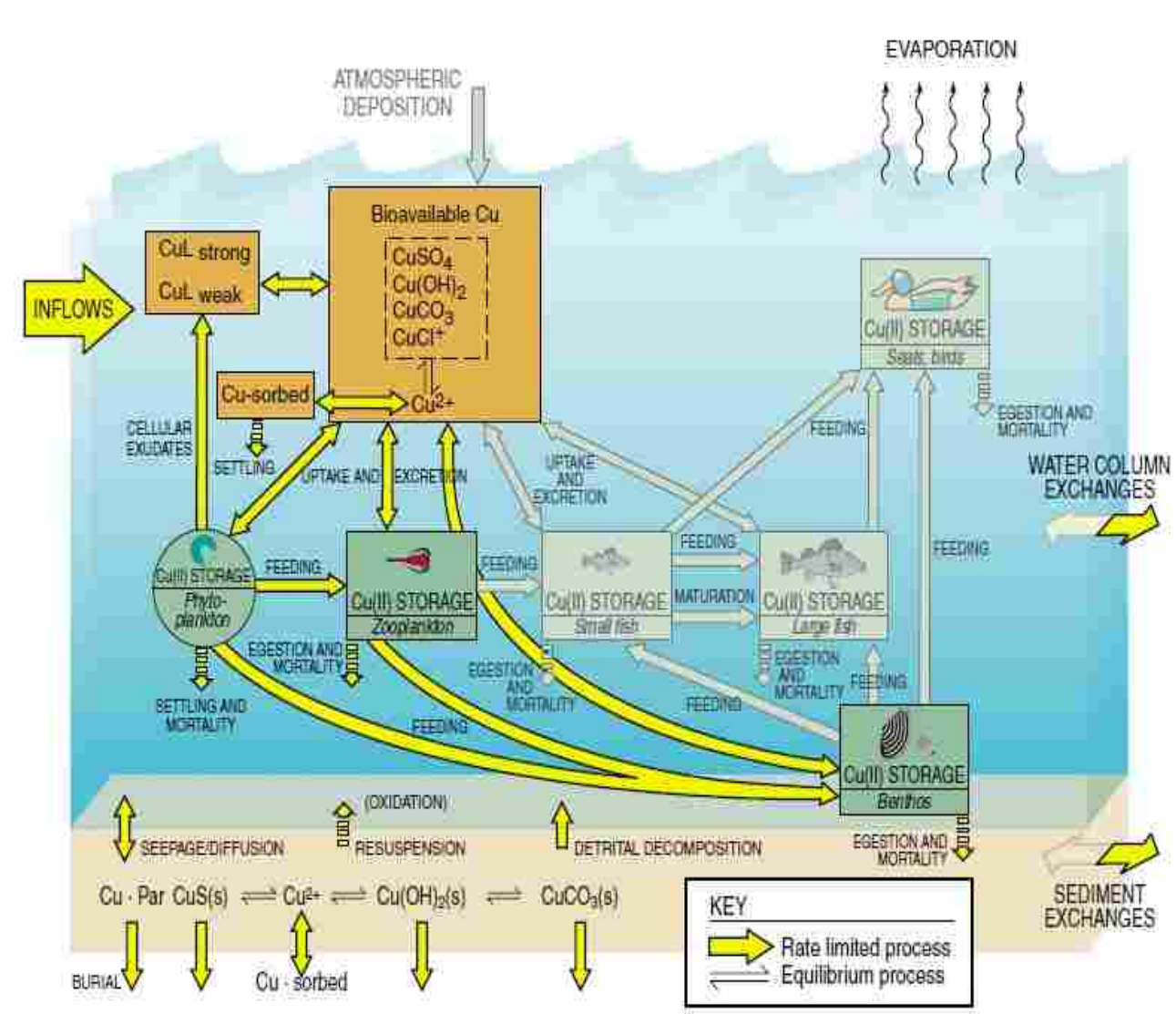

Figure 3: Schematic diagram showing Cu cycling in San Diego Bay as developed by Tetra Tech, 1999. The diagram shows different bioavailable forms of Cu that can be taken up biota as well as bio-accumulation through the food chain. The diagram illustrates abiotic cycling as well as the physiological mechanism through which Cu is cycled through organisms

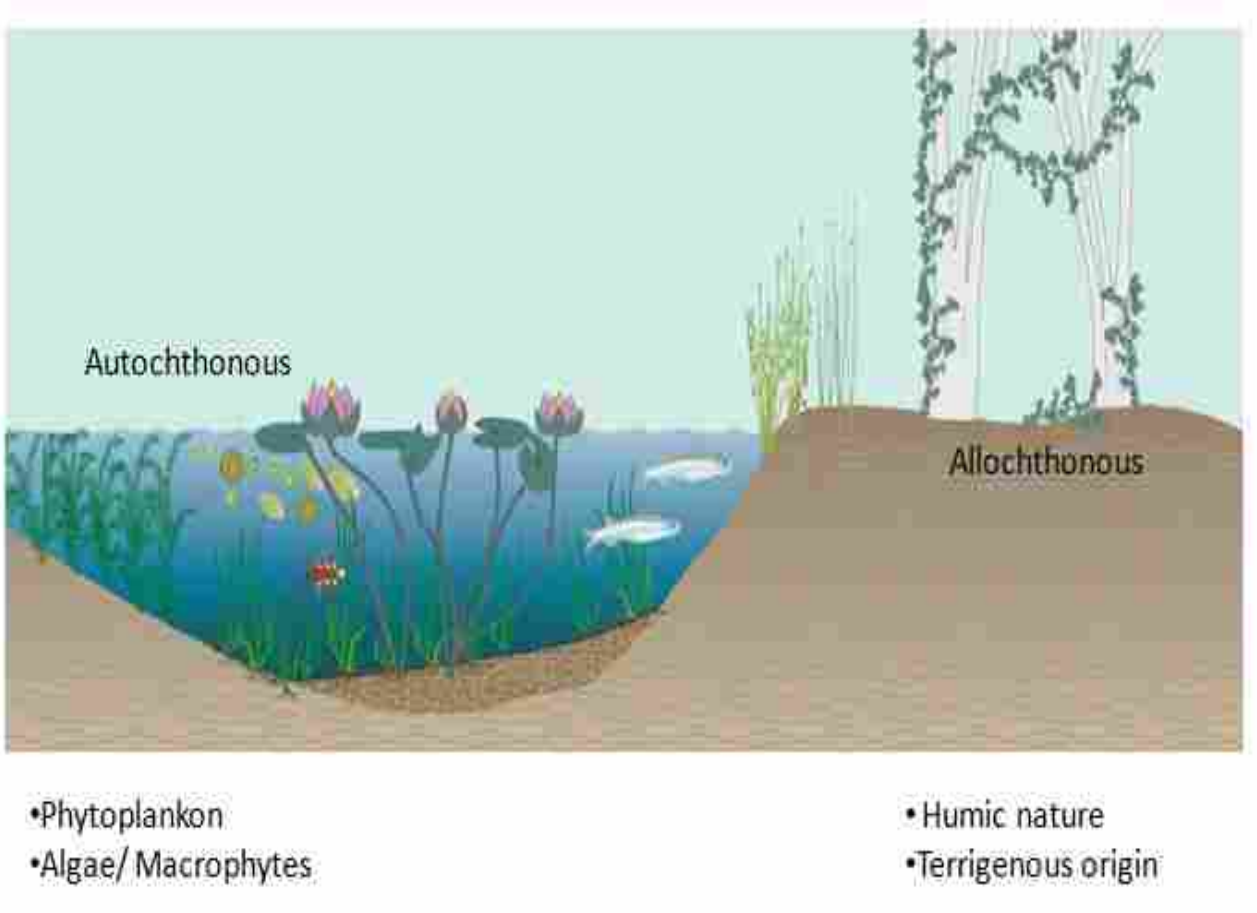

Figure 4: There are two different forms of DOM found in surface waters. Allochthonous DOM is humic in nature and of terrigenous origin whereas autochthonous DOM is produced within the water body itself (Wood et al., 2011; Lozovick et al., 2005). DOM concentrations are found to decrease in marine waters, but are usually highly variable in estuarine waters due to potential direct input from anthropogenic sources.

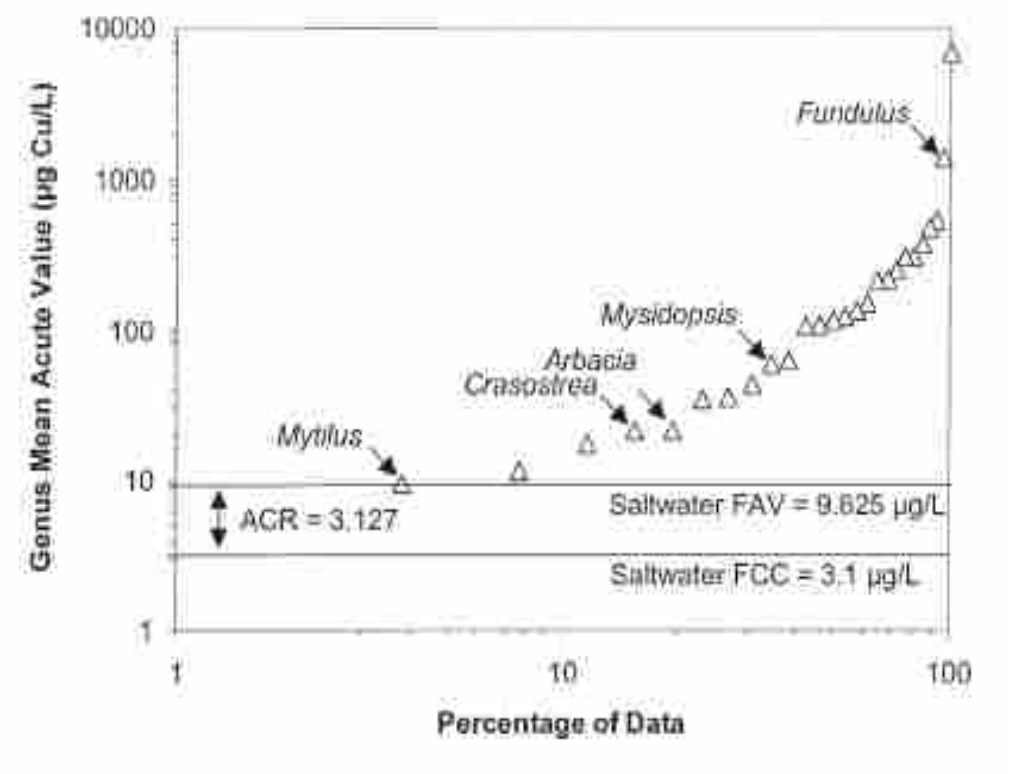

Figure 5: Species sensitivity distribution by USEPA with saltwater copper criteria genus mean acute values. The plot shows species most sensitive (*Mytilus*), intermediate (*Mysidospis)* and tolerant (*Fundulus*) to Copper (Arnold, 2005).

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Chapter 2

Acute Toxicity of Cu to *Americamysis bahia:* Mitigating Effects of Salinity and Dissolved Organic Matter

2.1 Introduction

Cu is an essential element, actively taken up and regulated in all organisms (Uauy et al., 1998; Gaetke et al., 2003), however increased ambient levels in surface waters can result in toxicity to aquatic biota (Bambang et al., 1995; Pinho et al., 2010; Martins et al., 2011; Lauer et al., 2010; Bianchini et al., 2004). Cu is present in all aquatic systems as a result of both natural and anthropogenic activities (Canadian Council of Ministers of the Environment, 1999; United States Environmental Protection Agency, 2007). Estuarine environments are of specific interest as they are the hub of anthropogenic activity and as a result are exposed to various contaminants. Cu concentrations have particularly been shown to increase in the aquatic system as a result of extensive use of anti-fouling paints. Furthermore, the geochemistry of these environments complicates the speciation of Cu and understanding of its toxicity.

The environmental and chemical factors influencing Cu speciation define its bioavailability therefore its toxicity at the biotic ligand in an organism (Georgopoulus et al., 2001; Arnold et al., 2005). In aquatic systems the most toxic form of Cu is the free cupric ion Cu^{2+} (Blanchard et al., 2006; Paquin et al., 2002; USEPA, 2007; Hall et al., 2008). Cu^{2+} not only reacts at the biotic ligand but also interacts with inorganic/organic anions and as a result becomes less bioavailable (Flemming et al., 1989; Arnold et al., 2010; Allen et al., 1996; Blanchard et al., 2006). Anions such as OH^-, HCO_3^-, NH_3 and Cl^- act as competitors actively binding to Cu while limiting the concentration of bio-available Cu^{2+} and increase the dissolved concentration of Cu (as total) required to result in toxicity (Kramer et al., 2004; De Schamphelaere et al., 2004; Hall et al., 2008; Arnold

et al., 2010; Erickson et al., 1996, USGS, 1997; CCME, 1997). Similarly cations like Ca^{2+}, H^+ and Na^+ compete with the free metal for uptake at the biotic ligand thereby reducing toxicity (Paquin et al., 2000; Blanchard et al., 2006; Flemming et al., 1989).

Increasing salinity and hence the Na^+ and Cl^- concentration had been shown to result in decreased Cu toxicity (Arnold et al., 2010, Mochida et al., 2006 and Bambang et al., 1995, Pinho et al., 2010, Lussier et al., 1985 and Ho et al., 1998). Protective effects of salinity on acute Cu toxicity have been observed in the estuarine copepod *Acartia tonsa* as a 1.8-fold increase in 48h EC_{50} values as salinities increased from 5 and 15ppt (Pinho et al., 2010) and *Neomysis integer* as a decrease in Cu toxicity was observed at 25ppt as the LC_{50} doubled from 41µg/L at 5ppt to 83 µg/L at 25ppt (Verslycke et al., 2003). For sheepshead minnow (*Cyprinodon variegates*) a 4-fold increase in percent survival was observed as the salinity was increased from 2.5ppt to 18.5ppt and this was associated with a decrease in whole body Cu from 200 µg/g at 2.5ppt to 75 µg/g (Adeyemi et al., 2012). Similar protective effects were observed in *Callinectes sapidus* (Martins et al., 2011), *Acartia tonsa* (Lauer et al., 2010) and *Branchinous plicatilis* (Cooper et al., 2014). This protective effect of salinity, however, is not consistent across the salinity gradient that occurs in estuarine environments.

Estuarine environments range in salinity from 1ppt up to full strength sea water and this changing salinity can have a dramatic effect on Cu toxicity. The mechanism of toxicity for Cu has now been widely established to be disruption of ion (Na^+) regulation, which leads to disruption of osmoregulation resulting in mortality (Grosell et al., 2007). In estuarine waters, osmo-regulatory capabilities of euryhaline organisms therefore may also determine the extent of Cu toxicity. Once a certain threshold of salinity is reached by

an organism the protective effects have been shown to be reduced (Blanchard et al., 2006, Grosell et al., 2007, Pinho et al., 2010 and Hall et al., 2008). At low range of salinity there appears to be a direct relation between EC_{50} and salinity (Grosell et al., 2007) however this protective effect of salinity plateaus as the organism reaches its iso-osmotic point and at salinities past the osmoregulatory threshold an increased in Cu sensitivity is observed (Blanchard et al. 2006; Grosell et al., 2007; Adeymi et al.,2012). In killifish at the iso-osmotic salinity, disruption of osmoregulation capabilities will have minimal effects because internal and external Na concentrations are similar (Grosell et al., 2007). The inability of the organism to regulate internal Na, Cl concentrations therefore results in death of the organism, past the osmoregulatory threshold (Bambang et al., 1995, Adeyemi et al., 2012, Grosell et al., 2007 and De Lisle et al., 1986, 1987). Cu toxicity in estuarine waters may therefore be dependent on the reduced uptake of metal associated with Na^+ and Cl^- effects and on the physiological capabilities of the organisms at different salinities.

In marine waters complexation of Cu with dissolved organic matter can further reduce Cu toxicity (Arnold, 2005; De Palma et al., 2011; Arnold et al., 2006; Nadella et al., 2009). A limited number of studies, such as Hall et al. (2008) and Arnold et al. (2010), have tested the potential changes in the protective effect of DOC over a range of salinities. DOM entering estuaries can be modified in several ways such as photolytic reactions (Amon and Benner, 1996), production of autochthonous material (Raymond and Bauer, 2001) as well as further degradation and homogenization of DOM from different sources (McCallister et al., 2006). This may alter the quality of DOM and its capacity to protect against metal toxicity. Characterizing techniques such as absorbance

at 340 nm as well as Fluorescence excitation emission matrix spectroscopy (EEM) can also help distinguish DOM of a variety of composition and from a range of sources (Murphy et al., 2008; Al-Reasi et al., 2011). Furthermore, dissolved organic matter concentrations can vary across the salinity gradient. An overall decrease in DOC concentrations is observed as salinity is increased (McCallister et al., 2006, Gerringa et al., 1998, Shank et al., 2004 and Ndung'u et al., 2003). Currently DOM is used as a homogenous entity in toxicity predictions and is assumed to have the same Cu-complexation characteristics, regardless of source and composition however, different source and quality differences of DOM have been reported to provide unique site-specific protection from metal toxicity (Ryan et al., 2004; McKnight et al., 1983; De Schemphelaere et al., 2004; Shwartz et al., 2004; Brooks et al., 2007). Further research is required to build an understanding of the interactive effects of salinity and DOM (both source and quality) in estuarine waters.

The USEPA publishes ambient water quality criteria for both fresh and marine conditions. The fresh water criteria are based on application of the BLM (USEPA, 2007). The Biotic Ligand Model is a model that predicts the toxicity of a metal based on its bioavailability and geochemical speciation (Di Toro et al., 2001). The BLM incorporates the biology (the biotic ligand) with the water chemistry (both inorganic and organic (i.e. DOM)) to determine the toxicity of metals in a variety of organisms (Arnold et al., 2005; Paquin et al., 2000). A marine BLM has been proposed but at this time single values for criteria are still used.

Water quality guidelines and criteria for estuarine conditions are often generated using both fresh water and marine guidelines. For example, the CCME freshwater

guideline is applied to waters up to 1ppt salinity and no marine and estuarine guideline is currently available. USEPA criteria defines marine waters as having salinity higher than 5ppt and the saltwater acute dissolved copper criterion is 4.8 µg/L, while chronic value is defined as 3.1 µg/L (USEPA, 2007). Therefore there appears to be little recognition of the variability in toxicity responses for estuarine conditions and this introduces uncertainty in toxicity prediction and setting WQ guidelines/criteria. Increasing salinity and other changes in water chemistry as fresh water flows into salt water will alter the toxicity of metals and therefore neither fresh water criteria/guidelines nor ones for full strength sea water assess the potential for impacts in estuarine conditions. The lack of estuarine specific criteria/guidelines that account for a wide range of salinities represents a gap in the understanding of the potential environmental impacts of metals. At least in part, the lack of water quality guidelines and criteria for estuarine waters results from the fact that toxicity prediction model are lacking. Models such as the BLM have been successfully applied to establish site specific thresholds for Cu in fresh waters (EPA 2007) and are being developed for marine waters (Aquatic Life Ambient Freshwater Quality Criteria—Copper 2007 Revision).

The purpose of this study was to build an improved understanding of the acute toxicity of Cu in estuarine conditions. The toxicity mitigating effects of both salinity and DOM (source and concentration) were studied using *Americamysis bahia. A bahia* is a euryhaline invertebrate organism from the order of mysida and has been established as an indicator organism by the USEPA for standardized acute and chronic toxicity testing (De Lisle et al., 1986; USEPA, 1990). They are tolerant of a wide array of salinities and have the ability to iono-regulate successfully (Lussier et al., 1988; De Lisle et al., 1987). The

data developed through this research will contribute to broader interdisciplinary efforts to develop prediction models for application to environmental protection of estuarine ecosystems.

2.2 Methodology

2.2.1 Americamysis bahia culturing

Culturing of mysids was carried out following Standard EPA Methods (EPA-505/8-90-006b) using organisms purchased from a commercial supplier (Aquatic Research Organisms, Hampton, New Hampshire, USA) and held in 10 L aquaria with synthetic (reconstituted) water at 25°C. Cultures were establish at salinities, either 15 or 25ppt, made by reconsituting sea salts (Kent Marine Reef Salt Mix, Big Als Canada Inc, Kitchener ON). Culture water was prepared in 400 L batches and aged over at least 5 days before use. Salinity and temperature were monitored daily using a salinity meter (YSI 30, YSI Inc., Yellow Springs, OH). A 16:8 (light:dark) photoperiod was used and freshly hatched *Artemia nauplii* (Brine Shrimp Direct, Ogden, UT) were fed at a rate of 150 per *A. bahia* per day. Culture water was renewed (80%) daily at which time neonates were collected to maintain broodstock and for subsequent testing.

2.2.2 DOM Collection

DOM collections were carried out in November of 2011 and May of 2012 at coastal locations in New Brunswick and Prince Edward Island Canada (Table 1). Collection was by reverse osmosis concentration (see below), which can only be done in fresh water, and to provide maximum similarity to natural DOC in estuaries collection sites were selected from sources with no anthropogenic influences upstream that flowed directly into salt water. As much as possible collection sites were in close proximity to an

estuary. All DOM sources were collected and stored following the methods described by Schwartz et al. (2004) with 200 to 500 L reduced to approx. 8 L of concentrate using a custom built portable reverse osmosis system. DOM concentrates were resinated to pH of 2 using a cationic exchange resin to remove all residual metals and cations and stored at 4°C (Schwartz et al., 2004).

2.2.3 A. bahia Toxicity Tests

96 h toxicity tests were carried out to assess the acute toxicity of Cu. Synthetic sea water (Kent Marine Reef Salt Mix) used for mysid cultures was also used for all test solutions to maintain consistency in test medium. All tests were carried out following USEPA Standard Test Methods (EPA 712-C-96-136). Tests were static renewal in nature and were carried out at a temperature of 25^{o}C ± 0.5. The tests were carried out in 350 mL crystallizing dishes (Pyrex, Fisher Scientific, Ottawa, ON, Canada) with 250 mL of test solution. Test solutions were prepared to allow for a 48 hour test solution renewal using synthetic Kent sea salt mixed with appropriate AAS standard Cu stock solution (TraceCERT, Sigma-Aldrich Co., Oakville, ON, Canada). All test solutions were prepared 24h prior to the start of the test to allow the solution to reach equilibrium. pH, salinity and temperature were recorded at the beginning and end of each test. The tests were done in duplicate with 10 neonates per replicate and included unexposed controls. The end point for all 96h acute toxicity tests was chosen to be mortality. Water samples were collected at 0, 48 and 96 hours in scintillation vials. All dissolved Cu samples were collected using a 0.45µM Acrodisc (Pall Co.) syringe filter (VWR, Missisauga, ON, Canada) in falcon tubes. Mysids were fed *Artemia nauplii* daily for the duration of all

testing and dead artemia and debris were removed daily as well as during the 48h solution renewal.

Effects of Acclimation

The test acclimation salinities were chosen to be 5, 15, 25 and 35ppt, however due to a lack of reproduction at 5 and 35ppt all cultures were acclimated to 15 and 25ppt for the duration of all testing. The neonates were collected from both in lab cultures and used in testing. Individuals from all salinities were tested at salinities of 5, 15 and 25ppt. The tests were 96h acute toxicity tests and were carried out using standard EPA test methods (listed above). The salinity was monitored carefully throughout the tests for accuracy of any salinity effects on toxicity testing. The results for each salinity were compared to those of other acclimation salinities to examine if acclimation had an effect on metal (Cu) toxicity.

Effect of Salinity on Cu

The chosen test salinities were 5, 10, 15, 20, 25, 30 and 40ppt to allow for a complete understanding of how salinity affects Cu toxicity in estuaries. AAS standards (TraceCERT, Sigma-Aldrich Co., Oakville, ON, Canada) for Cu (1g/L) were used to make standard and test solutions. Cu test concentrations were chosen to be 0, 50, 100, 200, 400, 600, and 800 µg/L. 2 L of each test solution was made using a volumetric flask and the remainder of the solution after start of the test was stored in nalgene bottles for 48h test solution renewals. Salinity and temperature were measured at 24h intervals and recorded. Any mortality was recorded at both 48 and 96h intervals and dead individuals were removed. The tests were done in duplicate and water samples from all replicates

were collected at 0, 48 and 96h intervals. All collected samples were stored in the fridge until further analysis.

Effects of DOC

For DOM 96 h acute toxicity tests the DOC concentrations tested to determine any protective effect of DOC ranged from 2 to 10mg/L. The effect of DOC concentration was tested at 15, 25 and 35ppt while salinities of 15 and 25ppt were used to test site-specific differences in DOC. DOC was measured on a TOC analyzer to determine the concentration of the collected concentrate. Diluted samples of the concentrates were then obtained to get the required concentration. A Cu negative control was used, with no added metal to the salt water as well as DOC positive control where the DOC concentration being tested was added without any input of metal to see any toxic effects of DOC alone. Cu concentrations used ranged from 100 µg/L to approximately 2 mg/L. The mysids were fed throughout the duration of the 96h acute toxicity test and the debris was removed during the 24h test solution renewal. Dissolved and total Cu water samples as well as DOC samples for each concentration were collected at 0, 48 and 96h intervals.

2.2.4 Statistical Analysis and measurements

LC 50

Lethal Concentrations 50 (LC_{50}) values were calculated using mortality data representative of each acute test. The LC_{50}s as well as the 95% confidence limits for all tests were determined using Spearman Karber method using a commercial software package (CETIS; Comprehensive Environmental Toxicity Information System™). The generated data was plotted using SigmaPlot™ (ver.11) to determine dose response curve and mortality trends. Data was considered significant if the 95% confidence intervals did

not overlap; if the confidence intervals overlapped significance was determined using Litchfield-Wilcoxon statistical analysis method (Environment Canada, 2005).

Cu Measurements and Analysis

Water samples taken at 0h, 48h and 96h were measured to determine total and dissolved Cu concentrations. Since most equipment does not provide an accurate measurement of Cu in salt water an extraction process is required to eliminate the effect of salt on Cu readings. Water samples were prepared using lanthanum oxide precipitation (Nandella et al., 2009; Toyota at ela., 1982). 10 mL of each sample was mixed with 10 µL of Lanthanum oxide and Sodium carbonate. The pH for all samples was adjusted to 9.8 using sodium hydroxide. The samples were then placed in a water bath for 30 min and subsequently centrifuged for 15 min. The supernatant was discarded and the precipitate re-suspended in 1N nitric acid. The samples were then vortexed and subsequently measured by flame atomic absorption spectrometry (SpectraAA 880 with GTA100, Varian Inc., Palo Alto, CA).

DOC measurements and characterization

50 mL filtered DOC samples were taken from the test solutions prior to the start and end of the tests for DOC analysis. The samples were taken from controls as well as each test concentration. DOC concentrations were measured using a total carbon analyzer (TOC) analyzer (Shimadzu TOC-$L_{CPH/CPN}$, Shimadzu Corporation, Kyoto Japan). TOC standards at 5 and 10 mg C/L concentrations were used as reference and were prepared using a 1g/L stock of potassium hydrogen phthalate which was added to artificial sea water. All the samples and standards were brought to room temperature, transferred to

TOC vials and spiked with 2-3 drops of concentrated Hydrochloric acid (Sigma-Aldrich, Oakville, ON, Canada).

Optical characterization of DOC was carried out by EEMS using a fluorescence spectrometer (Cary Eclipse, Varian, Victoria, Australia). The samples were measured using a quartz cuvette (Hellman Canada Ltd., Concord Canada). The measurements were done using the excitation wavelength range of 200-450 nm with 10 nm increments while the emission was measured from 250- 600 nm. The generated data was then analyzed using PARAFAC analysis as implemented in MATLAB^{TM} to distinguish any differences between sources.

Calculations and Statistics

EEMS was used to conduct optical characterization of the DOM sources. The generated data was sub sequentially analyzed using PARAFAC analysis through Eigenvector Research Inc. PLS toolbox (The MathWorks, MA, USA) to derive and quantify the relative amounts of the four major fluorescent components, humic acid-like, fulvic acid-like, tryptophan and tyrosine, to understand any quantitative DOM characteristics (De Palma et al, 2011). The concentrations for all components were added together to allow for the calculation of the relative percent of each component found within each source of DOM using in house MATLAB scripts. MATLAB^{TM} was also used to create two-dimensional contour plots to visualize any fluorescence differences between the sources, after removal of any Rayleigh-Tyndall scattering. Both the quantitative and qualitative data were then used to form any linkages between biological toxicity (LC_{50}s) and chemical and optical properties of DOM.

DOM quality was further assessed by measuring the absorbance of each source at 340 nm and 370 nm in order to examine the aromaticity (darkly colored DOM) and origin of DOM (terrigenous and autochthonous) consecutively of each source. Absorbance measurement at 340 nm for each DOM source was converted into specific absorption coefficient values (SAC_{340}) as described by Schwartz et al (2004) as per Equation 2.1:

$$SAC_{340} = [2303 \times (Abs_{340})]/DOC) \qquad \text{Equation 2.1}$$

Where Abs_{340} is the absorbance at 340 nm and DOC is the measured DOC concentrations (mg C/L). The FI was also calculated according to Equation 2:

$$FI = (EI_{450} / EI_{500}) \text{ at } 370 \text{ nm} \qquad \text{Equation 2.2}$$

Where EI is the emission intensity at either 450 or 500nm after excitation at a wavelength of 370 nm.

The EEMS data was evaluated using PARAFAC analysis through MATLAB PLS toolbox (The MathWorks, MA, USA) to derive and quantify the relative amounts of the four major fluorescent components, humic acid-like, fulvic acid-like, tryptophan and tyrosine (Al-Reasi et al., 2011; De Palma et al. 2011; Stedmon et al., 2003, 2005). The concentrations of these components within each sample were determined using the resolved component concentrations from the daily standards. MATLAB™ was also used to create two-dimensional contour plots to visualize presence of different fluorophores in the DOC samples to distinguish between the sources.

Optical characteristic (SAC340, FI and 4 fluorophores) were then correlated to 96h LC50 values to determine any links between DOM protective capabilities and

toxicity (Ryan et al., 2004). Correlation coefficients (r) were determined by the Pearson product moment method (n=4) and significance was determined at $p<0.05$.

2.3 Results

Toxicity tests met the EPA standard method for a valid test. Acute Cu toxicity tests at each salinity were repeated 3 times over the course of this study and demonstrated that *A. bahia* were relatively consistent in their response, the difference between LC50 values from all replicates for each test was around 15%.

Samples collected, from each replicate of each concentration before during and after all tests, were subsequently measured for both total and dissolved Cu concentration. In general nominal values were close to measured values. Dissolved Cu concentrations were 80 % of total Cu (n = 528). The measured DOC concentration in test solutions without added DOM was 0.8 ± 0.5 mg C/L. The reported LC_{50} values were calculated from measured dissolved Cu concentrations.

2.3.1 Effect of salinity on acute Cu toxicity

The LC_{50} values varied significantly over the salinity range tested (5-40ppt) with a protective effect observed up to 30ppt (Figure 1). There was a significant increase in LC_{50} values from 5 to 15ppt and while there was a continuing trend for reduced toxicity up to 25ppt it was not significant. This protective effect was lost as the salinity continued to increase to 35ppt. Tests were also conducted at 40 ppt and inconsistent results were observed as majority (5 out of 6) of the test failed to meet the acceptability criteria due to high mortalities in controls and therefore an LC_{50} value could not be calculated.

In the second test series *A.bahia* neonates from cultures acclimated to either 15 or 25ppt were tested at salinities of 5, 15 and 25ppt to investigate whether culturing salinity influenced responses. The protective effect of salinity was observed with significant increases in LC_{50} values between 5 and 15ppt (Fig. 2) but there was little difference between acclimation salinities (Fig. 2).

2.3.2 Effect of DOM Cu Toxicity

DOM provided a variable site dependent protective effect (Figure 3). In tests at 25ppt the DOM from Rankin Brook provided the highest protection while Cape Enrage provided no protection compared to test with no added DOM (Figure 3). The sources from Northlake and Kelly's Bog provided an intermediate mitigation of acute Cu toxicity. At 15ppt the variation among sources was less distinct however Kelly's Bog and Rankin Brook provided a significant protective effect compared to tests with no added DOM (Figure 3). Significant protection was provided by some DOM sources (at 15 and 25ppt with 4 mg C/L) compared to controls with no added DOC, whereas minimal significant differences (Northlake vs. Rankin Brook at 25ppt) in LC_{50} values were found among the sources at either salinity. A final comparison was conducted to determine any differences among the 4 DOM sources. At 25ppt, Cape Enrage was determined to provide a significantly different protection from Kelly's Bog as well as Rankin Brook while Northlake was determined to be significantly different from Rankin Brook. No difference in protection were found between the three DOM sources at 15ppt.

2.3.3 Optical Characterization of DOM

EEMS data analysis through PARAFAC was used to derive humic acid-like, fulvic acid-like, tryptophan-like and tyrosine-like fluorophores (Table 2). These four components accounted for 97% of the data. Sources had very similar fluorescence profiles and there was very little variation among sources (Table 2). Two-dimensional contour plots were created using EMMS data to visualize the fluorophores and determine any site-specific differences (Figure 6). SAC_{340} and fluorescence index (FI) values were calculated for the 4 DOM sources. Cape Enrage had the lowest SAC_{340} value at 19.5 (higher SAC_{340} values signify lighter DOM) while Kelly's bog has the highest. Northlake and Rankin Brook provided median values of FI, Cape Enrage the highest and Kelly's Bog provided the lowest FI at 1.06 .

2.3.4 Correlation of LC50 values to DOM Optical Characteristics

Correlation coefficients (calculated using Pearson Product Moment) for all variables tested were used to determine any relationships between toxicity and protective capacity of DOM. A positive correlation (r = 0.78, 0.86; 15, 25ppt) was found between the SAC_{340} (Figure 4) and the calculated 96h LC_{50} values. Similarly, a negative correlation was found (r = 0.75, 0.75; 15, 25ppt) between FI and the LC_{50} values (Figure 5). The four optically derived components (humic acid, fulvic acid, tryptophan and tyrosine) were also analyzed to determine any correlation to toxicity. At 25ppt no correlation was found between humic and fulvic acid contents, however a negative correlation was found for both tryptophan (r = 0.66) and tyrosine (r = 0.75) with the 96h LC_{50} values. At 15ppt, a positive correlation was found between fulvic-acid content (r =

0.95) for acute toxicity while negative correlations (r= 0.95) were found for tryptophan and humic-acid.

2.4 Discussion

The toxicity of Cu can be influenced by salinity as a result of complexation and the presence of competing cations (Allen et al., 1996; Arnold et al., 2005; Blanchard et al., 2006; Arnold et al., 2010: Pinho et al., 2010; Martins et al., 2011). In estuarine waters, osmo-regulatory capabilities of euryhaline organisms may determine the extent of Cu toxicity. The mechanism of toxicity for Cu has now been widely established to be disruption of ion (Na^+) regulation, which leads to disruption of osmoregulation resulting in mortality (Grosell et al., 2007). *A. bahia* is an osmoregulator and is able to adjust to salinity changes within 95 min of transfer (De Lisle et al., 1987). It is therefore the model organism to test toxicity of contaminants in estuarine environments, where such salinity changes are common. Tests were conducted at 3 different salinities: 5, 15 and 25ppt to determine if culturing acclimation salinity had any effect on Cu toxicity at different test salinities. Neonates cultured at both 15 and 25ppt were directly transferred to the required test salinity at the start of each test and 96h LC_{50}s were recorded. The results were unable to reject the null hypothesis as the results showed that acclimation salinity had no significant difference on Cu toxicity (Figure 1). The neonates were able to successfully adjust to abrupt salinity changes and therefore no differences were seen in toxicity of Cu whether the neonates were cultured at 15 or 25ppt. LC_{50} values, however, showed a linear protective effect of salinity as salinity was increased from 5 ppt to 25ppt. The results found in this study were in agreement with the literature where the authors showed

that acclimation salinity has minimal effect on Cu toxicity at intermediate salinities (10.5-18.5ppt), however, a strong effect is observed at either extremes of the salinity tolerance limit of the sheepshead minnow (Adeyemi et al., 2012). Culturing acclimation of 5ppt and 35ppt was also conducted, however, due to low survival and poor reproduction functioning mysid cultures were not possible and therefore no neonates were derived to conduct further testing.

Salinity provided a protective effect on acute Cu toxicity as it was increased from 5ppt to 30ppt (Figure 2). This protective effect has been associated with a decreased bioavailability of Cu^{2+} as a result of increased competition due to Na^{+} ions at the site of toxic actions as well as binding to Cl^{-} ions as the salinity increases (Arnold et al., 2005 and 2010 and Blanchard et al., 2006). Similar protective effects of salinity on acute Cu toxicity have also been observed in the estuarine copepod *Acartia tonsa* as a 1.8 fold increase in 48h EC_{50} values as salinities increased from 5 and 15ppt (Pinho et al., 2010). For sheepshead minnow (*Cyprinodon variegates*) a 4 fold increase in percent survival was observed as the salinity was increased from 2.5ppt to 18.5ppt and this was associated with a decrease in whole body Cu from 200 µg/g at 2.5 ppt to 75 µg/g (Adeyemi et al., 2012). *Brachionus plicatilis* showed a salinity dependent increase in its 24 h Cu LC_{50} (from 38.2 µg/L to 78.4 µg/L) moving across a salinity gradient from 6-29ppt (Arnold et al., 2010). Similarly acute dissolved copper toxicity to *Callinectes sapidus* was higher at a salinity of 2ppt (5.3 µM Cu) than at 30ppt (53 µM) of Cu (Martins et al., 2011). Increased protective effect was observed for salinities between 15 to 25ppt which is considered to be the optimal range of salinity for *A. bahia.*

As *A. bahia* exhibited sensitivity to Cu at both high as well as low salinities (Figure 2), our results agreed with previous literature (Grosell et al., 2007; Pinho et al., 2010). At an approximate iso-osmotic point of 10ppt a decrease in Cu toxicity in killifish was observed as there was minimal disruption of osmoregulation because internal and external Na concentrations were similar (Grosell et al., 2007). When salinity was lower (17ppt) and closer to the iso-osmotic point the same Cu exposure concentration had a much smaller disruptive effect on Na balance due to minimal osmoregulation required for survival by the organism (Bambang et al., 1995). Reduced Cu toxicity was also observed for sheepshead minnow at 10.5ppt, their iso-osmotic point and no net increase or decrease in whole body Na levels was found (Adeyemi et al., 2012). However, this protective effect of salinity was lost at salinity extremes (5, 10, 35 and 40ppt) as *A.bahia* exhibited increased sensitivity to Cu which was attributed to the inability of the organism to maintain its homeostasis and death resulted due to osmoregulatory failure (Figure 2). A similar trend has been shown in the literature for several different species and mortality has been linked to osmoregulatory stress in addition to contaminant which ultimately collapses the regulatory system. In full strength sea water (37ppt), *Penaeus japonicas*, completely lost their ability to maintain internal Na concentrations at Cu concentrations of 1000 and 1500 µg Cu/L while a 73% reduction in internal Na was observed at low concentrations (500 µg Cu/L) (Bambang et al., 1995). Pinho et al. (2010) also found a significant change in the 48h EC_{50} of Cu to *A. tonsa* in salinities from 5-15ppt (see above), EC_{50} was reduced when it was further increased to 30ppt. Acute Cu toxicity to *E. Affinis* was reduced at 5ppt (104 µg/L) in comparison to 15ppt (67.6 µg/L) and 25ppt (58.1 µg/L, Hall et al., 2008).

Dissolved organic matter acts as a modifying agent in aqueous systems by binding free metal ions, generally considered to be the most toxic form of metal to biota (De Schamphelaere et al., 2004). DOM composition varies between sites and this variability may result in differences in its protective capacity, subsequently influencing Cu toxicity. DOM sources were strategically collected from various sources of fresh water that flowed right into salt water; to have environmentally relevant conditions and composition of DOM. Currently in toxicity predictions models, DOM is used as a single homogenous value, however several studies have shown site-specific protective capacities of DOM (See above). A protective effect was observed for three of the four DOM sources at a concentration of 4 mg C/L (Fig. 6). Up to a 2 fold difference in LC_{50} was observed for Cu as DOM concentrations increased from 0-4 mg C/L at 25ppt (Fig. 4). No salinity differences in protection were observed, however, Kelly's bog and Rankin Brook DOM were determined to be more protective than other sources. Site-specific protective effect of DOM have been demonstrated in freshwater for rainbow trout (Richards et al., 2001; Schwartz et al., 2004), Daphnia (De Schamphelaere et al., 2004; Glover et al., 2005) and fathead minnows (Ryan et al., 2004, Sciera et al., 2004 ; VanGenderen et al., 2003), although there are very little data available to show such source dependent protection of DOM in estuarine and sea water. Nadella et al. (2009) showed a DOM based site specific difference in toxicity of Cu, Zn, Ni and Cd, when freshwater DOM sources (similar to this study) were used to determine toxicity at different salinities, however the results from the present study were equivocal. This study helps further the understanding of source dependent differences in protective capacity of DOM. All tests were conducted at 4 mg C/L concentrations at either 15 or 25ppt, further tests incorporating a matrix of different

salinities, concentrations and sources will be extremely beneficial in better understanding DOM interactions in toxicity mitigation of all metals in estuarine and marine environments.

DOM samples were also optically characterized to determine any source/composition dependent differences, similar characterization has previously been done to show relationships between DOM quality/source and toxicity (Al-Reasi et al., 2011, De Palma et al., 2011; Hicks, 2009; De Shamphelaere et al., 2004). SAC_{340} values were measured for all four DOM sources (Table 2) as well as FI. The FI exhibited a negative correlation ($r = -0.8324$) to LC_{50} values, the lower the FI the higher the binding capacity of Cu which in turn results in a higher protective capacity, however this relationship was not significant. Similar relationships between FI and toxicity have been demonstrated (McKnight et al., 2001; Brooks et al., 2007; Hicks, 2009) but this relationship is not considered to be a universal measure of DOM composition. A positive correlation was observed between toxicity and SAC_{340} values ($r = 0.8614$). SAC_{340} is a measure of the color and the light is usually absorbed by the aromatic components of the DOM. Darker DOM sources (Kelly's Bog) provided a higher SAC_{340} value as well as a higher LC_{50}. The results from this research show some support of SAC340 a very good indicator of protective capacity of different DOM sources but were not significant.

PARAFAC analysis was performed on all the samples and 97 % the data was summed up in to four fluorophores (HA-like, FA-like, Tyr and Trp). All sources exhibited a high humic acid-like content but very low tryptophan and tyrosine content. Correlation coefficients were calculated to determine any relationships between protective capabilities of DOM and measured toxicity. Negative correlations were found

between tryptophan and tyrosine concentrations and the calculated LC_{50}s for 25ppt, however no correlation was observed for 15ppt. No differences in fluorescence of the four fluorophores was determined when 2-D contour plots were created. No correlations were found between humic and fulvic acid content and toxicity differences for various DOM sources. Previously, differences in the concentrations of the four fluorophores, particularly humic acid-like substances, have been associated to differences in protection derived from each unique source (Schwartz et al., 2004; Al-Reasi et al., 2012; Ryan et al., 2004), however this was not found in our study.

2.5 Conclusion

The results from this study show that Cu toxicity varies considerably across the salinity gradient found in estuaries. Salinity acclimation prior to testing had no effect on Cu toxicity within the salinity tolerance limit of *A. bahia.* Cu toxicity decreased with increasing salinity up to 30 ppt. *A. bahia* was found to be more sensitive to Cu at higher and lower salinities and that was attributed to the organism reaching a critical osmoregulatory threshold. This threshold is essentially defined as the salinity at which the internal and external ion concentration is equivalent, resulting in minimal requirement for ionoregulation which reduces Cu toxicity. Salinity change below or above this osmotic threshold result in increased physiological stress in addition to Cu induced result and the inability to maintain an ionic balance by the organism results in toxicity. DOM was determined to provide a protective effect but no significant site-specific difference could be found as some sources imparted greater protective effect than others and the results were variable. This protective capacity of DOM was not always salinity

dependent. The optical characteristics of DOM (SAC_{340} and FI) were correlated with test LC_{50} values but the relationships were not significant and could not be deemed accurate predictors of DOM capacity in estuarine systems.

Overall, as salinity increases the geochemical speciation of Cu is altered as a result of organic/inorganic complexation and is further challenged due to the wide array of osmoregulatory capabilities of euryhaline organisms. This chemical-biological interaction complicates the understanding of the impacts of Cu in estuarine waters. More research is required to better understand metal toxicity in estuarine systems to move towards developing an estuarine Biotic Ligand Model.

Acknowledgements

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2.6 Figures

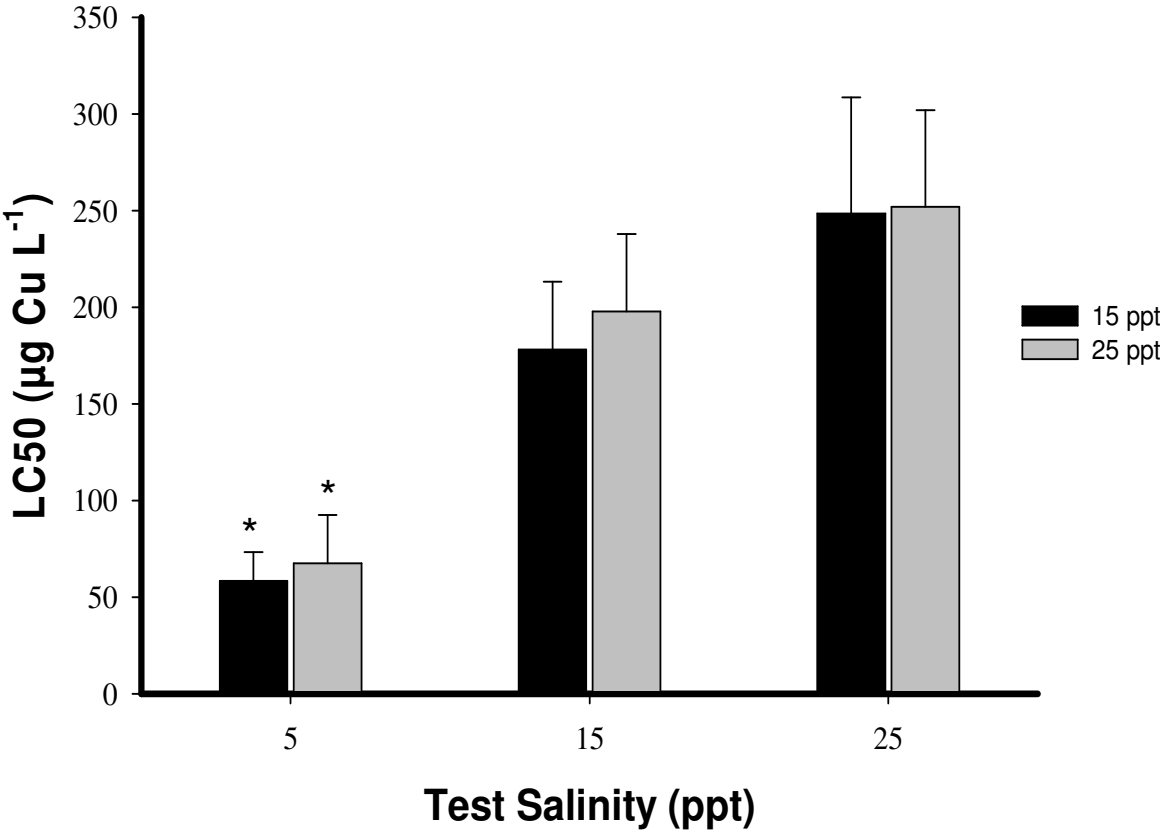


Figure 1: Comparison of acute Cu LC_{50} values for test salinities of 5, 15 and 25ppt in mysids acclimated to 15 or 25ppt. A * represents a significant difference from 25ppt.

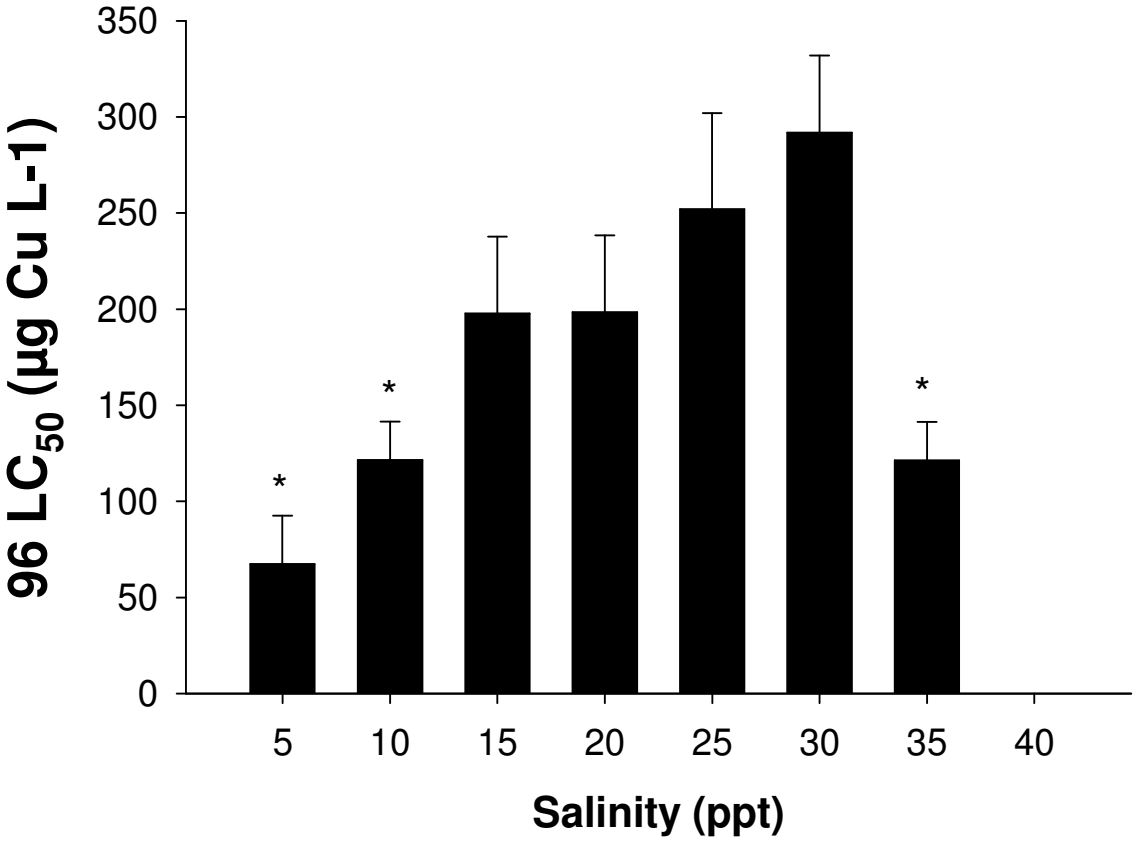


Figure 2: The 96 h LC_{50} values (µg Cu/L) for *A. bahia* tested at different salinities. The error bars show the upper 95% confidence limit, toxicity values are given for measured dissolved Cu and * indicates a significant difference in other LC_{50} values than that calculated for 25 ppt.

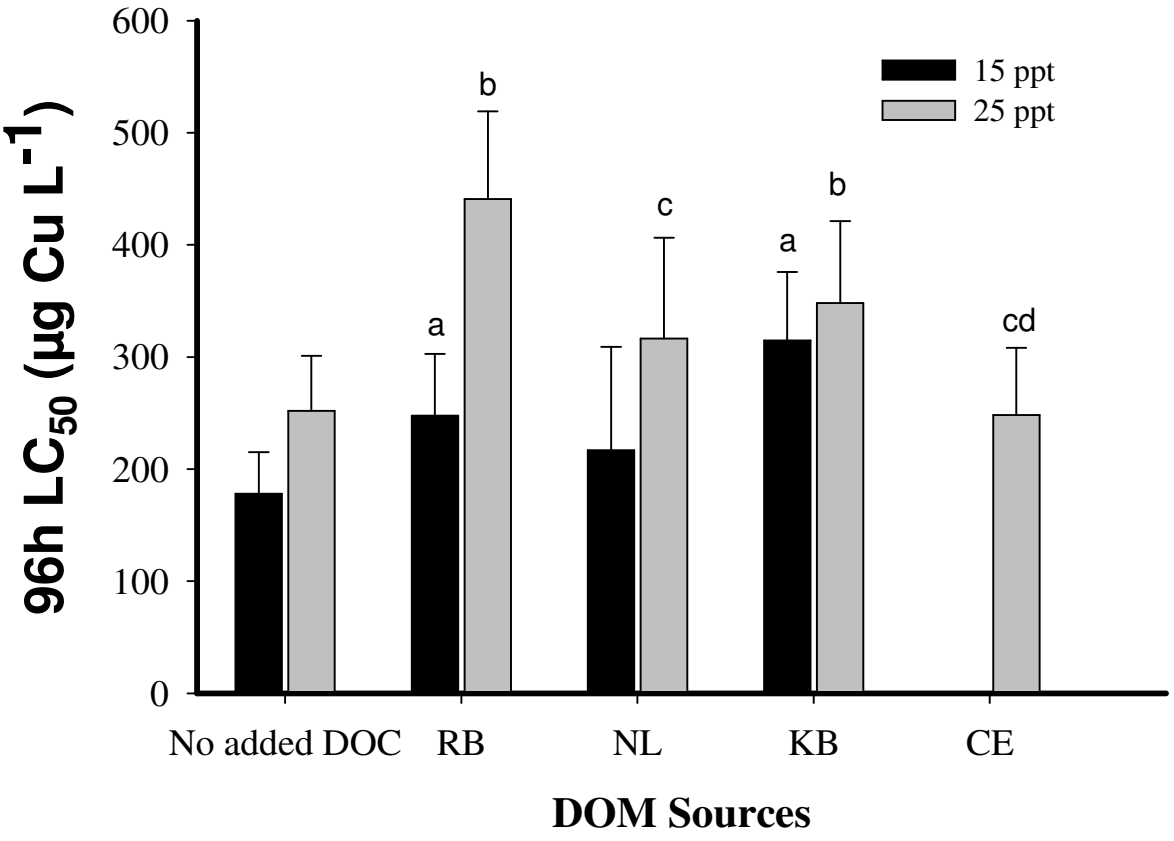


Figure 3: Site-specific differences of DOM in 96h acute Cu toxicity to *A. bahia* at 15 and 25ppt with a DOC concentration of 4 mg C/L. Rankin Brook and Kelly's Bog DOM provided a significant protective effect from Cu toxicity at 15 ("a" significant difference from no added DOC) and 25ppt ("b" significant difference from no added DOC). Northlake was significantly different from Rankin Brook ("c") while Cape Enrage was different from both Rankin Brook and Kelly's Bog ("cd")

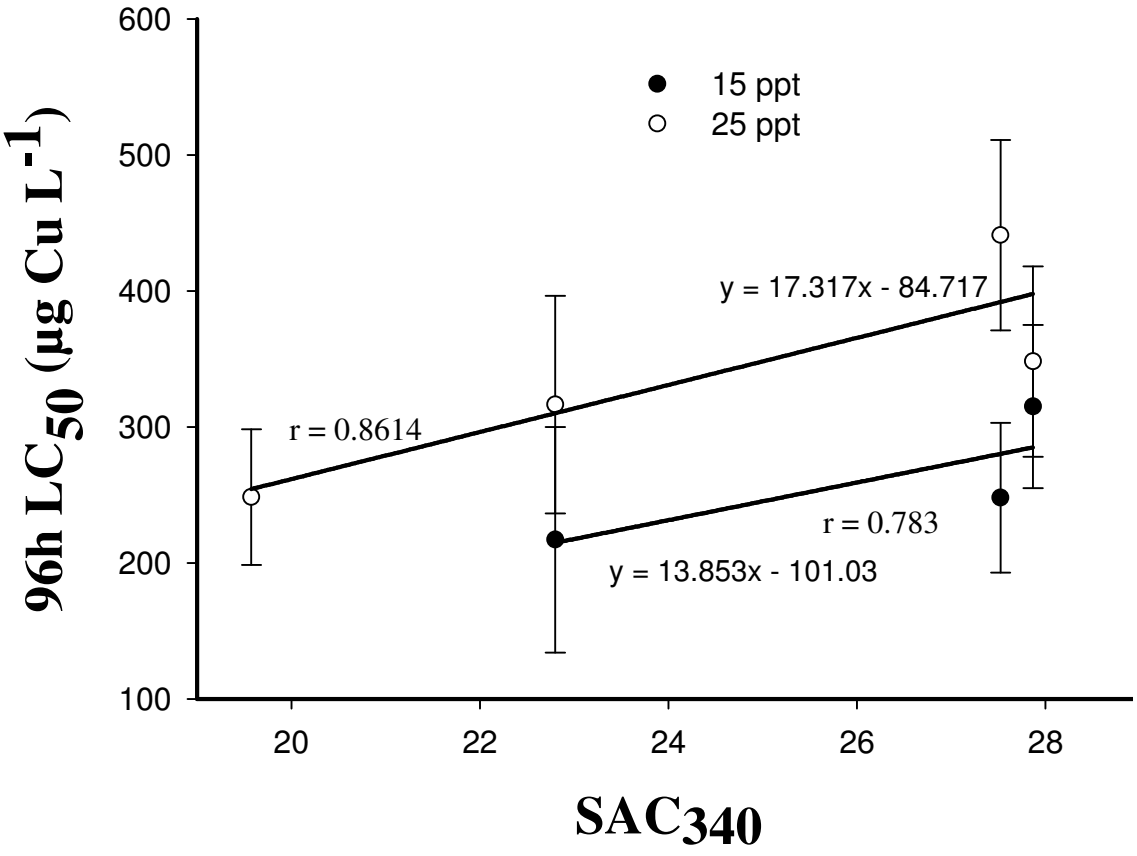


Figure 4: Linear correlation of 96 h LC_{50} values for various DOM sources at 25ppt (white fill) and 15ppt (black fill) in increasing order of protective capability with 4 mg DOC/L added and specific absorption coefficient (SAC) at 340 nm for DOM sources.

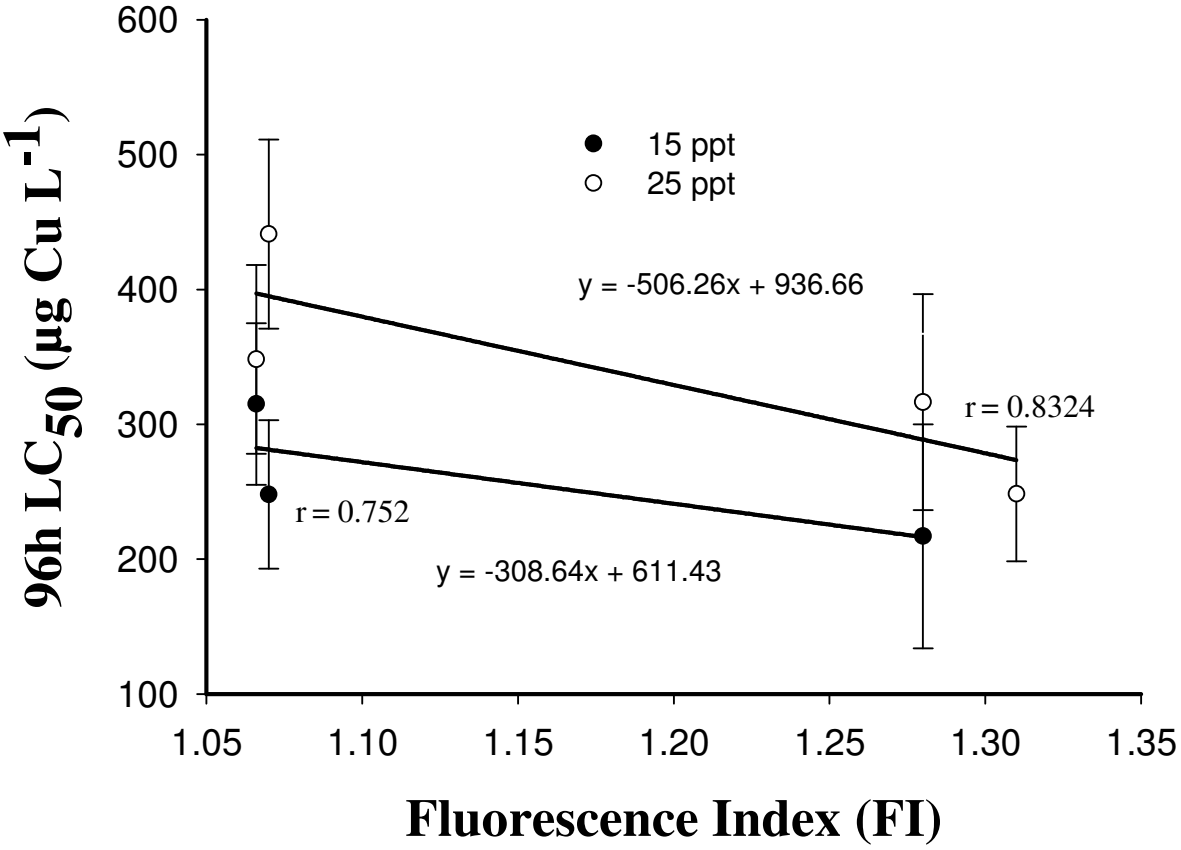
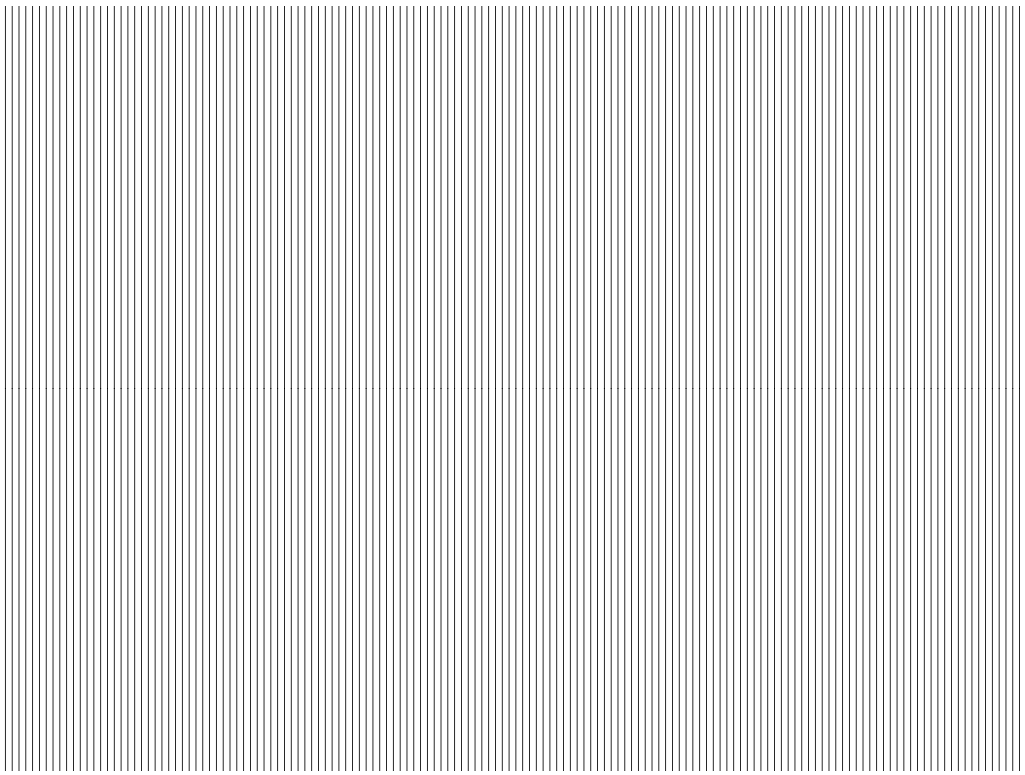
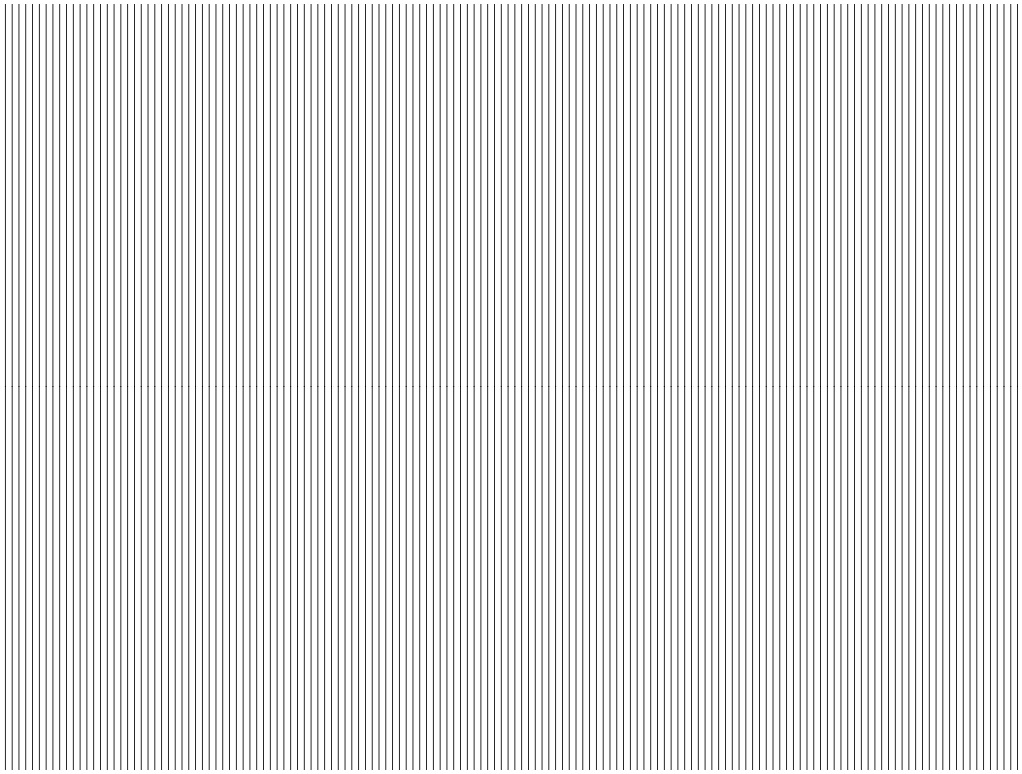


Figure 5: Negative correlation of 96h LC_{50} values for various DOM sources in decreasing order of protective capability with 4 mg DOC/L added at 25ppt (white fills) and 15ppt (black fills) and fluorescence index (FI) at excitation wavelength of 370 nm for DOM sources.



(A)



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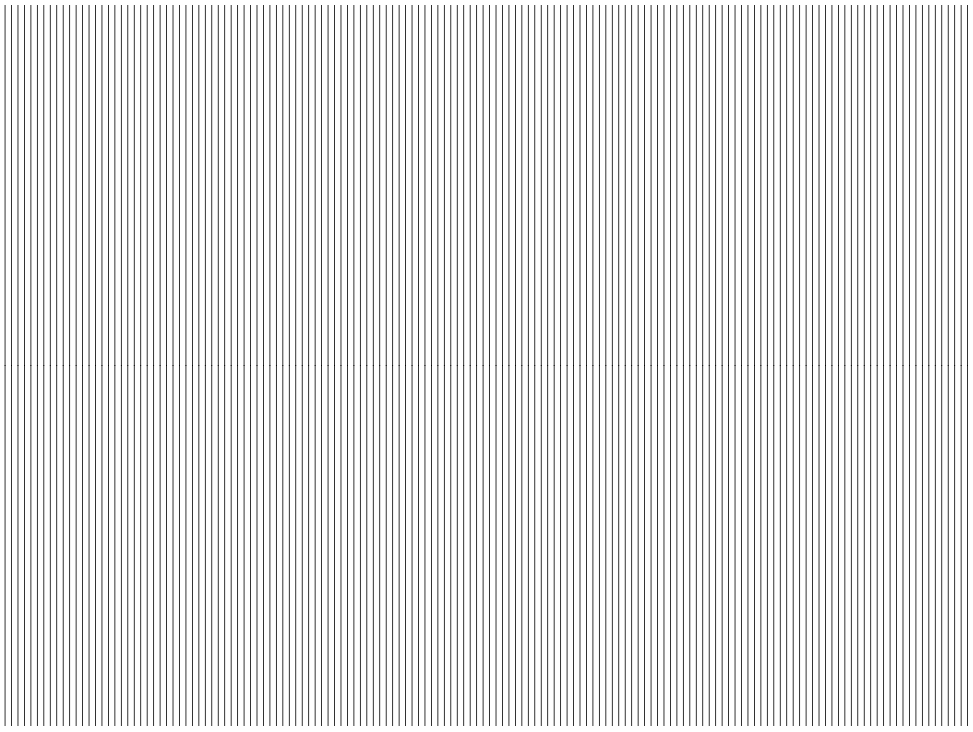

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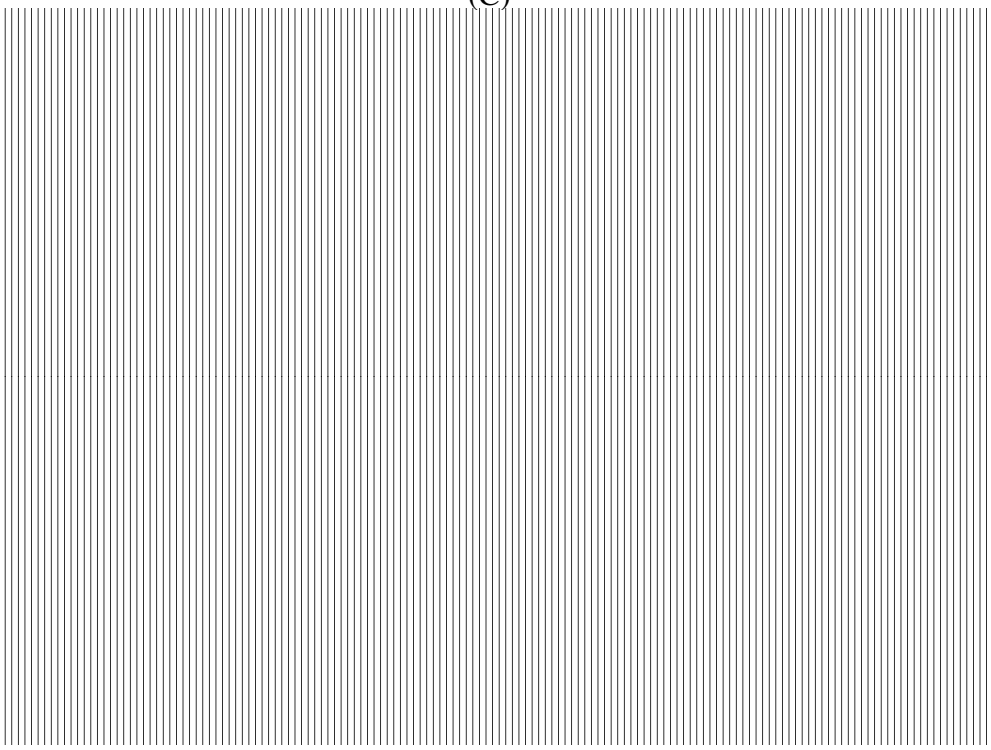

(D)

Figure 6: Fluorescence excitation-emission contour plots for A) Cape Enrage, (B) North Lake, (C) Kelly's Bog and (D) Rankin Brook.

2.7 Tables

Table 1: Sampling location information for the collected DOM sources.

Source name	Location	Date	Coordinates	Additional site details
Cape Enrage (CE)	Cape Enrage Road, Cape Enrage, NB	Nov. 2011	45° 37' 26" -64° 47' 13"	Un-named creek draining into salt marsh.
Northlake (NL)	Northside Road, Northlake, PEI	2011	46° 27' 30" -62° 05' 50"	From freshwater lense at upper end of North Lake
Kelly's Bog (KB)	Kouchibouguac National Park, NB	May 2012	46° 48' 53" -64° 54' 58"	un-named stream draining Kelly's Bog
Rankin Brook (RB)	NB highway 117, Kouchibouguac National Park, NB	May 2012	46° 49' 13" -64° 55' 5"	downstream of Rankin Bog

Table 2: Correlation between 96 h LC_{50}s and optical characteristics of 4 DOM sources. Four components of the DOM sources were determined by PARAFAC analysis. %HA is humic acid component, %FA is fulvic acid, %trp tryptophan, and %tyr is the tyrosine component. These four components account for 97% of data variability.

DOM Source	LC_{50} (µg/L)		% HA	% FA	% trp	% tyr	SAC_{340}	FI
	15 (ppt)	25 (ppt)						
Cape Enrage	N/A	248	72	23	2	0.1	19.6	1.31
North Lake	217	316	73	23	1.5	0	22.8	1.28
Kelly's Bog	315	348	70	27	1.3	0	27.9	1.07
Rankin Brook	248	441	73	23	1.5	0	27.5	1.07
Correlation Coefficient	15ppt		-0.95*	0.95*	-0.95*	N/A	0.78	-0.75
	25ppt		0.18	0.08	0.66	0.75	0.86	-0.83

Table 3: Lethal concentration 50 for 96h acute toxicity tests over a wide range of estuarine salinities. LC_{50} values and CI were calculated using the Trimmed Spearman-Karber method. LC_{50}s are given as µg Cu/L and are based on measured dissolved (0.45 µM) Cu concentrations.

Test Salinity (ppt)	**Measured Salinity (ppt)**	**Temperature (^{o}C)**	**pH**	**96h LC_{50}**	**96h CI**
5	5.0 ± 0.2	24.8 ± 0.3	7.8 ± 0.2	67.46	45.8-99.4
10	10.0 ± 0.3	25.1 ± 0.2	7.9 ± 0.1	121.6	101-146.4
15	15.0 ±0.5	24.9 ± 0.2	8 ± 0.0	197.8	160.8-243.2
20	20.0 ± 0.1	25.0 ± 0.2	8.0 ± 0.1	198.4	162.3-242.7
25	25.0 ± 0.5	25.0 ± 0.1	8.2 ± 0.4	252	210.7-301.3
30	30.0 ± 0.3	24.9 ± 0.2	8.0 ± 0.3	291.9	247.6-344.3
35	35.0 ± 0.3	25.2 ± 0.3	7.9 ± 0.2	121.3	102.9-142.9
40	40.0 ± 0.4	25.0 ± 0.2	8.0 ± 0.1	CND	CND

*CND = could not be determined; CI = Confidence Intervals

Table 4 – Water chemistry parameters at culturing salinities of 15 and 25ppt. Effect of culturing salinity on Cu toxicity was tested at three different test estuarine salinities (5, 15 and 25ppt). Test exposure concentrations are given as measured dissolved Cu concentration

Culture Salinity	15ppt					25ppt				
Test Salinity (ppt)	**Measured Salinity (ppt)**	**T (^{o}C)**	**pH**	**Nominal Cu (µg/L)**	**Dissolved Cu (µg/L)**	**Measured Salinity (ppt)**	**T (^{o}C)**	**pH**	**Nominal Cu (µg/L)**	**Dissolved (µg/L)**
5	5.0 ± 0.4	24.7 ± 0.3	7.8 ± 0.1	0	13 ± 2	5.0 ± 0.2	24.7 ± 0.3	7.8 ± 0.1	0	10 ± 2
				50	31 ± 7				50	33 ± 3
				100	62 ± 12				100	67 ± 9
				200	182 ± 19				200	159 ± 18
				300	269 ± 20				300	250 ± 24
15	15.0 ± 0.2	25.1 ± 0.1	8.1 ± 0.4	0	0.8 ± 0.2	15.0 ± 0.3	25.1 ± 0.1	8.1 ± 0.4	0	3.5 ± 3
				100	86.3 ± 12				50	48 ± 9
				200	193 ± 21				100	98 ± 35
				300	261 ± 8				200	181 ± 13
				400	351 ± 13				400	347 ± 15
				600	531 ± 85				800	678 ± 31
25	25.0 ±0.1	24.9 ± 0.3	7.9 ± 0.3	0	1.5 ± 0.5	25.0 ±0.4	24.9 ± 0.3	7.9 ± 0.3	0	8 ± 5
				100	102 ± 7				100	73.7 ± 9
				200	192.7 ± 5				200	152 ± 13
				300	304 ± 14				400	420 ± 29
				400	379.7 ± 30				600	564 ± 37
				600	585.3 ± 45				800	771 ± 73

2.8 References

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Chapter 3

Short-Term Chronic Cu Toxicity to *Americamysis bahia* in Estuarine Environments: Effects on Growth, Survival and Sexual Maturation

3.1 Introduction

Estuaries and coastal regions are areas of interest as they are frequently impacted by anthropogenic activities. One of the main concerns in these areas is the presence of metals, such as copper which are of concern in these environments, particularly in boat basins as a result of the use Cu as a biocide in anti-fouling paints (De Polo et al., 2012; Matthiessen et al., 1999). Cu is an essential element (Gaetke et al., 2003) actively taken up by organisms, but inreased levels in ambient waters can result in toxicity to aquatic biota (Pinho et al., 2010; Martins et al., 2011). The most bioavailable form of Cu is the free ion (Cu^{2+}; Paquin et al., 2002) and it induces acute toxicity by disrupting internal ionic balance, particularly Na and Cl (Grosell et al., 2002, 2004). The proportion of Cu^{2+} in surface waters can vary tremendously, dependent on the geochemical speciation of the source waters. Interactions with inorganic ions (Blanchard et al., 2006; Flemming et al., 1989) and organic compounds (Arnold, 2005; McGeer et al., 2002; Santore et al., 2001) alter the geochemical speciation of dissolved Cu through complexation and thereby alter bioavailability and toxicity. As well, the impacts of Cu^{2+} can be altered via competition with cations at sites of uptake/toxicity.

Increased competition with Na^{+} and complexation to Cl^{-} occurring as a result of increasing salinity have the potential to provide protection against Cu toxicity across the salinity gradient that occurs in estuarine waters (Grosell et al., 2007; Blanchard et al., 2006; Grosell et al., 2004). For example, *Cyprinodon variegatus* (Adeyemi et al., 2012), *Brachionus plicatilis* (Arnold et al., 2010), *Callinectes sapidus* (Martins et al., 2011), *Acartia tonsa* (Pinho et al, 2010; Lauer et al., 2010) and *Neomysis integer* (Verslycke et

al., 2003) all showed decreased sensitivity to Cu at increased salinity in acute toxicity assays. Toxicity-salinity relationships are also partially related to the ability of Cu^{2+} to induce ionic imbalances and osmoregulatory disruption. The toxic nature of Cu^{2+} is reduced when organism are in an iso-osmotic environment while at higher and lower salinities the potential for osmoregulatory disruption and thus sensitivity to Cu^{2+} increases (Grosell et al., 2007; Blanchard et al., 2006). Cu toxicity in estuarine waters therefore may be dependent on complexation of Cu^{2+}, cationic competition and the capacity to cause osmoregulatory disruption as it relates to internal (organism) vs external (environment) salinity with Na and Cl effects.

As mentioned previously, dissolved organic matter (DOM) has been established as an important modifier for Cu toxicity (Arnold et al., 2010; Nadella et al., 2009; Arnold et al., 2005). As DOM enters estuaries, it's quality and capacity to protect against metal toxicity can be modified in several ways (McCallister et al., 2006) to better understand its function and structure to determine any toxicity mitigating qualities (Murphy et al., 2008; Brooks et al., 2007; Stedmon et al., 2003, 2005; McKnight et al., 2001). While sufficient data is available to understand the effects of DOM in sea-water (Arnold, 2005; De Palma et al., 2011; Arnold et al., 2006; Nadella et al., 2009), relatively few studies have examined the protective effects of Cu over a range of estuarine salinities (Hall et al., 2008; Arnold et al., 2010).

Estuaries are usually defined with having a wide variability in composition and concentration of DOM. Furthermore, as salinity increases a general decrease in DOM concentration has been observed (McCallister et al., 2006, Gerringa et al., 1998, Shank et al., 2004 and Ndung'u et al., 2003). DOM has also been established to exhibit source

(Ryan et al., 2004; McKnight et al., 1983; De Schemphelaere et al., 2004; Shwartz et al., 2004; Brooks et al., 2007) and concentration (Arnold, W.R., 2005, 2006 and 2010; De Palma et al., 2011; Nadella et al., 2009; Hall et al., 2008) dependent protection from Cu toxicity, however it is currently being used as a singular numerical value in toxicity prediction. Further research is required to build an understanding of the interactive effects of salinity and DOM (both source and quality) in estuarine waters to better establish water quality guidelines/criteria.

Americamysis bahia are small arthropods commonly found in estuaries and marine waters at salinities ranging from 15 to 30ppt, with an iso-osmotic point of 25ppt (De Lisle and Roberts, 1987). They were chosen for this study because they tolerate a wide range of salinities and are moderativelysensitive to metals (Lussier et al., 1988; De Lisle et al., 1987). *A bahia* are established as an indicator organism and there are standardized methodologies for culturing the organisms (EPA-505/8-0-006b) and toxicity testing (EPA 712-C-96-136; EPA 1007.0) (USEPA, 1990).

While studies have looked at acute (96h) metal toxicity (Hunt et al., 2002; Lussier et al., 1985; Lussier et al., 1999; Toussaint et al., 1985) to *Americamysis bahia* there is a lack of data on chronic Cu toxicity. In addition to understanding Cu speciation in natural waters and metal bio-availability, effects of long-term Cu exposure on sub-lethal end-points are critical to understanding the potential impact of Cu in estuarine systems. Standardized test methodologies for assessing chronic (lethal an sub-lethal) effects on *A. bahia* include the 28d (OPPTS 850.1350) life-cycle toxicity test (Ward et al., 2002; Hunt et al., 2002, Lussier et al., 1985; Breteler et al., 1982) and also the 7d growth and survival (EPA 1007.0). The latter has been shown to provide comparable results to the longer test

and it provides greater flexibility and opportunities to test natural water and DOM (Lussier et al., 1999; Ward et al., 2002; Khan et al., 1992).

The purpose of this research was to understand the effect of Cu on *A. bahia* at two salinities and the ameliorative effects of DOM on toxicity. All tests were conducted at salinities of 15 or 25 ppt as they were selected as the most representatives of estuarine environments and well within the tolerance limits of mysids. We used short-term (7d) tests with *A. bahia* to measure the effects of Cu on survival and growth as end-points following standard methods. We also developed an index of female sexual maturation based on relative brood sac size as it develops and applied this as a third endpoint. The overall objective of this study was to generate data towards developing an improved understanding of the potential impacts of Cu in estuarine waters.

3.2 Methodology

3.2.1 Test Organism

Test organisms (*A. bahia*, 4d old*)* were obtained from Aquatic Research Organisms (Hampton, New Hampshire, USA) and acclimated to lab conditions following standard EPA method (EPA-505/8-0-006b) for 3d in reconstituted salt water using synthetic sea salt (Kent Marine Reef Salt Mix). Salinity and temperature was monitored daily using a handheld conductivity meter (YSI 30, YSI Inc., Yellow Springs) and kept constant at 25ppt and 26^{o} C (± 0.5). The mysids were fed *Artemia nauplii* (Brine Shrimp Direct, Ogden, UT, USA) at a density of 150 artemia/day/neonate.

3.2.2 DOM Collection

(Refer to section 2.2.2 in Chapter 2)

DOM collections were carried out in November of 2011 and May of 2012. The collection sites were selected to provide minimal anthropogenic influences and maximum similarity to natural DOC entering estuaries. The DOM sources were collected using a portable reverse osmosis system (Refer to Chapter 2) and stored following the methods described by Schwartz et al. (2004). Three DOM samples were chosen, Kelley's Bog, Northlake and Rankin Brook, for all the testing (Refer to Chapter 2; Table 1)

3.2.3 A. bahia Toxicity Tests

Seven day toxicity tests were carried out to assess the short-term chronic toxicity of Cu following standard EPA method (EPA-821/R-02-014; 1007.0). All test solutions were prepared using Kent Marine Reef Salt Mix 24 h before test initiation to allow solutions to reach equilibrium. Tests were static renewal in nature with daily renewals. The tests were carried out in crystallizing dishes (Pyrex, Fisher Scientific, Ottawa, ON) with 250 mL of test solution. Test solutions were prepared to allow for a daily test solution renewal using synthetic Kent sea salt mixed with appropriate Atomic Absorption Spectroscopy (AAS) standard Cu stock solution (TraceCERT, Sigma-Aldrich Co., Oakville, ON). Salinity, pH and temperature were monitored and recorded on a daily basis. Each test consisted of four replicates with 10 neonates per replicate and included unexposed controls. Samples for total (unfiltered) and dissolved Cu (using 0.45 µM filters; Acrodisc HT tuffryn membranes, Pall Corp., Ann Arbor, MI) were collected on days 1, 3, 5 and 7 for Cu analysis in addition to DOC samples.

The end points were chosen to be mortality, growth as well as a new end-point sexual maturity. Observation for dead organisms was done daily until test completion and the dead individuals were removed. The final number of surviving mysids was used to calculate mortality LC_{50} values. Growth was measured as a second end-point and was determined both as percent body weight. It was also calculated as biomass (total dry weight for surviving mysids per concentration and replicat). Surviving organisms were individually weighed to the nearest µg using a Sartorius SE2 Ultra Microbalance (Sartorius Mechanatronics Corp., Bohemia, NY). Images of surviving mysids were taken using an inverted microscope to determine sex of the organism as well as to determine a sexual maturation score. Sexual maturity in females was quantified using a brood-sac scoring system that identifies the presence of clearly distinguished gonads, testis or brood-sac. The score ranges from 0 to 5, 0 being not sexually mature and 5 representing a fully mature female with eggs in the brood pouch (Figure 3.11).

3.2.4 Effect of Salinity on Cu toxicity

AAS standards (TraceCERT, Sigma-Aldrich Co., Oakville, ON, Canada) for Cu (1g/L) were used to make standards and test solutions. Tests were conducted ta 15 and 25ppt and Cu concentrations ranged from 0 to 160 µg/L. 10 L of test solution was made for each test concentration and stored in plastic carboys until test renewal. Salinity, pH and temperature were measured at 24h intervals and recorded and mysids were fed after every water renewal. Any mortality was recorded daily and dead individuals were removed.

3.2.5 Effects of DOC on Cu toxicity

All tests were carried out with measured DOC concentration of either 0 or 4 mg C/L. The effect of DOM was tested at both salinities, 15 and 25ppt with three DOM sources to determine any site-specific differences. The test Cu concentrations ranged from 30 to 320 µg/L and were prepared using standard AAS Cu standards. DOM concentrates were diluted accordingly to obtain the desired DOC concentration. A Cu negative control was used, with no added metal to the salt water as well as DOC positive control where the DOC concentration being tested was added without any input of metal to see any toxic effects of DOC alone. All solutions were prepared 24h in advance to allow for a homogenous mixture to be made with Cu and DOC. The mysids were fed throughout the duration of the test with daily water renewals and the debris was removed. Dissolved and total Cu water samples as well as DOC samples for each concentration were collected for analysis.

3.2.6 Measurements and Analysis

3.2.6.1 LC 20 &50

Lethal concentrations (LC_{50}) values were calculated using mortality data representative of each short-term chronic test using Spearman Karber Analysis in CETIS. The 95% confidence limits for all samples were also determined. Dry weight was used on an average dry weight basis and all calculations were done as a percent of control to eliminate any differences in initial weights of the mysids. Effect concentrations 20 (EC_{20}) and 50 (EC_{50}) were also calculated for growth and sexual development data using CETIS. The generated data was plotted using SigmaPlot™ (ver.11) to determine dose response curve, growth and sexual maturity trends. Data was considered significant if the 95%

confidence intervals did not overlap; if the confidence intervals overlapped significance was determined using Litchfield-Wilcoxon statistical analysis method (Environment Canada, 2005). Data for growth, biomass as well as sexual maturation, also represented as a percent of control, was compared using one-way ANOVA, in SigmaPlot^{TM} (ver. 11), to determine significance differences within each test. T-tests were utilized to compare different salinity and DOM tests ($p<0.05$).

3.2.6.2 Cu Measurements and Analysis

Water samples were measured to determine total and dissolved Cu concentrations. A lanthanum oxide precipitation process to eliminate salt from sample (Toyota et al., 1983) was used on all samples. The samples were subsequently measured by flame atomic absorption spectrometry (SpectraAA 880 with GTA100, Varian Inc., Palo Alto, CA).

3.2.6.3 DOC measurements and characterization

Filtered DOC samples from all concentrations and controls were used for all DOC reading using non-purge able organic carbon (NPOC) analysis. DOC concentrations were measured using a total carbon analyzer (TOC) analyzer (Shimadzu TOC-$L_{CPH/CPN}$, Shimadzu Corporation, Kyoto Japan). All the samples and standards were brought to room temperature, transferred to TOC vials and spiked with 2-3 drops of concentrated Hydrochloric acid (Sigma-Aldrich, Oakville, ON, Canada) prior to sample analysis.

Optical characterization of DOC was carried out by EEMS using a fluorescence spectrometer (Cary Eclipse, Varian, Victoria, Australia). The samples were measured in a quartz cuvette (Hellman Canada Ltd., Concord Canada). The measurements were done

using the excitation wavelength range of 200-450 nm with 10 nm increments while the emission was measured from 250- 600 nm. The generated data was then analyzed using MATLAB^{TM} through PARAFAC analysis to determine the four components to distinguish any differences between sources (Refer to 2.2.4).

3.2.6.4 DOM source and quality analysis (Refer 2.2.4)

EEMS was used to conduct optical characterization of the DOM sources. This data was analyzed using PARAFAC to determine components of the DOC samples and relative mounts of fluorophores were determined. The absorbances and scans were also used to form contour plots to determine any visual difference between DOM sources. Furthermore absorption coefficients at 340 nm (SAC_{340}) and fluorescence index (FI) were determined to further optically characterize the DOM (Refer to Chapter 2). The distribution of the four components within each DOM source was then correlated to effect concentrations for survival, growth and sexual maturation to determine toxicity. Finally, the SAC340 values as well as FI were examined in conjunction with LC/EC values to observe potential relationships between DOM source quality and corresponding protective capacity (Refer to 2.2.4). The correlations were determined using Pearson Product Moment and were considered significant when $p<0.05$.

3.3 Results

3.3.1 Test Conditions/Water Samples Analysis

Temperature, salinity and pH were monitored throughout the duration of each test. The temperature for all tests was 25.6 ± 0.3 (n =1536), pH was measured (n =240) to

average 7.9 ± 0.4 (Table 3.1). The salinity for all tests conducted at 15ppt was 15 ± 0.2 ppt through the duration of the test; whereas salinity was 25 ± SEM 0.3 for tests conducted at 25ppt (n = 2304). Measured dissolved Cu concentrations were determined to be 97 ± 5% of the nominal Cu concentrations (n = 312) . To determine if there were changes in Cu concentration over the duration of the tests, samples were also collected at the termination of each test and dissolved Cu concentrations on Day 7 were measured to be 90 ± 5 % of those on Day 1 (n = 144). DOC measurements in tests with no added DOC provided a background level of 0.81-1.22 mg C/L and DOC additions for all three sources were determine to be within 100 ± 12 % (n = 280) of nominal DOC concentrations (Table 3.1)

3.3.2 Short-term Chronic Toxicity

3.3.2.1 Survival

There was a concentration dependent effect of Cu, with higher concentrations causing increased mortality (Figure 3.1). Although not significant, higher mortality was observed at 15ppt (Figure 3.1). All three DOM sources provided a significant protective effect from Cu toxicity at 15ppt and LC50s ranged from 226-309 µg Cu/L (Figure 3.2). Kelly's Bog provided the highest protection against toxicity while Northlake was least protective (Figure 3.2). The protective effects of DOM sources were variable at both 15 and 25ppt and no significant source dependent differences were found. No significant protection of DOC on survival was found at 25 ppt as 50 % mortality was not reached in all the test and the LC_{50} values were determined through extrapolation.

3.3.2.2 Growth

In all experiments, except Kelly's bog, increasing Cu concentrations resulted in a decrease in the average mysid dry weight (Table 3.2). No salinity differences were discovered for percent dry weight between 15 and 25ppt Cu only tests (Figure 3.3a). The results were equivocal for dry weight and neither of the three DOM sources provided any protection from Cu toxicity at 15 or 25ppt. Since no significant trends were determined using dry body weight as an indicator, biomass was chosen as a second growth end-point as it accounts for both survival and body weight. Biomass at 15ppt was significantly lower than at 25ppt therefore expressed as percent control (Figure 3.3b). There was a significant decrease in biomass between control and the highest Cu concentration for all tests. The results were equivocal for biomass at a salinity of 25ppt and neither of the three sources provided significant protection (Figure 3.5), however Kelly's Bog had the highest EC_{20} at 140 µg Cu/L (Figure 3.6). At 15ppt, EC_{20} values for Kelly's Bog and Rankin Brook were significantly different from those with no added DOC, while Northlake DOC provided no extra protection (Figure 3.4). EC_{50}s for all sources could not be determined as at some sources a 50 percent reduction in body weight or biomass did not occur over the course of the tests and any EC_{50} values would have to be extrapolated.

3.3.2.3 Sexual Maturation (Brook-sac Development Score)

Sexual Maturation decreased with increasing Cu concentration (Table 3.2). There was a strong salinity difference between the brood-sac scores from mysids acclimated to 15 and 25ppt independent of Cu (Figure 3.7). Brood-sac differences were therefore calculated within 15 and 25ppt as a percent of control to eliminate any starting

differences for each test. No source-dependent protective effect of DOM was found on brood-sac development score at either 15ppt (Figure 3.8) or 25ppt (Figure 3.9). EC_{20}s also failed to exhibit any differences in protective ability of the three DOM sources at either salinity (Figure 3.10).

3.3.2.4 DOM Quality vs Toxicity

Several correlations were found between EC/LC values and optical characteristics for both 15 and 25ppt (Table 3.3). A significant negative correlation was found between humic acid, tryptophan content and the LC_{50} and both EC_{20} and EC_{50} values for growth at 15ppt and a positive correlation was found with the fulvic acid for the same three values. Similarly for 25ppt, a positive correlation was determined between fulvic acid content an EC_{20} biomass while negative correlations were determined between humic and tryptophan. No correlation was found between sexual maturation EC_{50} concentrations and any of the optical properties. While positive correlations were found between SAC_{340} and FI values and effect concentrations, none of these correlations were statistically significant.

3.4 Discussion

This study examined the toxicity ameliorating effects of salinity and DOM on Cu toxicity to *Americamysis bahia* in estuarine environments. Both DOM and salinity provided a protective effect from Cu toxicity during the 7-day toxicity tests. The three DOM sources were protective to varying degrees, however there was no conclusive evidence of site-dependent protection from Cu toxicity at either salinity. Optical

characteristics were also unable to provide adequate explanation for observed differences in toxicity among various sources.

As mentioned previously, there are plenty of studies examining acute Cu toxicity in sea water, however there is a lack of chronic studies looking at survival and sub-lethal effects of Cu in estuarine environments. The present study was designed to better understand short-term chronic toxicity of Cu and demonstrated deleterious effects of a chronic Cu exposure. An appropriate comparison between 7-day toxicity tests to 28 day life cycle tests could not be carried out due to a lack of sufficient available 7-day test data examining Cu toxicity in estuarine environments.

Overall a decrease in survival was observed as Cu concentrations were increased as has been observed in literature (Morrison et al. 1989; Chen et al., 2001). Two estuarine salinities were used to determine the effects of salinity on short-term chronic exposures of Cu. Overall the organisms were approximately 1.5 times more sensitive during the chronic exposure (115 µg Cu/L) in comparison to acute Cu exposures (178 µg Cu/L), very similar to what was observed by Lussier et al. (1985). In general *A. bahia* was determined to be more sensitive to Cu toxicity at 15ppt in comparison to 25ppt, a trend very similar to what was found for tiger shrimp (Chen et al. 2001) as well as acute Cu toxicity due to stress on its osmoregulatory system (Refer to chapter 2). Blanchard et al. (2006) and Grosell et al. (2007) observed an increased sensitivity of the organisms to metal toxicity below or above the iso-osmotic point as well (25ppt for *A. bahia*; De Lisle et al., 1986, 1987). However, there was no statistical difference between the LC_{50} values at 15ppt, 116 µg/L CI (64-214) or 25ppt, 186 µg/L CI (121-444). One of the few studies to test the chronic effect of Cu on *A. bahia* (Lussier et al., 1985) tested effects of Cu at

30ppt using a 28-day toxicity test and an LC_{50} value of 104 (77-144) µg/L Cu was calculated. Morrison et al. (2007) also tested the effects of Cu at 30ppt using the same 7d test and calculated an LC_{50} of 169 (137-196) µg/L. The results obtained from this study were in agreement with the LC_{50} values obtained by Morrison et al. (2007) and Lussier et al. (1985) as no significant differences were found between the LC_{50} values.

Effects of Cu were also tested on growth (measured as body weight as a % of control) and no salinity difference was observed, which demonstrates that even though fewer mysids survived at 15ppt, the ones that did survive appeared to grow at the same rate as those at 25ppt. To better illicit the differences that may be present in growth total, biomass was calculated at each exposure concentration. Biomass is a better end-point representing growth to exhibit differences as it accounts for both survival as well as body weight. This end-point revealed that biomass at both 15 and 25ppt was significantly lower at the highest Cu concentrations in comparison to controls. A similar pattern was observed in a studies by Chen et al. (2001) on shrimp, Erickson et al. (1996), in fat head minnows, Munari et al. (2007) for clams and Lorenzo et al. (2002) for sea urchins all of which observed a decreased biomass with increasing Cu concentrations. A significant protective effect of salinity was observed as the salinity was increased from 15 to 25ppt, similar to literature (Riedel et al., 1995). EC_{20} values were determined for growth based on biomass and *A.bahia* exhibited a significantly lowered sensitivity to Cu at 25ppt in comparison to 15ppt with no addition of DOC.

Finally, brood-sac development score was determined to exhibit a very strong salinity dependence, independent of Cu exposure, as there was a significant difference between the control group at both 15 and 25 ppt. Values calculated to be significantly

different from control from this test (> 80 µg Cu/L) were compared to those (for delay of reproduction; > 70 µg Cu/L) by Lussier et al. (1985) and were determined to not be significantly different. Brood-sac development was therefore deemed unsuitable as an end-point to determine between salinities, however it was determined to be an appropriate end-point to use amongst different treatments within a salinity.

In addition to salinity, effects of DOM (source and concentration) on short-term chronic Cu toxicity were also determined. A protective effect of DOM similar to that on acute toxicity was observed for short-term chronic toxicity. Additions of DOM added at both 15 and 25ppt resulted in increased LC50 values, hence providing protection from Cu toxicity. This effect of DOM on toxicity mitigation is similar to the effect observed in *M. galloprovincials* (Nadella et al., 2009; Arnold et al., 2005), *E. affinis* (Hall et al., 2008) and *B. plicatilis* (Arnold et al., 2010). All tests with DOM added at a measured concentration of 4 mg C/L were significantly protective at 15ppt as up to a 2.5 fold difference in LC50 values was observed. The results were equivocal at 25ppt and a linear increase in protective capacity of DOM with increasing salinity was not observed. The reason for this lack of increase in protective capacity is suggested to be attributed to the formation of salt induced colloid formation (Cooper et al. 2014; Wood et al., 2011). It is proposed that at high ionic strength a decrease in Van der Wall forces and results in inter-particle attraction resulting in colloid formation (Cooper et al., 2013). It can also be as a result of the fact that 15 to 25ppt is the optimal range of salinities and thus requires minimal active osmotic gradient changes.

Minimal protective effects on dry weight of mysids were observed after the addition of DOM, however no statistical differences were calculated between no added

DOC tests and three DOM sources for dry weight as a percent of control at both 15 and 25ppt. To further understand any differences that may be present, bio-mass as a percent of control, was also calculated. Rankin Brook and Kelly's bog were shown to provide a significant protection from Cu toxicity at 15ppt while Northlake provided no protection. There were no significant differences in Cu toxicity at 25ppt for any of the sources.

The final end-point measured was sexual maturation (as measured by brood-sac development score) and the comparison amongst tests was only conducted within each salinity to avoid strong salinity effects. Broods-sac development score was also calculated as a percent of control to eliminate differences between controls. The average brood-sac development score calculated at 15ppt for all controls in the tests (no added DOC and DOM sources at 4mg C/L) was 1.5 and DOM did not provide any source-dependent protection from toxicity. Similarly, at 25ppt DOM did not mitigate Cu toxicity in a source-dependent manner after addition of 4 mg C/L DOC and the average score for the controls was calculated to be 3.

The lack of effect of DOC on sexual maturation is consistent with what Lorenzo et al. (2002) found. Growth was determined to be a more sensitive end-point than embryogenisis and a protective effect was observed on growth at much lower DOC concentrations than those required to result in reduced toxicity on embrogenisis. The EC_{20} values in this experiments also exhibit a similar trend where the growth is shown to be a much more sensitive end-point than sexual maturation and DOM provides a significant reduction in toxicity whereas no protective effect of DOM is observed on sexual maturation.

Calculated effect concentrations for survival, growth and sexual maturation values were then compared to determine correlation between toxicity and optical characteristics of DOM. All sources exhibited a high humic-acid and fulvic acid content, which has previously been linked to protective capacities of DOM (Cooper et al., 2014; Arnold et al., 2010; Nadella et al., 2009; Zitko et al., 1973, Schwartz et al., 2004). A general protective effect of DOM was observed as higher Cu concentrations were required to result in the same mortality. Unlike freshwater it has been proposed that higher fulvic content of DOM results in increased protective effect (Nadella et al., 2009; Lorenzo et al., 2006). Negative correlations were determined between LC/EC_{50} or 20 values and humic acid and tryptophan, while a positive correlation was observed between the same parameters and fulvic acid exhibiting a protective effect of fulvic acid, similar to that of Nadella et al. (2009) however these correlations were not deemed significant. Strong correlation were found between acute LC_{50} values and SAC_{340} (i.e the color of DOM) as well as FI (i.e DOM origin; terrigenous vs autochthonous), while no strong correlation were determined for chronic values. These relationships were deemed insignificant as all sources exhibited very similar SAC_{340} values as well as FI values despite the geographical separation and these values covered a very small range of values associated with each index (SAC_{340}: 3 – >80, Al-Reasi et al., 2011; FI: 1-1.9, McKnight et al., 2001).

Overall this study found that DOC is a good predictive measure of Cu toxicity in estuarine systems independent of source, a result very similar to that found by DePalma (2009). Further research is required to clarify the interactions of Cu with DOM in salt

water environments and a wider array of optical techniques are required better differentiate any potential source dependent differences.

3.5 Conclusions

Overall 7-day short-term chronic tests proved to be an efficient and effective means of measuring Cu toxicity and DOC was established a good predictive measure of Cu toxicity. It is evident from this study that examining survival, growth and sexual maturation together provides a clearer and more robust understanding of the toxicity of metals in estuarine systems. Increasing salinity provided a protective effect from Cu toxicity similar to the literature (Grosell et al., 2007; Hall et al., 2008), but this effect is not as clear with the addition of DOM. DOM proved to be much more protective at 15ppt than at 25ppt, this can be attributed to the organism already being at its iso-osmotic point at 25ppt as well as the potential of colloid formation of DOM with increasing salinity. Biomass was determined to be a much more effective means of determining differences in body weight in comparison to dry weight as it accounts of survival as well as weight. Biomass, similar to survival was a more sensitive end-point at 15ppt where a significant change in Cu toxicity was observed. Sexual maturation was deemed unsuitable as an end-point in predicting toxicity across salinities due to a significant salinity difference in controls. No source-dependent protection was observed for sexual maturation at either salinity.

No relevant and significant correlation could be made to optical characteristics and toxicity parameters. All sites were almost identical in chemical composition and optical characteristics despite the geographical distance and thus any differences

observed in toxicity tests could not be accurately understood and explained. The results from this study in agreement with literature (Al-reasi et al., 2011; De Palma et al., 2011) suggest that DOM quality does not need to be considered when developing an estuarine biotic ligand model. Further testing and analyses is warranted given the limited sources of DOM and limited sample size of current study to better understand effects of DOM quality on toxicity in estuarine conditions. More sources of DOM (from disturbed vs undisturbed sites) and salinities (representatives of estuaries), perhaps with a wide array of DOC concentrations need to be tested to gain a full understanding of interactive effects of DOM and salinity on metal toxicity.

3. 6 Tables and Figure

Table 3.1: Measured water chemistry parameters for 7-day short-term chronic toxicity tests at 15 and 25ppt. Salinity, temperature and measured background DOC values and additions of DOC are given as mean ± SEM for each test. Cu exposure concentrations are given as measured dissolved Cu concentrations (mean ± SEM; µg/L).

Test Salinity (ppt)	Exposure	Temperature (^{o}C)	Salinity (ppt)	Nominal Cu (µg/L)	Dissolved Cu (µg/L)	Measured DOC (mg C/L)
15	Cu Only	25.6 ± 0.3	15.0 ± 0.2	0	4 ± 2.1	1.22 ± 0.12
				20	13 ± 5.3	
				40	27 ± 7.8	
				80	73 ± 4.5	
				120	113 ± 10	
				160	158 ± 13	
	Northlake	25.5 ± 0.1	15.0 ± 0.5	0	3 ± 4.1	3.94 ± 0.21
				30	27 ± 6.3	
				60	61 ± 9.8	
				120	118 ± 19	
				200	203 ± 8.5	
				280	280 ± 3.3	
	Rankin Brook	25.6 ± 0.4	15.0 ± 0.4	0	12 ± 5.6	4.27 ± 0.081
				40	37 ± 7.2	
				80	75 ± 4.6	
				160	155 ± 9.1	
				240	219 ± 15	
				320	318 ± 7	
	Kelly's Bog	25.6 ± 0.2	15.0 ± 0.1	0	8 ± 3	3.71 ± 0.15
				40	44 ± 2.5	
				80	80 ± 10	
				160	140 ± 14.6	
				240	230 ± 9.5	
				320	308 ± 6.2	

25	**Cu Only**	25.6 ± 0.3	25 ± 0.1	0	2 ± 5.6	0.81 ± 0.05
				20	17 ± 2.4	
				40	35 ± 3.6	
				80	67 ± 6.7	
				120	116 ± 11	
				160	168 ± 9.6	
	Northlake	25.5 ± 0.1	25 ± 0.1	0	6 ± 6.2	3.87 ± 0.06
				30	30 ± 7.1	
				60	59 ± 5.8	
				120	118 ± 4.4	
				200	188 ± 15.7	
				280	289 ± 10.9	
	Rankin Brook	25.6 ± 0.4	25.0 ±0.5	0	6 ± 3.3	4.48 ± 0.33
				40	40 ± 6.9	
				80	78 ± 15.2	
				160	140 ± 12.4	
				240	228 ± 20.3	
				320	314 ± 8.4	
	Kelly's Bog	25.5 ± 0.2	25.0 ± 0.4	0	6 ± 1.6	4.05 ± 0.09
				40	23 ± 3.9	
				80	73 ± 3.8	
				160	157 ± 10.1	
				240	255 ± 11.7	
				320	326 ± 8.6	

Table 3.2: 7-day short-term chronic toxicity end-points: survival, growth (dry weight and biomass) as well sexual maturation measured as brood-sac development score. Dry weight and brood-sac development are shown as mean ± SEM individual weight/score of surviving mysids. Biomass is given as the mean ± SEM total biomass for each exposure concentration Exposure concentration are given as mean measured dissolved Cu concentrations for both salinities (15 and 25ppt). An * indicates a significant difference from controls (ANOVA $P<0.05$).

Salinity (ppt)	Exposure	Dissolved Cu (µg/L)	Dry Weight (mg)	Biomass (mg)	Brood-sac Development Score	n
15	Cu Only	4 ± 2.1	0.189 ± 0.012	1.65 ± 0.143	1.143 ± 0.378	35
		13 ± 5.3	0.179 ± 0.009	1.462 ± 0.247	0.714 ± 0.267*	31
		27 ± 7.8	0.183 ± 0.023	1.198 ± 0.231*	0.667 ± 0.258*	26
		73 ± 4.5	0.162 ± 0.018	0.981 ± 0.265*	0.5 ± 0*	24
		113 ± 10	0.15 ± 0.019*	0.633 ± 0.142*	0 ± 0*	17
		158 ± 13	0.142 ± 0.021*	0.487 ± 0.016*	0 ± 0*	14
	Northlake	3 ± 4.1	0.283 ± 0.015	2.403 ± 0.115	2.318 ± 0.929	34
		27 ± 6.3	0.214 ± 0.019*	1.773 ± 0.116*	1.278 ± 0.441*	33
		61 ± 9.8	0.205 ± 0.044*	1.727 ± 0.295*	1.278 ± 0.363*	34
		118 ± 19	0.196 ± 0.017*	1.375 ± 0.12*	0.857 ± 0.556*	28
		203 ± 8.5	0.156 ± 0.018*	0.78 ± 0.088*	0.75 ± 0.274*	20
		280 ± 3.3	0.147 ± 0.02*	0.442 ± 0.061*	0 ± 0*	12
	Rankin Brook	12 ± 5.6	0.178 ± 0.022	1.694 ± 0.279	1.5 ± 0.632	38
		37 ± 7.2	0.161 ± 0.02	1.434 ± 0.076	1.167 ± 0.764	36
		75 ± 4.6	0.145 ± 0.008	1.163 ± 0.18*	0.7 ± 0.274*	32
		155 ± 9.1	0.132 ± 0.019*	0.915 ± 0.04*	0.5 ± 0*	28
		219 ± 15	0.125 ± 0.022*	0.625 ± 0.109*	0.5 ± 0*	20
		318 ± 7	0.12 ± 0.024*	0.277 ± 0.145*	0.5 ± 0*	9
	Kelly's Bog	8 ±	0.231 ± 0.062	1.847 ± 0.498	1.577 ± 0.572	32
		44 ±	0.222 ± 0.062	1.661 ± 0.425	1.045 ± 0.416*	30
		80 ±	0.198 ± 0.024	1.683 ± 0.205	0.8 ± 0.274*	34
		140 ±	0.196 ± 0.025	1.565 ± 0.196	0.7 ± 0.274*	32
		230 ±	0.177 ± 0.019	1.012 ± 0.081*	0.5 ± 0*	23
		308 ±	0.163 ± 0.017	0.614 ± 0.116*	0.25 ± 0*	15

25	**Cu Only**	2 ± 5.6	0.322	±	0.016	2.898	±	0.223	3.5	±	0.972	36
		17 ± 2.4	0.322	±	0.032	2.881	±	0.186	3.219	±	0.816	36
		35 ± 3.6	0.318	±	0.031	3.017	±	0.224	3.22	±	0.778	38
		67 ± 6.7	0.278	±	0.038	2.419	±	0.469	2.188	±	0.704*	35
		116 ± 11	0.252	±	0.037*	1.831	±	0.328*	1.778	±	0.905*	29
		168 ± 9.6	0.226	±	0.031*	1.191	±	0.242*	1.071	±	0.432*	21
	Northlake	6 ± 6.2	0.234	±	0.008	2.218	±	0.125	2.625	±	0.582	38
		30 ± 7.1	0.209	±	0.026	1.878	±	0.298	1.75	±	0.463*	35
		59 ± 5.8	0.194	±	0.02*	1.738	±	0.122*	1.778	±	0.264*	37
		118 ± 4.4	0.171	±	0.015*	1.562	±	0.126*	1.357	±	0.378*	37
		188 ± 15.7	0.15	±	0.014*	0.907	±	0.186*	1	±	0.612*	24
		289 ± 10.9	0.144	±	0.026*	0.609	±	0.118*	0.583	±	0.204*	17
	Rankin Brook	6 ± 3.3	0.238	±	0.026	2.142	±	0.238	2.5	±	1.354	36
		40 ± 6.9	0.209	±	0.026	1.726	±	0.213*	1.167	±	1.155	33
		78 ± 15.2	0.188	±	0.024*	1.544	±	0.18*	0.833	±	0.577*	33
		140 ± 12.4	0.161	±	0.025*	1.289	±	0.202*	0.7	±	0.274*	32
		228 ± 20.3	0.156	±	0.017*	1.166	±	0.112*	0.625	±	0.25*	30
		314 ± 8.4	0.145	±	0.021*	0.942	±	0.189*	0.583	±	0.204*	29
	Kelly's Bog	6 ± 1.6	0.182	±	0.004	1.64	±	0.187	2.079	±	0.731	36
		23 ± 3.9	0.169	±	0.006	1.435	±	0.091	2.4	±	0.615	34
		73 ± 3.8	0.167	±	0.012	1.414	±	0.176	2.25	±	0.5	34
		157 ± 10.1	0.166	±	0.013	1.372	±	0.284	1	±	0.447*	33
		255 ± 11.7	0.164	±	0.012	1.179	±	0.268*	0.667	±	0.258*	29
		326 ± 8.6	0.154	±	0.02	0.769	±	0.101*	0.5	±	0*	20

Table 3.3: Correlation coefficients (r) calculated with Pearson Product Moment for optical characteristics of DOM with measured 7-day short-term chronic toxicity end-points. * denotes significance in relation to toxicity parameters.

	End Point	EC/LC	SAC340	FI	HA	FA	Trp	Tyr
15	Survival	**Day 7 LC50 (µg C/L)**	0.65	(-)0.62	(-)0.99*	0.99*	(-)0.99*	N/A
	Biomass	**Day 7 EC20 (µg C/L)**	0.75	(-)0.72	(-)0.96*	0.96*	(-)0.96*	N/A
		Day 7 EC50 (µg C/L)	0.72	(-)0.69	(-)0.98*	0.98*	(-)0.98*	N/A
	Sexual Maturation	**Day 7 EC50 (µg C/L)**	0.92	(-)0.90	(-)0.83	0.83	(-)0.83	N/A
25	Survival	**Day 7 LC50 (µg C/L)**	0.72	(-)0.75	0.18	(-)0.18	0.18	N/A
	Biomass	**Day 7 EC20 (µg C/L)**	0.37	(-)0.33	(-)0.98*	0.98*	(-)0.98*	N/A
		Day 7 EC50 (µg C/L)	0.92	(-)0.89	(-)0.84	0.84	(-)0.84	N/A
	Sexual Maturation	**Day 7 EC50 (µg C/L)**	(-)0.21	0.25	(-)0.69	0.69	(-)0.69	N/A

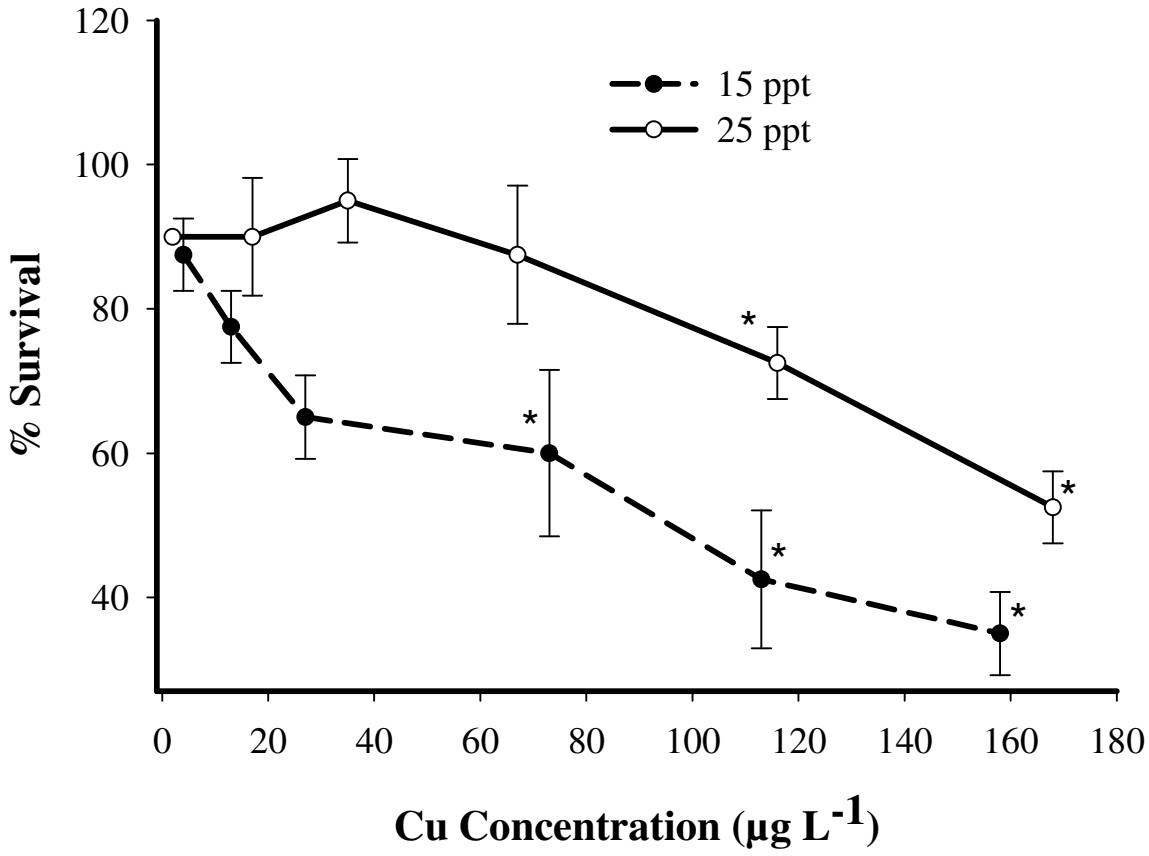

Figure 3.1: Exposure response relationship for survival of *A.bahia* exposed to Cu for 7-d at either 15 (filled circles) or 25ppt (open circles). Mean ± SEM are shown and n=40 for each test. A * indicates a significant difference from mean survival with no added Cu ($p<0.05$).

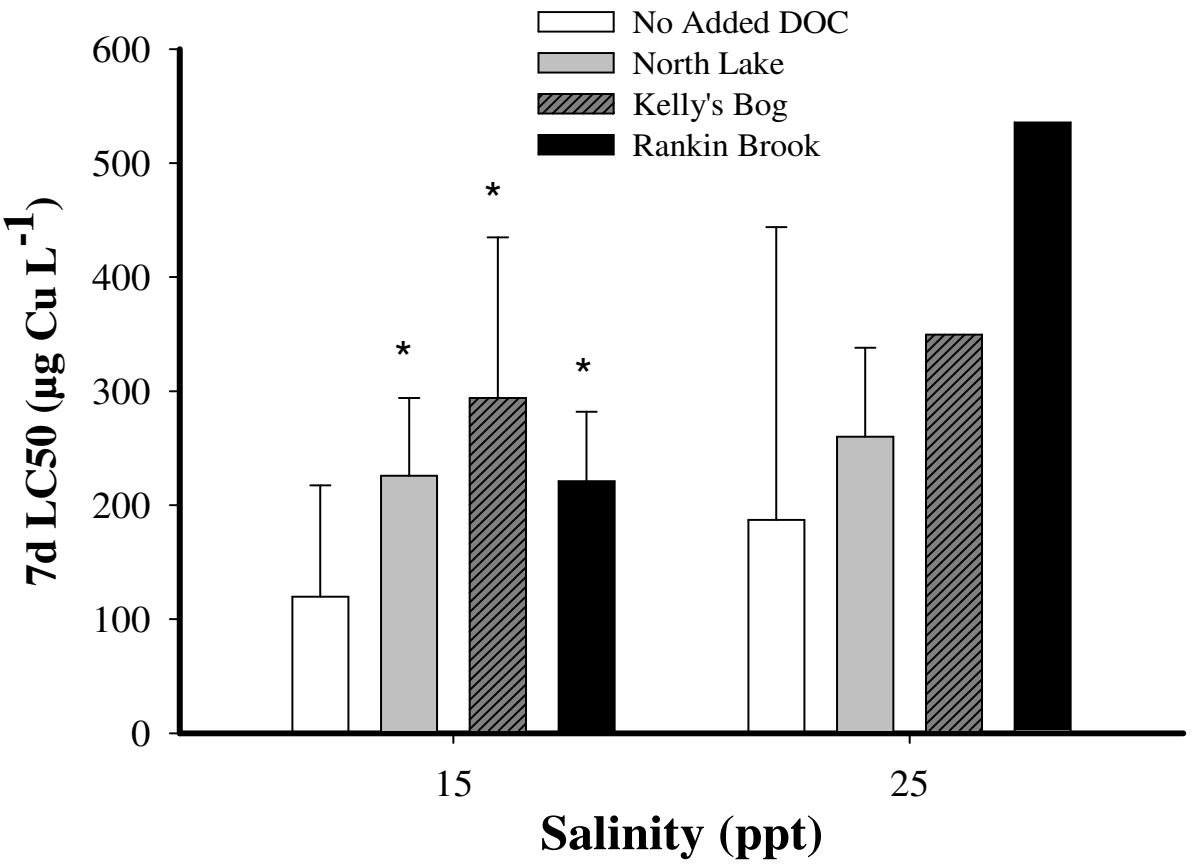

Figure 3.2: Effect of 4 mg DOC/L on 7-d LC_{50}. DOM sources were tested at 15 and 25ppt. A* indicates difference from no added DOC at 15ppt.

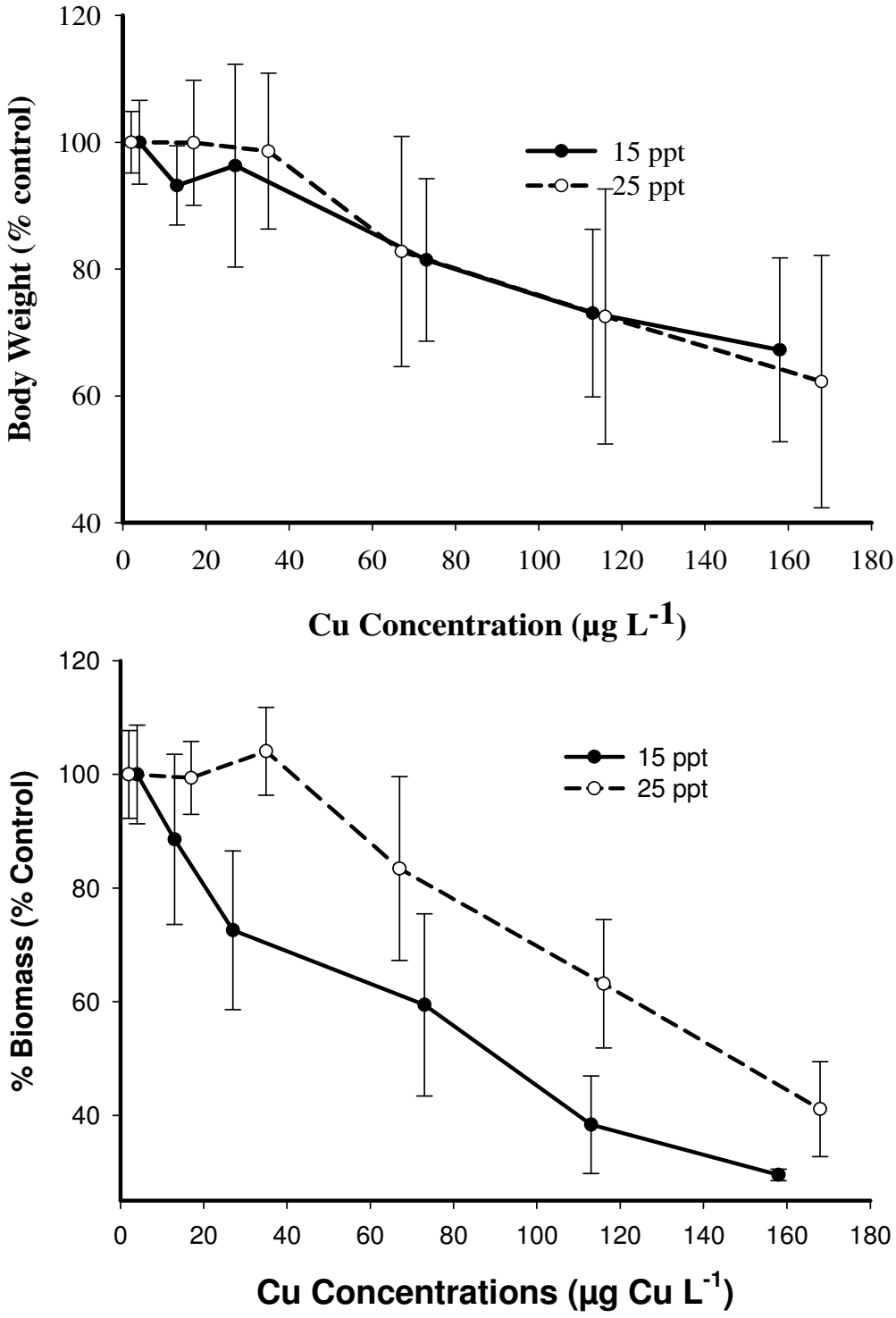

Figure 3.3: Effects of salinity on Cu toxicity as measured by growth on *Americamysis bahia*: A) body weight as a percent of control B) biomass as percent of control, at 15 (solid) and 25 (dashed) ppt.

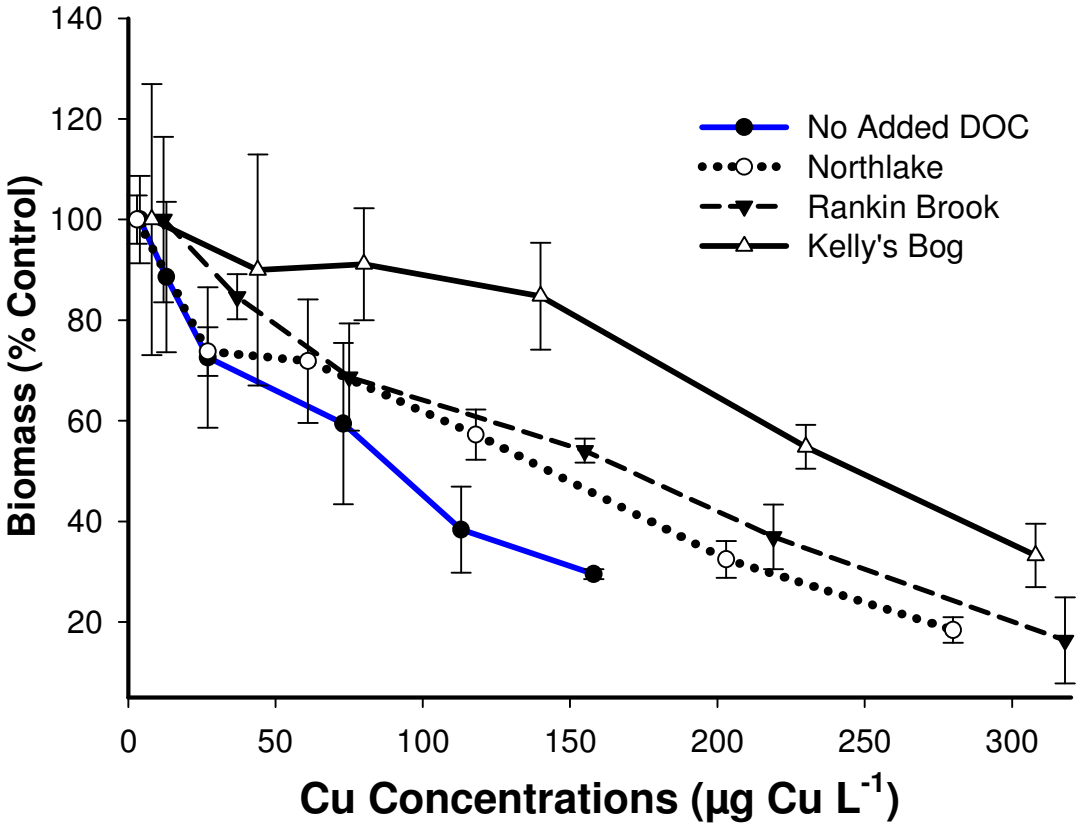

Figure 3.4: Comparison between three DOM sources (at 4 mg C/L) and no added DOC on biomass on day 7 at 15 ppt for short-term chronic Cu exposure. Rankin Brook and Northlake were determined to be significantly different from Kelly's Bog.

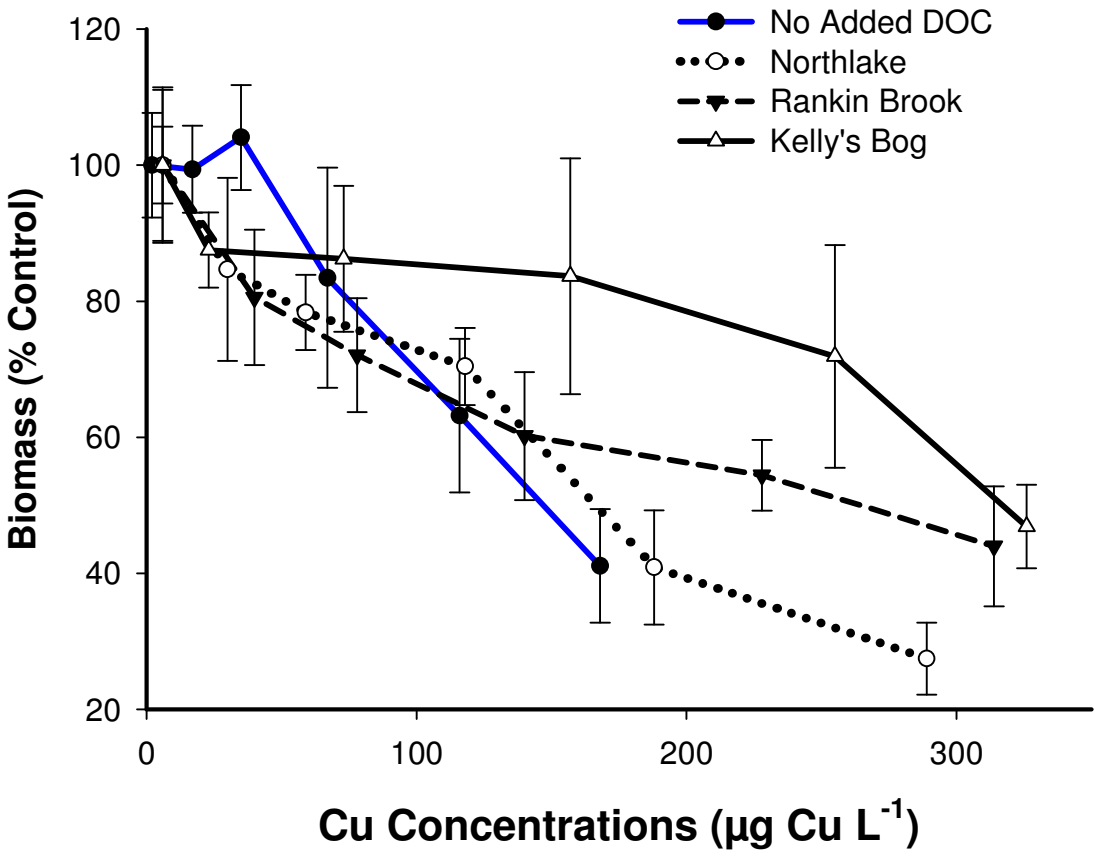

Figure 3.5: Growth dose response curve (represented as biomass as a percent of control) after 7-day short-term chronic Cu exposures at 25ppt. All DOM tests were conducted at a measured concentration of 4 mg C/L. No significant difference were found among the DOC sources.

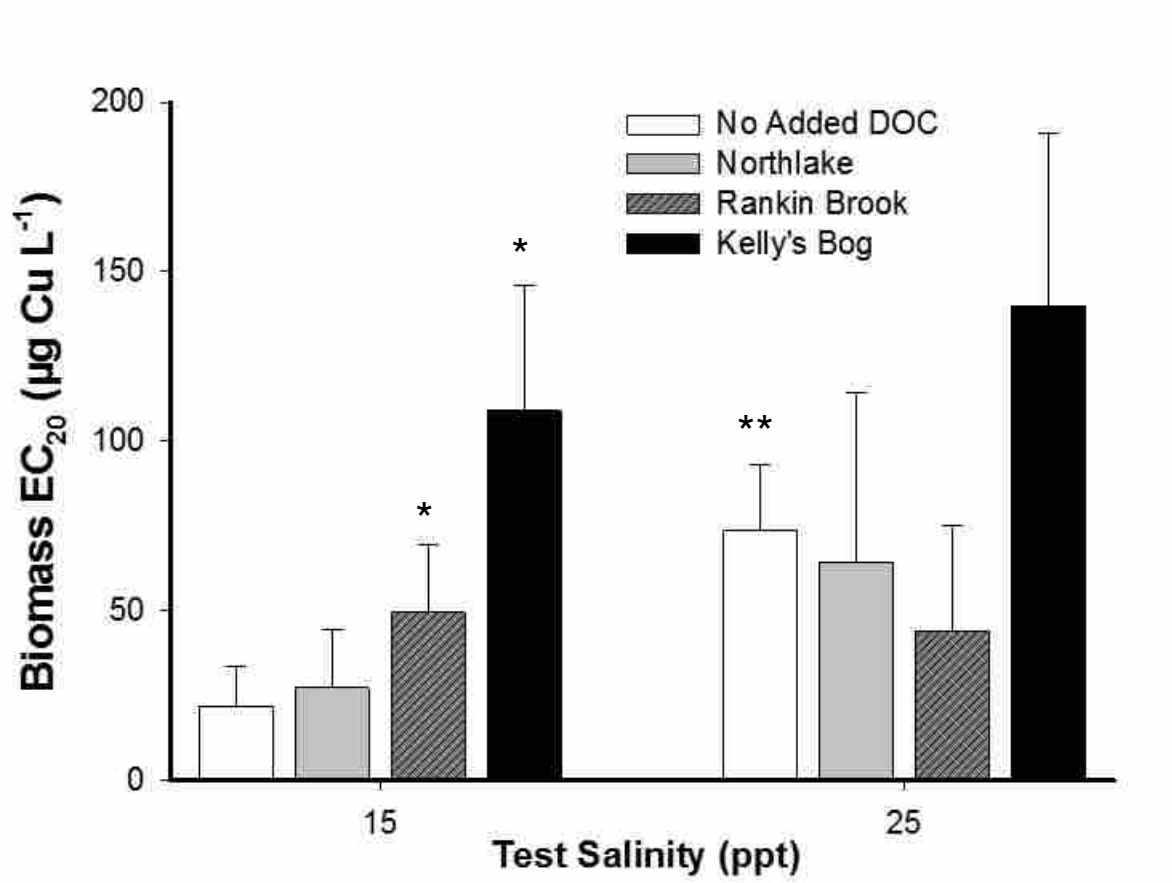

Figure 3.6: A comparison of 7-d Cu EC_{20} values for biomass at 15 and 25ppt. * Denotes a significant difference in toxicity compared to 15ppt (no added DOC) while ** represents a significant difference between 15 and 25ppt, Cu only exposures.

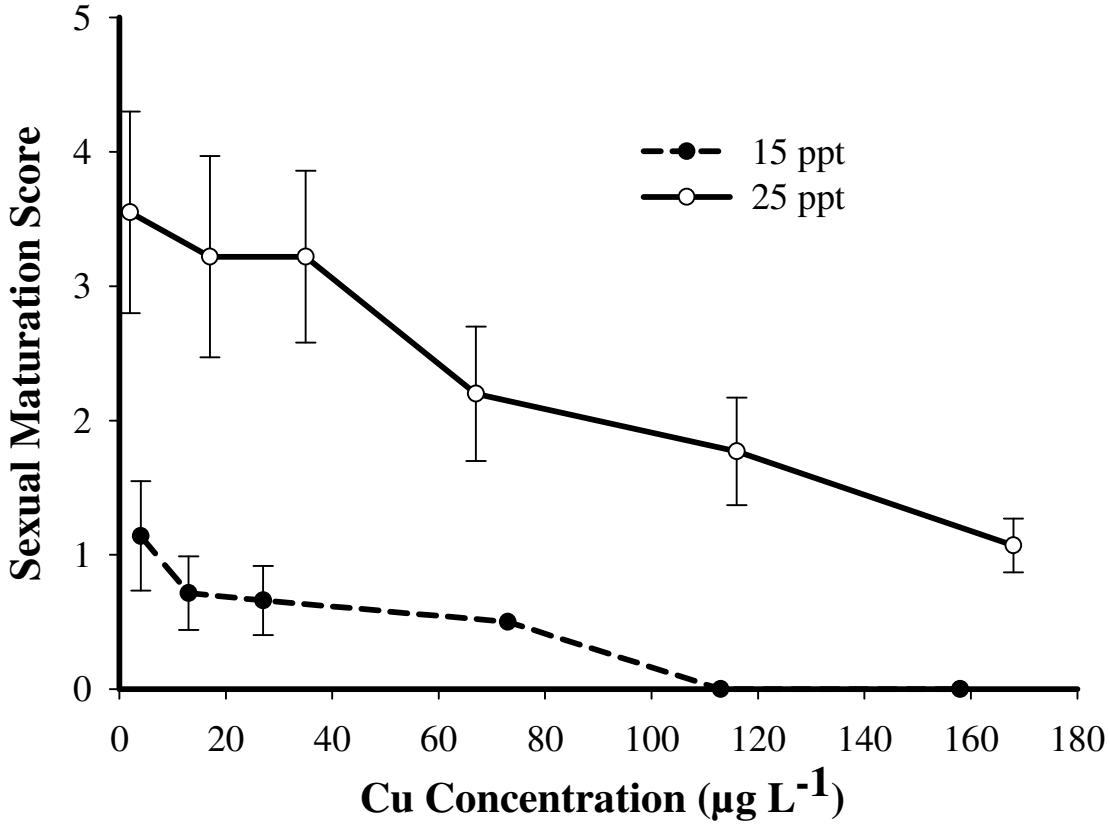

Figure 3.7: Effect of salinity on sexual maturation (calculated as brood-sac development) at 15 (dashed) and 25ppt (solid). The brood-sac score ranged from 0 (immature mysid) – 5 (fully mature female with eggs in brood-pouch).

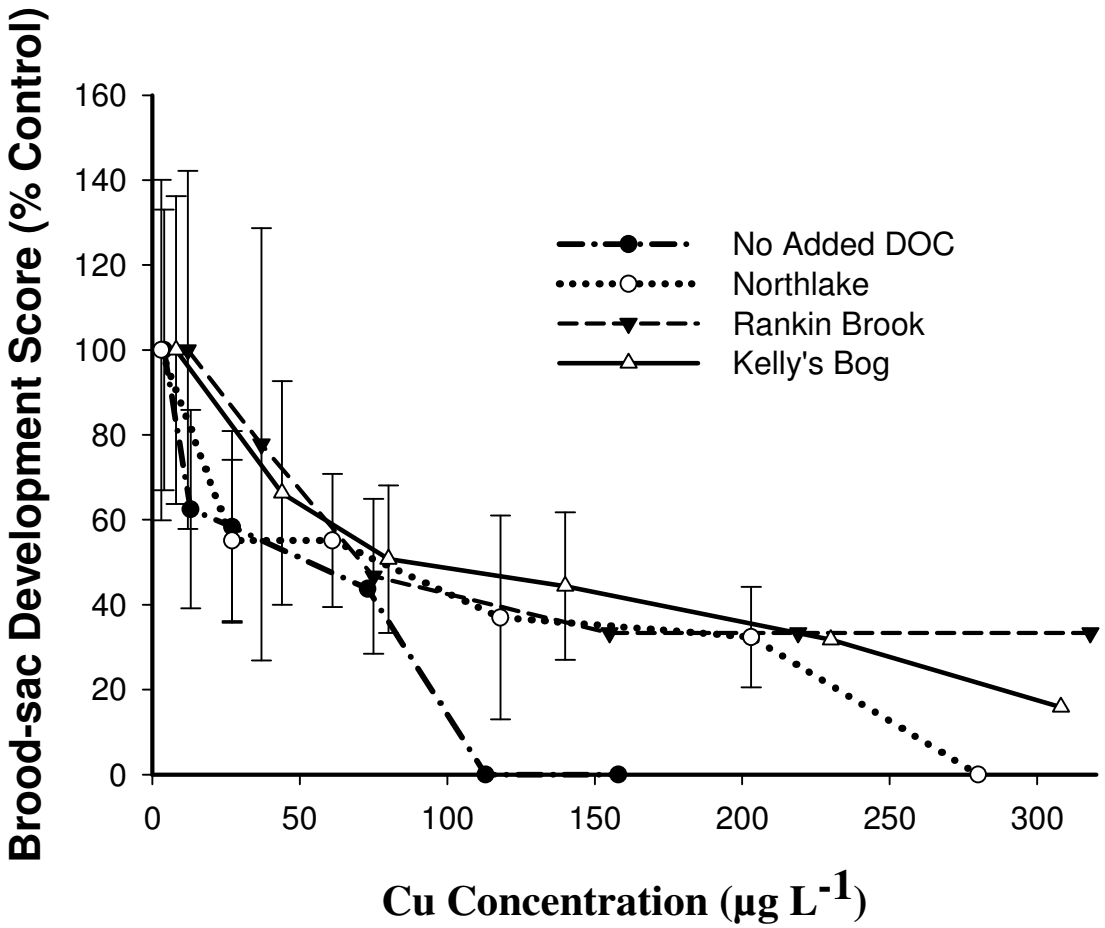

Figure 3.8: Intra-specific comparison of brood-sac development score at 15ppt. DOM was added at a measured concentration of 4 ± 0.5 mg C/L with a background level of 0.5 mg C/L.

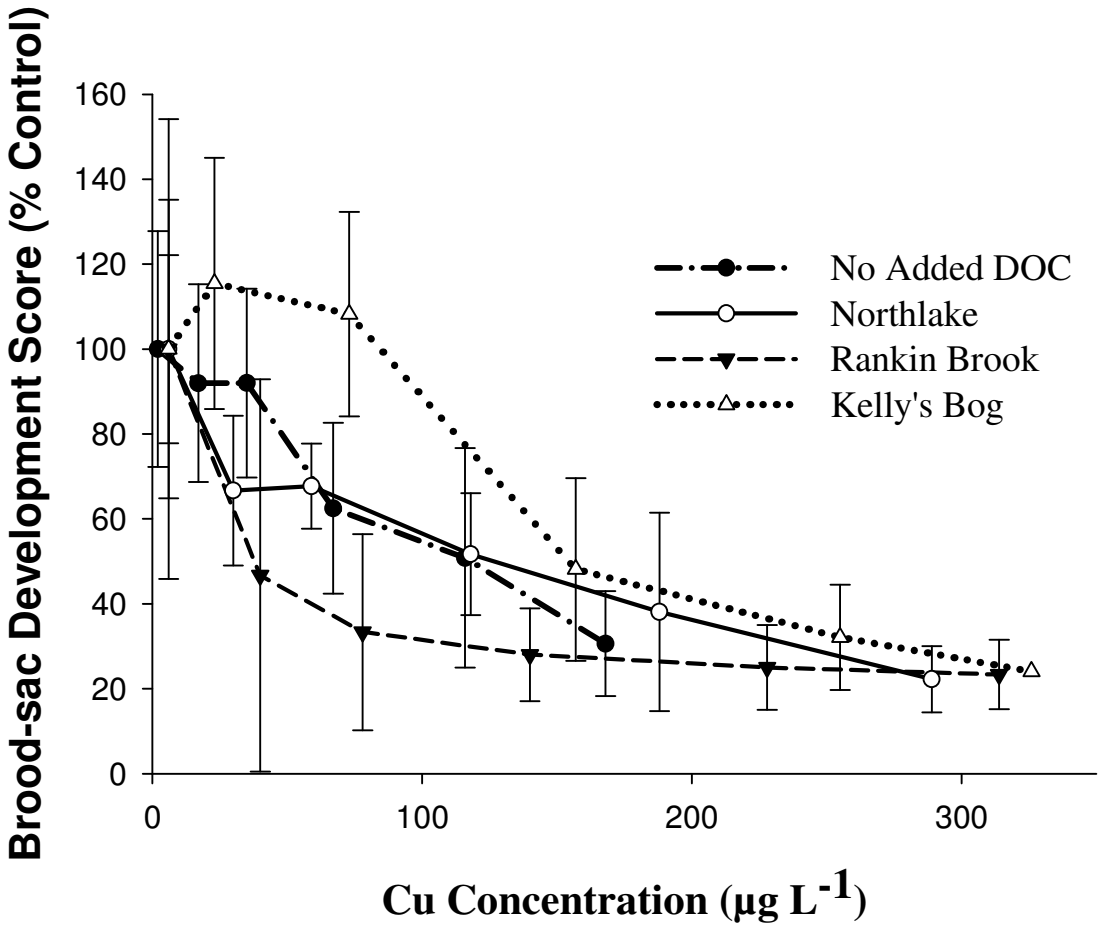

Figure 3.9: Interactive effect of salinity (25ppt) and DOM (4 mg C/L) on sexual maturation score. Brood-sac development is illustrated as a % of control.

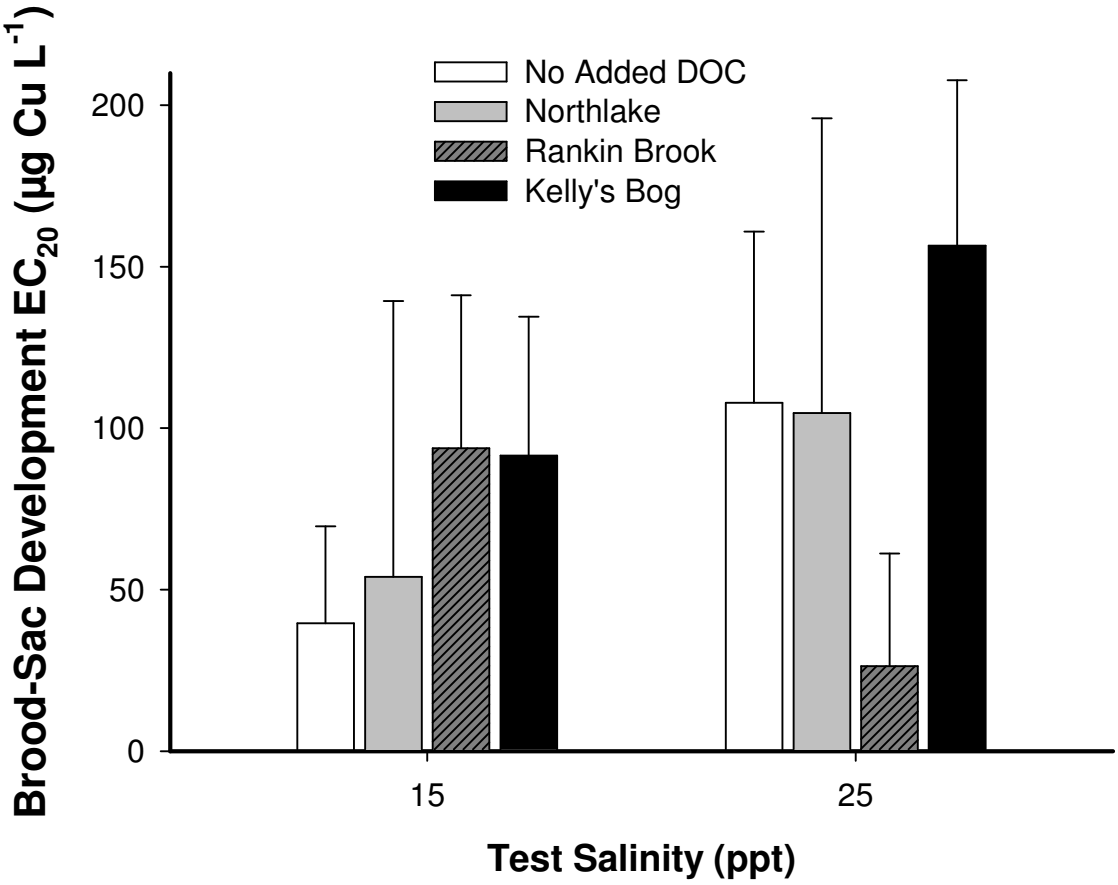

Figure 3.10: Effects of salinity and DOM (source) on effect concentration 20 for sexual maturation.

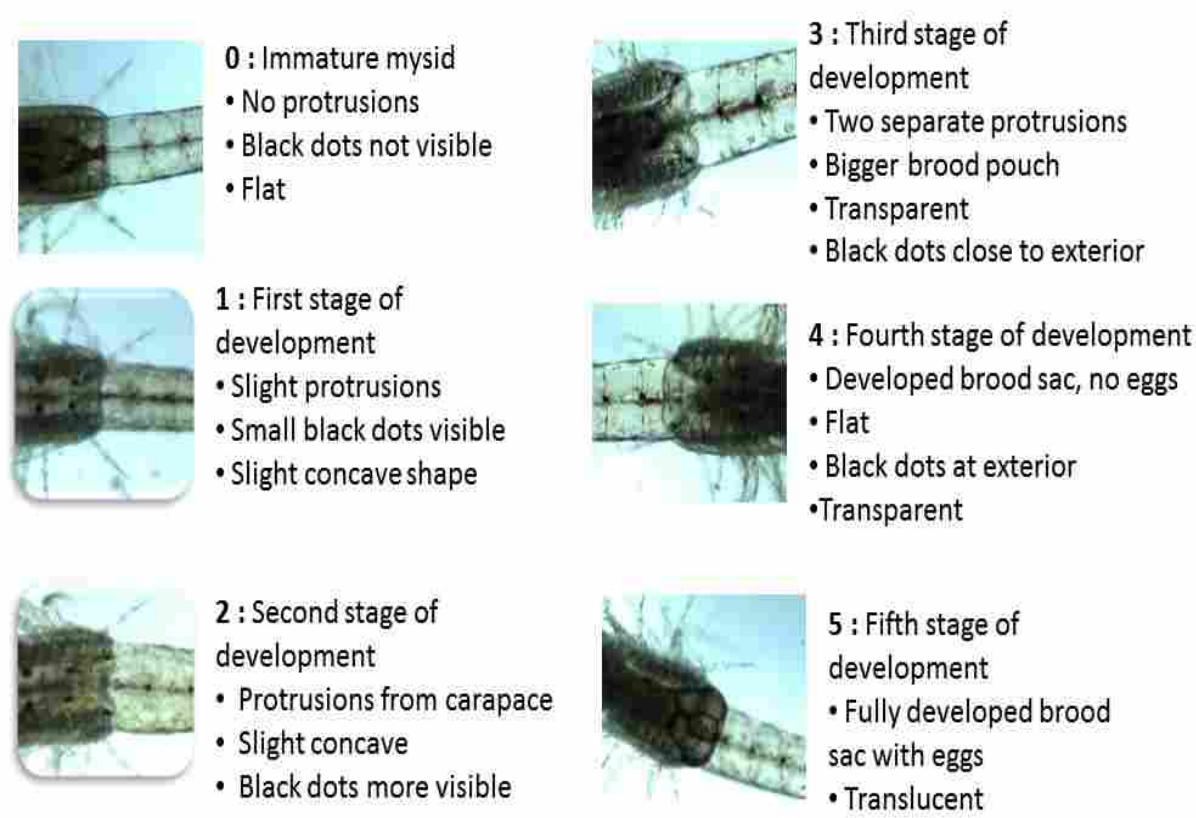

Figure 3.11: Brood-sac development score (as a measurement of sexual maturation score). Brood sacs are rated on a scale from 0 (immature) to 5 (fully mature female with eggs in the brood sac).

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Chapter 4

Establishing a Provisional BLM for Cu in Estuaries: Influence of Salinity and DOM Quality

4.1. Cu Toxicity: The Relative Importance of Cu bioavailability vs Osmoregulatory Demands in Changing Salinities

A wide range of salinities ranging from near fresh water (5 ppt) to marine (40 ppt) were tested in this thesis to gain a complete and thorough understanding of the impacts of Cu toxicity across different salinities. As salinity was increased from 5 ppt to 15 ppt a strong protective effect of salinity was observed against Cu toxicity. This protective effect was attributed to an increased in the Na^+ and Cl^- ion concentrations (Blanchard et al., 2006; Arnold et al., 2005, 2010). An increase in salinity results in a decrease in Cu bioavailability as high Na^+ concentrations provide an increased competition to the labile Cu^{2+} for uptake at the biotic ligand. Cu^{2+} is further rendered un-available due to complexation with Cl^- ions and overall higher Cu concentrations are required to result in a toxic action. The protective effect of salinity, however, was not linear, as a plateau (20-30 ppt) and then a decrease was observed in protection at higher salinities (>30 ppt). This plateau and tje increase in Cu sensitivity observed in the results from this study may have resulted from increased physiological stress and disruption of osmoregulation, as described previously in Killifish (Grosell et al., 2007), where increased sensitivity was observed at salinities lower and higher than its iso-osmotic point (10ppt), the same effect was observed for the copepods *E. affinis* (Hall et al. 2008) and *A. tonsa* (Pinho et al., 2010).

While many details regarding osmoregulation are worked out for different estuarine fish species (e.g. Marshall and Edwards, 2013; Wood et al., 2011). There remains a need to study the physiological mechanisms required to maintain internal osmotic balance by different invertebrate organisms across estuarine salinities. It is well established that Cu toxicity occurs through disruption of Na^+/K^+ ATPase and an overall disruption of ion regulation (Grosell et al., 2007). At

salinities below the iso-osmotic point, *A. bahia* would experience a loss of Na^+ ions to the external environment due to diffusion as well as a disruption of Na^+/K^+ ATPase, resulting in a decrease of Na^+ ions causing death. Above the iso-osmotic gradient, an influx of Na^+ ions would occur inhibiting of Na^+/K^+ ATPase resulting in a toxic effect. However, at iso-osmotic point, where minimal ion-regulation is required, a plateauing effect was observed for *A. bahia* around 20-30 ppt (iso-osmotic point at 25 ppt) illustrating decreased toxicity.

To further explore effects of salinity on organism physiology and acclimation experiments were conducted at 15 and 25 ppt. Acclimation to a salinity prior to testing has been shown to decrease toxic effects of Cu (Adeyemi et al., 2012), however, no such effects of acclimation on toxicity were found in this study. Acclimation to lower or higher salinities is supposed to result in an increased tolerance for Cu exposure as a result of increased expression of $Na^{+/}K^+$ ATPase (Adeyemi et al., 2012), but this phenomenon was not observed as acclimation to 15 ppt did not significantly affect survival at test salinities. This lack of change might be due to the fact that 15-25 ppt is the optimal salinity tolerance limit for *A. bahia,* so acclimation at either salinity will result in similar toxicity.

Short-term chronic tests were conducted at only 15 and 25 ppt, as higher and lower salinities negatively affect reproduction and growth in controls. Biomass as well as brood-sac development score were determined to be more sensitive end-points than survival, for the 7-day toxicity tests, as an increase in salinity provided a significant protective effect from Cu toxicity. This increased sensitivity to salinity at 15 ppt may be attributed to greater energy demands for maintenance of internal ionic-osmotic balance, which would divert energy resources from processes related to sexual maturation and growth.

According to the current fresh water BLM, toxicity prediction is simply a factor of metal speciation in aquatic systems (Paquin et al., 2000; Di Toro et al., 2001; Santore et al., 2001). However, a key findings in this study is hat the same does not hold true for Cu toxicity in estuarine and marine systems. In conclusion, toxicity in dynamic environments, such as estuaries, may be primarily governed by physiology in comparison to Cu geochemistry. Future estuarine toxicity prediction models need to incorporate the wide array of physiological differences present in estuarine organisms.

4.2 DOM Quality: Spatial Considerations in the Development of a Cu BLM in Estuaries

DOM is a well-established toxicity modifying factor in both fresh and marine environments (De Shamphelaere et al., 2004; Arnold et al., 2005). However, its protective capacity in estuarine system has not been completely studied, which was the basis of this research. Environmentally relevant salinities (15 and 25 ppt) for *A.bahia* were selected to test protective effects of DOM sources/quality on acute and chronic Cu toxicity. To accomplish these objectives optical characterization of DOM was completed. Fluorescence analysis was carried out to determine the four fluorescent components (humic-like, fulvic like, tryp and tyr). SAC_{340} and FI were also calculated to determine the origin of DOM sources and finally all these quality indices were related back to toxicity end-points. During acute exposures, addition of 4 mg C/L provided significant protection from Cu toxicity for some DOM sources (15 and 25 ppt: Kelly's Bog and Rankin Brook) while others did not provide any protection. A similar trend was observed for the chronic exposures, but no significant source dependent protection was observed for survival, growth or sexual maturation at 25 ppt.

Increasing salinity from 15 to 25 ppt did not provide significant protection of DOM at 4 mg C/L for 96 h acute Cu toxicity. Overall, DOM was much more protective at 15 ppt for both acute and chronic exposures than a higher salinity of 25 ppt, a trend similar to what has previously been observed (Nadella et al., 2013; Cooper et al., 2013). The lack of a linear increase in protective capacity of DOM as salinity is increased from 15 to 25 ppt could be due to salt-induced colloid formation. As DOM-DOM interactions increase with increasing salinity, this decreases the overall Cu-binding capacity of DOM molecules for Cu. This results in a plateauing effecting and prevents a linear increase in protection offered by DOM (Figure 4.1). Similarly, this plateauing effect has also been observed when DOM concentrations are increased at higher salinities (Nadella et al., 2013; Cooper et al., 2013). This result is important for a better understanding of DOM speciation and metal-DOM interactions across the salinity gradient, as this defines the toxicity prediction of Cu when DOM is present in aquatic systems. When considering an estuarine BLM, this salinity-DOM interaction will allow for a more accurate prediction of Cu toxicity and combined protective effects of salinity as well as DOM at higher salinities.

Since DOM is a strong ligand for Cu and an effective toxicity modulator, another relationship between DOM concentrations and predicted/expected LC50 can be extrapolated from the data. This relationship was determined to relate LC_{50} values obtained in this study to DOC concentrations where LC_{50} ($\mu g\ L^{-1}$) = $319.19DOC^{0.1026}$. Similar relationships between LC_{50} and DOC have been observed in other marine organisms (Arnold 2005; Arnold et al., 2006, Arnold et al., 2010; DePalma et al., 2011) and provide reasonably accurate predictions of metal toxicity based only on DOM concentrations. This has important implications, as toxicity can simply be predicted for a species based on the DOM concentrations for the environmental site. A

wider range of DOC concentrations need to be studied using *A. bahia* to determine a robust equation, representative of the relationship between toxicity and DOC concentrations.

Correlations were also calculated between quality indices and toxicity end-points. All four sources of DOM were determined to be of similar composition as they exhibited a very similar fluorescence profile. Significant correlations were found between LC_{50} values and HA and FA (acute: $r = 0.95$; chronic: $r = 0.99$; $p<0.05$) concentrations. A strong positive correlation was found between acute LC_{50} values and SAC_{340} (color of DOM; 19.6-27.5) values, indicating that SAC_{340} is a good measure in predicting toxicity. A strong negative correlation was also determined between acute LC_{50} values and FI (origin of DOM; 1.06-1.31) indicating that FI may be used to determine the origin of DOM sources. Correlations between SAC_{340} and FI were also present for chronic toxicity tests, however, these were not as strong as acute toxicity. The optical quality index correlations while strong were not significant as they cover a very narrow range of possible values for each index (SAC_{340}: 3 – 80 (Al-Reasi et al., 2011); FI: 1-1.9 (McKnight et al., 2001)) and the same was true for fluorescence data as humic, fulvic and proteinaceous content of the four sources was 95 percent similar. All sources were collected from un-disturbed sites, representative of DOM entering estuaries, but the optical similarity of all DOM sources was unexpected given the geographical distances between sampling locations with in eastern Canada. Tait et al. (2013) found similar results when a wide variety of sampling locations comprising of both disturbed and undisturbed sites from the same geographical location (eastern Canada) were tested for their protective effects on Cu toxicity to rotifers. This suggests that at least on eastern Canada shores the DOM entering estuaries may relatively homogenous. In order to determine if quality of DOM really affects Cu toxicity, a wider range of DOM sources, that have been confirmed to be optically different, need to be tested.

Overall, the results from this study in conjunction with previous literature suggest that while protection warranted by DOM is a good predictive measure of metal toxicity, it may not source dependent in estuarine systems. This means that toxicity prediction models for estuarine environments may not need to incorporate a DOM quality factor into an estuarine-Cu-BLM for Cu toxicity predictions.

4.3 An Overview of an Estuarine BLM for Determining Cu Toxicity.

A thorough understanding of Cu interactions with inorganic and organic ligands in estuaries, and an understanding of the physiology of euryhaline organisms is necessary and is need to better understand how Cu adversely affects aquatic biota. This is one of the first studies to examine how Cu toxicity varies across the full range of salinity gradient in osmoregulating invertebrate, the copepod *A. bahia*. With respect to estuarine organisms and their wide range of osmoregulatory capabilities, physiology is pivotal to understanding the effects of salinity on Cu toxicity for development of water quality guidelines and criteria. The data from this study also contributes towards updating the species sensitivity distribution curve for euryhaline organisms. *A. bahia* is an iono-regulator, similar to several fish as well as estuarine and marine invertebrates, and this data will help regulators and aquatic toxicologists estimate/predict toxicity for other iono-regulators for which toxicity has not yet been tested.

The prediction of Cu toxicity is further complicated by presence of DOM as demonstrated in this study. DOM collected from a wide range of geographical areas had a similar chemical composition and provided similar protection against Cu toxicity. This findings suggests that as far as estuaries are concerned total DOM concentration alone may be sufficient for future toxicity predictions and developing an estuarine Cu-BLM. However, the interactive effects of

DOM and salinity also need to be understood when predicting toxicity in estuarine and marine systems because as salinity increases, colloid DOM may in fact increase Cu bioavailability. In other words, at higher salinities DOM exhibits a reduced protective effect, as a result of salt-induced colloid formation. Such interactions between salinity and DOM are critical for development of toxicity prediction models.

Future research should focus on using a wider geographical range of DOM samples to determine difference in DOM across estuaries. Sources that exhibit optical differences in DOM composition, can be used to conduct toxicity tests to determine site/quality dependent effects of DOM. Tests should also be conducted with organisms spanning a wider range of osmoregulatory capacities, to determine how Cu toxicity differs amongst osmoconformers, osmoregulators etc. Finally, bioaccumulation of Cu at different salinities with different DOM sources and concentrations needs to be determined to relate critical accumulation (LA_{50}) to toxicity (LC_{50} and EC_{50}). All of these tests will contribute to a more robust understanding of Cu toxicity in estuaries and will help create more accurate toxicity prediction models for development of guidelines/criteria.

Figure 4.2 depicts a proposed schematic of an estuarine BLM for Cu toxicity. Cu^{2+} is centered as the most labile form resulting in toxicity. DOM interacts with Cu to form organic bonds rendering Cu less bioavailable, reducing toxicity. Salinity also affects Cu^{2+} bioavailability through complexation with Cl^- ions and more importantly through competition with Na^+ ions, both of which result in reduced uptake of metal at the biotic ligand. Simultaneously a DOM and salinity interaction also occurs, resulting in salt induced colloid formation at higher salinities which also alters the concentrations of free Cu in surrounding system. Finally, changes in salinity result in critical alteration in the physiology (osmoregulatory changes). This results in a feedback

loop changing Cu uptake as a derivative of physiological reaction to the salinity (i.e osmoregulation).

In conclusion, the aim of this study was to help improve the understanding of Cu toxicity in estuarine environments. As estuaries present a very dynamic environment it was determined that both the geochemistry and biology (osmoregulatory mechanisms) of the organisms determine toxicity. The study successfully explored and defined how salinity affects Cu toxicity in estuarine environments. Moreover, the interactive effects of salinity and DOM on toxicity were also examined over a range of estuarine salinities. The data from this study and future studies will assist with the development of estuarine toxicity prediction models such as BLM, which can be used to set guidelines/criteria.

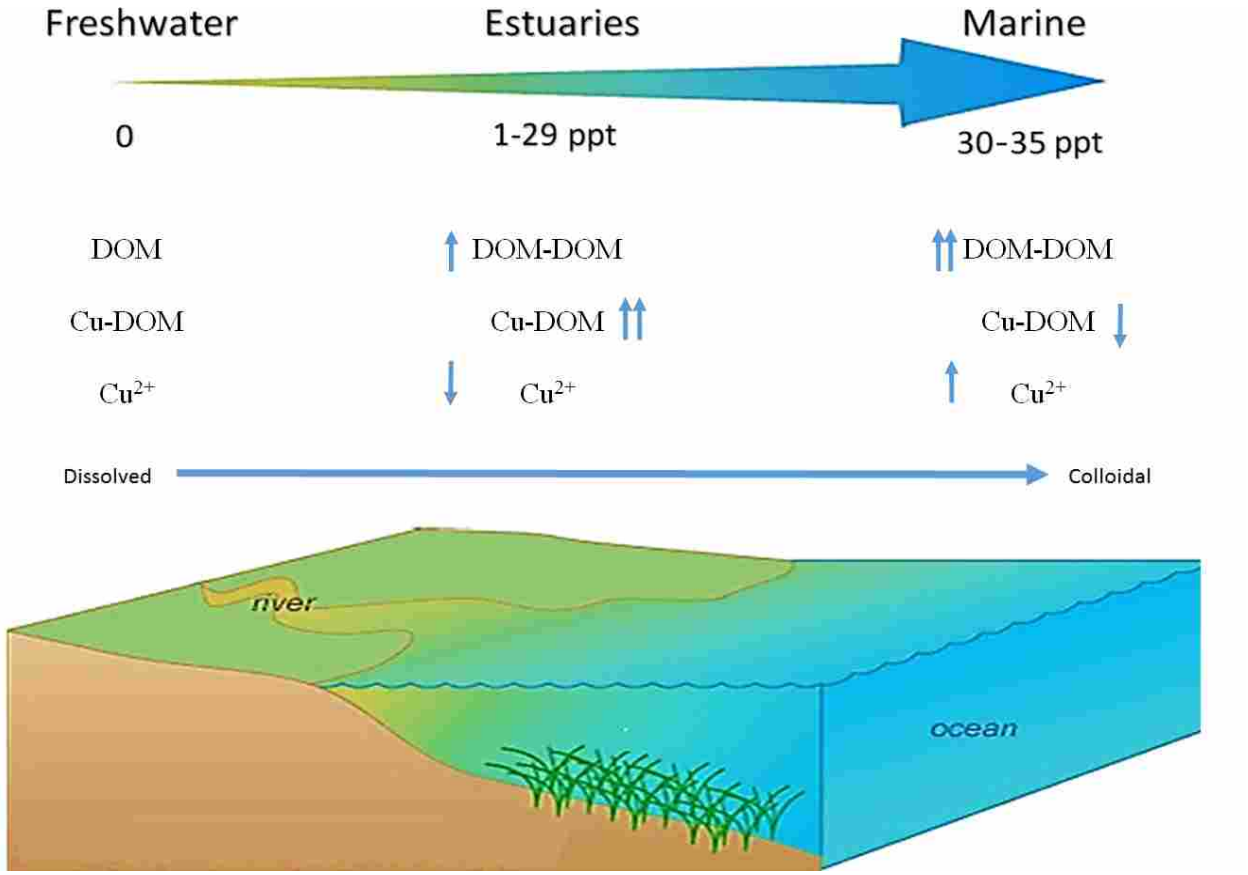

Figure 4.1: A schematic diagram of DOM speciation across the salinity gradient in aquatic systems. Copper is used to illustrate how salinity changes DOM interactions/phases: metal bound and dissolved in fresh water to DOM aggregates and particulate as a result of induced colloid formation. This change in form, from dissolved to colloidal, result in increased Cu^{2+} bioavailability as a result of decreased metal-DOM binding and this increases metal toxicity.

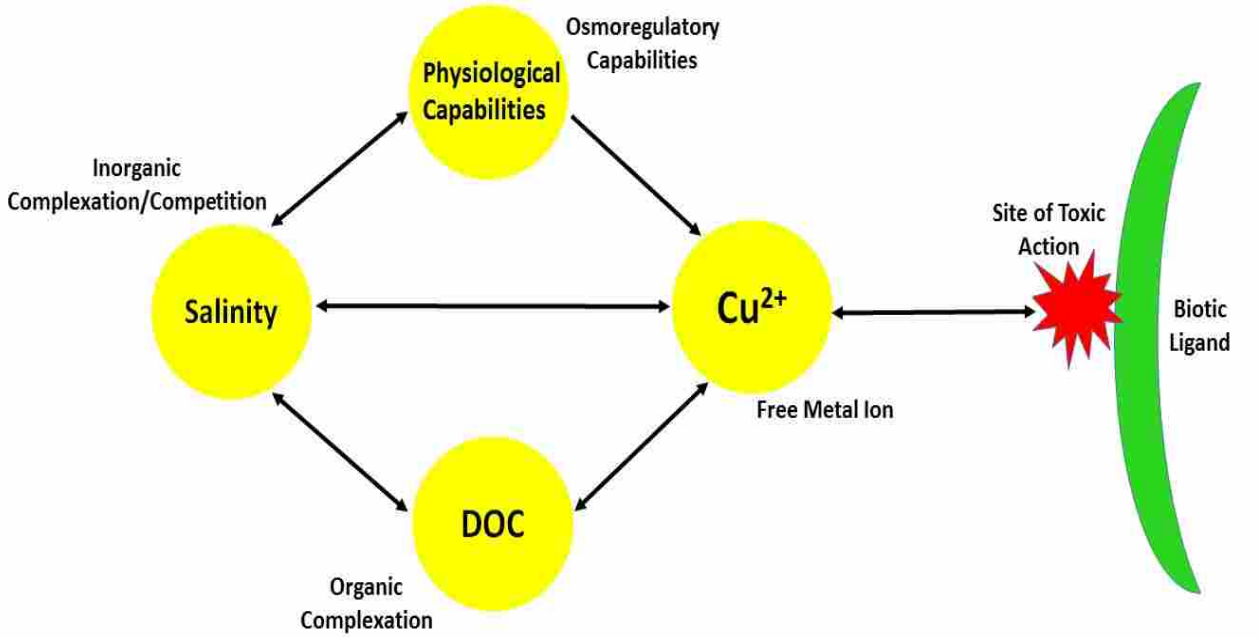

Figure 4.2: Schematic representation of a possible estuarine BLM. Toxicity is defined to be a derivative of metal speciation, which is altered by both inorganic and organic interactions, that results in changing metal bio-availability. In estuarine systems however, a wide array of physiological mechanisms (i.e osmoregulatory capabilities) play a major role in further altering metal toxicity. Therefore, physiology is proposed as a further component in addition to geochemistry (salinity and DOM) to consider when determining the extent of Cu toxicity in estuarine environments.

4.4 References

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