Evaluation of Chelex 100 ® and Assessing the Impact of Fulvic Acid (NOM) on Copper Toxicity and Bioavailability to *Americamysis bahia*

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Thesis submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree o

Masters of Science
In
Environmental Engineering

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June 14, 2001
Blacksburg, Virginia

Keywords: ion exchange, bioavailable copper, Suwannee River Fulvic Acid Reference (NOM), *Americamysis bahia* (mysid), toxicity

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ABSTRACT

A cation exchange method (Chelex 100 ®) that distinguishes weakly bound and strongly bound copper was investigated for its ability to measure bioavailable copper in estuarine waters. Copper bound to the Chelex 100 ® resin was operationally defined as bioavailable copper. Varying initial copper concentration from 195 to 495 µg/L at a constant 12.5 mg/L natural organic matter (NOM) did not affect percent bioavailability. There were also no noticeable effects when varying total Cu concentration in the presence of 0, 12, and 24 mg/L NOM. An increase in pH from 4 to 8.5 and NOM from 0 mg/L to 12.5 mg/L reduced percent bioavailability. Using the Chelex 100® resin to measure bioavailable copper, about 20 to 40% of the total copper was bioavailable in the absence of NOM, while about 15 to 20% was bioavailable when either 12 or 24 mg/L NOM was present.

Acute toxicity bioassays were performed with mysid shrimp (*Americamysis bahia*) to evaluate the toxic effects of copper in the presence of Suwannee River Fulvic Acid, which served as a source of NOM. Static or static renewal tests, based on EP method OPPTS 850.1035 with a minimum of 10 mysid shrimp per test condition, were used to determine the LC_{50} and EC_{50} of copper and the effects of NOM. Test solutions consisted of artificial synthetic seawater at 20 parts per thousand containing concentrations of 0, 100, 200, 400, 800 µg/L copper with either 0, 12, 24 mg/L NOM. Forty-eight hour acute toxicity tests were performed on larval (2 to 3 day) mysid shrimp that were fed *Artemia* (brine shrimp); mortality and immobilization were the endpoints. The 48 hour LC_{50} was 200 µg/L dissolved Cu and 94 µg/L bioavailable Cu without NOM, 340 µg/L dissolved Cu and 98 µg/L bioavailable Cu when 12 mg/L NOM was present, and 495 µg/L dissolved Cu and 105 µg/L bioavailable Cu at 24 mg/L NOM.

The consistency of the LC_{50} measurement using bioavailable Cu suggest that the Chelex 100 ® resin is a useful technique for toxicity analysis in saline water.

Keywords: ion exchange, bioavailable copper, Suwannee River Fulvic Acid Reference (NOM), *Americamysis bahia* (mysid), toxicity

Acknowledgements

I gratefully thank the Edna Bailey foundation for the financial support for this research. Thanks to my committee members for their advice, patience, and assistance during this project. Laboratory managers Julie Petruska and Jody Smiley provided invaluable assistance with equipment and analytical analysis. I also thank my friends a Virginia Polytechnic Institute for assistance, friendship, and laughs. Finally, I would to thank my family for their support, faith, and endless love.

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Author's Preface

The first chapter of this thesis is a literature review of copper toxicity and the natural environment. The final chapter is a separate manuscript entitled, "Evaluation of Chelex 100 ® and Assessment of Impact of Fulvic Acid (NOM) on Copper Toxicity and Bioavailability to *Americamysis bahia*," and is formatted to the specifications of *Environmental Toxicology and Chemistry*.

The first chapter is a comprehensive synopsis of copper in the natural environment. In this chapter, bioavailable (labile) copper is the most toxic copper species. Chapter 2 utilizes a Chelex 100 ® resin ion exchange column method for evaluating copper toxicity in the presence and absence of Suwannee River Fulvic Acid Reference (SRFAR) used as NOM. The research on copper bioavailability and copper toxicity to *Americamysis bahia* mysid shrimp was a contribution of research begun by Mr. Jeffery D. Snyder (M.S. ENE, 1999). In order to generate a more readable document, a portion of Mr. Synder's thesis has been reproduced. These sections include: Chelex 100 ® Preparation and Evaluations, mathematical modeling of Chelex 100 ® resin; pages 8 -12, results of mathematical modeling and affects of NOM; pages 14-19, and Figures 2-1, 2-3, and 2-4.

Research Project

Low concentrations of heavy metals such as copper can impart toxicity to natura waters. Consequently, during the 1980's, the United States Environmental Protection Agency (USEPA) set metal discharge criteria for industry and wastewater treatmen plants. Although the USEPA criteria for metals, including copper, are not enforceable, they establish guidelines for the implementation of state water quality regulations. These criteria were initially established in terms of total copper concentration because there was insufficient knowledge about copper speciation and toxicity. In the early 1990's, regulatory standards for metals were changed to dissolved metal concentrations because research reported that if a metal was strongly bound in the particulate phase, then it was not readily toxic (Renner, 1997).

Metals may also be bound to dissolved organic matter, also impacting the toxicity. Therefore, analysis of copper toxicity should be set in terms of species specific bioavailable copper. Conceptually, bioavailable metal is considered that portion of the total metal concentration (particulate, dissolved, or free metal) which can be taken up b organisms. If a metal is or becomes bioavailable, it can exert toxicity and should be regulated. Unfortunately, even in the early 21st century, metal bioavailability and toxicity are still not well understood. To completely understand copper as a toxin, the sources and fates of the metal and organic and inorganic materials which control the relationship between labile Cu^{2+} and dissolved and total copper must be evaluated. Bioavailability may be easier to understand if a bioassay is utilized with study of the chemistry of an ecosystem.

Bioavailability is defined as the degree of sorption or uptake to which chemicals in the environment (e.g. water, sediment, food items) can actually be taken up by organisms (Lagos, 1997). Bioavailability changes with factors such as water characteristics, specific metal, organism type, and organism stage. The measurement o potentially harmful, or bioavailable copper, is a difficult task due to these intricacies. Thus, bioavailable metal concentrations are typically operationally defined based on measurement technique. Although, current bioavailable metal measurement methods do not consider every possible form of copper in the environment, they are still valuable for

understanding how the dissolved fraction of bioavailable copper changes in natural water given characteristics such as pH, NOM, and hardness/alkalinity.

As of 2000, there were no standard methods to perform dissolved bioavailable metal analyses. A Chelex 100 ® ion exchange resin technique can be used to measure bioavailable dissolved copper. Chelex 100 ® resin is a styrene-divinylbenzene copolymer that has imminodiacetate functional groups that are able to complex with free metals through ion exchange. Furthermore, the copper affinity of the resin may be great enough to dissociate and retain copper ions that were previously weakly complexed (such as copper hydroxide complexes) in solution. These weak copper complexes are generall considered bioavailable whereas copper strongly bound to organic ligands that pass through the resin is not. One major advantage of the Chelex 100 ® resin technique is tha samples may be processed through the resin in the field, and the actual metal analysis can occur in the laboratory.

The amount of bioavailable copper in a natural environment is subject to changes in water characteristics such as pH and NOM, and the measurement of bioavailable copper by a resin should respond in a similar manner to be useful for measuring bioavailable copper. Furthermore, the resin measurement should not respond to changes in water characteristics that do not effect bioavailable copper concentrations in the natura environment.

Bioavailable copper measurements would be useful in any natural aquatic environment. Acute toxicity tests were performed with Suwannee River Fulvic Acid and larval *Americamysis bahia* to evaluate the Chelex 100 ® resin.

The objectives of this research were to:

- Determine if the Chelex 100 ® resin responded to changes in water characteristics in a logical and consistent manner when measuring bioavailable copper.

  Characteristics studied were:
  - Copper concentration
  - pH
  - Natural Organic Matter (NOM)

- Conduct 48-hour to 96-hour acute bioassays wit *Americamysis bahia* to determine LC_{50} values for copper toxicity under varying fulvic acid levels.

- Investigate a relationship between copper toxicity in the presence of fulvic acid and bioavailable copper measurements using the Chelex 100® resin.

I. Literature review

Sources of Copper in the Natural Environment

Low concentrations of heavy metals such as copper can impact the ecology o natural water. Consequently, regulations for discharge of copper to the environment have recently become more stringent. An important step in the development of managemen strategies to meet regulatory limits, is to consider sources of copper and the perceived benefits and detriments (Table 1-1). Agricultural nonpoint source (NPS) runoff and WWTP effluents may result in significant discharges of heavy metals and suspended sediments into adjacent waters. Copper enters surface water through two primary sources including storm water from transportation areas, lawns, and fields or discharge of treated wastewater. Copper inputs include industrial and commercial, CCA (copper chromium and arsenic) treated wood, corrosion, application of liquid manure, algaecides, and pesticides (Merkel, 1994, Xue, 2000, Sprague, 1999). Exposure to toxic levels result in fish and shellfish mortality. Proposed management practices and knowledge of metal toxicity in natural waters reduce contaminant risk from these discharges.

Copper Speciation in the Natural Environment

In this section, the chemical and physical properties of copper speciation are reviewed to better understand and increase knowledge of cupric ion and weakly bound complexes. Bioavailable or labile copper as cupric ion (Cu^{+2}) or CuOH is potentially toxic to aquatic organisms (Allen and Hansen, 1996). Copper in aquatic environments is routinely classified as particulate and dissolved, with the dissolved fraction further classified as free, inorganically or organically complexed (Figure 1-1). However, copper strongly bound (complexed) to organic ligands is usually not considered toxic. For this reason, it is beneficial to know which species of copper form in natural aquatic systems.

Table 1-1. Sources of Copper in the Natural Environmen

Source	Description	Perceived Benefits	Perceived Detriments
Industrial	Used in the metal plating/finishing, circuit board production, and paint products. Produced through smelting and used in heat transfer applications such as cooling towers	Unique benefits and characteristic	Release into storm sewers
Commercia	In products used by metal fabricators, vehicle service facilities, medical facilities, construction areas, laboratories, automobile parts stores, plumbing businesses, hardware stores and others	N/A	Discharge directly to sewers and storm drains
Residential	Corrosion of washing machines, present in consumer products, foods, fecal matter, and runoff from lawn care and car washing	Essential in the human diet, sometimes useful in consumer product	Might be detrimental when consumed in hi concentrations (10-20 grams)
Plumbing Materials	Corrosion by-product release from copper plumbing	Pathogen control properties, relatively high resistance to corrosion	Source of copper loading through sewers
Brake pads	Contain copper which is generally introduced to the environment in a particulate form through wear	Material in brake pads	Particles washed int storm drains
Algaecides	Algaecides include copper sulfate pentahydrate (blue stone), copper enolate (Cutrine Plus), and copper citrate (Cupeos)	Control of THM potential and taste and odor problems	Copper addition to drinking water source
Pesticides	Pesticides including copper hydroxide, copper sulfate, copper carbonate, copper oxide, and copper oxychlorides	Crop protectant in agricultural operations	Surface and storm water runoff and infiltration into groundwater
Root Killer	Commercially sold copper sulfate solid dosed to toilets	Slowly kills roots clogging sewage pipes	Contributes copper spikes to WWTPs

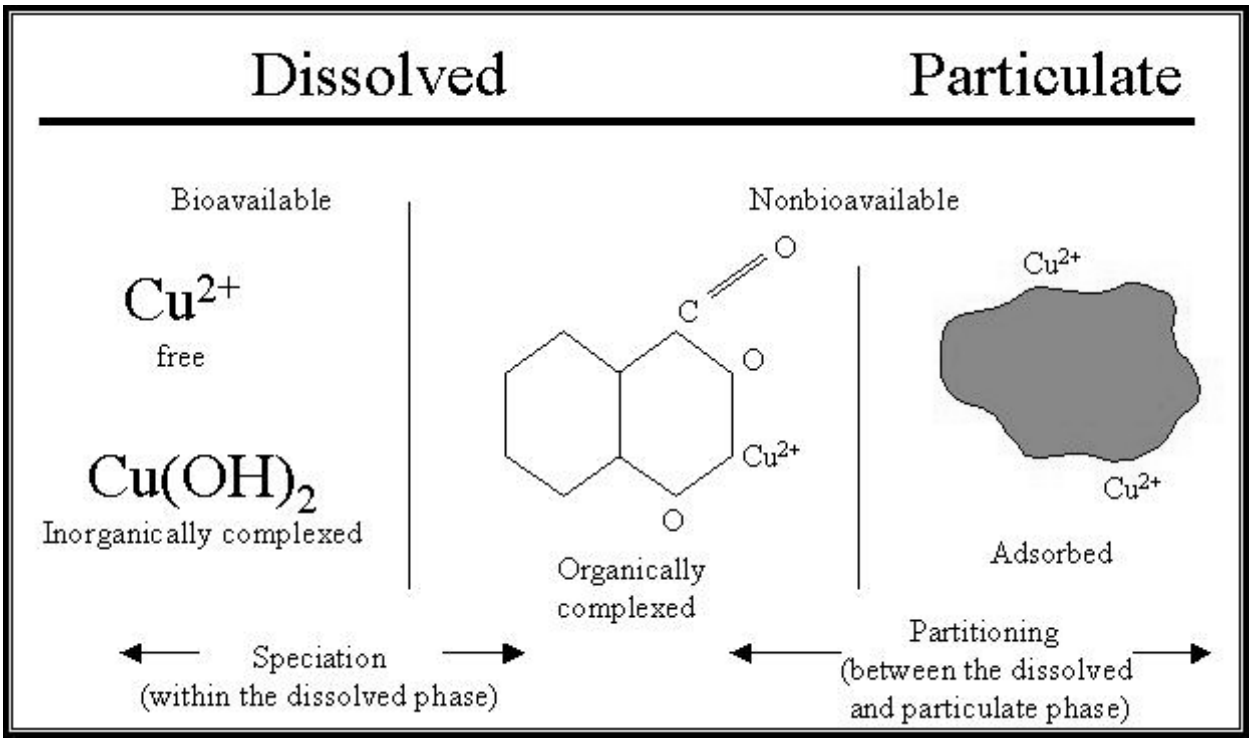

Figure 1-1. Forms of Copper present in Aquatic Environments (from Paulson *et al.*, 1993)

Chemical and Physical Properties of Copper

The chemical and physical properties of surface waters strongly influence copper toxicity. Since some copper complexes are toxic to aquatic organisms whereas others are not, it is beneficial to know what copper species will be present in natural waters. For example, Cu^{2+} and $CuOH^{+}$ are considered highly toxic, whereas $[Cu_2(OH)_2]^{2+}$ is sometimes considered toxic (Meador, 1991). The most widely bioavailable form o copper that is toxic to aquatic life is often referred to as labile copper which includes free copper and easily dissociable and exchangeable inorganic complexes (Sloo *et al.*, 1989). However, speciation changes with water quality can make copper more or less bioavailable. Table 1-2 depicts typical water characteristics that effect copper speciation and therefore toxicity in natural waters. Seawater shows less variation than freshwater.

This is because freshwater is generally poorly buffered and constituents can vary greatl in nature (Tessier *et al.*, 1995).

Table 1-2. Water quality characteristics that affect copper speciation and typical values for natural waters (from Buchwalter *et al*, 1996)

Characteristic	Seawater	High Alkalinity River Water[a]	Low Alkalinity River Water[a]
Ionic Strength	0.7 M	0.004 M	0.001 M
Salinity	35 mg/L	0.144 mg/L	0.041 mg/L
pH	8.1	8.4	<7.5
DOC[b]	0.4 - 2.5 mg/L	2 - 100 mg/L	2 - 100 mg/L
SPM[c]	0.020 - 0.050 g/L	0.08 - 38 g/L	0.08 - 38 g/L

[a]As defined by Dyrsen and Wedborg (1980)
[b]Dissolved organic carbon; data from Buffle (1988)
[c]Suspended particulate matter; seawater data from Whitfield and Turner (1987), river water from Martin and Whitfield (1983)

One of the most influential water quality parameters is the amount of complexing material present (Hodson *et al.* 1979). The more organic matter present, for example, the more bound copper and the lower the copper toxicity. For example, high alkalinity waters complex carbonate with copper to form non-toxic complexes. Copper-carbonate species are generally not considered toxic (Meador, 1991). Increases in carbonate species are usually associated with increases in alkalinity. Therefore, alkaline waters have more of a potential to form copper-carbonate species, thus reducing copper toxicity (Snoeyink and Jenkins, 1980). Because inorganic calcium and magnesium ions compete with copper for binding sites on organisms, high water hardness also tends to reduce copper toxicity (Brezonik *et al*., 1991). In general, the more saline the water, the less toxic the copper due to inorganic complexation of copper. Dissolved elements make up the background electrolyte in saline waters. This background electrolyte not only defines the ionic strength of the water, but also provides inorganic ligands which may complex trace copper (Tessier *et al*., 1995). If a given trace metal does not interact with organic ligands, the percent of free or weakly complexed metal is fixed given water characteristics such as pH, alkalinity, total suspended solids, and hardness. Metals such as manganese and cadmium only form inorganic complexes (Mackey *et al.*, 1989). In general, these inorganic complexes form rapidly. However, increased salinity and

temperature might also stress the organisms lowering their natural defenses agains copper toxicity.

Copper toxicity is also affected by the mode of toxic action in the organism. For example, toxic effects experienced by fish depend on the extent to which copper binds to the gills of the fish. Receptor sites on the gill surface, which are negatively charged ligands, bind with organic matter, inorganic ions such as sodium, calcium and magnesium, and metals such as copper. The extent to which gill sites are available for binding with copper will determine the extent of copper toxicity (Allen and Hansen, 1996).

pH

Copper can also adsorb onto the surface of particles. These particles usually contain hydroxyl groups on their surface, which can gain or lose protons, thus resulting in a net positive or negative charge. This surface complexation is highly pH dependent. pH change of a few units can change sorption from 0 to 100%. For example, a solution containing 5×10^{-7} M copper does not surface complex in the presence of 10^{-3} M total particulate ferric oxide at pH 4.0, but when the pH was increased to 6.0 over 90% of the copper was surface bound (Dzombak and Morel, 1990). Thus, pH and hardness o natural water affect metal complexation.

The pH of a saline water has seemingly contradictory effect on copper toxicity. Hydrogen ions compete with copper ions for organism binding sites decreasing toxicity at low pH, but a large fraction of the copper is free at low pH, increasing toxicity (Dietrich *et al*., 2001). Renner 1997, found that the amount of total copper required to kill 50% of fathead minnows increased with pH indicating that the effect of the cupric ion is dominant over the effect of hydrogen ion competing for binding sites in this case.

In addition, laboratory experiments have shown that clay and mineral oxide particles can have organic coatings (Hunder *et al*., 1979). The NOM on the particle surface enhances the adsorption of copper, and is pH dependent with the greatest NOM sorption occurring at pH 4 to 6 (Davis *et al*., 1982 and 1984).

However, as the pH of a natural water is lowered, copper has a greater tendency to form inorganic complexes that are generally considered toxic. Hydrogen concentrati

plays a pivotal role in the activity and toxicity of copper. Buchwalter *et al* (1996) reported that pH highly determines the Cu^{2+} concentration and fulvic acid behavior.

Natural Organic Matte

Copper metal ions not only can form well-quantified complexes with inorganic ligands such as OH^-, HCO_3^-, NH_3, and Cl^-, but also can form stable complexes with organic ligands such as Ethylenediaminetetraacetate (EDTA) and NOM (Raspor *et al*., 1980). Natural environments contain a multitude of dissolved or colloidal organic matter (NOM). Natural organic ligands, also referred to as natural organic matter (NOM), usually consist mainly of humic material which can be further operationally classified into three categories. These categories are fulvics, humic, and humin. Fulvic acids are soluble at any pH, humic acids are soluble above pH 1.0, and humin is insoluble at any pH. Dissolved natural organic material or (NOM) consists of both humic and fulvic acids, where fulvic acids are of a lesser molecular weight (<1000). Humics derive from the degradation of plant debris and from the excretion of organic matter from aquati organisms (Tessier *et al.*, 1995). The different ligand classes (strong or weakly complexed) control the overall speciation of Cu in aquatic environments (Skrabal*et al*., 1997).

The typical concentrations of total dissolved organic matter (DOM) in rivers and estuaries are 2 to 20 mg/L; and humic substances account for a significant fraction of NOM (Kogut and Voelker, 2001). Humics account for approximately 60% of the tota NOM in southeastern U.S. estuaries.

DOC interacts with organic chemicals by various methods. These methods are binding and adsorption, such as ion exchange, hydrogen bonding, charge transfer, covalent binding and hydrophobic adsorption and partitioning. These interactions have enhanced the concentration of pollutants in water, reduce volatilization, increase photolysis rates, alter bioconcentration and affect toxicity of organic compounds (Haitzer *et al*. 1998).

In general, copper complexes with organic ligands more than any other divalent metal (Irving *et al.*, 1953). Therefore, when considering dissolved metal speciation in estuarine water the researcher or regulator must have knowledge that complexation

fluctuates with the nature and concentration of inorganic and organic compounds present (Baeyen *et al.*, 1998). Recent experiments suggest that greater than 99% of the tota amount of copper in seawater is complexed by organic ligands (Sunda *et al.*, 1987).

Copper can form strongly bound species in the presence of dissolved natura organic carbon (NOM) and suspended particulate matter (SPM). These complexes are not considered as toxic as copper in the ionic or free form. These interactions affec bioavailability of organic compounds in aquatic organisms, because only freely dissolved and weakly complexed compounds are assumed to bioaccumulate in organisms (Haitzer *et al*., 1998). Although organic copper species are generally considered non-toxic, they do have the potential to bioaccumulate which can then lead to toxicity (Stumm and Morgan, 1996). Therefore, increasing the amount of DOC or SPM in a natural water ma reduce the amount of toxic copper.

Copper Ligand Binding

Much effort has been spent on modeling natural waters in order to determine which forms of copper will be present in a natural aquatic system. With inputs of water characteristics such as pH, ionic strength, alkalinity, and hardness, computer models can predict copper speciation. Copper forms well-defined inorganic hydroxide and carbonat complexes, and a water that contains these species exclusively can be modeled with a high degree of accuracy. However, copper-organic complexes are more difficult to model. As binding sites on the organic matter are filled, the energetic properties of the complex change. The value of the first dissociation constant (K_{A1}) will depend on which binding site is involved, and will also affect the second dissociation constant (K_{A2}). Therefore subsequent dissociation constants are not only a function of the binding sites involved, but also depend on the previous dissociation constant. Considering that an organic molecule may have over a hundred binding sites, modeling is extremely difficult (Schecher *et al*., 1998). Furthermore, natural waters may contain multiple organic ligands which may compete for metals, resulting in mixed complexes, which are strongly favored over complexes involving only one type of ligand (Buffle, 1988).

Although the exact nature of copper-organic ligand complexation is not well quantified, copper-organic ligand species can still be predicted through empirical

modeling. Models can be established through mathematical calculations given reasonable chemical assumptions. A model can also be established and calibrated directly from an experimental data set. For example, the nature of copper binding to fulvic acid was investigated by titrating a known solution with copper and measuring the resulting amount of free copper (Cabaniss and Shuman, 1987a and 1987b). These data were then used to derive and calibrate a model that predicted copper speciation in natura waters given NOM content, pH, ionic strength, and total copper concentration with a high degree of accuracy.

Binding capacities are very important for characterizing free metal concentrations. Humic and fulvic substances are contributors to both strong L_1 and weak L_2 strength ligands in coastal systems (Bruland, 2000 and Kogut and Voelker, 2001). Future research related to cation competition and ionic strength effects are of equal importance, as well as the possible release of copper in estuarine ecosystems. A titration method was developed which directly models copper-ligand binding as: $[Cu] + [Li]_T \leftrightarrow [CuL]$ yielding a stability constant. The binding strength of such copper ligand with respect to free copper is represented by its binding constant, $_{CuLi}$, defined by the equation:

$$K_{CuLi} = [CuLi]/([Cu^{2+}][Li] \quad (1)$$

in which[CuLi] is the concentration of copper bound to organic and inorganic ligands of Li. The total ligand concentration is also determined, and is defined as $[Li]_T$. Li is defined as:

$$[Li] = [Li]_T - [CuLi \quad (2)$$

The [CuLi] concentration can be calculated from Eqs. 1 and 2 (Kogut and Voelter 2001).

Using FITEQL (a program for the determination of chemical equilibrium constants from experimental data) Suwannee River Humic and Fulvic Acids (SRHA and SRFA) were modeled to contain 8 to 10 nM copper or metal binding ligand per mg/L o humic material (Kogut and Voelter, 2001).

Much research has been performed in order to quantify copper's ability to complex a given organic ligand through modeling. A simple 1:1 complexation model has been shown to give reasonable results when predicting copper-organic ligand complexes (Ruzic, 1982).

The method plots inorganic copper vs. the amount of inorganic copper divided by the amount of ligand-bound copper. If 1:1 complex formation is expected, the plot should form a straight line with slope [L_T] and intercept –1/[$_L$]. A linear trend suggests that only complexes involving one trace metal and one ligand form over the concentration range of interest (Snyder, 1999).

Several authors have demonstrated Ruzic's method to be accurate for measuring 1:1 copper complexation in saline waters (Coal *et al*.,1988, van den Berg *et al*., 1989). Using differential pulse anodic stripping voltammetry (DPSV) to measure inorganic copper, Coale *et al*., 1988 were able to titrate a copper-EDTA system. Titration results were compared to calculated results with a good degree of accuracy. The added EDT concentration was 10.0 nM, whereas the titration-derived concentration was 10.4 +/- 1.6 nM. The conditional stability constant derived from the titration was 8.6 +/-0.1. B taking into account inorganic side reactions (αCu) and also ionic strength corrections (γCu) for the saline system, the calculated stability constant was determined to be 9.2. This value can vary, based on the numbers chosen for αCu and γCu.

Once the total organic ligand concentration ([L_T]) is well quantified, along with its affinity for copper complexation (K_L), computer programs such as MINEQL+ version 4.0 by Schecher and McAvoy (1998) can predict both organic and inorganic metal complexation. In this paper the fraction of inorganic copper measured in the Ruzic (1982) method described above was operationally defined as the amount of copper exchanged onto the Chelex 100 ® resin surface; the derived K_L and L_T values were strictly conditional. These values were dependent on resin characteristics such as sample retention time in the resin and the amount of resin used. Therefore, applying the conditionally measured Ruzic K_L and L_T values to a mathematical model to predict resin results would be illogical. However, the values determined by the Ruzic method would be compared to other natural estuarine K_L and L_T values in the literature, in order to indicate if the resin is complexing the correct fraction of copper.

Copper Toxicity in Natural Waters

The toxicity of a given copper concentration in aquatic systems is influenced by many factors including the pH, salinity, and temperature of the water, the age and type o the aquatic species, the concentration of mineral and organic matter and whether or not the copper is bioavailable. The latter criterion has recently received a large amount o scrutiny because surface water regulations are based on dissolved copper concentrations rather than bioavailable (Allen and Hansen, 1996).

The more bioavailable a chemical, the higher the toxic potential. The bioavailability of metals in aquatic environments is currently a heavily debated topic, mostly due to the fact that metal toxicity appears to be site and species specific. Copper in the aquatic environment is routinely classified as particulate and dissolved, with the dissolved fraction further classified as free, inorganically complexed and organicall complexed (Figure 1-1).

Copper is an essential element to most aquatic organisms at very low concentrations. Concentrations as low as 8 µg/L have been known to have negative effects on larval shellfish (LaBreche, 1998). In general, the toxicity of copper to aquatic species is orders of magnitude higher than it is for humans. Low dissolved copper concentrations, on the order of 1-10 µg/L, tend to affect smaller aquatic organisms such as diatoms and invertebrates while higher concentrations, in the range of 100-1000 µg/L dissolved copper, are typically necessary to produce toxic effects in fish Table 1-3. The toxicity of copper depends on many water characteristics, as described in Table 1-2. Increased amounts of natural organic matter, carbonate, and higher pH levels may reduce the toxic effect of copper.

Table 1-3. Summary of toxic effects expected for various copper concentrations in water with moderate to high bioavailability.

Total dissolved copper Conc. Range (µg/L)	Characterization of Toxic Effec
1-10	Significant effects are expected for diatoms and sensitive invertebrates, notably freshwater cladocerans. Effects on fish could be significant in freshwaters with low pH and hardness.
10 - 100	Significant effects are expected on various species of microalgae, some species macroalgae, and a range of invertebrates, including crustaceans, gastropods, and sea urchins. Survival of sensitive fish will be affected and a variety of fish should have sublethal effects.
100 - 1000	Most taxonomic groups of macroalgae and invertebrates will be severely affected. Lethal levels for most fish species are reached.
> 1000	Lethal concentrations for the most tolerant organisms

Major factors that influence the bioaccumulation of copper in saltwater organisms include: size and age of the organism, concentration and speciation in water and food, exposure routes, and physical environmental characteristics such as pH, temperature, alkalinity, DOC, and turbidity (Pelletier, 1995). Some aquatic organisms are more susceptible to copper. The concentration required to kill 50 percent (LC_{50}) of the marine mussel *Mylitus edulis* in 30 days is 2 µg/L (Luoma and Carter, 1991). Ionic copper concentrations as low as 5.8 µg/L stunts the growth of the bay scallop *Argopecten irradians.* The 42 day LC_{50} for this organism is 9.3 µg/L ionic copper (Pesch *et al.*, 1979). The soft shell cla *Mya arenaria* experiences a 7 day LC_{50} of 35 µg/L added copper during the summer (Eisler *et al*., 1994).

When a toxic metal in the dissolved form first reaches an aquatic organism, it usually first comes in contact with a protective layer. In microorganisms and some plants, this is usually the cell wall; in animals it is the mucus layer. After passing through this protective layer, the dissolved metal then encounters the plasma membrane. The plasma membrane varies considerably from one organism to another, but all have similar characteristics. It is hydrophobic in nature, and contains transport proteins an/or ion channels which transport ions across the membrane. Thus, the metal has various potential binding sites. The variability in toxicity values is partly due to the ability of each organism to regulate internal copper concentrations. If the metal ion binds at physiologically inert sites it may accumulate without adversely affecting the organism.

However, if the metal ion binds at a physiologically active site, it may affect cell metabolism directly. Furthermore, metal ions can interact with a large number o intracellular sites, thus adversely effecting metabolic activity (Williams, 1981).

In aquatic organisms, the cell membranes discussed above are usually the gills or body tissue. However, some organisms, including filter feeders such as larval clams and Oligochaete worms, not only take up soluble metals, but also ingest particulate-bound metals through the food chain (LaBreche, 1998, Baerselman *et al*. 1998). Marine phytoplankton may accumulate metals from the water (Tessier *et al*., 1995). Once these plankton are inside the stomach, the acidity of the system can reduce the particulate copper to a form that will readily pass through the cell membrane, potentially causing a toxic effect.

Copper toxicity also depends on the organism considered. Some aquatic organisms are more susceptible to copper than others. The concentration required to kill 50 percent (LC_{50}) of the marine mussel *Mylitus edulis* in 4, 10, 14, and 30 days is 200 to 300, 90, 15, and 2 µg/L total copper respectively (Luoma and Carter, 1991). Furthermore, ionic copper concentrations as low as 5.8 µg/L stunts the growth of the bay scallop *Argopecten irradians.* The 42 day LC_{50} for this organism is 9.3 µg/L ionic copper (Pesch *et al*., 1979). The soft shell clam *Mya arenaria* experiences a 7 day LC_{50} of 35 µg/L added copper during the summer. However, the organism's tolerance to copper increases as the weather becomes colder. The 21 day LC_{50} at 17°C during the fall is 86 µg/L added copper. The 14 day LC_{50} at 4°C is greater than 3000 µg/L added copper during the winter (Eisler *et al*., 1994). The larval form of the hardshell clam *M. mercenaria* experiences an 8 to 10 day LC_{50} of 16.4 µg/L added copper (Calabrese *et al.*, 1977).

The toxic effect of copper also depends on the organism's life stage. The larval stage of the marine mussel *Mylitus edulis* cannot survive total copper concentrations exceeding 400 µg/L. This organism is even more susceptible to copper in the embryonic form, where the organism cannot survive in waters containing 5 to 6 µg/L total copper (Luoma and Carter, 1991). The embryonic form of the American oyster *C. virginica* experiences a 12 day LC_{50} of 103 µg/L added copper, whereas the larvae form 12 da

LC_{50} is only 32.8 µg/L added copper (Calabrese *et al*., 1973). Aquatic organisms can also bioaccumulate copper over time, leading to toxic responses and even death (Luoma and Carter, 1991).

Research conducted wit *Crassostrea virginica*, American oyster, result concluded copper accumulation rates were decreased with the addition of dissolved organics. The presence of dissolved organic reduced the accumulation or toxicity o copper, although there may be some organic copper complexes that are directly available to aquatic organisms (Zamuda *et al*., 1985). These weakly bound and free ions are operationally deemed as bioavailable. Winner (1984) reported that the effect o increasing the humic acid concentration reduced the bioaccumulation of copper the significantly below the level which bioaccumulates in the absence of humic acid because of an increase in the number of binding sites.

Copper Regulations

Since aquatic organisms exhibit toxicological responses to copper in the µg/L range, criteria have been established to help regulate copper discharge to natural waters. In 1972, the Clean Water Act was established, and in 1977 the United States Environmental Protection Agency (USEPA) was required to set criteria for toxi pollutant discharge for industry and wastewater treatment plants. Although the USEPA criteria are not enforceable, they provide guidelines for the implementation of state water quality regulations. These criteria were initially established in terms of total meta concentrations. The established total copper criteria are shown below, where hardness was set in terms of mg/L as calcium carbonate (USEPA, 1985).

- Saltwater 24 hour average: 4.0 µg/L total copper
- Saltwater average not to exceed 23 µg/L total copper at any time
- Freshwater 24 hour average: 5.6 µg/L total copper
- Freshwater average not to exceed $e^{(.94(\ln \text{hardness})-1.23)}$ total copper at any time

Research has shown that if a metal was strongly bound in the particulate phase, i was not readily toxic (Renner, 1997). Therefore, soluble copper was deemed the better parameter in copper toxicity. In the early 1990's criteria for metals were generally

changed to dissolved metal concentrations. In 1995 the USEPA published a saltwater draft in terms of dissolved copper (USEPA, 1995). The dissolved copper criteria are shown below, and are not to be exceeded more than once every three years on average.

- Acute (24hr average): 4.8 µg/L dissolved copper
- Chronic: 3.1 µg/L dissolved copper

The State of Virginia regulates dissolved copper concentrations, as shown below:

- Saltwater
  - Acute (1 hr average): 2.9 µg/L dissolved copper
  - Chronic (4 day average): 2.9 µg/L dissolved copper
- Freshwater
  - Acute (1 hr average): $< e^{(.9422(\ln \text{hardness})-1.464)}$
  - Chronic (4 day average): $< e^{(.8545(\ln \text{hardness})-1.465)}$

These values are not to be exceeded more than once every three years on average. Again, hardness values were set in terms of mg/L as calcium carbonate ($CaCO_3$).

Even within the dissolved phase, metals may be bound to dissolved organic or inorganic components, which impacts toxicity. Therefore, copper regulations and guidelines should consider bioavailable copper concentrations. Conceptually bioavailable metal is considered that portion of the total metal concentration that is available to aquatic organisms. If a metal is or is made to be bioavailable, it can exer toxicity, and therefore should be regulated.

Methods to Measure Bioavailability

In order to determine bioavailable Cu, it is necessary to utilize methods which determine specific copper species. Copper specific electrodes, voltammetry and ion exchange are analytical methods used to determine bioavailable copper concentrations. Copper specific electrodes are utilized to measure labile copper concentrations in freshwater systems. The use of ion selection in seawater is not advisable due to strong Cl^- interference which stabilizes Cu (I) formed at the electrode surface. Therefore voltammetry and ion exchange methods are utilized for seawater matrices.

Electrochemical methods, such as voltammetry and potentiometry, use electrodes to determine metal species (Ruzic, 1982), (Coale *et al*.,1988), (Van Den Berg *et al*.,

1984), and (Buffle *et al*., 1979). Non-electrochemical methods such as dialysis, ion exchange resins, ligand competition methods, and size-based separations are also used to determine metal species (Mazidj *et al.*, 1992) and (Tubbing *et al*., 1994). Furthermore, mass spectrometry and high-performance liquid chromatography have been applied to metal speciation studies (Marshall, 1988).

Although a wide range of analytical methods are available for measuring trace metal species, voltammetric techniques with low detection limits have been extensively used. Furthermore, ion exchange resins have often been implemented in speciation studies. These techniques are widely used for determination of trace metals in artificial seawater matrices.

Application of Voltammetry

A wide range of analytical methods are available for measuring trace meta species, voltammetric techniques with low detection limits have been extensively used. Voltammetry uses an electrode in order to distinguish between various metal species. Solutes come in contact with the working electrode, which is usually a mercury drop. The solute then undergoes either oxidation or reduction reactions on the electrode which produce a current that is measured while keeping the electrode potential constant. Voltammetry can also be accomplished by keeping the current constant and varying the electrode potential (Tessier *et al*., 1995). The reactions at the surface of the working electrode are a function of the solution's thermodynamic equilibria, the mass transfer rate of the solute from the bulk solution to the electrode, and the adsorption of surface active species at the electrode. Solution composition is related to the difference in potential (or current) between the working electrode and a reference electrode, where the reference electrode potential is assumed to remain constant during the measurement process.

Voltammetric methods, such as anodic stripping voltammetry (ASV) and adsorptive cathodic stripping voltammetry (ACSV) can determine free ions in the presence of ligands and differentiate between the various valence states of a specific element. These procedures are directly applied to determine trace metal speciation in saline waters. However, because the reactions at the working electrodes are a function o many parameters as discussed above, the information obtained from voltammetric

methods is usually highly complex, making it very difficult to interpret. Furthermore, voltammetry requires that samples be transported to the laboratory prior to analysis. During this time crucial water characteristics such as pH may change in the sample, thus changing metal speciation (Tessier *et al*., 1995).

Although voltammetric methods are among the most powerful techniques in speciation studies for heavy metals in natural water, further developments are required in order to provide data less influence by experimental parameters. Performing *in situ* measurements will minimize erroneous data. These errors occur due to losses b adsorption and contamination, and chemical and physical changes. New technologies in microelectrodes are also leading to new applications of the study of natural waters, not accessible to electrodes of conventional size.

Bioavailable copper measurements utilizing atomic adsorption and differential pulse anodic stripping voltammetry (DPASV) were compared to *Hyalella azteca* Saussure responses to copper in freshwater (Deavers and Rodgers, 1996). In thi experiment, DPASV was useful for measuring biavailable copper in toxicity tests. Cathodic stripping voltammetry (CSV) is another common technique for metal complexation measurements (van den Berg and Donat, 1991). Previous research has shown that in a study of organic complexation of copper in estuarine conditions that the detection window of CSV could be varied by varying the degree of ligand competiti with different ligand concentrations. The results for this study suggest that it is necessary to model the complexation data of copper in natural seawater which includes severa complexing ligands to completely understand complexed and free fractions (van de Berg and Donat, 1991).

Application of the Chelex 100 ® Resin

A field portable approach uses a Chelex 100 ® ion exchange resin to bind ionic and weakly bound copper. Samples may be processed in the field and the actual meta analysis can occur later using standard atomic absorption equipment. Chelex 100 ® resin is a styrene-divinylbenzene copolymer with imminodiacetate functional groups that ar able to form complex compounds with free metal species and weakly bound metal ion complexes through ion exchange. Complex formation is referred to as chelation.

Functional groups in the resin, referred to as ligands, form coordination compounds which bind the central metal ion using multiple sites. The resin is usually pretreated in order to convert the resin to the hydrogen, calcium, or ammonium form. Figure 1-2 depicts the mechanics of ion exchange in a calcium-based resin, as used in this research.

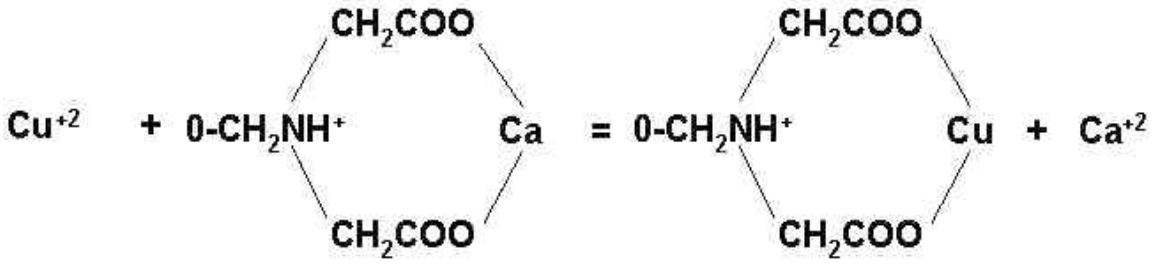

Figure 1-2. Chelex 100 ® Ion Exchange Mechanism shown for Sorbing Copper

Frequently the resin is used in the hydrogen form, although the effluent pH may be as low as 2.8. Research showed that the hydrogen form did not completely remove labile zinc, cadmium, lead, or copper from seawater until the passage of 500 mL o sample had increased the pH of the effluent to 6.5. Converting the resin to the calcium or ammonium form increases copper retention onto the resin, thus helping to alleviate this problem (Florence and Batley, 1976).

The Chelex 100 ® ion exchange resin has been put to use in many different ways. The resin has been used to determine trace metal concentrations in natural waters, when they are too low for detection by other means. A large volume of water is passed through the resin allowing metals to concentrate onto the resin. The metal ions are then recovered from the resin using a small volume of acid. By using a pre-concentration factor equal to the ratio of the volume of sample to the volume of elutent, the concentration of metal in the initial water can be determined using flame or graphite atomic-absorption spectrometry. However, the presence of fulvic and humic acids can inhibit metal ion complexation (Florence *et al.*, 1976; Figura *et al.*, 1977). This can be overcome by pre-treating the water sample through irradiation with ultraviolet light (Leyden, 1982).

Metals that are strongly bound to sediments or organics are not chelated by Chelex 100 ® (Figura *et al.*, 1979). Hence the resin will retain only free metal ions and

some weakly bound metal species. The species retained are potentially bioavailable. B measuring the amount of copper in the water sample both before and after contact with the resin, the amount of bioavailable copper can be determined by difference.

If the rate of metal uptake by a biological system is defined by the kinetics o dissociation of metals across the cell membrane, the Chelex system can be used to measure bioavailable metals. This assumes the resin mimics the key binding sites on the biological membrane. However, these dissociation kinetics will only be important when the metal binding reactions at the cell surface are slow in comparison to the transport o metal across the membrane. Current thinking is that this is usually not the case (Turner, 1984). For this reason, bioavailable metals are operationally defined as the ionic and weakly bound metal species retained on the Chelex 100 ® resin.

Chelex 100 ® measurements of bioavailable metal concentrations are affected b various parameters. The resin is available in various particle sizes (50-100, 100-200, and 200-400 mesh). Chakrabartl and Lu (1994) reported that copper uptake rates decrease with increasing mesh sizes. While it is generally known that exchange is more efficien with smaller beads, it has been shown that a larger size is preferred if the exchanged metals are to be recovered from the resin. Elution of metals with acid is particularly inefficient with the 100-200 mesh size (Pakalns, 1978).

The contact time between the resin and the water may determine which species chelate. Tubbing *et al.* (1994) found that contact times ranging from 0.3 to 6 seconds showed no increased retention of copper. However, very long contact times, i.e. 60 hours, showed an increase in copper retention of 10-20%. This is further complicated when the water contains organic matter. Copper uptake rate by the Chelex 100 ® resin slowed with increasing ratios of [EDTA]/[Cu] or in the presence of Cu-fulvic complexes (Chakrabartl and Lu, 1994). Table 1-4 summarizes past research with the Chelex 100 ® resin, and shows how the detention time may effect copper sorption onto the resin.

Table 1-4. Past research Chelex 100 ® resin: detention time, water type studied, and copper retention to the resin

Paper	Column Setup	Water Characteristics	Cu binding affinity
(Rasmussen, 1981)	Ammonium For	Seawater	Retained 100 to 107% of 1.87 µg/L Cu
	Flowrate: 0.1 ml/min until resin had shrunk. Flow was then increased to 1 ml/min	pH adjusted to 5.0	
	Detention time not given		

Paper	Column Setup	Water Characteristics	Cu binding affinity
(Chakrabartl *et al.*, 1994)	Resin was used in a batch Setup	Rideau river water	Cu(II) total = 133.5 µg/L
		pH was adjusted to 5.0	[EDTA]/[Cu(II)] = 0; retained 99% Cu after 1000 second
	1% resin on a weight basis used		[EDTA]/[Cu(II)] = .38; retained 63% Cu after 2000 second
		DOC: 6.6 mg/L	[EDTA]/[Cu(II)] = .77; retained 45% Cu after 2000 second
			[EDTA]/[Cu(II)] = 2.3; retained 7% Cu after 2000 second

Paper	Column Setup	Water Characteristics	Cu binding affinity
(Kuhn *et al*., 1998)	Sodium for	Artificial Brine	retained a mean of 84.4%Cu, given 10 mg/L Cu
	0.9g resin	Total Dissolve Solids (TDS): 35 to 250 mg/L	
	flow = 1.8 ml/min	(NaCl, CaCl2, MgCl2, Na2SO4)	
	detention time: 3.7 seconds		
		pH: 4-6	

Paper	Column Setup	Water Characteristics	Cu binding affinity
(Chakrabarti *et al.*, 1993)	detention time: 6-9 seconds	Rain Water, pH 4.7	Rain: retained 82.9% of 24.7 μg/L Cu
	5 g resin	Snow Samples, pH 5.3	Snow: retained 83.3% of 4.0 μg/L Cu

Paper	Column Setup	Water Characteristics	Cu binding affinity
(Tubbing *et al.*, 1994)	1.5 mg resin	River Rhine	50 to 60% Cu retained over the range of 15.9 to 99.8 μg/L Cu
	Calcium form		
	flow: 1.7 ml/min		
	contact time: 33 seconds		

Paper	Column Setup	Water Characteristics	Cu binding affinity
(Ryan *et al.*, 1985)	calcium form		
		pH 6.0	75% of 100 μg/L Cu retained in setup 1
	Bore: 7mm		
		25 mg/L soil-derived fulvic acid	61.9% of 27.8 μg/L Cu retained in setup 2
	setup 1:		
	10cm length, Flowrate3 ml/min		
	detention time: 77 seconds		
	setup 2:		
	30cm length, Flowrate 2 ml/min		
	detention time: 346.5 second		

Paper	Column Setup	Water Characteristics	Cu binding affinity
(Miwa *et al.*, 1989)	30mm length; 3 mm id	Laboratory reagent water	no DOC: retained 100% of 10 μg/L Cu
	detention time: 5.1 seconds		0.5 mg/L Humic Acid: retained 48% of 10 μg/L Cu
	calcium form	pH 7.0	
	2.5 ml/min		

Previous research has shown that alkaline earth metals ion sorbed on Chelex 100 at pH high than 3 by complexation with the iminodiacetic acid groups inside the resin. Divalent cations such as Ca^{2+} and Mg^{2+} are common in water and may compete with heavy metals for active sites on chelating resins, so that they could be an important interference (Pesavento and Biesuz, 1997). Pesavento and Biesuz concluded that the presence of alkaline earth metal ions, which are sorbed on Chelex 100 at a pH higher than 2.5 do not interfere with the sorption of copper.

Chelating resins were able to complex 3.5 mg/L copper up to a hardness of 195 mg/L as $CaCO_3$. As hardness was increased to 254 mg/L as $CaCO_3$, elevated effluen copper concentrations were observed, indicating some breakthrough of the resin (Mazidji *et al.*, 1992). Furthermore, pH may affect the Chelex 100 ® resin performance. Mazidj *et al.* (1992) and Kuhn *et al.* (1998) both found that pH values near 4 result in decreased resin performance compared to higher pHs.

No noticeable chelating changes were seen in brines ranging from 35 to 250 g/L in total dissolved salts (Kuhn *et al.*, 1998). This suggested that chelating resins may be used in estuarine waters, although typical salinity ranges in estuaries are generally lower than those tested by Kuhn *et al*. (1998).

Domestic and industrial discharges of ionic and non-ionic detergents, formulated detergents, and detergent additives may affect also Chelex performance. Pakalns and Batley (1978) reported better than 95% recovery of metals, but this recovery was diminished to less than 50% in the presence of 20 mg/L soap and 10 mg/L nitrotriacetic acid (NTA) for metals such as 0.5 mg/L zinc, 1.0 mg/L nickel, 1.0 mg/L cobalt, and 3.0 mg/L lead. Furthermore, 1.0 mg/L copper was not affected by NTA, but cationic surfactants were totally retained by the resin, taking up 13% of the total resin capacity. I must be noted that Pakalns and Batley (1978) worked with a cationic surfactant concentration of 100 mg/L in order to note possible interference. This concentration is much higher than would be seen in polluted waters.

As discussed above, the Chelex resin has been implemented to measure bioavailable copper. Current regulations are in terms of dissolved copper, but researchers are striving to understand bioavailable copper. The Chelex 100 ® resin technique measures the amount of free copper and weakly bound copper in a natural water.

However, as shown above, the metal complexing ability of the ion exchange resin depends upon many different factors. Resin characteristics such as flowrate and resin form can affect metal complexation. Water characteristics such as pH, alkalinity, and NOM can affect metal complexation. Furthermore, the Chelex resin technique must be calibrated for a specific species type and maturity. Because of the many factors listed above, the copper that binds to the Chelex 100 ® resin is operationally defined as bioavailable copper, and does not relate to a specific water or aquatic organism.

In the past, some researchers have made attempts to relate Chelex 100 ® performance to toxicity for a given aquatic organism. In a study using hatchery-reared juvenile coho salmon (*Oncorhynchus kisutch*) in river water, there was a significant correlation between truncated mortality (partial kills) and the fraction of Chelex 100 ® bound cadmium (r = .681) (Buckley, 1985). However, in a similar study no correlati was seen between the fraction of copper bound to the Chelex 100 ® resin and the fraction of copper inhibiting the growth of the marine diatom *Nitzschia closterium*. Furthermore, some researchers concluded that the copper-Chelex fraction overestimated toxicity (Florence *et al*., 1983). Tubbing (1992) found a significant correlation between Chelex 100 ® copper and the percent reduction in photosynthesis for the algal *S. capricornutum*. This correlation was actually better than that seen with free copper.

Bioassays: Acute Toxicity

Aquatic toxicity tests have been performed in order to observe the impact o contaminates on aquatic organisms (Buikema *et al*, 1982). There are several importan criteria commonly used by researcher to select toxicity test organisms. These include sensitivity, lab adaptability, biological requirements, regulatory acceptability, and availability. Toxicity tests are commonly performed using organisms at various life stages, because larval organisms tend to be more sensitive to toxic responses than adult organisms.

These tests allow researchers and regulators to assess the potential and existing harmful effects of contaminates in the environment. Toxicity tests are utilized to answer many watershed questions: 1) what concentration of toxicant is lethal to organism, 2) contaminate species is most toxic, 3) organism sensitivity, 4) under which environmental

conditions are the contaminant most toxic, 5) impacts of the receiving stream, and 6) acute and chronic affects (Buikema *et al*., 1982). The results from toxicity testing assist regulators in development of NPDES (National Pollutant Discharge and Elimination System) permit requirements. Management strategies are designed from the knowledge obtained from toxicity testing to minimize possible environmental impacts.

Many different biological responses to contamination (end points) are applied in bioassays. Tests may utilize one species, an array of single species tests, or multiple species exposed to the same test environment. Toxicants are presented in water only, mixtures of water and sediment, or mixtures of food and water. Toxicants in known concentrations may be spiked into experimental media of known composition, or the toxicity of ambient water/sediment (from an ecosystem) may be tested.

Bioassays are an essential tool in the development of a conceptual model of how pollutants influence natural systems. Currently, chemical analysis, ecological studies in polluted environments, and bioassays are utilized to evaluate the damage caused by trace element contamination as a result of human activities. An element must be biologically available to cause an adverse effect (Luoma, 1995). Concentration, geochemical speciation, and biological processes influence biological availability. The influence o contamination is limited by the complexity of ecosystems thus, demonstrating the difficulties of proving cause and effect. More advanced experimental laboratory studies are necessary to complement ecosystem studies.

In order to verify the health of the culture, standard 48 hour acute reference tests were conducted during the experiment. The standard toxicant was an EPA certified solution of sodium lauryl sulfate. The health of the laboratory cultures is verified fro literature reports of LC_{50} for reference toxicant solutions.

Test Organism: Americamysis bahia

Mysid shrimp are small shrimp crustaceans found in marine and freshwater environments. Figure 1-3 illustrates an immature mysid. The mysid that currently is of primary interest in the NPDES program is the estuarine species *Americamysis bahia*. Mysid shrimp thrive in greatest abundance at salinities near 30 ppt in a wide range o salinities above 15 ppt to (Price, 1982). Other marine mysids that are used in toxicit

testing include *Metamysidopsis elongata*, *Neomysis amerciana*, *Neomysis awatchenis*, *Neomysis intermedia*, and for the pacific coast, *Holmesimysis sculpta*, and *Meomysis mercidis*..

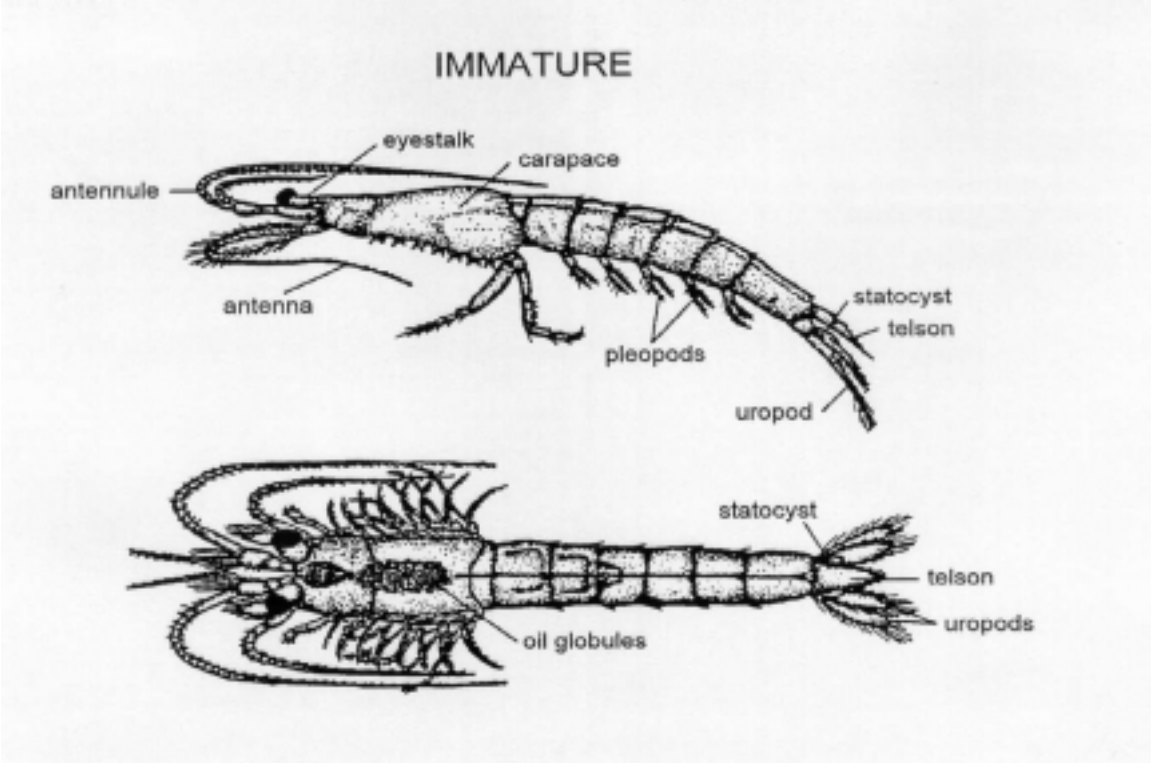

Figure 1-3. Immature mysid, *Americamysis bahia*, (A) lateral view, (B) dorsal view. From USEPA (1987d).

In laboratory cultures, Americamysis bahia reach sexual maturity in 12 to 20 days, depending on water temperature and diet (Nimmo *et al*., 1977). The female has eggs in the ovary at approximately 12 days of age. Unlike Daphnia, the eggs will not develop unless fertilized. Mating takes place at night and lasts only a few minutes.

Brood pouches are normally fully formed at approximately 15 days as shown in Figure 1-4, and young are released in 17 to 20 days (Price, 1982 and Nimmo, 1977). The number of eggs in the brood and the number of young produced per brood are a direc function of body length and environmental conditions. Mature females produce about 25

stage I larvae (egg-shaped embryo) per brood in natural and artificial seawater, but average 11$^{\pm}$ 6 stage III larvae. A new brood is produced every 4 to 7 days.

At the time of emergence, juveniles are immobile, thus susceptible to predation by adult mysids. The 24 to 48 hour juveniles feed upon plankton, then begin pursuing food organisms such as *Artemia* and rotifers. Adults range in length from 4.4 mm to 9.4 mm, measured from the anterior margin of the carapace to the end of uropods (Figure 1-4 and 1-5). The mature females are normally larger than the males and the pleopods of the female are smaller than those of males (Price, 1982 and Nimmo, 1977). Living organisms are usually transparent, but may be tinted yellow, brown or gray.

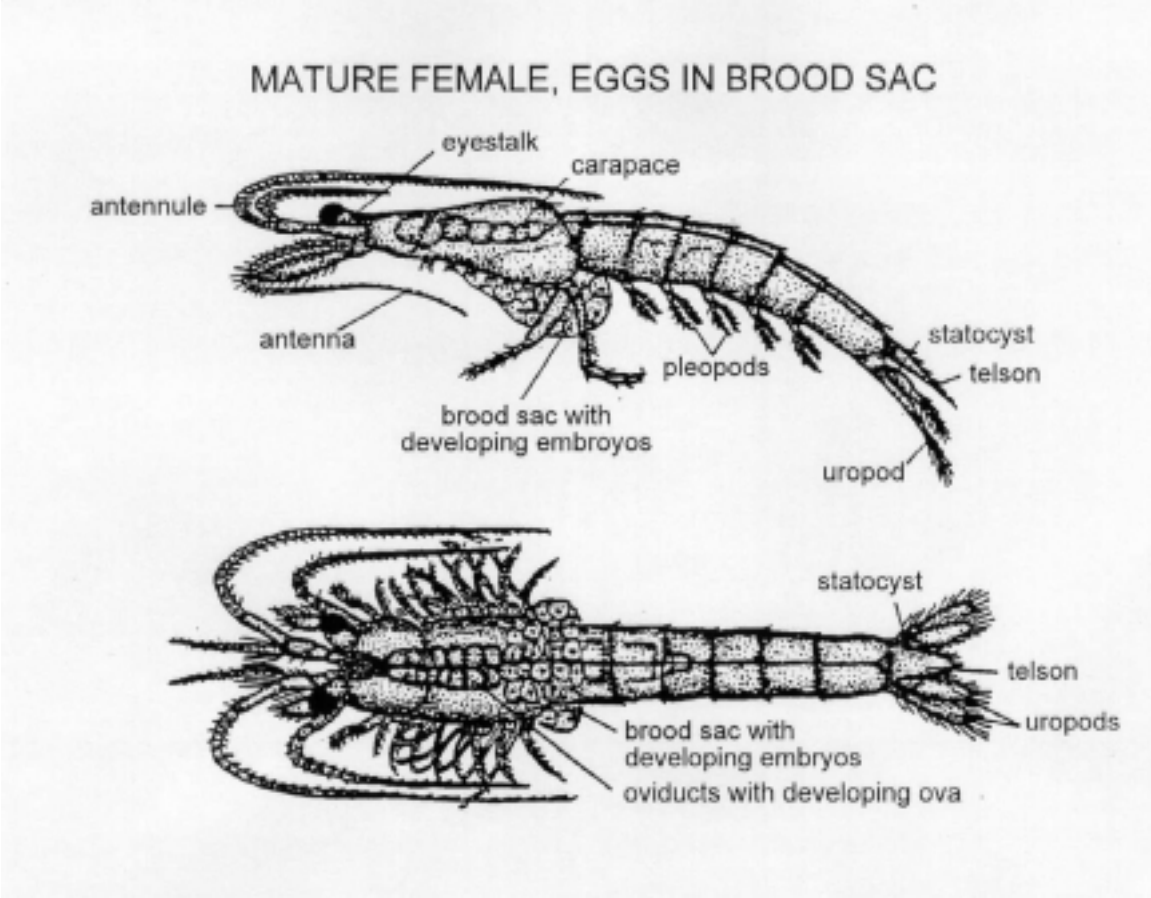

Figure 1-4. Mature female mysid, *Americamysis bahia*, with eggs in oviducts and developing embryos in the brood sac. Above: lateral view. Below: dorsal view. Fro USEPA (1987d).

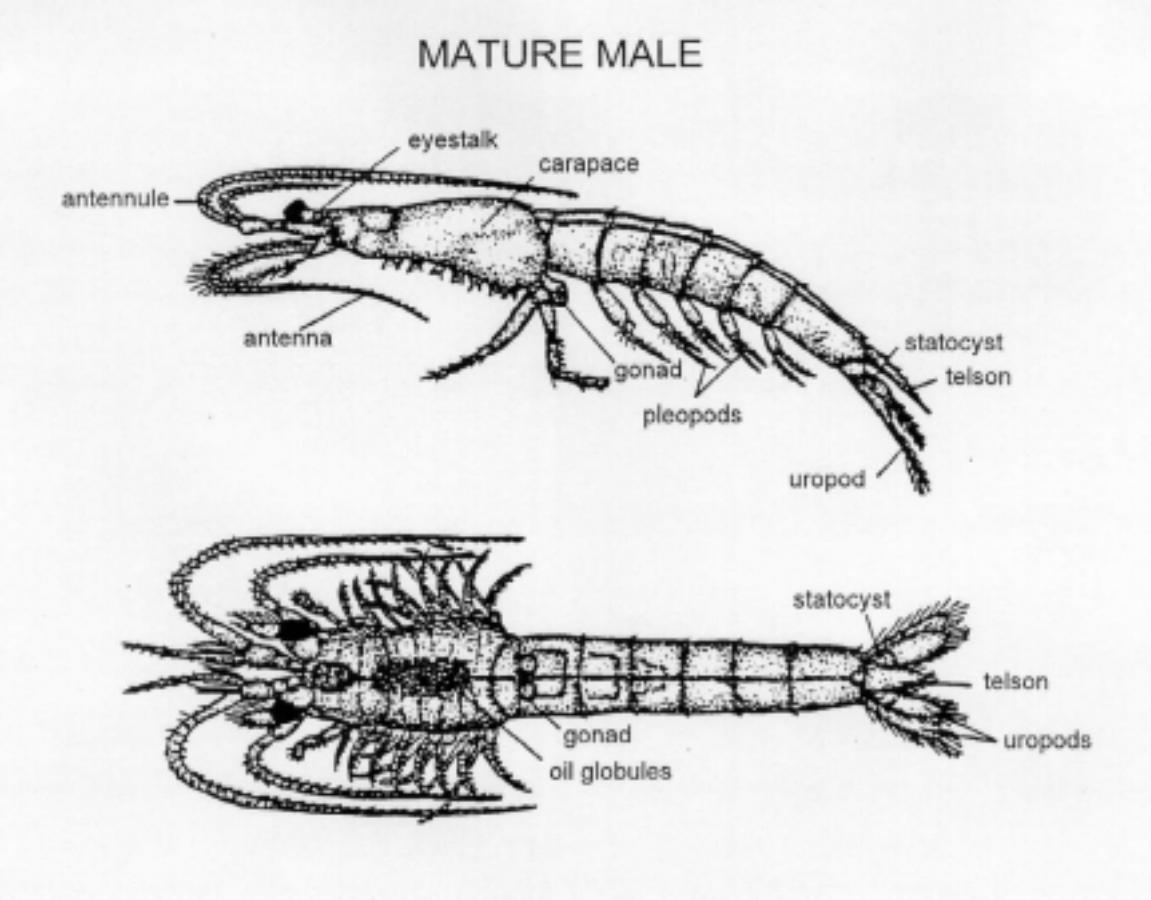

Figure 1-5. Mature male mysid, *Americamysis bahia*. From USEPA (1987d).

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Evaluation of Chelex 100 ® for Assessing the Impact of Fulvic Acid (NOM) on Copper Toxicity and Bioavailability to *Americamysis bahia*

Niel Holland Postlethwait

(ABSTRACT)

A cation exchange method (Chelex 100 ®) that distinguishes weakly bound and strongly bound copper was investigated for its ability to measure bioavailable copper in estuarine waters. Copper bound to the Chelex 100 ® resin was operationally defined as bioavailable copper. Varying initial copper concentration from 195 to 495 µg/L at a constant 12.5 mg/L natural organic matter (NOM) did not affect percent bioavailability. There were also no noticeable effects when varying total Cu concentration in the presence of 0, 12, and 24 mg/L NOM. An increase in pH from 4 to 8.5 and NOM from 0 mg/L to 12.5 mg/L reduced percent bioavailability. Using the Chelex 100® resin to measure bioavailable copper, about 20 to 40% of the total copper was bioavailable in the absence of NOM, while about 15 to 20% was bioavailable when either 12 or 24 mg/L NOM was present.

Acute toxicity bioassays were performed with mysid shrimp (*Americamysis bahia*) to evaluate the toxic effects of copper in the presence of Suwannee River Fulvic Acid, which served as a source of NOM. Static or static renewal tests, based on EP method OPPTS 850.1035 with a minimum of 10 mysid shrimp per test condition, were used to determine the LC_{50} and EC_{50} of copper and the effects of NOM. Test solutions consisted of artificial synthetic seawater at 20 parts per thousand containing concentrations of 0, 100, 200, 400, 800 µg/L copper with either 0, 12, 24 mg/L NOM. Forty-eight hour acute toxicity tests were performed on larval (2 to 3 day) mysid shrimp that were fed *Artemia* (brine shrimp); mortality and immobilization were the endpoints. The 48 hour LC_{50} was 200 µg/L dissolved Cu and 94 µg/L bioavailable Cu without NOM, 340 µg/L dissolved Cu and 98 µg/L bioavailable Cu when 12 mg/L NOM was present, and 495 µg/L dissolved Cu and 105 µg/L bioavailable Cu at 24 mg/L NOM.

The consistency of the LC_{50} measurement using bioavailable Cu suggest that the Chelex 100 ® resin is a useful technique for toxicity analysis in saline water.

Keywords: ion exchange, bioavailable copper, Suwannee River Fulvic Acid Reference (NOM), *Americamysis bahia* (mysid), toxicity

Introduction

Copper (Cu) is plentiful in the environment and essential for the normal growt and metabolism of all living organisms [1]. The sources of copper in surface waters include storm water runoff from transportation areas, lawns, fields, or discharge of treated wastewater. Other inputs of copper are contributed from metal plating/finishing circuit board industry effluents, copper from brake linings, CCA (copper, chromium, arsenic) treated wood, natural copper cycling corrosion, application of liquid manure, pesticides and algaecides such as copper sulfate addition to reservoirs for algae contro [2,3,4]. Heavy metal contamination of aquatic ecosystems is potentially detrimental to aquatic life. Copper is among the most toxic of the heavy metals to freshwater and marine biota [1]. Low dissolved copper concentrations, on the order of 1-10 μg/L, tend to affec smaller aquatic organisms such as diatoms and invertebrates while higher concentrations, in the range of 100-1000 μg/L dissolved copper, typically produce toxic effects in fish [5].

In the early 1990's, regulatory standards for metals were changed from total meta to dissolved metal because some research showed that if a metal was strongly bound in the particulate phase, it was not readily toxic [6]. Even within the dissolved phase, metals may bind to dissolved organic matter or inorganic complexes, which impac toxicity. Analysis of copper toxicity should be set in terms of species specific bioavailable copper. Conceptually, bioavailable metal is considered that portion of the total metal concentration which interacts with a given organism. Free metal ions and weakly complexed metals, as well as metals that are sorbed and released, are bioavailable and exert toxicity, and therefore should be regulated.

Bioavailability is defined as the degree to which chemicals in the environmen (e.g. water, sediment, and food items) can actually be taken up by organisms [5]. The more bioavailable a chemical, the greater the toxic potential. Toxicity is better correlated to bioavailable, labile, or free (Cu^{2+}) copper concentration than to total copper concentrations [7].

The toxicity of Cu to aquatic organisms varies with chemical form and species [7-9]. In seawater, the major chemical species of copper are cupric ion, Cu^{2+}, Cu(OH)Cl,

copper carbonate, $CuCO_3$, and copper hydroxide, $Cu(OH)_2$ [7]. Labile copper and copper hydroxides are considered highly toxic copper species. $[Cu_2(OH)_2]^{2+}{}_{aq}$ is sometimes considered bioavailable depending upon natural water conditions. Copper-carbonate species and copper-organic ligand species are considered nontoxic [8]. Chelators, such as EDTA, and more alkaline pH increase the survival and larval developmental rates o copepods challenged with copper through increased complexation of cupric ions [9].

The bioavailability of metals to aquatic organisms is influenced by meta speciation. Numerous species of copper coexist simultaneously under natural environmental conditions. These species are known to be controlled by pH, organic complexes, inorganic ligands such as phosphates, carbonates, etc., and sediment [8].

In order to determine bioavailable Cu, it is necessary to utilize methods which determine specific copper species. Copper specific electrodes, voltammetry and ion exchange are analytical methods used to determine bioavailable copper concentrations. Copper specific electrodes are utilized to measure labile copper concentrations in freshwater systems. The use of ion selection in seawater is not advisable due to strong Cl^- interference which stabilizes Cu (I) formed at the electrode surface. Therefore voltammetry and ion exchange methods are utilized for seawater matrices. Bioavailable copper measurements utilizing atomic adsorption and differential pulse anodic stripping voltammetry (DPASV) were compared to *Hyalella azteca* Saussure responses to copper in freshwater [10]. In this experiment, DPASV was useful for measuring biovailable copper in toxicity tests.

Chelex 100 ® resin, a styrene-divinylbenzene copolymer with imminodiacetate functional groups, can form complexes with free metals through ion exchange. The copper affinity of the resin may be great enough to dissociate and retain copper ions (i.e., copper hydroxide complexes) that were weakly complexed in solution. These weakl bound copper complexes are generally considered bioavailable, whereas copper strongly bound to organic or inorganic ligands that pass through the resin is complexed and not considered bioavailable. One major advantage of the Chelex 100 ® resin technique is tha samples may be processed through the resin in the field, and the actual metal analysis can occur later using standard techniques.

To completely understand copper as a toxin, the sources and fates of the metal and organic and inorganic materials which control the relationship between labile Cu^{2+} and dissolved and total copper must be evaluated. Bioavailability may be easier to understand if a bioassay is utilized with study of the chemistry of an ecosystem.

Major factors that influence the bioaccumulation of copper in saltwater organisms include: size and age of the organism, copper concentration and speciation in water and food, exposure routes, and physical environmental characteristics such as pH, temperature, alkalinity, NOM, and turbidity [11]. Because of these intricacies, the measurement of potentially harmful copper is a difficult task.

Some aquatic species are more sensitive to heavy metal toxicity than others. The LC_{50} of the marine mussel *Mylitus edulis* in 4, 10, 14, and 30 days is 300, 90, 15, and 2 µg/L total copper [12]. The soft shell clam *Mya arenaria* 7 day LC_{50} during the summer is 35 µg/L copper [13]. *A. bahia*, an estuarine mysid shrimp, has a 2 day LC_{50} of 150 µg/L dissolved Cu [14].

The toxic effect of copper also depends on the organism's life stage. The larval stage of the marine mussel *Mylitus edulis* cannot survive total copper concentrations greater than 400 µg/L. This organism is even more susceptible in the embryonic form, where water containing 5 to 6 µg/L total copper is toxic [12]. For crabs and shrimp more frequent molting may explain some of the sensitivity differences between adults and larvae of the same species. Larval crabs (*Cancer magister*) and sand shrimp (*Crangon crangon*) were 1 to 2 orders of magnitude more sensitive than adults to metals [14].

Furthermore, some organisms, including filter feeders such as larval clams and oligochaete worms, not only take up soluble metals, but also ingest particulate-bound metals through the food chain [15,16]. Once inside the stomach, enzymatic activity has the potential to release bound particulate copper to a form that is readily bioavailable, thus causing a toxic effect that would not be directly measured in a water sample. Although current bioavailable copper measurement methods do not consider ever possible form of copper in the environment, they are still valuable for understanding how the dissolved fraction of bioavailable copper changes in natural water given characteristics such as pH, NOM, and hardness.

In coastal waters, copper is sorbed to the organic suspended material and transferred to the sediment [17]. The sorption reduces bioavailability and toxicity to aquatic organisms [18]. The typical concentrations of total natural organic matter (NOM) in freshwater rivers and estuaries are 2 to 20 mg/L. Organic matter content varies between 0.3 and 5 mg/L of carbon in seawater. About 90% of dissolved copper species are found to be sorbed to natural organic matter in seawater [19]. Humic substances account for approximately 60% of the total NOM in southeastern US estuaries [20].

It is suggested that natural ligands control the speciation of heavy metals and thus the bioavailability of these metals in natural water. Copper binding behaviors of a water sample can be calculated if the total concentration of the ligands present $[Li]_T$ and their copper binding strengths are known. The binding strength of such copper ligand wit respect to free copper is represented by its binding constant, $_{CuLi}$, defined by the equation:

$$K_{CuLi} = [CuLi]/([Cu^{2+}][Li] \quad (1)$$

in which[CuLi] is the concentration of copper bound to organic and inorganic ligands of Li. Li is defined as

$$[Li] = [Li]_T - [CuLi \quad (2)$$

The [CuLi] concentration can be calculated from Eqs. 1 and 2 [20].

Using FITEQL (a program for the determination of chemical equilibrium constants from experimental data) Suwannee River Humic and Fulvic Acids (SRHA and SRFA) were modeled to contain 8 to 10 nM copper or metal binding ligand per mg/L of humic material [21]. Humic and fulvic substances are contributors to both strong (L_1) and weak (L_2) strength ligands in coastal systems [20,21].

Bioavailable copper measurements would be useful in any natural aquatic environment, and are especially relevant to coastal areas, where discharges of POTWs effluents, agriculture, and aquaculture are located very close to one another. Metals desorb from sewage particulate matter in seawater and promote the release of copper and other metals. Farmers routinely apply copper-based pesticides to crops and in animal feed, and studies have shown that 16-240 µg/L of dissolved copper are present in the runoff from plasticulture tomato fields, and concentrations of dissolved copper in the

receiving stream can be as high as 120 µg/L [17]. Bioavailable or free metal imparts toxicity to aquatic organisms.

The objectives of this paper were to: 1) evaluate the response of Chelex 100® to measure bioavailability of toxic copper species under varying pH and Aldrich Humic Acids (NOM) conditions, 2) perform acute toxicity experiments with mysids under conditions of varying Suwannee River Fulvic Acid Reference (NOM), 3) determine total, dissolved, and bioavailable copper LC_{50} and EC_{50} values for *A. bahia* in the presence of 0, 12, and 24 mg/L of SRFAR (NOM).

Methods

Chelex 100 ® Ion Exchange

Preparation and Evaluation

Fisherbrand ® Fisher Scientific (Pittsburgh, Pennsylvania), chemicals, sample containers, and glassware were used exclusively for all laboratory analyses. The sample containers and glassware were soaked in 10% trace metal grade nitric acid for at least 8 hours, washed three times with distilled water, and then washed three times using Nanopure ™ water.

Chelex 100 ® ion exchange resin, Bio-Rad (Hercules, California) #142-2842, was prepared in accordance with Miwa *et al*. [22]. The resin slurry was packed into columns for sampling. These columns were constructed using liquid chromatography components and plastic tubing. Figure 2-1 depicts a typical column.

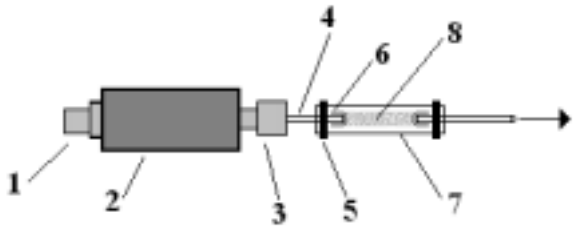

Figure 2-1. Chelex 100 ® Column Apparatus

Where 1: Luer Lock, Upchurch Scientific (Oak Harbor, Washington) #P 624
2: Plastic Union, Upchurch Scientific (Oak Harbor, Washington) #P 623
3: Male Connector, Upchurch Scientific (Oak Harbor, Washington) #P 345
4: 1/16" id Teflon Tubing, Upchurch Scientific (Oak Harbor, Washington) #1523
5: Zip Tie, GB brand (Milwaukee, Wisconsin) #45-308
6: ¼" * ¼" .35 µm Polyester Nytex Cloth Frit
7: 1/8" id clear PVC Tubing Fisher Scientific (Pittsburgh, Pennsylvania) #141697A
8: 200-400 mesh Chelex 100 ® Resin, Bio-Rad (Hercules, California) #142-2842

Items 1 through 7 in Figure 1 were soaked in 10% trace metal grade nitric acid bath for two hours and rinsed with reagent water. The columns were then submerged three times in distilled water, rinsed with 30 mL of Nanopure ™ water, air dried, and then weighted using a Model 100A Denver Instrument Company analytical balance. The resin slurry was then drawn into the columns using a 60 cc plastic syringe that was acid washed in the same manner as described above. Excess slurry water was drawn out of the packed column and each column was adjusted to 0.085 g on a dry weight basis. second ¼" × ¼" polyester frit, 1/16" id Teflon tubing, and zip tie were then added to the column to retain the resin as shown in Figure 1. Using a KD Scientific (Boston, Massachusetts) 220 screw-driven syringe pump set to 60 mL/min, 4 mL of 2 M calciu chloride was passed through each column, followed by 30 mL of Nanopure ™ water to precondition the resin.

Chelex 100 ® Sampling

Samples were first treated to remove particulate matter before passage through the Chelex 100 ® resin. Samples with a pH of 8.0 or greater were passed through the 3.5 µm glass filter were operationally deemed as dissolved. Previous research has shown tha copper sorbed to a 0.45 µm filter at pH values above 8. Samples were passed through a 0.45 µm Fisherbrand ® number 09-719-2D filters when the pH was less than 8. After filtering, samples were passed through the Chelex 100 ® resin at 60 mL/min, yielding a sample detention time of approximately 0.1 seconds. The first 5 mL of sample was wasted, and then a 15 mL sample was collected for analysis. Dissolved copper retained by the Chelex 100 ® resin columns was operationally defined as bioavailable copper. To

determine the amount of resin-bound bioavailable copper, dissolved copper was measured both before and after passing through the resin. This was accomplished using either graphite furnace atomic adsorption (GFAA)(Perkin-Elmer Model 5100 PC) or a Perkin-Elmer Model 703 flame atomic adsorption (FAAS), following the manufacturers' recommendations and in accordance to Standard Methods for the Examination of Water and Wastewater 1995 [23].

Preparation of ASW

The typical salinity range for estuarine waters is 15 ppt to 25 ppt. Four days prior to use, 50 liters of 20 ppt salinity ASW was prepared by adding 24 g/L Instant Ocean™ 40 Fathoms Crystal Sea™ to Nanopure™ water as recommended by the EPA [24]. Salinity of 20 ppt was confirmed by refractometer; background copper levels ranged from 1-3 µg/L. ASW was filtered through a 41 Whatman Filter, aerated with filtered house air, and stored in 50-gallon carboy that was protected from the light. ASW was discarded after 14 days to prevent contamination from bacterial, fungal, and algal growth.

Assessment of Chelex 100 ® Performance Using Aldrich Humic Acid

Initial laboratory testing was performed in order to compare the degree of Chelex 100 ® resin copper retention in a fresh water (300 µg/L dissolved copper, pH 6.5 buffered with 30 mg/L as calcium carbonate, n=3) to a saline water (300 µg/L dissolved copper, 25 ppt salinity, pH 8). To assess copper-organic ligand binding competitive effects, 20 mg/L Aldrich Humic Acid (Aldrich Chemical Co.) as a source of natural organic matter (NOM) was also added to each solution. The sample flow rate through the Chelex 100 ® resin was also varied between 60 and 10 mL/min to determine its effect, for a solution consisting of µg/L Cu, 21 ppt salinity, pH 4, and 12.5 mg/L NOM.

Preliminary laboratory tests varied water characteristics such as copper concentration, salinity, pH, and NOM. A 1000 mg/L copper nitrate Fisher certified copper reference solution was used to adjust the amount of copper. The salinity of the solution was adjusted as needed using Instant Ocean ® (Mentor, Ohio); the pH of the

water was adjusted using either 10 M NaOH or 10 M HCl; the NOM was adjusted b diluting with 21 ppt salinity ASW. The variable in question was modified while keeping the remaining sample matrix constant for all tests. T-tests and ANOVAs were performed using NCSS 97 to test for significant difference.

Mathematical Modeling of Chelex 100 ® Resin Using Aldrich Humic Acid

A copper-organic ligand model was established using MINEQL$^+$ version 4.0, [25], which predicted copper-NOM species in natural estuarine waters. A five-ligand model developed by Cabaniss and Shuman [26,27] from experimental data was implemented utilizing MINEQL$^+$. All of the copper-organic ligand complexes predicted by the mathematical model were assumed strongly complexed and therefore not bioavailable in terms of the Chelex 100 ® ion exchange resin.

Suwannee River Fulvic Acid (SWFA) stability constants from Cabaniss and Shuman for copper-ligand (Cu-L) complexation applied in MINEQL+ were as follows: CuL_1 (log K = 3.90), CuL_2 (log K = 1.494), CuL_3 (log K = -0.364), CuL_4 (log K = -7.483), CuL_5 (log K = -10.05). MINEQL$^+$ also required the total molar concentration for each ligand. To convert the copper-NOM complexes from mg/L to moles/L, the NOM concentrations were multiplied by constants of 5.0×10^{-6}, 1.9×10^{-7}, 1.1×10^{-6}, 1.4×10^{-7}, and 9.6×10^{-6} moles/mg respectively [26,27].

Bioassay: *Americamysis bahia* Toxicity Test

Test Conditions

The bioassay was designed to determine the concentration at which copper is toxic, determine the toxicity in natural waters, and understand the constituents that determine toxicity. All test were conducted according to EPA guidelines; specifically the acute methods are presented in EPA method OPPTS 850.1035. Toxicity tests were performed in 500 ml polypropylene beakers with 250 ml of test solution. All tests were static tests, 10 mysid shrimp per replicate, fed *Artemia* once a day with survival and immobilization as the endpoint. The light regime was 16 hours light/ 8 hours darkness,

the pH ranged from 7.8 to 8.0, with a temperature of 20°C ± 1°C. The dissolved oxygen for all tests remained above 4.0 mg/L.

Organism source

Larval (2 day) *Americamysis bahia* were purchased from AquaTox Inc., Hot Springs, Arkansas. Organisms were cultured in 10 gallon tanks at salinities between 25 and 28 ppt. Shrimp were acclimated to 20 ppt artificial seawater over a 24 hour period b addition of 20 ppt artificial seawater (ASW) dilution water. 10 ± 1 larval shrimp were carefully pipetted to the five test solutions in quadruplicate; this method reduced impingement stress. The shrimp were fed approximately 100 to 150 *Artemia* daily. In order to verify the health of the culture, standard 48 hour acute reference test was conducted using an EPA-certified solution of sodium dodecyl sulfate or sodium lauryl sulfate ($C_{12}H_{25}NaO_4S$) lot number 706674 . Sodium lauryl sulfate was diluted with 20 ppt ASW at concentrations of 0, 1.25, 2.5, 5, 10, 20 mg/L. The LC_{50} was determined to be appropriate based on studies performed by Burton and Fisher [28].

Test Solutions

A dilution series of 800, 400, 200, 100, and 0 µg/L added soluble copper was prepared in ASW from Fisherbrand ® copper 1000 mg/L copper solution (Pittsburgh, Pennsylvania). Suwannee River Fulvic Acid Reference (SRFAR) 1R101F was obtained from the International Humic Substances Society, (Denver Co.). The source of NOM was switched from Aldrich Humic Acid to SRFAR to obtain a natural synthesized source o NOM for conducting the bioassay. The SRFAR is well characterized and its isolation method and chemical properties have been reported: the organic carbon content is approximately 53.3% Lin [29]. The TOC measurement for the 12 mg/L FA was 6.0 mg/L and at 24 mg/L FA was 12.0 mg/L, measured using a Sievers 800 TOC analyzer (Boulder, Co.). Treatments contained concentrations of 0, 12, and 24 mg/L SRFAR in respective test chambers.

Artemia Cultures

Artemia Cysts (brine shrimp) were purchased from Argent Chemical (Redmond, WA). *Artemia* were cultured in a 1L conical glassware at temperature of 28°C, a 24 hour light cycle, and 2 tablespoons ASW to 1 teaspoon of cysts. The *Artemia* were first rinsed with deionized water then rinsed with 20 ppt ASW. To feed the mysid shrimp, two to three drops o *Artemia* were added to each 500 ml polypropylene test chamber daily, to produce approximately 100 to 150 *Artemia* per mysid shrimp daily.

Statistical Analysis

After tests were completed, survival data was analyzed to determine the LC_{50} and EC_{50}. The number of mortalities from the acute testing was used to determine the LC_{50} (lethal concentration). EC_{50} data was measured using swimming and feeding as an activity. Methods for determination of mysid EC_{50} (median effective concentration) were derived by observation [30]. The EC_{50} was determined based on mysid immobilization. Survival and activity data were analyzed using US EPA Toxicity Data Analysis Software ® (Cincinnati, OH) to determine the LC_{50} and EC_{50}. The analysis scheme suggested in EPA report no 600/4-90/027 was used to determine the LC_{50}. Because of observed mortality in the controls the Trimmed Spearman-Karber (TSK) method was used to calculate LC_{50} values and confidence limits for the toxicant exposures. The LC_{50} values were graphically compared for toxic effects at 0, 12, and 24 mg/L of NOM.

Results

Chelex 100 ® Evaluation Using Aldrich Humic Acid as source of NOM

Assessment of Chelex 100 ® performance

Initial laboratory testing indicated that the percent of copper bound to the resin did not differ between a freshwater solution (pH 6.5 buffered with 30 mg/L as calcium carbonate) and a saline solution (25 ppt salinity at pH 8), ($p = 0.31$). Adding 20 mg/L

natural organic matter (NOM) to each solution lowered the amount of copper sorbed to the resin in both cases by an equal amount ($p = 0.736$), (data not shown).

Figure 2-2 illustrates the effects of varying salinity, copper concentration, pH, and NOM on the percent Chelex bound copper measured by the resin. In each case, the solution matrix was held constant while the variable in question was modified. Varying the salinity to 16, 21, and 30 ppt and copper concentration from 195 to 579 µg/L (Figure 2-2) had no effect ($p = 0.051$ and $p = 0.66$ respectively). Lowering pH and NOM (Figure 2-2) statistically increased percent bioavailability, ($p = 0.01$ and $p = 0.007$ respectively).

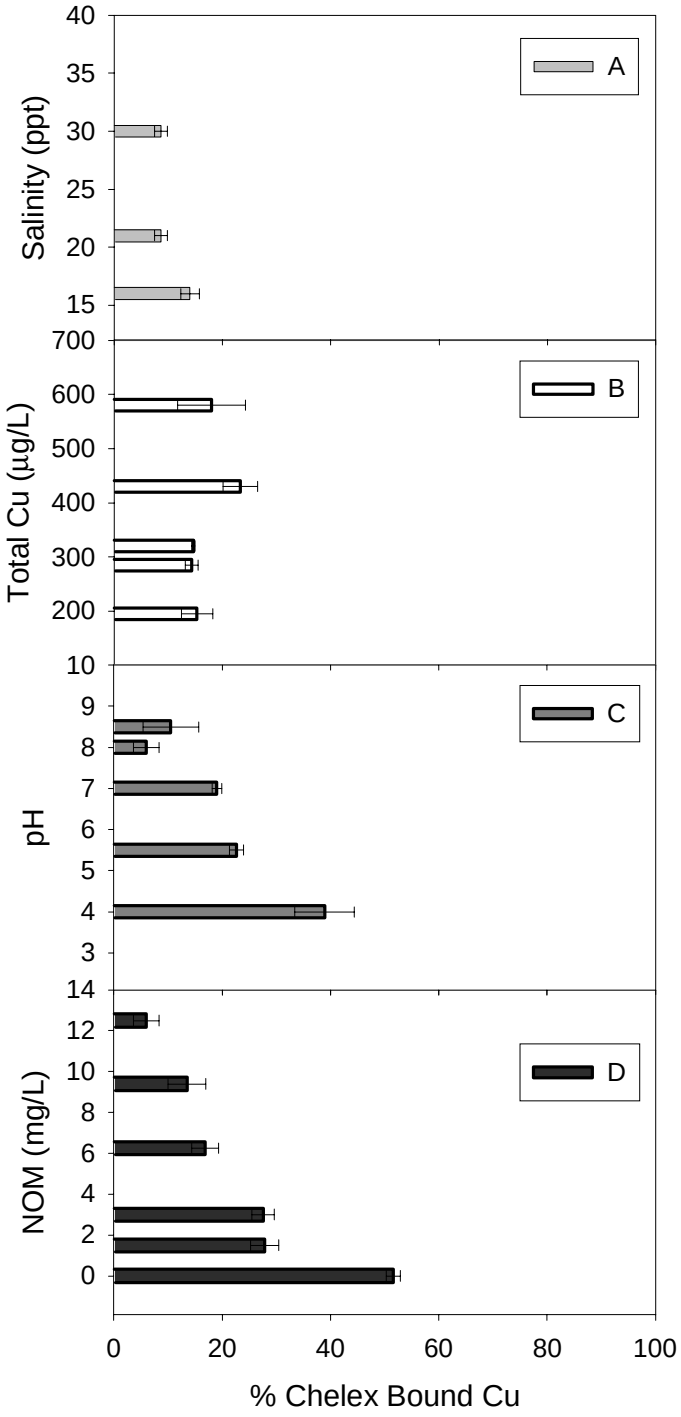

Figure 2-2. Effect of various parameters on Chelex 100 ® Binding of Copper. Error bars are +/- standard deviation (n=3). (A) Varying Salinity (ppt) (150 µg/L Cu and 12.5 mg/L NOM AHA), (B) Varying Cu concentration, (pH 7.3, 15 ppt salinity, 12.5 mg/L NOM AHA), (C) pH, (150 µg/L Cu, 21 ppt) and (D) NOM, (150 µg/L Cu, 21ppt salinity, pH 8.1).

Figure 2-3 and 2-4 compare MINEQL$^+$ model predictions to the experimental resin pH and NOM titrations respectively. In Figures 3 and Figure 4, the individual model-predicted copper-ligand species are shown (i.e.- CuL, Cu_2L, CuL_2), along with the total amount of model-predicted copper-bound ligand, denoted by "Sum of all Cu-Ligands". The ligand bound copper predicted by MINEQL+ is a calculation of the complexed or non-Chelex bound copper in the system. Figure 2-3 illustrates that at pH values between 5.5 and 8.5 there was a satisfactory agreement between the MINEQL$^+$ prediction and the experimentally measured complexed copper as determined by the Chelex 100 ® resin. Figure 2-4 suggests good agreement between the MINEQL$^+$ prediction and the experimentally measured "non-Chelex bound" copper at NOM concentrations greater than 6 mg/L.

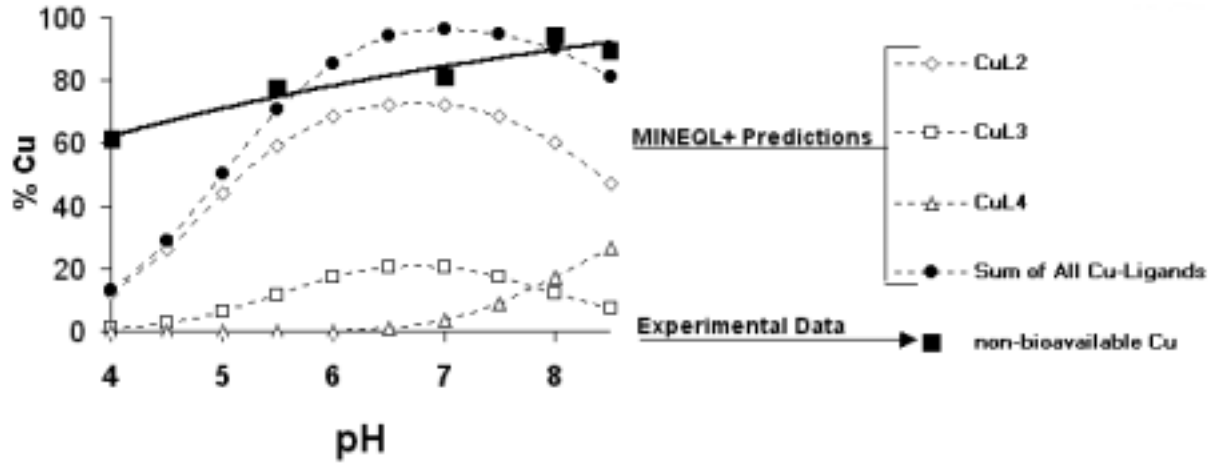

Figure 2-3. pH Titration: MINEQL+ prediction of non-bioavailable Cu-ligand species (CuL, Cu_2L, and CuL_2) vs. Chelex 100 ® Experimental Results (150 µg/L Cu, 21ppt salinity, and 12.5 mg/L NOM (Aldrich Humic Acid). MINEQL$^+$ complexation constants were calibrated using data from Cabaniss and Shuman [26,27].

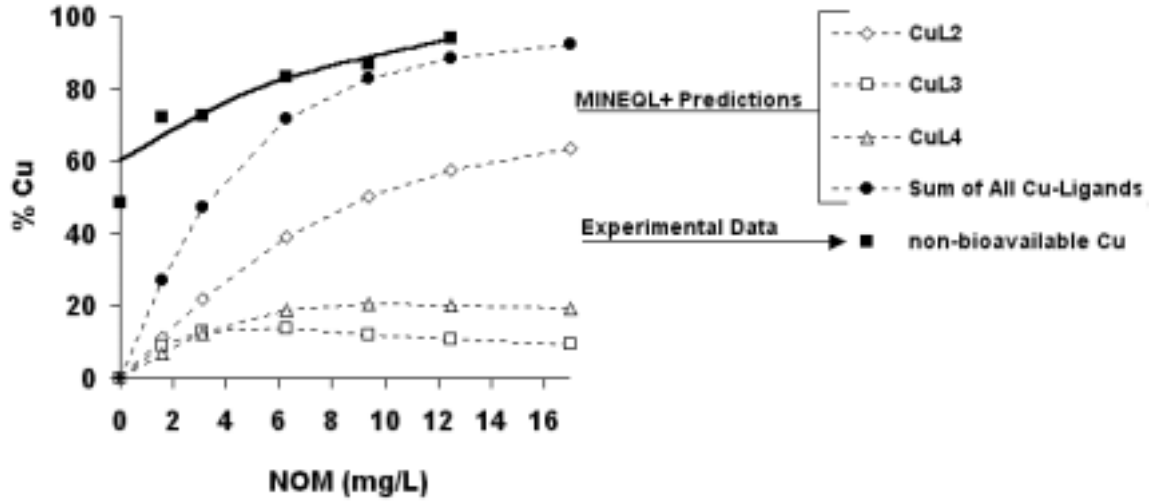

Figure 2-4. NOM Titration: MINEQL+ prediction of non-bioavailable Cu-ligand species (CuL, Cu_2L, and CuL_2) vs. Chelex 100 ® Experimental Results (150 µg/L Cu, 21ppt salinity, pH 8.1, NOM (Aldrich Humic Acid). MINEQL+ complexation constants were calibrated using data from Cabaniss and Shuman [26,27].

Bioassay: Americamysis bahia Toxicity Tests

Acute Results

A dilution series of 100, 200, 400, and 800 µg/L Cu and 0, 12, and 24 mg/L SRFAR was tested in quadruplicate. The solutions were tested to compare survival and immobilization. Figure 2-5 illustrates the percent mysid shrimp survival during a 48 hour static toxicity test with 0, 12, 24 mg/L SRFAF. Ninety-seven percent survival was observed in control test chambers. Complete mortality was observed at less than 48 hours for mysids treated with 800 µg/L Cu and 0 mg/L SRFAR. A survival of greater than 60% was maintained through 48 hours for 100, 200, and 400 µg/L Cu groups in the presence of 12 or 24 mg/L SRFAR. The presence of 12 or 24 mg/L SRFAR reduces toxicity at the 100, 200, and 400 µ/L Cu concentrations. Mysid shrimp exposed to 800 µg/L Cu and 0, 12, and 24 SRFAR displayed higher mortality than mysids exposed to 100, 200, and 400 µg/L. The presence of 12 or 24 mg/L SRFAR did not completely protect organisms from mortality at higher concentrations as observed at 800 µg/L Cu.

After 48 hours approximately 70% mortality was observed in mysids exposed to 800 µg/L Cu and 12 or 24 mg/L SRFAR.

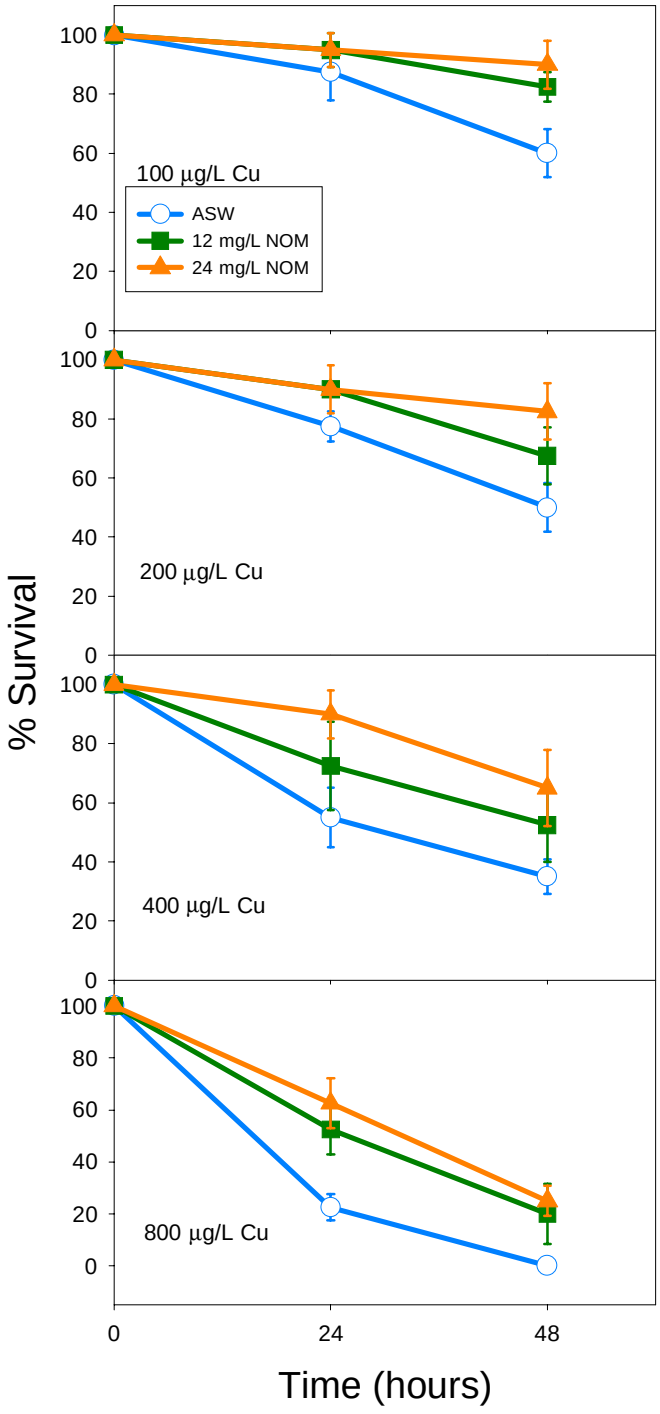

Figure 2-5. Percent survivorship in 48 hour static toxicity test with 0, 12, 24 mg/L SRFAF EPA method OPPTS 850.1035 (pH 7.7-8.0 and 20 ppt). Error bars are +/- standard deviation (n=40).

LC_{50} and EC_{50} values were calculated for mysid shrimp exposed to copper using USEPA Toxicity Data Analysis Software. Based on the 48-hour and 96-hour, toxicity test with mysid shrimp, dissolved copper LC_{50} values were calculated at 24, 48, 72, and 96 hours. LC_{50} values of 390, 200, 120, and 105 µg/L Cu were determined at 24, 48, 72, and 96 hours. The 48-hour LC_{50} for the mysid shrimp was determined to be 200 µg/L dissolved Cu in 0 mg/L SRFAR as NOM (Figure 2-6), which is similar to the 48 hour LC_{50} of 181µg/L Cu LC_{50} for mysid shrimp reported by EPA (EPA 1985)[31]. Cripe [14], reported an 48 hour LC_{50} of 150 µg/L using copper chloride for a 96-hour static acute toxicity tests with mysid shrimp at 25°C and 25 ppt. EC_{50} values (based on immobilization) were lower than the LC_{50} prior to 72 hours.

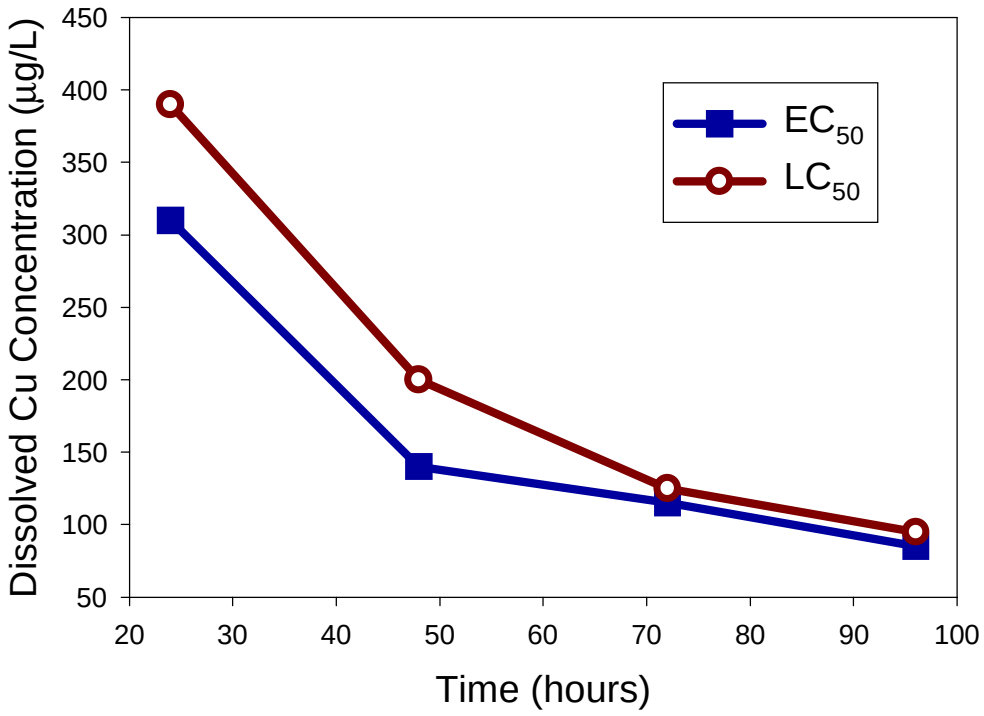

Figure 2-6. *Americamysis bahia* LC_{50} and EC_{50} values for dissolved Cu in 0 mg/L SRFAR (pH 7.9, 20 ppt).

Evaluation of Bioavailable Copper

Figure 2-7 shows the average percent bioavailable copper at various 0, 12, 24 mg/L SRFAR and copper concentrations. There was no significant difference in % bioavailable copper over the copper concentration treated, although there is a slight trend that percent bioavailable copper increases with an increase in total copper concentration. Figure 2-7 also displays that a larger percent of copper is bioavailable in 0 mg/L SRFAR than 12 and 24 mg/L SRFAR.

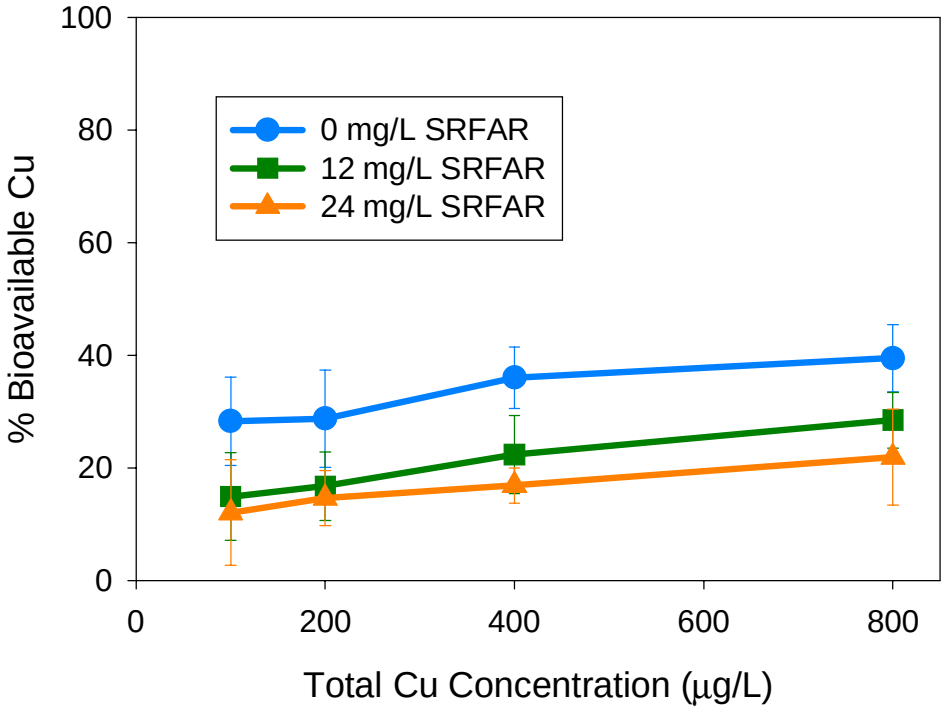

Figure 2-7. Percent Bioavailable Copper at various SRFAR and Copper Concentrations (pH 7.8, 20 ppt, and 0, 12, 24 mg/L SRFAR). Error bars are +/- standard deviation (n=9).

The 48 hour LC_{50} values for the mysid shrimp at three SRFAR levels are presented in Figure 2-8. Figure 2-8 illustrates the LC_{50} values for total, dissolved, and bioavailable copper. The 48 hour total and dissolved copper LC_{50}s increased at

increasing SRFAR. Total copper LC_{50} calculations were 230 µg/L (150-350) in the presence of 0 mg/L SRFAR, 400 µg/L (315-510) in 12 mg/L SRFAR, and 540 µg/L (400 –720) in 24 mg/l SRFAR. Dissolved copper LC_{50} concentrations were 200 µg/L at 0 mg/L SRFAR, 340 µg/L when 12 mg/L SRFAR was present, and 495 µg/L at 24 mg/L SRFAR. The 95% confidence intervals for the dissolved Cu were (135-310) 0 mg/ L, 12 mg/L (260-445), and 24 mg/L (360-675). These results indicate that the presence of 12 mg/L and 24 mg/L significantly reduce tota and dissolved copper toxicity. The LC_{50} values based on bioavailable copper were consistent at 0, 12, and 24 mg/L NOM concentrations. The 48-hour values at each concentration of NOM in terms o bioavailable copper were 94 µg/L (68-130) for 0 mg/L NOM, 98 µg/L (73-130) for 12 mg/L NOM, and 105 µg/L (68-150) for 24 mg/L NOM. The bioassay toxicity LC_{50} results indicate that Chelex 100 ® bound copper is a practical measure of bioavailable copper.

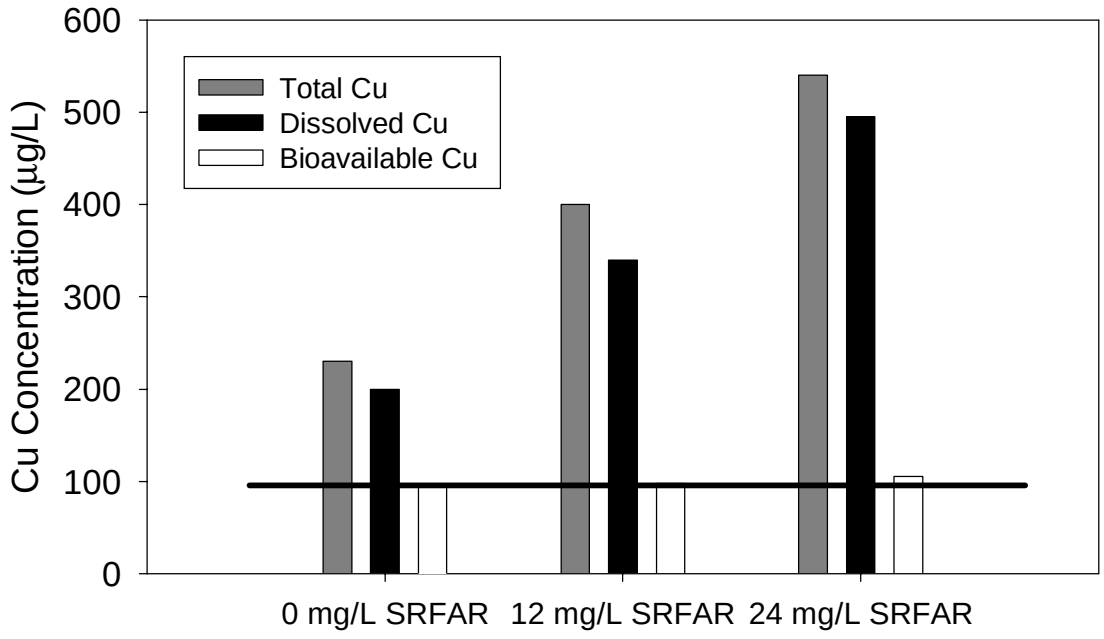

Figure 2-8. Total, dissolved, and bioavailable LC_{50}s for 0, 12, and 24 mg/L SRFAR. The black line represents the calculated mean bioavailable Cu concentration of 100 µg/L in the presence of 0, 12, and 24 mg/L SRFAR (pH 7.7-8.0, 20 ppt).

Copper binding by SRFAR was also observed during the bioassay. Table 2-1 displays the initial bioavailable copper concentration, the calculated total complexed and calculated organic complexed and free estimated at 100, 200, 400, and 800 µg/L Cu in the presence of 0, 12, 24 mg/L SRFAR. Total dissolved complexed copper estimates were assumed from pre resin analytical results. Organic complexed copper estimates were calculated subtracting the total complexed fraction from the total complexed 0 mg/L SRFAR, assuming that only inorganic complexes form when no SRFAR was present. In addition, the calculation assumes that there was no competition between inorganic and organic binding sites. For example, the calculation of the total organic fraction at 100 µg/L Cu in the presence of 12 mg/L SRFAR was calculated subtracting the tota complexed fraction at 100 µg/L Cu from the total complexed at 100 µg/L in the presence of 0 mg/L SRFAR. Test chambers which contained 24 mg/L SRFAR displayed more copper binding capacity or available sites than 0 and 12 mg/L SRFAR. Approximately 3 to 9 µg/L of Cu was organically complexed or bound per 1 mg/L SRFAR during the 48 hour bioassay.

Table 2-1. Initial Bioavailable, Total and Organic Complexed at 100, 200, 400, and 800 µg/L Cu in the presence of 0, 12, 24 mg/L SRFAR

Total Cu 100 ug/L		0 mg/L SRFAR	12 mg/L SRFAR	24 mg/L SRFAR
	Bioavailable (µg/L)	33	12	14
	Calculated Total Complexed (µg/L)	67	88	86
	Calculated Organic Complexed (µg/L)		21	19
Total Cu 200 ug/L		0 mg/L SRFAR	12 mg/L SRFAR	24 mg/L SRFAR
	Bioavailable (µg/L)	59	43	38
	Calculated Total Complexed (µg/L)	141	157	162
	Calculated Organic Complexed (µg/L)		16	21
Total Cu 400 ug/L		0 mg/L SRFAR	12 mg/L SRFAR	24 mg/L SRFAR
	Bioavailable (µg/L)	147	113	67
	Calculated Total Complexed (µg/L)	253	287	333
	Calculated Organic Complexed (µg/L)		34	80
Total Cu 800 ug/L		0 mg/L SRFAR	12 mg/L SRFAR	24 mg/L SRFAR
	Bioavailable (µg/L)	333	224	172
	Calculated Total Complexed (µg/L)	467	576	628
	Calculated Organic Complexed (µg/L)		109	161

Discussion

Chelex 100 ® Evaluation Using Aldrich Humic Acid

Initial experiments proved that copper binding to the Chelex 100 ® resin responded to environmental changes in a logical manner. Varying salinity from 16 to 30 ppt did not affect percent bioavailability. This indicated that the ions typically found in seawater did not compete with copper for ion exchange sites on the resin. Kuhn *et al.* [32] also found little to no chelating changes in brines ranging from 35 to 250 g/L in total dissolved salts. Also MINEQL$^+$ modeling of the system did not show any changes in copper speciation with increase in salinity.

The fraction of bioavailable copper exchanged onto the resin remained constan even as the total and dissolved copper concentration increased (Figure 2). If the system was exclusively comprised of inorganic compounds, this result would be expected considering simple water chemistry theory. For example, the formation of $CuCO_3$ is expressed as: $Cu^{2+} + CO_3^{2-} \leftrightarrow CuCO_3$, yielding an equilibrium constant of: $K = [CuCO_3]/[Cu^{2+}][\ CO_3^{2-}]$. Considering that K is constant, if the amount of Cu^{2+} increases, and the concentration of CO_3^{2-} is also held constant in an open system, the amount of $CuCO_3$ must also increase proportionally, thus not affecting percent bioavailability. However, this scenario is complicated when NOM was present. As more copper binds to NOM, the active sites may change, thus affecting Cu-NOM complexation. Although not shown in this paper, the MINEQL$^+$ model indicated that copper speciation in this estuarine water did not change as the total copper concentration was increased over the range studied. This is in accordance with the Chelex 100 ® resin experimental results presented in Figure 2-2.

By decreasing pH, an increase in % bioavailability was observed (Figure 2-2). As the pH of natural water is lowered, copper has a better chance to form inorganic species that are potentially bioavailable. MINEQL$^+$ modeling in Figure 2-3 also showed that as the pH of natural water was decreased, the percent of copper bound to NOM decreases. However, the Chelex 100 ® resin did not accurately follow MINEQL$^+$ predictions when the pH was below pH 5.5. At pH below 5.5, the functional groups on the resin change, and ion exchange becomes less efficient, thus lowering the amount of bioavailable copper

measured. Mazidji *et al.* [33] and Kuhn *et al.* [32] both found that pH values near 4 result in decreased resin performance compared to higher pHs.

Decreasing the amount of natural organic matter increased the percent o bioavailable copper in the resin system (Figure 2-2). As fewer sites were available for strong copper complexation with organic ligands, copper could be free or complexed with weaker inorganic species, thus forming potentially bioavailable species. This trend was further noted in MINEQL$^+$ modeling, as shown in Figure 2-4. However, as the NOM content was lowered below 6 mg/L, the experimental results started to deviate greatl from the model results. The resin did not retain all of the inorganic copper present. Further investigation into copper speciation as a function of NOM content indicated that the concentration of $Cu(OH)_2$ increases dramatically as the NOM content is lowered. The results of this model indicate that the resin may not be retaining as much as 55% of this complex, even though it has been deemed bioavailable by researchers in the past [8].

In this research a 60 mL/min flowrate with a 0.1 sec detention time was utilized. When the flowrate was decreased to 10 mL/min, there was no change in copper retention (data not shown). Previous researchers have been able to retain close to 100% copper for various waters. Chakrabartl *et al*., [34] was able to retain 99% of 133.5 µg/L copper using a batch process with a 1000 second detention time. Samples were collected from the Rideau river, and adjusted to pH 5.0. Miwa *et al.*, [22] prepared reagent water at pH 7.0, which did not contain any dissolved organic carbon. Using a detention time of 5.1 seconds, approximately 100% of 10 µg/L copper was retained by the Chelex 100 ® resin. Rasmussen *et al.*, [35] implemented flow rate of 0.1 mL/min until the resin had swollen. Afterwards the flow rate was increased to 1.0 mL/min. A detention time was not given. However, the researchers were able to retain 100% of 1.87 µg/L copper from sea water, adjusted to pH 5.0. Seawater samples passed through a Chelex 100 ® column at natural pH at a flowrate between 1 and 3 mL/min were shown to contain a significant fraction of metal that could not be removed by the column. Buckley *et al*. [36] compared Chelex liable measurements with fish toxicity measurements. Free or liable cadmium separated from river water and sewage treatment effluents by batch experiments correlated wel with the toxicity of salmon. The application of Chelex 100 ® separation provides a

simple and effective method for measuring free or weakly complexed meta concentrations [37].

Chelex 100 ® Evaluation Using Americamysis bahia Acute Toxicity Test

Results of the bioavailability measurements and toxicity tests with the mysid shrimp indicated that NOM bound copper reduced copper toxicity. Approximately 3 to 9 µg/L of Cu was complexed or bound per 1 mg/L SRFAR during 48 hour the experimental bioassay. Using the Chelex 100® resin to measure bioavailable copper, about 20 to 40% of the total copper was bioavailable in the absence of NOM, while about 10 to 20% was bioavailable when either 12 or 24 mg/L NOM was present. Addition of Suwannee River Fulvic Acid Reference as NOM decreased bioavailable copper. Similar trends were also displayed in Figures 2-2 through 2-5 utilizing Aldrich humic acid. *Daphnia magna* and *D. pulex* were used in a study of the effects of Aldrich humic acid on the toxicity and bioaccumulation of Cu, Zn, and Cd [38]. Winner [38] reported for Cu and Zn, prolonged survival of 7-day old *D. magna* and increased LC_{50} values, and decreased toxicity in the presence of humic acid. The mean LC_{50} increased from 28.3 µg/L Cu in water containing 0 mg/L HA to 53.2 µg/L in water containing 1.5 mg/L HA. The results of the acute toxicity of Cd show increased toxicity in the presence of HA. The LC_{50} decreased from 87.7 µg/L Cd 0 mg/L HA to 71.1 µg/L Cd 1.5 mg/L HA [38]. Ma [39] also reported the presence of HA decreased the toxicity of copper. The addition of 300µg/L Cu to 20 mg/L HA solution resulted in the same *C. dubia* survival as 88 µg/L Cu to 5 mg/L HA.

The results for the toxicity tests also displayed that percent bioavailable copper was not significantly different with varying total copper concentration. These results were shown in Figure 2-7. The presence of 12.5 mg/L concentration of commercial humic acid (Figure 2-2) also displayed no noticeable effect of increased percen bioavailable copper concentration with increasing copper concentration. Varying initial copper concentration from 195 to 495 µg/L for Aldrich Humic Acid (NOM) content of 12.5 mg/L did not effect percent bioavailability, indicating that as the copper concentration of this water is increased over the range studied, the percentage of

potentially toxic copper is constant, but at higher total copper concentrations a greater concentration is bioavailable and potentially toxic. The results of the NOM and saltwater experiments suggest that at different concentrations of NOM, copper may exhibit different toxicity.

Conclusions

The Chelex 100 ® resin has been shown to complex copper consistently under varying salinity, Cu concentration, pH, and Aldrich Humic Acid as depicted in this research. Changes in salinity and total copper concentration did not affect percen bioavailability, whereas decreases in NOM and pH increased percent bioavailability.

Acute toxicity testing concluded that SRFAR binds copper and thus reduces copper toxicity. SRFAR also chelates copper complexes and reduces the amount o bioavailable copper similar to the results using Aldrich Humic Acid. The results of the SRFAR and saltwater experiments suggest that at different concentrations of SRFAR, copper may exhibit different toxicity. Using the Chelex 100® resin to measure bioavailable copper, about 20 to 40% of the total copper was bioavailable in the absence of NOM, while about 10 to 20% was bioavailable when either 12 or 24 mg/L NOM was present. This research has also shown that even i n the presence of NOM and copper concentrations may be high enough to cause significant toxicity to aquatic organisms. Initial experiments and the *Americamysis bahia* bioassay results determine that Chelex 100 ® resin ion exchange method is a useful method for measuring bioavailable copper in natural estuarine and seawaters.

Acknowledgement - I gratefull thank the Edna Bailey foundation for the financial support for this research. Thanks to my committee members for their advice, patience, and assistance. Laboratory managers Julie Petruska and Jody Smiley provided invaluable help assistance with equipment and analytical analysis. I also thank friends at Virginia Polytechnic Institute for assistance, friendship, and laughs. Finally, I would to thank my family for their support, faith, and endless love.

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III. Appendix

Abbreviations

AHA	Aldrich Humic Acid
ASW	Forty Fathoms ™ Artificial Seawater
ASV	Anodic Stripping Voltammetry
CCA	Copper Chromium Arsenic wood treatment
CSV	Cathodic Stripping Voltammetry
Cu	Copper
Cu^{2+}	Free or Labile Copper
CuLi	Copper bound to Organic and Inorganic Ligands
DPASV	Differential Pulse Anodic Stripping Voltammetr
DPSV	Adsorptive Cathodic Stripping Voltammetry
EC_{50}	Median Effective Concentration based on 50% Immobilization
EDTA	Ethylenediaminetetraacetate
HA	Humic Acid
K_{CuLi}	Copper Binding Constant
LC_{50}	Lethal Concentration to Observed 50% Mortality
Li	Organic and Inorganic Ligands
Li_T	Total Ligand Concentrati
NOM	Natural Organic Matter
NPS	Non Point Source Pollution
NTA	Nitrotriacetic Acid
ppt	parts per thousand
PVC	Polyvinyl Chloride
SRFAR	Suwannee River Fulvic Acid Reference
TSK	Trimmed Spearman-Karber
USEPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant

Forty Fathoms Crystal Sea

Table 3-1. Instant Ocean ® species and their respective concentrations at 21 ppt salinity. Assume ½ of all trace amounts

ion	g/moles	mg/L (at 20ppth salinity)	mg/L (at 21ppth)	M (at 21ppth)
Chloride	35.45	11324	11890	3.35E-01
Sodium	22.99	6327	6644	2.89E-01
Sulfate	32.07	1564	1642	5.12E-02
Magnesiu	24.31	774	813	3.35E-02
Potassium	39.1	236	248	6.35E-03
Calcium	40.08	234	245	6.13E-03
Carbonate/Bicarb	60	112	118	1.98E-03
Strontium	87.62	5.1	5.3	6.06E-05
Boron	10.81	3.3	3.5	3.20E-04
Bromide	79.9	1.4	1.4	1.78E-05
Iodide	126.9	0.13	0.14	1.07E-06
Lithium	6.94	0.11	0.11	1.60E-05
Copper	63.55	Trace (<.03)	0.02	2.36E-07
Iron	55.85	Trace (<.03)	0.015	2.69E-07
Nickel	58.69	Trace (<.04)	0.02	3.41E-07
Zinc	65.39	Trace (<.02)	0.01	1.53E-07
Manganese	54.94	Trace (<.01)	0.005	9.10E-08
Molybdenu	95.94	Trace (<.01)	0.005	5.21E-08
Cobalt	58.93	Trace (<.05)	0.025	4.24E-07
Vanadiu	50.94	Trace (<.04)	0.02	3.93E-07
Selenium	78.96	Trace	Trace	---
Fluorine	19	Trace (<.05)	0.025	1.32E-06
Lead	207.2	Trace (<.005)	0.0025	1.21E-08
Arsenic	74.92	Trace (<.0002)	0.0001	1.33E-09
Cadmium	112.41	Trace (<.02)	0.01	8.90E-08
Chromium	52	Trace (<.0006)	0.0003	5.77E-09
Aluminum	26.98	Trace (<.03)	0.015	5.56E-07
Tin		Trace	Trace	---
Antimony		Trace	Trace	---
Rubidium		Trace	Trace	---
Barium	137.34	Trace (<.03)	0.015	1.09E-07

Chelex 100 ® laboratory data

For all tables, blank samples were attained by passing Nanopure ® water through prepared columns containing Chelex 100 ® resin. All laboratory experimental blanks were below 5 µg/L copper.

Table 3-2. The effect of sample flow rate through the Chelex 100 ® resin in a system containing 150 µg/L dissolved copper, 12.5 mg/L NOM, pH 8.1, and 21 ppt salinity

Flow Rate	Post Resin (µg/L)	Pre Resin (µg/L)
60 mL/min	99	156.5
60 mL/min	86.5	157.5
60 mL/min	102.5	157.5
10 mL/min	82	164.5
10 mL/min	97.5	166
10 mL/min	99	159

Table 3-3. The effect of copper concentration on Chelex 100 ® binding of copper in a system containing 12.5 mg/L NOM, pH 7.3, and 15 ppt salinity

Sample type	Post Resin (µg/L)	Pre Resin (µg/L)
Concentration 1	163.1	196.7
Concentration 1	172.2	195.3
Concentration 1	158.9	192.5
Concentration 2	291	334
Concentration 2	218	257
Concentration 2	225	264
Concentration 3	271	317
Concentration 3	271	319
Concentration 3	270	316
Concentration 4	335	430
Concentration 4	330	417.5
Concentration 4	317.5	435
Concentration 5	437.5	565
Concentration 5	510	572.5
Concentration 5	480	600

Table 3-4. The effect of salinity on Chelex 100 ® binding of copper in a syste containing 12.5 mg/L NOM, pH 8.1, and 150 µg/L dissolved copper

Salinity (mg/L)	Post Resin (µg/L)	Pre Resin (µg/L)
16	124.5	146.5
16	120.5	141.5
16	128	144.5
21	123.5	130
21	123	128.5
21	120	131.5
30	131.5	146
30	132.5	143.5
30	133	144

Table 3-5. The effect of pH on Chelex 100 ® binding of copper in a system containing 12.5 mg/L NOM, 21 ppt salinity, and 150 µg/L dissolved copper

pH	Post Resin (µg/L)	Pre Resin (µg/L)
4	99	156.5
4	86.5	157.5
4	102.5	157.5
5.5	117	148.5
5.5	116	152
5.5	116.5	151.5
7	121.5	151
7	119	148
7	121	147.5
8	123.5	130
8	123	128.5
8	120	131.5
8.5	126	141.5
8.5	115.5	136.5
8.5	131.5	138.5

Table 3-6. The effect of NOM on Chelex 100 ® binding of copper in a system containing pH 8.1, 21 ppt salinity, and 150 µg/L dissolved copper

NOM (mg/L)	Post Resin (µg/L)	Pre Resin (µg/L)
12.5	123.5	130
12.5	123	128.5
12.5	120	131.5
9.38	125.5	146.5
9.38	121.5	145.5
9.38	130.5	144.5
6.25	120.5	145
6.25	125	146
6.25	117	145
3.13	102.5	137
3.13	99.5	140.5
3.13	100	139.5
1.56	91.5	132
1.56	95.5	131.5
1.56	95	127.5
0	56	119.5
0	61	123.5
0	62	127

Table 3-7. The effect of SRFAR (NOM) on Chelex 100 ® resin binding of copper using acute bioassay. (Initial Concentrations, pH 7.7-8.0, 20 ppt)

Sample type	Sample Location/Replicate #	Post Resin (µg/L)	Pre Resin (µg/L)
No NOM	1	0.3	0.5
No NOM	2	1	1.2
No NOM	3	0	0.4
No NOM 100 µg/L Cu	1	53	85
No NOM 100 µg/L Cu	2	54	86
No NOM 100 µg/L Cu	3	58	93
No NOM 200 µg/L Cu	1	113	172
No NOM 200 µg/L Cu	2	115	180
No NOM 200 µg/L Cu	3	128	181
No NOM 400 µg/L Cu	1	215	372
No NOM 400 µg/L Cu	2	233	381
No NOM 400 µg/L Cu	3	248	385
No NOM 800 µg/L Cu	1	432	765
No NOM 800 µg/L Cu	2	465	783
No NOM 800 µg/L Cu	3	474	822
12 mg/L NOM	1	1.2	1.6
12 mg/L NOM	2	1.5	1.8
12 mg/L NOM	3	1.8	2
12 mg/L NOM 100 µg/L Cu	1	64	73
12 mg/L NOM 100 µg/L Cu	2	65	76
12 mg/L NOM 100 µg/L Cu	3	69	85
12 mg/L NOM 200 µg/L Cu	1	140	182
12 mg/L NOM 200 µg/L Cu	2	142	184
12 mg/L NOM 200 µg/L Cu	3	142	186
12 mg/L NOM 400 µg/L Cu	1	258	350
12 mg/L NOM 400 µg/L Cu	2	259	380
12 mg/L NOM 400 µg/L Cu	3	266	391
12 mg/L NOM 800 µg/L Cu	1	501	753
12 mg/L NOM 800 µg/L Cu	2	567	765
12 mg/L NOM 800 µg/L Cu	3	599	822
24 mg/L NOM	1	0.8	1.2
24 mg/L NOM	2	0.6	0.8
24 mg/L NOM	3	1	1.2
24 mg/L NOM 100 µg/L Cu	1	69	79
24 mg/L NOM 100 µg/L Cu	2	71	80
24 mg/L NOM 100 µg/L Cu	3	71	95
24 mg/L NOM 200 µg/L Cu	1	132	167
24 mg/L NOM 200 µg/L Cu	2	145	180
24 mg/L NOM 200 µg/L Cu	3	168	212
24 mg/L NOM 400 µg/L Cu	1	296	363
24 mg/L NOM 400 µg/L Cu	2	299	364
24 mg/L NOM 400 µg/L Cu	3	308	378
24 mg/L NOM 800 µg/L Cu	1	546	774
24 mg/L NOM 800 µg/L Cu	2	583	782
24 mg/L NOM 800 µg/L Cu	3	703	794

Table 3-8. The effect of SRFAR (NOM) on Chelex 100 ® resin binding of copper using acute bioassay. (48 Hour 0 mg/L SRFAR, pH 7.7-8.0, 20 ppt)

Sample type	Sample Location/Replicate #	Post Resin (µg/L)	Pre Resin (µg/L)
No NOM	1	0	0
No NOM	2	2	2.5
No NOM	3	0.5	0.7
No NOM 100 µg/L Cu	1	61	65
No NOM 100 µg/L Cu	2	51	65
No NOM 100 µg/L Cu	3	62	59
No NOM 200 µg/L Cu	1	91	176
No NOM 200 µg/L Cu	2	110	161
No NOM 200 µg/L Cu	3	98	139
No NOM 400 µg/L Cu	1	237	303
No NOM 400 µg/L Cu	2	194	333
No NOM 400 µg/L Cu	3	256	389
No NOM 800 µg/L Cu	1	361	667
No NOM 800 µg/L Cu	2	370	652
No NOM 800 µg/L Cu	3	366	643

Table 3-9. The effect of SRFAR (NOM) on Chelex 100 ® resin binding of copper using acute bioassay. (48 Hour Renewal 0 mg/L SRFAR, pH 7.7-8.0, 20 ppt)

Sample type	Sample Location/Replicate #	Post Resin (µg/L)	Pre Resin (µg/L)
No NOM	1	0	0
No NOM	2	13	39
No NOM	3	25	0
No NOM 100 µg/L Cu	1	98	138
No NOM 100 µg/L Cu	2	82	89
No NOM 100 µg/L Cu	3	84	140
No NOM 200 µg/L Cu	1	199	183
No NOM 200 µg/L Cu	2	129	175
No NOM 200 µg/L Cu	3	133	173
No NOM 400 µg/L Cu	1	223	356
No NOM 400 µg/L Cu	2	229	355
No NOM 400 µg/L Cu	3	238	354
No NOM 800 µg/L Cu	1	534	701
No NOM 800 µg/L Cu	2	447	751
No NOM 800 µg/L Cu	3	505	834

Table 3-10. The effect of SRFAR (NOM) on Chelex 100 ® resin binding of copper using acute bioassay. (48 Hour 12 mg/L SRFAR, pH 7.7-8.0, 20 ppt)

Sample type	Sample Location/Replicate #	Post Resin (µg/L)	Pre Resin (µg/L)
12 mg/L NOM	1	0.4	0.6
12 mg/L NOM	2	0.8	1.2
12 mg/L NOM	3	0.2	0.6
12 mg/L NOM 100 µg/L Cu	1	77	88
12 mg/L NOM 100 µg/L Cu	2	80	100
12 mg/L NOM 100 µg/L Cu	3	82	109
12 mg/L NOM 200 µg/L Cu	1	125	152
12 mg/L NOM 200 µg/L Cu	2	133	161
12 mg/L NOM 200 µg/L Cu	3	135	162
12 mg/L NOM 400 µg/L Cu	1	240	316
12 mg/L NOM 400 µg/L Cu	2	264	319
12 mg/L NOM 400 µg/L Cu	3	290	380
12 mg/L NOM 800 µg/L Cu	1	467	714
12 mg/L NOM 800 µg/L Cu	2	512	736
12 mg/L NOM 800 µg/L Cu	3	545	746

Table 3-11. The effect of SRFAR (NOM) on Chelex 100 ® resin binding of copper using acute bioassay. (48 Hour 24 mg/L SRFAR, pH 7.7-8.0, 20 ppt)

Sample type	Sample Location/Replicate #	Post Resin (µg/L)	Pre Resin (µg/L)
24 mg/L NOM	1	0	0.4
24 mg/L NOM	2	0.2	0.4
24 mg/L NOM	3	0.6	0.8
24 mg/L NOM 100 µg/L Cu	2	77	77
24 mg/L NOM 100 µg/L Cu	3	77	92
24 mg/L NOM 100 µg/L Cu	1	78	104
24 mg/L NOM 200 µg/L Cu	2	141	162
24 mg/L NOM 200 µg/L Cu	3	151	168
24 mg/L NOM 200 µg/L Cu	1	153	172
24 mg/L NOM 400 µg/L Cu	2	278	363
24 mg/L NOM 400 µg/L Cu	3	291	374
24 mg/L NOM 400 µg/L Cu	1	307	497
24 mg/L NOM 800 µg/L Cu	3	600	725
24 mg/L NOM 800 µg/L Cu	1	611	738
24 mg/L NOM 800 µg/L Cu	2	619	739

Table 3-12. The effect of SRFAR (NOM) on Chelex 100 ® resin binding of copper using acute bioassay. (96 Hour 0 mg/L SRFAR, pH 7.7-8.0, 20 ppt)

Sample type	Sample Location/Replicate #	Post Resin (µg/L)	Pre Resin (µg/L)
No NOM	1	0.2	0.4
No NOM	2	0.8	1.2
No NOM	3	3	2
No NOM 100 µg/L Cu	1	68	75
No NOM 100 µg/L Cu	2	70	77
No NOM 100 µg/L Cu	3	87	112
No NOM 200 µg/L Cu	1	101	134
No NOM 200 µg/L Cu	2	110	141
No NOM 200 µg/L Cu	3	119	141
No NOM 400 µg/L Cu	1	180	298
No NOM 400 µg/L Cu	2	190	303
No NOM 400 µg/L Cu	3	200	309
No NOM 800 µg/L Cu	1	358	602
No NOM 800 µg/L Cu	2	391	610
No NOM 800 µg/L Cu	3	397	615

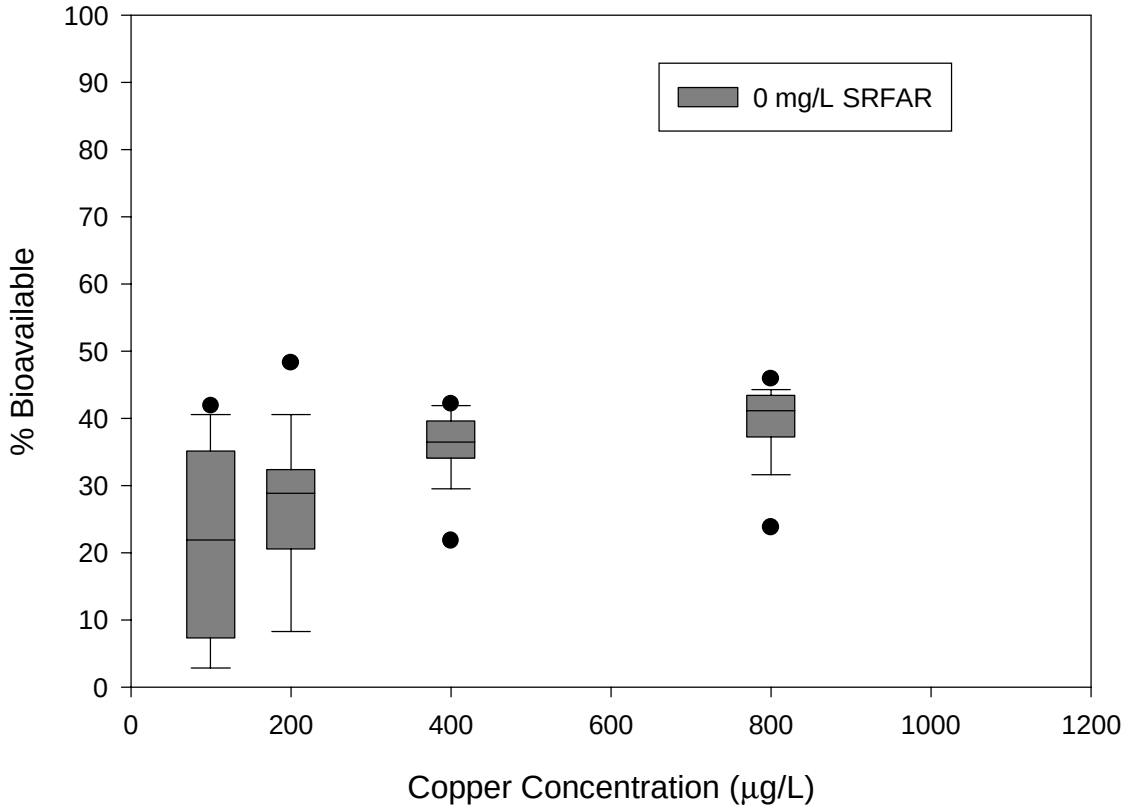


Figure 3-1. Percent Bioavailable Cu and Cu concentration (µg/L) in 0 mg/L SRFAR (pH 7.7-8.0 and 20 ppt).

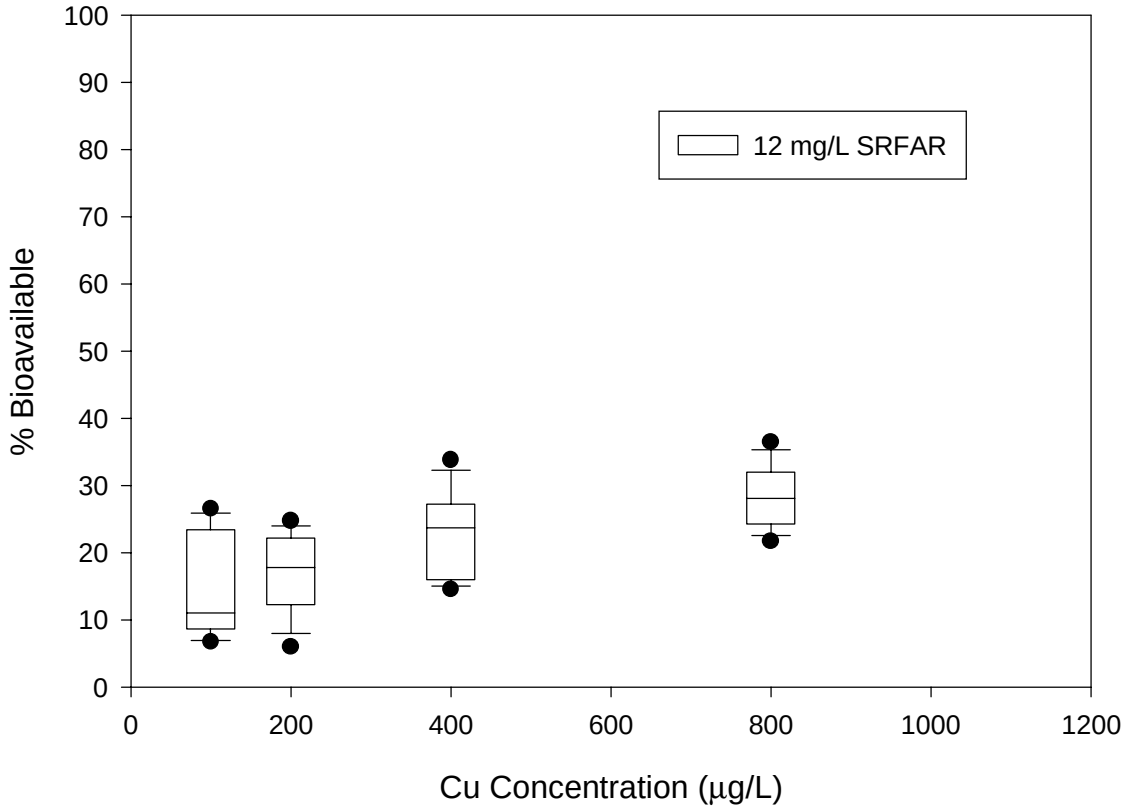


Figure 3-2. Percent Bioavailable Cu and Cu concentration (µg/L) in 12 mg/L SRFAR (pH 7.7-8.0 and 20 ppt).

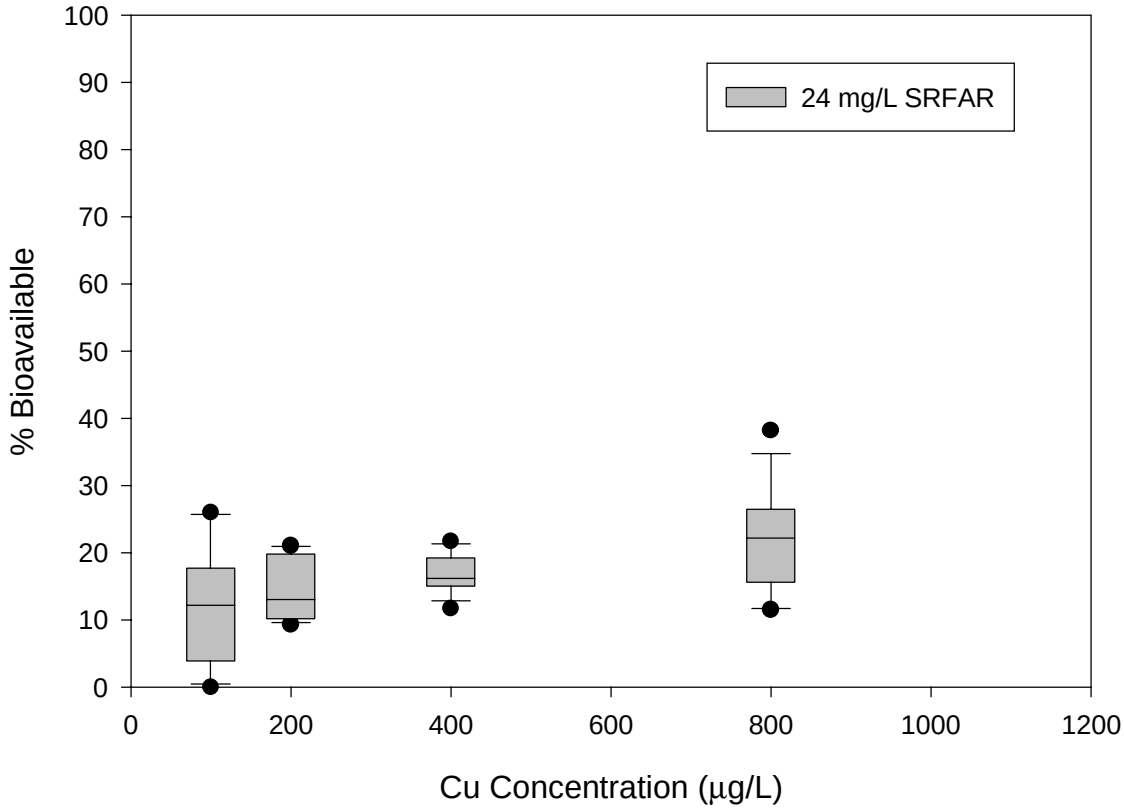


Figure 3-3. Percent Bioavailable Cu and Cu concentration (µg/L) in 24 mg/L SRFAR (pH 7.7-8.0 and 20 ppt).

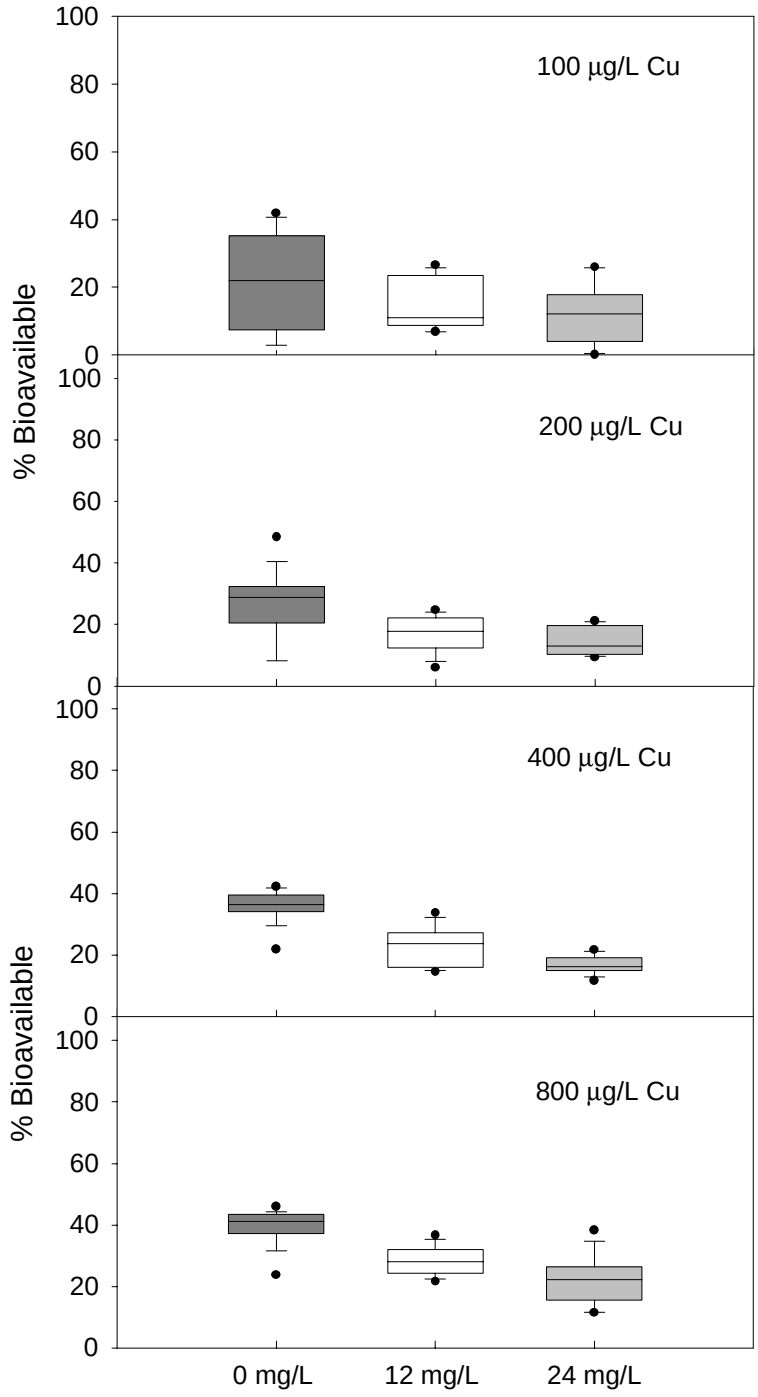


Figure 3-4. Percent Bioavailable Cu for Cu concentration 100, 200, 400, and 800 (µg/L) in 0,12, 24 mg/L SRFAR (pH 7.7-8.0 and 20 ppt).

Bioassay: Assessment of Bioavailable Copper Toxicity to Americamysis bahia

Table 3-13. Raw Data for *Americamysis bahia* 48 and 96 Hr Acute Toxicity Tests

	0 mg/L SRFAR							12 mg/L SRFAR				24 mg/L SRFAR			
Concentration(µg/L)/replicate	0 hr	24 hr	48 hr	48 survival	72 hr	96 hr	96 survival	0 hr	24 hr	48 hr	48 Survival	0 hr	24 hr	48 hr	48 Survival
0A	10	10	10	97.50%	10	10	92.50%	10	10	10	95%	10	10	10	95%
0B	10	10	9		9	9		10	9	9		10	9	9	
0C	10	10	10		10	9		10	10	10		10	10	10	
0D	10	10	10		9	9		10	9	9		10	9	9	
100A	10	10	6	60%	4	4	45%	10	9	8	82.50%	10	10	9	90%
100B	10	8	5		5	3		10	9	8		10	9	9	
100C	10	8	6		5	5		10	10	8		10	9	8	
100D	10	9	7		7	6		10	10	9		10	10	10	
200A	10	8	5	52.50%	2	1	25%	10	9	6	72.50%	10	9	7	82.50%
200B	10	8	5		4	3		10	9	6		10	9	9	
200C	10	7	6		3	3		10	9	8		10	8	8	
200D	10	8	4		3	3		10	9	9		10	10	9	
400A	10	7	4	35%	1	0	0%	10	5	4	52.50%	10	9	5	65%
400B	10	5	3		0	0		10	8	5		10	9	7	
400C	10	5	4		2	0		10	8	5		10	10	8	
400D	10	5	3		1	0		10	10	7		10	8	6	
800A	10	3	0	0%	0	0	0%	10	6	1	20%	10	5	2	25%
800B	10	2	0		0	0		10	6	3		10	7	2	
800C	10	2	0		0	0		10	5	1		10	7	3	
800D	10	2	0		0	0		10	4	3		10	5	3	

Table 3-14. LC_{50} and EC_{50} Data for Total, Dissolved, and Bioavailable Copper

ASW Test Solution	LC_{50}[1], µg/L Cu			EC_{50} Activity, µg/L Cu		
	Total	Dissolved	Bioavailable	Total	Dissolved	Bioavailable
0 mg/L NOM 48 hour	230 (150-350)	200 (135-310)	94 (68-130)	185	140	70
0 mg/L NOM 96 hour	115	95	20	110	85	< 20
12 mg/L NOM 48 hour	400 (315-510)	340 (260-445)	98 (73-130)	360	310	85
24 mg/L NOM 48 hour	540 (400-720)	495 (360-675)	105 (68-150)	510	470	95

[1] LC_{50} value with 95% CI in parentheses

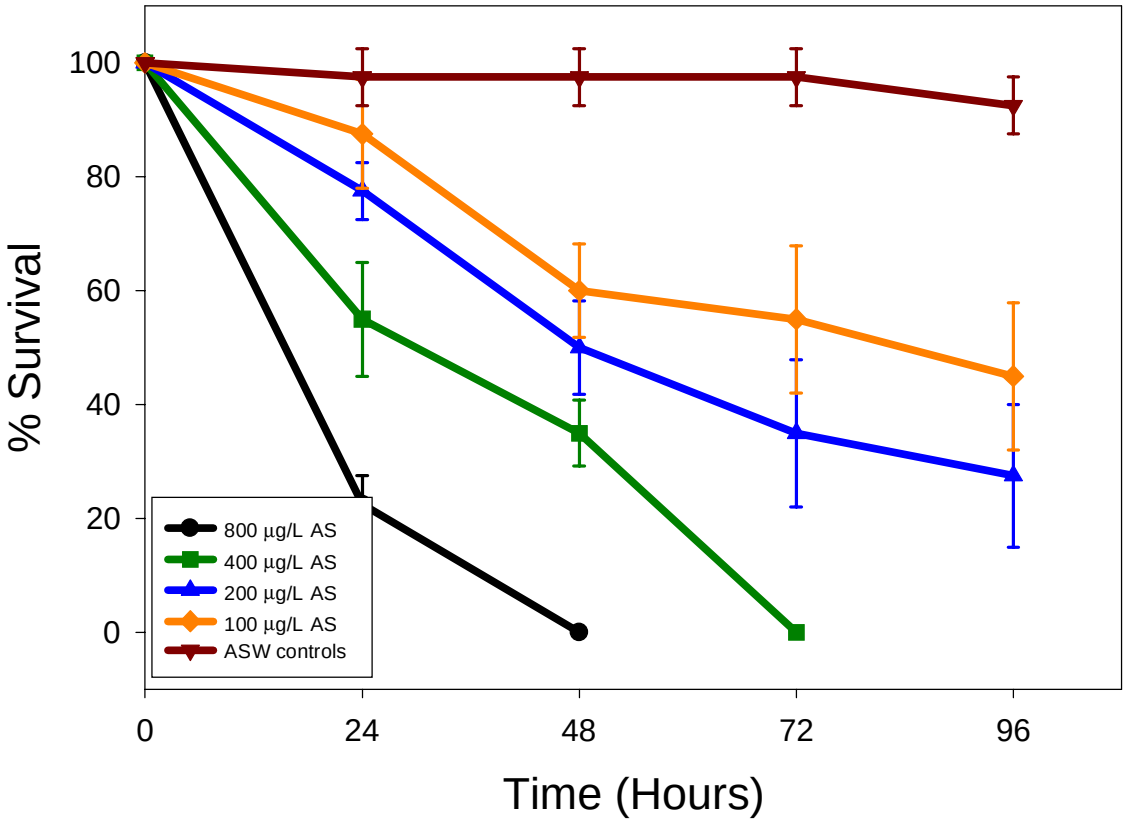


Figure 3-5. Copper Toxicity in the presence of 0 mg/L SRFAR (NOM). Ninety seven percent survival in the control test chambers. (pH 7.7-8.0 and 20 ppt). Error bars are +/- standard deviation (n=40).

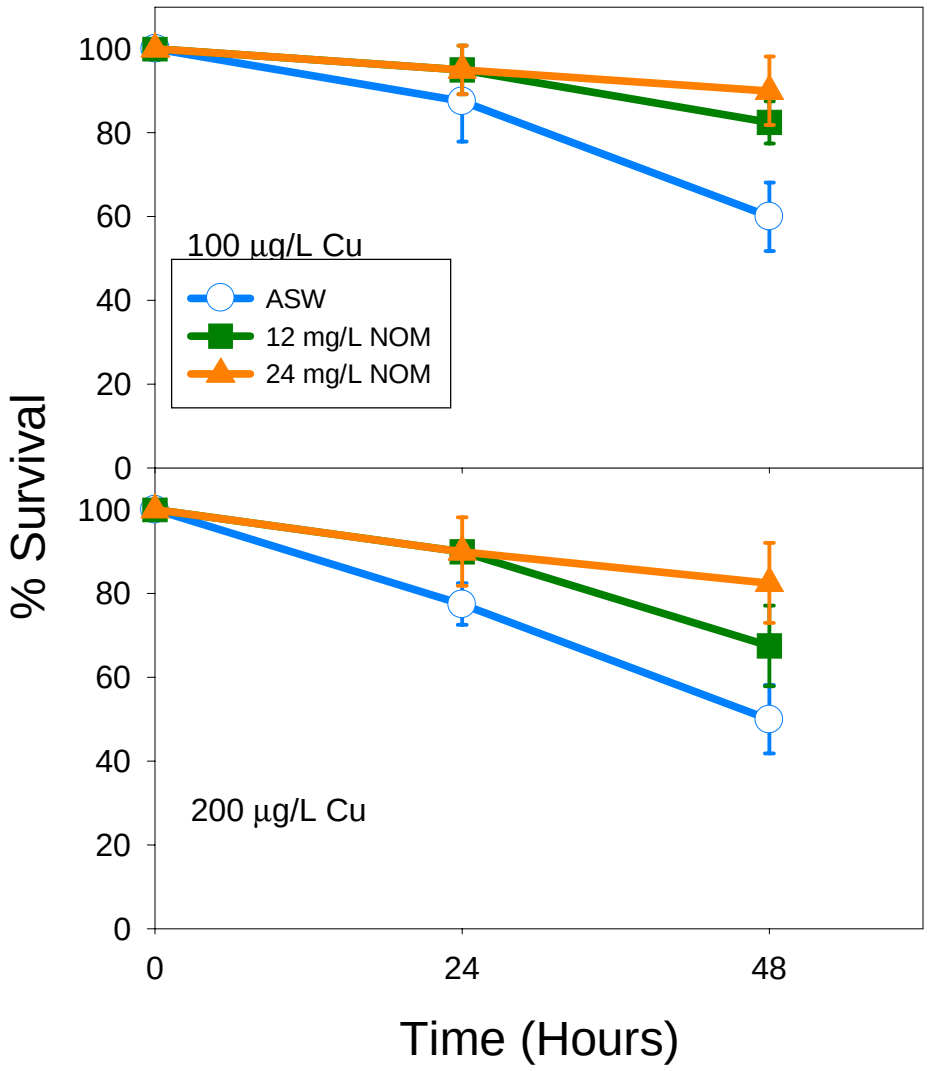


Figure 3-6. 100 and 200 µg/L Cu toxicity to *A. bahia* in 0, 12, and 24 mg/L SRFAR (pH 7.7-8.0 and 20 ppt). Error bars are +/- standard deviation (n=40).

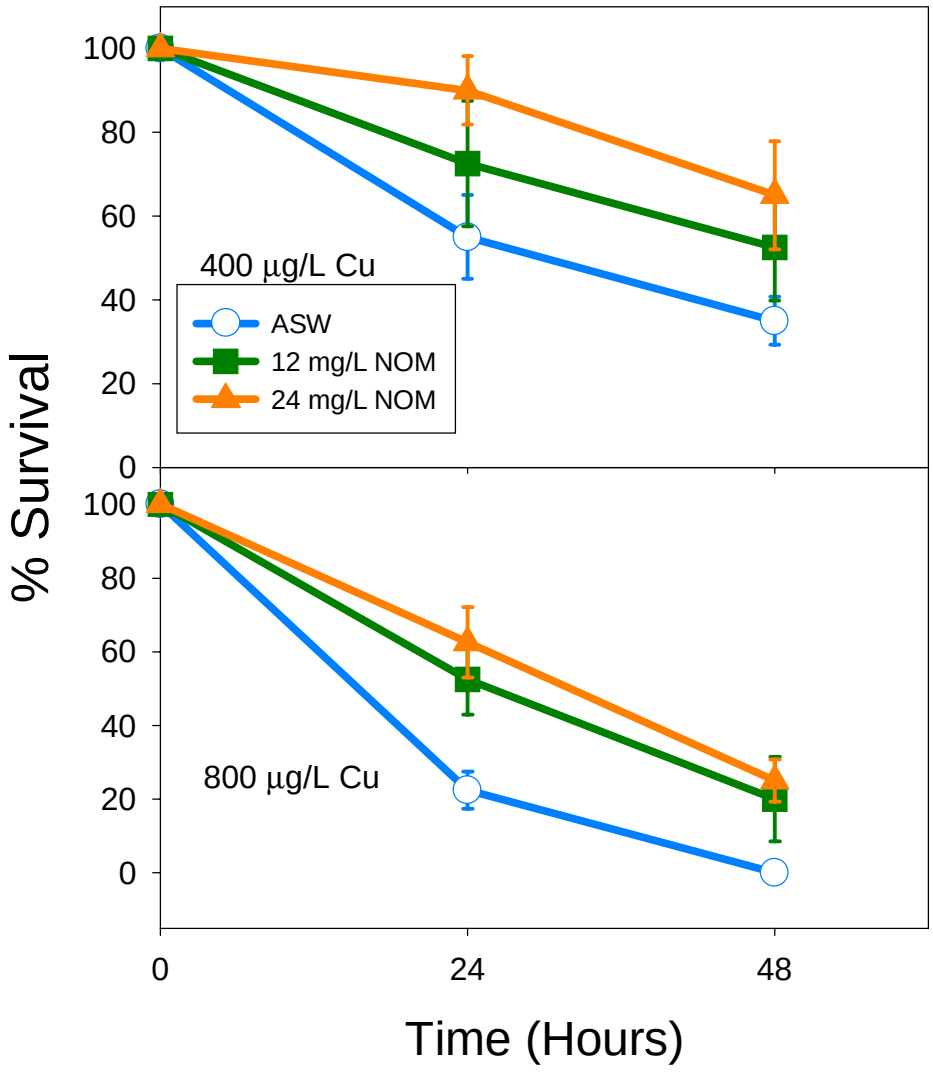
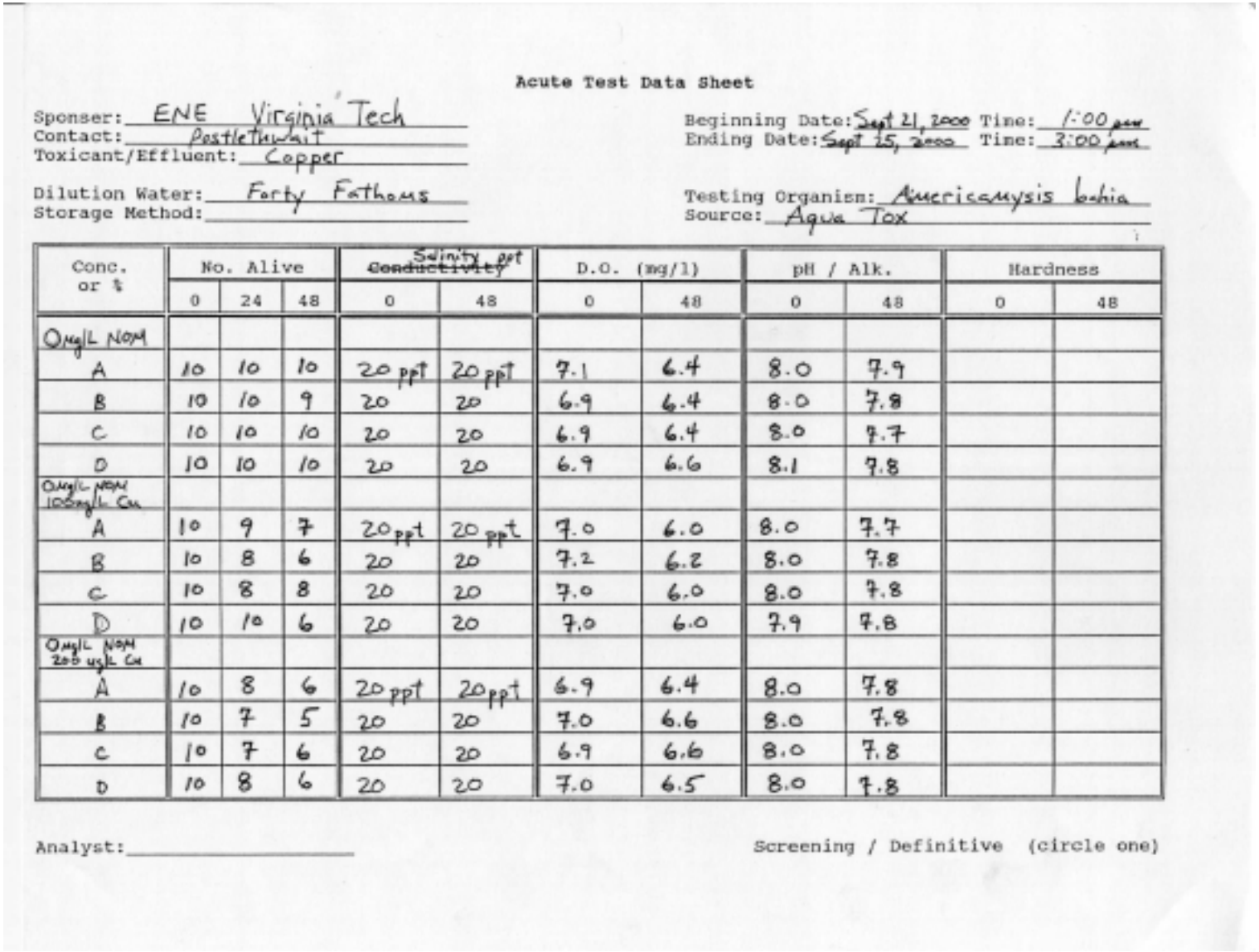


Figure 3-7. 400 µ/L and 800 µ/L Cu toxicity to *A. bahia* in 0, 12, and 24 mg/L SRFAR (pH 7.7-8.0 and 20 ppt). Error bars are +/- standard deviation (n=40)

Acute Toxicity Bench Sheets for the Mysid Shrimp (Americamysis bahia)



Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: Postlethwait
Toxicant/Effluent: Copper

Beginning Date: Sept 21, 2000 Time: 1:00 pm
Ending Date: Sept 25, 2000 Time: 3:00 pm

Dilution Water: Forty Fathoms
Storage Method:

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			~~Conductivity~~ Salinity ppt		D.O. (mg/l)		pH / Alk.		Hardness	
	0	24	48	0	48	0	48	0	48	0	48
0 ug/L NOM											
A	10	10	10	20 ppt	20 ppt	7.1	6.4	8.0	7.9		
B	10	10	9	20	20	6.9	6.4	8.0	7.8		
C	10	10	10	20	20	6.9	6.4	8.0	7.7		
D	10	10	10	20	20	6.9	6.6	8.1	7.8		
0 ug/L NOM 100 ug/L Cu											
A	10	9	7	20 ppt	20 ppt	7.0	6.0	8.0	7.7		
B	10	8	6	20	20	7.2	6.8	8.0	7.8		
C	10	8	8	20	20	7.0	6.0	8.0	7.8		
D	10	10	6	20	20	7.0	6.0	7.9	7.8		
0 ug/L NOM 200 ug/L Cu											
A	10	8	6	20 ppt	20 ppt	6.9	6.4	8.0	7.8		
B	10	7	5	20	20	7.0	6.6	8.0	7.8		
C	10	7	6	20	20	6.9	6.6	8.0	7.8		
D	10	8	6	20	20	7.0	6.5	8.0	7.8		

Analyst: _____

Screening / Definitive (circle one)

Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: Fathead Minnow
Toxicant/Effluent: Copper

Beginning Date: Sept 21 Time: 1:00 pm
Ending Date: Sept 25 Time: 2:00 pm

Dilution Water: FF
Storage Method:

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			Salinity ~~Conductivity~~		D.O. (mg/l)		pH / Alk.		Hardness	
	0	24	48	0	48	0	48	0	48	0	48
0 mg/L NOM 400 mg/L Cu											
A	10	7	4	20 ppt	20 ppt	6.9	6.6	7.9	7.8		
B	10	5	3	20	20	6.9	6.7	7.8	7.8		
C	10	5	3	20	20	7.0	6.7	8.0	8.0		
D	10	5	4	20	20	6.9	6.4	7.8	7.8		
0 mg/L NOM 800 mg/L											
A	10	3	0	20 ppt	20 ppt	6.8	6.7	7.9	7.8		
B	10	2	0	20	20	6.9	6.7	7.8	7.8		
C	10	2	0	20	20	6.8	6.7	7.8	7.8		
D	10	2	0	20	20	6.8	6.6	7.9	7.8		

Analyst:

Screening / Definitive (circle one)

Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: PestleThwait
Toxicant/Effluent: Copper

Beginning Date: Sept 1 2000 Time: 1:00 pm
Ending Date: Sept 25 2000 Time: 3:00 pm

Dilution Water: FF
Storage Method:
48 hr Renewal

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			Salinity 20 ppt Conductivity		D.O. (mg/l)		pH / Alk.		Hardness	
	48 hr	72 24	96 48	48 hr	96 48	48 hr	96 48	48 hr	96 48	0	48
0 mg/L NOM											
A	10	10	10	20 ppt	20 ppt	6.4	6.2	7.9	7.8		
B	9	9	9	20	20	6.4	6.1	7.8	7.8		
C	10	10	9	20	20	6.4	6.0	7.7	7.7		
D	10	9	9	20	20	6.8	6.0	7.8	7.7		
0 mg/L NOM 100 mg/L Cu											
A	7	4	4	20 ppt	20 ppt	6.8	6.5	7.7	7.7		
B	6	5	3	20	20	6.8	6.7	7.8	7.7		
C	8	7	7	20	20	6.8	6.7	7.8	7.7		
D	6	6	6	20	20	6.8	6.9	7.8	7.7		
0 mg/L NOM 200 mg/L Cu											
A	6	2	1	20 ppt	20 ppt	6.7	6.8	7.8	7.8		
B	5	4	4	20	20	6.7	6.7	7.8	7.8		
C	6	3	3	20	20	6.7	6.7	7.8	7.8		
D	6	3	3	20	20	6.6	6.7	7.8	7.8		

Analyst:

Screening / Definitive (circle one)

Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: Postlethwait
Toxicant/Effluent: Copper

Beginning Date: Sept 21, 2000 Time: 1:00 pm
Ending Date: Sept 25, 2000 Time: 3:00 pm

Dilution Water: FF
Storage Method:
48 hr Renewal

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			Salinity ppt ~~Conductivity~~		D.O. (mg/l)		pH / Alk.		Hardness	
	48 hr	72-24	96-48	48 hr	96-48	48 hr	96-48	48 hr	96-48	0	48
0 mg/L NOM 400 mg/L Cu											
A	4	1	0	20 ppt	20 ppt	6.8	6.7	7.8	7.8		
B	3	0	0	20	20	6.7	6.7	7.8	7.8		
C	3	2	0	20	20	6.7	6.7	7.8	7.8		
D	4	1	0	20	20	6.7	6.7	7.8	7.8		
0 mg/L NOM 800 mg/L Cu	48										
A	0	0	0	20 ppt	20 ppt	6.75	6.5	7.8	7.7		
B	0	0	0	20	20	6.7	6.7	7.8	7.8		
C	0	0	0	20	20	6.7	6.7	7.8	7.7		
D	0	0	0	20	20	6.7	6.7	7.8	7.7		

Analyst: _____

Screening / Definitive (circle one)

Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: Postlethwait
Toxicant/Effluent: Copper

Beginning Date: Sept 21, 2000 Time: 1:00 pm
Ending Date: Sept 25, 2000 Time: 3:00 pm

Dilution Water: FF
Storage Method:

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			Salinity ppt ~~Conductivity~~		D.O. (mg/l)		pH / Alk.		Hardness	
	0	24	48	0	48	0	48	0	48	0	48
12 mg/L NOM											
A	10	10	10	20 ppt	20 ppt	7.0	6.8	7.8	7.7		
B	10	9	9	20	20	6.9	6.4	7.8	7.7		
C	10	10	10	20	20	6.8	6.4	7.8	7.7		
D	10	9	9	20	20	6.9	6.4	7.8	7.7		
12 mg/L NOM 100 ug/L Cu											
A	10	9	6	20 ppt	20 ppt	6.8	6.1	7.7	7.7		
B	10	9	6	20	20	6.7	6.0	7.8	7.7		
C	10	10	7	20	20	6.7	5.9	7.7	7.7		
D	10	10	8	20	20	6.8	6.2	7.8	7.7		
12 mg/L NOM 200 ug/L Cu											
A	10	9	8	20 ppt	20 ppt	6.7	6.0	7.8	7.7		
B	10	9	8	20	20	6.6	5.9	7.7	7.7		
C	10	9	8	20	20	6.7	6.2	7.7	7.7		
D	10	9	9	20	20	6.6	6.0	7.8	7.7		

Analyst:_____

Screening / Definitive (circle one)

Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: Postlethwait
Toxicant/Effluent: Copper

Dilution Water: FF
Storage Method:

Beginning Date: Sept 21 2000 Time: 1:00 pm
Ending Date: Sept 25 2000 Time: 3:00 pm

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			~~Conductivity~~ Salinity ppt		D.O. (mg/l)		pH / Alk.		Hardness	
	0	24	48	0	48	0	48	0	48	0	48
12 mg/L NOM 400 ug/L Cu											
A	10	5	4	20 ppt	20 ppt	6.6	6.1	7.9	7.8		
B	10	8	5	20	20	6.5	6.2	7.8	7.8		
C	10	8	5	20	20	6.7	6.2	7.8	7.8		
D	10	10	7	20	20	6.8	6.2	7.7	7.7		
12 mg/L NOM 800 ug/L Cu											
A	10	6	1	20 ppt	20 ppt	6.8	6.4	7.7	7.7		
B	10	6	3	20	20	6.7	6.4	7.8	7.7		
C	10	5	1	20	20	6.8	6.3	7.8	7.8		
D	10	4	3	20	20	6.8	6.4	7.7	7.7		

Analyst:

Screening / Definitive (circle one)

Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: Postlethwait
Toxicant/Effluent: Copper

Dilution Water: FF
Storage Method:

Beginning Date: Sept 21, 2000 Time: 1:00 pm
Ending Date: Sept 25, 2000 Time: 3:00 pm

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			~~Salinity~~ ~~Conductivity~~ ppt		D.O. (mg/l)		pH / Alk.		Hardness	
	0	24	48	0	48	0	48	0	48	0	48
24 mg/L NOM											
A	10	10	10	20 ppt	20 ppt	6.6	6.0	7.7	7.7		
B	10	10	9	20	20	6.7	6.2	7.7	7.7		
C	10	10	10	20	20	6.8	6.0	7.7	7.7		
D	10	10	9	20	20	6.7	6.0	7.7	7.7		
24 mg/L NOM 100 mg/L Cu											
A	10	10	9	20 ppt	20 ppt	6.5	5.8	7.8	7.8		
B	10	9	9	20	20	6.6	6.0	7.7	7.7		
C	10	9	8	20	20	6.5	6.0	7.8	7.7		
D	10	10	10	20	20	6.5	5.8	7.7	7.7		
24 mg/L NOM 200 mg/L Cu											
A	10	9	7	20 ppt	20 ppt	6.5	5.8	7.7	7.8		
B	10	9	9	20	20	6.6	5.8	7.7	7.8		
C	10	8	8	20	20	6.7	6.0	7.8	7.7		
D	10	10	9	20	20	6.7	5.9	7.7	7.8		

Analyst: _____

Screening / Definitive (circle one)

Acute Test Data Sheet

Sponser: ENE Virginia Tech
Contact: Postlethwait
Toxicant/Effluent: Copper

Beginning Date: Sept 21, 2000 Time: 1:00 pm
Ending Date: Sept 25, 2000 Time: 3:00 pm

Dilution Water: FF
Storage Method:

Testing Organism: Americamysis bahia
Source: Aqua Tox

Conc. or %	No. Alive			Salinity ppt ~~Conductivity~~		D.O. (mg/l)		pH / Alk.		Hardness	
	0	24	48	0	48	0	48	0	48	0	48
24 mg/L NOM 400 ug/L Cu											
A	10	9	5	20 ppt	20 ppt	6.8	6.4	7.8	7.7		
B	10	9	7	20	20	6.8	6.5	7.7	7.7		
C	10	10	8	20	20	6.7	6.4	7.7	7.7		
D	10	8	6	20	20	6.8	6.3	7.8	7.8		
24 mg/L NOM 800 ug/L Cu											
A	10	5	2	20 ppt	20 ppt	6.8	6.6	7.7	7.7		
B	10	7	2	20	20	6.7	6.6	7.7	7.7		
C	10	7	3	20	20	6.7	6.5	7.8	7.7		
D	10	7	3	20	20	6.8	6.4	7.8	7.8		

Analyst:

Screening / Definitive (circle one)

48 HOUR STATIC ACUTE REFERENCE TOXICANT TEST

Laboratory Name: Virginia Tech Norris Hall 319 Species: Amesicamysis bahia

Test Start Date/Time: Sept 21 / 2000 1:00pm Organism Source: Aqua Tox

Test End Date/Time: Sept 25 / 2000 3:00pm Organism Age: 2 to 3 day ID#: _____

Dilution Water: DM - Diluta Mineral Water
GP - Modified GP2
MH - Moderate Hard Synthetic
TW - Tap Water, Dechlor
~~EV - Evaporated Seawater~~
FF - Forty Fathoms
~~ME - Marine Environment~~
HM - Hawaiian Marine Mix
Other - _____

Reference Toxicant: Sodium Lauryl Sulfate

Analytical Conc Ref Tox: _____ mg/L

	Alkalinity	Hardness	Chlorine
Diluent	_____ mg/L	_____ mg/L	_____ mg/L
Ref Tox 100%	_____ mg/L	_____ mg/L	

CONC & REP	Survival			DO, mg/L			pH			~~Conductivity~~ Or Salinity (ppt)			Temp, °C			Percent Survival per Conc.
	0	24	48	0	24	48	0	24	48	0	24	48	0	24	48	
Control	10	10	10	7.1		6.4	8.0		7.9	20 ppt	20 ppt	20 ppt	20°	20°	20°	
	10	10	9	6.9		6.4	8.0		7.8	20	20	20	20	20	20	97.5%
	10	10	10	6.9		6.4	8.0		8.0	20	20	20	20	20	20	
	10	10	10	6.9		6.6	8.0		7.9	20	20	20	20	20	20	
100%	10	5	0	6.9		6.7	8.0		7.8	20	20	20	20	20	20	
	10	4	0	6.8		6.6	8.0		7.9	20	20	20	20	20	20	0%
50%	10	8	5	6.8		6.7	7.9		7.8	20	20	20	20	20	20	
	10	8	6	6.9		6.6	7.8		7.7	20	20	20	20	20	20	65%
25%	10	9	8	7.0		6.5	7.9		7.9	20	20	20	20	20	20	
	10	9	9	6.8		6.4	7.9		7.9	20	20	20	20	20	20	85%
										20	20	20				
12.5%	10	10	9	7.0		6.0	8.0		7.9	20	20	20	20	20	20	
	10	10	10	7.2		6.0	8.0		8.0	20	20	20	20	20	20	95%

LC 50 12.5 mg/L

Vita

Niel Holland Postlethwait

Niel Holland Postlethwait was born 21 May 1975 in Biloxi, Mississippi. He lived in Dover, Delaware where he graduated from Caesar Rodney High School in 1993. Niel attended Elon College in Elon College, NC from 1993-1997 and received a Bachelor o Science degree in Environmental Studies. Following the summer of 1998, Niel attended the graduate school of Virginia Polytechnic Institute and State University and received a Master of Science degree in Environmental Engineering in July of 2001. Upon graduation, Niel will pursue a career as an environmental engineering consultant.