EVALUATION OF DIFFUSIVE GRADIENTS IN THIN-FILMS FOR PREDICTING BIOACCUMULATION OF COPPER IN AQUATIC ANIMALS

by

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(Under the Direction of Robert Bringolf)

ABSTRACT

Due to metals posing a risk to aquatic organisms, regulators have been interested in monitoring metals in aquatic environments, primarily determining the influence of speciation and bioavailability. Passive diffusion devices, like Diffusive Gradients in Thin-films (DGT), could be used as a simple, cost-effective, *in situ* sampling device that allows regulators to measure the free and labile metal fraction in natural waters. We evaluated whether DGT can accurately predict copper bioavailability to two freshwater species, fathead minnow (*Pimephales promelas*) and yellow lampmussel (*Lampsilis cariosa*). Organisms and DGT were exposed to environmentally relevant copper concentrations over a range of water chemistries: soft and hard water, addition of natural organic matter, and metal mixture with lead. Effect of deployment duration was also explored. Correlations of organism accumulated copper with DGT measured fraction suggested strong predictive ability for fathead minnow (r^2 0.662-0.929), but less for yellow lampmussel (r^2 0.224-0.711).

INDEX WORDS: copper; bioavailability; bioaccumulation; DGT; fathead minnow; freshwater mussels; freshwater organisms; metal speciation

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Metals, known to cause toxicity to a wide range of aquatic organisms, are frequently released into the environment through anthropogenic actions. It is in the interest of regulators to have accurate and precise methods for measuring the amount of metal in a freshwater environment that can adversely affect aquatic organisms (i.e. the bioavailable fraction of total metal). This introduction and literature review will delve into why assessing metal bioavailability has been problematic by discussing the influence of water chemistry on metal bioavailability, the recent research on metal bioavailability and the relevance to regulatory agencies, and where current research is focusing. The emphasis will largely be on copper in freshwater, but the same principles can be extended to other divalent cationic metals.

Influence of Water Chemistry on Metal Bioavailability

The term bioavailability can elicit many definitions, based on the background of the researcher. As a whole, the concept of bioavailability indicates that the total metal concentration in an aquatic system is not always (or usually) an acceptable predictor of that metal's biological effect. The chemical reactivity of the metal, measured by the free metal ion activity, determines the bioavailability (Morel 1983, Campbell 1995). The free metal ion activity represents how much the metal will react with cellular sites on the organism, independent of the reaction mechanisms (Morel 1983). For the remainder of

this document bioavailability of a metal will indicate the metal that could be absorbed by the organism, based on reactivity.

Before we continue on with bioavailability, it is imperative to discuss the bulk water chemistry and how that will lead to differences in metal bioavailability and metal speciation. Total metal in a bulk water solution is a function of all metal species, and the types of metal species are a function of the greater water chemistry.

$$[\text{Total metal}] \text{ in solution} =$$

$$[\text{free metal ion}] + \Sigma\,[\text{metal in all metal ligand complexes}]$$

Species, in terms of chemical speciation, refers to the actual physical form that an ion or molecule is in at the present time in the bulk water solution (Stumm and Morgan 1996). Oxidation state, precipitation and sorption, complexation, and the formation of organometallic compounds are all factors that affect metal speciation (Allen and Hansen 1996).

Ligand denotes an anion or other molecule that cationic metals can form coordination complexes with (Stumm and Morgan 1996). In natural surface waters, the primary ligands can be separated into four groups. The first group are simple anions, Cl^-, F^-, Br^-, I^-, which besides Cl^- are typically in too low of concentration to be considered a significant part of metal binding (Morel 1983). Complex inorganics, including NO_3^-, CO_3^{2-}, SO_4^{2-}, NH_3, S^{2-}, PO_4^{3-}, SO_3^{2-}, are often a large component of metal species. Organic molecules with functional groups have an oxygen, nitrogen or sulfur atom(s) to donate electron pairs for coordination complexes. Finally, acid-base reactions are included in metal speciation by including OH^- as a ligand.

Metal species are formed by metals reacting with ligands and thereby increasing the stability of their valence electron configuration (Stumm and Morgan 1996). These reactions are either electrostatic interactions, covalent bonds or a mixture of both (Morel 1983). Electrostatic interactions form ion pairs where opposite charged atoms are drawn towards one another, but still maintain one or more water molecules between the ions (Stumm and Morgan 1996). Complexes are formed by covalent bonds when a metal and an electron-donating ligand interact. In the case of complexes, the ligand and metal are adjoining, without any water molecule separation (inner-sphere complex). The strength of the metal to ligand interactions are quantified by stability constants and binding affinities of which the literature is prolific, so they will not be reiterated here.

Metal species can be separated into multiple categories. The first dichotomy is between particulate and dissolved species. Particulates are solids or surfaces that sorb metals, removing them from the soluble pool, and thus not contributing to bioavailability (Allen and Hansen 1996). Dissolved species are operationally defined as the metal fraction passing through a 0.45μm filter (Stumm and Morgan 1996). The operationally defined dissolved species are further categorized into colloidal particles, free metal ions, and metal-ligand complexes. The metal-ligand complexes include inorganic complexes and organic complexes as mentioned above.

The primary inorganic complexes with copper around circumneutral pH are $CuOH^+$, $Cu(OH)_2^0$, $CuCO_3^0$ and $Cu(CO_3)_2^{2-}$ (Allen and Hansen 1996), with $CuOH^+$ and $CuCO_3^0$ being the most predominant (Morel 1983). The proportion of copper in each complex can be computed using hand calculations (Stumm and Morgan 1996) or more typically a computer modeling system such as MINTEQ, PHREQUE, CHESS, or

WHAM. Copper and other divalent metals have little impact on the overall inorganic ligand concentration, due to an order of magnitude difference in concentrations, resulting in a constant ratio between the concentration of inorganic copper species and the free copper ion (Allen and Hansen 1996). As the total copper concentration fluctuates, the proportion of copper that is in any of the inorganic copper complexes will remain consistent. Thus, the inorganic metal speciation is well characterized and can be easily monitored and modeled in solution waters.

While inorganic metal complexes are well described with known stability constants, organic metal complexes alter metal speciation in a variety of ways, depending on the composition of the organic matter (Neubecker and Allen 1983). Natural dissolved organic matter (DOM) is a heterogeneous mixture with a variety of functional groups with different binding affinities over a range of ten orders of magnitude (Reuter and Perdue 1977, Craven et al 2012). The characterization of DOM is still under development, leading to complications when modeling metal speciation. In general, binding of copper to DOM may change the free metal ion reactivity, decreasing its bioavailability (Allen and Hansen 1996). In contrast to inorganic complexes, the proportion of copper that is complexed with DOM will fluctuate as the total concentration of copper in the system varies, and it often changes nonlinearly.

Further confounding the determination of metal bioavailability and impacting metal speciation is the competition of other cations within the bulk water solution for ligands (Pagenkopf 1983, Meyer et al 2007). The competition by Ca^{2+}, Mg^{2+}, H^{+}, Na^{+}, or K^{+} for metal binding sites is dependent on pH, alkalinity, and hardness of the system. H^{+} is the primary competitor at lower pH and low alkalinity, and Ca^{2+} and Mg^{2+} being the

main competitors at high alkalinity, high hardness (Meador 1991, Meyer et al 2007). Due to these influences, pH, alkalinity, and hardness impact metal bioavailability in relatively predictable ways, but often co-vary with one another and other water parameters (Lu and Allen 2002).

Natural surface waters are dynamic systems, where, as eloquently stated by Morel, "everything depends on everything else" (1983). To predict and assess metal bioavailability in a surface water, all of the factors outlined above should be monitored or controlled. Even after the water chemistry has been taken into account, the interactions of the metal species with the organism further affect bioavailability. Metals can be accumulated by organisms via the water column (uptake, absorption, and/or adsorption) or through the ingestion of food and other particles (Phillips and Segar 1986, Meyer et al 2007). Due to the lower accumulation from dietary exposure at toxic levels (Minghetti et al 2008, Ates et al 2015), the limited relationship to the water chemistry, low probability of biomagnification (Cardwell et al 2013), and the assumption of the main site for metal interactions with organisms being the plasma membrane (Campbell 1995), this review will focus solely on the exposure and accumulation via the water column exposure.

Metals must undergo a variety of interactions with the surface of the organism prior to being absorbed by the organism. Initially, the metal (as free metal ion or metal-ligand complex) diffuses from the bulk solution and binds to a reactive site on the cellular surface. It is at this reactive site that other aqueous cations may compete with the metal (Campbell 1995). If the metal binds to the reactive site it then diffuses across the thin mucus layer of the organism to reach the plasma membrane of the organism (Campbell 1995, Meyer et al 2007). If the metal is a free metal ion, once it reaches the plasma

membrane it has two courses of action (Campbell 1995). The free metal ion might bind to a receptor at the plasma membrane surface, potentially causing physical or physiological damage. The second option is for the metal to impact an internal site by being transported across the plasma membrane. If the metal initially crossing the mucus layer was part of a metal-ligand complex, the ligand may exchange the metal for a cation at the plasma membrane surface, then the new "free metal" would continue with the two options listed above. The metal-ligand complex may also form a bond with the organism surface, potentially causing similar damage as a free metal ion.

It has been shown that variation of water chemistry, including water hardness, alkalinity, organic matter content, and pH, impact the bioavailability of metals by either binding directly to the metal or by interfering with the ability of the metal to bind to a ligand (inorganic, organic, or organism) by binding preferentially to the ligand before the metal can bind (Meyer et al 2007). Due to the large number of water chemistry combinations, the lab results and methodologies are not readily comparable to many field locations and thus, extrapolating these results to the natural environment continues to present difficulties. In addition, the laboratory results often differ in their conclusions of the effect the water chemistry parameter has on the metal bioavailability (Meyer et al 2007). Modeling systems or controlled bioassays are currently employed in attempt to elucidate the bioavailability, and resulting toxicity of copper and other metals to aquatic organisms.

Review of Recent Research on Metal Bioavailability and Relevance to Regulations

Knowledge of metal speciation has progressed since the early 1970's. Researchers discovered that complexes between cationic metals and water constituents such as

inorganic anions and organic matter can decrease the toxicity of metals to aquatic organisms (Reuter & Perdue 1977, Andrew et al 1977, Vuceta & Morgan 1978). In particular, pH and water hardness (concentrations of Ca and Mg ions) were identified as attenuators of metal toxicity (Howarth & Sprague 1978, Chakoumakos et al 1979).

The first regulatory inclusion of a water quality adjustment was the 1980 ambient water quality criteria for cadmium by the U.S. EPA, with the inclusion of a hardness factor (USEPA 1980). At this time the U.S. EPA regulated metals based on the concentration of the total recoverable metal (TRM) which included all free metal in solution, metal complexes of dissolved inorganics and organics, and all metals sorbed to particles, i.e. the total metal in solution. In 1993, the Prothro memorandum by the U.S. EPA allowed states the option of using dissolved metal concentration (<0.45 μm) rather than the TRM for regulating metals. Using dissolved metal concentration assumes that the particulate fraction (fraction associated with suspended solids) does not contribute to toxicity. While the use of the dissolved metal concentration was a step forward from the TRM, studies at the time were beginning to show that not all of the dissolved metal contributes to toxicity (Chakoumakos et al 1979).

The Free-Ion Activity Model (FIAM) progressed from the observation that the dissolved metal in its entirety does not always contribute to toxicity. According to FIAM the bioavailability of copper to aquatic organisms has been determined to be linked to the concentration of the free metal ion, Cu^{2+}, not on the total copper concentration, dissolved copper concentration, or other copper species (Morel 1983, Campbell 1995, Martin and Goldblatt 2007). Morel made a point that the reaction of the free metal ion with water

constituents is not unique, but that the free metal ions react with organismal cellular surfaces at the same rate as they do with the bulk water ligands (Morel 1983).

With the development of the Free-Ion Activity Model came interest in developing a device that could directly measure or determine the free metal ion. The goal was for a device that could be used in situ to decrease sampling and handling error, have acceptable analytical sensitivity, and be relatable to one or more of the chemical species in the bulk water (Sigg et al 2006). Some examples of these device/techniques are gel integrated microelectrode arrays-voltammetry (GIME-V), stripping chronopotentiometry at gel integrated single microelectrodes (SCP), fiber permeation liquid membranes (FTPLM), competitive ligand exchange stripping voltammetry (CLE-SV), and ion selective electrode (ISE: Luider et al 2004, Meylan et al 2004, Sigg et al 2006) . Of these five, only one can be used in situ, GIME-V. In addition, the results from these techniques may be biased based on water chemistry and operator error or technique (Meylan et al 2004) especially the ISE (Hales et al 1999).

The same year as the FIAM was developed, 1983, Pagenkopf proposed the Gill Surface Interaction Model (GSIM: Pagenkopf 1983). The GSIM predicts metal toxicity to fish using chemical principles including pH, hardness, and (as stated by Pagenkopf) trace-metal complexation. Key factors for the development of the GSIM were the ideas that gills are: altered by trace metals, form complexes with metal species in bulk water, can readily exchange metals with the bulk water, and have a limited capacity for binding trace metals. Pagenkopf also took into account the ideas that toxicity varies among metal species and hardness can inhibit the toxicity of metals. Overall, the GSIM was the first model that took into account all important water quality parameters (free metal, cations,

pH, alkalinity, hardness, and dissolved organic matter), but the final value predicted, termed the effective toxicant concentration, was impractical, as it could not be correlated with any physical metal measurement. Yet, the GSIM set the stage for subsequent models including Playle et al's expansion of the GSIM, altering the gill into just a particle competing for the metal in the bulk water (Playle et al 1993).

In the early 2000's the general Biotic Ligand Model (BLM) was developed (Di Toro et al 2001). The BLM is an integration of both the FIAM and GSIM; it expands the idea of the gill surface to be any biological surface that is then factored into the geochemical model as if it were a suspended particle in the bulk water competing for metals. The geochemical speciation portion (MINTEQ and WHAM) of the BLM calculates the amount of metal complexed with the biotic ligand after accounting for competition. Aqueous ligands compete with biotic ligands to bind free metal ions, while cations in the bulk solution compete with free metal ions to bind to biotic ligands (Di Toro et al 2001). FIAM is included in the BLM; it is implied that the free-ion activity can be a good predictor of organismal toxic response when the concentration of competing cations is constant. The concentration of competing cations is often not constant, so the BLM incorporates other water quality parameters to help predict metal speciation, organism accumulation, and acute toxicity.

The operation of the BLM requires a variety of water quality input parameters, and parameterization based on the organism and metal of interest. The required water quality inputs are temperature, pH, dissolved organic carbon (DOC), major cations (Ca, Mg, Na, K), major anions (e.g. SO_4^{2-} and Cl^-), alkalinity, and sulfide (BLM users guide and reference manual 2007). Additionally, the dissolved metal fraction can be entered into

the program, based on the output of interest. Initially the BLM was applied to fathead minnows with copper and silver, but has since been applied to a variety of organisms and metals via the addition of appropriate stability constants for metal complexes with inorganic, organic, and membrane ligands. The use of the BLM for predicting acute toxicity was validated by comparing the output results of the BLM to LC50s (lethal concentration to 50 percent of test organisms) of a particular metal and organism.

As of 2014 the BLM has been accepted as the most suitable method available for assessing the combination of bioavailability of metals, metal speciation and acute toxicity due to metal exposure. Consequently, the BLM is currently used for assessing water quality by most governmental agencies and environmental monitoring companies; the U.S. EPA utilizes the BLM for determination of copper toxicity to aquatic organisms (EPA 2007). Yet, the BLM, as a model, is a simplification of reality and will continue to require experimentation to increase its effectiveness in predicting bioavailability and sublethal effects to organisms. Challenges to the BLM have been identified (Erickson 2013, De Schamphelaere & Janssen 2004). One such challenge is that the effect of the composition of organic matter on its complexation with metals is not reflected by the BLM modeling (Slaveykova & Wilkinson 2005, Craven et al 2012, Bringolf et al 2006). Also, the BLM is constructed as an equilibrium model, so it may not accurately address the kinetics of either geochemical or toxicodynamic processes (Niyogi & Wood 2004, de Polo & Scrimshaw 2012, Slaveykova & Wilkinson 2005).

While there have been great improvements in the study of metal speciation and bioavailability in terms of modeling programs and direct measurements, more research and refinement in this area is needed to increase efficiency, effectiveness, and accuracy,

and to decrease cost for regulators. In the 2007 Aquatic Life Ambient Freshwater Quality Criteria for Copper published by the U.S. EPA, the following requests were made in relation to the need for an approach to assess bioavailability of metals (US EPA 2007): (1) the approach explicitly and quantitatively accounts for the effects of individual water quality parameters that modify metal toxicity; (2) it can be applied more cost-effectively and easily, and hence more frequently across spatial and temporal scales.

Diffusive Gradients in Thin-Films

Diffusive gradients in thin-films (DGT) were designed as a passive diffusion, in-situ sampling device with the goal of providing a rapid method for determining the free and labile portions of dissolved metal in surface waters, i.e. the bioavailable fraction (Davison & Zhang 1994). DGT are robust samplers that develop a concentration gradient with the bulk solution by allowing metal species to diffuse through a filter membrane and diffusive gel and subsequently, bind to a resin gel impregnated with Chelex 100, all housed in a plastic deployment device (Figure 1). The concentration gradient results in a consistent diffusion flux, causing a higher accumulation of metals in the resin gel over time. Post retrieval, the deployment housing is opened, the resin gel removed, eluted with acid, and the metal concentrations determined with an ICP-MS (inductively coupled plasma-mass spectrometer) or similar instrument. Equations provided by Davison and Zhang (1994) are used to calculate the concentrations of free and labile metal from the measured concentration.

The principle behind DGT is based in the free ion activity model and Fick's first law of diffusion; the metal species that are bioavailable are those that are not strongly complexed (Martin 2008), and that developing a diffusion gradient (via the gels) will

inhibit the movement of those strongly complexed species through the DGT device (Davison and Zhang 1994). The result is that only the free and labile metal species that can dissociate in a few minutes are able to diffuse through the DGT layers to bind to the final cation-exchange resin layer (Zhang et al 1995). The diffusion occurs through the 0.45μm pore size, hydrophilic polyethersulfone filter membrane followed by the diffusive gel of polyacrylamide gel cross-linked with an agarose derivative, pore size 2-5 nm (Davison and Zhang 1994, Davison and Zhang 2012). An additional diffusion layer can develop between the bulk water and the DGT device, termed the diffusive boundary layer (DBL), which further restricts movement of ions to only molecular diffusion. The thickness of the DBL can vary, but in well mixed or flowing solutions the impact of the DBL is negligible (Davison and Zhang 2012). Metal species diffuse through the DBL (if applicable), membrane filter, and diffusive gel to the resin layer (see Figure 1-1) within a few minutes of deployment, resulting in steady state levels of diffusion (Zhang et al 1995, Martin 2008).

DGT measured values are translated into mass, concentration, or flux of metal through calculations based on Fick's first law of diffusion (Davison and Zhang 1994).

$$flux = DC_b/\Delta g$$

Where D is the effective diffusion coefficient through the diffusive gel, C_b is the metal concentration in the bulk solution, and Δg is the thickness of the diffusive gel and membrane filter, typically 0.8 mm and 0.13 mm respectively. To determine the mass of ions in the resin layer, the resin layer is eluted with a known volume (V_e) of 1M HNO_3 for at least 24 hours. The eluent is then measured for metal concentration, C_e. The

elution factor, f_e, is based on the amount of metal that is eluted compared to bound on the resin layer. For Zn, Cd, Cu, Ni, and Mn a f_e of 0.8 is used (Zhang 2003).

$$Mass\ of\ ions\ in\ resin\ layer\ (M) = \frac{C_e(V_g + V_e)}{f_e}$$

V_g is the volume of the resin layer gel. The concentration of the free and labile metals in the bulk solution (C) is calculated from the mass of ions in the resin layer (M), the diffusive layer (Δg), diffusion coefficient (D), time of deployment (t), and the area of the exposed diffusive layer (A).

$$C = \frac{M\Delta g}{DtA}$$

Since the initial development of DGT in the early 1990's, the technique has been well examined, especially in relation to metal speciation and comparisons to the existing metal speciation devices. In a 2004 study by Meylan et al, DGT and voltammetric measurements of copper and zinc had comparable results when tested in natural freshwaters. In the same study DGT results were compared to multiple computer models (WHAM Model VI, NICA-Donnan and Stockholm Humic Model) where DGT-Zn compared well with the models, while DGT-Cu did not, due to complexation differences. In a study with high levels of natural organic matter, DGT and ion selective electrode (ISE) had similar speciation of copper (Luider et al 2004). DGT rivaled and improved upon detection of Cu, Cd, Pb, Zn, and Ni when compared to gel integrated microelectrodes combined to voltammetric in situ profiling system (GIME-VIP), stripping chronopotentiometry (SCP), flow through fiber permeation liquid membranes (FTPLM), hollow fiber permeation liquid membranes (HFPLM), Donnan membrane

technique (DMT), and competitive ligand-exchange/stripping voltammetry (CLE-SV: Sigg et al 2006).

The technique of DGT has been used by researchers to both acclaim and critique. The advantages of DGT include improved detection limits, time averaged measurements, capturing of unusual or cyclic events, decreased chance of sample contamination, and a wide range of deployment capabilities (Peijnenburg et al 2014). Due to the diffusive gradient that develops, and the large number of binding sites on the resin gel, a large concentration amplification occurs during short deployment times, thus improving the detection limit while not altering the analytical chemistry equipment (Davison and Zhang 1994). In typical water quality sampling a single grab sample is taken at a point during the day, this can lead to missing a contamination event or diurnal effects. DGT can be deployed for days or weeks, then the average concentration during that time is measured, thus capturing events that may be missed by grab sampling. Deployment capabilities of DGT include freshwater, estuarine, or saltwater, along with sediments or soils, and a wide range of deployment durations.

Limitations of DGT are most often related to the unknowns of the device, and the assumptions that are required. The appropriate area and thickness of gels for calculation purposes, the possible inadvertent charge of the gel, best deployment time, and the question of what DGT is actually measuring are all subjects that have been presented and discussed since the initial use of DGT, and are addressed in detail in a recent paper by Davison and Zhang (2012). Other limitations of DGT include biofouling of the device over long deployments and issues due to small sample volumes (Peijnenburg et al 2014). Unfortunately all methods available for assessing metal speciation and bioavailability

require assumptions and have limitations that must be recognized. It is the responsibility of researchers to attempt to understand these limitations and further the knowledge of the techniques' capabilities.

Use of DGT for monitoring bioavailability of metals as a bio-mimetic tool has begun gaining research popularity in the last decade. Studies comparing DGT to aquatic plants have concluded both adequate and poor correlations to DGT values for a variety of metals (Zhang 2001, Meylan et al 2003, Diviš et al 2007, Töpperwien et al 2007, and Ferreira et al 2013). In a 2007 study by Diviš et al, the aquatic moss's (*Fontinalis antipyretica)* concentration of Cu and Ni did not correlate well with DGT values, but Cd, Pb, Cr, and Zn did have good correlations. In another study using *Fontinalis antipyretica*, the bioaccumulation of Cu was found to correlate well with DGT if an additional cation-effect model was implemented (Ferreira et al 2013). In Meylan et al's 2004 study, it was postulated that DGT would be practical for predicting copper bioavailability to periphyton, based on their study of DGT compared to voltammetric measurements and chemical equilibrium models. In a comparison of algal (*Scenedesmus vacuolatus*) Cd uptake to DGT measured Cd, there was no relationship found (Töpperwien et al 2007).

DGT has also been compared with aquatic invertebrates, primarily bivalves (Webb and Keough 2002, Jordan et al 2008, Schintu et al 2008), *Daphnia magna (*Tusseau-Vuillemin et al 2004, Buzier et al 2006), and shrimp (Wang et al 2014). Significant correlations were found between DGT and transplanted shrimp (*Litopenaeus vannamei*) and transplanted oysters (*Saccostrea glomerata*) for Cu *in situ* in seawater (Jordan et al 2008, Wang et al 2014). Significant correlations were also found for Cd and Pb in DGT

and transplanted mussels (*Mytilus galloprovincialis*) in seawater (Schintu et al 2008). In the study by Webb and Keough (2002) DGT were compared to transplanted saltwater mussels (*Mytilus galloprovincialis*), but results were not definitive due to high levels of biofilms on DGT after 1 month of deployment. *Daphnia magna* were compared to DGT uptake of copper in lab studies where the organisms and DGT were exposed in separate beakers for similar deployment durations (Tusseau-Vuillemin et al 2004, Buzier et al 2006). In both cases the results required further exploration, and the researchers postulated that the DGT had accumulated labile organic complexes, which would not be traditionally classified as a bioavailable species.

Two studies have been identified that compare DGT to uptake of metal by vertebrates in surface waters; both studies used trout. Rainbow trout (*Oncorhynchus mykiss)* gill bound copper correlated well ($r^2 = 0.477$) with DGT labile copper in systems with and without natural organic matter (Luider et al 2004). When brown trout (*Salmo trutta)* and DGT were exposed to aluminum spiked acidic waters in flow-through outdoor mesocosms, the DGT aluminum and gill aluminum uptake correlated well ($r^2 = 0.57$-0.73) (Røyset et al 2005). These studies have encouraging results, but further research is necessary if DGT are to be implemented in a bio-mimetic capacity.

Fathead minnows (*Pimephales promelas*) as experimental organisms

Fathead minnows (FHM: *Pimephales promelas*) are the most commonly used small fish in ecotoxicology due to their large natural range, ease of culturing, tolerance of a wide range of water quality, small size, and a well-defined life cycle (Ankley and Villeneuve 2006). FHM are members of the family Cyprinidae. They are distributed across the center of North America, from below the Mexican-US border up to the Hudson

Bay, and extending from the Appalachian Mountains to the Rocky Mountains (Etnier and Starnes 1993). They are opportunistic omnivores, with a diet ranging from detritus and algae, to aquatic insects and zooplankton. Maximum total length of an adult FHM is 101 mm with males being longer and larger; males are typically 3-5 grams, whereas females are 2-3 grams (Etnier and Starnes 1993, Ankely and Villeneuve 2006).

The lifecycle of FHM has been defined over many studies since the first use of FHM in research in the 1950s (Ankely and Villeneuve 2006). In natural systems there is an elaborate courtship leading to spawning, after which the males guard the nest. In cultured systems pairs or groups of FHM can be placed in tanks with appropriate spawning substrate (typically hard surfaces such as rocks or logs) under controlled conditions. The eggs, which hatch 4-5 days post spawn (at 25°C), are transparent with several visible development stages. For the first 30 days post-hatch, the young FHM feed upon live food, with an optional switch to pellet food as they age. Sexual maturity can be reached in 4-5 months. Based on temperature and photoperiod of the tank, spawning can occur frequently for several months. At their prime, females produce 4,000 to 5,000 eggs in a year (Etnier and Starnes 1993). FHM average lifespan is from 1 to 3 years, depending on conditions.

An abundance of literature is available about FHM copper binding affinities (Playle et al 1993, Brooks et al 2006) and the impact of many water chemistry parameters on the uptake and toxicity of copper (Erickson et al 1996, Playle 1998, USEPA 2003, Ryan et al 2004, Sciera et al 2004, Meyer et al 2007, USEPA 2015 ECOTOX database). Hardness, sodium, DOM, total suspended solids (TSS) and pH all decreased toxicity of Cu as they increased in static and flow-through 96 hour exposures, while alkalinity had

no effect (Erickson et al 1996). In a static renewal 96 hour exposure, hardness, pH, and DOC all decreased toxicity of Cu, and this decrease was not accurately predicted by the BLM (Sciera et al 2004). Fish physiology, primarily gill physiology, is thought to be the source of the observed outcomes of the water chemistry parameters (Paquin et al 2002).

Fish gills are responsible for many physiologic processes including ion regulation, acid-base regulation, gas exchange, and waste excretion (Paquin et al 2002). Metals primarily impact the ability of fish to regulate ions; copper specifically influencing ionoregulation by creating sodium imbalances (Meyer et al 2007). Membrane surfaces, including gills (i.e., biotic ligands) have negatively charged proteins that bond with positively charged ions (e.g., $Ca^{2+,}$ Cu^{2+}, H^{+}). Due to the site specificity of binding, metals can interfere with the influx and efflux of necessary ions. For example, Cu^{2+} can displace Ca^{2+} from fish gills, causing an increase in Na^{2+} and K^{+} efflux, which further increases the permeability of the gills to ion efflux (Lauren and McDonald 1985, Meyer et al 2007).

Three mechanisms of metal toxicity related to ionoregulation have been identified (Paquin et al 2002). Sodium transport is affected by monovalent metals (e.g., Cu^{+}), Ca^{2+} is disrupted by divalent metals (e.g., Zn^{2+}), and some metals directly impact the fish after traversing the gill (e.g., Hg^{2+}, Pb^{2+}). It should be noted that the free copper ion in the bulk water is Cu^{2+}, but transport across biological membranes reduces the Cu^{2+} to Cu^{+} (Campbell et al 1999, Paquin et al 2002, Meyer et al 2007). The disruption of ion regulation caused by monovalent and divalent metals cause levels of sodium, chloride and other ions in the plasma and other tissues to decrease or become imbalanced, often leading to death.

The general knowledge of these physiological effects caused by metals exposure is imperative to the understanding of how metals bioaccumulate in differing water chemistries. In models like the BLM, the complexation parameters (binding affinities, binding site densities) are the link between the physiological and geochemical aspects of metal bioaccumulation (Brooks et al 2006).

Freshwater mussels as experimental organisms

The interest in use of freshwater mussels (Family Unionidae) for ecotoxicology studies has grown since the early 1990's after the significant decrease in their natural populations was noticed (Farris and Van Hassel 2007). Population declines are attributed to anthropogenic actions, including habitat destruction and fragmentation, sedimentation, invasive species, and pollution. As of 2015, 54 species of Unionidae mussels were considered endangered, 65 critically endangered, and 29 extinct (IUCN 2015). In 2001, a standard ASTM test method for in situ studies with freshwater bivalves was developed. Use of freshwater bivalves for other types of toxicity tests such as use of biomarkers, as biomonitors of sublethal effects and in bioaccumulation studies are gaining acceptance (Farris and Van Hassel 2007). Hindrances to the use of mussels in ecotoxicology include their slow growth, complex reproduction, and difficulties with culturing (due to complex reproduction; Farris and VanHassel 2007).

Lampsilis cariosa (LMP) is a freshwater bivalve of the family Unionidae, tribe Lampsilini that range along eastern North America from Georgia to Nova Scotia (Sabine et al 2004, Haag 2012). It naturally inhabits large streams and rivers where it buries in the sediment. LMP average 75 mm in length, with a smooth yellowish outer shell. LMP exhibit sexual dimorphism, with males being more elongate than rounded (UGA Museum

of Natural History 2008). Females also have a modified mantle flap that resembles a small fish with eyespots that are used as lures in reproduction (Figure 1-2).

Like other freshwater bivalves, adult LMP filter feed, taking in water, toxins, detritus, and microorganisms through their incurrent siphon, washing it over their gills. Waste, as feces and/or pseudofeces, is released by the excurrent siphon. The exact food material needed by freshwater mussels is unknown, with evidence of diatoms, bacteria, protozoans, detritus, and zooplankton found in the gut (Bisbee 1984, Watters 2007).

The life cycle and reproduction of *Lampsilis cariosa* is reputed to be similar to other members of the genus *Lampsilis*; LMP are dioecious with three distinct life stages, glochidia, juvenile, and sexually mature adult (UGA Museum of Natural History). Adult male *Lampsilis* release sperm into the water column. Sperm are then pulled into the female via the incurrent siphon to fertilize the eggs held in chambers of the gills (Watters 2007). This typically occurs in the summer, with the fertilized eggs being held by the female until the following spring. In spring, the gravid females release the developed glochidia. The glochidia are a parasitic stage that must attach to the gills or fins of an appropriate fish host to continue its development. The white (*Morone americana*) and yellow perch (*Perca flavescens*) are believed to be hosts for *Lampsilis cariosa* (Werner 2014). The length of the glochidia stage is unknown, but after an appropriate amount of time metamorphosis to the juvenile stage occurs, then the juvenile drops from the gills or fin of the fish to the sediment. The juvenile then burrows into the sediment to mature to an adult. The average lifespan of *Lampsilis cariosa* is unknown, but has been reported to possibly be 7.8 years (Werner 2014).

Adult mussels are often used for bioaccumulation and bioavailability studies, especially when related to metals (Armstead and Yeager 2007). As discussed above, metal bioavailability, bioaccumulation, and uptake is affected by metal speciation, resulting in complex interactions. For bivalves, aside from the effects of metal speciation and environmental conditions such as water chemistry, the metal uptake is related to the physiology including homeostatic regulation of essential elements and interactions between essential and nonessential metals and the condition of the organism (Thorsen et al 2007). Metals are taken up by endocytosis, active transport, and facilitated diffusion primarily via the active sites on the gills, but also by the mantle, foot, kidney, hepatopancreas, and the digestive tract. Thus, metal concentrations in the tissue are not directly related to the aqueous metal concentration.

The BLM has gained popularity in determining bioavailability of metals to bivalves, but the organism species, condition, and physiology need to be taken into account to more accurately determine the possible effects. As discussed above, DGT have been used in multiple studies with transplanted marine bivalves with considerable success (Webb and Keough 2002, Jordan et al 2008, Schintu et al 2008). Little work has been done with comparing DGT to freshwater mussels.

Figures and Tables

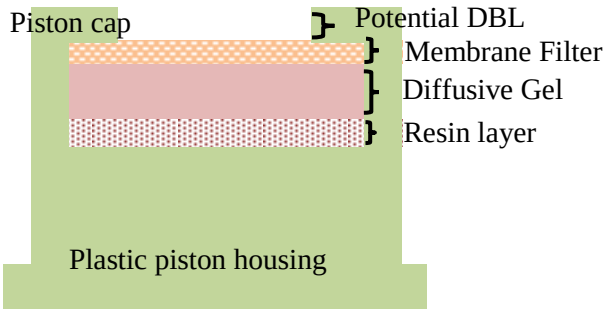

Figure 1-1: cartoon of DGT cross section

Figure 1-2: Female *Lampsilis cariosa* displaying modified mantle flap lure

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CHAPTER 2

EVALUATION OF DIFFUSIVE GRADIENTS IN THIN-FILMS FOR PREDICTION OF COPPER BIOACCUMULATION BY YELLOW LAMPMUSSEL (*LAMPSILIS CARIOSA*) AND FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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Abstract

Using a coupled method of Diffusive Gradients in Thin-films (DGT) exposure with aquatic organism bioassays, we assessed the use of DGT as a tool for estimating copper bioavailability in contaminated waters. The metal species believed to be accumulated by DGT, free copper ions and labile copper complexes, are generally considered to be the bioavailable metal species. DGT accumulated copper fraction could possibly be used as a surrogate for other assessments of metal bioavailability. Fathead minnow (*Pimephales promelas*) and yellow lampmussel (*Lampsilis cariosa*) soft tissue copper concentrations were compared with DGT accumulated copper after two, four, and six days of exposure to a copper concentration series in static, water-only assays. DGT accumulated copper was found to include free copper ions, labile inorganic copper complexes, and labile dissolved organic matter copper complexes when compared to the copper speciation output from the Biotic Ligand Model. Regressions of DGT and fathead minnow accumulated copper at four and six days of exposure demonstrated linear relationships (r^2 = 0.601 and 0.771, respectively). Similarly, regressions of DGT and yellow lampmussel accumulated copper at four and six days of exposure demonstrated linear relationships (r^2 = 0.428 and 0.711, respectively). Yellow lampmussel bioaccumulated copper was over-predicted by DGT at copper concentrations greater than 10 μg L^{-1}. The speciation component of the Biotic Ligand Model predicted inorganic copper had similar relationships to fathead minnow and yellow lampmussel as DGT at all deployment durations. DGT appears to provide a good estimate of metal bioavailability to fathead minnow at the exposure concentrations, but the relationship is not linear for the yellow lampmussel.

Introduction

The amount of aqueous metal that is available for uptake by an organism at any given time is a function of the metal speciation, and is not reflected by the total metal or dissolved (<0.45 μm) metal concentrations [1-4]. Copper (Cu) is cited as one of the most highly reported causes of surface water impairment by metals [5]. Cu speciation is affected by water chemistry, including metal to ligand bonding and competition with cations (Ca^{2+}, Mg^{2+}, Na^{+}, H^{+}, K^{+}) [6, 7]. Accurate and efficient methods for assessing metal bioavailability are necessary for regulation of metals in surface waters. Geochemical modeling, biological, and passive sampling techniques for assessing trace metal bioavailability and bioaccumulation have been developed. The Biotic Ligand Model (BLM) incorporates water chemistry and metal complexation to predict the amount of metal that will bind to a biotic ligand after extensive parameterization of the computer model [8]. Biomonitors, often bivalves or plants, are used *in situ* to determine bioavailability of metals by measuring bioaccumulation [9-11]. In addition, passive sampling devices, like Diffusive Gradients in Thin-films (DGT) can be used to measure the free and easily dissociated metal species *in situ* [12].

The fundamentals of DGT are extensively described in the literature [13]. In summary, DGT consist of three thin layers: a filter (0.45 μm pore size) membrane, a diffusive gel (open pore size 2-5 nm), and a resin gel, which when measuring Cu is impregnated with Chelex 100, all housed in a plastic deployment device. Upon deployment, a concentration gradient, with a quantifiable diffusion flux, develops with the bulk solution allowing free and labile metal species to diffuse through the filter membrane and diffusive gel and bind to the Chelex resin layer [14]. After the DGT are

retrieved, the resin layer is extracted and eluted with acid, and then the amount of metal accumulated is measured. The mass, concentration, and flux can be calculated and related back to assess metal speciation and/or bioavailability. DGT have been compared to other metal speciation techniques with similar results, however less work has been done comparing DGT to aquatic organism bioaccumulation [15-17].

The fraction of metal believed to be measured by DGT, the free and labile species, is generally considered to be an approximation of the bioavailable fraction [18, 19]. Recent studies comparing DGT accumulated metals to aquatic plant bioaccumulated aqueous metals have had varied results [15, 20-23]. When DGT was compared to aquatic invertebrates, including bivalves, shrimp, and *Daphnia*, significant correlations were found for Cu, cadmium (Cd), and lead (Pb) [19, 24-27]. As of 2015, only two studies have been identified that compare DGT to uptake of metal by vertebrates in surface waters. Both studies used trout; rainbow trout (*Oncorhynchus mykiss)* and brown trout (*Salmo trutta)* had significant correlations with DGT when exposed to Cu and aluminum, respectively [16, 28]. These studies have encouraging results, but further research is necessary if DGT are to be implemented as a bioavailability monitoring tool.

In the studies comparing DGT with aquatic organisms a large range of deployment durations were utilized, from two hours to one month [15-16, 19-21, and 23-28]. Theoretically, any DGT deployment up to saturation should have a linear accumulation, due to the uptake being based on kinetics, not equilibrium, as long as biofouling is not an issue [29]. However, the effect of deployment duration has not been explicitly examined, and has led to discussion in the literature. Studies have looked at multiple deployment times, but results were not described [19, 30]. In Buzier et al 2006,

three deployment durations (3, 6, and 24 hours) were examined, and found to have a linear accumulation of Cu in a non-evolving solution [26]. Gimpel et al [31] also looked explicitly at effect of time in DGT accumulation of Cd over 33 days, finding a linear increase in the mass of Cd accumulated, indicating the DGT measured concentration did not vary over the exposure durations.

The intention of this study was to examine the validity of DGT as a tool for assessing bioavailability to different aquatic organisms by coupling DGT exposure to Cu with fathead minnow (*Pimephales promelas:* FHM) and yellow lampmussel (*Lampsilis cariosa*: LMP) Cu accumulation bioassays. Three deployment durations were used to evaluate the most appropriate deployment duration for paralleling DGT with organisms in a controlled water only system. In addition, the results were compared with the BLM metal speciation output to further evaluate the species that DGT accumulated.

Materials & Methods

Experimental Design

The exposure trial was conducted at the University of Georgia's Savannah River Ecology Lab (SREL), Aiken, SC, in a climate controlled animal care facility (16 hour light: 8 hour dark, 27°C ± 3°). A full two-way factorial design of three Cu treatments (0, 10, 20 μg L^{-1}; Cu-0: 0 μg L^{-1}, Cu-10: 10 μg L^{-1}, Cu-20: 20 μg L^{-1}) and three exposure duration treatments (2, 4, or 6 days; D-2, D-4, D-6) with 5 replicates of each was employed, for a total of 45 tanks. Synthetic water was prepared in 20 liter carboys following USEPA (2002) requirements for soft water (0.960 g $NaHCO_3$, 0.600 g $CaSO_4$, 0.600 g $MgSO_4$, 0.040 g KCl per 20 liters) and spiked with $CuSO_4$ to yield final Cu concentrations of 0, 10 and 20 μg L^{-1} [32]. The Cu concentrations were chosen to be

below the 96 hour LC50 (lethal concentration to 50 percent of test organisms) for *P. promelas* of 25 μg L^{-1} for similar pH and alkalinity [4]. Plastic (HDPE) food-grade containers (20 L) with covers were used as exposure tanks and arranged in five blocks on laboratory benches, with each randomly assigned treatment represented once per block. Seventeen liters of synthetic soft water containing the appropriate Cu addition was added to the respective exposure tanks. An aerator was inserted into each tank to maintain dissolved oxygen levels and facilitate solution mixing. All tanks were allowed to equilibrate for 24 hours prior to introduction of fish, mussels and DGT. Each tank was allocated 4 sub-adult FHM (111 days old, average weight: 24.7 mg, standard error of the mean (SEM) 1.18), 3 adult LMP (average weight: 1.28 g, SEM 0.466) and 1 DGT.

On the sampling day, the fish, mussels, DGT and water samples were collected as explained below. Water temperature (27°C ranged ± 3°) and pH (7.34 ± 0.5) were recorded every 60 minutes for the entirety of the study with a 21x Campbell Scientific data logger. Every 24 hours pH (Mettler Toledo), temperature (°C), and ORP (mV, Double Junction ORP Testr) measurements were taken by handheld meters and recorded. Water samples were collected at intervals of 48 hours or less, and analyzed for acid soluble metal (TM: [Cu_{tot}]), dissolved (<0.45μm) metal (DM: [Cu_{diss}]), dissolved organic carbon (DOC), and alkalinity concentrations. Mortalities were noted and removed from the tank daily.

Aquatic Animals

FHM and LMP were obtained from clean laboratory stocks; Aquatic Bio Systems (Fort Collins, Colorado) and North Carolina State University (Raleigh, NC) respectively. Upon arrival at SREL, FHM and LMP were tempered and combined into 40 gallon

containers of aerated synthetic soft water for holding. On day 0 (June 8, 2014) of the study they were taken from the containers and randomly placed in treatment tanks (4 FHM and 3 LMP per tank). Feeding ceased 48 hours prior to placing the organisms in experimental tanks. Feeding did not occur during the exposure period.

On the appropriate sampling day (2, 4, or 6) all FHM and LMP were removed from their tank, euthanized with MS-222, rinsed with 1% nitric acid and ultra-pure water (Barnstead Nanopure Analytical Ultrapure Water System, Series 1367, >18.2 MΩ cm^{-1}), then placed in clean, labeled, pre-weighed whirl bags. LMP were prodded with a sharp forceps prior to exposure to MS-222 to assess survivorship. All samples were stored at -80°C until they were freeze dried (Labconco, Freezone 4.5) to constant weight (Denver Instrument Company, TR-204). Prior to freeze-drying, mussel in-shell volumes were obtained then soft tissues were extracted with acid cleaned spatulas, and wet weight of soft tissue measured and recorded.

Organisms were digested (CEM MARSxpress) using 1 mL of ultrapure HNO_3; FHM were composited by tank and whole body digested, LMP soft tissues were digested individually. Tort-3 (Lobster Hepatopancreas Reference Material for Trace Metals, National Research Council of Canada (497 ± 22 mg kg^{-1} Cu)), blanks, and replicates were all included twice in each digestion cycle. Digested samples were diluted with ultra-pure water prior to analyses by inductively coupled plasma – optical emission spectroscopy (ICP-OES; Perkin Elmer, Optima 4300 DV). Spikes (addition of 50 µL ICP-200.7-6, High Purity Standards, Charleston, SC), double dilutions (14.5 mL ultra-pure water and 0.5 ml digest) or replicates were included every 10 samples.

DGT

DGT components (filter membrane, diffusive gel, Chelex resin gel, and plastic housing) were obtained from DGT Research Ltd (Lancaster, UK). They were assembled using acid cleaned forceps in a laminar flow bench according to DGT Research Ltd. protocols [33]. DGT were suspended to 40% depth of the tank using fishing line, 1 DGT per tank. Deployment time was recorded to the nearest minute. On respective final sampling days, DGT were removed from the tank, time to the nearest minute recorded, rinsed with MQ water, shaken dry, placed in clean plastic bags with limited air space, placed in cooler with ice for the remaining sampling period, then transferred to a refrigerator (13 °C) until the resin layer could be extracted (less than 2 hours). The chelex resin layer was then extracted by popping the DGT housing, removing the chelex resin layer with acid washed plastic forceps, placing in acid washed 1.5 mL micro-centrifuge tubes, and eluting with 1 mL of 1 M HNO_3. The micro-centrifuge tubes were left for 23 to 24 hours before pulling 0.9 mL aliquots. Aliquots were then transferred to 15 mL trace metal free centrifuge tubes (VWR product information) and refrigerated until analysis by ICP-OES. Aliquots were diluted 5:1 (900µL eluent, 3.6 mL MQ) prior to analysis.

Time averaged DGT-Cu concentrations ($[Cu_{DGT}]$) were calculated using the equations and appropriate parameter values according to DGT research LTD specifications [33]. Specifically,

$$M = \frac{Ce\ (V_{HNO3} + V_{gel})}{fe}$$

where *M* is total mass of metal in bound in the resin gel, *Ce* is the concentration of metals in the 1 M HNO_3 elution solution as determined by the ICP-OES, V_{HNO3} is the volume of

HNO_3 added to the resin gel (1.5 mL), V_{gel} is the volume of the resin gel (0.16 mL), and fe is the elution efficiency factor for Cu (0.8). The concentration of bioavailable Cu ($[Cu_{DGT}]$) in the exposure solution (i.e., operationally defined by the DGT) is

$$Cu_{DGT} = \frac{M\Delta g}{DtA}$$

where Δg is the thickness of the diffusive gel and filter membrane (0.93 mm), D is the diffusion coefficient of Cu in the gel (6.06E-06 cm^2 sec^{-1}), t is the deployment time, and A is the exposure area on the resin gel (3.14 cm^2).

Water Analysis

Water samples were taken from each tank prior to initial exposure, every 24 hours, and prior to removing organisms and DGT. Temperature (°C, Mettler Toledo), pH (Mettler Toledo), ORP (mV, Double Junction ORP Testr), and dissolved oxygen (mg L^{-1}, Mettler Toledo) were measured and recorded before collecting water samples. Samples were obtained for alkalinity, DOC, DM, TM, and cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}). They were transported in a cooler with ice back to the main lab for analysis immediately following collection. Samples for anions (chloride and sulfate) were obtained before and after exposure periods. Alkalinity was determined by titration with H_2SO_4 to pH of 4.5 (mg L^{-1} as $CaCO_3$, Hach kit). DM and DOC samples were hand filtered using 0.45 µm Environmental Express syringe filters. The DM and TM samples were acidified with HNO_3 (Fisher Scientific, trace metal free) to pH 2. Filtered DOC samples and anion samples were refrigerated prior to analyses using a TOC analyzer (Shimadzu, Tokyo, Japan) and an ion chromatograph (Dionex, USA), respectively, at the Stable Isotope and Soil Biology Laboratory, UGA. Cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}) were determined by ICP-OES using the filtered, acidified DM samples.

Metals Analysis

All tubes, bottles, and vessels used for metals analysis were either certified trace metal free (vendor info), or acid cleaned with 3% HNO_3 (Fisher Scientific, trace metal free) for at least 5 days. Samples for metals analysis (FHM, LMP, DGT, DM, and TM) were analyzed by ICP-OES for Cu using wavelength of 324.752 nm, with 3 runs per sample, averaged for final concentration. Spikes (addition of 10 (DM) or 20 (TM) µL ICP-200.7-6, High Purity Standards) and replicates were included every 10 samples. Certified reference material, TM 27.3 (National Research Council of Canada), and SLRS-5 (National Research Council of Canada) were included, two per run. Linear calibrations of R^2>0.995 were achieved for all analyte calibration curves (ICP-200.7-6, High Purity Standards).

Data Analysis

The Biotic Ligand Model (Hydroqual, Inc.) speciation component was used to determine the free Cu ([Cu_{free}]) and inorganic Cu (sum of free Cu^{2+}, $CuOH^+$, $Cu(OH)_2$, $CuSO_4$, $CuCO_3$, $Cu(CO_3)_2^{2-}$, CuCl, and $CuHCO_3^+$: [Cu_{inog}]) for all tanks using the above water quality parameters. The Cl^- and SO_4 were averaged and applied for all daily samplings.

Two-way factorial with block design ANOVAs were conducted for the response variables of [Cu_{DGT}], FHM Cu concentration (Cu_{FHM}), and LMP Cu concentration (Cu_{LMP}) with an α of 0.05. All assumptions of ANOVA were assessed and met prior to analysis. Linear regression modeling (SigmaPlot 12) was employed for assessing correlations of Cu_{FHM} and Cu_{LMP} vs. [Cu_{DGT}] and [Cu_{inorg}] using non-transformed data.

Results

Water Chemistry

Water chemistry stayed consistent throughout the exposure period (Table 2-1). Statistically significant differences between the starting measurement and the final sampling day measurement occurring in four of the eleven parameters measured; temperature, DOC, K, and sulfate (Figure 2-3).

Average [Cu_{tot}] for the entire exposure duration were 24% below their target values, with Cu-0 being below method detection limit (3.21 ± 0.41 μg L^{-1}), Cu-10 at 7.87 ± 0.67 μg L^{-1}, and Cu-20 at 15.7 ± 0.88 μg L^{-1}. The decrease in [Cu_{tot}] occurred over the exposure duration, due to uptake of Cu by LMP, FHM, and DGT. Average [Cu_{diss}] ranged from 71-78% of [Cu_{tot}]. For [Cu_{diss}] the Cu-0 was below method detection limit (2.81 ± 0.26 μg L^{-1}), Cu-10 at 5.57 ± 0.43 μg L^{-1}, and Cu-20 at 12.3 ± 0.98 μg L^{-1}. Twenty-two to 29 percent of the Cu was in the particulate phase.

[Cu_{free}] and [Cu_{inog}] are given in Table 2-2 with [Cu_{inog}] ranging from 3 to 7% of the [Cu_{tot}]. The distribution of Cu among inorganic species is shown in Table 2-4. The general trend for all Cu treatments was $CuHCO_3^+$ > $CuCO_3$ > $CuOH^+$ > free Cu^{2+} > $Cu(OH)_2$ > $CuSO_4$ > $Cu(CO_3)_2^{2-}$ > CuCl.

Aquatic Animals

FHM mortalities occurred at similar rates in each treatment, of the total mortalities 6.6% were in Cu-0 (12 of 60), 5.0% in Cu-10 (9 of 60), and 6.67% (12 of 60) in Cu-20. Neither Cu treatment ($F_{(1,39)}$ = 0.000, p = 1.0) nor deployment duration ($F_{(1,39)}$ = 0.256, p = 0.616) had a statistically significant effect on the mortality. There were no LMP mortalities during the exposure period.

The Cu_{FHM} and Cu_{LMP} are shown in Figure 2-1. The pre-deployment Cu values were 16.6 μg g^{-1} and 16.7 (± 0.619) μg g^{-1} for FHM and LMP respectively. All Cu-0 treatments had final Cu_{FHM} and Cu_{LMP} concentrations below this initial value (Figure 2-1). The interaction terms of Cu treatment and deployment duration were found to be significant using an α of 0.05 for FHM ($F_{(4,32)} = 7.86$, $p < 0.01$, $n = 45$) and LMP ($F_{(4,32)} = 11.7$, $p < 0.00001$, $n = 45$).

DGT

As with FHM and LMP, there was a significant interaction of Cu treatment and deployment duration for [Cu_{DGT}] ($F_{(4,32)} = 2.99$, $p < 0.05$, $n = 45$). The [Cu_{DGT}] by treatment are illustrated in Figure 2-2. [Cu_{DGT}] for treatments Cu-0 at all deployment treatments were at or below the method detection limits (4.46 μg L^{-1}) and are not shown.

Comparison of Techniques

[Cu_{DGT}] in the Cu-10 and Cu-20 treatments averaged 51.6% ± 0.05 and 59.2% ± 0.05 of the [Cu_{tot}], respectively. The percentage of [Cu_{diss}] as [Cu_{DGT}] were 75.5% ± 0.09 for Cu-10 and 76.4% ± 0.06 for Cu-20, indicating than on average, less than 25% of the [Cu_{diss}] was non-labile, with variation based on deployment duration (Table 2-3). The Cu-0 values were below method detection limit. Deployment durations were as follows: D-2 45.97 ±0.12 hours, D-4 89.0 ± 0.031 hours, D-6 139.2 ± 0.036 hours.

Regressions of [Cu_{DGT}] with Cu_{FHM} and Cu_{LMP} (Figure 2-4) indicate a linear relationship between [Cu_{DGT}] and Cu_{FHM} for four (Figure 2-4, b: $r^2 = 0.601$, $p < 0.001$) and six (Figure 2-4, a: $r^2 = 0.771$, $p < 0.0001$) day deployments. Similarly, regressions of [Cu_{inog}] and Cu_{FHM} have linear relationships at four (Figure 2-5, b: $r^2 = 0.607$, $p < 0.001$) and six (Figure 2-5, a: $r^2 = 0.703$, $p < 0.0001$) days of deployment. Also, regressions of

[Cu_{DGT}] and Cu_{LMP} for four (Figure 2-4, b: $r^2 = 0.428$, $p < 0.01$) and six (Figure 2-4, a: $r^2 = 0.7111$, $p < 0.0001$) day deployments; while, regressions of [Cu_{inog}] and Cu_{LMP} have linear relationships at four (Figure 2-5, b: $r^2 = 0.454$, $p < 0.01$) and six (Figure 2-5, a: $r^2 = 0.595$, $p < 0.001$) days of deployment. Two day deployment for both [Cu_{DGT}] and [Cu_{inog}] do not establish linear relationships.

Discussion

Comparison of Techniques

DGT, as a surrogate for metal bioavailability, offer the potential to predict bioaccumulation and thus could provide an easy, cost effective tool for regulators. The validation of using DGT for this purpose requires studies comparing DGT to metal bioaccumulation by aquatic organisms at environmentally relevant concentrations and conditions. This study compared [Cu_{DGT}] to Cu accumulation by fish and freshwater mussels in coupled exposures. The results suggest that [Cu_{DGT}] can be used to predict FHM and LMP whole body accumulation of Cu, based on highly significant positive regressions after four and six days of exposure. These results are similar to other fish and DGT experiments [16, 28]. In Luider et al's 2004 study, rainbow trout (*Oncorhynchus mykiss*) and DGT were exposed to Cu in waters with and without added natural organic matter, in a controlled laboratory setting [16]. The [Cu_{DGT}] compared to rainbow trout gill bound Cu across the natural organic matter treatments had similar patterns of Cu. In a study by Røyset et al, brown trout (*Salmo trutta*) gill accumulation of aluminum and DGT measured aluminum ([Al_{DGT}]) were compared in acidic waters in an outdoor flow-through system [28]. Differences in regression results are likely due to the large amount of methodological variation between the two trout experiments and this FHM experiment,

including the use of different fish species. Both trout studies looked at gill accumulation, while we used total body accumulation. Exposure durations of 3 hours to 14 days were used throughout the three experiments. Also, water quality and chemistry varied between all experiments. Difficulty arises when non-standard methods are used for assessing new techniques; true comparisons between studies are limited. Yet, it is important for DGT to be tested as an estimation of bioavailability of metals in a variety of water chemistries with many different aquatic organisms.

Soft tissue accumulation by *Lampsilis cariosa* was linearly related to [Cu_{DGT}], but at lower r^2 compared to FHM. At the highest Cu concentration (Cu-20) [Cu_{DGT}] over predicted the Cu_{LMP} concentrations (Figure 2-4). Cu accumulation between Cu-10 and Cu-20 for LMP was not significantly different ($p < 0.55$, paired t-test), despite the doubled Cu exposure, suggesting a physiological control of Cu accumulation and/or increased elimination, and consequently, reducing the importance of bioavailable metal concentration. In a 2008 study by Casas et al, Cu accumulation in soft tissue of a marine mussel (*Mytilus galloprovincialis*) reached a similar asymptote after a period of rapid accumulation [34]. Cu has a strong affinity for metallothionein proteins which could increase detoxification and elimination in mussels [35].

Total inorganic Cu (Cu^{2+}, $CuOH^{+}$, $Cu(OH)_2$, $CuSO_4$, $CuCO_3$, $Cu(CO_3)_2^{2-}$, CuCl, and $CuHCO_3^{+}$: [Cu_{inorg}]), calculated using the speciation component of the BLM, similarly was linearly correlated with Cu_{FHM} and Cu_{LMP}. Based on the regression statistics, the [Cu_{inorg}] better predicted Cu_{FHM} accumulation than [Cu_{DGT}] at six days of deployment, but at four days of deployment [Cu_{DGT}] was a better predictor than [Cu_{inorg}]. In this experiment, neither technique appears to be superior with regard to the predictive

ability of either abiotic technique for biotic accumulation. This experiment was conducted using basic water chemistry (soft water, circumneutral pH, no added organic matter, only one metal). With more complex water chemistry a dichotomy may arise, necessitating further research.

Deployment Duration

As shown in Figures 2-5 and 2-6, a two day deployment duration does not provide enough time for a linear relationship to develop between the [Cu_{DGT}] and Cu_{FHM}/Cu_{LMP}, or [Cu_{inorg}] and Cu_{FHM}/Cu_{LMP}. This is likely due to the additional time needed for fish to develop pseudo-equilibrium with their environment at these Cu concentrations [36]. Both four and six day deployment durations were sufficient for developing significant linear relationships between Cu_{FHM}/Cu_{LMP} and both [Cu_{DGT}] and [Cu_{inorg}], indicating that either deployment duration would be acceptable for future comparisons of organisms with [Cu_{DGT}] accumulation.

Metal Speciation

[Cu_{DGT}] was found to be greater than 10x the [Cu_{inorg}], for the same treatment (Figure 2-6). Open pore diffusive gels used in DGT allow the diffusion of both inorganic and organic metal complexes through the gel to the Chelex resin layer [37]. It appears that a large portion (49 to 78%) of the [Cu_{DGT}] was labile Cu-dissolved organic matter ([Cu_{DOM}]) complexes. Use of restricted diffusive gel DGT in addition to the open pore diffusive gels used in this study could facilitate more complete speciation information, than solely using DGT [19]. Although in some cases, the restricted diffusive gels allow accumulation of labile organic complexes, in addition to the inorganic complexes [26].

Overall, the DOM concentrations were low, yet in this study [Cu_{diss}] was primarily found as labile [Cu_{DOM}]. The non-labile [Cu_{DOM}] ranged from 17.5 to 45.3 percent of the [Cu_{diss}]. Greater non-labile [Cu_{DOM}] percentages were observed in longer deployment duration treatments indicating either changes in the DOM characteristic over time or slow reaction kinetics for Cu complexation at stronger binding sites. This is potentially a result of increasing the quantity or quality of organic matter due to animal excretion or defecation, resulting in alteration of metal speciation. No organic matter was added to the exposure tanks, resulting in DOM being primarily from autochthonous origins. Dissolved organic matter interaction with Cu is complex and varies by DOM source due to the heterogeneity of DOM [38]. Terrigenous DOM, organic matter from allochthonous or terrestrial origins, are often found to be more protective against Cu accumulation and toxicity than autochthonous DOM [39, 40]. The large percentage of [Cu_{diss}] found as labile [Cu_{DOM}] may be due to a high proportion of the DOM being less firmly binding autochthonous DOM. Further studies assessing the influence of DOM source on DGT measured Cu compared to aquatic organism accumulated Cu are needed.

Applications of DGT

DGT appears to be a simple technique that can measure labile metals in the water column and could be used to predict metal bioavailability and bioaccumulation by fish. This technique could potentially be used in place of lethal methods of sampling fish gills or blood for determining metal bioavailability. DGT were originally designed as a tool for quantitatively assessing the labile species in freshwater [29]; yet, within six years researchers, including the inventors of DGT, Davison and Zhang, were researching the use of DGT as a bio-mimetic tool [20]. Due to the rapid assessment of metal

bioavailability in situ, potential applications of DGT include monitoring of natural and contaminated environments. DGT could be employed to assess differences in metal bioavailability between system compartments, such as upstream versus downstream of an effluent or in constructed treatment wetlands.

Universal use of DGT for any metal bioavailability assessment is likely ill advised. As was shown with the yellow lampmussel (LMP), DGT do not always provide the same linear prediction of metal bioavailability to all species. This disparity between FHM and LMP could be due to a variety of intrinsic factors, including different physiology, allometry, metabolism, and elimination capabilities. With a different deployment duration (i.e., shorter LMP with longer DGT) or exposure system the correlations between LMP and DGT may improve. More research using other species and metals is required for DGT to be considered a robust technique for predicting metal bioavailability and bioaccumulation to an aquatic organism.

Conclusion

The experimental results of a coupled exposure to Cu by DGT, FHM and LMP indicate a significant linear relationship between DGT and FHM Cu accumulation after four and six days of exposure. Exposure duration of two days was concluded to not be of sufficient length for Cu accumulation by FHM to arrive at pseudo-equilibrium where DGT could be used as bio-mimetic tool. In contrast to FHM results, DGT was found to be a good estimate for LMP Cu bioaccumulation, but not as highly correlated as FHM at total Cu concentrations greater than 10 $\mu g\ L^{-1}$. At these greater Cu concentrations DGT over-predicted LMP soft tissue Cu concentrations.

Similarly, [Cu_{inorg}] had a significant linear relationship with Cu_{FHM} at four and six day exposures, no relationship at two day exposure, and over-predicted Cu_{LMP}. Little difference between [Cu_{DGT}] and [Cu_{inorg}] ability to estimate organism Cu bioaccumulation was found in the study conditions. In similar studies with different water chemistry the DGT technique may prove to be advantageous. In this experiment, both techniques are adequate tools for assessing Cu bioavailability to FHM. But further studies are needed to elucidate any advantages/disadvantages of using DGT rather than the speciation component of BLM for different water chemistries and metals.

Figures

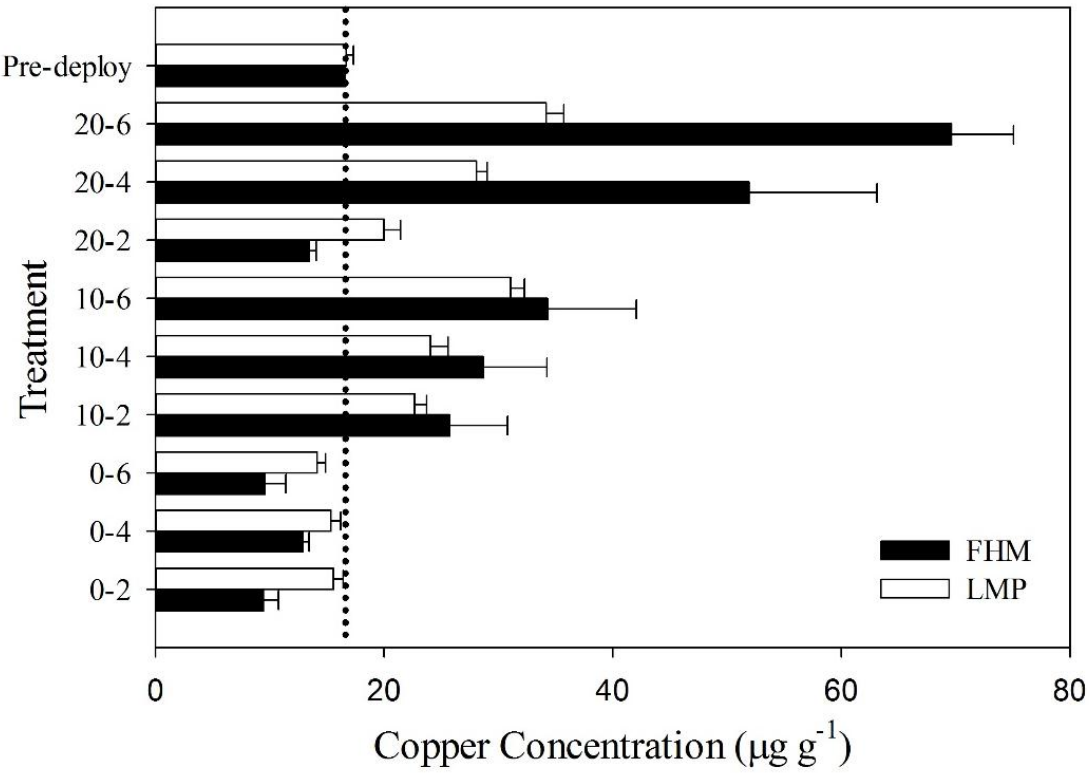

Figure 2-1: Fathead minnow (FHM) and *Lampsilis cariosa* (LMP) total body concentrations of copper based on treatment. First number of treatment indicates copper treatment target (0, 10, or 20 ppb) and second number indicates the deployment duration (2, 4, or 6 days). Pre-deployment FHM and LMP were taken and euthanized on the initial day of the experiment to assess background body burdens.

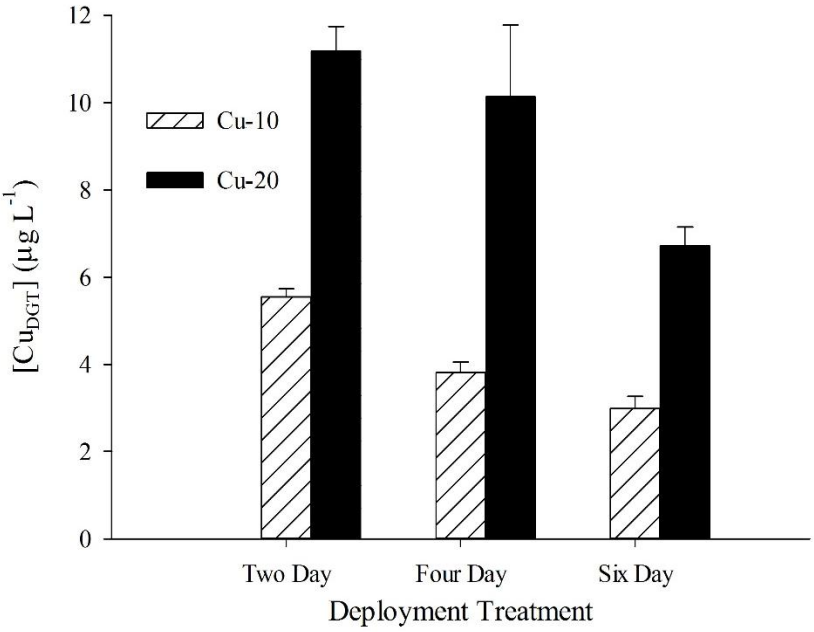

Figure 2-2: DGT measured and calculated copper concentrations, averaged by 5 replicates per treatment ± standard error of the mean.

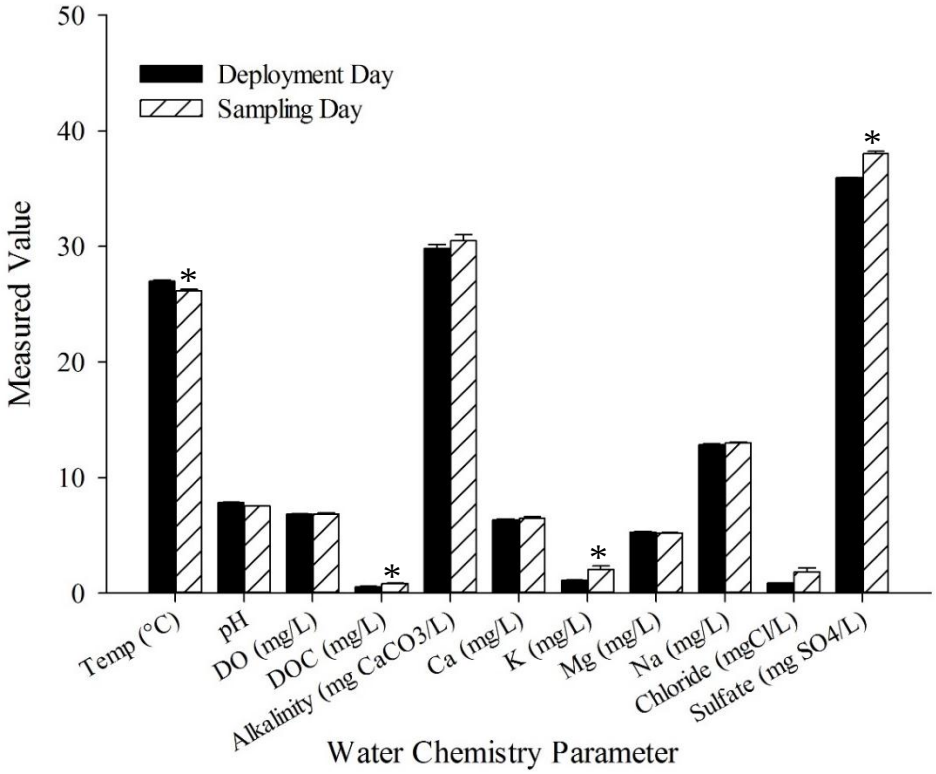

Figure 2-3: Average water chemistry for all tanks and treatments comparing deployment day to final sampling day water chemistry. An asterisk above the sampling day bar indicates a statistical difference (two tailed t-test, α 0.05) from the deployment day average. Error bars are standard error of the mean.

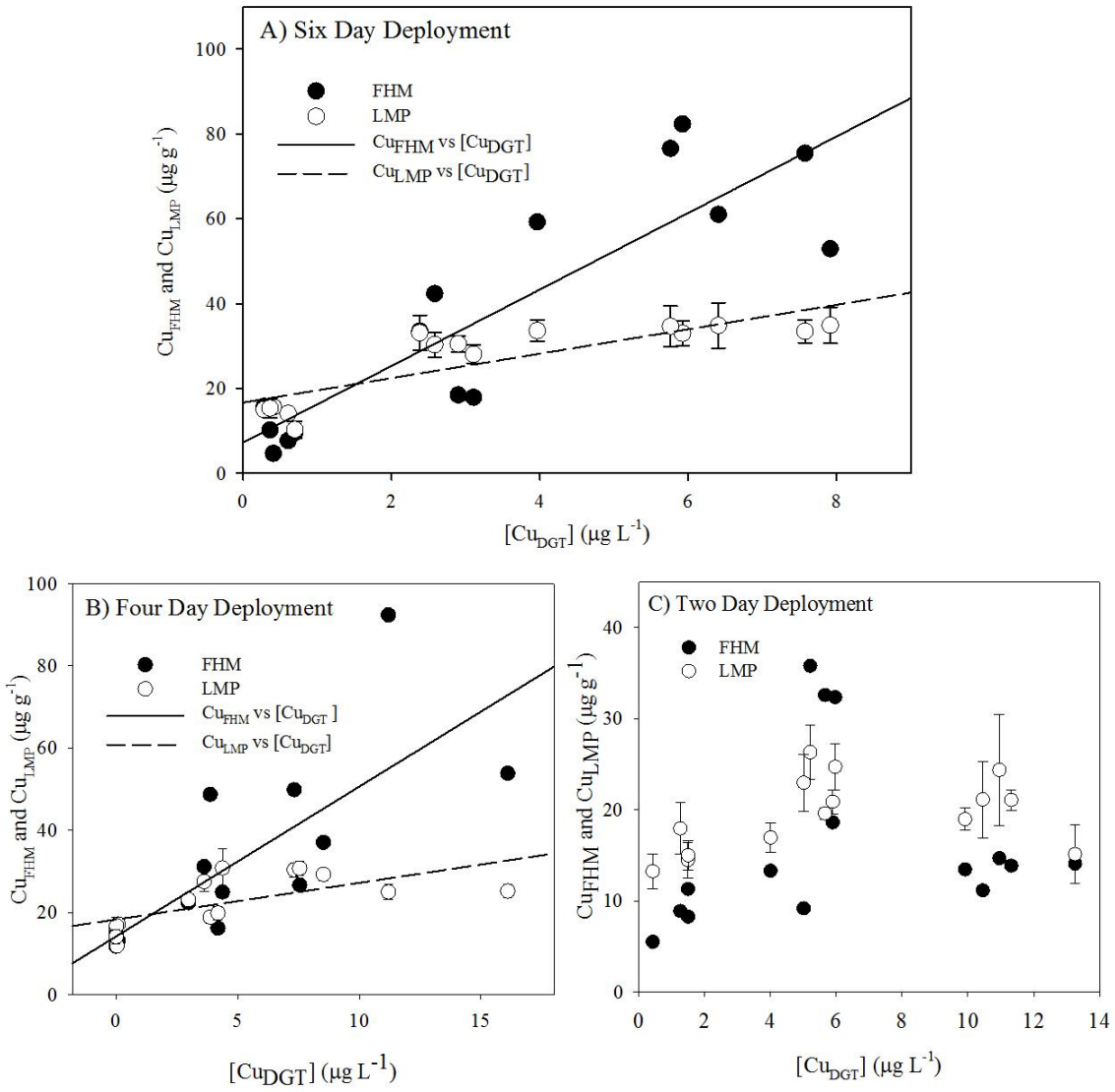

Figure 2-4: [Cu_{DGT}] and fathead minnow (FHM) and *Lampsilis cariosa* (LMP) copper concentrations separated by exposure duration treatment a) six day deployment, b) four day deployment, c) two day deployment. Regression of DGT six day deployment: FHM: y=7.28+9.02x, r^2 = 0.7710, p < 0.0001, n = 15; LMP: y=16.69+2.88x, r^2 = 0.7111, p < 0.0001, n = 15. Regression of [Cu_{DGT}] four day deployment: FHM: y = 14.17+3.65x, r^2 = 0.6010, p = 0.0007, n = 15; LMP: y=18.3+0.89x, r^2 = 0.4275, p = 0.0082, n=15.

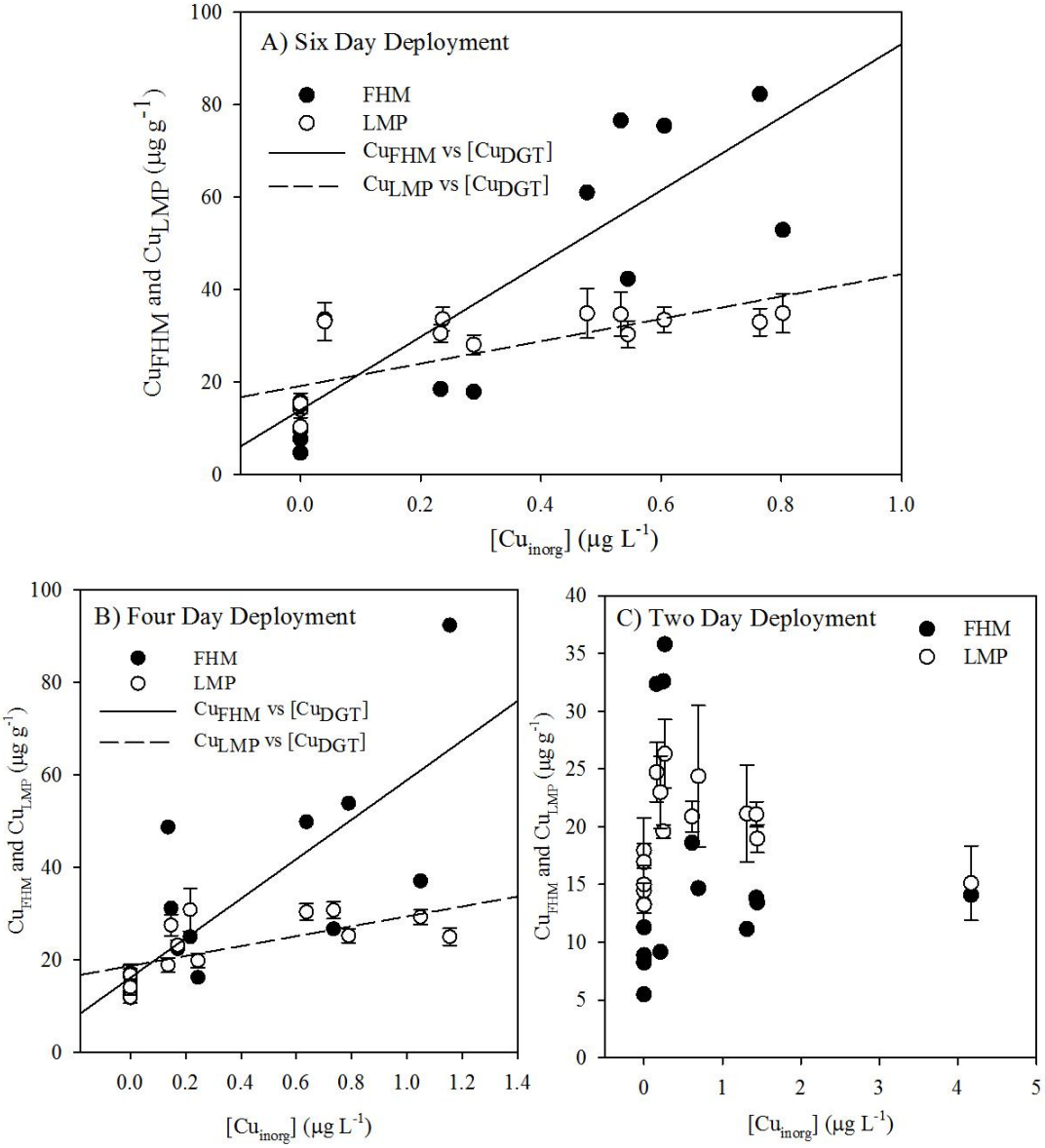

Figure 2-5: BLM speciation component inorganic-Cu ([Cu_{inorg}]) and fathead minnow (FHM) and *Lampsilis cariosa* (LMP) copper concentrations separated by exposure duration treatment a) six day deployment, b) four day deployment, c) two day deployment. Regression of [Cu_{inorg}] six day deployment: FHM: y=13.97+79.15x, r^2 = 0.703, p < 0.0001, n =15; LMP: y=19.15+24.2x, r^2 = 0.5945, p = 0.0008, n = 15. Regression of [Cu_{inorg}] four day deployment: FHM: y=16.11+42.79x, r^2= 0.607, p = 0.0006, n=15; LMP: y=18.68+10.71x, r^2 = 0.4535, p = 0.0059, n=15.

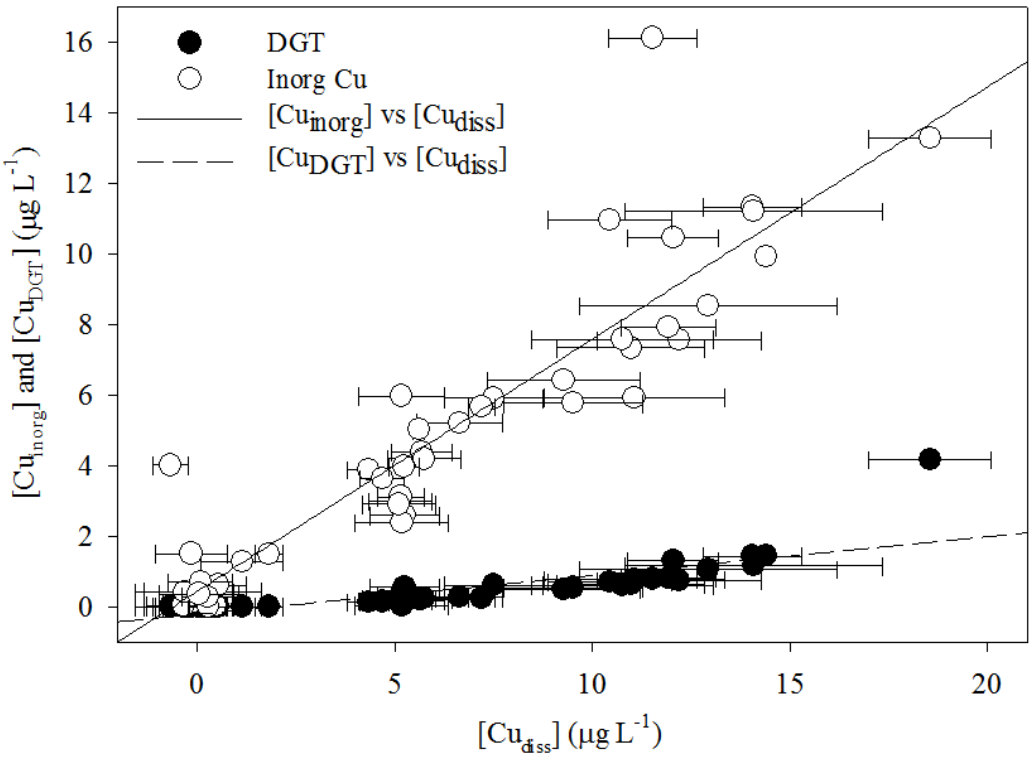

Figure 2-6: Measured dissolved copper (<0.45 µm: [Cu_{diss}]) compared to [Cu_{DGT}] and [Cu_{inorg}] for deployment durations four and six days. Regression of [Cu_{diss}] and [Cu_{DGT}], $y = -0.209+0.1098x$, $r^2 = 0.6508$, $p<0.0001$. Regression of [Cu_{diss}] and [Cu_{inorg}], $y=0.437+0.714x$, $r^2 = 0.8378$, $p< 0.0001$.

Tables

Table 2-1: Water chemistry averaged by all tanks over all exposure days. SEM is standard error of the mean

Parameter	Mean	SEM	n
Temperature (°C)	27.0	1.22	482
pH	7.61	0.01	222
DOC (mg C L^{-1})	0.70	0.02	90
Calcium (mg L^{-1})	6.42	0.08	135
Magnesium (mg L^{-1})	5.20	0.02	135
Sodium (mg L^{-1})	12.9	0.06	135
Potassium (mg L^{-1})	1.42	0.11	135
Sulfate (mg SO_4 L^{-1})	37.0	0.13	90
Chloride (mg Cl L^{-1})	1.35	0.16	90
Alkalinity (mg $CaCO_3$ L^{-1})	30.2	0.27	90
ORP (mV)	222	1.66	222
DO (mg L^{-1})	6.68	0.03	222

Table 2-2: Calculated free copper values ([Cu_{free}]) and total inorganic ([Cu_{inorg}]) values from BLM. [Cu_{inorg}] BLM modeled species Free Cu, CuOH, $Cu(OH)_2$, $CuSO_4$, $CUCO_3$, $Cu(CO_3)_2$, CuCl, and $CuHCO_3$. All five replicates for each treatment were averaged for treatment values ± standard error of the mean. Treatments are denoted by copper treatment target, hyphen, deployment duration

Treatment (Cu - DD)	[Cu_{free}]		[Cu_{inorg}]	
	Average (µg L^{-1})	± SEM	Average (µg L^{-1})	± SEM
0 - 2	1.60E-13	1.31E-14	2.75E-12	2.07E-13
0 - 4	1.72E-13	9.22E-15	2.92E-12	9.32E-14
0 - 6	1.86E-13	4.45E-15	2.96E-12	9.30E-14
10 - 2	1.78E-02	5.79E-03	2.98E-01	8.34E-02
10 - 4	1.08E-02	1.11E-03	1.83E-01	2.06E-02
10 - 6	1.55E-02	5.14E-03	2.69E-01	8.10E-02
20 - 2	9.47E-02	3.04E-02	1.81E+00	6.06E-01
20 - 4	4.75E-02	4.51E-03	8.73E-01	9.84E-02
20 - 6	3.34E-02	3.32E-03	6.36E-01	6.40E-02

Table 2-3: Percent of dissolved copper ([Cu_{diss}]) represented by metal species. Inorganic copper calculated by Biotic Ligand Model, [Cu_{inorg}]. Labile Cu-DOM (dissolved organic matter: [Cu_{DOM}]) calculated from [Cu_{DGT}] concentration minus [Cu_{inorg}]. Non-labile Cu-DOM is the remaining measured [Cu_{diss}], not measured by [Cu_{DGT}].

Treatment (Cu-DD)	**10-2**	**20-2**	**10-4**	**20-4**	**10-6**	**20-6**
Inorganic Cu	4.43%	11.5%	3.46%	6.60%	4.93%	5.71%
Labile Cu-DOM	78.1%	59.7%	68.4%	70.1%	49.8%	54.6%
Non-Labile Cu-DOM	17.5%	28.8%	28.1%	23.3%	45.3%	39.7%

Table 2-4: Biotic Ligand Model speciation output of inorganic copper species ([Cu_{inog}]) , averaged by copper treatment (Cu-10 and Cu-20), n = 15. SEM is standard error of the mean. Cu-0 treatment was not included due to all species being less than 1E-11 μg L^{-1}.

μg L^{-1}	Free Cu	CuOH	$Cu(OH)_2$	$CuSO_4$	$CuCO_3$	$Cu(CO_3)_2$	CuCl	$CuHCO_3$	[Cu_{inorg}]
Cu-10	0.0147	0.0208	0.0022	0.0021	0.1380	0.0004	0.0000	0.2850	0.4630
±SEM	0.0021	0.0026	0.0003	0.0003	0.0176	0.0001	0.0000	0.0435	
Cu-20	0.0585	0.0976	0.0113	0.0085	0.6790	0.0024	0.0000	1.1900	2.0500
±SEM	0.0185	0.0327	0.0040	0.0027	0.2240	0.0008	0.0000	0.3810	

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CHAPTER 3

BIOACCUMULATION OF COPPER AND LEAD BY FATHEAD MINNOW (*PIMEPHALES PROMELAS)* AND YELLOW LAMPMUSSEL (*LAMPSILIS CARIOSA*): EVALUATION OF DGT TECHNIQUE FOR PREDICTING UPTAKE OF METAL MIXTURES

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Abstract

Diffusive gradients in thin-films (DGT) were assessed for their predictive capability of fathead minnow and yellow lampmussel sublethal bioaccumulation in copper (Cu) and lead (Pb) mixed metal exposures. Nine treatments with a matrix of three Cu concentrations (0, 15, 25 μg L^{-1}) and three Pb concentrations (0, 30, 50 μg L^{-1}) were utilized. Exposures were coupled, with organisms and DGT exposed together in tanks, for six day durations. Copper measured in fathead minnow, yellow lampmussel, and by DGT was found to not be influenced by lead treatment, whereas accumulated lead was impacted by the interaction of Cu treatment and Pb treatment. DGT significantly correlated with fathead minnow accumulated Cu ($p < 0.0001$) and Pb ($p < 0.0001$). Yellow lampmussel bioaccumulated metal were not as highly linearly correlated with DGT (Cu: $p < 0.0001$, Pb: $p = 0.105$). In this experiment, DGT was determined to better predict aquatic organism bioaccumulation of copper than the inorganic Cu fraction modeled by the Biotic Ligand Model speciation component.

Introduction

In natural aquatic environments, metals are often found in complex mixtures. In contrast, toxicity testing and regulations are based on single contaminants. This can potentially lead to a disparity between the regulations of surface waters and the protection of fauna. Metal bioaccumulation can be used to monitor aquatic organism exposure and predict toxicity because metals are not readily metabolized [1, 2]. Geochemical and biological aspects of the environment and organisms affect bioaccumulation and bioavailability in single metal exposures. When metal mixtures are taken into consideration, the interactions among the metals and between the metals and organism

add additional complexity that has been difficult to predict or model [3-6]. Uptake and other biological effects of metal mixtures are dependent on the bulk solution water chemistry, metals in the mixture, concentrations and ratios of those dissolved metals, and affinity of each metal for the organism [7].

Metals often act on different membrane surface sites and have different modes of action with regards to toxicity [8-10]. Copper (Cu) is an essential element that is actively taken up by aquatic organisms, but at high concentrations copper interrupts Na^+ homeostasis [9, 7]. Lead (Pb) is a nonessential element that has been found to affect both Ca^{2+} homeostasis and Na^+ and Cl^- balance [8-10]. Interactions between essential and nonessential metals can impede nonessential metal uptake by out-competing for binding sites on freshwater bivalves [11], but in other organisms the interactions may result in different patterns of uptake. In a 2009 study of Pb and Cu uptake by zebrafish (*Danio rerio*), Pb facilitated Cu uptake while Cu resulted in nonlinear uptakes of Pb [3]. In a simultaneous exposure Cu and Pb facilitated the uptake of the other metal by *Paracheirodon innesi*, but when exposed sequentially only Cu facilitated increased accumulation of Pb [4]. When green alga (*Chlamydomonas reinhardtii*) was exposed to Pb and Cu, bioaccumulation varied with concentration; at low Cu concentrations (<1 μM) Pb had no effect on Cu uptake, but at higher Cu concentrations Pb decreased Cu bioaccumulation [6].

Due to the variability of metal mixture effects, modeling has been employed to predict toxicity. One commonly used approach is concentration addition, where individual metal concentrations are expressed as toxic units (TU), then summed [12]. Concentration addition is only appropriate when all metals in the mixture have the same

mode of action [10]. As research with metal mixtures has progressed, many mixtures have been shown to not respond additively [7, 13]. The Biotic Ligand Model (BLM) has also been expanded for use in metal mixture situations [5], but its feasibility as a tool for metal mixture bioaccumulation assessment is still under scrutiny [6, 9].

In situ measurements of the bioavailable metals could potentially be used as a non-lethal method for predicting bioaccumulation by aquatic organisms. Diffusive gradients in thin-films (DGT) is a passive diffusion device that measures the free and easily dissociated metal species, i.e., those that are bioavailable [14]. DGT measured metals in mixture solutions should theoretically account for metal interactions, allowing the free and labile species of all metals to diffuse through the membranes. The DGT resin membrane layer for trace metals is impregnated with Chelex 100, and is capable of accumulating aluminum, cadmium, cobalt, chromium, Cu, iron, manganese, nickel, and zinc [15].

Comparisons have been made between DGT measured metals and other metal bioavailability techniques. A validation study comparing DGT measured Cu and Zn to voltammetric measurements in natural freshwaters showed good agreement between the two techniques [16]. DGT has been compared with biomonitors in natural waters polluted with mixtures of metals [17-19]. When compared with an aquatic moss (*Fontinalis antipyretica)*, DGT concentrations corresponded well for Cd, Pb, Cr, and Zn, but not for Cu and Ni [17]. Two studies used transplanted mussels (*Mytilus galloprovincialis*) to assess Cd, Pb, Cu, Ni, and Zn levels in seawater when exposed in parallel with DGT [18, 19]. Significant correlations were found between the mussels and DGT for Cd and Pb, but not for Cu and Ni [18]. Only one study was identified where

DGT was used to assess metal bioavailability in a laboratory [20]. DGT and *Daphnia magna* accumulated Cu and cadmium were assessed, and found to not correlate significantly.

While DGT has been used to monitor metal mixtures in natural environments, the systematic use of DGT in controlled conditions is lacking. The knowledge gained in a controlled study can be used to further understand DGT's capabilities in natural waters. In addition, the investigation of DGT as a bio-mimetic device for predicting bioaccumulation of metal mixtures by aquatic animals has been limited [17-20]. This study aims to assess Cu and Pb bioaccumulation by fathead minnow (*Pimephales promelas:* FHM) and yellow lampmussel (*Lampsilis cariosa:* LMP) and Cu and Pb measured by DGT in a series of controlled exposures. The accumulated metal concentrations will be compared and used to assess DGT's ability to predict Cu and Pb bioaccumulation to aquatic organisms.

Materials & Methods

Lab Setup

The full two-way factorial with block design exposure trial was conducted at the University of Georgia's Savannah River Ecology Lab (SREL), SC, in a climate controlled animal care facility (16 hour light: 8 hour dark, 28°C ± 0.06). Nine treatments were established on three Cu concentrations (0, 15, 25 μg L^{-1}) and three Pb concentrations (0, 30, 50 μg L^{-1}) with five replicates per treatment. Synthetic water (2.4 g $NaHCO_3$, 1.5 g $CaSO_4$, 1.5 g $MgSO_4$, 0.1 g KCl per 50 liters) was prepared in carboys following USEPA requirements for soft water [21]. Acid-cleaned plastic (HDPE, 20 liters) covered food grade containers were used as tanks and arranged in five blocks on

laboratory benches, with each randomly assigned treatment represented once per block. Twenty liters of synthetic water was added to each tank and spiked with $CuSO_4$ and $PbNO_3$ 2000 mg L^{-1} stock solution to yield final Cu and Pb concentrations listed above. Cu and Pb concentrations were chosen to be well below the predicted BLM LC50s for FHM for both Cu and Pb, due to the possibility of synergistic or additive effects (Cu 76.34 μg L^{-1}, Pb 535.6 μg L^{-1}). A plastic aerator was placed in each tank to facilitate mixing and allow water chemistry to equilibrate.

After 24 hours of water chemistry stabilization, each tank was allocated 5 sub-adult FHM (97 to 101 days old on day 0, average weight: 27.65 mg, standard error of the mean (SEM) 1.43), 3 adult LMP (average weight: 1.44 g, SEM 0.035) and 1 DGT. Initial deployment of fish, mussels, and DGT were staggered on two days due to physical and time constraints. After the exposure period, fish, mussels, DGT and water samples were collected as explained below. A 21x Campbell Scientific data logger recorded water temperature (28°C range ± 0.06°) and pH (7.28 ± 0.01) every 60 minutes for the entire six days of the study. Every 48 hours pH, temperature (°C), and ORP measurements were taken by handheld meters and recorded. Water samples were taken for acid soluble metal (TM), dissolved (<0.45μm) metal (DM), dissolved organic carbon (DOC), and alkalinity analyses. Mortalities were noted and removed from the tank daily.

Aquatic Animals

Fathead minnow and LMP were obtained from clean laboratory stocks; FHM were cultured at the Warnell School of Forestry and Natural Resources, Aquatic Toxicology Lab (University of Georgia, Athens, GA) and LMP at North Carolina State University (Raleigh, NC). Fathead minnow and LMP were acclimated and held for greater than 48

hours in aerated synthetic soft water after arrival at SREL. Five FHM and 3 LMP were placed in each tank on day 0 (August 14, 2014 and August 15, 2014) of study. Organisms were not fed prior to or during exposure.

After the exposure (7.0 days ± 0.008) FHM were removed from their tank, euthanized with MS-222, rinsed with 1% nitric acid and ultra-pure water (Barnstead Nanopure Analytical Ultrapure Water System, Series 1367, >18.2 MΩ/cm), and then placed in clean, labeled, pre-weighed whirl bags. Next, LMP were removed from their tank, prodded with sharp forceps to evaluate survivorship, prior to following the same steps as with FHM. FHM and LMP were immediately transferred to an ultra-cold (-80°) freezer. LMP were removed from their shells and wet weights were obtained for both LMP and FHM before being freeze dried (Labconco, Freezone 4.5) to constant weight (Denver Instrument Company, TR-204).

Freeze dried organisms were digested (CEM MARSxpress) using 1 mL of ultrapure HNO_3. Fathead minnow were composited by tank and whole bodies digested. Yellow lampmussel were digested individually, soft tissues only. Tort-3 (Lobster Hepatopancreas Reference Material for Trace Metals, National Research Council of Canada (497 ± 22 mg/kg Cu, 0.225 ± 0.018 mg/kg Pb)), blanks, and replicates were all included twice in each digestion cycle. Digested samples were diluted with ultra-pure water for analyses by ICP-OES (inductively coupled plasma- optical emission spectrometry, Perkin Elmer, Optima 4300 DV). Spikes (addition of 50 µL ICP-200.7-6, High Purity Standards, Charleston, SC), double dilutions (14.5 mL ultra-pure water and 0.5 ml digest) or replicates were included every 10 samples.

DGT

DGT components were purchased from DGT Research Ltd (Lancaster, UK). Components for each DGT device include one 0.45 μm pore size hydrophilic polyethersulfone filter membrane, one polyacrylamide diffusive gel cross-linked with an agarose derivative (open pore size 2-5 nm), and one resin gel impregnated with Chelex 100. These components were assembled in a plastic deployment device in a laminar flow bench according to DGT Research Ltd. Protocols [15].

On initial deployment day, DGT were suspended to 40% depth of the tank using fishing line, 1 DGT per tank. Deployment time was recorded to the nearest minute. On the final sampling day, DGT were removed from the tank and time to the nearest minute recorded. DGT were then rinsed with MQ water, shaken dry, placed in clean plastic bags with limited air space, placed in cooler with ice for period of continued sampling, and then transferred to a refrigerator until the resin layer could be extracted (less than 2 hours). The Chelex resin layers were then extracted and placed in individual acid washed 1.5 mL micro-centrifuge tubes, and eluted with 1 mL of 1 M HNO_3. The Chelex resins were left in the 1M HNO_3 for 64 to 65 hours before pulling 0.9 mL aliquots. Aliquots were transferred to 15 mL trace metal free centrifuge tubes, diluted 5:1 and refrigerated until analysis by ICP-OES.

ICP-OES measured Cu and Pb were used to calculate time averaged DGT mass of metal and concentration of metals. The following calculations were used according to DGT research LTD specifications [15].

$$M = \frac{Ce\ (V_{HNO3} + V_{gel})}{fe}$$

where *M* is mass of metal in the resin gel, *Ce* is the concentration of metals in the 1 M HNO_3 solution as determined by the ICP-OES, V_{HNO3} is the volume of HNO_3 added to the resin gel (1.5 mL), V_{gel} is the volume of the resin gel (0.16 mL), and *fe* is the elution factor for Cu and Pb (0.8).

$$C_{DGT} = \frac{M\Delta g}{DtA}$$

Where C_{DGT} is the concentration of metal measured by the DGT, Δg is the thickness of the diffusive gel and filter membrane (0.93 mm), *D* is the diffusion coefficient of Cu in the gel (6.06E-06 cm^2/sec), *t* is the deployment time, and *A* is the exposure area (3.14 cm^2). C_{DGT} calculation was used to calculate the concentration of Cu measured by DGT ($[Cu_{DGT}]$) and the concentration of Pb measured by DGT ($[Pb_{DGT}]$).

Water Analysis

Temperature, pH, ORP, dissolved oxygen, alkalinity, DOC, DM, TM, and cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}) measurements or samples were taken from each tank prior to initial exposure, every 24 hours, and prior to pulling organisms and DGT. Samples were transported in a cooler with ice back to the main lab for analysis immediately following collection. Samples for anions (chloride and sulfate) were obtained before and after exposure periods. Alkalinity was determined by titration with H_2SO_4 to pH of 4.5 (mg L^{-1} as $CaCO_3$, Hach kit). DM and DOC samples were hand filtered using 0.45 µm Environmental Express syringe filters. DM and TM samples were acidified with trace metal free HNO_3 to pH 2. Filtered DOC samples and anion samples were refrigerated prior to analyses by TOC analyzer (Shimadzu, Tokyo, Japan) and an ion chromatograph (Dionex, USA), respectively, at the Stable Isotope and Soil Biology Laboratory, UGA.

Cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}) were determined by ICP-OES using the acidified, filtered DM samples.

Metals Analysis

All tubes, bottles, and vessels used for metals analysis were either certified trace metal free, or acid cleaned with 3% HNO_3 for at least 5 days. Samples for metals analysis (FHM, LMP, DGT, DM, and TM) were analyzed by ICP-OES for Cu and Pb with wavelengths of 324.752 and 220.353 respectively, with 3 runs per sample, averaged for final concentration. Spikes (addition of 10 (DM) or 20 (TM) µL ICP-200.7-6, High Purity Standards) and replicates were included every 10 samples. Certified reference material, TM 27.3 (National Research Council of Canada), and SLRS-5 (National Research Council of Canada) were included, two per run. Linear calibrations of $R^2 >$ 0.995 were achieved for all analyte calibration curves (ICP-200.7-6, High Purity Standards).

Data Analysis

Free Cu and Pb ($[Cu_{free}]$, $[Pb_{free}]$) and inorganic Cu and Pb ($[Cu_{inorg}]$, $[Pb_{inorg}]$) were determined using the BLM speciation mode for all tanks over the exposure duration using the above water quality parameters. Inorganic Cu species include free Cu^{2+}, $CuOH^{+}$, $Cu(OH)_2$, $CuSO_4$, $CuCO_3$, $Cu(CO_3)_2^{2-}$, CuCl, and $CuHCO_3^{+}$. Inorganic Pb species include free Pb^{2+}, $PbOH^{+}$, $Pb(OH)_2$, $Pb(OH)_3$, $PbSO_4$, $PbCO_3$, $Pb(CO_3)_2^{2-}$, $PbCl^{-}$, and $PbCl_2$. The Cl^{-} and SO_4 were averaged and applied for all daily samplings.

Paired, two sample t-tests (R) were used for assessing differences in water chemistry values between initial deployment day and final exposure day. Alpha was set at 0.05. Equality of variances and normality were assessed prior to use of t-test.

Two-way factorial with block design ANOVAs ($\alpha = 0.01$; R statistical program) were conducted for the response variables of Cu and Pb accumulated or measured by DGT ([Cu_{DGT}], [Pb_{DGT}]), FHM (FHM-Cu, FHM-Pb), and LMP (LMP-Cu, LMP-Pb). Regression modeling (SigmaPlot 12) was employed for assessing the following correlations: [Cu_{DGT}] vs FHM-Cu and LMP-Cu; [Pb_{DGT}] vs FHM-Pb and LMP-Pb; [Cu_{inorg}] vs FHM-Cu and LMP-Cu; [Pb_{inorg}] vs FHM-Pb and LMP-Pb.

Results

Water chemistry

On the initial deployment day (day 0), total Cu ([Cu_{tot}]) and total Pb ([Pb_{tot}]) were 84 to 95 percent of the target concentrations. Dissolved (<0.45µm) Cu ([Cu_{diss}]) and Pb ([Pb_{diss}]) were 71 to 90 percent of the target concentrations. On day 0, [Cu_{diss}] was 87 to 97 percent of [Cu_{tot}], and [Pb_{diss}] was 79 to 85 percent of [Pb_{tot}].

Throughout the exposure duration, for all 45 tanks, the water chemistry (temperature, pH, DOC, alkalinity, Ca, K, Mg, Na, Cl^-, and SO_4) remained constant (Table 3-1); however, the [Cu_{diss}] decreased to 32 to 39 percent of the target Cu concentration and [Pb_{diss}] decreased to 1 to 18 percent of the target Pb concentration. At higher Cu target concentrations (Cu-15, Cu-25) the Pb concentrations decreased less than at Cu-0 (Table 3-2). When averaged for the entire exposure duration [Cu_{tot}] ranged from 57 to 73 percent of target concentration, while [Pb_{tot}] ranged from 55 to 70 percent of target due to uptake by aquatic organisms and DGT. Particulate Cu ranged from six to 13 percent of [Cu_{tot}], and particulate Pb ranged 20 to 40 percent of [Pb_{tot}].

[Cu_{inorg}] for Cu-15 and Cu-25 ranged from four to six percent of the [Cu_{tot}]. [Pb_{inorg}] for Pb-30 and Pb-50 ranged from three to six percent of the [Pb_{tot}] (Table 3-3).

As a further breakdown, [Cu_{free}] averaged 6.93 ± 0.22 percent of [Cu_{inorg}] while the fraction of [Pb_{inorg}] as [Pb_{free}] was more variable than Cu ranging from nine to 17. Block effect was found to not be statistically significant for all response variables.

DGT

DGT measured metals corresponded to the treatment targets, with a stepwise increase in metal concentration measured by DGT as the target treatment concentration increased (Figure 3-2). All [Cu_{DGT}] for treatment Cu-0 were below method detection limits (4.6 μg L^{-1}). Also, all [Pb_{DGT}] for treatments of Pb-0 were below method detection limits (13.0 μg L^{-1}).

[Cu_{DGT}] was found to only be significantly affected by Cu treatment ($F_{(2,32)}$ = 33.4E+2, p < 2E-16). [Pb_{DGT}] was significantly affected by Cu (p < 1 E-12), Pb (p < 2E-16), and the interaction of Cu and Pb treatments ($F_{(4,32)}$ = 25.0, p < 1 E-9).

Aquatic Animals

Fathead minnow mortalities throughout the exposure from all tanks and treatments were 4.44%, with only 0.44% in control (Cu-0, Pb-0) treatments. Of the total 135 LMP in the experiment, 4 mortalities were observed, 2.96% of the total LMP.

Figures 3-3 and 3-4 illustrate the body tissue concentrations for FHM and LMP accumulated Cu and Pb. Fathead minnow Cu was significantly affected by Cu treatment ($F_{(2,32)}$ = 43.4, p < 1 E-9, n = 45) at α 0.01, and not affected by Pb treatment ($F_{(2,32)}$ = 0.556, p = 0.58) or the interaction of Cu and Pb treatments ($F_{(4, 32)}$ = 0.85, p = 0.51). In contrast, FHM-Pb was significantly influenced by both Pb treatment ($F_{(2,32)}$ = 4.53, p < 0.05) and Cu treatment ($F_{(2.32)}$ = 20.1, p < 0.00001). The Cu and Pb interaction was not significant at α 0.01, p = 0.057 ($F_{(4,32)}$ = 2.57).

Similar to FHM, LMP-Cu was significantly affected by Cu treatment ($F_{(2,32)}$ = 84.7, p < 1 E-12) and not influenced by Pb treatment ($F_{(2,32)}$ = 2.72, p = 0.08). Copper treatment ($F_{(2,32)}$ = 9.3, p < 0.001), Pb treatment ($F_{(2,32)}$ = 38.2, p < 1E-8), and the interaction of Cu-Pb ($F_{(4,32)}$ = 11.1, p < 0.00001) all significantly influenced LMP-Pb.

Comparison of Techniques

Percent [Cu_{diss}] as [Cu_{DGT}] averaged 38.2% ± 0.02 for Cu-15 at all Pb treatments, and 47.6% ± 0.01 for Cu-25 at all Pb treatments. Percent [Pb_{diss}] as [Pb_{DGT}] varied according to Cu treatment rather than Pb treatment. Cu-0 resulted in 44.7% ± 0.3 of [Pb_{diss}] as [Pb_{DGT}] for Pb-30 and Pb-50. Similarly, Cu-15 resulted in 67.4% ±0.004, and Cu-25 in 81.4% ± 0.003 of [Pb_{diss}] as [Pb_{DGT}] for Pb-30 and Pb-50. All [Cu_{DGT}] and [Pb_{DGT}] for zero μg L^{-1} target treatments were below method detection limits. Metal species were separated into inorganic, labile DOM complexes, and non-labile DOM complexes (Table 3-5). A similar trend of Cu treatment influencing labile Pb-DOM and non-labile Pb-DOM fractions rather than Pb treatment is apparent.

A significant linear relationship was found between FHM metal accumulation and both [Cu_{DGT}] (r^2=0.770, p<0.0001) and [Pb_{DGT}] (r^2=0.552, p<0.0001; Figure 3-5). Also, FHM-Cu and –Pb were linearly related to [Cu_{inorg}] (r^2=0.692, p<0.0001) and [Pb_{inorg}] (r^2=0.625, p<0.0001, Figure 3-6). In contrast, LMP metal accumulation was only linearly related to [Cu_{DGT}] (r^2 = 0.463, p<0.0001) and [Cu_{inorg}] (r^2=0.327, p<0.0001), but not [Pb_{DGT}] (r^2=0.0598, p=0.105) , or [Pb_{inorg}] (r^2=0.075, p=0.0726). Based on the correlation coefficients, abiotic metal measuring techniques result in better linear predictions of FHM-Cu ([Cu_{DGT}] 0.770, [Cu_{inorg}] 0.692) than FHM-Pb ([Pb_{DGT}] 0.552, [Pb_{inorg}] 0.625).

Discussion

Metal Mixture Relationships

The influence of metals in mixtures on the speciation and resulting bioaccumulation of other metals is largely under-explored, and primarily explained with inclusive terms such as additive or synergistic [4]. It is recognized that when evaluating the response of aquatic organisms to metal mixtures that the interactions between the metals will have an effect on the magnitude of exposure the organisms receive [3, 12]. The full factorial ANOVAs indicated that for the set of exposures in this experiment Cu was only significantly affected by Cu treatment, while Pb was influenced by Pb treatment along with either the Cu treatment or the Cu-Pb interaction for all response variables.

As visually evidenced in Figure 3-2, there was no change in the DGT measured fraction of Cu with any Pb concentration, indicating that the free and labile Cu concentration was primarily dependent on Cu concentration. Whereas, the fraction of lead measured by DGT was increased as both Pb and Cu concentrations increased. This could be due to Cu facilitating an increase in free Pb ions in the solution by binding to ligands preferentially over Pb. In studies of complexation of metals by organic (NTA and fulvic acid) and inorganic ligands, the stability constants were higher for Cu^{2+} than Pb^{2+} for all ligands [22, 23]. In another study where the percent of the metal complexed by a variety of inorganic ligands (OH^-, HCO_3^-, CO_3^{2-}, $(CO_3^{2-})_2$, SO_4^{2-}, and Cl^-) was quantified, 97 percent of the Cu^{2+} was complexed while only 72 percent of the Pb^{2+} was complexed [23]. Similarly, the effect of the complex stability could also be a reason for both FHM and LMP accumulated Cu not being affected by the Pb treatment, and the organism accumulated Pb was affected by both Pb and Cu treatments.

A similar trend was observed in the FHM total body metal concentrations as for DGT. The FHM figure (Figure 3-3) does not as clearly illustrate the interaction between Cu and Pb as did DGT, likely due to variation for individual fish. The interaction of the Cu and Pb treatments had a p-value of 0.057 for the response variable of FHM-Pb, which was not statistically significant using the a priori α of 0.01. The FHM results were in contrast to a previous study looking at Cu and Pb accumulation in zebrafish, where increasing Pb concentrations in the bulk water increased Cu uptake, whereas Cu effect on Pb uptake was more variable [3]. A sequential exposure of Cu, then Pb to tetra resulted in Pb uptake being facilitated by the prior exposure to Cu [4]. The researchers hypothesized this synergistic response could be due to a physiological change in the metal absorptive process. As the exposure of Cu and Pb to the FHM in this experiment was simultaneous, a combination of complexation competition and physiological change is likely responsible for the synergistic bio-accumulative response.

Bioaccumulation of Cu and Pb by the freshwater mussel LMP resulted in an order of magnitude lower concentrations than FHM. Mussels have been found to actively regulate the uptake of some metals, resulting in their body concentrations not being directly proportional to the bulk water concentration [24]. Even with this extra complexity, the same trend was found for the LMP soft tissue metal concentrations; LMP-Cu was only affected by Cu treatment and LMP-Pb was affected by Cu, Pb and the Cu-Pb interaction. The differences between individual treatments were less distinct (Figure 3-4), potentially due to that active regulation. In the case of Cu and Pb in this study, the interaction between the essential and non-essential metals did not result in a decrease in the non-essential metal uptake, as was found previously in freshwater mussels

[11]. Rather, the accumulation pattern was too variable to make a concise description of the impact of the essential metal (Cu) on the non-essential metal (Pb). Overall for the Cu there appears to be a concentration limit around 35 μg g^{-1} that the mussel soft body concentrations do not exceed, regardless of the Cu or Pb concentrations (Figure 3-4). A similar result was found in a previous study with LMP (Chapter 2) and *Mytilus galloprovincialis* [25].

Effect of Metal Speciation

The results of DGT, FHM, and LMP uptake of Cu and Pb indicate that more Pb was accumulated than Cu for most treatments with spiked concentrations (non-zero Cu or Pb target concentrations; Figures 3-2 and 3-3), in direct contrast to the previous findings that mixtures of essential and nonessential metals can decrease nonessential metal uptake [11]. This trend of less Pb accumulated than Cu is especially apparent in the DGT values. If the bioavailability of Cu and Pb were similar in the exposures and uptake only related to metal concentrations, it would be expected that a Cu:Pb ratio of 0.5 would result in, $[Cu_{DGT}]$:$[Pb_{DGT}]$ close to 0.5, rather than the observed values of 0.42 for 15-30 and 0.36 for 25-50. Similarly, the FHM-Cu:FHM-Pb ratio for 15-30 and 25-50 were 0.33 and 0.42 respectively. In an exposure with Cu and Pb concentrations nearly equal, 25-30, the Cu:Pb ratio was 0.83 and the $[Cu_{DGT}]$:$[Pb_{DGT}]$ was 0.66, further indicating that the bioavailable fraction of Cu is less than that of Pb across the range of experimental concentration ratios.

This difference in bioavailability and accumulation can be linked to the metal speciation observed in this experiment. Both Cu and Pb had similar percentages of $[Cu_{tot}]$ and $[Pb_{tot}]$ as inorganic metal, but the percentage of the inorganic metal as the free

metal ion was significantly higher for Pb than Cu. Also, the percent of dissolved metal as labile DOM complexes was considerably higher for Pb than Cu across treatments (Table 3-5). Often bioavailability is used to refer to the free metal ion and labile inorganic complexes, yet in recent studies it was concluded that weakly bound metal and DOM complexes may also be bioavailable [26-29], thus the higher percentage of labile Pb-DOM complexes may have added to the increased uptake of Pb over Cu.

Comparison of Techniques

We used multiple metal speciation techniques to assess DGT's potential use for predicting bioaccumulation of metal mixtures to aquatic organisms. These techniques included monitoring total metal and operationally defined dissolved (<0.45μm) metal from grab samples, bioaccumulation bioassays of FHM and LMP, speciation modeling with the BLM, and time-integrated measurement of the bioavailable fraction using DGT. The coupled nature of the exposures, where FHM, LMP, and DGT were all exposed to the same bulk solution allows for thorough analysis of the metal speciation, while avoiding potential nuances of speciation difference if the organisms and DGT had been in separate exposure vessels. This is also the most relevant use for DGT to regulators; in the environment DGT would be exposed in situ, in natural waters occupied by aquatic organisms.

When compared to the total metal or dissolved metal fractions, DGT metal concentrations were always lower. This is expected as the DGT fraction should only be a portion of the total or dissolved metal due to the exclusion of large humic molecules or smaller strongly complexed metals from DGT. Comparisons of [Cu_{tot}] and [Cu_{diss}] with [Cu_{DGT}] had highly significant correlations, but were considerably less than a 1 to 1 ratio

(Figure 3-7). The [Cu_{DGT}] were more highly correlated with [Cu_{diss}] than [Cu_{tot}], as would be predicted. The [Pb_{DGT}] had a closer to 1 to 1 relationship to [Pb_{tot}] and [Pb_{diss}] than did Cu, suggesting once again that more of the total and dissolved lead was in bioavailable forms.

Fathead minnow and LMP accumulated metals were relatively well predicted by the total and dissolved fractions. As with DGT, FHM Cu and Pb were better predicted by the dissolved fraction than total. Yellow lampmussel accumulated Cu was significantly predicted by both dissolved and total Cu, but Pb was not.

The validity of DGT for predicting metal bioaccumulation by FHM and LMP was suggestive in the study results (Figure 3-5). DGT proved to be very successful at predicting FHM bioaccumulated Cu in a mixed metal exposure over the concentrations used in this study. While the fit between LMP-Cu and [Cu_{DGT}] was less strong, DGT still significantly correlated with the LMP accumulated Cu. The predictive strength for Pb was less than for Cu, but nevertheless correlated for FHM. It appears that DGT would not be a good predictor of LMP bioaccumulated Pb under these exposure conditions. These results are in contrast to previous studies using DGT and aquatic organisms, including *Daphnia magna*, mosses, and mussels, where DGT did not correlate with organism Cu, but did with Pb [17-20]. The success of DGT predicting FHM-Cu could be related to species or fish specific uptake physiology that was not comparable for the invertebrates and plants in those studies. When exposed to a single metal, Cu, rainbow trout were found to have analogous correlations with DGT as did FHM in this mixed metal study [30]. Moreover, the mussels in the previous studies were marine species,

exposed to metals in seawater, which has considerably different metal speciation than freshwaters [31].

Similar to DGT, the inorganic concentrations, as modeled by BLM speciation component, successfully predicted FHM-Cu and FHM-Pb, with considerably lower predictive strength for LMP-Cu and LMP-Pb. When [Cu_{DGT}] and [Cu_{inorg}] are compared for their fit to the organism bioaccumulated Cu, it is apparent that [Cu_{DGT}] better fits the data and has lower error associated with each treatment. The same conclusion cannot be made for [Pb_{DGT}] and [Pb_{inorg}]. For the results of this experiment, it appears that DGT, which also incorporate the labile metal-DOM complexes, better predict the aquatic organism Cu accumulation than just the calculated inorganic Cu fraction. This could be due to the time-integrated nature of DGT, whereas the inorganic fraction was calculated from a large set of water samples. Another possibility is that the organisms were accumulating a similar portion of labile Cu-DOM complexes as DGT, as suggested by Ferriera et al in their 2008 study [26]. More research is needed to discern the reason behind these observed differences.

Suggested Use of DGT for Metal Mixtures

Diffusive Gradients in Thin-films appears to discriminate the effects of Cu and Pb interactions in a similar fashion as the metals were bioaccumulated by FHM and LMP. While the mechanisms behind this discrimination may be different between the DGT and organisms, it does not appear to lessen the predictive ability of the DGT for bioaccumulation. Overall, FHM are likely a better model organism for DGT metal bioavailability and bioaccumulation prediction, based on the significant correlations, but due to their high mobility in natural environments other, more stationary, organisms may

be more useful in discerning micro-habitat variation. The LMP are relatively stationary organisms that could prove useful in future *in situ* field experiments of Cu, despite the lower correlations than FHM. More studies incorporating other metals are necessary for DGT to be considered a tool for regulators to use in mixed metal situations.

Conclusion

Diffusive gradients in thin-films were assessed for their predictive capability of FHM and LMP sublethal bioaccumulation in environmentally relevant Cu and Pb mixed metal exposures. For DGT, FHM, and LMP, Cu accumulated was found to only be dependent on Cu treatment concentration, while Pb accumulated was affected by Cu treatment, Pb treatment, and the interactions of Cu and Pb. With increasing Cu treatment concentration, more Pb was accumulated by FHM and LMP and measured by DGT. This is suggested to be due to metal speciation differences where Cu binds preferentially to ligands, decreasing its bioavailability an, concomitantly, displacing Pb from complexing sites.

Based on regressions of DGT with FHM and LMP for Cu and Pb, DGT is a good predictor of FHM bioaccumulated Cu and Pb, while only Cu_{LMP} was significantly predicted by DGT. The free metal ion, inorganic metal fraction, and labile metal-dissolved organic matter complexes were found to be measured by DGT. The DGT metal fraction was also found to better predict organism bioaccumulation for both metals than$[Cu_{inorg}]$, modeled by the BLM speciation component, suggesting that more than the inorganic metal fraction was accumulated by the organisms. With further research, DGT could prove to be a simple, cost effective technique for monitoring water quality and predicting aquatic organism bioaccumulation in metals impacted areas.

Figures

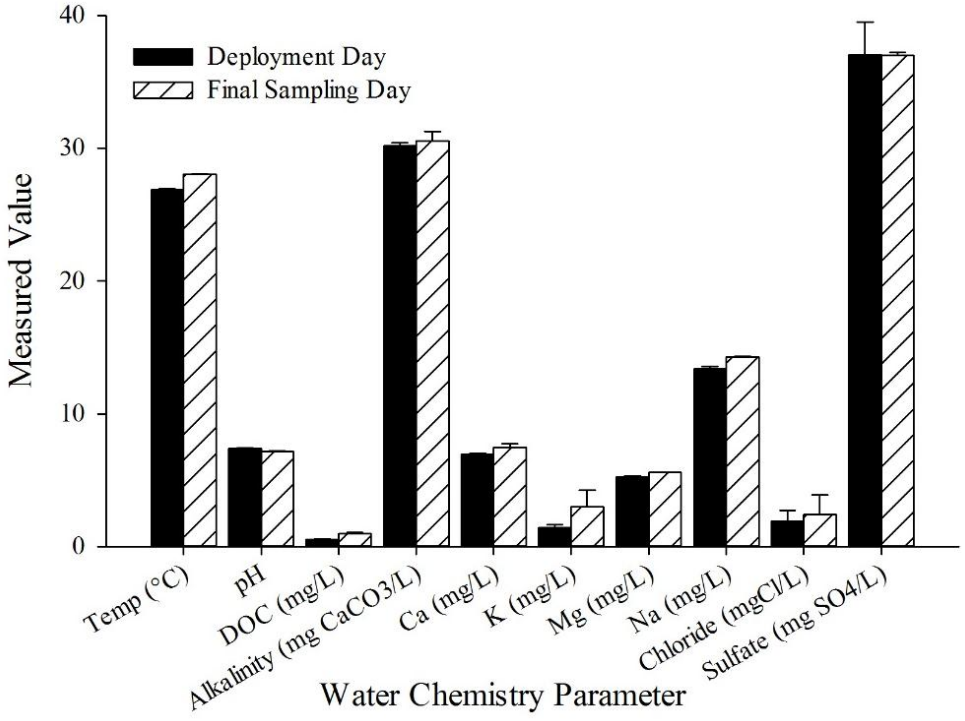

Figure 3-1: Average water chemistry for all tanks and treatments comparing initial deployment day to final exposure day water chemistry. Error bars indicate standard error of the mean.

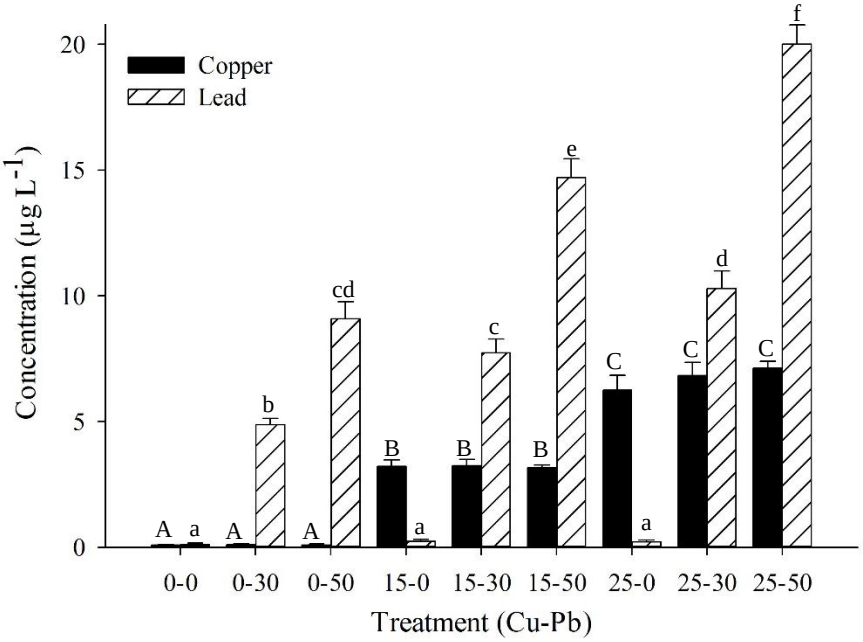

Figure 3-2: DGT measured and calculated copper [Cu_{DGT}] and lead [Pb_{DGT}] averaged by replicates for each treatment. Treatment notation indicates target copper and lead concentrations (µg L^{-1}). Upper case letters represent significant differences between copper concentrations in DGT according to TukeyHSD. Lowercase letters represent significant differences between lead concentrations in DGT.

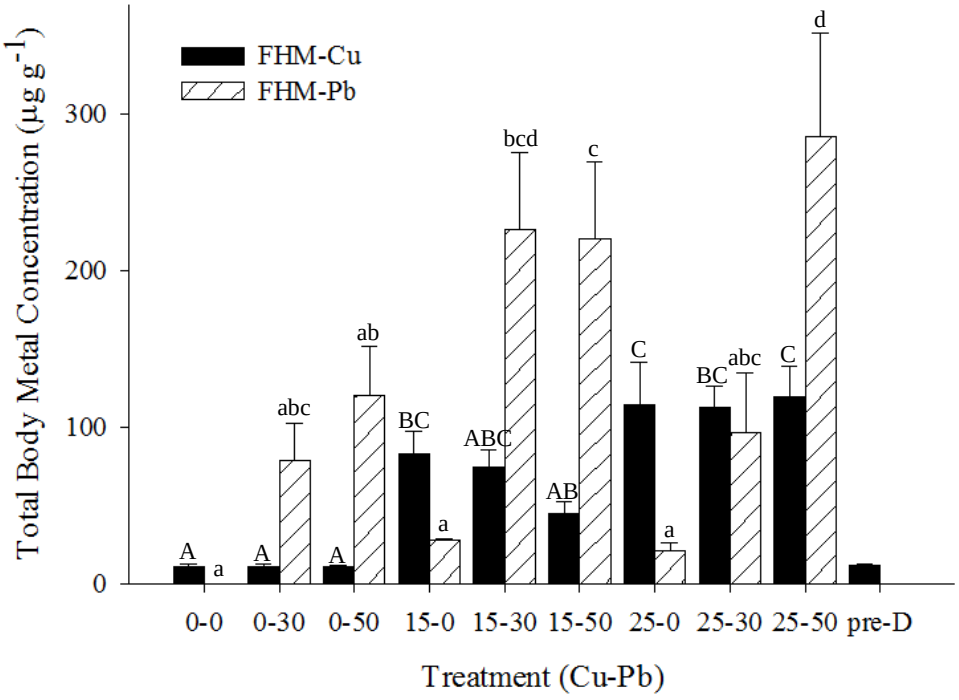

Figure 3-3: Fathead minnow (FHM) total body copper and lead concentrations averaged by treatment (n=5). Bars are standard error of the mean. X-axis treatment notation indicates target copper and lead concentrations (µg L^{-1}). Uppercase letters signify significant differences between copper concentrations in treatments according to Tukey HSD post hoc test. Lowercase letters signify significant differences between lead concentrations in treatments.

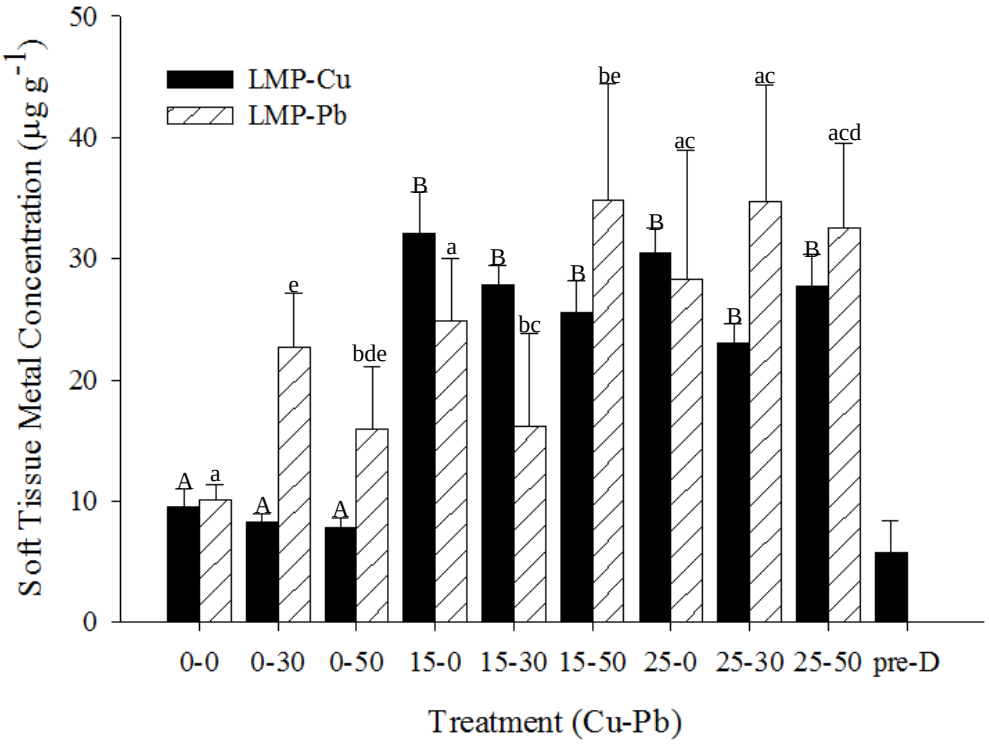

Figure 3-4: Lampsilis cariosa (LMP) soft body copper and lead concentrations averaged by treatment (n=5). X-axis treatment notation indicates target copper and lead concentrations (µg L^{-1}). Bars are standard error of the mean. Uppercase letters denote significant differences between copper concentrations and lowercase letters for lead concentrations in treatments according to Tukey HSD post hoc test.

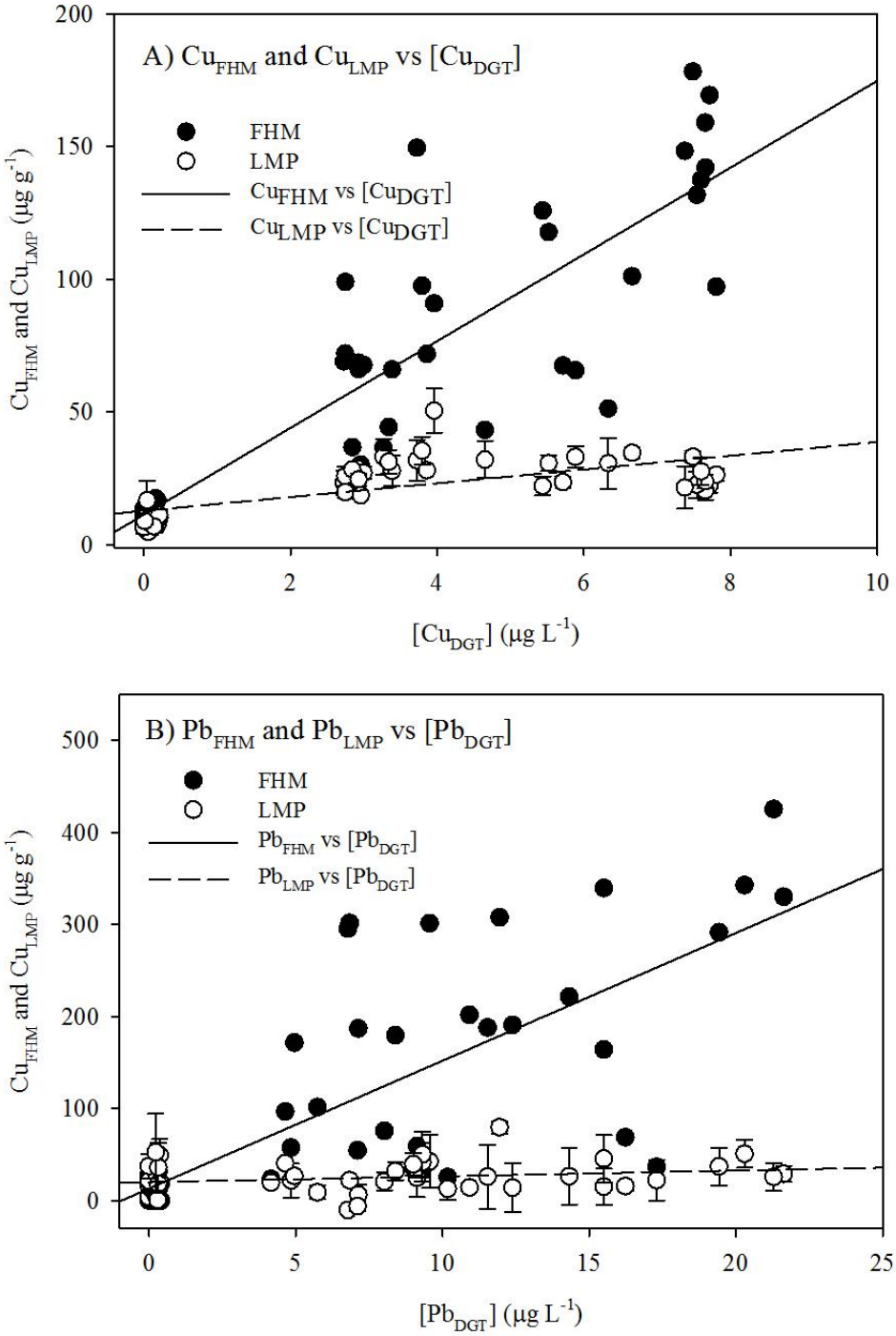

Figure 3-5: A) DGT copper concentrations ($[Cu_{DGT}]$) regressed to fathead minnow (FHM: r^2=0.7701, y=11.39+16.35x, $p < 0.0001$) and *Lampsilis cariosa* (LMP: r^2 =0.463, y=12.84+2.596x, $p < 0.0001$) total body copper concentrations. B) DGT lead concentrations ($[Pb_{DGT}]$) regressed to FHM (r^2 =0.552, y=13.21+13.89x, $p < 0.0001$) and LMP (r^2=0.0598, y=20.19+0.63x, p=0.1053) total body lead concentrations. Error bars are standard error of the mean.

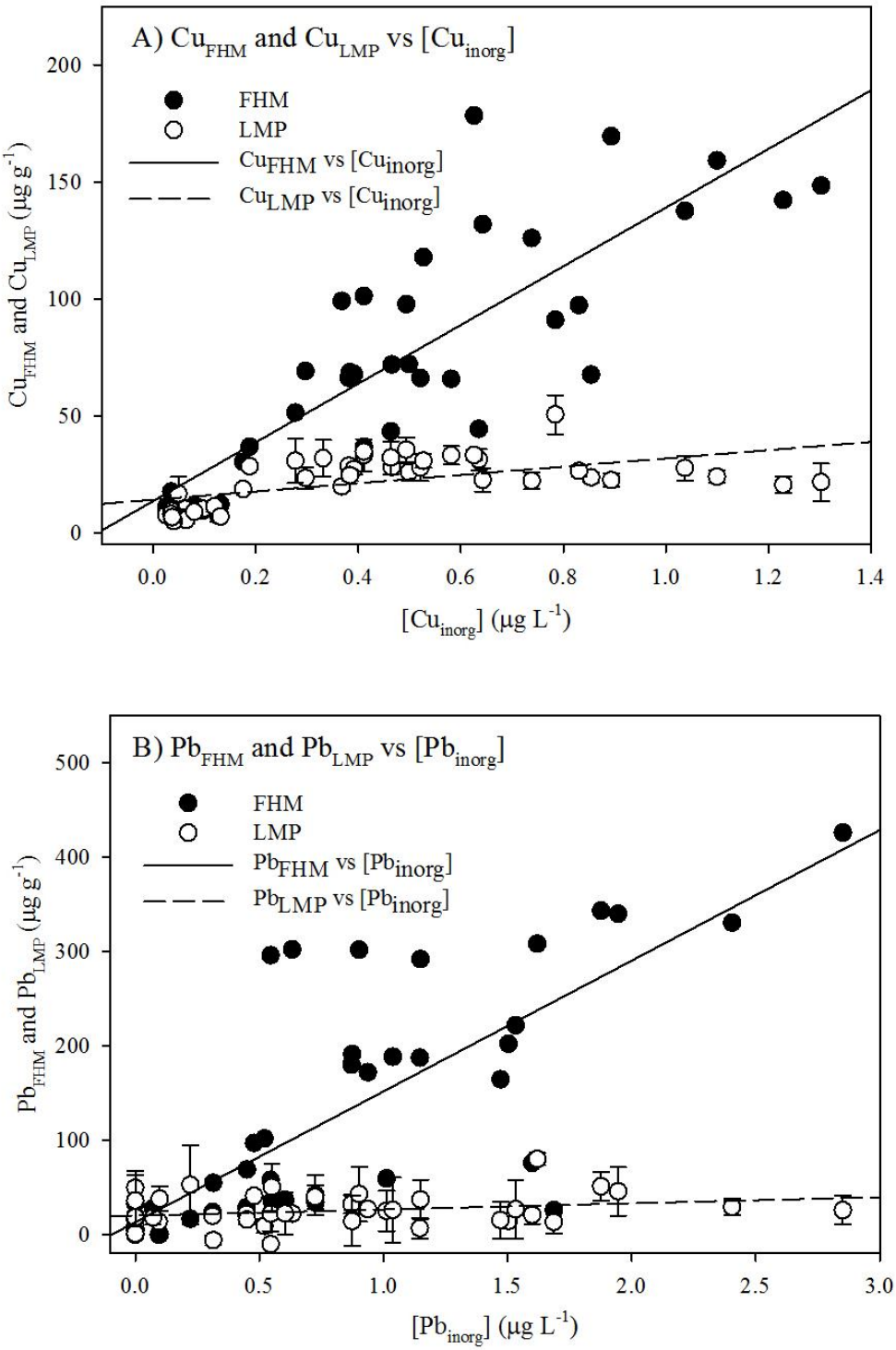

Figure 3-6: A) Biotic Ligand Model speciation component predicted inorganic copper concentrations ($[Cu_{inorg}]$) regressed to fathead minnow (FHM: $r^2 = 0.692$, $y=13.57+125.4x$, $p < 0.0001$) and *Lampsilis cariosa* (LMP: $r^2 =0.327$, $y=14.14+17.65x$, $p < 0.0001$) total body copper concentrations. B) Biotic Ligand Model predicted inorganic lead concentrations ($[Pb_{inorg}]$) regressed to FHM ($r^2 =0.625$, $y=13.19+138.4x$, $p < 0.0001$) and LMP ($r^2 =0.073$, $y=20.01+6.51x$, $p = 0.0726$) total body lead concentrations. Error bars are standard error of the mean.

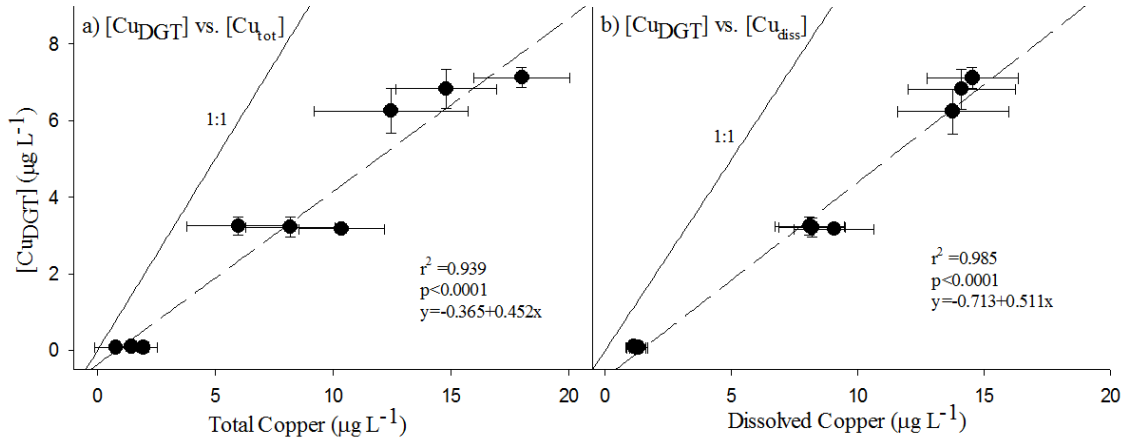

Figure 3-7: Copper measured by DGT ([Cu_{DGT}]) in all treatments compared with the measured total (a) and dissolved (b) copper concentrations. The dashed lines are regressions of the total or dissolved copper to [Cu_{DGT}]. Regression statistics are reported within the body of the representative graph. The solid line is a 1 to 1 representation of total or dissolved copper to [Cu_{DGT}].

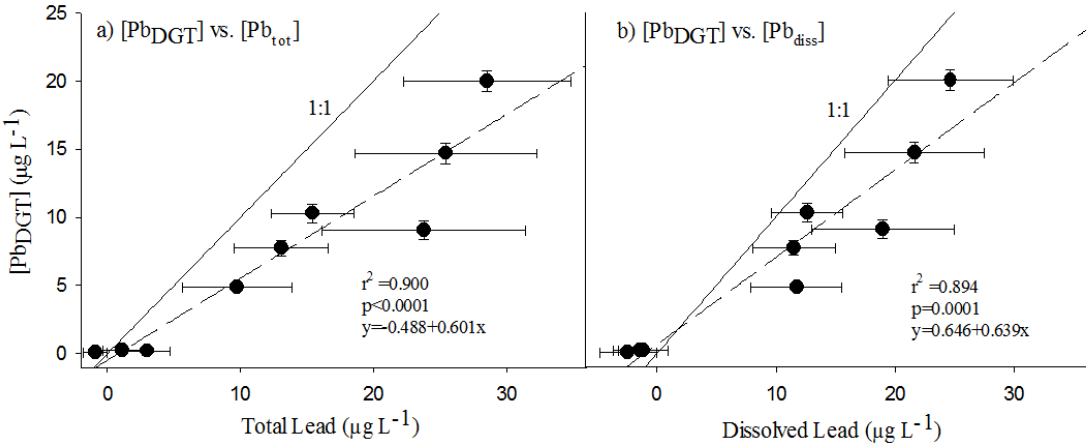

Figure 3-8: Lead measured by DGT ([Pb_{DGT}]) in all treatments compared with the measured total (a) and dissolved (b) lead concentrations. The dashed lines are regressions of the total or dissolved lead to [Pb_{DGT}]. Regression statistics are reported within the body of the representative graph. The solid line is a 1 to 1 representation of total or dissolved lead to [Pb_{DGT}].

Tables

Table 3-1: Water chemistry averaged by all tanks over entire exposure duration. SEM is standard error of the mean.

Parameter	Mean	SEM	n
Temperature (°C)	28.0	0.06	180
pH	7.28	0.01	180
DOC (mg C/L)	0.78	0.06	90
Calcium (mg/L)	7.46	0.12	135
Magnesium (mg/L)	5.46	0.05	135
Sodium (mg/L)	13.9	0.10	135
Potassium (mg/L)	2.07	0.43	135
Sulfate (mg SO4/L)	37.0	0.12	28
Chloride (mg Cl/L)	2.12	0.74	28
Alkalinity (mg CaCO3/L)	30.9	0.30	135
ORP (mV)	216	2.29	180
DO (mg/L)	5.50	0.12	180

Table 3-2: Dissolved metal percent difference from target treatment concentrations for day 6, final sampling day. Treatment is denoted as target concentrations (µg L^{-1}) of copper and lead (Cu-Pb). <MDL indicates the measured copper or lead concentration was below the method detection limit for ICP-OES.

Treatment	$[Cu_{diss}]$	$[Pb_{diss}]$
0-0	<MDL	<MDL
0-30	<MDL	1.37%
0-50	<MDL	2.18%
15-0	32.56%	<MDL
15-30	31.59%	3.89%
15-50	34.28%	8.37%
25-0	31.64%	<MDL
25-30	34.51%	12.77%
25-50	39.17%	18.42%

Table 3-3: Biotic Ligand Model predicted free copper [Cu_{free}] and lead [Pb_{free}] concentrations, and total inorganic copper [Cu_{inorg}] and lead [Pb_{inorg}] concentration averaged by treatment (Cu-Pb). Treatment is denoted as target concentrations (μg L^{-1}) of copper and lead (Cu-Pb). SEM is standard error of the treatment mean.

Treatment	μg L^{-1}	[Cu_{free}]	[Pb_{free}]	[Cu_{inorg}]	[Pb_{inorg}]
0-0	Average	0.0052	0.0023	0.0674	0.0190
	SEM	0.0013	0.0023	0.0158	0.0190
0-30	Average	0.0040	0.0766	0.0520	0.5589
	SEM	0.0011	0.0106	0.0134	0.1040
0-50	Average	0.0064	0.2333	0.0818	1.3900
	SEM	0.0016	0.0370	0.0199	0.1465
15-0	Average	0.0310	0.0159	0.4587	0.1042
	SEM	0.0065	0.0143	0.0910	0.0873
15-30	Average	0.0244	0.0648	0.3814	0.6533
	SEM	0.0033	0.0097	0.0544	0.1093
15-50	Average	0.0280	0.1729	0.4255	1.4043
	SEM	0.0051	0.0274	0.0777	0.2550
25-0	Average	0.0388	0.0056	0.6183	0.0642
	SEM	0.0042	0.0039	0.0746	0.0439
25-30	Average	0.0540	0.0773	0.8589	0.7828
	SEM	0.0059	0.0073	0.1029	0.0841
25-50	Average	0.0557	0.2041	0.8255	1.7776
	SEM	0.0146	0.0506	0.2040	0.4112

Table 3-4: DGT measured and calculated copper ([Cu_{DGT}]) and lead ([Pb_{DGT}]) averaged by replicates for each treatment. Treatment is denoted as target concentrations (μg L^{-1}) of copper and lead (Cu-Pb). SEM is standard error of the treatment mean.

Treatment	[Cu_{DGT}] (μg L^{-1})	±SEM	[Pb_{DGT}] (μg L^{-1})	±SEM
0-0	0.08	0.04	0.09	0.08
0-30	0.10	0.03	4.87	0.26
0-50	0.08	0.04	9.08	0.69
15-0	3.22	0.26	0.22	0.09
15-30	3.25	0.24	7.74	0.54
15-50	3.18	0.10	14.7	0.75
25-0	6.25	0.59	0.20	0.09
25-30	6.83	0.52	10.3	0.69
25-50	7.12	0.27	20.0	0.78

Table 3-5: Percent of dissolved metal ([M_{diss}]; [Cu_{diss}], [Pb_{diss}]) represented by metal species. Inorganic metals ([M_{inorg}]) were calculated by Biotic Ligand Model. Labile metal-dissolved organic matter (DOM) was calculated from [M_{DGT}] minus [M_{inorg}]. Non-labile M-DOM is the remaining measured [M_{diss}], not measured by [M_{DGT}]. Treatment is denoted as target concentrations (µg L^{-1}) of copper and lead (Cu-Pb). <MDL indicates that [M_{diss}] or [M_{DGT}] were below the ICP-OES method detection limit.

Treatment	**[Cu_{inorg}]**	**Labile [Cu_{DOM}]**	**Non-Labile [Cu_{DOM}]**	**[Pb_{inorg}]**	**Labile [Pb_{DOM}]**	**Non-Labile [Pb_{DOM}]**
0-0	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
0-30	<MDL	<MDL	<MDL	4.77%	36.75%	58.48%
0-50	<MDL	<MDL	<MDL	7.34%	40.61%	52.05%
15-0	5.60%	33.67%	60.73%	<MDL	<MDL	<MDL
15-30	4.71%	35.42%	59.87%	5.67%	61.53%	32.80%
15-50	4.70%	30.35%	64.96%	6.50%	61.52%	31.98%
25-0	4.50%	40.95%	54.55%	<MDL	<MDL	<MDL
25-30	6.09%	42.34%	51.57%	6.21%	75.50%	18.29%
25-50	5.68%	43.33%	50.99%	7.21%	73.94%	18.85%

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CHAPTER 4

IMPACT OF NATURAL ORGANIC MATTER AND INCREASED WATER HARDNESS ON DGT PREDICTION OF COPPER BIOACCUMULATION BY YELLOW LAMPMUSSEL (*LAMPSILIS CARIOSA*) AND FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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Abstract

We conducted a copper exposure experiment with Diffusive Gradients in Thin-Films (DGT), fathead minnow (*Pimephales promelas*), and yellow lampmussel (*Lampsilis cariosa*) with three different water chemistries. Nine copper-water chemistry combinations were used to assess the effect of water chemistry on DGT's predictive capability for aquatic organism copper bioaccumulation. Water chemistries utilized were addition of natural organic matter (NOM: Suwannee River, DOC 2 mg C/L), hard water (alkalinity 109.4 mg $CaCO_3$/L), and control (soft water, alkalinity 29.8 mg $CaCO_3$/L, DOC < 1 mg C/L) with a copper range of 0 to 60 μg/L. NOM treatments resulted in decreased DGT measured Cu. Both hard water and NOM treatments had reduced free and inorganic Cu compared to control waters. Linear regressions of DGT measured Cu and fathead minnow bioaccumulated Cu demonstrated significant linear relationships (r^2 = 0.661-0.929) that were higher than regressions of fathead minnow accumulated Cu with predicted inorganic Cu (r^2 = 0.573-0.815). Despite having significant linear regressions for two of the water chemistries tested (control and hard water), yellow lampmussel bioaccumulated Cu was deemed to not be well predicted by DGT (r^2 0.224-0.300).

Introduction

Copper (Cu) is an essential element for aquatic organisms that occurs naturally in environments, but can be toxic at higher concentrations often created by anthropogenic actions. Metal toxicity is a function of the metal bioavailability, which is controlled by metal speciation [1-3]. Metal to ligand bonding and competition with cations for bonding sites (e.g., Ca^{2+}, Mg^{2+}, Na^{+}, H^{+}, K^{+}) impact Cu speciation and decrease bioavailability [4]. Natural organic matter (NOM) is one type of complexing ligand that has been shown

to have ameliorative effects on Cu bioaccumulation and toxicity to a wide range of aquatic organisms [3, 5-10]. Other inorganic ligands, including carbonate and hydroxide can also bind Cu, altering speciation, but the effect on bioavailability is less clear [11].

Due to the importance of metal speciation on metal bioavailability and toxicity, regulators have begun considering water chemistry in making metal regulations. The US EPA first incorporated water chemistry through addition of a hardness factor in 1980 [12]. Additional tools, including geochemical modeling, biological, and passive sampling techniques, have been developed to increase the accuracy and efficiency of assessing metal bioavailability. Controlled toxicology studies and biomonitors have been used to assess the biological response (toxicity and accumulation) at various water chemistries, at considerable cost, effort, and variable accuracy [13-15]. The Biotic Ligand Model (BLM) was developed to bridge the gaps of water chemistry and aquatic organism toxicology by using metal complexation parameters to predict metal speciation and membrane binding [3]. The BLM was adopted by the US EPA for development of sit-specific Cu water quality criteria in 2003 [16]. At various water chemistries, the BLM has been found to over or under-predict toxicity and bioavailable metal concentrations [5, 17, and 18]. One alternative to determining metal speciation or metal bioavailablility via the BLM is the use of passive sampling devices. One such device is Diffusive Gradients in Thin-films (DGT). Diffusive Gradients in Thin-films are used as metal speciation devices, and can be used to determine metal bioavailability, as they are designed to measure the free and easily dissociated metal (i.e., labile) species *in situ* [19-21].

The premise behind DGT is that a concentration gradient develops between the bulk solution and the device, allowing only the free and readily dissociated metals to

diffuse through the two diffusive membranes, and bind to the Chelex resin [19, 22]. The value of metals bound to the Chelex resin can be quantified and related back to the bioavailable concentration in the bulk solution during the period of deployment. Water chemistry should have a similar effect on the DGT measured metal as it would for the bioavailable metal in the system; as metals are complexed by NOM or other strongly binding large organic molecules they become less likely to diffuse through the DGT membranes.

While DGT has been primarily used as a metal speciation tool, recent studies have begun comparing DGT with metals bioaccumulated by a variety of aquatic organisms [11, 20, 23-28]. When DGT were compared to *Daphnia magna* exposed to Cu with EDTA, NTA, and humic acids, the lability of the organic matter influenced the DGT Cu measurement [25]. In another study comparing DGT to *Daphnia magna*, where four types of wastewater with varying water chemistries (DOC, cations, and anions) were used, it was concluded that DGT may be useful as an operational tool, but that more than the bioavailable metal was measured [27]. Higher correlation ($p < 0.00001$) was found when DGT was compared to bioaccumulation of Cu by rainbow trout (*Oncorhynchus mykiss*), when exposed to different NOM solutions [11]. This study also showed increasing NOM concentrations decreased Cu measured by DGT, and the Cu accumulation in rainbow trout gill. These and other studies demonstrate both the usefulness and limitations of DGT for use as a bioavailability and biomimetic device; however, more research on controlled exposure comparisons are clearly needed to better understand the potential applications of this technology.

The objectives of this study were three fold. First, we determined how the addition of NOM or use of hard water chemistry altered the fraction of Cu measured by DGT. The hard water treatment water chemistry included the natural increase in pH, alkalinity, and cations as these parameters co-vary; the focus was on environmentally relevant water chemistries, rather than elucidating the effect of just one parameter. Second, we compared the alteration of DGT measured Cu to the response (i.e., accumulation) of Cu by fathead minnow (*Pimephales promelas*; FHM) and/or yellow lampmussel (*Lampsilis cariosa*; LMP) to determine if DGT mimicked the organism response. Finally, when compared with the free and inorganic Cu predicted by BLM speciation component, we evaluated if DGT better predicts the organism response under the varied water chemistries.

Materials & Methods

Experimental Design

The exposure trial was conducted in a climate controlled animal care facility (16 hour light: 8 hour dark, 22.5°C ± 0.03) at University of Georgia's Savannah River Ecology Lab. Nine treatments were established with 5 replicates each for a total of 45 tanks. The treatments include one factor with three levels of Cu concentrations, and a second experimental factor of water type with three types of water: control, increased natural organic matter (NOM), or hard water (Table 4-1).

Plastic (HDPE) food-grade containers (20 L) with covers were used as exposure tanks and arranged in five blocks on laboratory benches, with each randomly assigned treatment represented once per block. Nineteen liters of synthetic soft water containing the appropriate Cu addition was added to the respective exposure tanks. Synthetic water

was prepared in 20 liter carboys following USEPA ASTM requirement guidelines [29] for soft water (48 mg $NaHCO_3$, 30.0 mg $CaSO_4$, 30.0 mg $MgSO_4$, 2.0 mg KCl per liter) for control and NOM treatments, and hard water (192 mg $NaHCO_3$, 120.0 mg $CaSO_4$, 120.0 mg $MgSO_4$, 8.0 mg KCl) per liter for the hard water treatment. Tanks were spiked with $CuSO_4$ to yield final Cu concentrations of 0, 30 and 60 μg L^{-1} (Cu-0, Cu-30, Cu-60). The Cu concentrations were chosen to be at or below the predicted BLM LC50s for FHM under each treatment condition. Natural organic matter was added to appropriate tanks using reconstituted Suwanee River NOM (International Humic Substances Society) [30]. The Suwanee River NOM was isolated using a RealSoft Co. reverse osmosis system as described by Serkiz and Perdue [31]. A plastic aerator was inserted into each tank to maintain dissolved oxygen levels and facilitate solution mixing.

All tanks were allowed to equilibrate for 24 hours prior to introduction of fish, mussels and DGT. Each tank was allocated 5 sub-adult FHM (14.3 mg, SEM 1.2), 2 adult LMP (1.87 g, SEM 0.045) and 1 DGT. Water temperature (22.5°C ± 0.03) and pH (7.80 ± 0.02) were recorded every 60 minutes for the entirety of the study using a 21x Campbell Scientific data logger. Every 48 hours pH, temperature, and ORP measurements were taken by handheld meters and recorded. Water samples were collected and analyzed for acid soluble metal (TM: [Cu_{tot}]), dissolved (<0.45μm) metal (DM: [Cu_{diss}]), dissolved organic carbon (DOC), and alkalinity concentrations. Mortalities were noted and removed from the tank daily.

Aquatic Animals

Fathead minnows and LMP were obtained from clean laboratory stocks; Warnell School of Forestry and Natural Resources, Aquatic Toxicology Lab (University of

Georgia, Athens, GA) and North Carolina State University (Raleigh, NC) respectively. Upon arrival at SREL, FHM and LMP were acclimated and held for more than 48 hours in aerated synthetic soft water. On day 0 (December 14 or 15, 2014) of study FHM and LMP were taken from the containers and randomly placed in tanks (5 FHM and 2 LMP per tank). Feeding did not occur during the exposure period.

After the exposure (6.0 days ± 0.003) FHM were removed from their tank, euthanized with MS-222, rinsed with 1% nitric acid and ultra-pure water (Barnstead Nanopure Analytical Ultrapure Water System, Series 1367, >18.2 MΩ/cm), and then placed in clean, labeled, pre-weighed whirl bags. Yellow lampmussel were removed from their tank following FHM, prodded with a sharp forceps to evaluate survivorship, prior to following the same steps as with FHM. Fathead minnow and LMP were immediately transferred to an ultra-cold (-80°) freezer. Yellow lampmussel were removed from their shells and wet weights were obtained for both LMP and FHM before being freeze dried (Labconco, Freezone 4.5) to constant weight (Denver Instrument Company, TR-204).

Organisms were digested (CEM MARSxpress) using 1 mL of ultrapure HNO_3. FHM were composited by tank and whole bodies digested. LMP were digested individually, soft tissues only. Tort-3 (Lobster Hepatopancreas Reference Material for Trace Metals, National Research Council of Canada (497 ± 22 mg/kg Cu)), blanks, and replicates were all included twice in each digestion cycle. Digested samples were diluted with ultra-pure water for analyses by ICP-OES (inductively coupled plasma- optical emission spectrometry, Perkin Elmer, Optima 4300 DV). Spikes (addition of 50 µL ICP-

200.7-6, High Purity Standards, Charleston, SC), double dilutions (14.5 mL ultra-pure water and 0.5 ml digest) or replicates were included every 10 samples.

DGT

DGT components were purchased from DGT Research Ltd (Lancaster, UK). Components for each DGT device include one 0.45 μm pore size hydrophilic polyethersulfone filter membrane, one polyacrylamide diffusive gel cross-linked with an agarose derivative (open pore size 2-5 nm), and one resin gel impregnated with Chelex 100. These components were assembled in a plastic deployment device in a laminar flow bench according to DGT Research Ltd. Protocols [32].

On initial deployment day, DGT were suspended to 40% depth of the tank using fishing line, 1 DGT per tank. Deployment time was recorded to the nearest minute. On the final sampling day, DGT were removed from the tank and time to the nearest minute recorded. Diffusive Gradient in Thin-films were then rinsed with MQ water, shaken dry, placed in clean plastic bags with limited air space, placed in cooler with ice for period of continued sampling, and then transferred to a refrigerator until resin layer could be extracted (less than 2 hours). The Chelex resin layers were then extracted and placed in individual acid washed 1.5 mL micro-centrifuge tubes, and eluted with 1 mL of 1 M HNO_3. The Chelex resins were left in the 1M HNO_3 for 26 hours before pulling 0.9 mL aliquots. Aliquots were transferred to 15 mL trace metal free centrifuge tubes, diluted 5:1 and refrigerated until analysis by ICP-OES.

ICP-OES measured Cu was used to calculate time averaged DGT mass of metal and concentration of metals. The following calculations were used according to DGT research LTD specifications [32].

$$M = \frac{Ce\ (V_{HNO3} + V_{gel})}{fe}$$

where *M* is mass of metal in the resin gel, *Ce* is the concentration of metals in the 1 M HNO_3 solution as determined by the ICP-OES, V_{HNO3} is the volume of HNO_3 added to the resin gel (1.5 mL), V_{gel} is the volume of the resin gel (0.16 mL), and *fe* is the elution factor for Cu (0.8).

$$C_{DGT} = \frac{M\Delta g}{DtA}$$

Where C_{DGT} is the concentration of metal measured by the DGT, Δg is the thickness of the diffusive gel and filter membrane (0.93 mm), *D* is the diffusion coefficient of Cu in the gel (6.06E-06 cm^2/sec), *t* is the deployment time, and *A* is the exposure area (3.14 cm^2). The C_{DGT} calculation was used to calculate the concentration of Cu measured by DGT ($[Cu_{DGT}]$).

Water Analysis

Temperature, pH, ORP, dissolved oxygen, alkalinity, DOC, DM, TM, and cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}) measurements or samples were taken from each tank prior to initial exposure, every 48 hours, and prior to pulling organisms and DGT. Samples were transported in a cooler with ice back to the lab for analysis immediately following collection. Samples for anions (chloride and sulfate) were obtained before and after exposure periods. Alkalinity was determined by titration with H_2SO_4 to pH of 4.5 (mg L^{-1} as $CaCO_3$, Hach kit). Samples for DM and DOC samples were hand filtered using 0.45 µm Environmental Express syringe filters. DM and TM samples were acidified with trace metal free HNO_3 to pH 2. Filtered DOC samples and anion samples were refrigerated prior to analyses by TOC analyzer (Shimadzu, Tokyo, Japan) and an ion

chromatograph (Dionex, USA), respectively, at the Stable Isotope and Soil Biology Laboratory, UGA. Cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}) were determined by ICP-OES using the acidified, filtered DM samples.

Metals Analysis

All tubes, bottles, and vessels used for metals analysis were either certified trace metal free, or acid cleaned with 3% HNO_3 for at least 5 days. Samples for metals analysis (FHM, LMP, DGT, DM, and TM) were analyzed by ICP-OES for Cu with wavelength 324.752, with 3 runs per sample, averaged for final concentration. Spikes (addition of 10 (DM) or 20 (TM) µL ICP-200.7-6, High Purity Standards) and replicates were included every 10 samples. Certified reference material, TM 27.3 (National Research Council of Canada), and SLRS-5 (National Research Council of Canada) were included, two per run. Linear calibrations of $R^2 > 0.995$ were achieved for all analyte calibration curves (ICP-200.7-6, High Purity Standards).

Data Analysis

The BLM (Hydroqual, Inc.) was used to determine the free Cu ($[Cu_{free}]$) and inorganic Cu (sum of free Cu^{2+}, $CuOH^{+}$, $Cu(OH)_2$, $CuSO_4$, $CuCO_3$, $Cu(CO_3)_2^{2-}$, CuCl, and $CuHCO_3^{+}$: $[Cu_{inog}]$) for all tanks using the above water quality parameters. The Cl^{-} and SO_4 were averaged and applied for all daily samplings.

Paired, two sample t-tests (R) were used for assessing differences in water chemistry values between initial deployment day and final exposure day. Alpha was set at 0.05. Equality of variances and normality were assessed prior to use of t-test.

Two-way factorial with block design ANOVAs were conducted for the response variables of $[Cu_{DGT}]$, FHM Cu concentration (Cu_{FHM}), and LMP Cu concentration

(Cu_{LMP}) using an α of 0.05. Regression modeling (SigmaPlot 12) was employed for assessing correlations of Cu_{FHM} and Cu_{LMP} vs. [Cu_{DGT}] and [Cu_{inorg}].

Results

Water Chemistry

Bulk solution water chemistry varied between the hard water treatment and the control and NOM addition treatments (Figure 4-1, Table 4-2). Aside from DOC, water chemistry was similar for control and NOM addition treatments (Figure 4-1 a, Table 4-2). DOC was higher at the end of exposure in all treatments, except 30-hard, compared to day 0 (Figure 4-2). Both NOM addition and hard water treatments had significantly higher DOC values than corresponding control treatments (Figure 4-3). NOM addition and hard water treatments had similar DOC concentrations throughout the exposure. This does not indicate that the hard water treatment had similar levels of NOM as the NOM addition treatment; DOC is an operational technique for measuring NOM, but DOC is only a portion of the total NOM [4]. Suwannee River NOM is 50.7% carbon, so the actual NOM would be double the DOC concentrations.

Total Cu concentrations ([Cu_{tot}]) averaged for the entire exposure ranged from 75 to 83 percent of the target concentration for the Cu-30 and Cu-60 treatments (Table 4-3). All Cu-0 treatments were below method detection limit (5.8 μg L^{-1}). The [Cu_{diss}] ranged from 89 to 99 percent of [Cu_{tot}] for Cu-30 and Cu-60 treatments. The [Cu_{tot}] decreased in all tanks between day 0 and day 6. The decrease ranged from 13 to 51 percent, with greater decreases in hard water and control treatments than NOM addition treatments.

The [Cu_{inorg}] ranged 1 to 19 percent of [Cu_{tot}]. Treatment 60-0 (60 μg/L Cu and control water chemistry) [Cu_{inorg}] was 19% of [Cu_{tot}], while all other treatments were less

than 5.8% (Table 4-4). The [Cu_{free}] averaged 3.69 ± 0.63 percent of [Cu_{inorg}]. Natural organic matter addition treatments had lower percentage of [Cu_{inorg}] as [Cu_{free}] than hard water or control treatments, with an average of 1.43 ± 0.0005% for NOM compared to 4.82 ± 0.004% for both hard water and control. The [Cu_{free}] and [Cu_{inorg}] were both significantly affected by the interaction of Cu treatment and water chemistry treatment ([Cu_{free}]: $F_{(4,32)}$=6.93, p<0.0005; [Cu_{inorg}]: $F_{(4,32)}$=6.83, p<0.0005).

DGT

The [Cu_{DGT}] corresponded to the Cu treatment target ($F_{(2,32)}$=407, p < 2E-16). All [Cu_{DGT}] for treatment Cu-0 were below method detection limits (4.77 μg L^{-1}). Water chemistry treatment also significantly affected [Cu_{DGT}] ($F_{(2,32)}$=67.3, p < 1E-11), as did the interaction of Cu and water chemistry treatment ($F_{(4,32)}$=24.6, p < 1E-8). Natural organic matter addition resulted in lower [Cu_{DGT}] than in control or hard water treatments for corresponding Cu treatment (Figure 4-4). The differences between hard water treatment and control treatment for [Cu_{DGT}] were not significant. Using an alpha of 0.05, the block effect was not significant, so all DGT figures present averages of replicates per treatment.

Aquatic Animals

Mortalities throughout the exposure were only FHM, and primarily in the 60-0 treatment. Mortalities of FHM from all tanks and treatments was 8.4%, though 7.6% were from the 60-0 treatment, while all other treatments had a combined mortality rate of 0.9%. Treatment 60-0 had a FHM mortality rate of 68%. Due to the high mortality rate in this treatment, the 60-0 treatment was excluded from [Cu_{DGT}] and Cu_{FHM} regressions.

Figure 4-5 illustrates the whole body concentrations for FHM (a) and soft tissue concentrations LMP (b) separated by treatment combination of Cu and water chemistry. The pre-deployment Cu concentration was similar for both FHM (11.85 μg g^{-1}) and LMP (11.27 ± 1.94 μg g^{-1}). The Cu-0 treatment for all water chemistry treatments had Cu_{FHM} and Cu_{LMP} near to the respective pre-deployment Cu concentration.

Fathead minnow Cu was significantly affected by the interaction of Cu and water chemistry treatment ($F_{(4.32)}$=9.35, $p < 0.0001$). In contrast, Cu_{LMP} was only influenced by the Cu treatment ($F_{(2.32)}$=23.8, $p < 0.000001$) and not by the water chemistry treatment ($F_{(2.32)}$=1.19, p=0.318). The block effect was not significant for either Cu_{FHM} or Cu_{LMP}.

Comparison of Techniques

Percent of [Cu_{diss}] as [Cu_{DGT}] averaged 52.2 ± 8.81 for all Cu-30 and Cu-60 treatments. Control Cu treatments were below method detection limit for [Cu_{diss}] and [Cu_{DGT}] regardless of water chemistry treatment. The percent of [Cu_{diss}] as [Cu_{DGT}] was significantly lower in the NOM addition treatments (25.1 ± 0.33%) compared to the control (71.0 ± 0.10%) and hard water (60.6 ± 2.43%) treatments. When metal species were separated into inorganic Cu, labile Cu-DOM complexes, and non-labile Cu-DOM complexes, 75% of the Cu in the NOM addition treatments was in the non-labile Cu-DOM complexes (Table 4-5). The hard water treatments also have a higher percentage of copper as non-labile (37-42%) in comparison to the control water chemistry treatments (29%). Also notable in Table 4-5 is the percent of Cu in the inorganic Cu species for treatment 60-0. Almost 20% of the Cu in the 60-0 treatment was inorganic Cu, which as a bioavailable form, explains the high FHM mortality observed for that treatment.

Significant linear relationships were found for [Cu_{DGT}] vs. Cu_{FHM} for all water chemistry treatments (Table 4-6, Figure 4-6). The correlation coefficients for [Cu_{DGT}] vs. Cu_{LMP} are considerably lower than those for Cu_{FHM}, but the relationships were found to be significant for both the control and hard water treatments, but not the NOM addition treatment at an alpha of 0.05 (Figure 4-6). Similarly, the [Cu_{inorg}] vs. Cu_{FHM} had significant linear relationships for all water chemistry treatments (Figure 4-7). None of the [Cu_{inorg}] vs. Cu_{LMP} regressions had significant linear relationships (Figure 4-7).

Discussion

NOM addition

Natural organic matter has been shown to have protective effects on aquatic organisms exposed to metals, both in terms of bioaccumulation and toxicity [3, 5-10]. The NOM added to the tanks in this experiment was a well characterized aquatic NOM from the Suwanee River, GA. The results of the metal speciation in this study indicate that the NOM addition decreased Cu bioavailability as measured by DGT and inorganic Cu predicted by BLM speciation component, compared to the control water chemistry treatment. The percent of [Cu_{diss}] as [Cu_{DGT}] was significantly lower in the NOM addition treatments compared to the control, indicating less free and labile Cu in those treatments. This was also demonstrated by the BLM metal speciation where the percent of [Cu_{inorg}] as [Cu_{free}] was reduced for NOM treatment compared to the other two treatments. When separated into free, labile, and non-labile Cu complexes, 75% of the operationally defined dissolved Cu was in non-labile Cu-DOM complexes, compared to 29 percent for the control treatment.

The [Cu_{DGT}] decreased with added NOM, illustrating a dependency on the overall metal speciation (Figure 4-4). This was also the case in Luider et al's study on the effect of NOM treatment from four different water bodies on [Cu_{DGT}], where at the same total Cu concentration all the NOM treatments resulted in less [Cu_{DGT}] than only Cu alone [11]. The Luider et al experiment also measured the free Cu ion by ion selective electrode (ISE) in the four NOM treatments. As with our predicted free Cu, the ISE measured free Cu was reduced by NOM, and that reduction was exhibited in [Cu_{DGT}] as well.

The FHM-Cu was significantly affected by the Cu and water chemistry interaction, but there was not a marked decrease in the total body Cu concentration for the NOM addition treatment as there was for DGT, when compared to the control Cu accumulation. Yet, when FHM-Cu was regressed to [Cu_{DGT}], they were found to be significantly linearly correlated. In a study with rainbow trout Cu gills accumulation, [Cu_{DGT}] was similarly found to significantly correlate ($p < 0.0001$) with the fish Cu accumulation [11]. The [Cu_{inorg}] was also significantly linearly correlated with FHM-Cu for the NOM addition treatment, but the correlation coefficient was decreased. Fathead minnow Cu was consistently greater than the corresponding [Cu_{DGT}] by a factor of 10,000 (or a factor of 10 with FHM-Cu as μg/g and [Cu_{DGT}] as μg/L), resulting in relatively easy predictability. Whereas [Cu_{inorg}] were not found to differ from FHM-Cu by a reliable factor.

Yellow lampmussel soft tissue accumulated Cu was found to not be influenced by the NOM addition treatment. As Figure 4-8 illustrates, LMP had a large increase in bioaccumulated Cu in the mid-range Cu treatment (Cu-30), regardless of water

chemistry, and then LMP bioaccumulation stayed relatively constant for the Cu-60 treatments. This result is in contrast to a 2013 study with juvenile *Lampsilis siliquoidea*, where at Cu concentrations of 12 μg L^{-1} the addition of dissolved organic matter decreased soft tissue Cu concentrations [6]. The rapid accumulation followed by a discernible leveling off of accumulation observed in this study occurred in other studies with mussels and metals, and is believed to be influenced by mussels ability to regulate essential metals to a high degree [Chapter 2, 3; 33]. In addition, in a study with *Mytilus galloprovincialis*, it was determined that the mussel Cu body burden was independent from metal speciation influenced by DOC, and was likely due to physiology instead [34].

Hard water

A water hardness factor was first included in the U.S. EPA water quality monitoring for metals in 1980. Since then numerous studies have pointed out that hardness alone is not the only water chemistry parameter that can influence metal toxicity [3, 5, 9, 14, and 35]. In a bioaccumulation study with a freshwater macrophyte it was suggested that a modification in hardness alone does not have ameliorative effects, but that the parameters that often co-vary with hardness, alkalinity and pH, couple to be protective despite having different modes of action [35]. We did not attempt to elucidate the influence of only true hardness on DGT, but rather the influence of environmentally relevant hard water, along with the co-varied water parameters [36].

As with the NOM addition treatment, the hard water treatment decreased the percentage of bioavailable Cu, albeit to a lesser degree. The [Cu_{inorg}] as [Cu_{free}] was found to be 3.9 and 4.1 percent respectively for Cu-30 and Cu-60, while the control treatment was 4.8 and 5.1 percent. The percentage of [Cu_{diss}] as [Cu_{DGT}] was 60.6% for

the hard water treatment, while the control was 71.0%. Due to the apparent decreased Cu bioavailability, the non-labile Cu-DOM complexes increased to 37-42% of the [Cu_{diss}] in the hard water treatment, compared to 29% in the control.

While the percent of [Cu_{diss}] as [Cu_{DGT}] was reduced for hard water treatment compared to the control, there was no significant difference between control and hard water treatments for the same Cu treatment, according to Tukey's HSD post-hoc analysis (Figure 4-4). It is likely that any additional complexes formed in the hard water treatment had a similar lability as those in the control treatment. One study looking at how cation and pH variability alters [Cu_{DGT}] concluded that in waters with high cationic compositions [Cu_{DGT}] alone is not a good predictor of aquatic moss Cu bioaccumulation [37]. In contrast to our results, there was an increase in [Cu_{DGT}] in water with higher pH, Ca^{2+}, Mg^{2+}, and Na^{+}, but the alkalinity was not reported.

When compared to the control treatment, FHM-Cu was increased by the hard water treatment. Yet, when compared to the high toxicity found in treatment 60-0, the hard water was protective. Increased water hardness and alkalinity have previously been found to be protective to FHM Cu [9] and Pb [38] toxicity. The increased bioaccumulated Cu in the hard water treatment may be due to accumulation of non-toxic forms of Cu, including $CuHCO_3^{+}$, $CuCO_3$, and $Cu(CO_3)_2^{2-}$ [39, 40]. Fathead minnow Cu was significantly correlated with [Cu_{DGT}] and [Cu_{inorg}] for the hard water treatment. There was greater variability among the FHM-Cu values than the [Cu_{DGT}], this was especially pronounced in the Cu-30 treatment (see Figure 4-6); potentially due to individual fish variations, including size and age. Similar to the NOM addition treatment, a ratio of 1:10,000 can be observed for [Cu_{DGT}]: FHM-Cu.

Yellow lampmussel Cu concentration for hard water treatments was not significantly different than control or NOM addition treatments for respective Cu concentration. In a study with freshwater clam (*Anodonta cygnea*) glochidia, water hardness did not protect the larvae from Cu, zinc, or cadmium toxicity [41]. Linear regressions between [Cu_{DGT}] and LMP-Cu for only the hard water treatment tanks indicate a correlation that is higher than for the pooled LMP-Cu data. Nevertheless, [Cu_{DGT}] would not be considered an appropriate technique for assessing LMP Cu bioaccumulation, due to the rapid accumulation, then leveling off observed for all LMP in all water chemistry treatments. The [Cu_{inorg}] did not significantly correlate with LMP-Cu.

Control treatment

Most notable in the control treatment is the metal speciation of the 60-0 treatment (60 μg L^{-1} Cu and control water chemistry) and the resulting FHM mortality. All Cu-60 treatments had similar [Cu_{tot}] and [Cu_{diss}], but 60-0 had 19% of [Cu_{tot}] as [Cu_{inorg}] while all other treatments were less than 5.8%. Apparently the increased percentage of inorganic Cu led to the high levels of toxicity, as all other water chemistry parameters were comparable across treatments. The low levels of FHM bioaccumulated Cu in the 60-0 treatment are consistent previous studies where an inverse relationship was found between whole-body accumulation and toxicity [40, 42, and 43]. When metals are available in toxic concentrations limited accumulation can occur due to the toxic actions occurring at external membranes, such as gills, rather than acting on internal systems post uptake [43].

When comparing the [Cu_{DGT}] and [Cu_{inorg}] regressions with organism bioaccumulated Cu, FHM-Cu regressions are significantly higher than LMP-Cu (Figures 4-6, 4-7). The control treatment is the only treatment where [Cu_{inorg}] had a higher correlation coefficient than [Cu_{DGT}], in the case of FHM-Cu (0.784, 0.661 respectively). Although for LMP, [Cu_{DGT}] r^2 was nearly double [Cu_{inorg}], and found to be statistically significant.

Impact of water chemistry on DGT predictive ability

The water chemistries used in this study do not appear to decrease the predictive power of DGT for FHM or LMP. In fact, NOM seems to increase the predictive power of DGT for FHM Cu accumulation in the concentration range utilized. Yellow lampmussel Cu accumulation is not as well predicted by DGT as FHM, but water chemistry has less of an impact as it appears that LMP regulate accumulated Cu, regardless of bulk solution concentrations. DGT correlated better with organism accumulated Cu than the predicted inorganic Cu for all but one water chemistry and organism combinations.

Diffusive Gradients in Thin-films is a simple tool and technique for evaluating metal speciation. For FHM, [Cu_{DGT}] are successfully correlated with the bioaccumulated Cu in the Cu range of zero to sixty $\mu g\ L^{-1}$. The higher correlation between [Cu_{DGT}] and FHM than [Cu_{inorg}] and FHM indicate that more than the inorganic complexes may be bioavailable, similar to observations in other studies [9, 34, 44].

Conclusion

In this study we had three objectives regarding the use of DGT for predicting bioaccumulation of Cu to aquatic organisms. We used a coupled method of Cu

exposures to assess in DGT can be used as a tool for estimating Cu bioavailability in metal contaminated waters. DGT is believed to accumulate the free Cu ions and labile Cu complexes, which are generally considered the bioavailable metal species. If found to be acceptably predictive, DGT could potentially be used as a surrogate for other assessments of metal bioavailability that are either more cost prohibitive, require more sampling effort, or are less accurate.

First we investigated how NOM and hard water change the fraction of Cu measured by DGT. We observed that NOM decreased the [Cu_{DGT}] significantly compared to both hard water and control treatments. The hard water treatment did not alter [Cu_{DGT}] compared to control, despite the percentage of dissolved Cu as inorganic Cu was greatly reduced in this treatment. Secondly, we endeavored to compare the DGT measured Cu with FHM and LMP accumulated Cu concentrations for all water chemistries. Significant correlations were found for [Cu_{DGT}] and FHM for all water chemistry treatments, with the best fit being for the NOM addition treatment. Yellow lampmussel had significant linear correlations with [Cu_{DGT}] for control and hard water treatments, but not for NOM addition. Finally, we compared the DGT correlations with those obtained by regressing the predicted inorganic Cu to FHM and LMP. Diffusive Gradients in Thin-films better fit the FHM and LMP for all but one water chemistry treatment-organism combinations.

In conclusion, DGT appear to be acceptable at predicting Cu bioaccumulation by FHM for all tested water chemistries, and are advantageous to predictions of inorganic Cu by the Biotic Ligand Model speciation component. Yellow lampmussel Cu bioaccumulation is not as well predicted by DGT or the inorganic Cu fraction. With

further research on DGT in other environmentally relevant water chemistries, compared to other aquatic organisms, and in natural environments, DGT could prove to be an effective technique for predicting aquatic organism bioaccumulation, monitoring water quality and determining metal bioavailability.

Figures

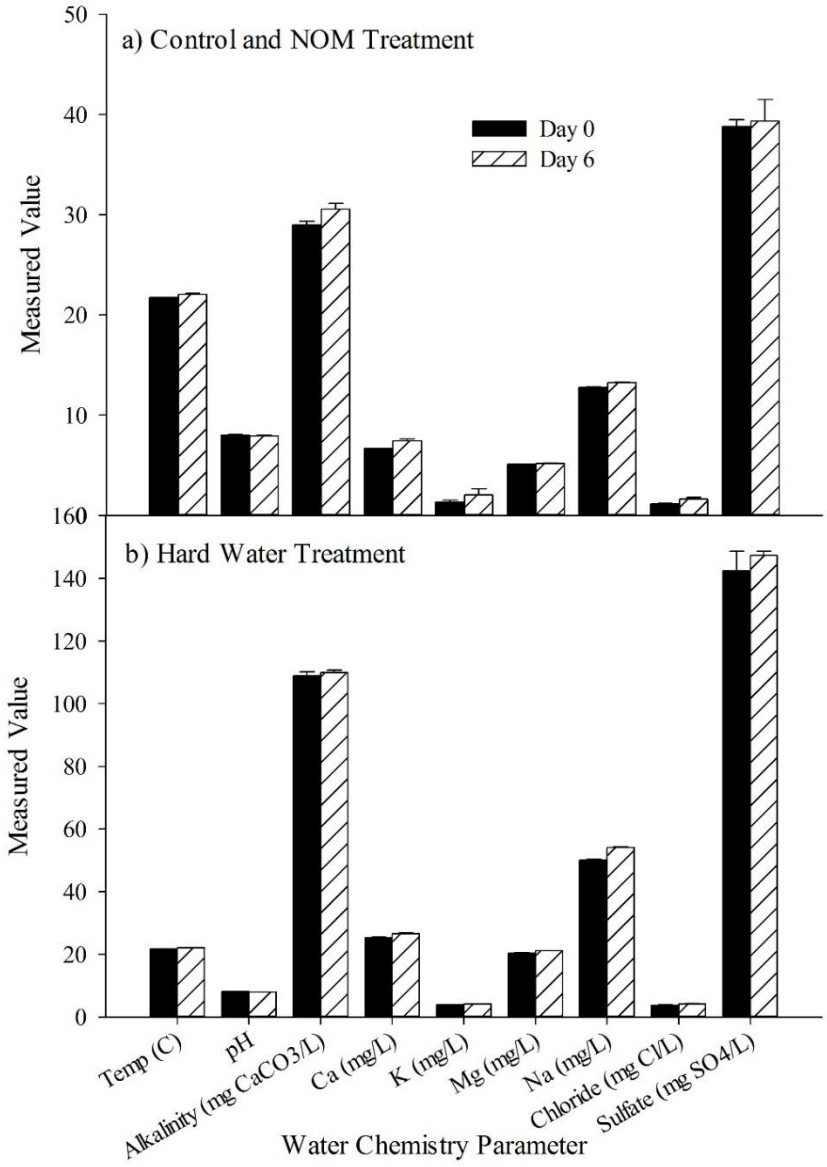

Figure 4-1: Water chemistry averaged for all tanks comparing initial deployment day to final exposure day water chemistry separated by treatments. a) NOM and control treatments b) hard water treatment. Error bars indicate standard error of the mean.

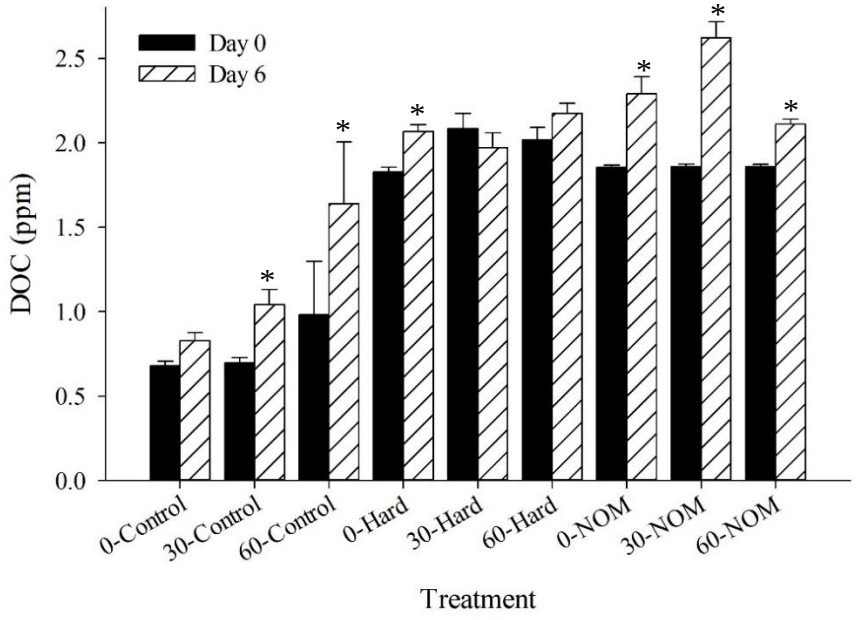

Figure 4-2: Dissolved organic carbon (DOC) as mg carbon per liter averaged by 5 replicates per treatment for initial deployment day (day 0) and final exposure day (day 6). Error bars indicate standard error of the mean. Asterisks indicate significant difference between day 0 and day 6.

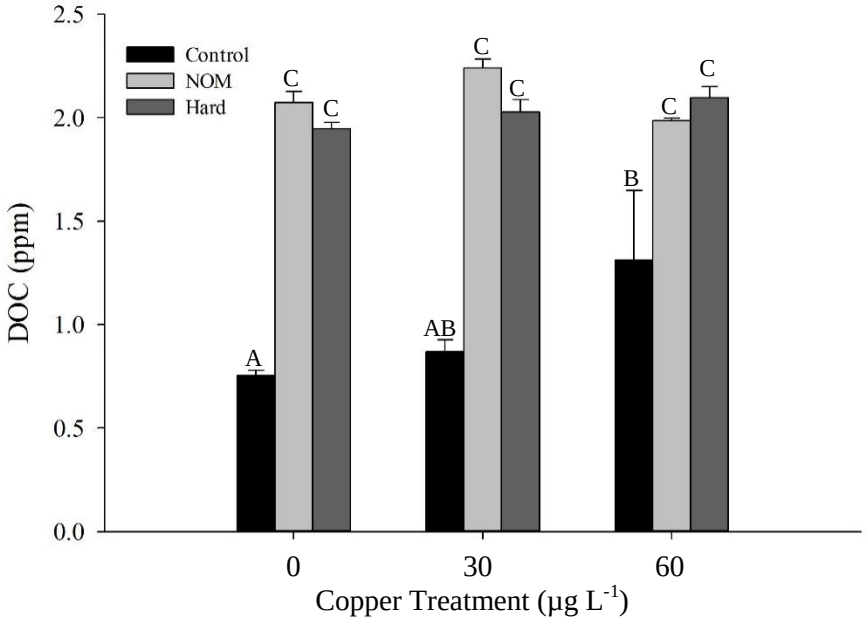

Figure 4-3: Dissolved organic carbon (DOC) averaged by treatment for all tanks and days sampled. Error bars indicate standard error of the mean. Letters indicate significant differences from other treatments, Tukey HSD post hoc multiple comparisons of means.

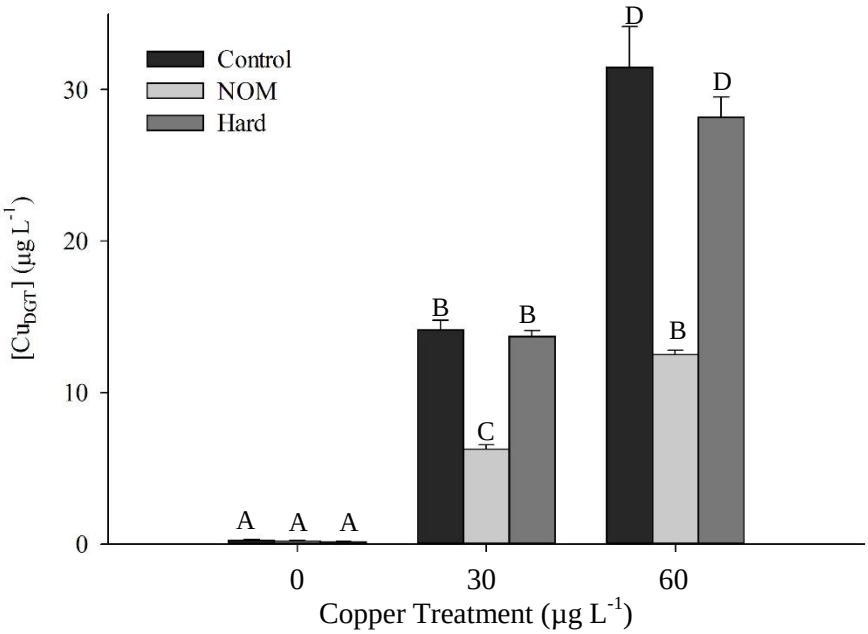

Figure 4-4: Copper accumulated, measured, and calculated by DGT [Cu_{DGT}] averaged by replicates per treatment (n = 5). Error bars indicate standard error of the mean. Letters indicate significant differences from other treatments, Tukey HSD post hoc multiple comparisons of means.

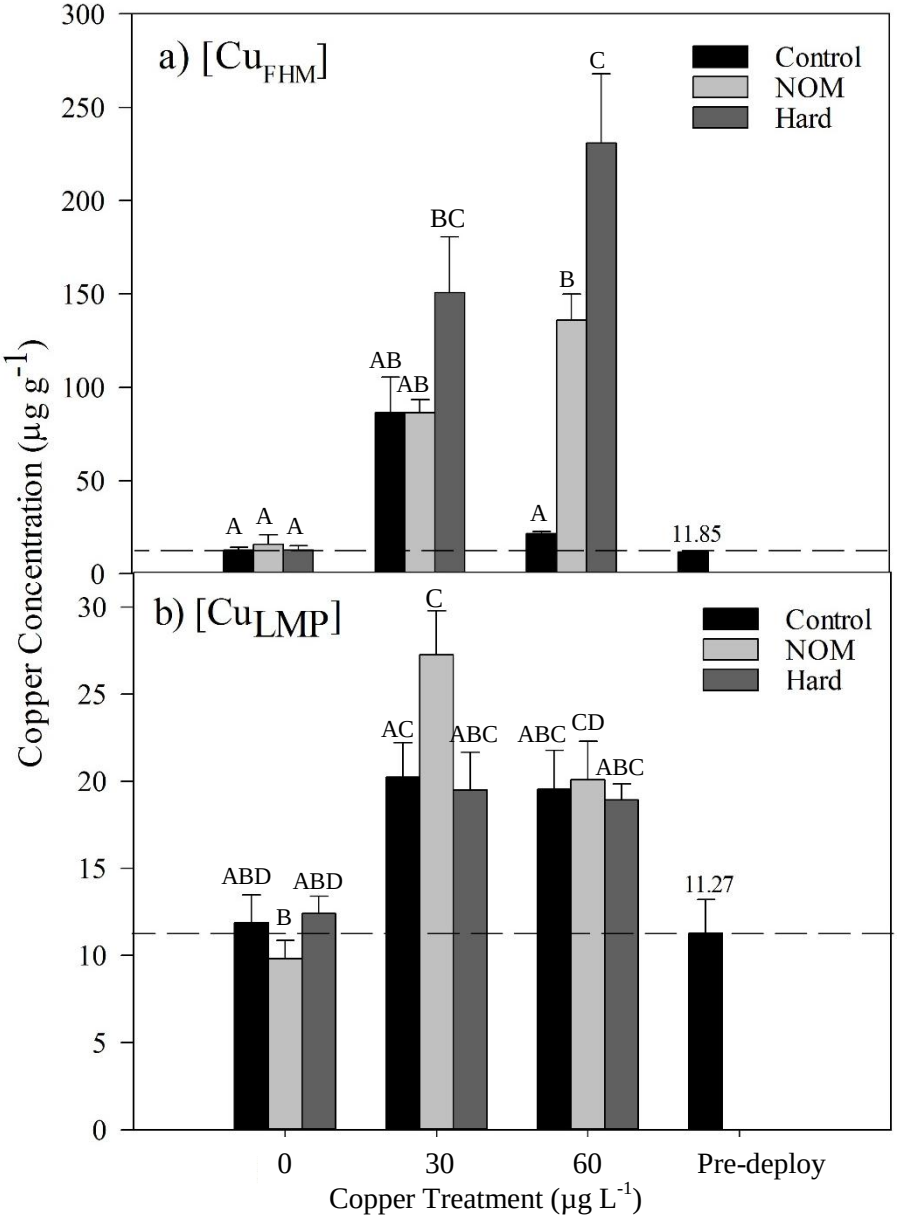

Figure 4-53: Fathead minnow (FHM: a) body and *Lampsilis cariosa* (LMP: b) soft tissue concentrations of copper averaged by treatment. Error bars are standard error of the mean. Letters indicate significant differences from other treatments, Tukey HSD post hoc multiple comparisons of means. Dashed line represents the average pre-deployment organism copper concentrations.

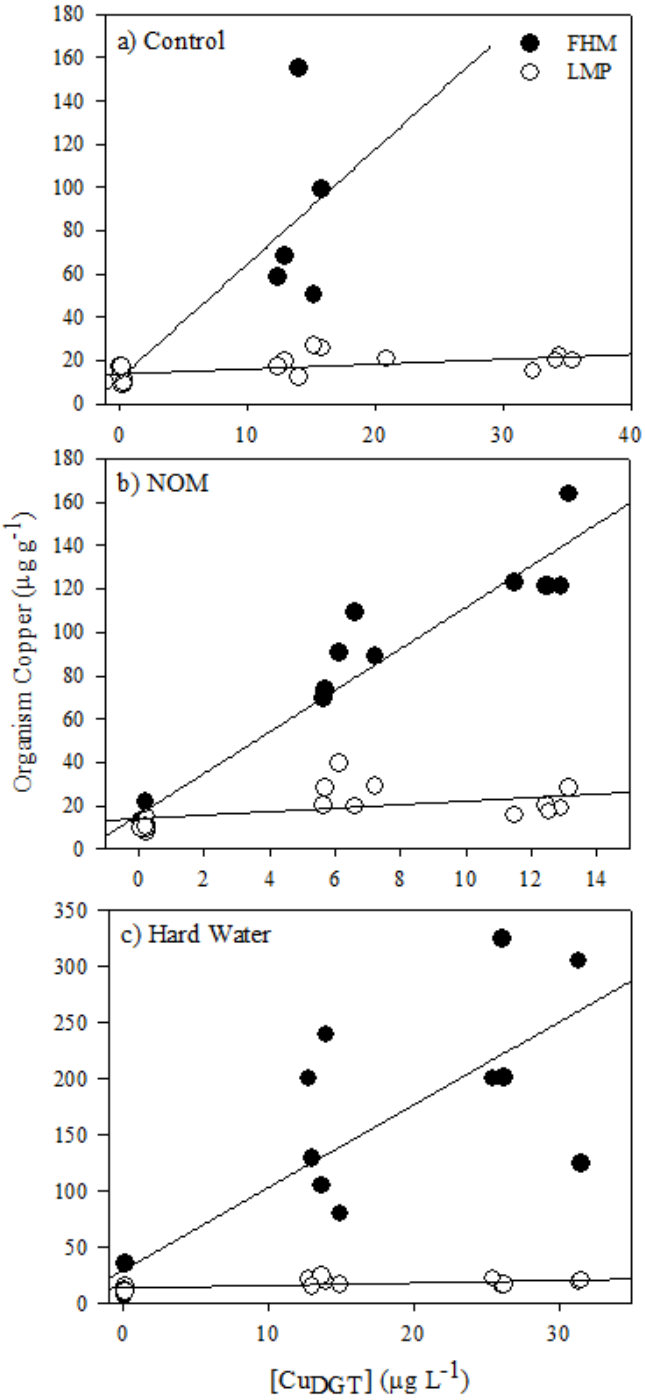

Figure 4-6: DGT Cu ([Cu_{DGT}]) regressed to fathead minnow and *Lampsilis cariosa* total body copper concentrations. a) Control treatments: FHM, without 60 µg L^{-1} control water treatment, r^2 =0.6609, p=0.0042, y=11.3+5.31x; LMP r^2 =0.300, p=0.0344, y=13.76+0.226x. b) Natural organic matter addition treatments: FHM r^2 =0.929, p<0.0001, y=15.87+9.57x; LMP r^2 =0.2237, p=0.0750, y=14.14+0.803x. c) Hard water treatments: FHM r^2 =0.6620, p=0.0002, y=29.32+7.37x; LMP: r^2 =0.412, p=0.0099, y=13.75+0.228x.

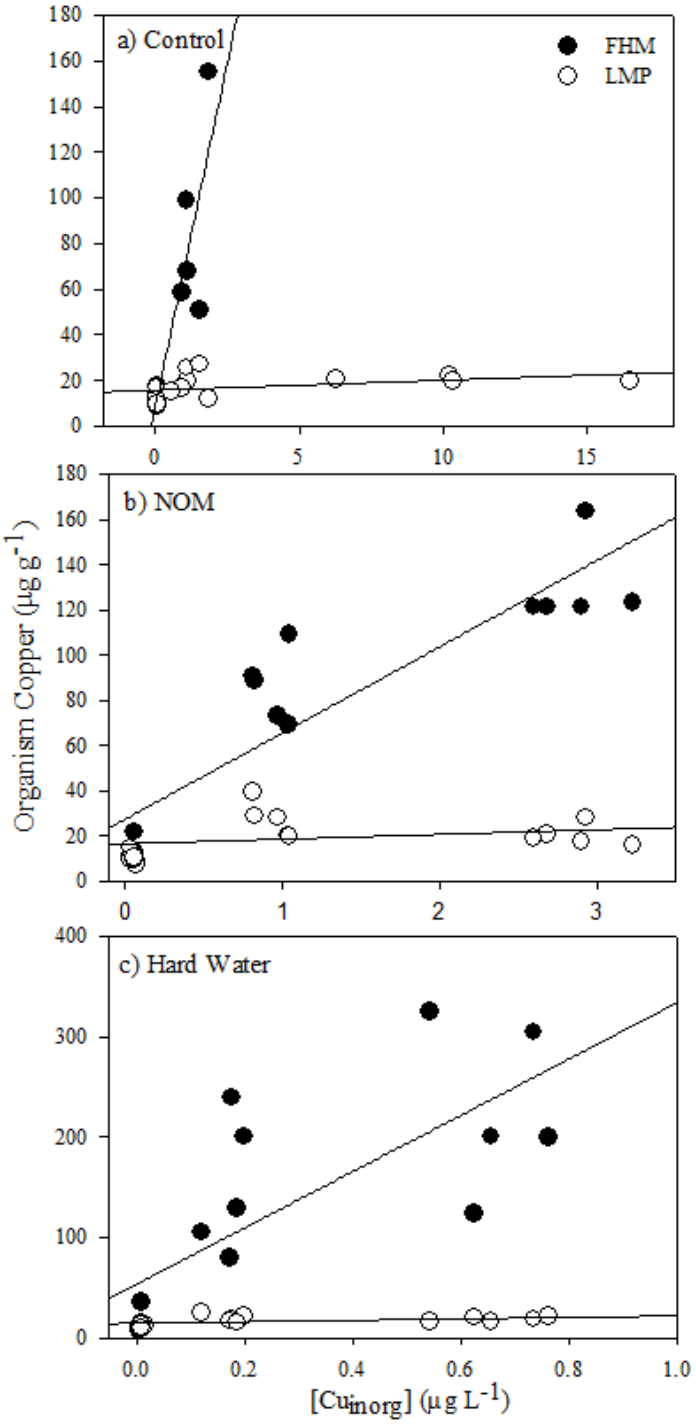

Figure 4-7: Inorganic Cu as predicted by biotic ligand model ([Cu_{inorg}]) regressed to fathead minnow and *Lampsilis cariosa* total body copper concentrations. a) Control treatments: FHM without 60-0 treatment r^2 = 0.7837, p=0.0007, y=8.96+60.3x; LMP r^2 = 0.1562, p=0.1448, y=15.74+0.436x. b) Natural organic matter addition treatments: FHM r^2 = 0.8145, p < 0.0001, y=27.42+38.15x; LMP r^2 = 0.0822, p=0.3001, y=16.56+2.07x. c) Hard water treatments: FHM r^2 = 0.5734, p=0.0011, y=53.49+281x; LMP: r^2 = 0.2557, p = 0.0545, y=14.87+7.34x.

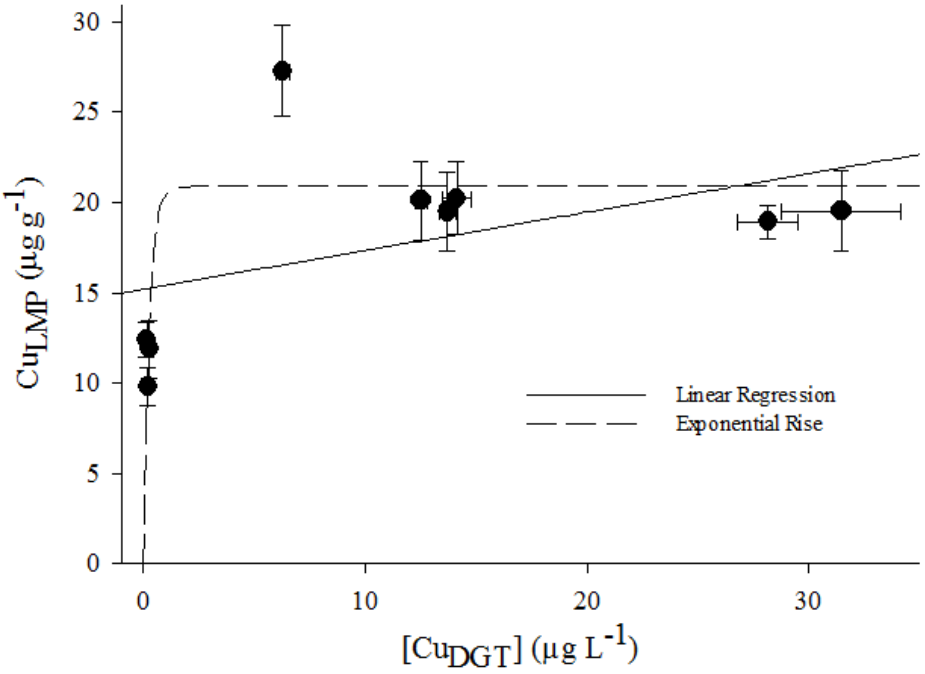

Figure 4-8: DGT Cu ([Cu_{DGT}]) regressed to *Lampsilis cariosa* soft tissue body copper concentrations for all water chemistry treatments. Each data point is an average of replicates per treatment (n=5) with error bars as standard error of the mean. Regression statistics for linear regression: p=0.213, y=15.2+0.213x, r^2=0.2116. Regression statistics for exponential rise regression: p=0.0031, y=20.9(1-$e^{-3.78x)}$, r^2=0.736.

Tables

Table 4-1: Exposure treatments. Treatment title is denoted by copper concentration (in column 2) and water chemistry treatment (column 3), Cu-WaterChem.

Treatment Title	Copper ($\mu g\ L^{-1}$)	Water Chemistry
0-0	0	Control
0-1	0	NOM addition
0-2	0	Hard Water
30-0	30	Control
30-1	30	NOM addition
30-2	30	Hard Water
60-0	60	Control
60-1	60	NOM addition
60-2	60	Hard Water

Table 4-2: Water chemistry averaged for all tanks over entire exposure duration. Temperature, pH, and ORP are averaged for all treatments. Dissolved organic carbon (DOC) is reported as mean of each water chemistry treatment. All other water chemistry parameters are separated into hard water treatment and non-hard water treatments (control and NOM addition). SEM is standard error of the mean.

Parameter	Mean	SEM	n
Temperature (°C)	22.50	0.03	503
pH	7.80	0.02	350
ORP (mV)	215.91	1.69	180
Control DOC (mg C/L)	0.98	0.10	30
NOM DOC (mg C/L)	2.03	0.03	45
Hard DOC (mg C/L)	2.10	0.06	30
Control and NOM Treatments			
Calcium (mg/L)	7.06	0.07	90
Magnesium (mg/L)	5.18	0.01	90
Sodium (mg/L)	13.12	0.04	90
Potassium (mg/L)	1.66	0.26	90
Sulfate (mg SO4/L)	39.22	0.28	24
Chloride (mg Cl/L)	1.50	0.10	24
Alkalinity (mg CaCO3/L)	29.77	0.36	60
Hard Water Treatment			
Calcium (mg/L)	25.99	0.14	45
Magnesium (mg/L)	20.81	0.08	45
Sodium (mg/L)	51.67	0.25	45
Potassium (mg/L)	4.05	0.02	45
Sulfate (mg SO4/L)	146.14	1.72	12
Chloride (mg Cl/L)	4.03	0.10	12
Alkalinity (mg CaCO3/L)	109.43	0.72	30

Table 4-3: Total [Cu_{tot}] and dissolved [Cu_{diss}] copper concentrations averaged for entire exposure by treatment (copper-water chemistry; 0=control, 1=NOM addition, 2=hard water; n = 5). <MDL indicates the copper concentration was below the ICP-OES method detection limit of 5.8 µg/L. SEM indicates standard error of the mean.

Treatment	**[Cu_{tot}]**	**SEM**	**[Cu_{diss}]**	**SEM**
	µg/L	µg/L	µg/L	µg/L
0-0	<MDL	na	<MDL	na
0-1	<MDL	na	<MDL	na
0-2	<MDL	na	<MDL	na
30-0	22.40	0.37	19.87	0.24
30-1	24.83	0.31	24.62	0.37
30-2	24.18	0.23	23.59	0.49
60-0	46.62	1.65	44.38	2.65
60-1	51.60	0.90	50.47	0.90
60-2	46.95	1.13	44.71	1.49

Table 4-4: Biotic Ligand Model predicted free copper [Cu_{free}] concentrations, total inorganic copper [Cu_{inorg}] concentrations, and percent of total copper concentration [Cu_{tot}] as [Cu_{inorg}] averaged by treatment (copper-water chemistry; 0=control, 1=NOM addition, 2=hard water). SEM is standard error of the treatment mean.

Treatment	**[Cu_{free}]** (ug/L)	**SEM**	**[Cu_{inorg}]** (ug/L)	**SEM**	**% [Cu_{tot}] as [Cu_{inorg}]**
0-0	0.003	0.001	0.048	0.012	<MDL
0-1	0.001	0.000	0.047	0.008	<MDL
0-2	0.000	0.000	0.010	0.002	<MDL
30-0	0.062	0.008	1.298	0.174	5.79%
30-1	0.014	0.001	0.937	0.053	3.77%
30-2	0.007	0.001	0.171	0.015	0.71%
60-0	0.450	0.149	8.783	2.636	18.84%
60-1	0.042	0.002	2.868	0.140	5.56%
60-2	0.026	0.001	0.664	0.045	1.41%

Table 4-5: Percent of dissolved copper ([Cu_{diss}]) represented by metal species. Inorganic copper ([Cu_{inorg}]) was calculated by the Biotic Ligand Model. Labile Cu-DOM (copper-dissolved organic matter complexes) were calculated by [Cu_{DGT}] minus [Cu_{inorg}]. Non-labile Cu-DOM is the remaining measured [Cu_{diss}], not measured by [Cu_{DGT}]. <MDL indicates that [Cu_{diss}] or [Cu_{DGT}] were below the ICP-OES method detection limit.

Treatment	Inorganic Cu	Labile Cu-DOM	Non-Labile Cu-DOM
0-0	<MDL	<MDL	<MDL
0-1	<MDL	<MDL	<MDL
0-2	<MDL	<MDL	<MDL
30-0	6.53%	64.59%	28.88%
30-1	3.81%	21.64%	74.55%
30-2	0.73%	57.39%	41.88%
60-0	19.79%	51.13%	29.08%
60-1	5.68%	19.11%	75.21%
60-2	1.48%	61.49%	37.02%

Table 4-6: Statistical results (correlation coefficient and p-value) of linear regressions. Regressions were performed on copper concentrations of FHM, LMP, DGT, and inorganic copper from all copper treatments separated by water chemistry treatment.

Statistic	Control	NOM	Hard Water
	[Cu DGT] vs. CuFHM		
r^2	0.661	0.929	0.662
p-value	0.0042	<0.0001	0.0002
	[Cu DGT] vs CuLMP		
r^2	0.300	0.224	0.412
p-value	0.0344	0.0750	0.0099
	[Cuinorg] vs CuFHM		
r^2	0.784	0.815	0.573
p-value	0.0007	<0.0001	0.0011
	[Cuinorg] vs Cu LMP		
r^2	0.153	0.082	0.256
p-value	0.1448	0.3011	0.0545

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CHAPTER 5

CONCLUSIONS

We investigated the potential use of the abiotic, passive diffusion, in situ sampling device Diffusive Gradients in Thin-films (DGT) for predicting copper bioaccumulation. DGT have the potential to be of considerable use to regulators for monitoring metal impacted surface waters. Studies have already demonstrated the advantages of DGT use over water spot sampling for capturing pollution events and discriminating temporal differences in metal concentrations, and the benefit of this for regulators (Allan et al 2008, Miège et al 2012). In this thesis we hoped to further the body of knowledge regarding DGT's use for predicting metal bioaccumulation using two aquatic animal species in four different water chemistry combinations over a range of copper concentrations from 0 to 60 μg L^{-1}

In all three experiments DGT measured copper had significant linear correlations with fathead minnow bioaccumulated copper. A pseudo-ratio of 1:10,000 was exhibited between fathead minnow Cu and DGT Cu for exposures greater than six days and the hard water and natural organic matter treatments. If this ratio holds true in other systems, it could be used as a straightforward, non-lethal method of prediction for copper bioaccumulation. Also, in the metal mixture experiment, the 1:10,000 pseudo-ratio predicts lead bioaccumulation by fathead minnows. DGT better predicted fathead minnow copper bioaccumulation than the inorganic fraction predicting using the Biotic

Ligand Model in all experiments. This suggests that more than just the inorganic copper is bioavailable to fathead minnow.

In contrast to the fathead minnow results, DGT did not highly correlate with yellow lampmussel copper concentrations in most of the experimental treatments. As was discussed in each chapter, this is potentially due to the yellow lampmussel having an increased ability to regulate metal accumulation. In all experimental treatments, the yellow lampmussel soft tissue concentrations were below 32 $\mu g\ g^{-1}$, regardless of treatment copper concentration. At all copper concentrations used in this thesis, DGT over-predict yellow lampmussel bioaccumulation, suggesting that if DGT were to be used in a bioaccumulation prediction capacity, yellow lampmussel would still be protected.

Overall this thesis demonstrates that more research on DGT is warranted. While DGT were determined to not be of use for predicting copper bioaccumulation by yellow lampmussel, they were highly effective for fathead minnow. The effectiveness of DGT for predicting bioaccumulation by other aquatic organisms and for other metals is yet to be determined. The results from the metal mixture experiment are especially promising, due to the dearth of an easy and effective technique for determining effects of metal mixtures, as the BLM for metal mixtures is still under development.

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