Improving Our Understanding of Cu Toxicity in Estuaries: The Effects of Salinity and Dissolved Organic Matter on Cu Toxicity to a Euryhaline Hydroid (*Eudendrium carneum*)

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Abstract

Currently, the effects of contaminants to estuarine biota are poorly understood. Estuaries are water bodies with salinities intermediate between fresh and salt water. It is important to understand how toxicity in estuaries may vary from freshwater or marine systems to adequately protect estuarine species. A common toxicant in estuaries is copper (Cu), which enters via both natural and anthropogenic sources. This research project developed an acute 48 hour toxicity test that measured sub-lethal and lethal response using a scoring system based on hydranth morphology. This project will help to better our understanding of the effects of intermediate salinities on Cu toxicity by using an estuarine hydroid, *Eudendrium carneum*. Small colonies of this hydroid were exposed to nominal concentrations of 10, 20, 35, 50, and 100µg/L of Cu at salinities of 30, 25, and 15ppt for 48 hours. Additionally, this hydroid was exposed to Cu at a salinity of 25ppt with 5mg/L of dissolved organic matter (DOM) for 48 hours to determine the effects of a common toxicity modifying factor in estuaries. The EC_{50} value at 15ppt was determined to be significantly different from 25 and 30ppt. There was a significant difference in the LC_{50} values between 15 and 25ppt. The EC_{50} and LC_{50} values at 48 hours at 25ppt with 5mg/L of dissolved organic carbon (DOC) were significantly different from no added DOC 25ppt. These results show that as salinity decreased, the sensitivity of this hydroid to Cu toxicity increased. In addition, DOM provided a significant protective effect against sub-lethal and lethal Cu toxicity. Understanding how toxicity changes in estuaries is invaluable in creating adequate water-quality guidelines for estuaries.

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1. Introduction

There is relatively little information on the effects of metals to estuarine biota. Previous research has shown that as salinity increases, toxicity to metals decreases and that osmoregulation is an important aspect of determining Cu toxicity in estuaries (Hall and Anderson, 1995; Grosell, *et al*., 2007; Pinho and Bianchini, 2010). As in freshwater systems dissolved organic matter, which has been shown to reduce toxicity in freshwater and saltwater, is present in various concentrations. This research project investigated the effects of salinity and dissolved organic matter on Cu toxicity to a euryhaline hydroid. The purpose of this project was to contribute to the understanding of how Cu toxicity varies in estuarine environments using an understudied species.

1.1 What is an Estuary?

Estuaries are semi-enclosed coastal areas that are connected to both freshwater sources (e.g. streams and rivers) and an ocean. These connections serve as sources for the influx of fresh and saltwater, which are responsible for the variable salinities characteristic of estuarine environments (Pritchard, 1967). Salinities in estuaries range between 0.5-35ppt and can change daily with the tides (Kennish, 2002). Water in these environments is often referred to as brackish water due to its intermediate salinity. A few well-known estuaries include the St. Lawrence estuary in Eastern Canada, the Hudson River estuary in the state of New York, and the Fraser River estuary in British Columbia.

Estuaries serve as natural filters removing sediment and nutrients from water, and large sources of organic matter (US EPA, 2006). Estuaries are habitats for many different species and

many marine species depend on estuaries at certain stages of their life cycle (US EPA, 2006). Estuaries are hubs of activity for fishing especially in the United States: with greater than 75% of commercial fish catches made in estuaries as well as over 75% of recreational fish catches in 1998 (US EPA, 2006). In addition, sea ports found in estuaries are used in international trade and commerce; two-thirds of the largest cities globally are found on/near estuaries (Ross, 1995).

1.2 Sources of Contaminants in Estuaries

The usage and proximity of estuaries to large cities subjects them to many environmental stressors that include pollution, overfishing, loss of wetland habitat, and habitat destruction (US EPA, 2006). Examples of pollutants in estuaries introduced by industrial and agricultural inputs include tributyltin, methylmercury, cadmium, lead, copper, and polycyclic aromatic hydrocarbons (Kennish, 2002). These contaminants do not necessarily have to enter estuaries directly from their source, since the contamination of upstream freshwater sources will carry contaminants downstream to the estuary.

Inputs of Cu from natural sources range can from 0.03 to 0.23µg/L in surface seawater and 0.2 to 30µg/L in freshwater systems (US EPA, 2003). Anthropogenic sources of Cu in estuaries including mine tailings, discarded electrical equipment, and anti-fouling paints (Patterson *et al*., 1998). Cu input from mine tailing discharge is usually less than 100µg/L; however, in some areas Cu input from discharges can be as high as 200,000µg/L (US EPA, 2003). Anti-fouling paints are used to prevent the adhesion and growth of aquatic organisms to the hulls of ships and their use is widespread because a fouled hull results in decreased speed, increased fuel consumption, and damage to the hull over time (Kennish, 2002).

1.3 The BLM and Metal Toxicity in Estuaries

There is a general lack of information on how contaminants affect estuarine biota. Due to tidal influences and the large presence of organic matter, estuaries are complex and dynamic environments that differ from freshwater and marine ecosystems. A method for trying to understand how these environmental factors affect metal toxicity to aquatic organisms is using the biotic ligand model (BLM). Generally, the BLM is used to make predictions on metal toxicity based on environmental factors in freshwater systems. The predictions the BLM makes for estuarine systems may not be accurate but it can still give an idea of how metal toxicity varies with different estuarine environments.

The BLM is used to predict metal toxicity to freshwater organisms based on the presence of different toxicity modifying factors (TMFs) in the environment (Figure 1). These TMFs include cations, anions, and dissolved organic carbon (DOC). Cations compete with metals for uptake into organisms, whereas anions and DOC bind to metals forming inorganic or organic complexes. Each of these can cause a reduction in Cu toxicity.

The BLM leads to the hypothesis that as salinity increases, Cu toxicity decreases. As salinity increases, the presence of Na^+ and Cl^- ions in solution increases. Na^+ acts as a competitor with Cu^{2+} for uptake into the organism and Cl^- forms inorganic complexes with Cu^{2+} which decreases bioavailability and in turn Cu toxicity (Arnold *et al*., 2005). Other studies have shown that generally, as salinity increased, toxicity to metals (including Cu) decreased (Hall and Anderson, 1995; Pinho and Bianchini, 2010; Grosell, *et al*., 2007; Nadella *et al.*, 2009). While Cu can be found as part of other compounds (e.g. $CuOH^+$, $CuHCO_3^-$, and $CuCO_3$), the focus of

the BLM is on Cu^{2+} because it is the form of Cu toxic to organisms ($CuOH^{+}$ is also a toxic form of Cu) (Grosell *et al.*, 2007).

Predictions about how DOC affects toxicity using the BLM propose that DOC will complex with free Cu ions in solution, which reduces the bioavailability of Cu (i.e. the amount of copper available to bind to the biotic ligand) (Arnold *et al.* 2005). This decrease in bioavailability should decrease the toxicity of Cu to estuarine organisms. DOC is a component of dissolved organic matter (DOM), which can be found in most aquatic environments.

1.4 Water Quality Guidelines and Estuaries

The USEPA sets water quality guidelines (WQGs) for metals based on dissolved concentrations of these metals. The only TMF that is considered is hardness in freshwater, while other components of water that affect metal bioavailability like DOM are not included (De Polo and Scrimshaw, 2012). Currently, the Canadian Council of Ministers of the Environment (CCME) has no WQG for marine or estuarine systems. To adequately protect estuarine species, understanding how metal toxicity may vary in different estuarine conditions is of paramount importance.

This study will look at understanding how salinity and dissolved organic matter affect Cu toxicity to the colonial hydroid *Eudendrium carneum*. The response of this hydroid to Cu, a common metal in estuaries, at different salinities will be quantified by using a morphological scoring system adopted from freshwater hydra (Trottier *et al*., 1996). In addition, the effect of DOM on the toxicity of Cu to this hydroid will be evaluated.

2.0 Materials and Methods

2.1 Test Organism and Culturing

E. carneum is a euryhaline hydroid from the phylum Cnidaria. This hydroid is found in estuaries in many areas including Atlantic Canada, the east coast of the United States, south-eastern Australia, the west coast of India, the Mediterranean Sea, and the east coast of South America, eastern Africa, and the western Mediterranean (Calder, 1989; Calder and Mayal, 1998; Calder 2010; Karlson and Osman, 2012; Nagale and Apte, 2013; Pecarevic *et al.*, 2013; Bavestrello and Piraino, 1991).

E. carneum is a colonial hydroid with an arborescent colony structure. Specialized polyps grow from the branches and take on a variety of forms (Figure 2). The male sporosacs and female gonophores are specialized polyps used for sexual reproduction (Bavestrello, 1991). Asexual reproduction by budding off of the main colony forms their tree-like appearance (Calder, 1989). The other specialized polyp is a feeding structure called a hydranth. Hydranths have a round, elongated body with 26-32 tentacles attached in a single whorl to the body and a trumpet shaped hypostome in the centre (Kelmo and De Santa-Isabel, 1998). Polyps among colonies are all connected by the coelenteron which is the gastric cavity of coelenterate.

This hydroid was chosen as the test organism for this research project because of its tolerance to a range of salinities (10-35ppt), wide distribution, and predicted sensitivity to metals. The predicted sensitivity of this hydroid to metals (e.g. Cu) was based on the fact that other marine hydroids are sensitive to metal toxicity (Stebbing, 1979; Stebbing, Stebbing, 1981). The predicted sensitivity of hydroids is also because they present a unique exposure system, in that

all of their cells are exposed to either the external medium or the water in the coelentreron (Schlichter, 1984). This continuous fluid-filled space allows for rapid exchanges of ions and nutrients to all cells (Schlichter, 1984). Colonies of *E. carneum* used for this research were provided by Gulf Specimen Marine Laboratory (Panacea, Florida).

Colonies of *E. carneum* were cultured in 10-20L tanks. Cultures were maintained with a temperature of 21^{o}C, a 16:8 day/night cycle, constant water movement, daily water changes of 20%, and daily feeding. Salinities were maintained by mixing Kent Sea Salt® with de-ionized water and adding this to the culture tanks. *E. carneum* colonies were fed once daily with *artemia* nauplii by mixing 50 mL of *artemia* solution with approximately 1.8L of salt water in a 2 L dish or beaker and submerging colonies in these solutions for 20 minutes. Solutions were stirred every 5 minutes to prevent clusters near the edges of the dish/beaker. Hydranths that had ingested any *artemia* are readily identifiable by their darkened and swollen appearance. *Artemia* were cultured in a 1L vessel at a salinity of 25ppt with 1g of *artemia* cysts. Aeration and water movement were used to prevent *artemia* cysts from accumulating at the bottom of the vessel.

It is important to note that the cultures of *E. carneum* are not monocultures. Among the extra inhabitants are skeleton shrimp, various types of algae, and crabs. The algae can be epibiontic (i.e. they grow on the surface of *E. carneum*), and unless treated appropriately will overtake even large colonies of hydroids. It appears that hydroids can tolerate some algal growth but not rampant algal presence.

2.2 Test Method Development

A method for measuring response in colonial hydroids that has yet to be explored is a scoring system based on the morphology changes of hydranths. Karbe (1972) looked at Cu toxicity to the marine hydroid *Eirene viridula* and the response of this hydroid was quantified using colony growth rates, while describing morphological changes in individual hydranths (Figure 4). This scoring system was later adapted for use in the freshwater hydra *H. attenuata* (Figure 3) (Trottier *et al.*, 1996). The freshwater hydra assay has been used with metals (Stebbing, 1979), wastewater (Fu *et al*., 1994), and pharmaceuticals (Karntanut and Pascoe, 2002; Quinn *et al*., 2009). Both of these scoring systems accurately represent the morphological changes in *E. carneum*. The scoring system presented by Trottier *et al*., (1996) will be used to assess toxicity due and the changes in hydranth morphology in *E. carneum* are given in Figure 5.

Previous test methods on colonial hydroids relied on large colonies that could be upwards of 300 individuals (Stebbing, 1981), which would make trying to identify morphological changes in single hydranths difficult. In *Campanularia flexuosa*, colony growth rate, gonozooid frequency, and stolon curves per colony were used as measures of toxicity (Stebbing, 1979; Stebbing and Santiago-Fandino, 1983). Colonial growth rate was found to decrease rapidly at 10-20µg/L of Cu (Stebbing and Santiago-Fandino, 1983). *C. flexuosa* colonies experienced an increased production of gonozooids when exposed to 0.1µg/L of Cu and that colony growth rate decreased when exposed to greater than 10µg/L of Cu (Stebbing, 1979). Stolons typically radiate linearly from the base of the hydroid, but stress causes the stolons curve, where the radius of the curve is inversely proportional to toxicity (Stebbing, 1979). This provides evidence that marine hydroids can be sensitive to low metal concentrations and shows what responses to toxicants in

colonial hydroids. In order to use the hydranth scoring system, the development of a new test design was required that allowed for morphological changes on individual hydranths to be readily identifiable. The test design took on a number of different setups to finally come up to a reliable design. The temperature (21^{o}C) was held constant across all experiment trials.

Initially, hydroids with 3-4 hydranths were placed in a 4mL micro-well plate (4x3 wells) and were conducted with and without feeding. Durations of these tests ranged from 48 hours to 11 days. These conditions were synonymous with freshwater hydra methods (Trottier *et al*., 1996). Unfortunately, hydranths in the controls showed regressed.

Next, hydroids were attached to slides and grown for 3 days in the culture prior to testing, after which they were placed in 400mL beakers. This method also showed regression in controls.

After, hydroids attached to microscope slides were grown in the culture tank for 3 days, and then put in 50mL falcon tubes. These hydroids were both fed and unfed but still showed regression in controls.

Another version of the experiment was where hydroids on microscope slides were put horizontally in a 200mL re-crystallization dish. Again, regression in controls was observed.

In the penultimate design, hydroids were fed 24 hours before the test, attached to microscope slides, and put in 50mL falcon tubes. Aeration and water movement was provided by a large pipe and the top of the falcon tube was sealed using parafilm. This method showed little regression in controls, but the design was inefficient and the parafilm did not prevent evaporation and loss of test medium.

It seemed that the critical factor that was missing from the majority of early test designs was water movement and aeration. The test design that shows very limited or no regression in controls, and was used for generating data in for this research, is described in the following section.

2.3 Test Methods

Glassware and materials necessary for making solutions were rinsed in 5% HNO_3 for 24 hours then rinsed thoroughly 3-times with both de-ionized water and epure. Hydroids were acclimated to the test salinity 1 week prior to the test. This was accomplished by increasing/ decreasing the salinity of the culture by approximately 1ppt per day. Hydroids were fed 24 hours prior to the initiation of the test (Trottier *et* al., 1996; Karntanut and Pascoe, 2002; Quinn *et al*., 2008; Quinn *et al*., 2009).

At 24 hours after feeding, hydroids were randomly excised from large colonies and attached to a microscope slide by either string or elastic band (Crowell, 1953; Fulton, 1960). Each hydroid consisted of 6-10 fully formed hydranths since this range allows for an adequate number of observations per replicate. The hydroids on these microscope slides were planar, to avoid any contact with the sides of the falcon tube which may induce hydranth regression (Fulton, 1960). These slides were then placed in a 50mL falcon tube with 50mL of solution. The caps of the falcon tubes were punctured with a large needle through which polyethylene tubing (0.58mm internal diameter) was fed through. Each polyethylene tube was attached to a small

needle, which was then used to puncture and rest inside a tube connected to an air pump to provide gentle aeration and current.

The duration of each exposure was 48 hours. Falcon tubes were kept at room temperature (21^{o}C) on a 16:8 light/dark cycle with constant water movement. Observations were made at 24 and 48 hours at which time scores were assigned accordingly. There were 2 replicates per Cu concentration for a total of 12-20 hydranths per concentration.

The observation times of 24 and 48 hours were chosen because they have been used as standard observation times in freshwater hydra tests (Trottier *et al.*, 1996). Hydranths of *E. carneum* showed similar morphological changes to freshwater hydra, so the same definitions of sub-lethal and lethal endpoints were adopted (i.e. clubbed tentacles and the tulip phase respectively).

2.4 Sampling and Analysis

Nominal Cu concentrations used were 0, 10, 20, 35, 50, and 100µg/L. This range of concentrations presented a varied response to Cu that ranged from minor (i.e. little or no effect) to major (i.e. 100% mortality). Test solution samples of 10mL were taken at the beginning of the test: one sample was for dissolved Cu and the other was for total Cu. To obtain the dissolved Cu water sample, 10mL of test solution was filtered through a 0.45µm filter. These samples were kept at -20^{o}C until they were measured. Cu concentrations were characterized using lanthanum oxide precipitation followed by graphite-furnace atomic-absorption spectrometry (GFAAS). Lanthanum oxide (LaOX) precipitation was performed as in Nadella *et* al. (2009). For LaOX precipitation, 1µL of lanthanum oxide and 7.5µL of 1 M Na_2CO_3 was added. Next, solutions

were placed in a hot water bath (80^{o}C) for 30 minutes after which samples were centrifuged for 15 minutes at 3000 *g*. The precipitate was saved, while the supernatant was decanted, and the precipitate was resuspended in 10mL of 1 N HNO_3. For GFAAS, the accuracy of concentrations were verified by using standard solutions TM15.2 and TM23.4 (Environment Canada Certified Reference Material). Salinities of 15, 25, and 30ppt were used to mimic various estuarine conditions while still satisfying the salinity tolerance of *E. carneum* and these salinities were verified using a YSI conductivity meter.

EC_{50}/LC_{50} values (i.e. the concentration of Cu responsible for 50% of hydranths expressing scores of 3 or less/scores of 1 or less) were used to measure the response in a given test condition. Comparison of these values was used to determine if there was a change in response across salinities or with the addition of DOM. EC_{50} and LC_{50} values were calculated using the software program CETIS (Tidepool Scientific, 2012) and significant difference between these values was determined by the Litchfield-Wilcoxon method.

3.0 Results

For the 25ppt, 30ppt, and DOM exposures all control hydranths showed no effect. In the 15ppt exposure, 11.8% of control hydranths regressed (2 of 17 total hydranths). The total and dissolved Cu concentrations are given in Table 1. The E/LC_{50} values and their associated 95% confidence limits are given in Table 2. An example of survival data plotted against Cu is illustrated in Figure 10 for all salinities used.

3.1 DOM and Cu Toxicity

With the addition of 5mg C/L (i.e. the DOM exposure), there was an increase in both EC and LC_{50} values (Figure 6). In both cases, the protective effect provided by DOM was significant (i.e. a significant increase in E/LC_{50}).

3.2 Salinity and Cu Toxicity

There was a difference in sub-lethal response of hydroids to Cu across the range of tested salinities (Figure 7). Hydroids were more sensitive at 15ppt than at 25 or 30ppt. Dissolved 48 hour EC_{50} values for 15, 25, and 30ppt were 14.1, 21.9, 22.6µg/L respectively. There were significant differences in EC_{50} values between 15 and 25ppt, as well as between 15 and 30ppt.

In addition, there was a difference in lethal response of hydroids to Cu across the range of tested salinities (Figure 8). Similar to the EC_{50} values, hydroids were most sensitive at 15ppt. Dissolved 48 hour LC_{50} values were 19.1, 31.3, and 25.9µg/L for 15, 25, and 30ppt respectively. There was only significant difference between the LC_{50} values at 15 and 25ppt. The LC_{50} value at 30ppt was greater than at 15ppt, but not significantly.

4.0 Discussion

The hypotheses made about the effects of increasing salinity and DOM presence on Cu toxicity based on the biotic ligand model suggest that the BLM is accurate at predicting how Cu toxicity to this hydroid can change based on DOM presence and when salinities increase from 15 to 25ppt; whereas it did not accurately predict the change in toxicity from 25 to 30ppt.

Increasing salinity provided a protective effect against both sub-lethal and lethal Cu toxicity to this hydroid. It seems likely that this is due to the increase of Na^+ and Cl^- ions at higher salinities, i.e. the combination of reduced bioavailability of the Cu free-metal ion by Cl^- binding and the increased competition from Na^+ decreased Cu toxicity when increasing salinity from 15 to 30ppt.

4.1 Physiology and Cu Toxicity in Estuaries

Basing our assumptions on how Cu toxicity changes across salinities solely on the BLM presents an issue. The E/LC_{50} values do not appear to increase at 30ppt when compared to 25ppt like we would expect with the BLM (Figure 7 and 8). This result is in agreement with a general survey of the effects of contaminants (e.g. metals, and organic chemicals) to estuarine biota conducted by Hall and Anderson (1995). In this study, the authors indicated that the physiology of an organism was important in determining toxicity in estuarine environments (Hall and Anderson, 1995). The lowest toxicity in euryhaline species was at their isosmotic point, and reduction in metal toxicity was attributed to low/absence of osmotic stress at the isosmotic point (Hall and Anderson, 1995).

A smaller survey on Cu toxicity to estuarine organisms by Grosell *et al* (2007) also shows that physiology is necessary to understand Cu toxicity to estuarine organisms. Among the toxicity datasets (9 species in total), every species was most sensitive at the lowest salinity tested and the majority of relationship between salinity and LC_{50} values plateaued as salinity increased (Figure 9) (Grosell *et* al., 2007). The conclusion of this paper was that in addition to the BLM

(i.e. abiotic factors), physiology was also important to understand Cu toxicity across salinities, specifically osmoregulation, acid-base disturbance, and ammonia excretion (Grosell *et al*., 2007).

In this same study, the authors also conducted experiments on Cu toxicity across salinities of 0 to 35ppt with the euryhaline killifish, *Fundulus heteroclitus*. These series of tests showed that as salinity increased from 0-10ppt, toxicity decreased and that as salinity increased from 10-35ppt, toxicity increased (Grosell, *et al.*, 2007). These results did not agree with the BLM framework of competing cations and metal complexation with anions reducing toxicity as salinity increased; however, they could be explained. *F. heteroclitus* has an isosmotic point at a salinity of approximately 12ppt. In this fish, Na^+ gradients between the blood and the water are disrupted by Cu in freshwater and sea water; whereas when salinity is closer to the isosmotic point, there is little net movement of Na^+ and therefore reduced Cu toxicity (Grosell, *et al.*, 2007).

Another study by Pinho and Bianchini (2010) found similar results. The test organism was the copepod *Acartia tonsa*, which was exposed to Cu at 5, 15, and 30ppt. A protective effect of salinity was observed from 5 to 15ppt and from 15 to 30ppt; however, the protective effect was less marked for the latter change in salinity (Pinho and Bianchini, 2010). A conclusion from this study was that salinity not only affected Cu bioavailability, but also altered ionic regulation demands in this copepod (Pinho and Bianchini, 2010). The evidence for this conclusion is based on the following: the isosmotic point of *A. tonsa* is 36ppt and this copepod was least sensitive to Cu toxicity at 30ppt (the salinity closest to the copepods isosmotic point). At the isosmotic point, the uptake of ions from water, including Cu^{2+}, is reduced (Pinho and Bianchini, 2010).

Yet another example of the effect of physiology on Cu toxicity is in the estuarine copepod *Eurytemora affinis* (Dr Polo and Scrimshaw, 2012). This study compared the predictions of the BLM over salinities of 0-35ppt versus the actual LC_{50} values at 96 hours. *E. affinis* has an isosmotic point of 10ppt and like in previous studies (Hall and Anderson, 1995; Pinho and Bianchini, 2010; Grosell *et al*., 2007), this copepod was least sensitive at its isosmotic point (De Polo and Scrimshaw, 2012). The LC_{50} values determined by the BLM differed from the LC_{50} values experimentally determined; salinities of 2-15ppt showed a protective effect that differed greatly from the prediction made by the BLM (Figure 12).

No data is available on the isosmotic point of *E. carneum*; however, based on the results of this study and the effects of physiology on metal toxicity reported in other studies it is likely that the isosmotic point for this species could be anywhere between 20-35ppt. This assumption is based on the recorded E/LC_{50} values since these values were almost identical at 25 and 30ppt. This assumption also includes 20 and 35ppt because they cannot be ruled out until tests have been conducted at these salinities. If the isosmotic point is 25ppt, then the increased sensitivity to Cu at 30ppt could be attributed to increased ion (including metal) exchange due to osmoregulatory stress.

4.2 DOM and Cu Toxicity

The presence of 5mg C/L provided a significant protective effect against both sub-lethal and lethal Cu toxicity to this hydroid. The prediction on how the presence of DOM would affect Cu toxicity based on the BLM was accurate. DOM has been shown to have a protective effect against metal toxicity in marine environments (Arnold *et al.*, 2009; Arnold *et al*., 2010; Wang *et*

al., 2011) and in freshwater systems (Kim *et al*., 1999). Arnold *et al.* (2009) investigated the effects of DOM to Cu toxicity to a variety of marine invertebrates and found that toxicity was correlated with DOM concentration.

The protective effect of DOM seems to play a larger role than the protective effect of salinity, at least in the range of 15-30ppt. The sources of DOM to marine environments are less diverse and plentiful than to freshwater environments since freshwater (as well as estuaries) has large allochthonous and autochthonous DOM sources. Highly concentrated DOM would be gradually diluted as it makes its way through estuaries and since salinities gradually increase down estuaries, protection by DOM will become less significant than protection from salinity near the mouth of an estuary. Interestingly, a study has shown that the influence of DOM on Cu toxicity to the mussel *Villosa iris* and the cladoceran *Ceriodaphnia dubia* was different in these species (Wang *et al*., 2011). EC_{20} values for the *V. iris* and *C. dubia* increased by factors of 5 and 17 respectively in acute exposures, showing varied protection by DOM (Wang *et al*., 2011).

The contrast between the BLMs reliability to predict changes in toxicity over a range of salinities and with the presence of DOM could be because the presence of DOM does not alter an organism's osmoregulatory activities like increasing salinity (i.e. Na^+ and Cl^-). In estuaries, more so than in fresh and saltwater, it appears that osmoregulation is important in understanding toxicity in addition to elements of the BLM.

4.3 The Development of an Estuarine BLM

The derivation of a marine and estuarine BLM is important to accurately predict metal toxicity in these environments. At present, a major challenge in the development of marine and

estuarine BLMs is incorporating an organism's physiology, which can be altered by salinity and affect metal toxicity (Hall and Anderson, 1995; Grosell *et* al., 2007; Pinho and Bianchini, 2010; De Polo and Scrimshaw, 2012). While incorporating physiology is important, the shorter-term goal would be to increase the number of species that have been tested. Some studies have suggested organisms useful to validate, calibrate, and help define an estuarine BLM (Pinho and Bianchini, 2010).

An important factor in determining such organisms is their sensitivity (Pinho and Bianchini, 2010). Based on the Cu species sensitivity distribution (SSD) presented by Arnold *et al*. (2005) (Figure 11), and the data from Grosell *et al* (2007) (Figure 13), it appears that *E. carneum* is among the more sensitive organisms tested in marine environments. It is important to add that this is using the test data based on a Cu exposure at 30ppt, not full strength seawater. For the development of an estuarine BLM , ideal test species should be euryhaline (Pinho and Bianchini, 2010). In this study, *E. carneum* was shown to tolerate salinities of 15-30ppt. The tolerable salinities of this hydroid are extended to 9-32ppt by surveys conducted along the east coast of the United States (Calder, 1990). These characteristic of *E. carneum* make it an ideal species to investigate effects of metals in estuarine environments.

Lastly, *E. carneum* is an ideal species for continued toxicity testing and development of an estuarine and marine BLM because hydroids, though ubiquitous, are not included in either of the two datasets above (i.e. Grosell *et al* 2007 and US EPA 2003). The data provided by the US EPA includes mussels, scallops, oysters, various species of fish, sea urchins, polychaete worms, squid, copepods, mysids, lobsters, shrimps, crabs and minnows (US EPA, 2003). The data

included in the summary by Grosell *et al* (2007) is an extension of the data from the US EPA (2003) and adds data on snails, shrimp, killifish, a prawn, and various sea urchins. The study by Hall and Anderson (1995) used mostly (70%) crustaceans or fish, and the remainder included species of molluscs, annelids, phytoplankton, and fungi. This lack of representation of hydroids, together with their sensitivity and wide salinity tolerance, make *E. carneum* an excellent species for continued progress towards an estuarine and marine BLM.

5.0 Conclusion

This study has demonstrated the sensitivity of a euryhaline hydroid to Cu. It has also demonstrated that, in agreement with other studies, the relationship between salinity and Cu toxicity is also dependent on an organism's physiology. This complex relationship is evident because of the lack of continuity in BLM predictions from 25 to 30ppt. *E. carneum* presents an excellent species for further study in estuarine environments because of its sensitivity to Cu, global distribution, wide salinity tolerance, and underrepresentation in toxicity datasets.

6.0 Future Directions

This study would benefit from toxicity data generated for 10, 20, and 35ppt to highlight how Cu toxicity varies across this hydroids tolerable salinity range. This data would also help to determine the isosmotic point for this hydroid. In addition, testing the effect of DOM at different salinities would help to understand what relationship salinity and DOM have that may affect Cu toxicity. The sensitivity of this hydroid to Cu could mean that this hydroid is also quite sensitive

to other metals (e.g. Ni and Zn). Applications of this test design could also be extended to effluent and more sources and types of DOM.

7.0 Figures

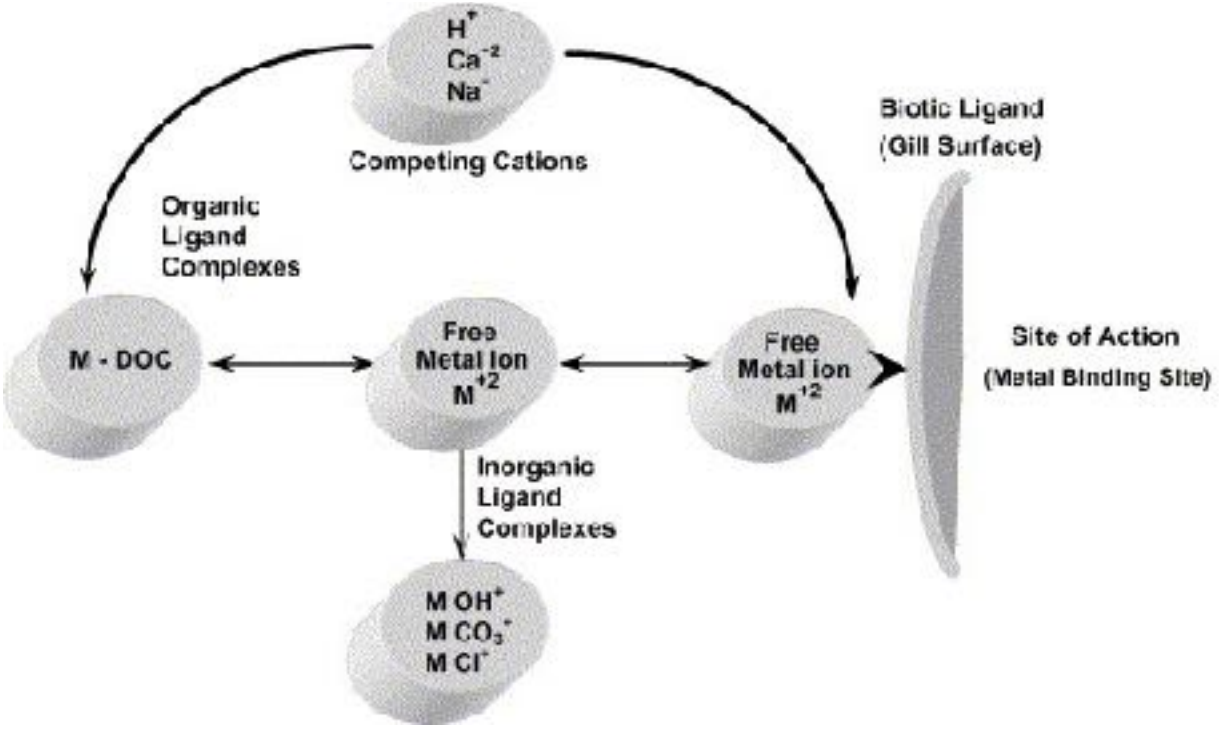

Figure 1. This figure represents the BLM for freshwater systems; it explains the interactions of free-metal ions with TMFs in the environment (Arnold *et al.*, 2007). The biotic ligand is a receptor where metals bind to produce acute toxicity responses.

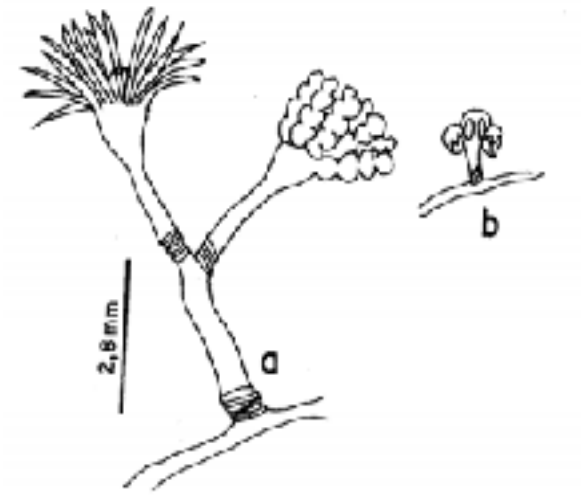

Figure 2. Diagram *a* shows a normal hydranth on the left and a male gonophore on the right, and diagram *b* shows a female gonophore (Kelmo and De Santa-Isabel, 1998).

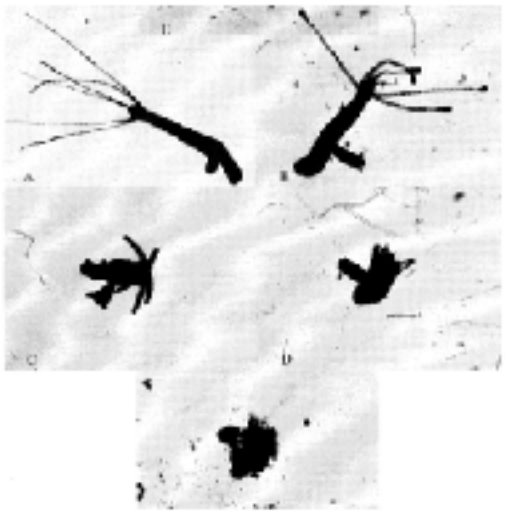

Figure 3. This figure shows the morphological changes in *Hydra* (Trottier *et al*., 1996). A: a normal *Hydra*; B: tentacles clubbed at the ends, the sub-lethal endpoint; C: tentacles are shortened; D: the tulip phase; E: the organism is disintegrated.

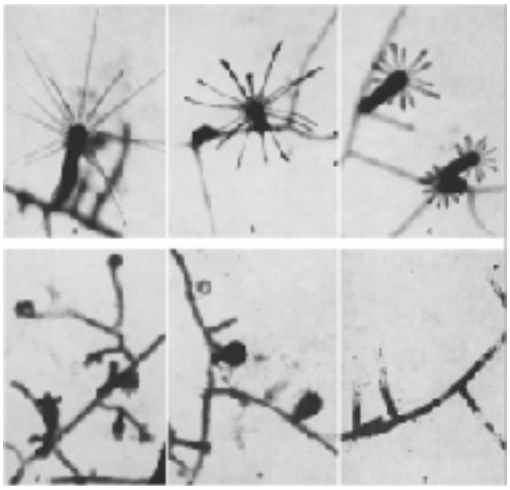

Figure 4. This figure shows the stages presented by Karbe (1972) in *E. viridula*. A: a normal

hydranth; B: hydranth with clubbed tentacles; C: hydranth with shortened tentacles; D: hydranth in the tulip stage; E: hydranth as a small bud; F: hydranth disintegrated.

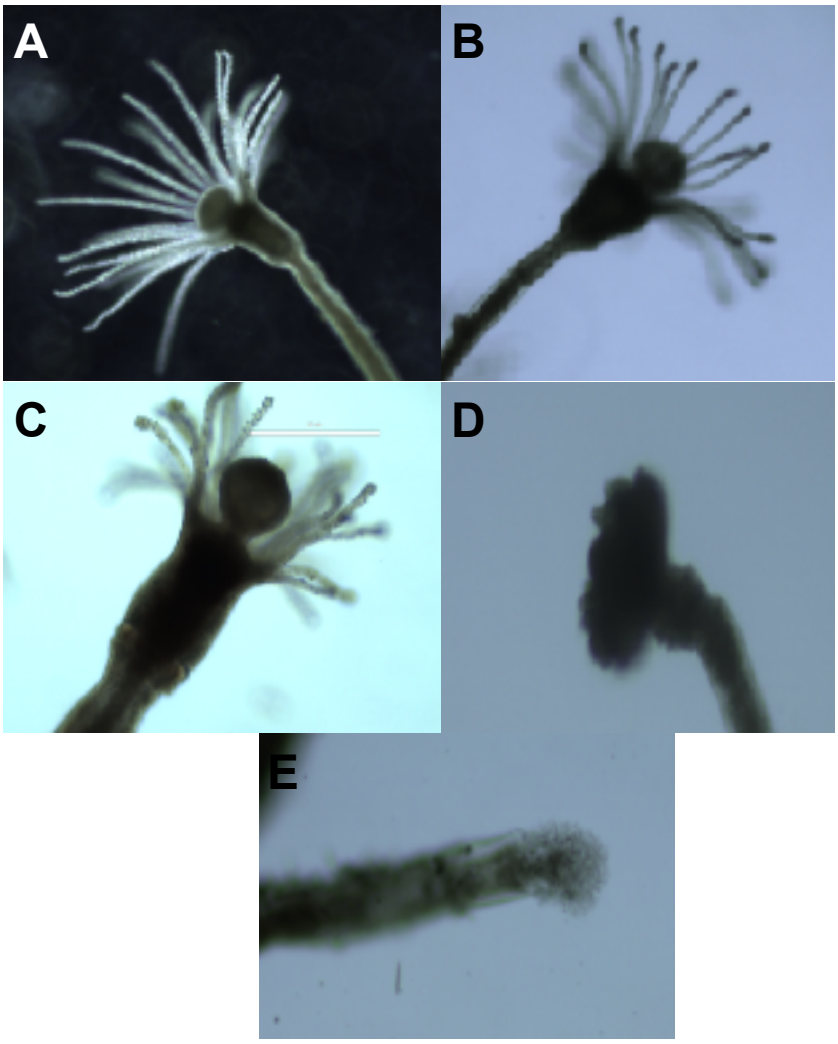

Figure 5. The morphological changes in hydranths of *E. carneum*. The sub-lethal endpoint is shown in panel B and the lethal endpoint is in panel D. The appropriate scores can be found in brackets. A: a normal hydranth (4); B: hydranth experiencing minimum effect of toxicity, i.e. clubbed tentacles (3); C: shortened tentacles (2); D: tulip stage (1); and E: disintegrated.

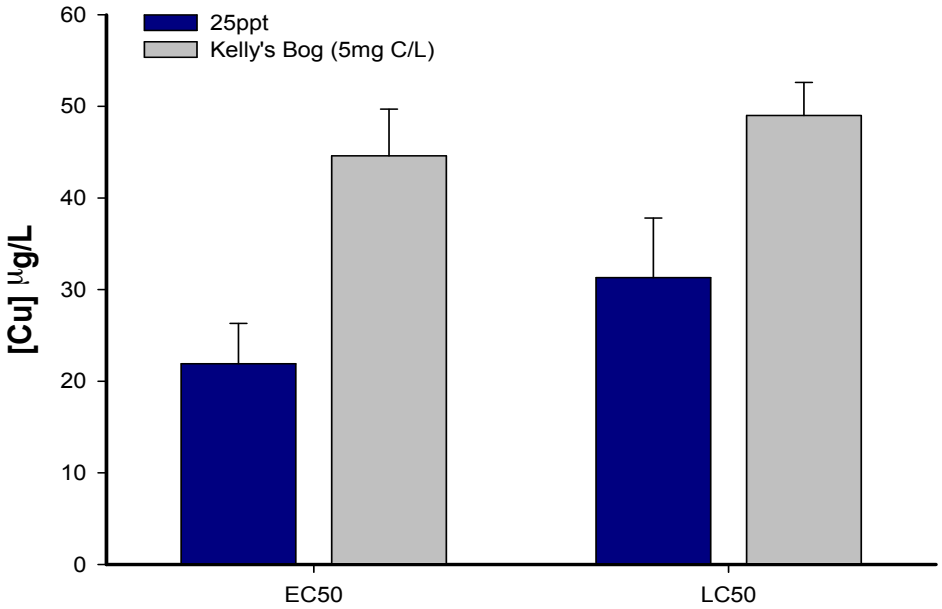

Figure 6. A comparison of the 48 hour EC_{50} and LC_{50} values for the response of *E. carneum* at 25ppt without DOM (blue bars) and with 5mg/L DOM (green bars). A * denotes a significant difference from the EC_{50} (or LC_{50}) value at 25ppt

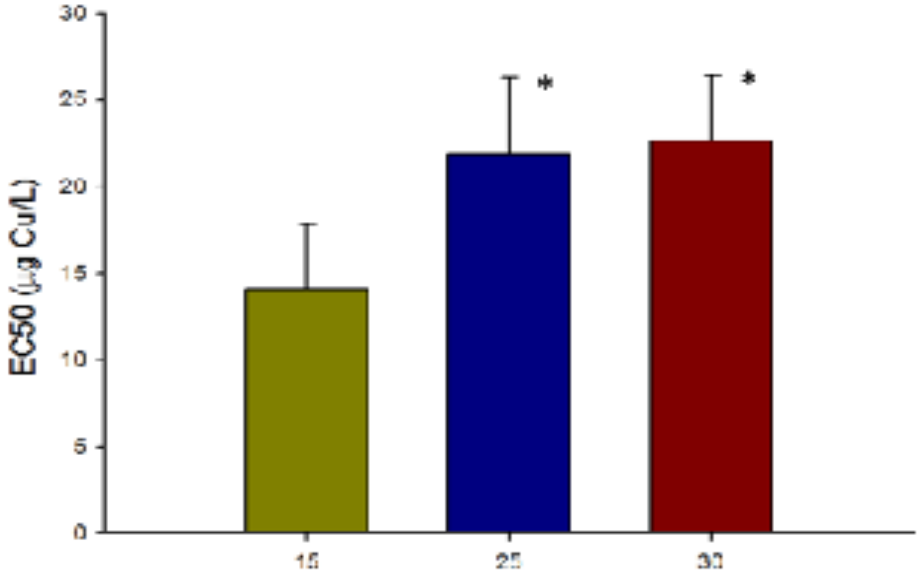

Figure 7. Forty-eight hour EC_{50} values at different salinities. A * indicates a significant difference from the EC_{50} at 15ppt.

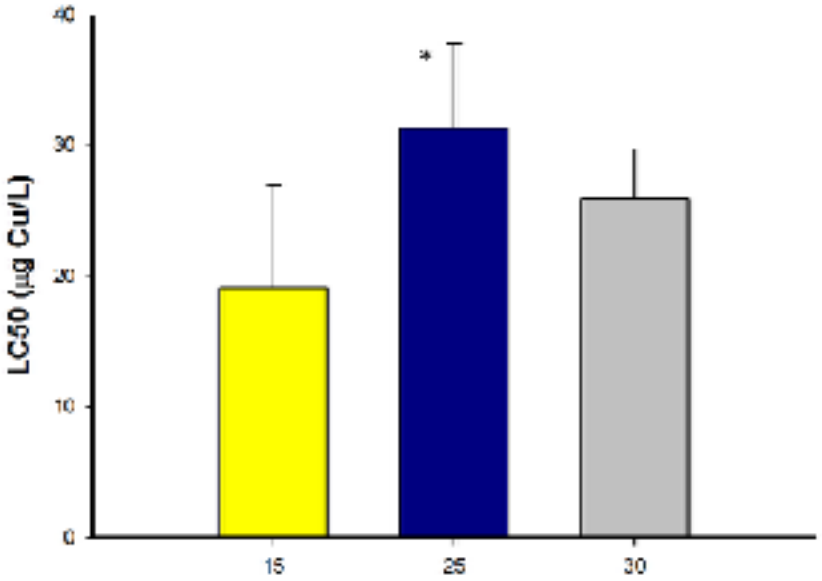

Figure 8. Forty-eight hour LC_{50} values for salinities of 15, 25, and 30ppt. A * indicates significant difference from 15ppt.

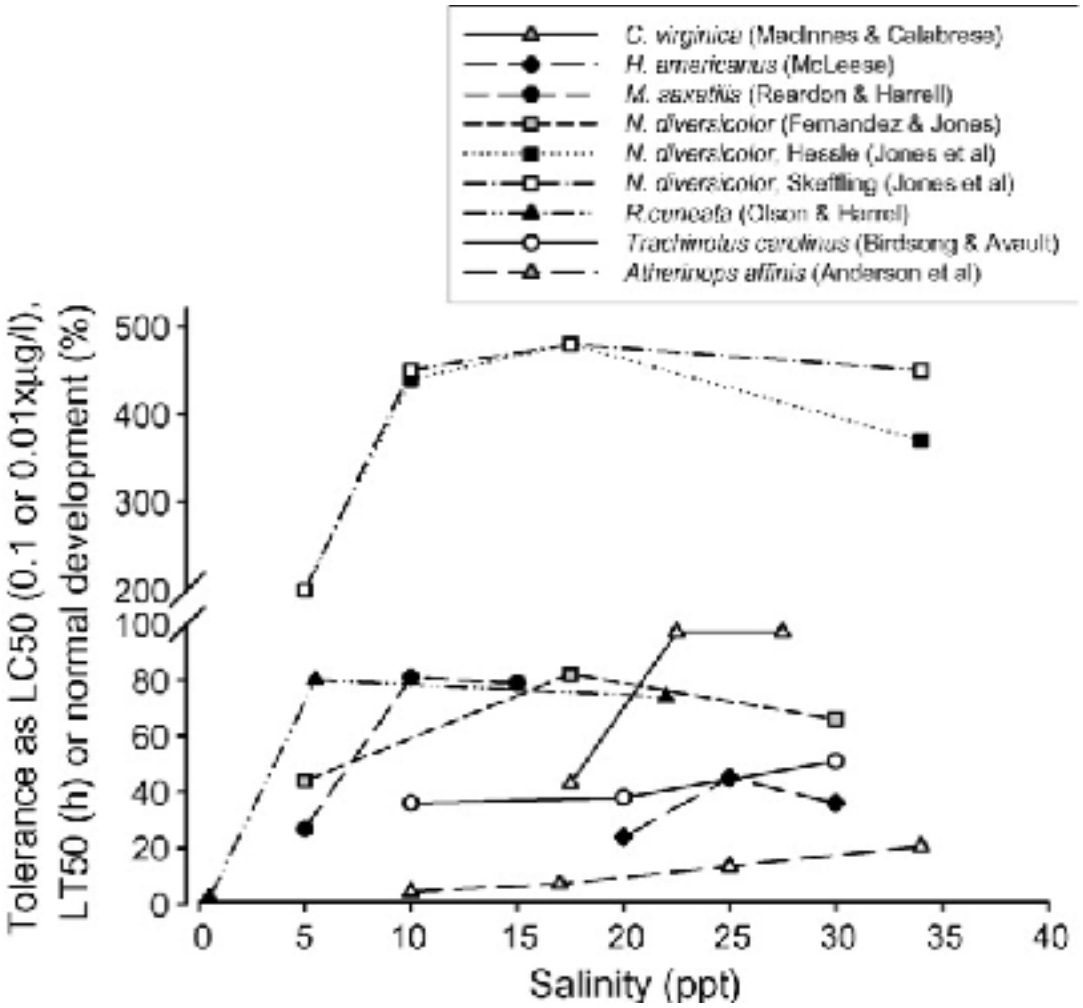

Figure 9. This figure summarizes the responses of estuarine organisms to Cu toxicity at different salinities. It appears there is uniformity in the responses: the lowest salinity is most sensitive and increasing salinity does not necessarily decrease sensitivity (Grosell, *et al*., 2007).

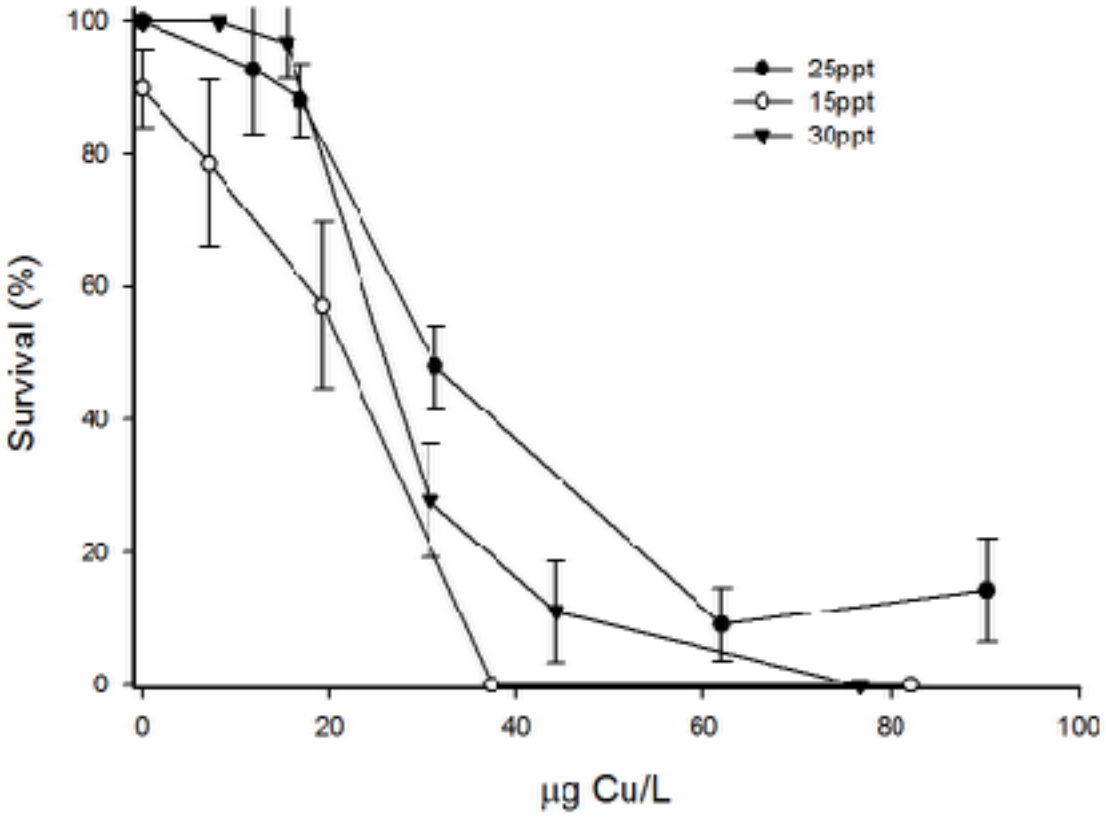

Figure 10. A plot of the mean survival at a given Cu concentration with *E. carneum*. Bars represent the standard error at each mean.

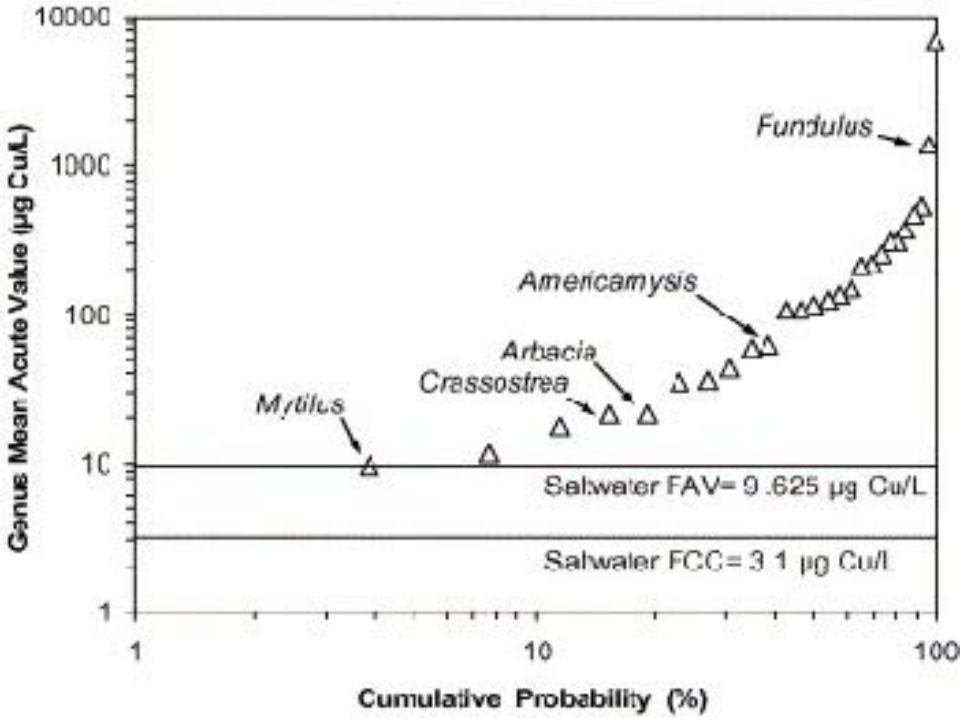

Figure 11. A species sensitivity distribution for Cu in marine environments shows the sensitivity of marine organisms to Cu and predicts the percent of affected species by different Cu concentrations (Arnold *et al*., 2005).Extrapolating from 30ppt suggests that *E. carneum* may be among the 10 most sensitive marine species to Cu.

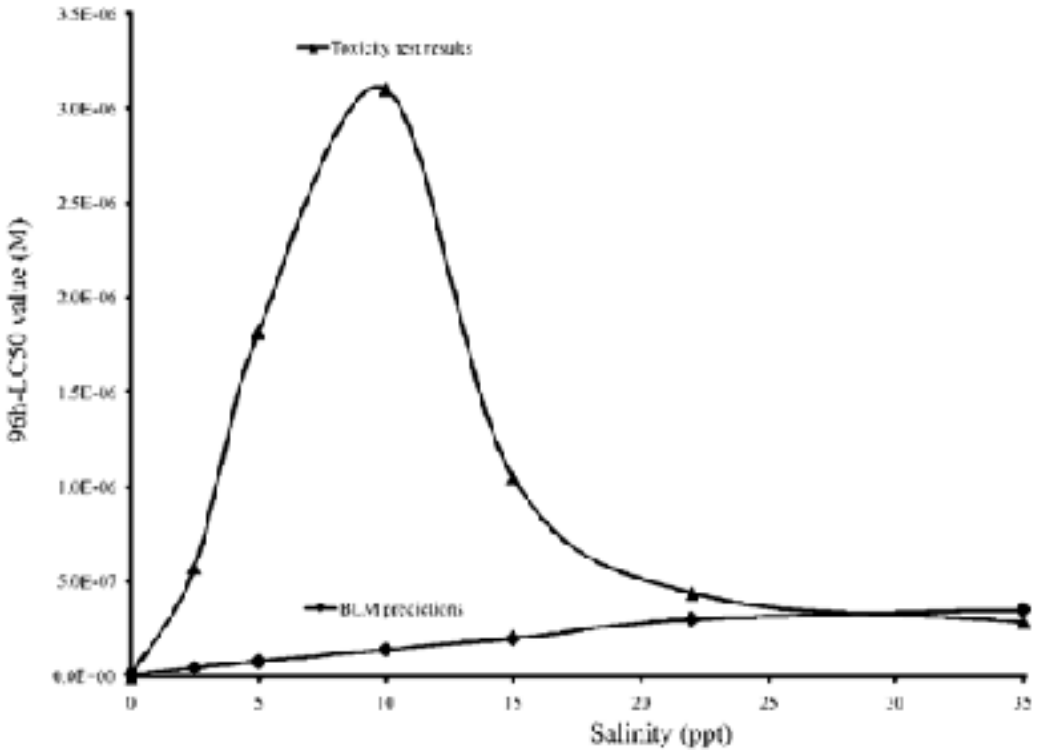

Figure 12. Comparing the 96 hour LC_{50} values of the euryhaline *E. affinis* determined by predictions of the BLM (diamond points) with the values determined by experiments (triangular points) (De Polo and Scrinshaw, 2012).

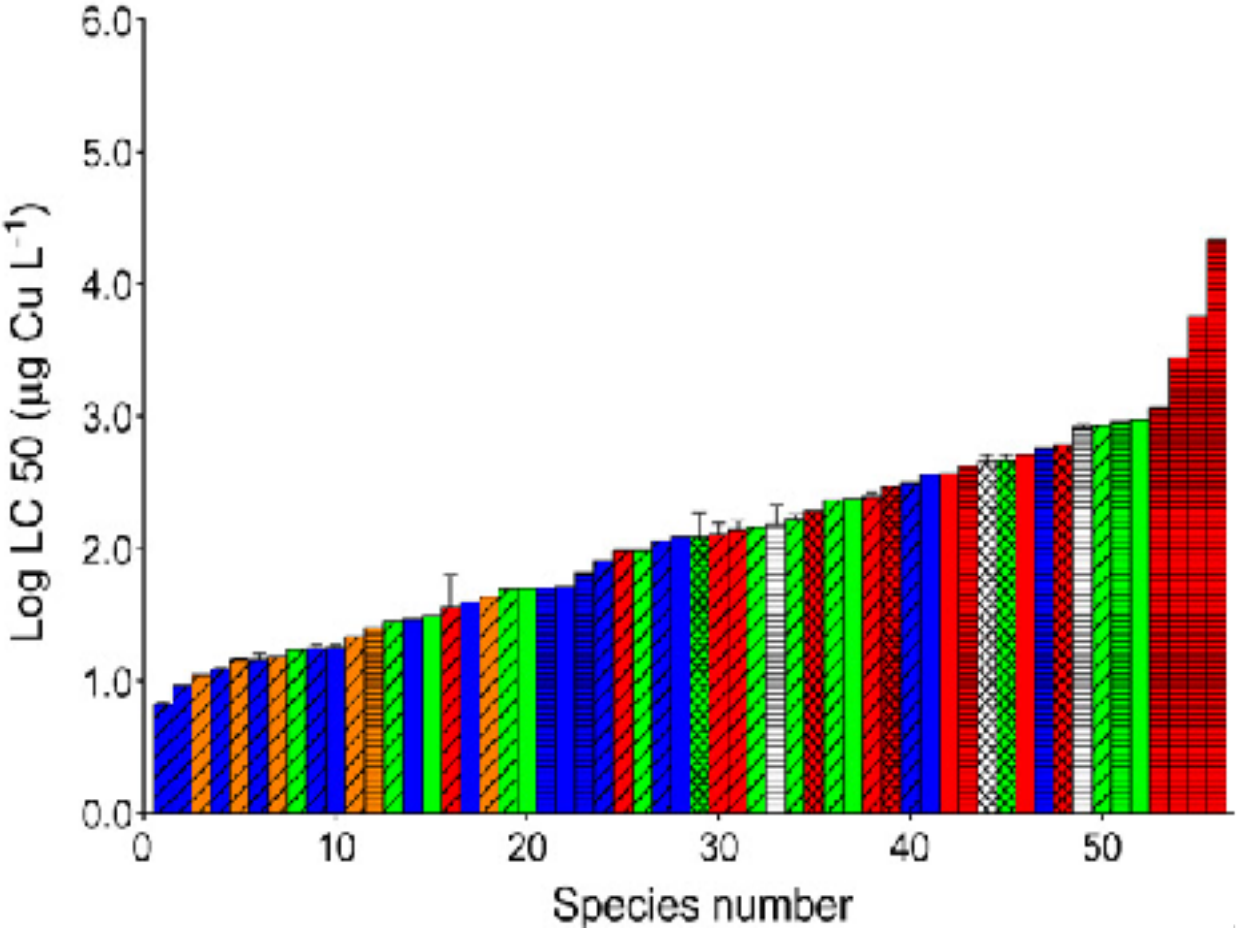

Figure 13. This species sensitivity distribution for Cu toxicity to marine organisms summarizes available marine Cu toxicity data (Grosell. *et al.*, 2007). Data uses toxicity data for salinities of over 25ppt, where single cross hatches represent toxicity data for larvae, eggs, or embryos, double cross hatches represent data for juveniles, and horizontal hatch bars represent data for adults.

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