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Novel Approaches For Assessment Of Copper Toxicity: Fast Scan Cyclic Voltammetry And Optical Bioassays

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NOVEL APPROACHES FOR ASSESSMENT OF COPPER TOXICITY: FAST SCAN CYCLIC VOLTAMMETRY AND OPTICAL BIOASSAYS

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A journey of a thousand miles begins with a single step
-Lao Tzu, Chinese philosopher

My journey has been challenging, demanding, exacting and most of all rewarding.

An enormous “thank you” to everyone who was part of my journey that assisted and inspired me along the way.

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CHAPTER 1: INTRODUCTION

Across the globe, freshwater ecosystems are negatively impacted by toxic contaminants released into surface waters through anthropogenic activities (Kolpin, Furlong et al. 2002, Camargo and Alonso 2006, Sarkar, Saha et al. 2007, Schwarzenbach, Egli et al. 2010). Assessment of environmental changes to aquatic ecosystems is essential to understanding the threat to water quality and biodiversity as a result of these human induced changes (Dudgeon, Arthington et al. 2006). Fresh water is a vital commodity worldwide, with inland lake and river surface waters comprising only a small fraction (0.01%) of the world's water (Gleick, Pacific Institute for Studies in Development et al. 1993).

Increasingly, aquatic ecosystems are stressed and water quality compromised as a result of a toxic contaminants being released into the environment (Gray 1998, Gobel, Dierkes et al. 2007). The discharge of toxic pollutants into aquatic ecosystems results from many sources, including manufacturing, agriculture, mining operations and urban growth. Catastrophic natural events (e.g. floods, tsunamis, hurricanes) have the potential to release toxic contaminants into surface water environments (Frickel and Vincent 2007, Bird and Grossman 2011, Magnuson, Ernst et al. 2014). Damaged industrial facilities and infrastructure can release vast amounts of toxic contaminants, including heavy metals, into surface waterways when containment is breached (Bird and Grossman 2011). Although typically rare events, as climate change escalates (Frich, Alexander et al. 2002) these scenarios may become a more frequent pathway for the release of pollutants and toxic metals into aquatic waterways.

Heavy metals are toxic contaminants to the environment and aquatic ecosystems. Although improving control of water contaminants at point sources has been the focus of many countries, toxic metals continue to impact aquatic ecosystems worldwide through both point and nonpoint sources (Schwarzenbach, Egli et al. 2010, Fu and Wang 2011). Metal polluted waste

water from industrial operations can be released into surface waters (Barakat 2011) with runoff a primary route for transporting heavy metals, including copper (Cu), to aquatic environments downstream (Fu and Wang 2011).

Upon entering aquatic food webs, Cu – like other heavy metals – is persistent with the potential to bioaccumulate (Moiseenko and Kudryavtseva 2001). In freshwater ecosystems, Cu toxicity is regulated by speciation predominately controlled by dissolved organic matter (DOM) (Linnik 2003, Hoffmann, Shafer et al. 2007). The ability to mitigate these Cu pollutants is largely determined by understanding the movement and complexation of these ions in the aqueous state with DOM and other competing ions (Baken, Degryse et al. 2011). Cu is highly toxic to *Daphnia*, fish and other aquatic biota, with toxicity decreasing as water hardness increases (Florence 1982, Pagenkopf 1983). Cu complexation with DOM has been shown to have a protective effect on aquatic biota toxicity (Richards, Curtis et al. 2001, Sciera, Isely et al. 2004) but further understanding is needed to evaluate Cu speciation and how different DOMs influence complexation.

Traditional toxicity bioassays evaluate the acute impact of Cu contaminants by assessing death at a specific concentration, often using a mean LC_{50} level as the endpoint. Although these bioassays have great utility, additional investigative bioassays are needed to evaluate alterations in behavior and physiology functions prior to the endpoint of immobility or death, providing more in-depth evaluation of the impact of Cu as an aquatic contaminant. Ecologically relevant consequences can develop well below the LC_{50} concentration levels for a pollutant with unknown impact to the ecosystem. Adapting new high through put, cost effective optical bioassays are needed to understand the impact of Cu and other contaminants on aquatic ecosystems.

The aim of this dissertation work is to develop and adapt environmental assessment tools evaluating Cu complexation and toxicity in an aqueous environment with *Daphnia* as the model aquatic species. The assessment techniques adapted and used in the dissertation research include fast scan cyclic voltammetry (FSCV) and optical behavioral (Zein, McElmurry et al. 2014) and physiology bioassays (Pitts 2013).

Chapter 1 introduces the dissertation work providing an overall presentation of the research and the challenges involved with Cu pollutants in aquatic ecosystems along with a general outline of the dissertation chapters.

Chapter 2 highlights knowledge relevant to new techniques developed as part of this dissertation to characterize Cu toxicity. It includes an introduction to water quality issues around the globe and the impact of Cu as a toxic pollutant.

Chapter 3 focuses on Cu-DOM interactions using FSCV. As a recently developed analytical technique, FSCV allows the study of metal speciation in aqueous solutions with high sensitivity and real-time resolution (Pathirathna, Yang et al. 2012). Initially used with biological applications, FSCV has been adapted to investigate environmental metal pollutants, including Cu (Cahill, Walker et al. 1996, Wood and Hashemi 2013). This novel technique was used to investigate Cu-DOM complexation, evaluating binding and release with competitive ions. This work characterizes and enhances understanding of Cu behavior in aquatic systems.

Chapter 4 investigates *Daphnia* exposure to Cu and toxicity mitigation with DOM. Resulting changes in behavior and physiology are quantified with optical bioassays. The optical behavioral bioassay adapted for this research evaluates *Daphnia* swimming behavior by quantifying distance travelled and degree of angle changed when exposed to Cu. The physiology optical bioassay takes advantage of the *Daphnia* translucent exoskeleton and quantifies changes

to heart rate (HR) and appendage beat rate (ABR) during Cu exposure providing more detailed information about the impact of Cu on this aquatic organism.

Chapter 5 investigates *Daphnia* in-vivo neurotransmitter release to photo stimuli using FSCV as the assessment technique. A carbon fiber microelectrode is placed in the brain ganglia of *Daphnia* which is then exposed to a light stimuli triggering a neurotransmitter like response captured by the FSCV system.

Chapter 6 takes the new knowledge developed in previous chapters and describes the relevance to aquatic ecosystems. This chapter also evaluates limitations of the research work performed and what additional research is needed to advance knowledge forward with the intent to improve water quality.

CHAPTER 2: BACKGROUND

Environmental Contaminants

Anthropogenic activities worldwide increasingly introduce toxic pollutants into the environment and waterways (Kolpin, Skopec et al. 2004, Schwarzenbach, Egli et al. 2010). Urban growth, global industrialization, mining operations, agricultural processes and catastrophic natural events are some of the routes promoting the release of environmental contaminants into global surface waters (Kolpin, Furlong et al. 2002, Sarkar, Saha et al. 2007, Clark, Steele et al. 2008, Yi, Kang et al. 2010, Pare and Bonzi-Coulibaly 2013). Water quality and fitness of aquatic ecosystems are threatened by environmental changes as a result of these activities (Jackson, Carpenter et al. 2001, Bunn and Arthington 2002). These human induced toxic contaminants impact all regions of the globe.

Once released into the environment, many pollutants are fundamentally persistent, posing potential risks to society and aquatic ecosystems (Srivastava and Majumder 2008, Fu and Wang 2011). Healthy and robust aquatic ecosystems are vital to the overall stability of the environment, as ecosystems are interconnected, with alterations in one region potentially generating a cascade of consequences within in another (Dudgeon, Arthington et al. 2006). These ecosystem alterations may have long term effects that may not be fully recognized at the onset. The long term effects may be subtle and initially not detected with unknown future implications to ecosystem fitness and water quality (Jackson, Carpenter et al. 2001).

Freshwater ecosystems are particularly vulnerable to environmental changes as these environments face an ever growing and increasing threat to biodiversity from anthropogenic activities (Vos, Dybing et al. 2000, Dudgeon, Arthington et al. 2006). Aquatic biodiversity is vital for ecosystem stability, as over 100,000 known species depend on freshwater resources for

survival with additional species unknown (Jackson, Carpenter et al. 2001). Freshwater lakes and rivers make up less than 0.01% of all the water on earth but with ever increasing demands on water needs around the world (Gleick, Pacific Institute for Studies in Development et al. 1993). Water quality and aquatic biodiversity continue to be threatened by alterations and changes in the environment with water scarcity issues occurring in some regions and flooding in others (Dudgeon, Arthington et al. 2006). Improved understanding of the effect of contaminants in the environment is vital to assessing the long term impact of water quality degradation on aquatic ecosystems.

The study of metal toxicity and speciation is critical to understanding the impact of metal pollutants on freshwater aquatic ecosystem. Heavy metals can be defined as elements having a molecular weight ranging from 63.5 to 200.6 g/mole with a specific gravity greater than 5 (kg/m^3 / kg/m^3) with Cu included in this group (Srivastava and Majumder 2008).

Cu is a known toxic aquatic contaminant (Erickson, Benoit et al. 1996). As an essential metal, Cu is an enzymatic cofactor and required by all living organisms for survival; however, excess amounts of this metal are toxic to humans and aquatic biota (Armendariz, Gonzalez et al. 2004, ATSDR 2004, Fu and Wang 2011). Over 1,400,000,000 lb of Cu were released into the environment in 2000 by industrial operations according to the U.S Agency for Toxic Substances & Disease Registry with some of it destined to reach and influence aquatic ecosystems (ATSDR 2004)

Improved understanding of how Cu impacts freshwater environments is needed as this metal pollutant is fundamentally persistent once introduced in the aquatic ecosystem (Tercier-Waeber, Confalonieri et al. 2008, Lin, Vogt et al. 2012). Cu discharged into aquatic ecosystems poses societal health risks through toxicity and potential for bioaccumulation.

Cu toxicity is regulated by speciation and complexation with dissolved organic matter (DOM) (Taylor, Kirwan et al. 2016). When Cu is complexed with DOM it reduces free Cu in aqueous solutions and reduces toxicity to aquatic organisms (Erickson, Benoit et al. 1996, Richards, Curtis et al. 2001, Sciera, Isely et al. 2004). However, other metals such as aluminum (Al) can compete with Cu for preferred binding sites on DOM, displacing Cu and potentially increasing the amount of free Cu in solution, particularly in soft water systems (Chappaz and Curtis 2013, Hoppe, Gustafsson et al. 2015, Hoppe, Gustafsson et al. 2015). Cu toxicity is a product of the complex aquatic ecosystem chemistry and the target organism, with toxicity increasing as water hardness is reduced. Calcium and magnesium ions reduce toxicity by limiting metal uptake (Villavicencio, Urrestarazu et al. 2005). Large variations in Cu toxicity can occur with differences in water hardness. It has been shown that Cu toxicity to fish can increase up to 20 times in soft water rather than in hard water (Taylor, McGeer et al. 2000). Hyne et al showed that in synthetic soft water 71% of Cu was in the free Cu^{2+} form responsible for toxicity (Hyne, Pablo et al. 2005). Generally, invertebrates are more sensitive to Cu toxicity than most fish and other vertebrates.

Dissolved Organic Matter (DOM)

Originating from partially decomposed organic matter including soil, plant and animal residues, DOM is a complex mixture of low molecular weight molecules in the 20-50 kDa range held together with hydrophilic and hydrogen bonding (Sutton and Sposito 2005). As a heterogeneous assemblage of different size organic molecules, DOM has distinctive properties that are determined by the aquatic system environment, as well as, the organic matter source (Tonietto, Lombardi et al. 2011, McElmurry, Long et al. 2014, International Humic Substances Society (IHSS) 2015). DOM is often present in aquatic systems in amounts greater than heavy

metal pollutants, thus, dominating the regulation of the chemical speciation (Dube, Zbytniewski et al. 2001, Tonietto, Lombardi et al. 2011).

DOM has an array of binding sites that complex with various organic and inorganic ligands. These binding sites compete for available cations in the aqueous environment. Sulfur and nitrogen binding sites are scarce, but bind ligands more strongly while phenolic and carboxylic sites are more abundant, but with weak ligand binding (Santschi, Lenhart et al. 1997). Conformation changes in the DOM macromolecule can take place over a matter of days with the potential for DOM structure to open up exposing additional ligand sites for stronger complexation (Tipping 2002). Cu is preferentially bound by certain DOM moieties and this alters behavior of the complexed metal with DOM (McElmurry, Long et al. 2010). Cu-DOM complexation is dynamic and difficult to characterize in real time, new and adapted methods are needed to evaluate the changes as they occur in-situ.

Fast scan cyclic voltammetry (FSCV)

Assessing environmental contaminants in relevant concentrations, cost effectively and in real-time, requires the development of innovative analytical methods (Buffle 2005). Analytical techniques that address metal speciation and complexation in environmental systems are often limited by methodology. Development of new techniques is needed to better assess metal speciation and ligand complexation without many of the significant limitations of currently available methods. Some of the current techniques available to analyze metal speciation include spectroscopy and anodic stripping voltammetry (ASV), but these methods have drawbacks associated with them.

Spectroscopic techniques readily detect metals with extreme sensitivity but limitations include portability, extensive sample handling and equipment expense (Wang and Marshall 1994, Harville and Marcus 1995). ASV is used in many environmental applications, but the

technique typically requires a preconcentration of mercury (Hg), an environmental contaminant, onto an electrode to increase sensitivity. Voltammetric techniques are well suited for application to environmental monitoring and assessment of metals and other pollutants, as these techniques are relatively inexpensive with the potential for portability for field applications, but adaptations are needed to minimize existing limitations (Buffle 2005).

A recently developed Hg free analytical technique is fast scan cyclic voltammetry (FSCV) at carbon fiber microelectrodes. Originally developed for biological applications, including the detection of neurotransmitters, this method is expanding to other applications including metal analysis (Cahill, Walker et al. 1996, Lama, Charlson et al. 2012, Wood and Hashemi 2013, Pathirathna, Samaranayake et al. 2014). FSCV microelectrodes, with a 10 µM carbon fiber diameter, lend themselves to applications where the microelectrode can be positioned, including in vivo applications (Hashemi, Dankoski et al. 2009, Samaranayake, Abdalla et al. 2015).

The FSCV technique has high sensitivity, in parts-per-billion (ppb), with a temporal resolution of 100 ms. Cyclic voltammograms can be acquired every 100 ms and in quick succession allowing the elimination of a high charging current through background subtraction. Scan rates range between 400 and 1000 V s^{-1} with a single cyclic voltammogram acquired within 20 ms (Pathirathna, Yang et al. 2012). Using an optimized waveform, FSCV voltammograms allow the identification and quantification of both deposition and stripping peaks, improving the detection of the target analyte. FSCV is a promising new technology to evaluate in-situ metal speciation and other environmental contaminants in real time (Yang, Pathirathna et al. 2013).

Optical Bioassays

Traditional toxicity bioassays evaluate the acute toxic effects on organisms, often using the LC_{50} (median lethal concentration for 50% of the target animals over a specified time course)

as the bioassay end point (Kimball and Levin 1985). Bioassays that assess acute toxic effects on target animals with death as the endpoint, do not adequately evaluate the effect of chronic, sub-lethal exposures of toxic contaminants that have the potential to alter animal physiology, behavior, including potential changes to reproduction, growth rates, motor functions and predator avoidance (Dodson, Hanazato et al. 1995).

Adverse effects can occur in receiving waterways even if discharging effluents meet local water quality criteria (Kim, Jun et al. 2008, Yoo, Ahn et al. 2013). Additional endpoints beyond animal immobility, which indirectly measures the concentration-dependent lethality, are needed to evaluate alterations in animal behavior prior to immobility and death (Dodson, Hanazato et al. 1995, Zein, McElmurry et al. 2014). *D. magna* exposed to Cu and Zn have been shown to exhibit signs of oxidative stress with a substantial increase in enzymes including superoxide dismutase, glutathione peroxidase, glutathione S-transferase, enzymes associated with reducing oxidative damage to cells, but without acute toxicity observed in the organism (Yoo, Ahn et al. 2013). New techniques for evaluating biological changes are essential to understanding the impact of chronic long term exposure of environmental contaminants to aquatic organisms. A novel behavioral bioassay with the potential for high through put screening has been developed recently (Zein, McElmurry et al. 2014) .

The optical bioassay developed by Zein et al. (2014) evaluates changes in swimming behavior by tracking the maximum distance travelled and how much the degree of angle changes over a specified time period using *Daphnia* as the target organism. Optical bioassays that investigate cardio-respiratory physiological changes to *Daphnia* heart rate (HR) and appendage beat rate (ABR) have been used to investigate exposure to emerging aquatic contaminants (Pitts 2013). Although not a high through put methodology this physiology bioassay complements and provides additional vital information obtained from the behavioral bioassay. Using these two

assays in conjunction together will provide an enhanced evaluation of sub-lethal toxic effects on *Daphnia* exposed to aquatic pollutants.

Model Organism: *Daphnia*

Daphnia, of the order Cladocera, are microcrustaceans that live in diverse freshwater aquatic habitats around the world, including the Great Lakes watershed. Considered a model organism for ecotoxicological research and evolutionary genomics, *Daphnia* are a sensitive indicator of environmental stressors (http://www.nih.gov/science/models/daphnia) (Colbourne, Pfrender et al. 2011). As a freshwater organism, *Daphnia* are filter feeders ingesting algae, organic detritus and are considered to be on the bottom of the food chain being a primary food source for larger aquatic species. Adult animals range in size from 1-5 mm in length, have one compound eye and a translucent exoskeleton allowing lifespan observation of motor functions. Easily cultured in synthetic medium, *Daphnia* typically reproduce by cyclic parthenogenesis, have large broods and reach maturity within 10 days (Ebert 2005), making them ideal for optical bioassays.

Daphnia exhibit diel vertical migration, where animals move up to shallow surface water at night to feed on algae, then, descend to darker depths during daylight hours to avoid predators (Cousyn, De Meester et al. 2001). Phototactic behavior is a function of diel vertical migration, with negative genotypes moving away from light stimuli and downward toward the deeper water levels where it is darker, a predator avoidance trait (Ringelberg 1995, Ebert 2005). An alteration in phototactic response is maladaptive for the organism making it more vulnerable to predation during daylight hours, thus, changing the predator-prey relationship within the aquatic environment (Cousyn, De Meester et al. 2001).

Considered a keystone species, *Daphnia* are often the dominant zooplankton in freshwater ecosystems and an essential part of the food web, with potential for the whole aquatic

ecosystems to be altered if the species declines or vanishes (Jeziorski, Tanentzap et al. 2015). Sensitive to environmental stressors, *Daphnia* are ideally suited for the investigation of toxic contaminants in an aquatic environment. *Daphnia* are impacted by aquatic environmental factors that include the influence of predators, oxygen content, food supply, pH, hardness and temperature among others with *Daphnia* developing the ability to make physiological and morphological changes in response to environmental stressors (Ebert 2005).

These specialized developmental processes have evolved allowing the *Daphnia* to adapt to environmental alterations as a result of epigenetic regulatory mechanisms (Coors, Hammers-Wirtz et al. 2004). Low oxygen levels cause *Daphnia* to upregulate hemoglobin (Hb) and anaerobic metabolism resulting in circulating hemolymph turning a red-orange hue (Zeis, Becher et al. 2003, Ebert 2005). Under environmental pressure *Daphnia* (Zeis, Lamkemeyer et al. 2009) also have the capability of converting from asexual (clonal reproduction) to sexual reproduction as a means of adaptation to stressors in the environment (Altshuler, Demiri et al. 2011). Morphological changes occur when *Daphnia* are exposed to predator released hormones called kairomones causing helmet and teeth-like formations to develop as a defense mechanism. With a high genome homology with humans, *Daphnia* are a sensitive ecoresponsive model with the capability of adapting to changing environmental conditions (Colbourne, Pfrender et al. 2011).

Neurotransmitter Release in *Daphnia*

Daphnia nervous and visual systems are important components of the phototacic response, however, relatively little is known about how these systems function alone or in conjunction with each other (McCoole, Baer et al. 2011). Neurotransmitters have been shown to play an important part in the visual system signaling in arthropods, (Stuart, Borycz et al. 2007), but relatively little is known about the neurochemistry component of *Daphnia* but knowledge in that area is increasing. McCoole et al illustrated that histaminergic signaling is extensive in

Daphnia with histamine playing a vital role in phototaxis (McCoole, Baer et al. 2011). The neuroanatomy of the optic ganglia and central brain of *Daphnia* has been investigated by Kress et al, including chemical markers for the neurotransmitter histamine (Kress, Harzsch et al. 2016).

Vertical migration in a water column is a bioassay that has been used to investigate behavioral changes in *Daphnia* phototactic response to light stimuli.(Ringelberg 1995) This optical bioassay evaluates *Daphnia* orientation changes to light intensity and light direction with movement away from the light considered to be a negative phototactic response (Dodson, Tollrian et al. 1997). Both photonegative and photopositive responses have been observed in *Daphnia*, however, exposure to ultraviolet light (UV) under normal conditions elicits a negative phototactic response (Poupa 1948, Lampert 1987). This adaptive behavior is theorized to reduce the risk of predation and exposure to UV light (Lampert 1993). Exposures to aquatic contaminants that reduce the phototactic response potentially leave *Daphnia* more susceptible to predators with a reduced ability to escape (Lampert 1993, Dodson, Tollrian et al. 1997). *Daphnia* exposed to sub-lethal Cu concentrations have been shown to have an altered phototactic response (Michels, Leynen et al. 1999, Yuan, Michels et al. 2003). With *Daphnia* as a keystone species these alterations in phototactic behavior could change the fitness of the aquatic ecosystem by changing predator-prey interactions (Cousyn, De Meester et al. 2001).

Additional neurotransmitters conserved regions identified in *Daphnia* include serotonin, dopamine and octopamine (McCoole, Atkinson et al. 2012). The suppression of the phototactic response has been identified in *Daphnia* exposed to Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) (Rivetti, Campos et al. 2016). SSRIs are termed “selective” as they work primarily on serotonin and not on other neurotransmitters. Used to treat clinical depression, SSRIs work by blocking serotonin reabsorption at neural synapses in the brain, making more serotonin available for circulation.

The FSCV analytical technique has been used with great success in detecting serotonin and histamine in small mammal brains, including rats and mice (Hashemi, Dankoski et al. 2009, Wood and Hashemi 2013, Samaranayake, Abdalla et al. 2015). Adaptation of this analytical technique for use in *Daphnia* in-vivo would promote the understanding of the role of serotonin and other neurotransmitters on the phototactic response.

CHAPTER 3: REAL - TIME VOLTAMMETRIC EVALUATION OF COPPER - DISSOLVED ORGANIC MATTER COMPLEXATION AND COMPETITIVE ALUMINUM BINDING

Introduction

Anthropogenic sources of copper (Cu) pollute the environment with global impact (Breault, Colman et al. 1996, Birsan, Luca et al. 2012, Soltani, Moore et al. 2014, Bui, Do-Hong et al. 2016, Gubelit, Polyak et al. 2016, Saglam and Akcay 2016). Improved understanding of this toxic metal is needed as Cu is fundamentally persistent once introduced into the environment with the potential for bioaccumulation and the ability to transport over long distances (Clark, Steele et al. 2008, McElmurry, Long et al. 2010). Industrialization, mining operations and urban growth have increased the release of Cu into the environment and to receiving surface waters (Ruello, Sani et al. 2011, Baralkiewicz, Chudzinska et al. 2014, Sharifi, Haghshenas et al. 2014, Shen, Wang et al. 2014). Understanding Cu toxicity is critical to assessing the impact of this pollutant on aquatic ecosystems and developing effective mitigation techniques for this toxicant (Tercier-Waeber, Confalonieri et al. 2008, Yang and van den Berg 2009, Mudhoo, Garg et al. 2012).

The toxicity of Cu is controlled by speciation; in freshwater systems, such as the Great Lakes, Cu speciation is dominated by organic complexation (Linnik 2003, Bigalke, Weyer et al. 2010, Baken, Degryse et al. 2011). Up to 99.99% of Cu in surface waters is complexed with dissolved organic matter (DOM) (Hoffmann, Shafer et al. 2007). Cu ions form ligands with DOM in aqueous solutions, impacting availability, toxicity and fate of the metal (McElmurry, Long et al. 2010). Cu-DOM complexation reduces free Cu in solution, limiting chemical and biological reactions with system biota (Kantar 2007) and typically decreasing Cu toxicity (Erickson, Benoit et al. 1996, Richards, Curtis et al. 2001, Sciera, Isely et al. 2004, Rogevich, Hoang et al. 2008). The ability to mitigate these Cu pollutants is largely determined by

understanding the movement and complexation of these ions in the aqueous state with DOM. While DOM is not a single macromolecule for which each binding site can be easily characterized: broad classification, such as molecular weight and general structural aromaticity have proven useful to describe DOM characteristics (Amery, Degryse et al. 2010, McElmurry, Long et al. 2010). DOM has a wide array of binding sites on each macromolecule. Carboxylic and phenolic binding sites are abundant on DOM while other less abundant binding sites containing sulfur and nitrogen form stronger metal bonds (Santschi, Lenhart et al. 1997). These DOM binding sites compete for available cations with many factors impacting and affecting this Cu-DOM complexation; often through opposing interactions.

Over a period of days, conformational changes may occur in DOM complexes (Swift 1999). These conformational effects may induce rearrangement of organic molecules exposing additional binding sites which are thought to strengthen complexation, reducing Cu toxicity (Tipping 2002). However, Cu toxicity can simultaneously be enhanced as other multivalent cations, such as aluminum (Al), compete for preferred DOM binding sites, releasing Cu (Benedetti, Milne et al. 1995, Hoppe, Gustafsson et al. 2015). It is, therefore, critical to understand such complex chemical interactions, which occur dynamically, to fully characterize aquatic Cu speciation (Yang and van den Berg 2009). Dynamic characterization of Cu's chemical interactions with DOM has been limited by the lack of analytical technologies that can measure environmentally relevant Cu speciation in situ in real-time. The advancement of voltammetry methods with improved reliability and accuracy has great potential for the environmental metal analysis field as a means to assess ecological pollutants (Buffle 2005). Recently a powerful new method has been developed for real-time Cu analysis using Fast scan cyclic voltammetry (FSCV) (Pathirathna, Yang et al. 2012).

FSCV at carbon fiber microelectrodes (CFM) is an analytical technique that allows for the study of metal speciation in aqueous solutions in real time without the need for environmentally hazardous mercury (Hg) as an electrode stabilizer. Originally developed for biological applications (Cahill, Walker et al. 1996, Lama, Charlson et al. 2012, Wood and Hashemi 2013, Samaranayake, Abdalla et al. 2015), FSCV has been modified as a means to investigate environmental metal pollutants, including Cu (Pathirathna, Yang et al. 2012, Yang, Pathirathna et al. 2013, Pathirathna, Samaranayake et al. 2014). The FSCV method measures changes with deposition stripping voltammograms and provides robust metal detection by both identification and quantification. This potent technique has been shown to have a high sensitivity (ppb) for Cu^{2+} and a real time resolution of 100 (ms) allowing it to have the functionality to provide Hg-free, rapid (sub second) detection of trace Cu ion concentrations in aqueous solutions (Pathirathna, Yang et al. 2012). Recent work has included development of an experimental paradigm by monitoring thermodynamically consistent Cu-ligand binding in real-time (Siriwardhane 2016).

In this chapter FSCV's ability to investigate the temporal characteristics of Cu binding with natural ligands (DOM) and release, as the result of Al competition for DOM binding sites is demonstrated. It is hypothesized that (1) the type of DOM will determine the amount of Cu that is complexed, (2) that Cu-DOM complexation is a time dependent process and will strengthen with increased exposure, (3) that competitive ions, such as Al, will displace and release Cu bound to weak DOM binding sites.

Materials and Methods

Fast scan cyclic voltammetry (FSCV)

Carbon fiber microelectrodes (CFMs) were constructed by vacuum aspirating a single T-650 carbon fiber (Cytec Industries, NJ; 5 μm radius) into a 4-inch glass capillary tube (A-M

Systems, Sequim, WA; 0.60 mm outer diameter, 0.40 mm inner diameter). The carbon-filled capillaries were gravity tapered using a vertical micropipette puller (Narishige Group, Tokyo, Japan) with heat-sealing the carbon fiber and glass together. The exposed carbon fiber was cut under an optical microscope to approximately 150 µM.

Electrochemical experimental data was collected using custom built equipment (Department of Chemistry Electronics Facility, University of North Carolina at Chapel Hill) with a customized version of TH-1 software (ESA, Chelmsford, MA) written in LabVIEW 2009 (National Instruments, Austin, TX) for data acquisition, signal processing and waveform output. Optimization of a Cu specific electrochemical waveform for was utilized (600 V s-1 at 10 Hz, -1.4 V to 1.3 V, resting at 0 V) (Pathirathna, Yang et al. 2012). Potential values were measured against an Ag/AgCl reference electrode, created by immersing an Ag wire (A-M systems, Sequim, WA) into 1 M HCl for 5 s at +4.0 V vs. Tungsten.

As previously reported, (Pathirathna, Yang et al. 2012) a 6 point calibration (0.1 to 1.0 µM Cu in 2.5 mM NaCl) was performed on 5 electrodes, for which the responses were averaged together, resulting in a coefficient of determination (r2) of 0.9939. For experimental data three carbon fiber electrodes were utilized throughout. Data was pooled and presented with error bars symbolizing the standard error of the mean (SEM).

Reagents and Chemicals

Stock solutions were prepared by dissolving copper nitrate trihydrate (Ward Science, Rochester, NY) or aluminum nitrate nonahydrate (Alfa Aesar, Ward Hill, MA) in a 2.5 mM sodium chloride (Mallinckdrodt, St Louis, MO) in nanopure water (>18 MΩ conductivity). The initial pH of the sodium chloride solution was 5.8 ± 0.2. The pH of Cu and Al stock solutions were 5.7 ± 0.1 and 4.6 ± 0.1, respectively.

Six humic substances of known composition from the International Humic Substances Society (IHSS) were prepared in nanopure water (>18 MΩ) in acid washed glass bottles (International Humic Substances Society (IHSS) 2015). The six reference substances were: Nordic Lake Humic Acid (NLHA, cat no.1R105H); Nordic Lake Fulvic Acid (NLFA, cat no. 1R105F); Nordic Lake Natural Organic Matter (NLNOM, cat no. 1R108N); Suwannee River II Humic Acid (SRHA, cat no. 2S101H); Suwannee River II Fulvic Acid (SRFA, cat no. 2S101F) and Suwannee River Natural Organic Matter (SRNOM, cat no. 1R101N). Stock solutions were filtered through 0.2 µm pore Acrodisc syringe filters with Supor Membranes (Pall Corporation, Cornwall, UK), adjusted to a pH of 7.0 and stored in a darkened refrigerator (4 ± 1° C). DOM Samples were analyzed for Total Organic Carbon (TOC) by a Shimadzu TOC-VCSH Analyzer (Shimadzu Corporation, Kyoto, Japan).

Real-time Measurements

Experiments were conducted at a CFM immersed in a miniaturized continuously stirred-tank reactor (CSTR) containing 2.5 mM NaCl salt solution (26.17 - 29.75 mL dependent on DOM concentration) with a stir bar spinning at 500 rpm. For all experiments, the ratio of Cu to DOM was 1:100 based on the carbon content of DOM (International Humic Substances Society (IHSS) 2015). This ratio is on the high end of the range commonly observed in the environment. The molar ratio of Al to Cu was equal (1:1) during experiments evaluating the competitive displacement of Cu from DOM complexes. Other details describing the experimental setup are described elsewhere (Pathirathna, Yang et al. 2012).

Immediate Experiment

A baseline FSCV signal was captured by measuring the electrode response in the 2.5 mM NaCl solution for 30 s. After 30 s, 100 µL of 300 µM Cu was injected to produce a 1 µM Cu solution and monitored for another 30 s. Thirty seconds after the Cu injection (t = 60 s), 100 µM of the

test DOM was injected and the response was measured for an additional 30 s. The entire experiment and period of data acquisition lasted 90 s. Injected DOM volumes varied from 51 – 58 µL to ensure the same amount of organic carbon for each ligand was added to the sample solution. The final sample volume was 30 mL after all additions. Immediately following the first set of recordings, a second FSCV file was initiated. Following 30 s of baseline measurements, 100 µL of 300 µM Al was injected and the response was monitored for 30 s (t = 60 s). The pH of DOM mixtures after all additions was 5.7 ± 0.2. Data were normalized.

5 Day Experiment

Samples containing DOM were spiked with 1 µM Cu and refrigerated in acid washed glass beakers for 5 days prior to experimentation. The metal to DOM ratio was again 1:100. After 5 days, a 2.5 mM NaCl solution (~28 mL) was placed in a CSTR. A file was collected for 30 s to obtain a baseline reading before 151 – 158 µL of the Cu-DOM solution was injected (t = 30 s). Following 30 s of measurement (t = 60 s), 100 µL of 300 µM Al was injected and the measurement continued for an additional 30 s. The final volume of the solution was 30 mL and the total amount time data was recorded was 90 s. The final pH was 5.7 ± 0.1. Data was normalized.

Statistical Analysis

Statistical analysis was performed using SPSS (version 22, IBM) with an alpha-level of 0.05 selected for significance. A standard linear mixed effects model was used for statistical analysis:

$$Y = (\beta 1X1 + \beta 2X2 + \ldots \beta nXn) + (\gamma 1Z1 + \gamma 2Z2 \ldots + \gamma nZn) + \mathcal{E} \qquad (1)$$

where Y is the dependent variable, β is the vector of fixed effect variable, X is the fixed effect, Z is the random effect variable, γ is the vector of the random effect variable (intercept) and Ɛ is the error of residuals. Through the analysis, multiple models were evaluated utilizing the type of

ligand (humic acid, fulvic acid or NOM), the source of DOM (Suwannee River or Nordic Lake), the amount of time between binding and release (immediate or 5 day), the electrode number and Cu levels as fixed effects. The date and the order in which the experiment was run served as random effects, both of which were included in all models. The fixed and random effects were used to predict the concentration of free Cu in solution and the dependent variable, using the SPSS linear mixed models package with diagonal repeated covariance. An intercept was included for random effects along with the subject variable for subject combinations. Estimated marginal means for fitted models were determined for the fixed effects with comparison of main effects for confidence interval adjustment utilizing post hoc Bonferroni pairwise comparison for adjusted significance levels. Pearson correlation matrices were developed using averaged Cu bound and release data for each DOM type and comparing it with the elemental composition of the characterized IHSS humic substances.

For this analysis, fifty data points (5 s) were selected from each of the three quasi-equilibrium levels observed during experiments (Figure 1). The experimental setup for the immediate and 5 day experiments were different, thus the data obtained during the immediate experiments were grouped to have a three tiered structure similar to the 5 day experiments. Accordingly, for the immediate experiments, concentrations of Cu resulting from Al competition during the second set of data acquisition (level 3) were added to averaged concentrations of Cu observed during the first set of data acquisition (level 2). For the 5 day experiments, the baseline (level 1) was constructed using measurements made in the 2.5 mM NaCl solution. The second tier of measurements (level 2) was based on the amount of Cu present after Cu-DOM ligand complexes were assumed to reach equilibrium (after 5 days). The third data level reflected the amount of Cu released from the Cu-DOM complex following Al addition.

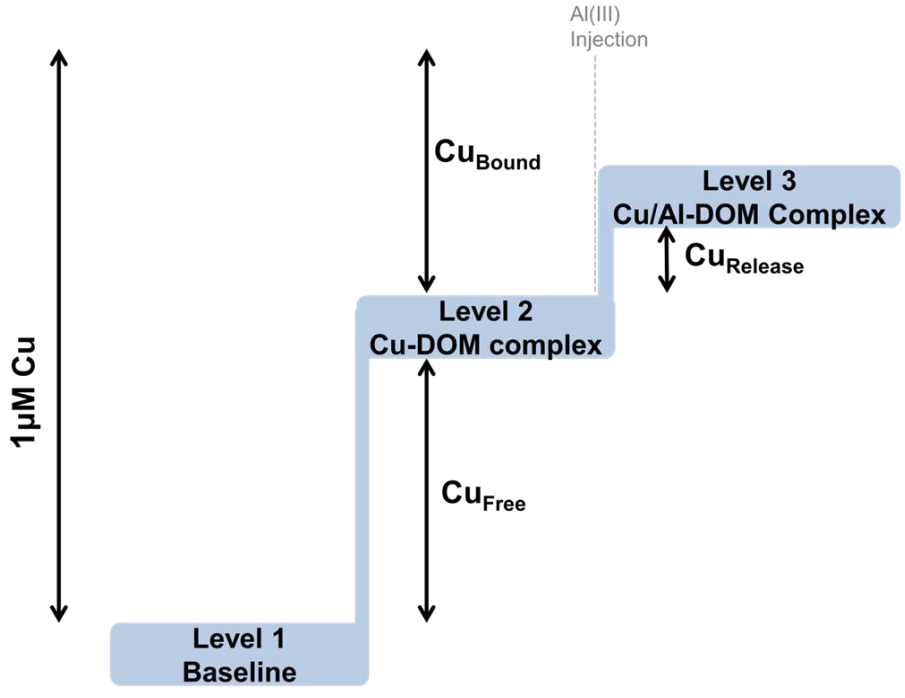


Figure 1. Three tiered design for statistical analysis. Fifty data points (5 s) were selected from each of the quasi-equilibrium levels observed during experiments and analyzed with a standard linear mixed effects model.

Results and Discussion

Cu-DOM complexation

DOM consists of heterogeneous aggregates of low molecular weight (20-50 kDa) originating from partially decomposed organic matter and held together with hydrophobic and hydrogen bonding (Sutton, Sposito et al. 2005). As a complex blend of organic compounds, DOM exhibits distinctive properties that are determined by the aquatic system environment, as well as, the source of organic matter (Tonietto, Lombardi et al. 2011). The source of DOM influences its composition and these compositional differences alter Cu bioavailability and toxicity (Stevenson, Fitch et al. 1993, Erickson, Benoit et al. 1996). For this study, we explored how DOM from two different sources (Nordic Lake and Suwannee River) influenced

complexation. From these two sources, we evaluated three different fractions with similar composition and structure: humic acid, fulvic acid and natural organic matter (NOM).

To begin the experiment, a CFM was lowered into a miniaturized CSTR of NaCl (2.5 mM) and file collection was initiated. FSCV for Cu detection has previously been described in detail elsewhere (Pathirathna, Yang et al. 2012, Pathirathna, Samaranayake et al. 2014, Siriwardhane 2016). In Figure 2, we followed a recently established protocol for assessing Cu complexation in real-time with FSCV (Siriwardhane 2016). As shown in Figure 2A, a Cu spike (1 µM) was added (illustrated by the vertical dashed line and injection artifact) to the CSTR. In Figure 2B an injection of DOM (SRHA, NLHA, NLFA, SRFA, SRNOM or NLNOM; all at 100 µM) was added 30 seconds after Cu addition (vertical dashed line).

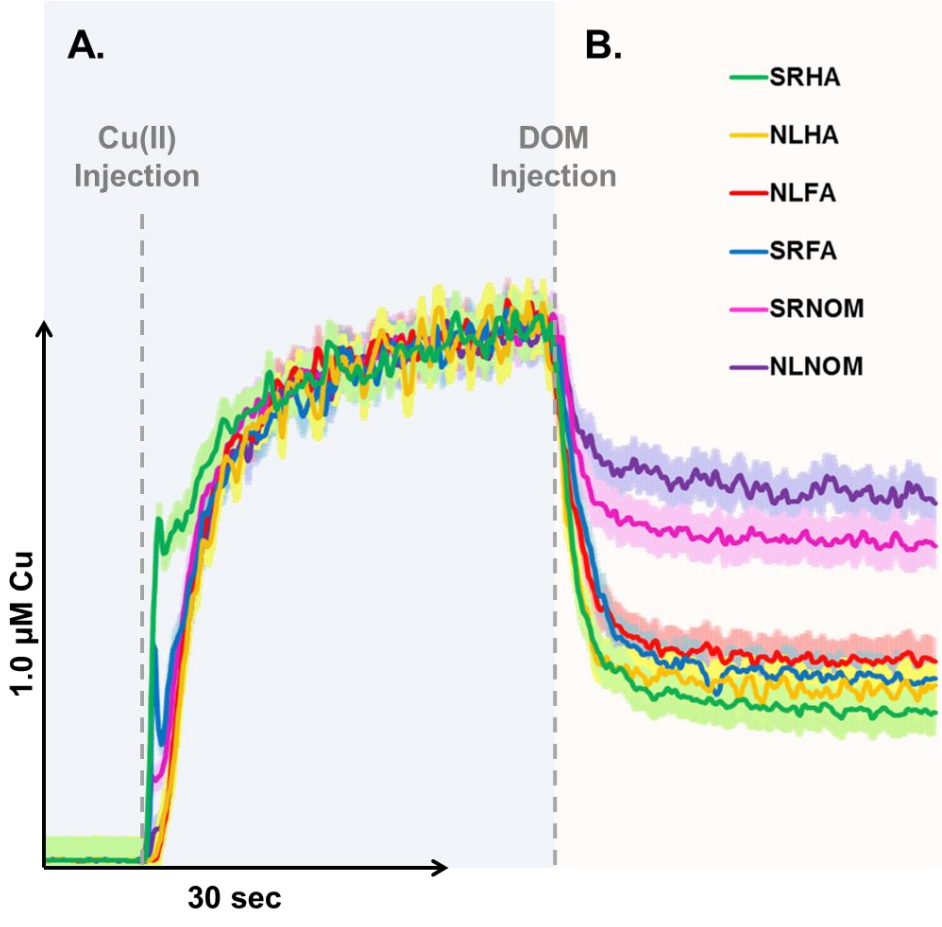


Figure 2. Cu detection and Cu-ligand complexation for the immediate experiment. (A) Normalized Cu response of 1.0 µM copper nitrate injection at 30 s marked by the first dashed line with error bars as standard error of the mean. (B) Cu-ligand complexation with 100 µM DOM injection at 60 s for six humic fractions indicated by the second dashed line.

After data collection, the measured current was converted to the molar Cu concentration versus time (Figure 3). All ligands followed Cu complexation with a hydrodynamic profile which had previously been characterized and modeled using known ligands (Siriwardhane 2016). This experiment allowed us to correlate real-time Cu complexation to composition differences in DOM. However, Cu-DOM complexation is known to be dependent on time and competition by other metals, therefore, next was explored the influence of these two factors using FSCV.

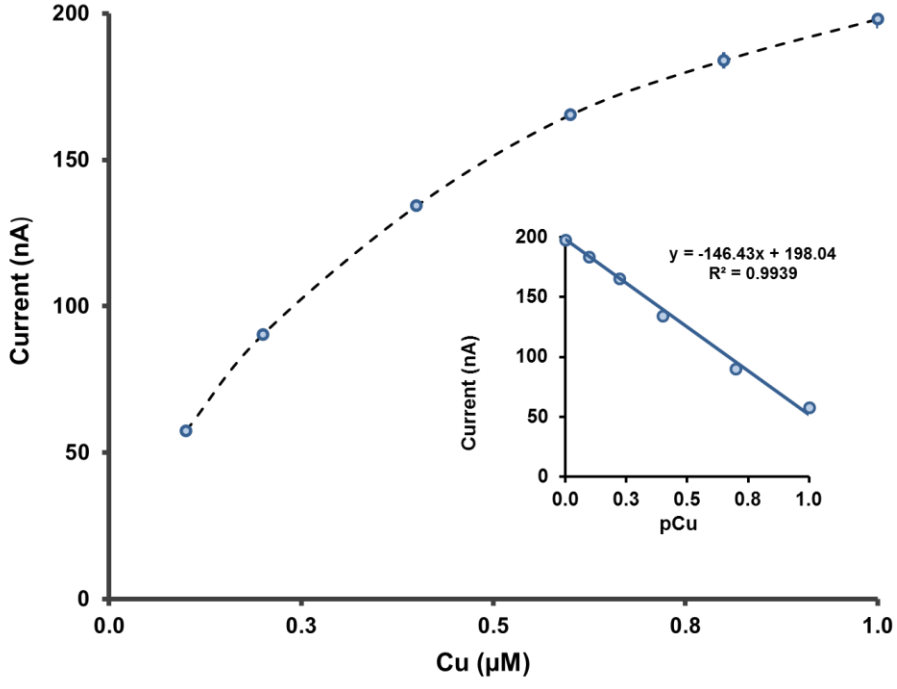


Figure 3. Cu Calibration Curve. The measured current (nA) was converted to molar Cu concentration (µM) with R^2=0.9939.

We observed two distinct complexation patterns. First, NLNOM and SRNOM complexed 0.325 ± 0.035 µM and 0.415 ± 0.011 µM Cu respectively (n=3, ± SEM). Importantly, while the method of isolation is the same for these fractions, we found a significant difference in complexation ($p<0.001$) (Appendix A, Table A-6). Second, NLFA, SRFA, NLHA and SRHA complexed 0.629 ± 0.009 µM, 0.665 ± 0.019 µM, 0.682 ± 0.034 µM and 0.725 ± 0.020 µM (n=3, ±SEM) Cu. There was a further pattern; the humic acids bound significantly more Cu than the fulvic acids ($p<0.05$) (Appendix A, Table A-4). Overall, the amount of Cu bound followed the order: NOM<FA<HA.

The similarities between how DOM types complexed Cu, reflects parallels in DOM composition (i.e. the number of binding sites). The procedures used to purify humic and fulvic acids are known to isolate DOM rich in carboxylic and phenolic groups that bind Cu (Benedetti,

Milne et al. 1995, Leenheer, Brown et al. 1998, Karlsson, Persson et al. 2006). The ability of humic acids to complex Cu has been described extensively by others (Stevenson, Fitch et al. 1993, Plaza, Senesi et al. 2005, Gondar, Lopez et al. 2006). Fulvic acids are generally smaller than humic acids, thus steric limitations are more likely to make binding sites slightly less available to Cu (Tipping 2002). Of the different forms of DOM, NOM bound the least Cu, likely because they are not refined as extensively during the DOM isolation process (reverse osmosis) and include carbohydrates and other compounds that serve as less effective ligands (i.e. the number of strong binding sites is less, diluting DOM's complexation capability (McElmurry, Long et al. 2010). This experiment allowed us to correlate real-time Cu complexation to composition differences in DOM. However, Cu-DOM complexation is known to be dependent on time and competition by other metals, therefore, next was explored the influence of these two factors using FSCV.

Dependence of time and Al competition on Cu-DOM complexation

Different environmental factors affect Cu-DOM complexation and hence, the availability and toxicity of Cu. In Figure 2, Cu-DOM complexation was evaluated immediately after DOM addition, however it is clear that equilibrium was not reached. This is echoed in the literature showing that Cu-DOM complexation requires hours to days to reach equilibrium (Rate, McLaren et al. 1992, Burba, Rocha et al. 1994, Bonifazi, Pant et al. 1996, Tipping 2002). Furthermore, the presence of other metals, in particular Al, affects Cu-DOM complexation due to DOM's high affinity for Al (Plankey and Patterson 1987, Roy and Campbell 1997, Sutheimer and Cabaniss 1997, Chappaz and Curtis 2013). Al is typically present in surface waters in the low micromolar concentration range (Drever 1988). Therefore, Al's ability to displace Cu from Cu-DOM complexes immediately and after 5 days is assessed.

In Figure 4, the unfilled bars represent the amount of Cu complexed by DOM following the experiment in Figure 2 (immediate complexation). The colored bars show the amount of Cu bound after 5-days of complexation with DOM. The bar graph illustrates the effect of increased exposure time on Cu-DOM complexation and reflects the elevated Cu binding following 5 days ($p<0.001$) (Appendix A, Table A-7). This trend was consistent across all six DOM isolates ($p<0.001$) (Appendix A, Table A-8). Over time, conformational changes can occur in DOM complexes, providing new bonding arrangements with sterically hindered but potentially more energetically favorable binding sites (Ghosh 1980, Choppin and Clark 1991, Korshin, Frenkel et al. 1998, Swift 1999, Lead, Wilkinson et al. 2000, Tipping 2002).

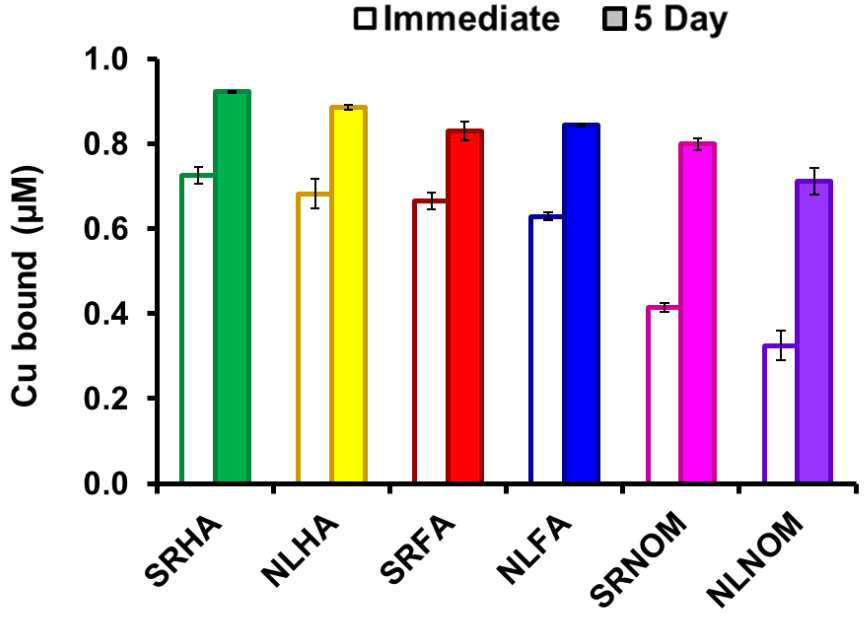


Figure 4. Comparison of Cu binding for immediate vs 5 day experiments. Increased exposure time of DOM with Cu is reflected in elevated Cu binding for 5 day data ($p<0.001$).

Al binds with DOM in a similar manner as Cu with a subsequent reduction in free Al ions (Sutheimer and Cabaniss 1997). Multivalent cations will potentially compete with each other for preferred DOM binding sites impacting displacement and binding of each other (Benedetti,

Milne et al. 1995, Hoppe, Gustafsson et al. 2015). Simulations with thermodynamic modeling software predict Al will strongly compete with Cu for DOM binding sites within a pH range of 4-9 (Chappaz and Curtis 2013). Al being one of the most abundant metals on earth may play an important role in understanding metal DOM interactions and complexation (Tipping, Rey-Castro et al. 2002). Given these two findings, that different DOMs bind varying amounts of Cu and that this is a time dependent process, how Al competes with Cu for DOM sites with time was evaluated next.

For this experiment Cu-DOM complexes formed immediately and during the 5 days prior to experimentation were placed into the CSTR where FSCV measurements were collected. FSCV is a background subtracted technique and can only detect changes in analytes; thus, the starting values shown in Figure 5 reflect an arbitrary basal free Cu in each vessel. Our primary interest was to investigate changes in Cu resulting from Al competition with Cu-DOM complexes that recently formed (immediate) versus those that were given enough time to mature (5 days). After an initial measurement period, Al (1 µM) was spiked into the CSTR (vertical dashed line) causing an increase in free Cu as it was displaced from DOM. Al is not detectable with our Cu specific waveform.

Distinct DOM patterns with the Al induced Cu release were observed. For immediately formed Cu-DOM complexes (Figure 5A), the amount of Cu released followed the order HA>FA>NOM (SRHA, NLHA, NLFA, SRFA, SRNOM and NLNOM; 0.075 ± 0.002 µM, 0.072 ± 0.002 µM, 0.067 ± 0.002 µM and 0.066 ± 0.001 µM, 0.054 ± 0.002 µM and 0.048 ± 0.002 µM (n=3, ± SEM)). This is the same order observed for Cu binding (Figure 2).

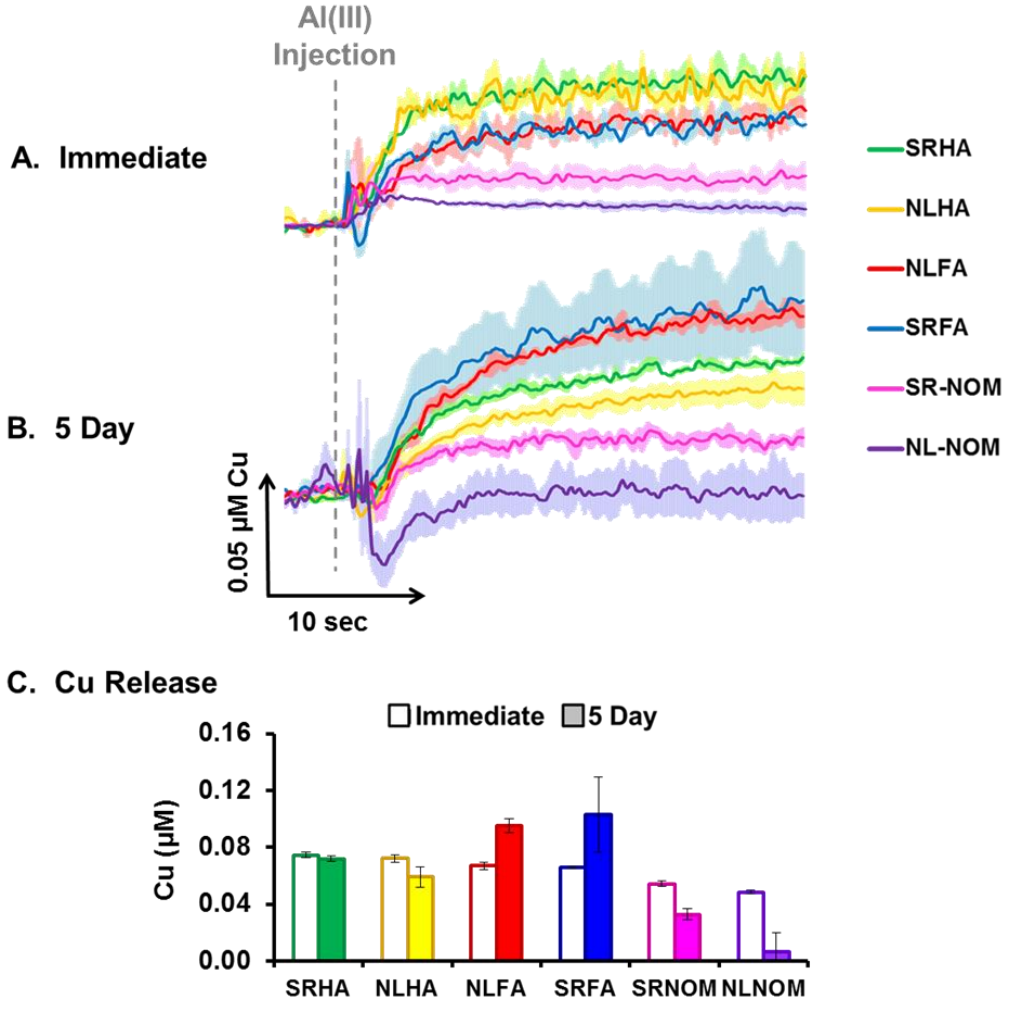


Figure 5. (A) Comparison between Cu released immediately and (B) 5 days after complexation following injection of 1.0 µM aluminum nitrate. Standard error of the mean is reflected by the shaded region. (C) Al induced Cu release is lower for 5 day humic acids.

The results of Al competition for Cu occupied binding sites in DOM complexes that were allowed to form over 5 days is shown in (Figure 5B). In contrast to previous experiments, the amount of Cu released followed the order FA>HA>NOM (SRFA, NLFA, SRHA, NLHA, SRNOM and NLNOM, with values of 0.103 ± 0.026 µM, 0.095 ± 0.005 µM, 0.072 ± 0.002 µM, 0.059 ± 0.007 µM, 0.033 ± 0.005 µM, and 0.007 ± 0.013 µM (n=3, ± SEM)). Further exemplifying this change in behavior is the histogram (Figure 5C) that shows a marked increase in the amount of Cu released from FA that was allowed to complex for 5 days rather than only a

few minutes. The same trend is observed in the percentage of Cu released (Table 1). The amount of Cu released from HA and NOM decreased for 5 day complexes relative to immediate complexes. These observed variations in behavior can be attributed to differences in the type and source of DOM, which we explore next.

DOM	Cu Release / Bound			
	Immediate		5 Day	
	(%)	SEM	(%)	SEM
SRHA	10.3	± 0.440	7.79	± 0.224
NLHA	10.6	± 0.546	6.66	± 0.839
SRFA	9.9	± 0.145	12.6	± 3.52
NLFA	10.6	± 0.258	11.3	± 0.515
SR-NOM	13.1	± 0.225	4.12	± 0.528
NL-NOM	15.2	± 1.518	1.03	± 1.98

Table 1. Immediate and 5 Day Cu binding and release with percent Cu release per amount bound for six DOM fractions reported ± standard error of the mean (SEM). Abbreviations are defined in methods.

DOM Structures and Binding Sites

After 5 days, Cu release decreased from humic acids and to a greater extent from NOM, and increased in fulvic acids. Based on stability constants, Al^{3+} is unable to out compete Cu^{2+} for amine and thiol binding sites (Tipping 2002, Tipping, Rey-Castro et al. 2002, Cabaniss 2011). While DOM contains an array of sulfur functional groups that have low affinity for Cu (e.g. sulfonates and sulfate esters), the presence of even low amounts of sulfhydryl groups are likely to impact Cu-DOM stability, as would amines. As suspected (Figure 6), the amount of Cu released by DOM after 5 days is inversely related to the amount of sulfur present in DOM (r^2=0.989, p=0.001). A similar trend was not observed based on nitrogen content. Consistent with the supposition that thiol binding is responsible for differences in Al mediate Cu release over time; SRNOM released the least amount of Cu after 5 days and contains the greatest

quantity of sulfur. Fulvic acids carry low amounts of sulfur which led to the opposite trend (increase Cu release with time). Here, over the 5 days of binding, Cu complexation appears to occur via other non-sulfur, binding sites. These sites, particularly carboxylics and phenolics, are susceptible to Al competition. Hence, the increase in Cu binding to these susceptible sites over time means Cu release is augmented over time.

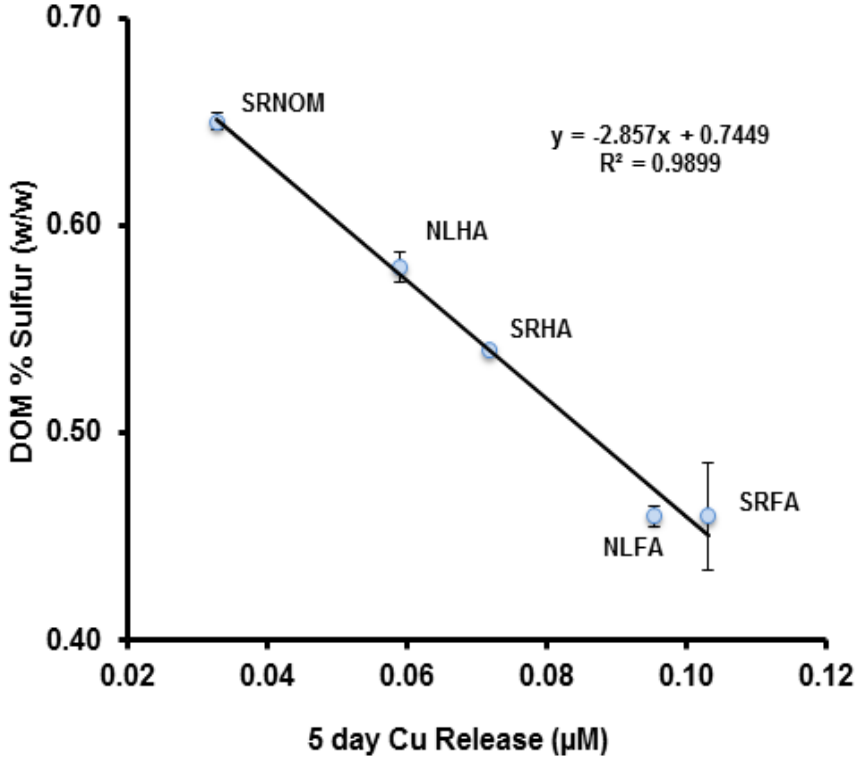


Figure 6. Cu release after 5 days correlated with sulfur content. DOM with lower sulfur content released greater amounts of Cu; fulvic acids showing the lowest sulfur and highest Cu release

Conclusion

The work demonstrated here is consistent with the initial hypotheses and has clearly shown the type of DOM influences the amount of Cu complexed, with humic and fulvic acids complexing significantly more Cu than natural organic matter. It has also been shown that Cu-DOM complexation is a time dependent process with DOM binding more Cu after 5 days than if

immediately combined. The competitive metal ion, Al, was shown to displace Cu from weak DOM binding sites releasing free Cu back into solution. Correlations between the amount of sulfur in DOM and Al induced Cu release highlighted the importance of this strong binding site in understanding Cu-DOM complexation. These results illustrated the utility of FSCV for investigating metal complexation and shed important new light on critical Cu-DOM interactions that ultimately impact Cu toxicity.

CHAPTER 4: EVALUATING DOM MITIGATED COPPER TOXICITY IN *DAPHNIA MAGNA* USING BEHAVIORAL AND PHYSIOLOGICAL OPTICAL BIOASSAYS

Introduction

Copper (Cu) is an environmental contaminant that impacts water quality across the globe. Human induced activities including agriculture, mining and industrial operations (Hoang, Schuler et al. 2009, Bui, Do-Hong et al. 2016, Saglam and Akcay 2016) have increased the release of Cu to surface waters contributing to the degradation of water quality (Clark, Steele et al. 2008). The discharge of Cu into surface waters poses risks to aquatic biota as Cu is environmentally persistent and has the ability to bioaccumulate (Mudhoo, Garg et al. 2012). In aquatic ecosystems Cu toxicity is controlled by Cu speciation and resulting complexation with available organic and inorganic ligands (Linnik 2003). Cu complexation with dissolved organic matter (DOM) plays a vital and well documented role in the mitigation of Cu toxicity to aquatic biota (Richards, Curtis et al. 2001, Taylor, Kirwan et al. 2016). DOM complexation with Cu limits the free Cu ions in solution and thus, limits potentially toxic interactions (Kantar 2007).

As a complex blend of partially decomposed organic matter, DOM is a loosely structured macromolecule held together with hydrophobic and hydrogen bonds (Sutton and Sposito 2005). Compositional differences between the type of DOM and the DOM organic matter source impacts Cu toxicity and bioavailability in aquatic ecosystems (Tipping 2002). Recent Cu-DOM experiments with fast scan cyclic voltammetry (FSCV) in Chapter 3 indicated that different DOM types bind Cu more readily suggesting that complexation is a function of the type of DOM and available binding sites with fulvic acid (FA) binding more Cu than natural organic matter (NOM) which includes both fulvic and humic acids.

As a surface water contaminant, Cu is toxic to many aquatic organisms including the cladoceran *Daphnia*. A keystone species of freshwater aquatic environments, the

microinvertebrate *Daphnia* play a vital role in the aquatic food chain and are extensively employed as monitors of ecotoxicological stressors (Kashian and Dodson 2004). Widely recognized as a model organism for studying aquatic ecosystems, *daphnia* have been used extensively in traditional toxicity assays, typically with median lethal concentration as the endpoint (LC_{50}) (Villavicencio, Urrestarazu et al. 2005). This well-established toxicity endpoint however, does not readily capture potential fluctuations and alternations in complex animal motor functions that can occur at lower concentrations (Christie 2011). These physical changes can influence development, animal survival and ultimately impact the fitness of an aquatic ecosystem.

Optical behavioral and physiological assays for environmental pollutants can provide advanced information beyond traditional protocol endpoints, by acquiring additional evidence of *Daphnia* sensitivity to xenobiotic stressors (Zein, McElmurry et al. 2014, Zein, McElmurry et al. 2015). *Daphnia* are ideal test organisms as they reproduce by cyclical parthenogenesis, are readily cultured in synthetic medium and sensitive to diverse xenobiotic stressors with the ability to adapt to environmental changes (Ebert 2005, Altshuler, Demiri et al. 2011).

Daphnia exposed to water with lower hardness, DOC and pH have been shown to result in a higher Cu toxicity (Bui, Do-Hong et al. 2016). The LC_{50} and EC_{50} for *D. magna* from different sources have been shown to reflect varying concentrations depending on the chemical parameters (Table 2). In natural soft water the Cu LC50 for *D. magna* ranged from 8-100 µg/L for a 48 hr assay with varying levels (0.1-5.2 µg/L of DOM) (Villavicencio, Urrestarazu et al. 2005). Bossyut et al determined the Cu 48 hr EC_{50} for *D. magna* in synthetic water ranged from 26-32 µg/L using ISO medium with 7.8 pH and hardness of 250 mg/L (Bossuyt and Janssen 2005, Bossuyt, Muyssen et al. 2005). Stoddard et al, found the Cu LC_{50} in *D. magna* to range from 25-38 µg/L (Stoddard 2007). However, Ferrando et al found the LC_{50} for a 24 hr assay to

be 0.38 mg/L a much higher value than other tests (Ferrando, Andreu-Moliner et al. 1992). These varying LC_{50} and EC_{50} toxicity levels are a function of the different parameters used in the bioassay and make comparison assessments more difficult and cumbersome for evaluating *Daphnia* toxicity in the aquatic environment.

Species	Cu Concentration µg/L	Endpoint	Hardness	DOC (mg/L)	pH	Reference
D. magna	18.1	LC50	50			Brix 2001
D. magna	30.0	EC50 - 48 hr	250	9.8		Bossuyt 2005
D. magna	40.6	EC50 - 48 hr	250	40.6		Bossuyt 2005
D. magna	53.2	EC50 - 48 hr	250	8.2		Bossuyt 2005
D. magna	380	LC50 - 24 hr				Ferrando 1992
D. magna	8.4	LC50 - 48 hr	42.7	0.1	7.9	Villavicencio 2005
D. magna	30.5	LC50 - 48 hr	42.7	1.1	7.84	Villavicencio 2005
D. magna	56.1	LC50 - 48 hr	42.7	2.1	7.85	Villavicencio 2005
D. magna	72	LC50 - 48 hr	42.7	3.1	7.87	Villavicencio 2005
D. magna	88.8	LC50 - 48 hr	42.7	4.2	7.91	Villavicencio 2005
D. magna	100	LC50 - 48 hr	42.7	5.2	7.87	Villavicencio 2005
D. magna	250-380	LC50 - 48 hr	160-180*		8.0 +/- 0.2*	Stoddard 2007

* EPA Synthetic Hard Freshwater

Table 2. Copper LC_{50}/EC_{50} for *D. magna* with varying DOC and water hardness

In this section a deeper look is taken at the mechanics of the toxicological response of *D. magna* to Cu and the interaction of dissolved organic matter (DOM) on Cu toxicity by evaluating both physiological and behavioral aspect of motor functions through two optical bioassays. It is hypothesized that (1) Cu induces sub-lethal changes in *D. magna* swimming behavior and motor functions, (2) the type and concentration of DOM in the system reduces the sub-lethal impact of Cu exposure.

Materials and Methods

Stock Solutions

Stock solutions of Cu were prepared by dissolving copper nitrate trihydrate $Cu(NO_3)_2*3H_2O$ (Ward Science, Rochester, NY) in nanopure water (>18MΩ). Serial dilutions of

Cu (0.1, 1.0 and 10.0 µM) were then made with EPA soft water (pH 7.4-7.6) prior to experiments.

From the International Humic Substances Society (IHSS), two reference humic substances were obtained: Nordic Lake Fulvic Acid (NLFA, cat no. 1R105F (Ca no 1R105H; Nordic Lake Natural Organic Matter (NLNOM) (Cat no. 1R108N). The composition of these two humic substances can be located at (http://www.humicsubstances.org/). Stock solutions of DOM were prepared with nanopure water (>18 MΩ) filtered with 0.2 µM Acrodisc syringe filters with Supor Membrane (Pall Corporation, Newquay, Cornwall, UK) and stored in a darkened refrigerator. Prior to experiments the pH of solutions were adjusted as needed to 7.6 ± 0.1. Serial dilutions of DOM were made with EPA Soft Water.

Preparation of synthetic assay media was carried out with reagent grade chemicals. Synthetic soft water assay media was prepared following U.S. EPA procedure. The synthetic water was made in nanopure water (>18MΩ) and contained 48mg/L NaHCO, 30 mg/L $CaSO_4 \cdot 2H_2O$, 30 mg/L $MgSO_4$ and 2mg/L KCl.

***Daphnia* Cultures**

Test specimens used in the study were selected from adult female laboratory clones of *D. magna* ranging in size from 2.8 to 3.4 mm. The *D. magna* clones were obtained from North Carolina State University, Department of Environmental & Molecular Toxicology in 2011. The *D. magna* were cultured in a 4-L clear glass container maintained in an incubator at 21°C with animals exposed to timed photoperiods of 16 hr light and 8 hr dark. The culture medium was synthetic lake water (COMBO) known to sustain growth of zooplankton and algae (Kilham, Kreeger et al. 1998). The synthetic COMBO water was changed on a weekly basis. The animals were fed a 50/50 mix of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardii* algae three

times per week. Animals were transitioned to EPA soft water for 1 hr prior to experiments. *Daphnia* were not fed during the optical bioassays

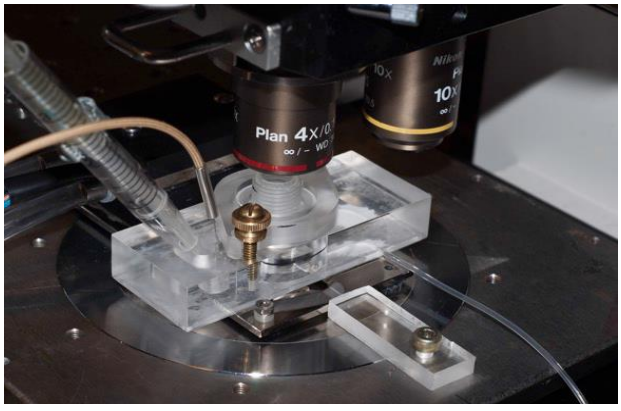
Physiology Optical Tracking Bioassay

A custom built Plexiglas® aquatic chamber (8.0 cm x 3.2 cm x 1.5 cm - LWH) was used for the physiology experiments (Figure 7). The Wayne State University, College of Engineering machine shop fabricated the aquatic chamber by drilling a 2 cm cylindrical opening in the center of a rectangular Plexiglas® base. A borosilicate glass microscopy slide sealed the bottom of the aquatic chamber with 23-gauge stainless steel hypodermic tubing forming the inlet and outlet ports allowing fluid perfusion of the chamber.

Fluid temperature in the aquatic chamber was monitored with a microprobe needle (Physitemp Instruments, Inc. Clifton, NJ) positioned at the exit end of the aquatic chamber in a small collection well. A threaded Plexiglas® cylindrical plunger with a rubber gasket sealed the opening of the perfusion chamber. Temperature adjusts within the chamber were achieved with a TS-4SPD heating and cooling stage (Physitemp Instruments) placed on top of the microstage and adjacent to the aquatic chamber.

A

B



C

D

Figure 7. Physiology optical bioassay design (A) Aquatic chamber side view. (B) Aquatic chamber top view. (C) Microscope configuration with custom built aquatic chamber, microscope, temperature probe, inlet and outlet tubes. (D) *Daphnid* in the aquatic chamber.

A single adult *D. magna* female was isolated and secured dorsal side up on a 10 mm piece of 33-gauge steel tubing positioned parallel to the anterior-posterior body axis using < 1 nL cyanoacrylate glue. The steel tubing was glued to the *Daphnid* head shield slightly posterior to the eye and anterior to the heart (Figure 7D). After allowing the glue to cure, the 33-gauge steel tubing with the animal attached was positioned into a 26-gauge steel tube fixed inside of the aquatic chamber. The chamber was filled with EPA soft water and closed with the threaded plunger. Once positioned in the aquatic chamber, animals freely moved swimming antennae and appendages. The temperature of the flow cell fluid was maintained at 20.0 °C ± 0.2. Animals were allowed to acclimate to the new environment for 10 minutes prior to obtaining video files.

Animals in the aquatic chamber were viewed with a Nikon FN600 light microscope with a 4X Nikon objective and an on-screen magnification of approximately 100X using a Lumenera Infinity 2M-1 monochrome camera (Ottawa, Canada) (Figure 7C). The aquatic chamber was connected to a Pharmacia LKB P-1 pump set at flow rate of 0.67 mL per minute and maintained at that rate for the duration of the experiment. Internal organs of the animal were observable

through the translucent exoskeleton allowing quantification of motor activities including heart rate (HR) and appendage beat rate (ABR). Motor activity was analyzed using Image Pro Premier 9, two dimensional (2-D) tracking software (Media-Cybernetics, Inc, Rockville, MD). Video recordings were captured at 10 minute intervals for the duration of 10 seconds each. Changes in motor activity were quantified by placing small regions of interest (ROI) within a specific animal movement and tracking optical density- intensity oscillations.

Statistical analysis of the data was performed using Statistica software (Statsoft Version 13.1) 2016 Tulsa, OK). Time (min) was used as the repeated measure for the analysis of motor activity. The dependent variables were HR and ABR. The independent variables were the categorical factors of Cu concentration, DOM concentration and DOM type. In selected cases, contrast analysis was used to compare a series of means across groups after the initial ANOVA analysis.

Behavioral Optical Tracking Bioassay

Prior to behavioral optical tracking bioassays, *Daphnia* were extracted from the COMBO solution and size selected with a mesh screen for uniformity of length (>1.4 mm) and approximate comparable age. For each assay, a single *Daphnid* was randomly selected and placed in an isolated chamber of a clear polyethylene plastic 12-well plate (Corning Costar, Sigma-Aldrich) using a pipette. Any fluid transferred with the animal was suctioned out to minimize dilution of the test medium. The individual chambers held a maximum of 7.0 mL of fluid with a surface area of 416 mm^2 exposed to the air. The well size of the 12-well plate allowed for free, if slightly limited vertical and horizontal swimming behavior of the *D. magna*. Upon placement of the animals in the 12 wells, the test medium in various concentrations and combinations was pipetted into the individual chambers. It was randomly determined which

chamber received a specific concentration of the test medium. The entire setup of the 12-well plate took approximately 10 min.

The 12-well plate was then transferred to an elevated Plexiglas® platform with an LED Light Pad A920 (Art Graph) positioned underneath. Ambient temperature of the laboratory averaged 21°C. *Daphnia* movements were tracked with video recordings using an Infinity 2-1M monochrome camera (Lumenera Corporation, Ottawa, Canada) with a telecentric lens, (Opto Engineering, Houston, TX) positioned above the platform at a fixed distance of 56 cm from the well plate surface (Figure 8).

Figure 8. Behavioral bioassay configuration (camera, lens and 12-well plate).

Daphnia movement was captured with live video recordings and analyzed with Infinity Capture software (Lumenera) and saved in AVI format. Video data was analyzed with Image Pro Premier 9.1 software (Media Cybernetics) using two dimensional (2-D) tracking. Video recordings were obtained over a time frame of 24 hr with 5 sec videos (145 frames) recorded

every 10 min for the initial 3 hr, followed by video recordings every 1 hr for the next 3 hr, then every 2 hr for the next 6 hr and final recordings were obtained every 4 hours for the last 12 hr.

Parameters analyzed and quantified in this bioassay evaluate swimming behavioral changes, including the maximum accumulated distance the *Daphnia* traveled during the experiment and the directional mean angle change of the *Daphnia (*Figure 9). The maximum accumulated distance was determined by calculating the difference of the *Daphnid* position between 2 sequential frames over the course of the experiment and measured in mm. The video frame rate was 28-29 frames/s. The *Daphnid* mean angle change measured in degrees was calculated using the movement detected over 3 consecutive frames. The positional changes were derived from the first vector determined between frames 1 and 2 with the second vector derived from frames 2 and 3. The difference in direction between vector 1 and vector 2 determines the mean angle change of the individual *Daphnid* (Zein, McElmurry et al. 2014).

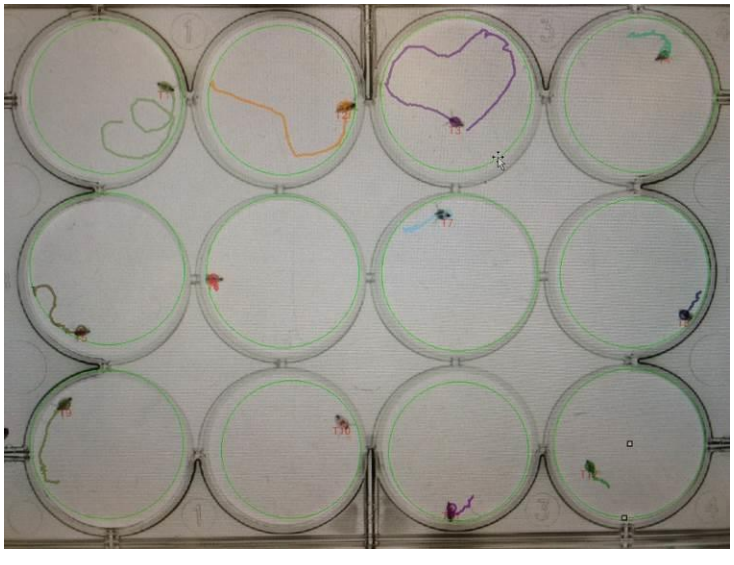


Figure 9. Behavioral bioassay 12-well plate with software tracking.

Two 12-well plate designs were utilized for the behavioral bioassay with each plate design repeated 6 times (n=6). For each plate design different chemical compositions were placed in each individual well and included EPA soft water, NLFA (100 and 1000 µM) NLNOM

(100 and 1000 µM), Cu (0.1, 1.0 and 10 µM), NLFA spiked with Cu, NLNOM spiked with Cu. The Cu to DOM ratios for combined chemical compositions ranged from 1:10 to 1:10000 and were kept in a darkened refrigerated at 4° C in glass beakers for 5 days prior to experimentation.

Statistical analysis was performed using repeated measures of analysis of variance (ANOVA) with time (min) as the repeated measure using Statistica software (Statsoft Version 13.1). Accumulated distance (mm) and mean angle (deg) were the dependent variables. The independent variables were Cu concentration, DOM concentration and DOM type (NLFA, NLNOM). Contrast analysis was used to compare a series of means across groups after ANOVA in selected cases to determine statistical significance.

Results and Discussion

Behavioral and physiology optical bioassays have the ability to provide more in-depth information on what is occurring within a target organism by evaluating alterations in swimming behavior and cardio-respiratory changes in *Daphnia*. For the behavioral bioassay, maximum accumulated swimming distance and mean angle change were the parameters providing the critical information on *Daphnia* exposure to Cu in aqueous solutions. EPA synthetic soft water was selected as the test medium as toxicity increases with a reduction in water hardness allowing potential changes to be observed more readily. The Cu concentrations selected for this the behavioral and physiological bioassay included 0.1 µM which is below the LC_{50}, 1.0 µM which is in LC_{50} range and 10 µM Cu which is well above the LC_{50}.

Daphnia response to Cu exposure and DOM mitigation of Cu toxicity is shown to have a strong concentration dependent response as seen by the repeated measures ANOVA statistical analysis. The behavioral bioassay analysis evaluating changes in *Daphnia* swimming angle is shown in Table 3 with 11 out of 15 effects significantly different (1 effect $p<0.05$, 10 effects $p<0.001$). The results for the behavioral cumulative distance analysis for swimming changes is

located in Appendix B, Table B-2 with 9 out of 15 effects found to be significant (5 effects $p<0.05$, 4 effects ($p<0.001$).

Behavioral Mean Angle

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
DOM Concentration (Conc)	629	1	629	0.2	0.680
Cu Conc	1560000	3	520000	141.4	0.000*
DOM	172000	2	86100	23.4	0.000*
DOM Conc x Cu Conc	9223	3	3074	0.8	0.476
DOM Conc x DOM Type	42900	2	21500	5.8	0.004*
Cu Conc x DOM Type	219000	6	36400	9.9	0.000*
DOM Conc x Cu Conc x DOM Type	31500	6	5246	1.4	0.210
Time	569000	28	20300	36.5	0.000*
Time x DOM Conc	35500	28	1269	2.3	0.000*
Time x Cu Conc	560000	84	6666	12	0.000*
Time x DOM Type	69200	56	1235	2.2	0.000*
Time x DOM Conc x Cu Conc	102000	84	1213	2.2	0.000*
Time x DOM Conc x DOM Type	33800	56	603	1.1	0.313
Time x Cu Conc x DOM Type	148000	168	882	1.6	0.000*
Time x DOM Conc x Cu Conc x DOM	124000	168	736	1.3	0.004

Table 3. Behavioral bioassay: Mean angle change with ANOVA repeated measures analysis. Cu concentration (0, 0.1, 1.0, 10 µM) x DOM concentration (100 and 1000 µM) x DOM type (NLFA and NLNOM) and time (min). Effects that have a significant effect are identified with an asterisk (*).

The behavioral assay evaluating the effect of Cu on degree of angle change over time is illustrated in Figure 10, with $F(84, 3360) = 11.97$ ($p<0.001$), ($n=6$). The Cu concentrations used in the analysis were 0, 0.1, 1.0 and 10 µM with a time course of 1440 min and included the mitigating effect of DOM. Six 12-well plates were run for each DOM concentration for a total of 12 plates. A strong concentration dependent response with time was seen for 1.0 and 10 µM Cu concentrations.

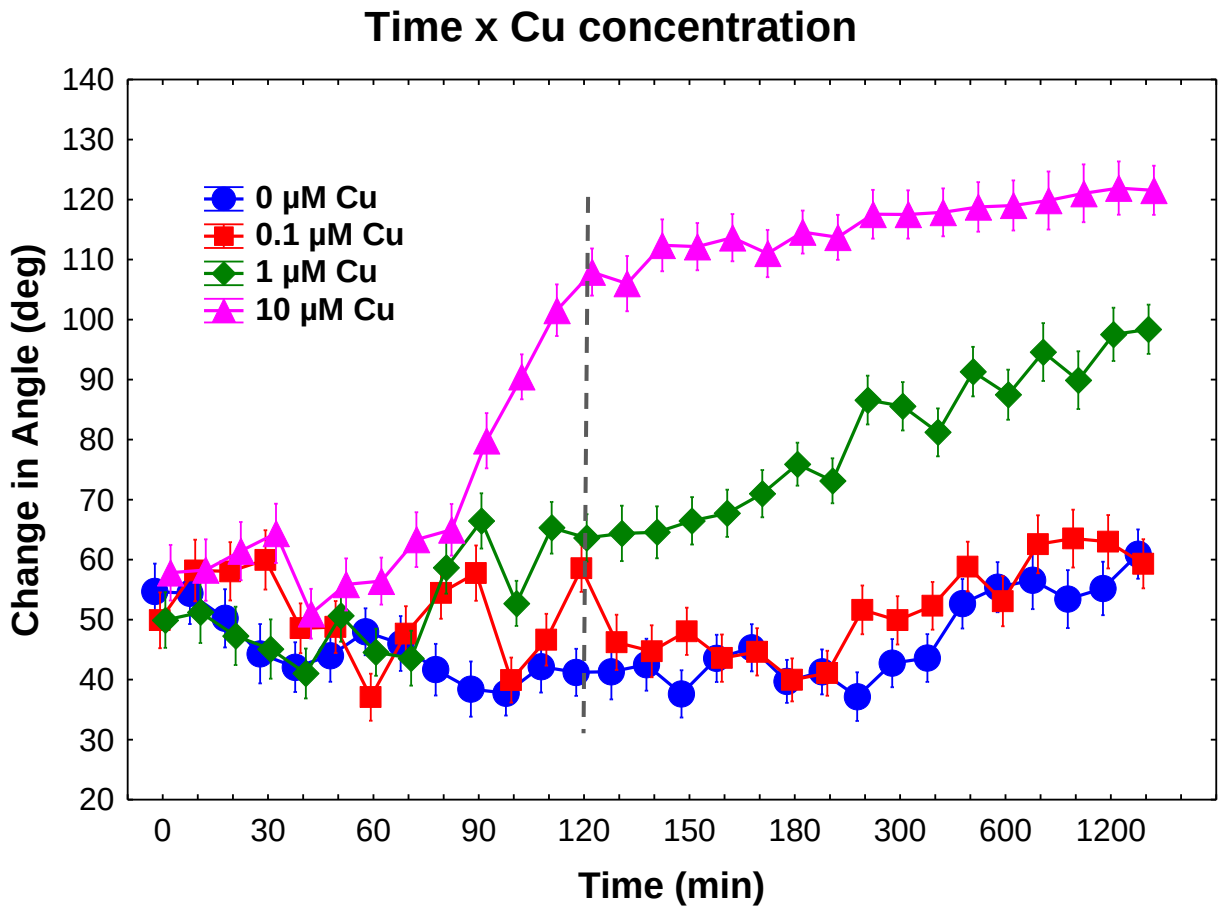


Figure 10. Behavioral bioassay: Mean angle change: in swimming behavior repeated measures ANOVA analysis (n=6) (p<0.001) Time x Cu concentration with a DOM mitigating effect. Dashed line indicates where contrast analysis started. Error bars ± SEM.

Contrast analysis between the soft water control and 1.0 and 10 µM Cu concentrations on change of angle in swimming behavior was significantly different ($p<0.001$) for both. The statistical analysis began at the dashed line on the graph, where significant changes were occurring in mean angle change or had initially plateaued. The 0.1 µM Cu closely matched the control between the 120-180 min interval, however, it varied from the control in the beginning of the time course with oscillations observed in mean angle. Contrast analysis between the control and 0.1 Cu was not significantly different starting at the dashed line, but was significant when all data points were included ($p<0.05$).

Table 4 shows the ANOVA statistical analysis for the mean angle change behavioral assay, with the analysis evaluating Cu conc with no DOM impact. All three effects analyzed were statistically significant ($p<0.001$).

Behavioral Mean Angle Change: Cu Only

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
Cu Concentration	1010000	3	338000	74.71	0.00*
Time	348000	28	12400	22.84	0.00*
Time x Cu Concentration	356000	84	4239	7.8	0.00*

Table 4. Behavioral bioassay: Mean angle change with ANOVA repeated measures analysis. Cu concentration (0, 0.1, 1.0 10 µM) x Time (min) with time as the repeated measure. Effects with a significant effect are identified with an asterisk (*).

The behavioral effect of Cu on degree of angle change over time is illustrated in Figure 11. A significant effect of Cu was observed with concentration-dependent changes in mean angle. The Cu concentrations used in the analysis were 0, 0.1, 1.0 and 10 µM with a time course of 1440 min (Time x Cu Conc) $F(84, 1904) = 7.80$, ($p<0.001$), ($n=6$). The effect of Cu exposure on the swimming behavior change of angle was significantly different from the soft water control using contrast analysis ($p<0.001$) for each Cu concentration. The dashed line on the graph indicates a selected point where active mean angle changes were occurring across all Cu concentrations with contrast analyses starting from this point using last 15 data points.

The 0.1 µM Cu concentration began at 50-70 deg mean angle change and was steadily higher than the control at 40-60 deg mean angle change. The control exhibited minor variations in mean angle change but remained stable through the time course. The 0.1 µM concentration exhibited strong oscillations in degree change throughout the bioassay with amplitude changes of 10-20 deg. The oscillations appeared to be a sub-lethal stimulatory effect on the *Daphnia* at 0.1

µM concentration, however; this effect was not observed with the control or 1.0 and 10 µM Cu concentrations.

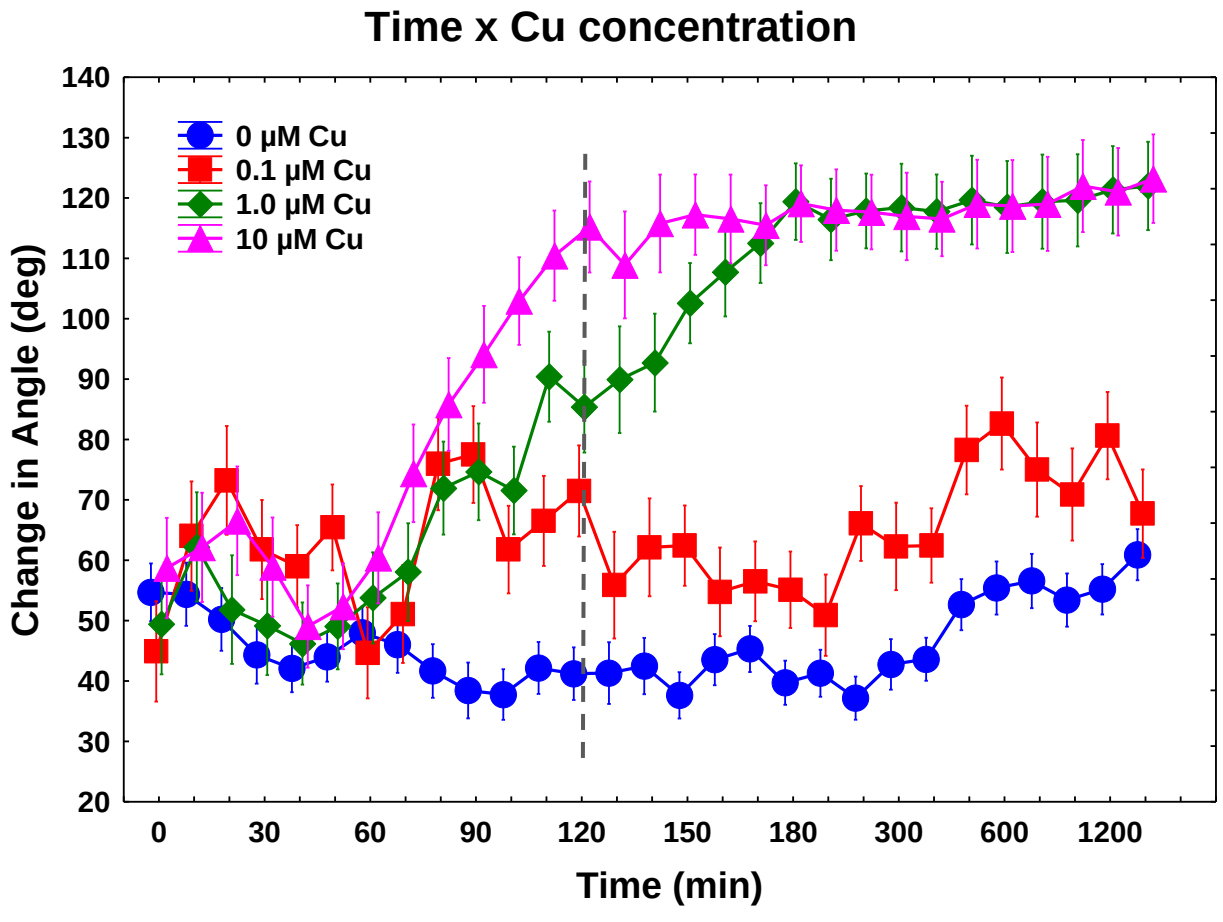


Figure 11. Behavioral bioassay: Mean angle change in swimming behavior repeated measures ANOVA analysis (n=6) (Time x Cu concentration) with no DOM effect ($p<0.001$). The dashed line marks where additional contrast analysis started for Cu concentrations. Error bars ± SEM.

The 1.0 µM Cu concentration followed the control fairly closely for the first 60 min with a mean angle change of between 50-60 deg. The 1.0 µM Cu concentration developed oscillations pattern between 70 and 120 deg followed by a steady rise in mean angle until a plateau was reached at approximately 180 min indicating animal movement was limited. Contrast analysis indicated that 1.0 µM was significantly different from 0.1 µM ($p<0.001$), but not significantly different from 10 µM ($p=0.122$). The 10 µM and 1.0 µM effects showed a

similar curve, but the 10 µM exhibited a threshold shift to the left with mean angle plateauing at 110 deg in 140 min. This threshold shift was the result of alterations in mean angle change induced earlier in the time course with the increased toxicity of the 10 µM Cu concentration.

In the physiology bioassay Cu elicited a strong concentration and time dependent cardio-respiratory effect on the *Daphnia* as illustrated in Figure 12. Cu concentrations (0. 0.1 and 1.0 and µM) x Time (min) x Parameter effect (HR and ABR) were evaluated in this analysis $F(42, 315) = 2.78$, ($p<0.001$), ($n=6$). Video files were collected every 10 min for 180 min once treatment was initiated. ABR began to be suppressed within 20 min of the initiation of the chemical infusion at Time 0. The 1.0 µM Cu experienced a dramatic decline after 60 min of chemical exposure. The ABR continued to drop with a steep negative slope leveling off around 120 min and the ABR sweep suppressed within 180 min. As a means to quantify this motor function, ABR was not counted once the appendages no longer made a complete sweep across the *Daphnia* body, although, the appendages may have continued to move slightly.

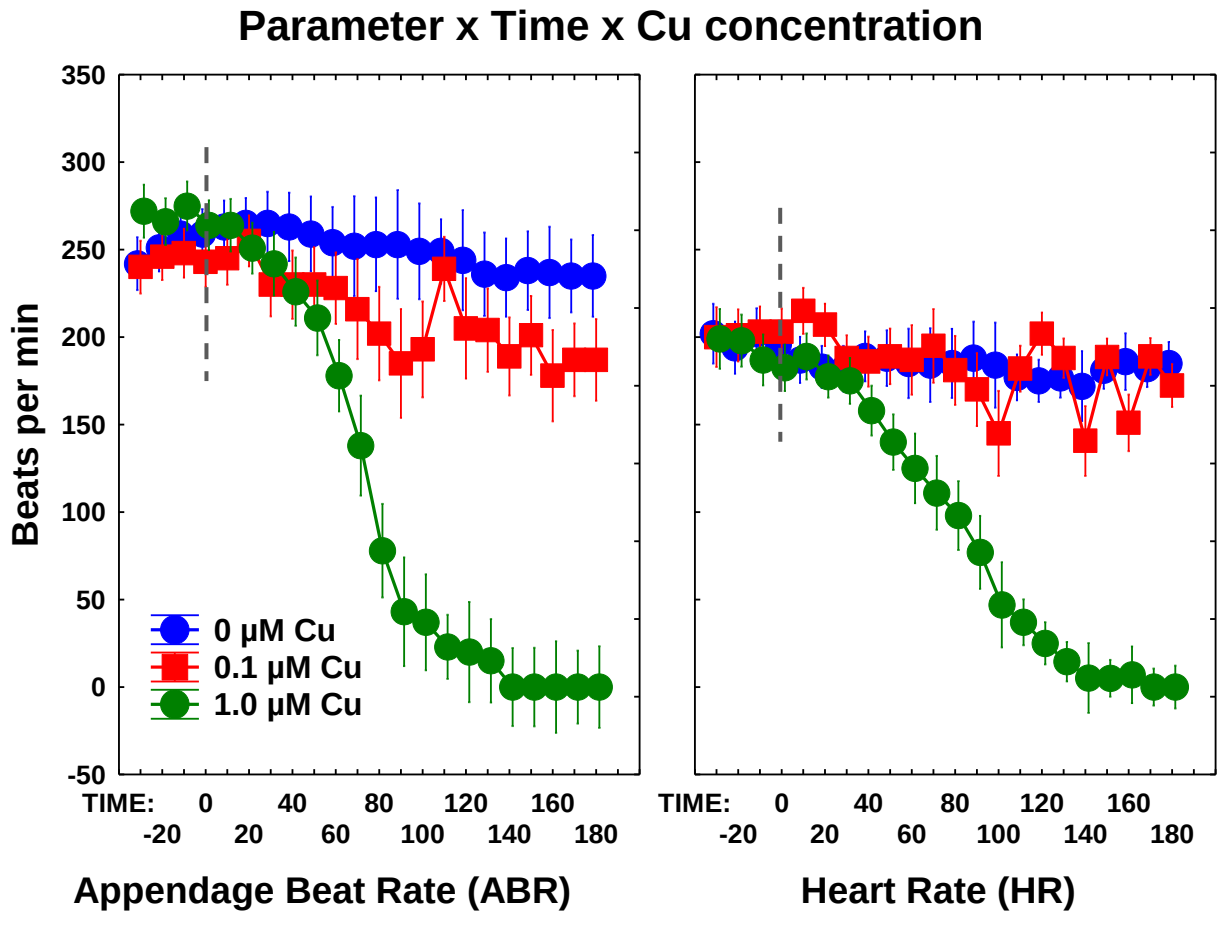


Figure 12. Physiology bioassay (n=6) ANOVA repeated measures analysis with Time (min) x Cu concentrations (0, 0.1, 1.0 µM Cu). Parameters: appendage beat rate (ABR) and heart rate (HR) parameters ($p<0.05$). The vertical dashed bar indicates when Cu infusion started and analysis began. Error bars ± SEM.

The 1.0 µM Cu effect on HR was similar to the ABR; however, the decline in HR was not as precipitous, starting after 40 min of Cu exposure and with a slightly more gradual slope in the beats per minute (BPM) suppression. The HR continued to decline steadily until reaching 140 min where it leveled off with the heart slowly and intermittently beating with apparent death due to cardiac arrest. The soft water control groups for ABR and HR were stable at approximately 250 and 200 BPM respectively with contrast analysis between the HR and ABR controls showing them to be significantly different at $p<0.05$.

Although, the 0.1 µM Cu exposure appeared to be steadily below the control ABR, contrast analysis indicated they were not significantly different ($p=0.21$). The dashed lines indicate when the Cu infusion started (Time 0) with analysis starting at the same point. HR at 0.1 µM Cu was also not significantly different from the control with ($p=0.99$). However, the 0.1 µM Cu exposure did affect the *Daphnia* HR with regular oscillations appearing after 60 min of Cu exposure not seen in the control. The oscillation pattern showed the HR declining and then accelerating above the control level, then repeating the pattern. This pattern was not flattened with multiple *Daphnia* ($n=6$) at 0.1 µM Cu concentration making it a cardio-respiratory phenomenon occurring at a sub-lethal exposure concentration. This oscillation phenomenon was also observed with ABR but with a lower peak amplitude. This pattern is likely a compensation mechanism to offset the sub-lethal Cu toxicity occurring within the *Daphnia* and was also observable in the behavioral bioassay (Figure 11) at the same concentration.

To evaluate whether *Daphnia* could potentially recover from Cu exposure, animals were exposed to 30 min and 60 min of 1.0 µM Cu, when toxicity changes were quantifiable (reduction in ABR and HR). At these time points Cu infusion was halted and switched to EPA soft water only with no Cu exposure for the duration of the experiment. Although toxicity was delayed for 1-2 hr it was not halted or reversed. Once Cu toxicity induced alterations to ABR and HR were initiated, the process was slowed by converting back to soft water, but not reversible with the endpoint remaining arrest of ABR and HR. This non-reversibly process is potentially a function of metabolic oxidative stress resulting in alterations of oxidative enzyme concentrations in the *Daphnia* (Yoo, Ahn et al. 2013).

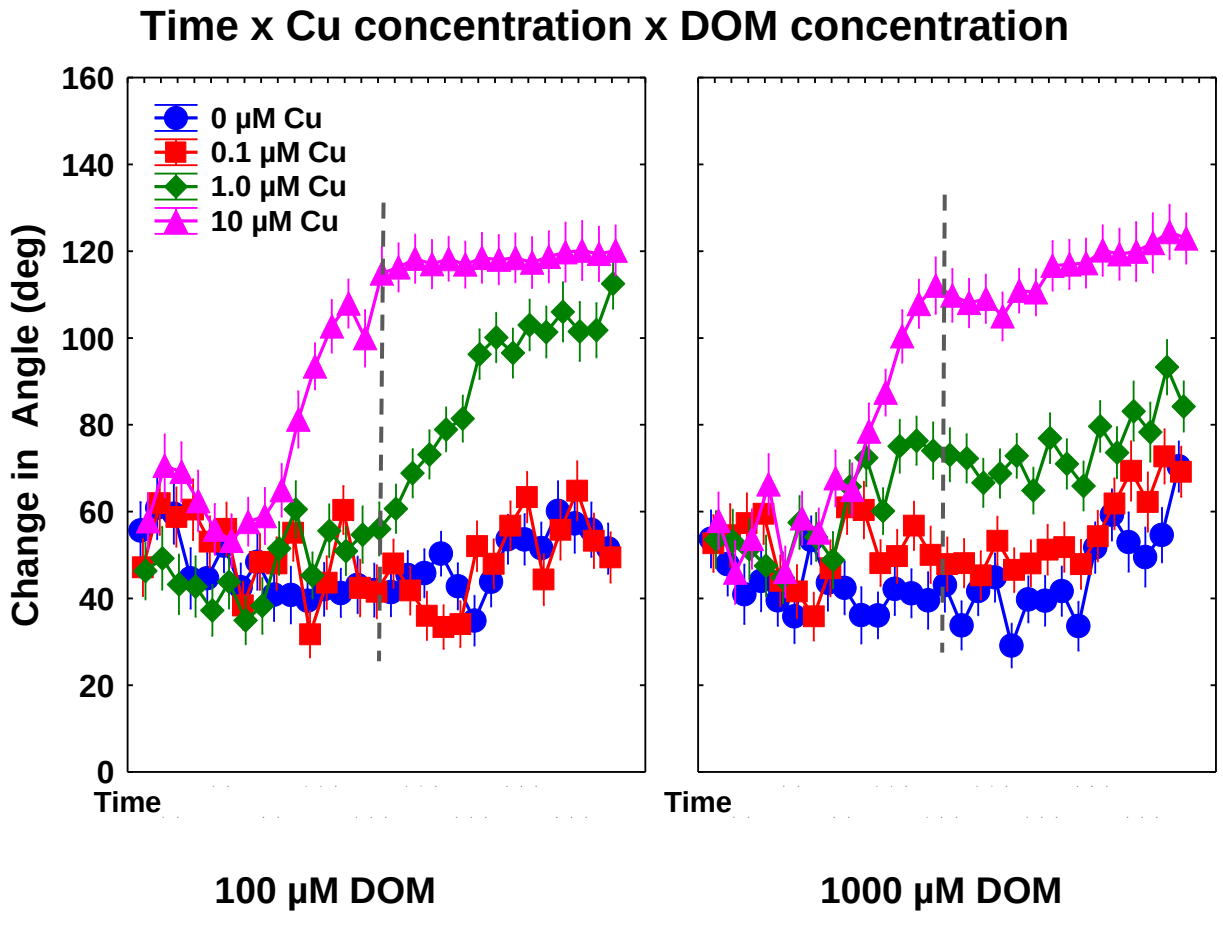


Figure 13. Behavioral bioassay (n=6) ANOVA repeated measures analysis for mean angle changes in *Daphnia* swimming behavior over time (min) with varying DOM and Cu concentrations (p<0.001). Contrast analysis was truncated to include the 15 end data points when Cu toxicity was rapidly changing or had initially plateaued. Error bars ± SEM.

The effects of Cu and DOM exposure on swimming behavior changes in degree of angle (deg) are illustrated in Figure 13 which shows Time (min) x DOM concentration (µM) and Cu concentration (µM) F (84, 3360) = 2.18 (p<0.001), (n=6). *Daphnia* in the control group were relatively stable between 40-60 degrees of angle change for both 100 and 1000 µM DOM. The 0.1 µM Cu line in 100 µM DOM concentration had periods of oscillation above and below the control. Contrast analysis between 0.1 µM and the control indicates that although there were regular oscillations the two lines were not significantly different (p=0.702). However, the *Daphnia* exposed to 0.1 µM Cu in 1000 µM DOM showed mean angle change steadily above the

control group with a contrast comparison showing a significant difference ($p<0.05$) between them. Contrast analysis was performed with the last 15 data points for this behavioral analysis at the point when mean angle was steadily changing or had initially plateaued across Cu concentrations. This increase in mean angle change at 0.1 µM may reflect a stimulatory effect of the higher 1000 µM DOM concentration on the *Daphnia* swimming behavior or a combined effect with the Cu that was not observed at 100 µM.

The 1.0 µM Cu exposure group in 100 µM DOM began to exhibit an increase in mean angle beginning at 40 deg at 110 min. The change in angle steadily increased until reaching approximately 110 degrees at 1440 min. The 1.0 µM Cu group in 1000 µM DOM at 110 min was at 60 deg and remained stable between 60 and 80 degrees for the duration of the experiment. This early increase to 60 degrees may reflect a stimulatory effect of DOM as it was not seen in the 100 µM DOM group. In the case of the 1000 µM DOM group with 1.0 µM Cu, the angle change increased earlier than the 100 µM DOM but remained stable across the experiment showing the protective effect of the DOM on the *Daphnia,* with Cu-DOM complexation reducing Cu exposure and toxicity. The 100 µM DOM with 1.0 µM Cu mean angle changes started later, but increased much more rapidly reflecting saturation of DOM binding sites as more Cu remained in solution becoming toxic to *Daphnia*. At 1000 µM DOM the 1.0 µM Cu showed a strong protective effect when compared with 100 µM DOM ($p<0.05$).

The 10 µM Cu concentration curves look similar in both 100 and 1000 µM DOM with the angle change starting at 55 deg at 60 min and rapidly escalating to 120 deg at 140 min. The similarity in the Cu concentration curves indicates that Cu was in excess of DOM binding sites for both concentrations leaving Cu toxicity unmitigated. Each concentration shows a slight dip in mean angle change at about 140 min. This alteration may be an attempt to compensate for Cu toxicity and potential oxidative changes, but ultimately the animal was unable to overcome the

Cu toxicity. Contrast analysis between 1.0 and 10 µM Cu at both 100 and 1000 µM DOM concentrations, indicated significant differences ($p<0.001$).

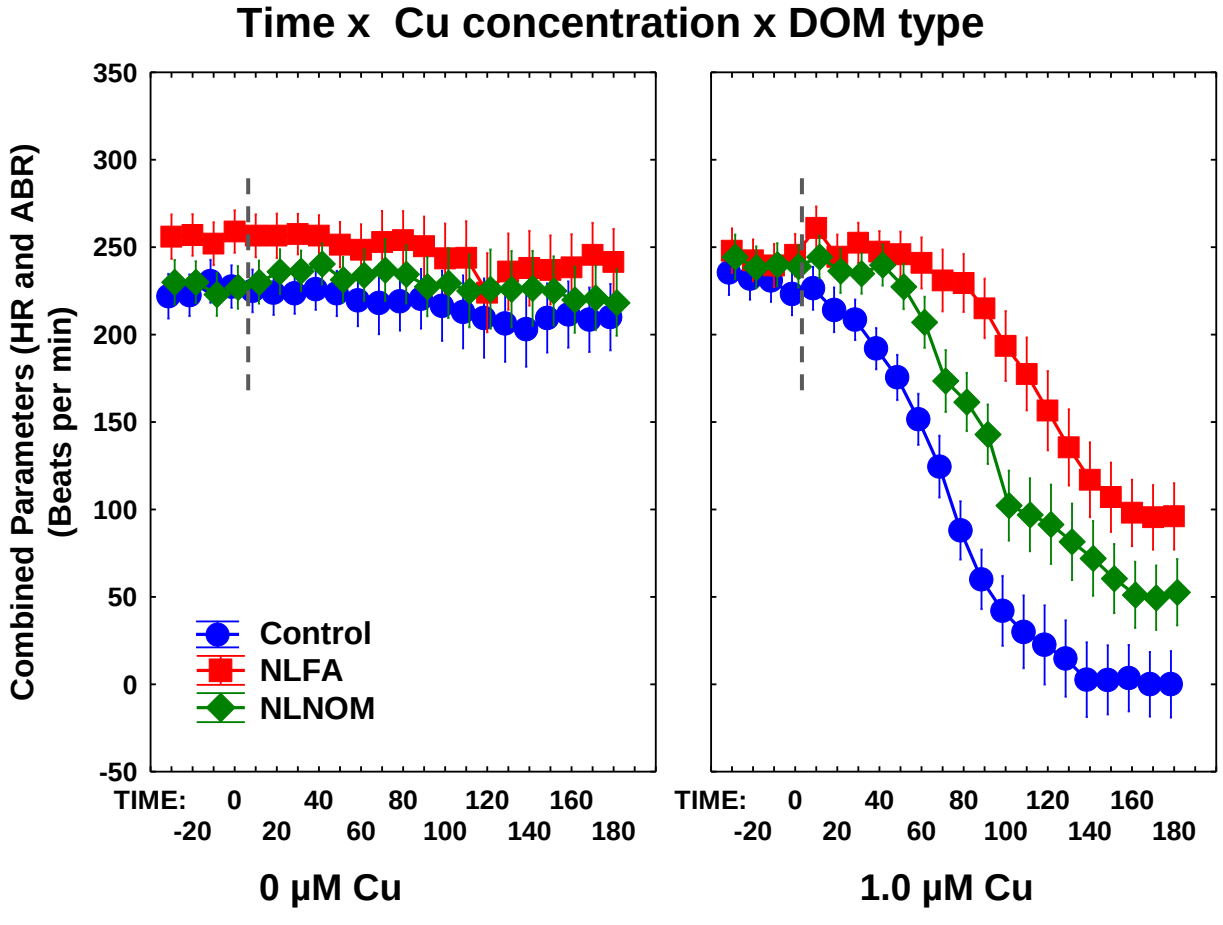


Figure 14. Physiology cardio-respiratory bioassay (n=6) at 100 µM DOM shows a mitigating effect on Cu toxicity over time (min) ($p<0.001$). The vertical dashed line represents where analysis began at time 0, at the point of Cu infusion. Error bars ± SEM.

The physiology bioassay results in Figure 14 shows a strong mitigating effect of the DOM (100 µM) on Cu toxicity, $F(42, 1260) = 3.38$, $p<0.001$, (n=6). The graph reflects cardio-respiratory changes with DOM type over time (min) with 0 µM and 1.0 µM Cu concentrations. A total of 36 animals were run through this bioassay with 6 animals for each DOM type and the control. A mitigating effect of DOM type on Cu toxicity is shown with 1.0 µM Cu exposure. NLFA provided the most protection from Cu toxicity, followed by NLNOM. Contrast analysis

showed a significant difference between NLFA and NLNOM ($p<0.05$). Significant differences were also noted between the control and DOM types, NLNOM ($p<0.05$) and NLFA ($p<0.001$). The vertical dashed line represents where analysis began at time 0, at the point of Cu infusion.

Although the DOMs and control stratify in a similar pattern with 0 µM Cu exposure, no significant difference was observed with contrast analysis between DOM types or between the DOMs and the control. NLFA with 1.0 µM Cu showed the beginning of ABR and HR suppression at 80 minutes with cardio-respiratory parameters leveling off at 100 BPM at the 160 min time point. NLNOM with 1.0 µM Cu started to reflect a decline in the same physiological parameters at 60 min and leveled off at 60 BPM after 160 min. The control group with 1.0 µM Cu exposure exhibited a more rapid decline in cardio respiratory parameters with suppression initiated at 20 min and leveling off at 10 BPM at 140 min.

This physiological bioassay analysis clearly showed that the two types of DOM, both impacted Cu toxicity by mitigating the metal's effect on *Daphnia* motor functions with NLFA having the strongest effect on limiting Cu toxicity, followed by NLNOM. This corresponded well with data obtained by FSCV analysis of Cu-DOM complexation with fulvic acids binding more than natural organic matter in Chapter 3. The NLNOM DOM likely bound less Cu due to differences in binding sites that result from not being as extensively refined during the fractionation process as NLFA. Critical information about cardio-respiratory effects taking place within the aquatic organism was obtained with this physiology optical bioassay.

Conclusion

This body of work illustrated that the behavioral and physiological bioassays used here have the ability to detect sub-lethal changes in *Daphnia* swimming behavior and motor functions when exposed to Cu. Alterations in ABR and HR were observed as well as changes in angle and maximum distance travelled at sub-lethal Cu concentrations. This study confirmed the initial

hypotheses by demonstrating that the type of DOM impacts Cu toxicity and that Cu, as an ecotoxicological stressor, is mitigated by the type and concentration of DOM present in the system with fulvic acid having a stronger mitigating effect than natural organic matter. This correlated well with what was observed in Chapter 3 which showed that NOMs bound less Cu than the fulvic acid or humic acids leaving more Cu in solution potentially increasing *Daphnia* Cu exposure.

CHAPTER 5: ASSESSING NEUROTRANSMITTER RELEASE IN *DAPHNIA* USING FAST SCAN CYCLIC VOLTAMMETRY

Introduction

Neurotransmitters are endogenous chemicals that carry signals along neuronal pathways (Hay-Schmidt 2000, Weiss, Tollrian et al. 2012). These biogenic signaling molecules control central physiological and behavioral processes in both vertebrates and invertebrates, including the microcrustacean *Daphnia* (McCoole, Atkinson et al. 2012, Robert, Monsinjon et al. 2016). *Daphnia* are a model species for ecotoxicological aquatic investigations and evolutionary genomics sharing a substantial genome homology with humans (Gunnarsson, Jauhiainen et al. 2008, McCoole, Baer et al. 2011, Seda and Petrusek 2011). The *D. pulex* genome was mapped in recent years, generating a cascade of research from this complete DNA sequencing (Shaw, Colbourne et al. 2007, Colbourne, Pfrender et al. 2011).

The *D. pulex* genome has demonstrated that recognized neurotransmitters in other crustaceans; histamine, serotonin, dopamine and octopamine, also have conserved regions in *Daphnia* (Lorenzon, Edomi et al. 2005, Christie, McCoole et al. 2011, McCoole, Baer et al. 2011, McCoole, Atkinson et al. 2012). Neurotransmitters play an important signaling role in the visual system of arthropods (Elias and Evans 1983, Stuart, Borycz et al. 2007, Rivetti, Campos et al. 2016). *Daphnia* nervous, visual and motor systems are important components of the phototacic response; however, relatively little is known about the neurochemistry of these systems and how they function in conjunction with each other (McCoole, Atkinson et al. 2012).

Diel vertical migration is a pattern of movement of *Daphnia* in the aquatic environment (Cousyn, De Meester et al. 2001, Ebert 2005). In response to light stimuli negative genotypes move down and away from the stimuli as a predator avoidance trait (Poupa 1948, Lampert 1993, Ringelberg 1995, Dodson, Tollrian et al. 1997). Alterations in this phototactic behavior are

maladaptive, making affected organisms more vulnerable to predators and thus, modifying the predator-prey relationship in the aquatic ecosystem (Lampert 1993, Dodson, Ryan et al. 1997, Cousyn, De Meester et al. 2001).

It has been illustrated that histaminergic signaling is extensive in *Daphnia* and plays an active role in phototactic behavior (McCoole, Baer et al. 2011). Serotonin also appears to function as part of the *Daphnia* phototactic response with suppression of phototaxis identified in *Daphnia* exposed to Fluoxetine, (IUPAC: N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine), a selective serotonin reuptake inhibitor (SSRI) (Rivetti, Campos et al. 2016). SSRIs treat clinical depression by blocking serotonin reabsorption at neural synapses in the brain, allowing more serotonin to remain available for re-circulation. Exposure to sub-lethal Cu concentrations has shown to reduce phototactic response in *Daphnia* (Michels, Leynen et al. 1999, Yuan, Michels et al. 2003).

FSCV at carbon microelectrodes has been used successfully to detect neurotransmitters in animal brains (Hashemi, Dankoski et al. 2009, Lama, Charlson et al. 2012, Wood and Hashemi 2013). Using optimized waveforms histamine and serotonin have been identified and quantified in small mammals in-vivo (Samaranayake, Abdalla et al. 2015). Adapting this technique to detect serotonin, histamine and potentially other neurotransmitters in *Daphnia* would greatly enhance understanding of these biogenic amines in-vivo and the role they play in phototactic behavior. The neuroanatomy of the optic ganglia and central brain of *D. magna* has been recently been explored in depth (Kress, Harzsch et al. 2016). *D. magna* have a single compound eye, with 22 individual ommitidium units sending a branching optic nerve composed of axon bundles into the lamina region of the optic ganglia that then descends into separated optic tecta (Sims and Macagno 1985, Smith and Macagno 1990, Kress, Harzsch et al. 2016). The optic nerve axons in the lamina have been shown to be histamine immunoreactive with this property used to map

histamine pathways in *D. magna* (McCoole, Baer et al. 2011, Kress, Harzsch et al. 2016). Serotonin and histamine have been found in similar regions of the brain of other animals and have been shown to be co-released upon stimulation (Moore, Halaris et al. 1978, Hashemi, Dankoski et al. 2011). Similar pathways for serotonin were likely to be present in the optic ganglia if this neurotransmitter was involved with phototaxis.

The goal of these experiments was to evaluate the hypotheses that (1) a light stimulated neurotransmitter release can be detected in *Daphnia* using the FSCV analytical technique, (2) Cu exposure will reduce *Daphnia* phototactic response by reducing neurotransmitter release with light stimulation and (3) that histamine and serotonin like neurochemicals are an integral component of the *Daphnid* phototactic response and released with light stimulation.

Materials and Methods

Stock solutions

Preparation of synthetic assay media was carried out with reagent grade chemicals. Synthetic soft water assay media was prepared following U.S. EPA procedure. The synthetic soft water was made in nanopure water (>18MΩ) and contained 48mg/L NaHCO, 30 mg/L $CaSO_4 \cdot 2H_2O$, 30 mg/L $MgSO_4$ and 2mg/L KCl.

Daphnia Cultures

Adult female laboratory clones of *D. magna* were size selected for this research application and ranged in size from 2.8 to 3.4 mm. The *D. magna* clones were obtained from North Carolina State University, Department of Environmental & Molecular Toxicology in 2011. Cultured in a 4-L clear glass container the *Daphnia* clones were maintained in an incubator at 21°C with animals exposed to photoperiods of 16 hr light and 8 hr dark. Known to sustain growth of algae and zooplankton, *Daphnia* clones were raised in a culture medium of synthetic lake water (COMBO) (Kilham, Kreeger et al. 1998). The synthetic COMBO solution was

changed weekly with animals fed a 50/50 blend of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardii* algae three times per week. Animals were transitioned to synthetic soft water for 1 hr prior to experiments. *Daphnia* were not fed during the FSCV analytical procedure.

Preliminary Studies

Preliminary studies were undertaken to investigate laboratory *D. magna* response to light stimulation to determine potential phototactic response. Five *D. magna* clones were placed into six glass cylinders with 70 ml of synthetic lake water (Figure 15).

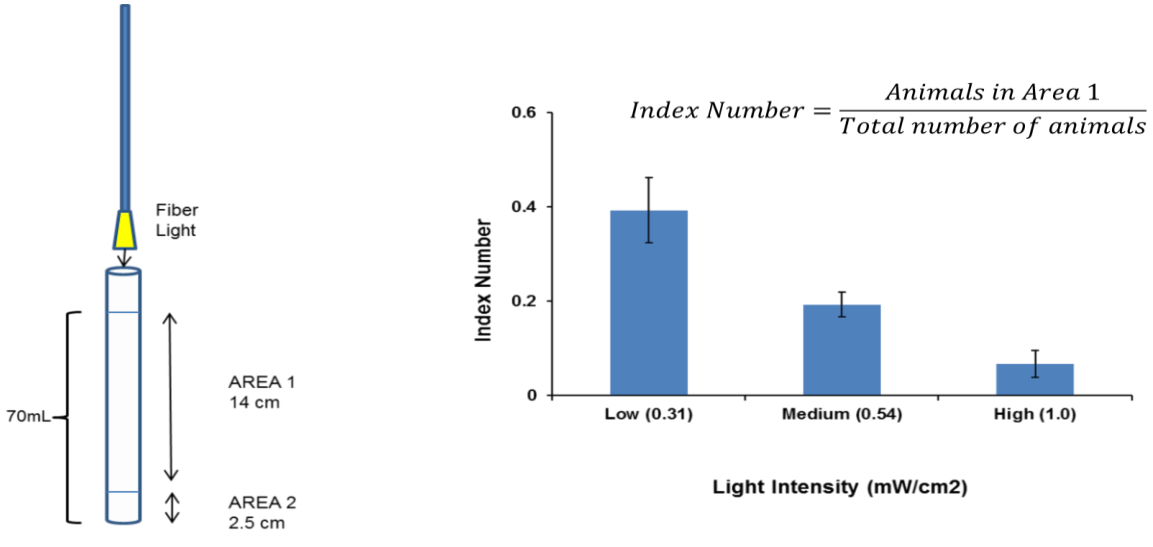


Figure 15. *D. magna* clones phototactic response to three levels of light stimuli (n=6) at 1-minute intervals over a 10 min continuous light exposure with significant differences between high and low intensity ($p<0.01$). Error bars ± SEM.

The cylinders were divided into two compartments: Area 1 (height of 14 cm) and Area 2 (height of 2.5 cm). Phototactic behavior was quantified by assessing the animals in Area 1 and dividing by the total animals in the cylinder creating an Index Number. The design of this experiment was based on a previous assay by Martins et al (Martins, Soares et al. 2007). Three levels of light intensity were used (high: 1.0, medium: 0.54 and low: 0.31 mW/cm^2). Animals in each area were determined at 1-min intervals over a 10 min period of continuous light exposure. The average Index Number for high intensity light (0.067 ± SEM 0.02), medium light (0.19 ±

SEM 0.03) and low light (0.39 ± SEM 0.16). A statistically significant difference was observed between all levels of light intensity (n=6) using t-test paired samples for means: high and low intensity light ($p<0.01$), high and medium ($p<0.01$), medium and low ($p=0.05$). The results indicated these laboratory *D. magna* clones had a negative phototactic response to light stimuli.

D. magna clones utilized for this experiment were also exposed to sub-lethal Cu concentrations of 0.05 and 0.1 µM over a time period of four hours to determine if there was an impact on phototactic response. A similar study design used previously for the initial phototactic response in *Daphnia* was used for this experiment. Five animals were place in columns (n=2) with synthetic soft water and exposed to light stimuli (high intensity light: 1.0 mW/cm^2). Animals in each area were determined at 1-min intervals over a 10 min period of continuous light following 1, 2, 3 and 4 hours of Cu exposure assessing changes in behavior (Figure 16). Phototactic behavior was again quantified by assessing the animals in Area 1 and dividing by the total animals in the cylinder creating an Index Number

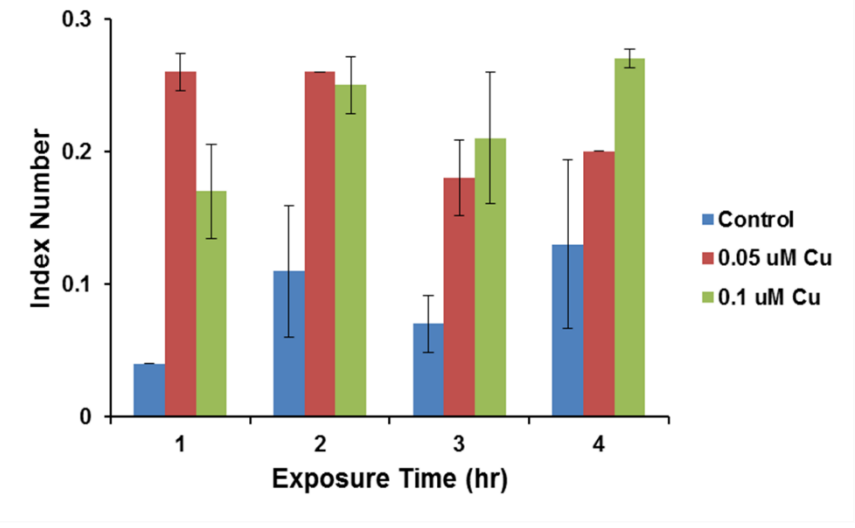


Figure 16. *D. magna* phototactic response in response to Cu exposure (n=2 for each concentration) with a significant difference ($p<0.05$). Error bars ± SEM.

There was some variability in the phototactic response as seen by the error bars, however, statistical analysis using t-test paired samples of the mean over four hours of Cu exposure

showed there was a significant difference between the control and Cu exposure ($p<0.05$) indicating that a reduction in phototactic response in *D. magna* exposed to Cu was observed.

Fast scan cyclic voltammetry (FSCV)

To construct the carbon fiber microelectrodes (CFMs) a single T-650 carbon fiber (Cytec Industries, NJ; 5 µm radius) was aspirated into a 4-inch glass capillary tube (A-M Systems, Sequim, WA; 0.60 mm outer diameter, 0.40 mm inner diameter). A vertical micropipette puller (Narishige Group, Tokyo, Japan) was used to gravity taper the carbon-filled capillary tubes by heat sealing the glass tube and carbon fiber together. The carbon fiber was then cut to 150 µM using an optical microscope. The microelectrode was electroplated with Nafion® for 30 s and placed in an oven for 10 min at 70° C to make it more selective for positively charged ions. (Hashemi, Dankoski et al. 2009) Nafion® is a chemically stable fluorocarbon-polymer with sulfonic groups that functions as an ion exchange membrane. When exposed to solution, pores in the Nafion® resin allow cations to pass through the membrane, but prevent anions from moving through, thus, increasing the selectivity with the FSCV technique.

Waveform generation was created using a PCIe-6341 DAC/ADC Card (National Instruments, Austin, TX) with a Chem-Clamp potentiostat (Dagan Corporation, MN) which measures the output current. Data acquisition, signal processing, waveform output and digital filtering were interfaced with the hardware using custom built software (Knowmad Technologies LLC, Tucson, AZ). All potentials were measured against a Ag/AgCl reference electrode, which was created by electrical deposition of Cl onto a silver (Ag) wire (A-M systems, WA) submerged in 1 M HCl at +4.0 V for 5 s. Optimized electrochemical waveforms for serotonin (1000 V s-1 at 10 Hz, -0.1 V to 1.0 V, -0.5 resting potential) and histamine (600 V s-1 at 10 Hz, -0.7 V to 1.1 V, -0.7 resting potential) were utilized to investigate neurotransmitter release in the *Daphnia*.

Experimental Design

A custom built Plexiglas® flow cell (9.1 x 5.1 x 1.8 cm LWH) was manufactured by the WSU engineering machine shop with a submersible Plexiglas® pedestal designed to fit within the flow cell (Appendix C, Figure C-1). A single adult *D. magna* female was isolated and secured on the animal's right lateral side to the pedestal top surface using < 1 nL cyanoacrylate glue placed on the dorsal side of the carapace. Swimming antennae were secured to avoid interference with the microelectrode position. After the glue cured, the pedestal with the *Daphnid* secured in place was placed in the flow cell and filled with synthetic soft water. The average time for the gluing procedure is < 5 min. Figure 17 shows the experimental design set up inside of a Faraday cage. Minimization of building vibrations was achieved with a nitrogen (N_2) floating base inside the cage.

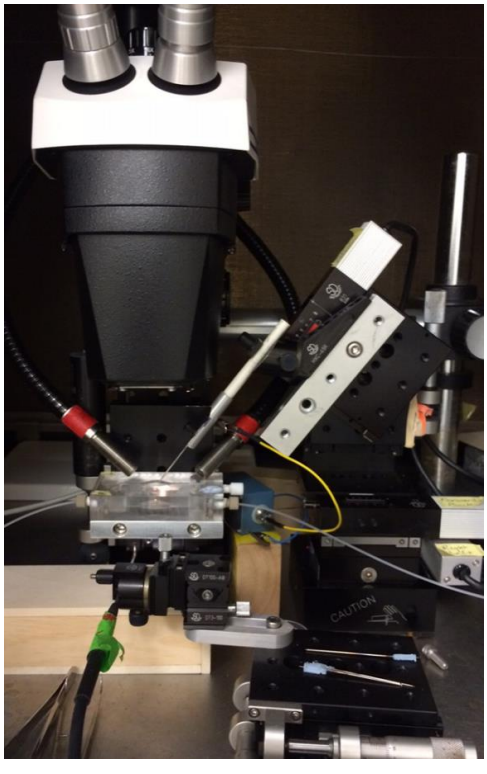


Figure 17. Experimental set up with custom flow cell for *Daphnia* in-vivo neurotransmitter detection (dissection microscope, microdrive and flow cell).

The animal heart rate (HR) and appendage beat rate (ABR) were observable with this positioning through the dissection microscope (Cambridge Instruments, Watertown, MA) and served as an indicator of health. A 33-gauge steel lancet (Lifescan, Tokyo, Japan) attached to a motorized microdrive was used to perforate the *Daphnid* carapace. A carbon fiber microelectrode was inserted into the animal's optic ganglia lamina through the carapace perforation. The Ag/AgCl reference electrode was positioned in the flow cell filled with synthetic soft water.

The Faraday cage housing the FSCV analytical equipment was shrouded in black material to prevent room light from reaching the animal during experiments. Cycling of the electrode prior to file collection was performed 5 min at 10 Hz and 5 min at 60 Hz for electrode stabilization. Data files were collected at 5 min intervals. A Fiber-Lite High Intensity Illuminator, Model 170D (Dolan-Jenner Industries, Boxborough, MA,) fiber was used for the light stimuli (light intensity - high: 1.1 mW/cm^2, medium: 0.54 mW/cm^2 and low: 0.31 mW/cm^2). Data files were obtained with the *Daphnid* exposure to discrete 10 s photo stimulation beginning at 10 s and terminating at 20 s with the data file continuing to completion. Files were selected with the following criteria: a relatively stable baseline (~10 s), a minimum of two files showing a response to light collected with the same electrode, a return to baseline after a phototactic response and minimal electrode artifacts from animal movement. Although secured to the pedestal for experiments the *Daphnia* were in continuous motion moving appendages, eye and anal claw during the file collection period which had the potential to introduce electrode artifacts into the files

Figure 18 shows the target brain region (optic ganglia) of the *D. magna* for microelectrode placement in the left panel and a carbon microfiber electrode inserted into the

target region in the right panel. Animal survivability varied after placing an electrode into the optic ganglion with *D. magna* surviving 6 hr observed.

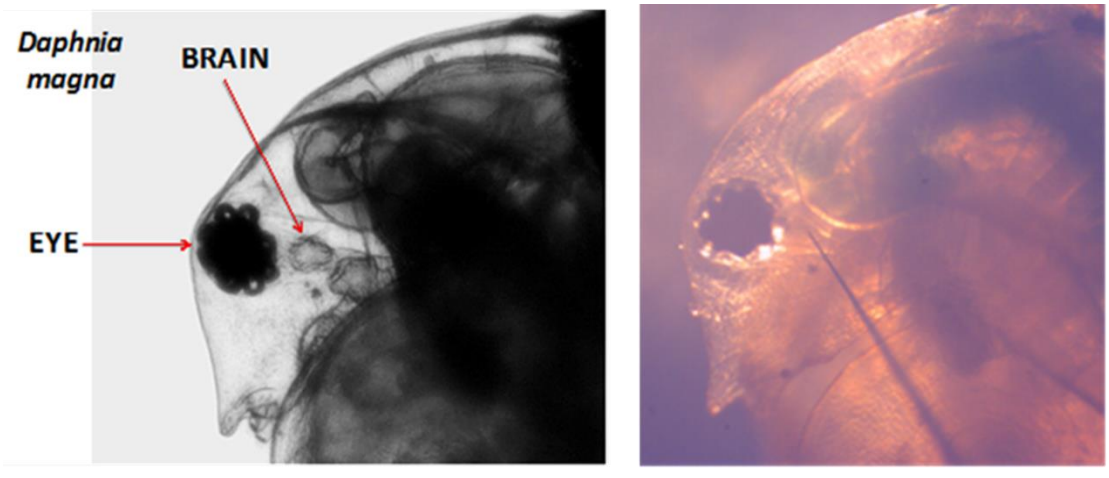


Figure 18. Right: *D. magna* optic ganglia (brain) target region for microelectrode placement. Left: *Daphnid* in flow cell with a microelectrode placed in the optic ganglia.

Figure 19 shows a *Daphnid* glued to the Plexiglas® pedestal and placed in the flow cell filled with synthetic soft water. The *Daphnid* translucent exoskeleton allowed monitoring of general animal health by observing motor functions (ABR and HR) throughout the experiment.

Figure 19. *Daphnia* in the flow cell with microelectrode. *Daphnia* appendages and heart rate were observable through the translucent exoskeleton.

Cu Exposure

For the Cu exposure experiment the serotonin waveform was utilized. Once a single file had been collected indicating a neurotransmitter response to photo stimulation (light on at 10 s off at 20 s), the flow cell was filled with a 0.1 µM Cu solution and a background file was collected. After the animal had been exposed to the Cu solution for 30 minutes another file was collected during light stimulation. File selection criteria included: relatively stable baseline (~10 s), response to light at 10 s, return to baseline following response to light by the end of the file (~30 s) minimal electrode artifacts from animal movement in the file and animal alive with relatively stable ABR. Following Cu exposure, a ten min window (30-40 min) was used to collect an acceptable file due to the potential for animal movement, unstable baseline or electrical interference disrupting the initial file. Statistical analysis of the data was performed using Statistica software (Statsoft Version 13.1) 2016 Tulsa, OK). Time (min) was used as the repeated measure.

Results and Discussion

A neurotransmitter release was observed with photo stimulation in *D. magna* using the FSCV system with the optimized waveforms for both serotonin and histamine. Figure 20 illustrates the *Daphnia* in-vivo response (current vs time) when exposed to a 10 s discrete continuous light with the current (nA) beginning to rise at the 10 s point when light stimuli was initiated after a stable baseline for the initial 10 s of the file collection. Replicates for the graph included 6 animals each (n=6) with error bars reflecting standard error of the mean (SEM).

The rise in current for the histamine oxidation peak had a steeper initial slope than the slope for the serotonin response. The current for both serotonin and histamine continued to increase during the photo stimuli reaching an oxidation peak near 4.5 nA and 9.0 nA for each neurotransmitter respectively at the 20 s mark in the file when the light stimulation was terminated. From this point, the current for histamine and serotonin began to drop off, returning

to base line level at the 30 s point in the file with an initial drop off for histamine becoming a more gradual negative slope than the initial rise for histamine. The serotonin returned to baseline in a similar pattern as the initial rise. This suggested that once neurotransmitter release was initiated it may continue to be detected for a short time after the stimuli had been terminated reflected by the steady return to baseline levels.

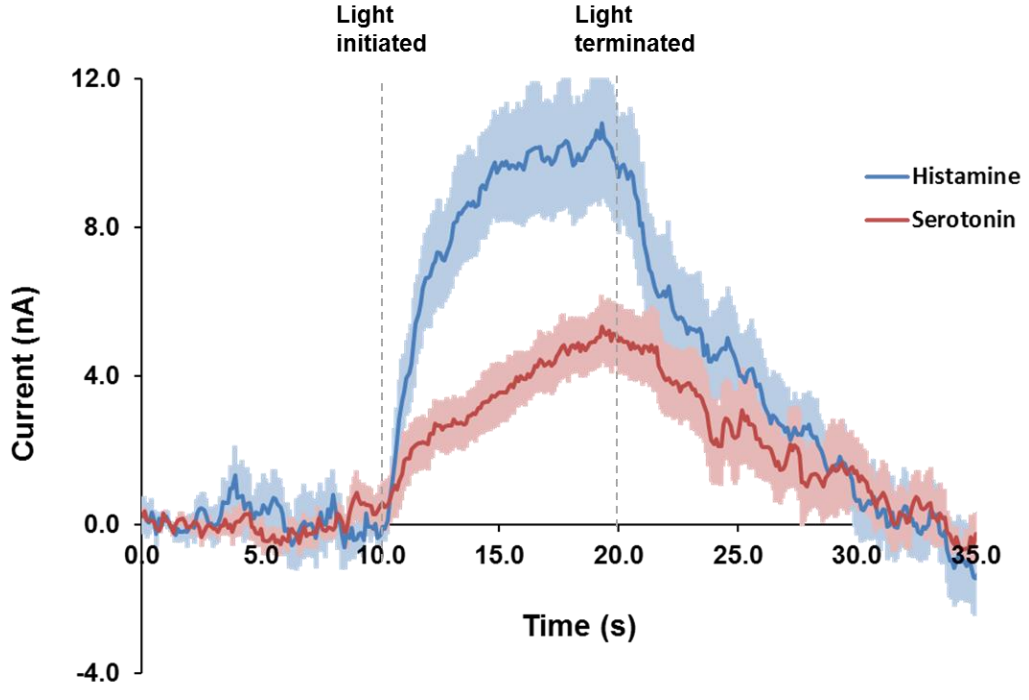


Figure 20. Serotonin and histamine response to a discrete 10 s light stimuli showing current (nA) vs time (s). The graph illustrates a photo response beginning when the animals were exposed to a light at 10 s (dashed line). The current rises steadily and at 20 s the light stimulation was terminated (dashed line) (n=6). Error bars reflect ± SEM.

The histamine oxidation peak was almost double the serotonin peak to the same stimuli. This difference may be a function of the role each neurotransmitter played in the phototactic response which may not be equivalent. However, the waveform for histamine is not as selective as the serotonin waveform allowing the possibility that multiple neurotransmitters or other neurotransmitter like chemicals may be released and detected during a file collection period. This

appeared to a possibility for the differences in peak magnitude for histamine and serotonin in Figure 20, suggesting that the histamine waveform peak may potentially include other positively charged neurochemicals beyond histamine, such as serotonin and dopamine. Literature indicates that the oxidation peak for histamine with flow injection analysis (FIA) is 0.3 V vs Ag/AgCl, with the oxidation peak for serotonin and dopamine at 0.6 V and 0.5 V respectively. (Samaranayake, Abdalla et al. 2015) Peaks can shift slightly as a function of the differences in the microenvironments, but CVs collected with the histamine waveform showed multiple peaks before the switching potential suggesting other neurotransmitters such as serotonin were released simultaneously as part of the response to photo stimuli. Although *Daphnia* exhibited a light-coupled neurotransmitter response, periodic bursts of serotonin and histamine were also observable in the background color plot files collected using the respective waveforms suggesting that neurotransmitter release was part of a complex interplay of *Daphnia* functional systems that included, but was not necessarily limited to the animal visual and motor systems.

Cyclic voltammograms (CV) were obtained at the vertical crosshair lines on the respective false color plots. The color plot is a visual representation of what is occurring at the CFM. Blue indicates a region where the current (nA) is less than the baseline level (reduction), while the green indicates regions with current above the baseline (oxidation). A representative color plot for serotonin release using the serotonin waveform is shown in Figure 21 with the white crosshairs indicating where the CV was obtained.

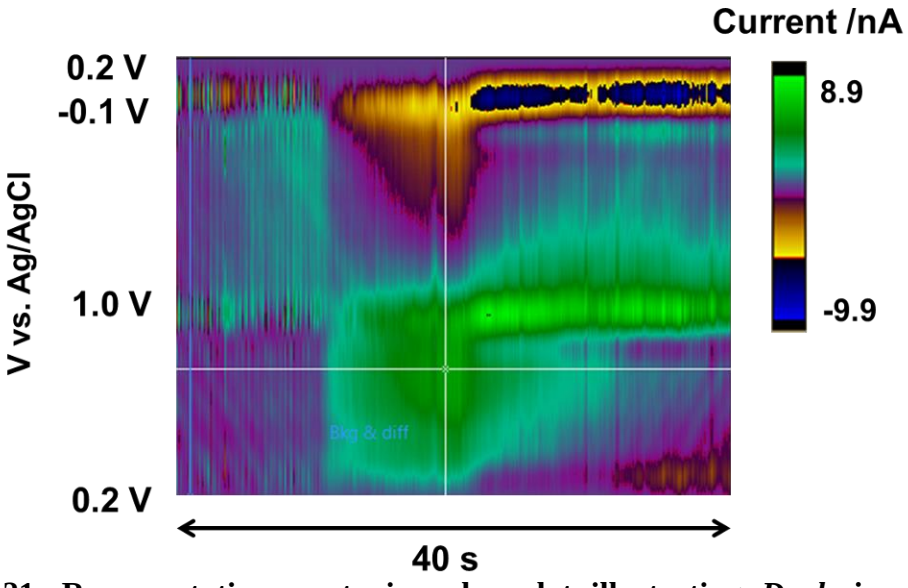


Figure 21. Representative serotonin color plot illustrating *Daphnia* response to light stimuli. Cyclic voltammograms (CVs) were obtained at the respective vertical white crosshairs. The x-axis reflects the length of the collected file in (s). The y–axis illustrates the waveform potential.

The serotonin waveform is more selective than the histamine waveform. The graph in Figure 22 showed a representative composite (n=6) serotonin CV for a *Daphnia* in-vivo response to photo stimuli (blue line) graphed simultaneously with a representative CV for serotonin hydrochloride (100 nm) by FIA in Tris buffer (dashed black line). Tris buffer is a biological buffer and is often used as a substitute to mimic biological systems. The serotonin oxidation peak for in-vivo *Daphnia* response occurred close to 0.61 V with the in-vitro serotonin hydrochloride oxidation peak occurring at 0.60 V. These two oxidation peaks correlate well considering the experiments were carried out in different microenvironments indicating that the response observed is likely serotonin, but not conclusive. Additional pharmacological studies are needed to confirm serotonin release.

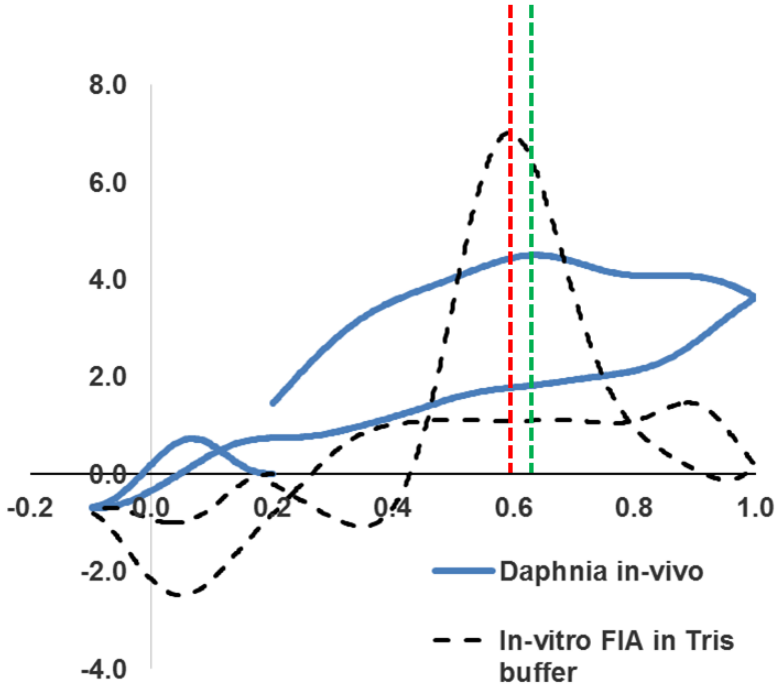


Figure 22. Serotonin Cyclic Voltammogram (CV) peak comparison for *Daphnia* in-vivo response to light stimuli and in-vitro serotonin hydrochloride (100 nm) in Tris buffer using flow injection analysis (FIA) (courtesy of Matthew Jackson, 2016). The red dashed line indicates the in-vitro serotonin oxidation peak at 0.60 V. The green dashed line indicates the oxidation peak for in-vivo *Daphnia* serotonin response at 0.61 V

To investigate the impact of sub-lethal exposure of Cu on *Daphnia* phototactic response initial FSCV files using the optimized serotonin waveform were obtained in response to photo stimuli. Animals were then exposed to 30 min of 0.1 µM Cu in the flow cell at which point FSCV files with light stimulation were obtained again. The same experimental protocol was observed as previously. This Cu concentration has been shown to be sub-lethal in experiments in Chapter 4 with the behavioral and physiological bioassays and preliminary studies performed.

A reduction in phototactic response was observed following 30 min of 0.1 µM Cu exposure as illustrated by Figure 23. The post exposure files showed a reduction in *Daphnia* response to light stimuli with a peak near 2.0 nA and returning to baseline closer to 25 s rather than 30 s observed with the pre-exposure (0 µM Cu) files. The pre exposure light response files

were similar to what was observed in Figure 20, with a current peak response near 4.5 nA and returning to baseline near 30 s. Statistical analysis of the comparison of pre and post Cu showed that the response to 0 uM and 0.1 uM Cu concentrations were statistically significant ($p<0.001$) from each other (Figure 23) using ANOVA repeated measures analysis. A sub-lethal Cu exposure was shown to reduce phototactic response in *D. magna* after 30 min of exposure to 0.1 uM Cu.

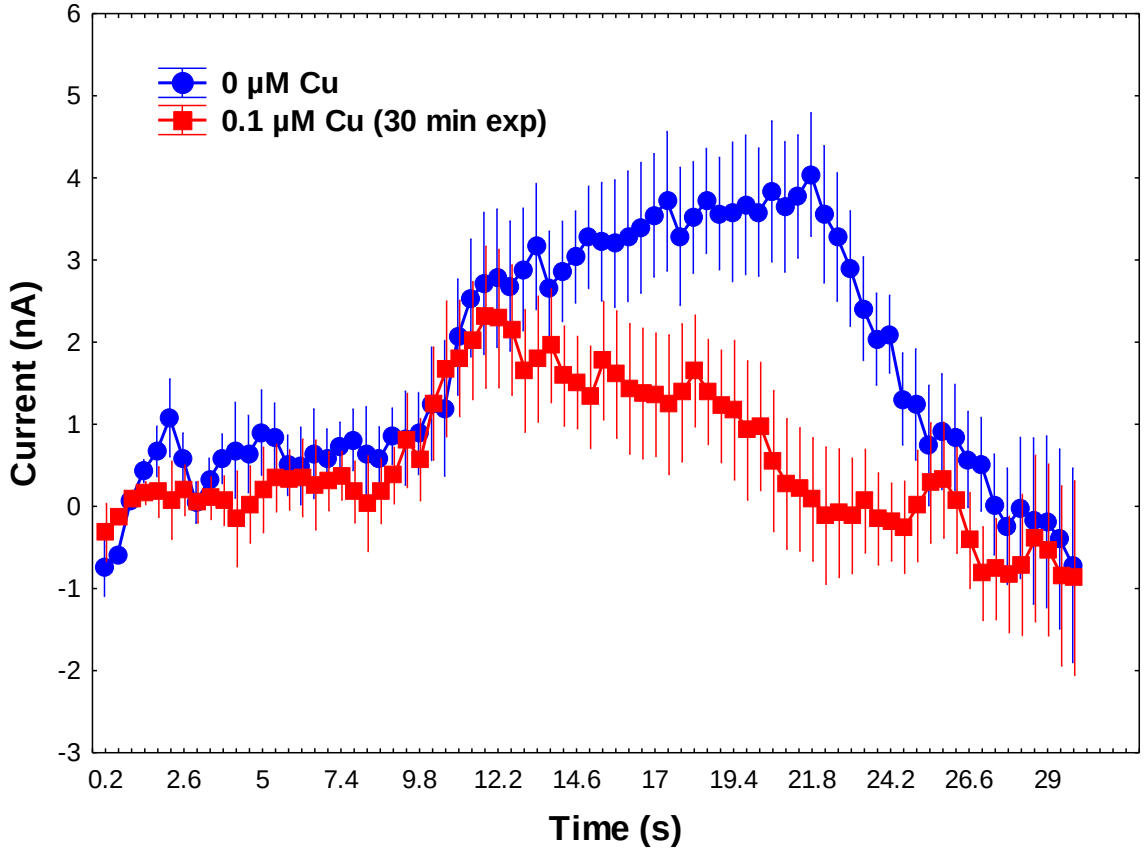


Figure 23. Phototactic response in *D. magna* (n=5) exposed to Cu. Comparison of animal post exposure response (30 min of 0.1 µM Cu) with pre exposure response (0 µM Cu) using FSCV. ANOVA repeated measures analysis ($p<0.001$). Light stimulation was initiated at 10 s and terminated at 20 s. Data was smoothed. Error bars ± SEM.

These observations of reduced phototactic response due to Cu exposure in *Daphnia* correlated well with the preliminary light studies performed for this research as well as phototactic studies performed by others (Michels, Leynen et al. 1999, Yuan, Michels et al.

2003); however, those studies used positive phototactic genotype *Daphnia* clones. This study showed that Cu exposure impacts negative genotype *D. magna* clones in a similar pattern by reducing neurotransmitter release following exposure.

Conclusion

The results of this study are consistent with the initial proposed hypotheses. The FSCV analytical technique has been shown to have the ability to detect a light-sensitive neurotransmitter release for the first time in *D. magna* using both the histamine and serotonin waveforms. It has been demonstrated that *Daphnia* phototactic response was altered by a sub-lethal Cu exposure with a significant reduction in neurotransmitter release with light stimulation for negative genotype clones. Histamine and serotonin like neurochemicals have been shown to be essential components of the phototactic response of *Daphnia* and released with light stimulation. A neurotransmitter response was observed when the light stimuli was initiated and dropped off when the light was terminated which is consistent with other observational experiments.

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CHAPTER 6: ENVIRONMENTAL IMPLICATIONS AND RELEVANCE

Water pollution is found worldwide with anthropogenic activities a primary contributor to the release of toxic contaminants into aquatic ecosystems (Daughton 2001, Kolpin, Skopec et al. 2004, Sarkar, Saha et al. 2007) Toxic pollutants negatively impact aquatic biodiversity and water quality. Improving water quality is a critical goal that needs to be approached in a comprehensive manner as fresh water is a vital commodity that sustains life. Fresh water ecosystems are increasingly threatened by human induced changes that have the potential for long term impacts with unintended consequences (Dudgeon, Arthington et al. 2006).

The research in this dissertation adds to the body of work that addresses water quality issues with the development of novel approaches to investigate the impact of toxic pollutants on aquatic ecosystems. Copper is the target pollutant in this research, but the novel approaches developed and adapted to investigate Cu toxicity can be used to assess the impact of numerous other aquatic contaminants including other metals, pharmaceuticals and emerging contaminants of concern.

Cu toxicity to aquatic biota has been identified for decades (Florence 1982, Iivonen, Piepponen et al. 1992, Weis and Weis 1992, Schubauerberigan, Dierkes et al. 1993). Progress has been made in many areas including waste water treatment for metals; however, (Fu and Wang 2011) new sources and pathways for these pollutants into the environment continue to emerge making Cu toxicity an ongoing challenge (Bird and Grossman 2011) (Adeleye, Oranu et al. 2016). The adapted approaches used in this research to investigate Cu toxicity in aquatic ecosystems include the FSCV analytical technique and two optical bioassays. These bioassays evaluate the behavioral and physiological changes observed in *Daphnia,* a model species for ecotoxicological studies.

Results from experiments described in Chapter 3 demonstrate the power and utility of the FSCV analytical technique by evaluating Cu-DOM interactions in-situ. Both the binding and release of Cu in the presence of competitive ions was investigated to better understand the speciation and thus the availability of Cu in aquatic ecosystems. The FSCV analytical technique was able to demonstrate that the type of DOM influenced the Cu binding capabilities and that Cu-DOM complexation was time dependent with increased complexation occurring over hours to days. The displacement of Cu from DOM by competitive Al ions was also shown and quantified with this method as well as the importance of strong DOM binding sites on Cu toxicity.

Although these Cu-DOM experiments were performed in an artificial laboratory environment, the research generated provided a better understanding of Cu speciation and the impact of DOM on Cu toxicity through alterations in Cu-DOM complexation. Improved understanding of these complex interactions has increased the ability to address Cu toxicity in the aquatic environment today. This research has strong implications for fresh water systems as many aquatic biota are highly sensitive to Cu with a low toxicity threshold including *Daphnia.* This organism is a keystone species in freshwater ecosystems making alterations in this animal's behavior potentially damaging to the entire aquatic ecosystem. The improved understanding of complex Cu-DOM interactions provides critical information to maintain and protect aquatic biodiversity ecosystem fitness.

Future work in this area with the FSCV technique and metals includes investigation of DOM from other natural sources and subsequent binding capabilities. Although, highly refined DOM was used in these experiments, the DOM was also highly characterized providing a basis for future explorations.

Chapter 4 demonstrated the Cu at sub-lethal concentrations had the ability to alter *Daphnia* physiological functions which were then shown to correspond to behavioral changes in the animal. Many traditional toxicity bioassays evaluate the effect of Cu and other contaminants by assessing the concentration at which death occurs, often utilizing the mean LC_{50} as the endpoint. Relevant changes to aquatic biota can occur at much lower chemical concentrations. The optical behavioral and physiological assays used in this research provided additional evidence of *Daphnia* sensitivity to Cu as a xenobiotic stressor. Alterations in *Daphnia* motor and cardio respiratory functions were used to evaluate Cu toxicity. This study demonstrated that type and amount of DOM available influenced Cu-DOM complexation and subsequent toxicity to *Daphnia*. The physiological bioassay showed that *Daphnia* HR and ABR were influenced by varying Cu concentrations inducing the changes in the animal motor functions. Alterations to Daphnia swimming behavior were evaluated with the behavioral bioassay by tracking changes in mean angle and accumulated distance over time evaluating sub-lethal Cu effects

These bioassays are environmentally relevant by providing additional knowledge on the impact of Cu toxicity to *Daph*nia at sub-lethal concentrations. This study evaluated Cu toxicity, but the bioassay methods can be used to evaluate other contaminants of concern that are in the environment. New chemicals are developed and put into use each year without understanding the long term implications or the toxicity of these chemicals to the aquatic environment and the impact on water quality. These cost effective optical bioassays can be used to assess the impact of sub-lethal pollutant concentrations on aquatic biota providing critical information that that can be used to protect water quality in aquatic ecosystems around the globe.

Chapter 5 demonstrated that the FSCV analytical technique has the ability to measure neurotransmitter release in *Daphnia* in response to photo stimulation. It was further shown that exposure to the environmental pollutant, Cu, altered the neurotransmitter response in *Daphnia*

and thus, the animal survival behavior. Neurotransmitters carry signals along neuronal pathways controlling behavior and physiological functions in invertebrates and vertebrate animals. *Daphnia*, a microcrustacean invertebrate exhibits a phototactic response when exposed to light. Animals typically move down and away from the light as a predator avoidance trait. Alterations in this behavior can change the predator-prey relationship and impact the fitness of freshwater ecosystems.

Understanding the neurotransmitters involved with phototactic response is vital to understanding the impact of external chemicals on this predator avoidance trait. The FSCV technique was adapted to investigate neurotransmitters in the *Daphnia* by placing a carbon microfiber electrode into the optic ganglia region of the animal and using optimized waveforms for serotonin and histamine. The FSCV analytical technique was shown to have the ability to detect neurotransmitters in response to light stimulation in near real time, with high resolution and high sensitivity making it a power tool for environmental assessment of electroactive chemicals. The impact of sub-lethal Cu concentrations was also investigated and shown to reduce the phototactic response of *Daphnia*. Future implications of this analytical technique include using this method to investigate other electroactive environmental pollutants and their influence on the phototactic response of *Daphnia*, such as pharmaceuticals and endocrine disrupting chemicals.

Water quality issues are ubiquitous in aquatic ecosystems around the world. To adequately confront and mitigate these global water problems, novel approaches and adaptation of methods are needed to be successful in addressing these challenges. Emerging contaminants along with known contaminants are continually introduced into the aquatic environment. An increase in knowledge is critical to reducing the impact of toxic chemicals on aquatic

biodiversity and improving water quality. The novel approaches used in this dissertation provide knowledge to help address challenges below the surface of the water.

APPENDIX A: SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Table A-1. Immediate and 5 day Cu binding and release

	Cu Bound				Cu Release			
	Immediate		5 Day		Immediate		5 Day	
DOM	(µM)	SEM	(µM)	SEM	(µM)	SEM	(µM)	SEM
SRHA	0.725	± 0.020	0.922	± 0.003	0.075	± 0.002	0.072	± 0.002
NLHA	0.682	± 0.034	0.885	± 0.005	0.072	± 0.002	0.059	± 0.007
NLFA	0.629	± 0.009	0.844	± 0.004	0.067	± 0.002	0.095	± 0.005
SRFA	0.665	± 0.019	0.830	± 0.022	0.066	± 0.001	0.103	± 0.026
SRNOM	0.415	± 0.011	0.799	± 0.013	0.054	± 0.002	0.033	± 0.004
NLNOM	0.325	± 0.035	0.712	± 0.032	0.048	± 0.001	0.007	± 0.013

Table A-2. Statistical models of fixed effects

Model	Source	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F-value	P-value
1	Intercept	1	100.025	1152.165	0.000
	Humic Fraction	2	100.025	42.908	0.000
	Electrode	2	100.025	0.31	0.734
	Level	1	100.025	9.915	0.002
	Experiment	1	100.025	111.748	0.000
2	Intercept	1	96.039	3444.82	0.000
	DOM Source	5	96.039	46.542	0.000
	Electrode	2	96.039	0.784	0.460
	Experiment level	3	96.039	149.719	0.000
3	Intercept	1	79.770	11792.802	0.000
	Experiment DOM level	25	79.770	220.511	0.000
	Electrode	2	79.770	2.577	0.082

Table A-3. Model 1 - Humic fraction estimates of fixed effects

Parameter	Estimate	95% Confidence Interval Lower Bound	Upper Bound	t-value	P-value
Intercept	-0.033	-0.073	0.006	-1.677	0.097
Fulvic Acid Fraction	0.330	0.282	0.379	13.548	0.000
Humic Acid Fraction	0.266	0.218	0.314	10.909	0.000
NOM Fraction	0.486	0.437	0.534	19.919	0.000
Level 1	0	.	.	.	.
Electrode1	-0.016	-0.055	0.024	-0.785	0.434
Electrode 2	-0.009	-0.048	0.031	-0.445	0.657
Electrode 3	0	.	.	.	.
Level 2	-0.063	-0.102	-0.023	-3.149	0.002
Level 3	0	.	.	.	.
Level 1	0	.	.	.	.
Immediate Experiment	0.172	0.140	0.204	10.571	0.000
5 day Experiment	0	.	.	.	.

*Note explanation of Levels (1-3) in Figure SI3

Table A-4. Model 1 – Humic fraction pairwise comparison

Fraction	Fraction	P-value
Fulvic Acid	Humic Acid	0.029
	NOM	0.000
	Level 1	.
Humic Acid	Fulvic Acid	0.029
	NOM	0.000
	Level 1	.
NOM	Level 1	.

Table A-5. Model 2 – DOM source estimates of fixed effects

Parameter	Estimate	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound	t-value	P-value
Intercept	0.053	0.030	0.075	4.598	0.000
NLNOM	0.655	0.614	0.696	31.551	0.000
NLFA	0.465	0.423	0.506	22.373	0.000
NLHA	0.410	0.369	0.451	19.758	0.000
SRNOM	0.575	0.534	0.616	27.705	0.000
SRFA	0.455	0.414	0.496	21.914	0.000
SRHA	0.381	0.339	0.422	18.331	0.000
Level 1	0	.	.	.	.
Electrode 1	-0.016	-0.040	0.009	-1.248	0.215
Electrode 2	-0.009	-0.034	0.016	-0.708	0.481
Electrode 3	0	.	.	.	.
5 day Level 2	-0.321	-0.356	-0.285	-18.103	0.000
5 day Level 3	-0.259	-0.294	-0.224	-14.61	0.000
Immediate Level 2	-0.064	-0.099	-0.028	-3.59	0.001
Immediate Level 3	0	.	.	.	.

Table A-6. Model 2 - DOM source pairwise comparison

DOM	DOM	P-value
NL-NOM	NLFA	0.000
	NLHA	0.000
	SR-NOM	0.006
	SRFA	0.000
	SRHA	0.000
	Level 1	.
NLFA	NLHA	0.209
	SR-NOM	0.000
	SRFA	1.000
	Level 1	0.003
NLHA	SR-NOM	0.000

	SRFA	0.625
	SRHA	1.000
SR-NOM	SRFA	0.000
	SRHA	0.000
SRFA	SRHA	0.013

Table A-7. Model 2 - Experiment level pairwise comparison

Bound-Release	Bound-Release	P-value
5 day Level 2	5 day Level 3	0.004
	Immediate Level 2	0.000
	Immediate Level 3	0.000
	Level 1	.
5 day Level 3	Immediate Level 2	0.000
	Immediate Level 3	0.000
	Level 1	.
Immediate Level 2	Immediate Level 3	0.003
	Level 1	.
Immediate Level 3	Level 1	.

Table A-8. Model 3 - Experiment DOM level estimates of fixed effects

		95% Confidence Interval			
Parameter	Estimate	Lower Bound	Upper Bound	t-value	P-Value
Intercept	0.053	0.037	0.068	6.590	0.000
5 day NLNOM Level 2	0.287	0.251	0.323	15.709	0.000
5 day NLFA Level 2	0.155	0.119	0.192	8.498	0.000
5 day NLHA Level 2	0.114	0.078	0.151	6.254	0.000
5 day SRNOM Level 2	0.201	0.165	0.237	11.005	0.000
5 day SRFA Level 2	0.168	0.132	0.205	9.217	0.000

5 day SRHA Level 2	0.092	0.055	0.128	5.016	0.000
5 day NLNOM Level 3	0.294	0.258	0.331	16.111	0.000
5 day NLFA Level 3	0.251	0.214	0.287	13.721	0.000
5 day NLHA Level 3	0.174	0.137	0.210	9.508	0.000
5 day SRNOM Level 3	0.234	0.197	0.270	12.799	0.000
5 day SRFA Level 3	0.272	0.236	0.309	14.910	0.000
5 day SRHA Level 3	0.164	0.127	0.200	8.962	0.000
5 day Level 1	0	-0.019	0.019	0.001	0.999
Imm NLNOM Level 2	0.673	0.637	0.709	36.838	0.000
Imm NLFA Level 2	0.372	0.335	0.408	20.341	0.000
Imm NLHA Level 2	0.320	0.284	0.356	17.519	0.000
Imm SRNOM Level 2	0.584	0.548	0.620	31.965	0.000
Imm SRFA Level 2	0.336	0.300	0.372	18.392	0.000
Imm SRHA Level 2	0.275	0.238	0.311	15.043	0.000
Imm NLNOM Level 3	0.723	0.687	0.760	39.595	0.000
Imm NLFA Level 3	0.438	0.402	0.474	23.970	0.000
Imm NLHA Level 3	0.390	0.354	0.426	21.356	0.000
Imm SRNOM Level 3	0.639	0.603	0.676	35.001	0.000
Imm SRFA Level 3	0.400	0.364	0.437	21.923	0.000
Imm SRHA Level 3	0.350	0.313	0.386	19.132	0.000
Imm Level 1	0	.	.	.	.
Electrode 1	-0.016	-0.029	-0.002	-2.264	0.026
Electrode 2	-0.009	-0.023	0.005	-1.284	0.203
Electrode 3	0	.	.	.	.

Imm = Immediate

Table A-9. Model 3 - Electrode pairwise comparison

Electrode	Electrode	P-value
1	2	0.991
	3	0.079
2	3	0.609

Statistical Analysis Methods - Experimental Details:

Statistical analysis of data was performed with Model IBM SPSS version 22 statistics software with a statistically significant determined alpha-level of 0.05. A standard linear mixed effects model was used for statistical analysis:

$$Y = (\beta_1 X_1 + \beta_2 X_2 + \ldots \beta_n X_n) + (\gamma_1 Z_1 + \gamma_2 Z_2 \ldots + \gamma_n Z_n) + \mathcal{E} \quad (1)$$

where Y is the dependent variable, β is the vector of fixed effect variable, X is the fixed effect, Z is the random effect variable, γ is the vector of the random effect variable (Intercept) and $\mathcal{E}$ is the error of residuals.

Through the analysis, three specific models were evaluated utilizing the type of ligand (humic acid, fulvic acid or NOM), the source of DOM (Suwannee River or Nordic Lake), the amount of time between binding and release (immediate or 5 day), the electrode number and levels as fixed effects. The date and the order in which the experiment was run served as random effects, both of which were included in all models. The fixed and random effects where used to predict the concentration of free Cu in solution and the dependent variable, using the SPSS linear mixed models package. The three models were as follows:

Model 1 - Humic fraction estimates of fixed effects (Table A-3)

$$Y = \beta_1(\textit{Humic Fraction}) + \beta_2(\textit{Electrode}) + \beta_3(\textit{Level}) + \beta_4(\textit{Experiment}) + \gamma_1(\textit{Date}) + \gamma_2(\textit{Order}) + \mathcal{E}$$

Humic Fraction included the ligand type as fulvic acids, humic acids and natural organic matter (NOMs). *Electrode* included the three electrodes 1, 2 and 3 used in immediate and 5 day experiments. *Level* included baseline (level 1) with no Cu, free concentration of Cu (level 2) (used to determine Cu Bound) and Cu released (level 3) with aluminum (Al) addition.

Experiment included immediate and 5 day experiment. *Date* included dates when experiments were performed (October 30, 2014 and November 15, 2014). *Order* specifies the order DOM was utilized in experiments. $\mathcal{E}$ included residual errors.

Model 2 – DOM source estimates of fixed effects (Table A-5)

$$Y = \beta_1(\text{DOM Source}) + \beta_2(\text{Electrode}) + \beta_3(\text{Experiment level}) + \gamma_1(\text{Date}) + \gamma_2(\text{Order}) + \mathcal{E}$$

DOM Source included the specific sources of DOM for experiments NLFA, NLHA, NLNOM, SRFA, SRHA and SRNOM. *Electrode* included: electrode numbers 1, 2 and 3 used in immediate and 5 day experiments. *Experiment level* included: Baseline (level 1) with no Cu present, Cu free (level 2) and Cu release (level 3) determined by the experiment type of 5 day or immediately after complexation. *Date* included dates when experiments were performed (October 30, 2014 and November 15, 2014). *Order* specified the numerical sequence DOM sources were utilized for experiments using numbers from 1 to 6. $\mathcal{E}$ included residual errors.

Model 3 – Experiment DOM Cu estimates of fixed effects (Table A-8)

$$Y = \beta_1(\text{Experiment DOM level}) + \beta_2(\text{Electrode}) + \gamma_1(\text{Date}) + \gamma_2(\text{Order}) + \mathcal{E}$$

Experiment DOM level included the DOM source further defined by experiment (immediate and 5 day) and by levels (1-3). *Electrode* included: electrode numbers 1, 2 and 3 used in immediate and 5 day experiments. *Date* included dates when experiments were performed (October 30, 2014 and November 15, 2014). *Order* specified the numerical sequence DOM sources were utilized for experiments using numbers from 1 to 6. $\mathcal{E}$ included residual errors.

APPENDIX B: SUPPLEMENTAL MATERIAL FOR CHAPTER 4

Table B-1. Behavioral: Change in Angle (Cu and DOM)

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
DOM Concentration (Conc)	629	1	629	0.2	0.680
Cu Conc	1560000	3	520000	141.4	0.000*
DOM	172000	2	86100	23.4	0.000*
DOM Conc x Cu Conc	9223	3	3074	0.8	0.476
DOM Conc x DOM Type	42900	2	21500	5.8	0.004*
Cu Conc x DOM Type	219000	6	36400	9.9	0.000*
DOM Conc x Cu Conc x DOM Type	31500	6	5246	1.4	0.210
Time	569000	28	20300	36.5	0.000*
Time x DOM Conc	35500	28	1269	2.3	0.000*
Time x Cu Conc	560000	84	6666	12	0.000*
Time x DOM Type	69200	56	1235	2.2	0.000*
Time x DOM Conc x Cu Conc	102000	84	1213	2.2	0.000*
Time x DOM Conc x DOM Type	33800	56	603	1.1	0.313
Time x Cu Conc x DOM Type	148000	168	882	1.6	0.000*
Time x DOM Conc x Cu Conc x DOM	124000	168	736	1.3	0.004*

Behavioral Analysis - repeated measures ANOVA analysis for mean angle change using Cu concentration, DOM concentration and DOM type with Time as the repeated measure. Effects that are significant ($p<0.05$) are indicated with an asterisk (*) with 11 out of 15 effects significant.

Contrast Analyses (Time x Cu Conc x DOM Conc)

Variable	Univariate Test of Significance for Planned Comparison (Cu_DOM_1 Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	12503.8	1	12503.76	4.665816	0.032757
Error	321583.8	120	2679.87		

Comparison between EPA soft water and 0.1 µm Cu at 1000 µM DOM. Significantly different at p<0.05 Truncated to last 15 data points

Variable	Univariate Test of Significance for Planned Comparison (Cu_DOM_1 Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	52108.0	1	52108.02	19.44427	0.000023
Error	321583.8	120	2679.87		

Contrast comparison at 1000 µM DOM between 0.1 and 1 µM Cu. Significant difference. p<0.001 Truncated to last 15 data points

Variable	Univariate Test of Significance for Planned Comparison (Cu_DOM_1 Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	227543.6	1	227543.6	84.90860	0.000000
Error	321583.8	120	2679.9		

Contrast comparison at 100 µM DOM between 0.1 and 1 µM Cu. Significant difference p<0.001 Truncated to last 15 data points

Variable	Univariate Test of Significance for Planned Comparison (Cu_DOM_1 Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	112990.0	1	112990.0	42.16255	0.000000
Error	321583.8	120	2679.9		

Contrast comparison at 100 µM DOM between 1 and 10 µM Cu. Significant difference p<0.001 Truncated to last 15 data points

Variable	Univariate Test of Significance for Planned Comparison (Cu_DOM_1(Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	222114.6	1	222114.6	82.88275	0.000000
Error	321583.8	120	2679.9		

Contrast comparison at 1000 µM DOM between 1 and 10 µM Cu. Significant difference p<0.001 Truncated to last 15 data points

Table B-2 Behavioral: Cumulative Distance (Cu and DOM)

Effect	**Sum of Squares**	**Degrees of Freedom**	**Mean Square**	**F value**	**P value**
DOM Concentration (Conc)	3427	1	3427	2.98	0.087
Cu Conc	283000	3	94300	82.07	0.000*
DOM	34500	2	17300	15.02	0.000*
DOM Conc x Cu Conc	2380	3	793	0.69	0.559
DOM Conc x DOM Type	9023	2	4512	3.93	0.022*
Cu Conc x DOM Type	39800	6	6633	5.77	0.000*
DOM Conc x Cu Conc x DOM Type	7313	6	1219	1.06	0.390
Time	72900	28	2605	11.45	0.000*
Time x DOM Conc	11500	28	410	1.8	0.006*
Time x Cu Conc	70100	84	834	3.67	0.000*
Time x DOM Type	18700	56	334	1.47	0.014*
Time x DOM Conc x Cu Conc	19800	84	236	1.04	0.389
Time x DOM Conc x DOM Type	11400	56	204	0.9	0.693
Time x Cu Conc x DOM Type	30100	168	179	0.79	0.978
Time x DOM Conc x Cu Conc x DOM	47500	168	283	1.24	0.021*

Behavioral Analysis - repeated measures ANOVA analysis for cumulative distance using Cu concentration, DOM concentration and DOM type with Time as the repeated measure. Effects that are significant ($p<0.05$) are indicated with an asterisk (*) with 9 out of 15 effects significant.

Table B-3. Physiology: Cu only

Effect:	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
Cu Conc	1690000	2	843000	21.11	0.000*
Parameter	345000	1	345000	18.81	0.001*
Parameter x Cu Conc	50000	2	25000	1.36	0.286
Time	1230000	21	58400	47.4	0.000*
Time x Cu Conc	1230000	42	29300	23.75	0.000*
Parameter x Cu Conc	64800	21	3085	5.32	0.000*
Parameter x Time x Cu Conc	67900	42	1616	2.78	0.000*

Contrast Analysis: B-3 (Parameter x Time x Cu Conc)

Univariate Test of Significance for Planned Comparison (Copy of DO
Tests for transformed variables

Variable	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	271617.5	1	271617.5	14.82260	0.001574
Error	274868.2	15	18324.5		

Parameter ABR: Compared EPA soft water and 0.1 µM Cu. Significantly different.

Univariate Test of Significance for Planned Comparison (Copy of DC
Tests for transformed variables

Variable	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	505837.6	1	505837.6	31.27262	0.000051
Error	242626.5	15	16175.1		

Parameter HR: Compared EPA soft water and 0.1 µM Cu. Significantly different.

Variable	Univariate Test of Significance for Planned Comparison (Copy of DO Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	69517.6	1	69517.64	1.652308	0.218137
Error	631095.7	15	42073.04		

Parameter ABR: Compared EPA soft water and 0.1 µM Cu. Not significantly different.

Variable	Univariate Test of Significance for Planned Comparison (Copy of DO Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	0.0	1	0.00	0.000000	1.000000
Error	242626.5	15	16175.10		

Parameter HR: Compared EPA soft water and 0.1 µM Cu. Not significantly different.

Variable	Univariate Test of Significance for Planned Comparison (Copy of DO Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	271617.5	1	271617.5	14.82260	0.001574
Error	274868.2	15	18324.5		

Comparison between ABR and HR control. Significantly different at $p<0.05$

TABLE B-4. Physiology: (Cu and DOM)

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
DOM Type	857000	2	428000	8.6	0.001*
Cu Conc	2270000	1	2270000	45.5	0.000*
Parameter	1290000	1	1290000	25.9	0.000*
DOM Type x Cu Conc	193000	2	96600	1.9	0.153
DOM Type x Parameter	21300	2	10600	0.2	0.808
Cu Conc x Parameter	150000	1	150000	3	0.088
DOM Type x Cu Conc x Parameter	59300	2	29600	0.6	0.555
Time	2640000	21	126000	106.5	0.000*
Time x DOM Type	159000	42	3779	3.2	0.000*
Time x Cu Conc	1910000	21	90900	77.1	0.000*
Time x Parameter	201000	21	9560	8.1	0.000*
Time x DOM Type x Cu Conc	167000	42	3987	3.4	0.000*
Time x DOM Type x Parameter	37900	42	903	0.8	0.860
Time x Cu Conc x Parameter	147000	21	7007	5.9	0.000*
Time x DOM x Cu x Parameter	33800	42	805	0.7	0.940

Physiology Contrast Analysis: B-4 (Time x Cu Conc X DOM Type)

Univariate Test of Significance for Planned Comparison (DOM Data_
Tests for transformed variables

Variable	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	214761	1	214760.6	4.415352	0.039826
Error	2918371	60	48639.5		

Compared NLNOM and NLF at 1 µM Cu. Analysis started at Time 0 when chemical treatment initiated (initial 3 data points not in analysis). Significant difference P< 0.05.

Variable	Univariate Test of Significance for Planned Comparison (DOM Data_ Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	305506	1	305506.2	6.281028	0.014933
Error	2918371	60	48639.5		

Compared EPA Control (no Cu) and NLNOM at 1 µM Cu concentration. Analysis started at Time 0 when chemical treatment initiated (first 3 data points not in analysis) Significant difference P< 0.05.

Variable	Univariate Test of Significance for Planned Comparison (DOM Data_ Tests for transformed variables				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	1032558	1	1032558	21.22880	0.000022
Error	2918371	60	48640		

Compared EPA Control (no Cu) and NLFA at 1 µM Cu concentration. Analysis started at Time 0 when chemical treatment initiated. Significant difference P< 0.001.

APPENDIX C: SUPPLEMENTAL MATERIAL FOR CHAPTER 5

Figure C-1. Flow cell computer-aided design (CAD) diagram

Figure C-2. Fast Scan Cyclic Voltammetry (FSCV) Analytical Technique

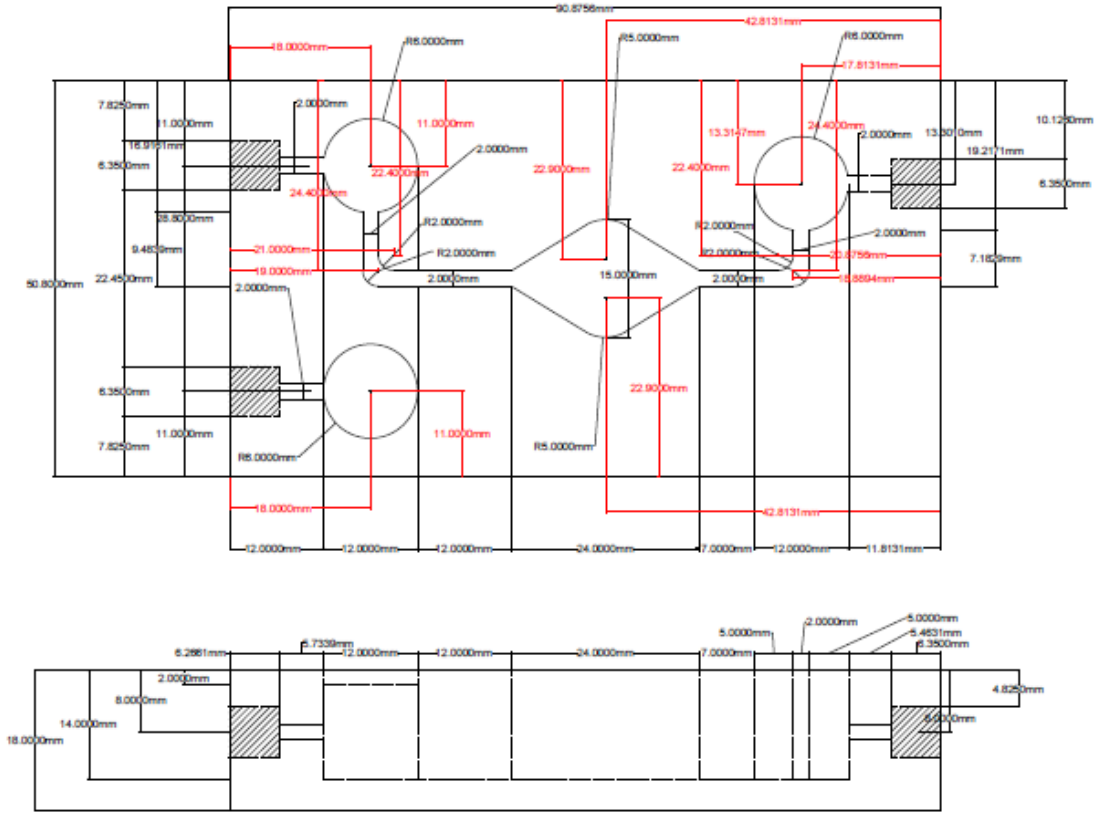

Figure C-1. Flow cell computer-aided design (CAD) diagram

Fast Scan Cyclic Voltammetry (FSCV) Analytical Technique

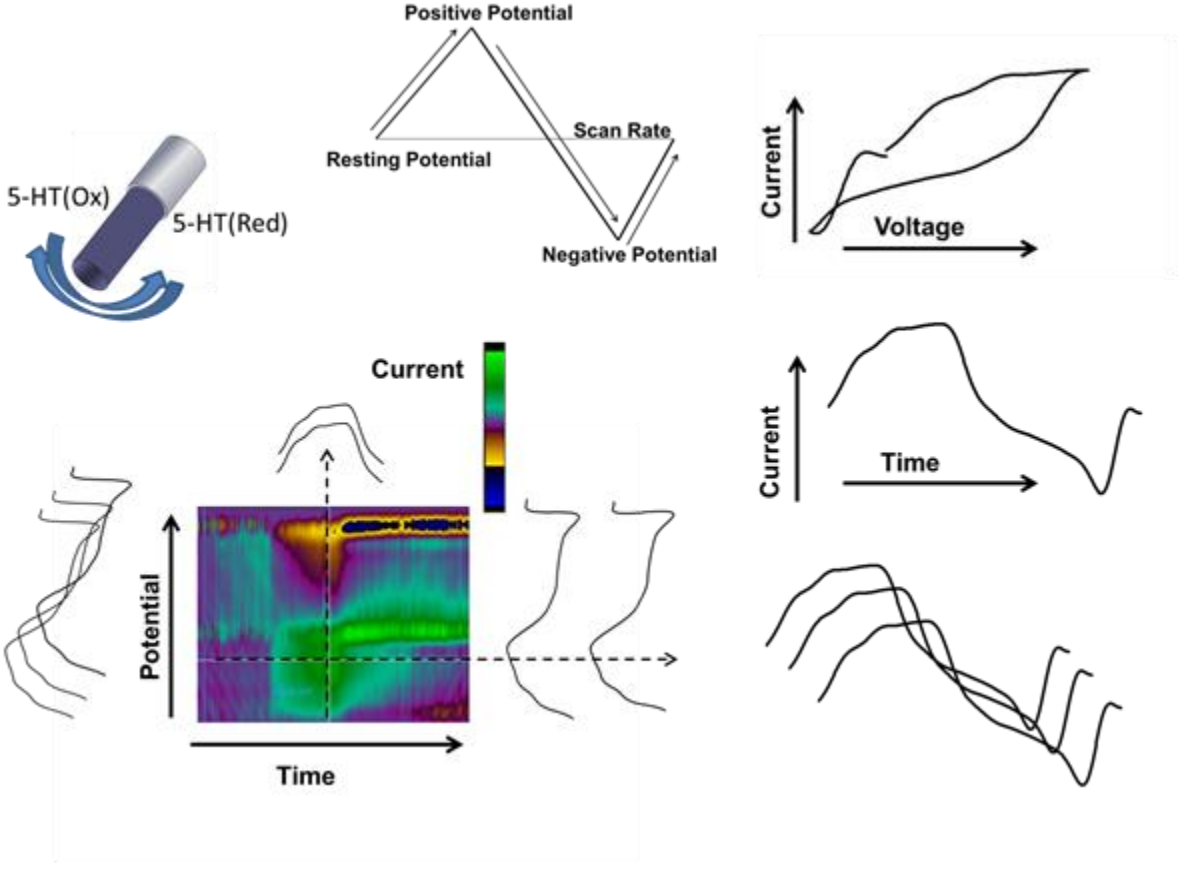

Figure C-2. Fast Scan Cyclic Voltammetry (FSCV) Analytical Technique. During each scan, a waveform is applied to the carbon fiber electrode resulting in rapid changes in potential. Electroactive species undergo oxidation/reduction and the resulting current is then measured and displayed as a cyclic voltammogram (CV). Electroactive species produce characteristic CVs. The color plot shows how the CV changes with time and is used to identify changes in electroactive species.

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ABSTRACT

NOVEL APPROACHES FOR ASSESSMENT OF COPPER TOXICITY: FAST SCAN CYCLIC VOLTAMMETRY AND OPTICAL BIOASSAYS

by

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Anthropogenic activities negatively impact fresh water ecosystems through toxic contaminants that are released into the environment. Copper (Cu) is a water contaminant that is fundamentally persistent once introduced into the environment that has the potential for bioaccumulation. Although Cu toxicity has been studied for decades, there is still a continuing problem with new sources and pathways. New approaches are needed to understand distribution and transport of Cu and its potential for complex biological impacts beyond the simple assessment of lethality. Several novel approaches were used in this research project to advance our understanding of Cu toxicity, including fast scan cyclic voltammetry (FSCV) and two optical bioassays. Fast scan cyclic voltammetry (FSCV) is a powerful new method for measuring electroactive species with high sensitivity and sub-second temporal resolution. The ability to mitigate Cu as an environmental pollutant is largely determined by understanding the movement and complexation of Cu ions in the aqueous state with dissolved organic matter (DOM) and other competing ions. The FSCV analytical technique was used to demonstrate that the type of DOM present influences the Cu binding capacity. In addition, the ability of another metal, aluminum (Al), to cause the release of DOM bound Cu was examined. This series of

experiments addressed factors that can influence the distribution of Cu in the aquatic environment and its bioavailability using a method (FSCV) with very high temporal resolution. *Daphnia magna, a* microcrustacean is a model freshwater species for commonly used in ecotoxicological studies. Novel optical bioassays were used to investigate both the behavioral and physiological changes resulting from exposure to Cu or Cu-DOM complexes. The behavioral bioassay examined swimming behavior by tracking two parameters: (1) maximum accumulated distance that *Daphnia* travel and (2) changes in mean angle that occur over time. The physiological bioassay investigated changes to *Daphnid* heart rate (HR) and appendage beat rate (ABR) after Cu exposure. These optical bioassays demonstrated that the type and amount of available DOM significantly affected the toxic effects of Cu in both the behavioral and physiological assays. Cu was also shown to significantly affect negative-phototactic behavior of *D. magna*. FSCV was used to measure the release of neurotransmitters from *Daphnia* in response to light stimulation. A carbon fiber microelectrode was placed in the brain of the *D. magna and* neurotransmitter release was measured using two different waveforms, one for histamine (histamine-like neurotransmitters) that was less selective, and one waveform that was more selective for serotonin. Light-sensitive neurotransmitter release was detected using both waveforms. When animals were exposed to 0.1 µM Cu there was a significant reduction in the light-coupled release of serotonin observed when using the serotonin waveform. This suggests that the serotonin may be involved in the effects of Cu on negative phototactic behavior. The use of FSCV and these optical assays are capable of advancing our knowledge of Cu distribution in the environment, its bioavailability and potential for sublethal toxicity.

AUTOBIOGRAPHICAL STATEMENT

Education and learning has always been important to me. I love to learn new things and the journey to my PhD has been filled with opportunities to stretch my mind and discover new avenues of knowledge and learning. My dissertation encompasses multiple disciplines including; engineering, chemistry and pharmaceutical science. I obtained a Bachelor's of Science degree from Georgia State University in Atlanta, Ga in and a Master's degree from Wayne State University in industrial hygiene and toxicology. I currently hold certifications as a Certified Industrial hygienist (CIH), a Certified Hazardous Materials Manager (CHMM) and a Registered Environmental Health Specialist (REHS).