8-21-2017

# Kinetics of Copper Uptake in Periphyton under Natural Stream and Wastewater Effluent Exposures

Katelyn Turpin-Nagel
katelyn.turpin-nagel@uconn.edu

# Kinetics of Copper Uptake in Periphyton under Natural Stream and Wastewater Effluent Exposures

**Katelyn Turpin-Nagel**

B.S., University of Wisconsin-Madison, 2013

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science at the

University of Connecticut

2017

# APPROVAL PAGE

Master's Thesis

Kinetics of Copper Uptake in Periphyton under Natural Stream and Wastewater Effluent Exposures

Presented by

Katelyn Turpin-Nagel, B.S.

Major Advisor _____

Dr. Timothy Vadas

Associate Advisor _____

Dr. Alexander Agrios

Associate Advisor _____

Dr. Melissa McKinney

University of Connecticut

2017

## Acknowledgements

I want to thank my advisor, Dr. Tim Vadas of the University of Connecticut's Environmental Engineering Department, for all his support, advice, and in-depth knowledge during my studies as a Master's student. Whenever I questioned my methods, discovered intriguing results, or needed help with a laboratory procedure, he was always willing to listen and lend a helping hand. The growth and knowledge I gained in this short 19$^{th}$ month span has far exceeded my expectations. I largely credit my success to the many hours Dr. Vadas and I spent contemplating and conferring over the numerous intricate topics in water quality, ecotoxicity, metal speciation, and biochemistry.

I would also like to thank Hongwei Luan for his instruction and support in the field and laboratory during the initial set-up of the experimental procedures and to April Doroski for her help with water sample analysis. Thank you to Randi Mendes, Dorottya Kelemen, and Thomas Funk for their assistance with analytical measurements and periphyton growth.

I would also like to extend a thank you to the Natural Science Foundation (NSF) who provided funding for this research.

A special thank you to Stephanie Kexel and Christopher Perkins from the University of Connecticut's Center for Environmental Sciences and Engineering for the chlorophyll-a measurements. Additionally, I would like to extend acknowledgment to Alex Valigosky and his team at EnviroScience, Inc. for their periphyton species-level analyses.

Furthermore, I would like thank Rian Savage, Todd Matthewson, and their team at the University of Connecticut's Waste Water Treatment Plant for waste water safety training and assistance with effluent collection for experimental runs.

Lastly, I would like to extend acknowledgements to Dr. Alexander Agrios of the University of Connecticut's Environmental Engineering Department and Dr. Melissa McKinney of the University of Connecticut's Department of Natural Resources and the Environment who stood on my Master's Thesis Board as associate advisors. I am very grateful for your invaluable comments and suggestions to improve my thesis.

## Table of Contents

## List of Figures

# List of Tables

## Abstract

The bioavailability of copper to aquatic organisms is largely dependent on water quality characteristics, which can influence the chemical speciation. In natural waters, a large percentage of copper is found complexed with inorganic and organic ligands leaving a small percentage available as free metal ions. Complexation with dissolved organic matter (DOM) is fundamental in controlling metal speciation and the source of DOM has been shown to influence the binding strength of the ligands. Wastewater treatment plant (WWTP) effluent DOM has been observed to contain high-affinity binding sites for copper and higher percentages of hydrophilic binding ligands leading to enhanced copper complexation. Thus, metal speciation differences are expected to vary between aquatic environments impacted by urban discharges and those more pristine. These variations in metal speciation between aquatic environments has the potential to impact copper uptake kinetics to photosynthetic organisms. The aim of the present study was to examine the uptake kinetics of copper to periphyton, a biofilm community of heterotrophic and autotrophic species that grow in running water ecosystems, under freshwater and WWTP effluent exposure conditions. Periphyton were colonized in indoor growth aquariums supplied with freshwater from the Fenton River (Connecticut, U.S.A). Copper uptake kinetic experiments were run using a constructed trickle apparatus, where short-term (90 – 380 minutes) exposures were completed using environmentally relevant total copper concentrations (2 – 16 μg/L). Fourteen exposure solutions were analyzed including six using natural stream water from the Fenton River, three consisting of WWTP effluent, and five containing mixtures of Fenton River and WWTP effluent (10%, 30%, and 50% WWTP effluent). The differences in periphyton surface bound copper and intracellular uptake kinetics were observed and modeled by examining periphyton copper content, periphyton cellular binding parameters, and metal speciation based on organic matter characteristics. First-order rate kinetics were used to analyze the differences in initial uptake rates between the different exposure waters over the first 30 – 60 minutes of exposure. It was found that as the percentage of WWTP effluent increases in the exposure waters that the periphtyon total and intracellular uptake rates decrease. Reduced periphyton copper content (surface bound and intracellular) was also observed for the exposure waters containing WWTP effluent. Water quality characteristics were investigated to further analyze the effects of source water on periphyton uptake kinetics. Free copper ion concentrations were estimated for the exposure waters by inputting measured water quality parameters and experimentally derived DOM conditional binding constants into Visual MINTEQ. An equilibrium ion exchange technique was used to investigate the exchangeable copper fractions of the exposure waters. Kinetics modeling using the total, free ion, and exchangeable copper concentrations allowed for examination of the bioavailable copper fraction. With reduced copper contents of periphyton under exposures containing WWTP effluent, this study illustrates a positive role that enhanced WWTP effluent DOM and inorganic complexation may play in limiting copper toxicity to periphyton.

# 1 Introduction

Streams and lakes serve important ecosystem functions such as providing habitats for organisms, transforming nutrients, offering flood and erosion control, and processing organic matter (Meyer et al., 2005). However, as urbanized areas expand, increasing the percentage of impervious areas in watersheds, waterbodies are becoming progressively impaired due to added stressors. Increased impervious areas decreases the amount of rain that is infiltrated into the soil and increases the volume of water and contaminant loadings discharging to nearby waterbodies. Metals are common stressors in aquatic ecosystems primarily entering during storm events and through wastewater treatment plant discharges (USEPA, 2002; Davis & Birch, 2010; Huber et al., 2016). A subset of metals (Cd, Hg, Pb, Ni) are included on the United States' and European's lists of priority pollutants (USEPA, 2002; European Commission, 2008). The US Environmental Protection Agency (EPA) and a subset of environmental agencies in EU member countries also list additional metals, such as Cu and Zn, as metals of concern (USEPA, 2006). High metal loadings to aquatic ecosystems are of particular concern due to their prevalence and toxicity to aquatic organisms. Copper, in particular, is an important metal to investigate in aquatic ecosystems. At low concentrations (on the order of a few μg/L), copper acts as a micronutrient to photosynthetic organisms playing important roles in photosynthesis, enzyme formation, and electron transport chain function (Pinto et al., 2003). However, at higher concentrations (on the order of 10s μg/L) or during prolonged exposure, copper can become a toxicant impacting growth, community function, chlorophyll content, and the distribution of species (Soldo & Behra, 2000; Pinto et al., 2003; Serra et al., 2009a).

The bioavailability of trace metals to aquatic organisms is largely dependent on water quality characteristics, which influences physical and chemical speciation. Metal ions can form complexes with a number of inorganic ligands, including hydroxide ions, carbonates, and chloride (Allen & Hansen, 1996), or bind with organic matter and particulates (Muller, 1995; Allen & Hansen, 1996; Breault et al., 1996; Meylan et al., 2003; Verheyen et al., 2014). In natural waters, a large percentage of copper is complexed with inorganic and organic ligands leaving a very small percentage available as free metal ions, which effects uptake potential (Muller, 1995; Meylan et al, 2003; Meylan et al., 2004). Nevertheless, as natural environments are dynamic in space and time, the concentration of bioavailable copper will fluctuate as water

parameters change (pH, alkalinity, temperature, hardness cations – $Ca^{2+}$, $Mg^{2+}$, and organic matter concentration and characteristics) (Muller, 1995; Breault et al., 1996; Lu & Allen, 2002; Sarathy & Allen, 2005; Worms et al., 2006; Chen et al., 2013; Tonietto et al, 2015). Thus, an in depth understanding of potential copper species that could be present in various natural aquatic environments and discerning which copper species are bioavailable under varying conditions will be pertinent to determining organism accumulation kinetics.

It is well established that dissolved organic matter (DOM) complexation is fundamental in controlling metal speciation (Muller, 1995; Allen & Hansen, 1996; Lu & Allen, 2002; Sarathy & Allen, 2005). The major chemical components of DOM (~50 – 90%) are humic substances. Humic substances, as defined by Natural Organic Matter Research (IHSS), are "complex and heterogeneous mixtures of polydispersed materials formed in soils, sediments, and natural waters by biochemical and chemical reactions during the decay and transformation of plant and microbial remains (2007)." Humic substances in soils and sediment can be divided into three main fractions, which include humic acids, fulvic acids, and humin. Aquatic humic substances generally consist only of humic and fulvic acids and can be separated from non-humic materials (amino acids, peptides, sugars) by lowering the pH and utilizing an absorption procedure to a resin column (IHSS, 2007).

Humic and fulvic acids contain a large variety of different functional groups that act as binding ligands (Allen & Hansen, 1996; Chen et al., 2013; Matar et al., 2015). The heterogeneous nature of organic matter is reflected not only in the concentration of binding ligands, but also in the diversity of binding functional groups each having a wide range of acidity constants (pKa) and affinities for binding ions (Thurman, 1985; Allen & Hansen, 1996; Lu & Allen, 2002; Al-Reasi et al., 2013). Recent research has stressed the significance of source water in determining the capacity of DOM-metal complexation. Effluent DOM is shown to have higher percentages of non-humic substances (Sarathy & Allen, 2005) and hydrophilic ligands than freshwater DOM (Pernet-coudrier et al., 2008; Quaranta, 2011; Matar et al., 2015). It is suggested that the higher percentage of hydrophilic fractions in effluent DOM likely correlates with high-affinity binding sites largely composed of protein structures, amines, and amide groups (Matar et al., 2015). These groups contain high contents of nitrogen, and sulfur that can strongly bind metal ions (Pernet-coudrier et al., 2008). With the greater percentage of high-affinity, hydrophilic binding ligands found in effluent DOM, the complexation of copper ions in

urbanized aquatic environments exposed to wastewater treatment plant (WWTP) discharges could be notably impacted. Thus, metal speciation and the bioavailability of copper has the potential to vary substantially between aquatic resources impacted by urban discharges and those more pristine.

Equilibrium models have been developed to help researchers and regulators determine the role of water quality characteristics and chemical speciation on metal bioavailability to organisms. Chemical equilibrium models for the calculations of metal speciation, solubility, sorption, and humic acid complexation have been used ubiquitously across a wide range of conditions. Some of the most popular models used include the Windermere Humic Aqueous Model (WHAM) and Visual MINTEQ. However, these models contain limitations. Although complexation with organic matter can be simulated, the existing models are based on isolated organic matter fractions from natural sources, which bias to larger molecular weight OM and humic like material (i.e., the lower molecular weight OM is not retained on the isolating resin). Differences in complexation potential due to variations in organic matter source or size are not fully represented in these chemical equilibrium models. Bioavailability models have also been developed, which use chemical equilibrium models with organic matter binding and organism binding components. The U.S. EPA has incorporated the biotic ligand model (BLM) into its regulatory framework for copper. The BLM is designed to predict interactions between metal species and organisms. It accomplishes this through assuming that the metal species of interest are in chemical equilibrium in solution and with the homogeneously distributed biological sites on organisms. The transfer of metal across the biological membrane is generally assumed a first-order process. Therefore, the internalization flux can be related to any metal species in equilibrium. Nevertheless, there exist many assumptions that must be fulfilled for the BLM to be valid. A few include, chemical homogeneousness of the organism plasma membrane, diffusion towards the cell and cell complexation are not rate limiting, a 1:1 binding ratio at cell binding sites is involved, and only a single compound is transported. While research suggests that the biotic ligand approach is a useful construct for determining the interaction of metals with biological organisms at equilibrium with their surroundings (such as those found in laboratory batch studies), several documented exceptions to the BLM exist. Currently the dynamic nature of a biological organism and fluctuating water quality parameters are not taken into account in the current model framework (Slaveykova & Wilkinson, 2005) and the BLM is focused more on

acute toxicity than chronic. Further research is needed to better understand under what circumstances dynamic models may be needed to predict bioaccumulation and to more accurately define biotic ligand binding parameters.

One class of organism particularly sensitive to the bioavailability of copper in aquatic ecosystems is periphyton. Periphyton are a complex, biofilm community of heterotrophic and autotrophic species that grow on various benthic substrates in running water ecosystems. They are fundamental in biogeochemical cycles and important primary producers responsible for the transfer of inorganic nutrients and organic carbon up the food chain (Stevenson et al., 1996). As a fundamental component of the food chain, copper toxicity impacts such as biomass reduction or species composition changes (Soldo & Behra, 2000; Pinto et al., 2003; Serra et al., 2009a) could impact the diets of higher trophic levels. Bioaccumulation of metals to higher trophic levels is also of notable concern (Pinto et al., 2003), specifically to aquatic insects. Metal ions can concentrate in periphyton by complexing with the extracellular matrix, adsorption to cell surface ligands, and through intracellular uptake (Campbell, 1995).

Previous algal and periphyton exposure scenarios have been completed, which investigated metal adsorption and internalization parameters to organisms under various natural water sources. Special attention has been invested to analyzing different water quality characteristics and the associated metal speciation. This has allowed for discoveries into probable metal species that control organism uptake. Bradac et al. (2009) investigated the kinetics of cadmium accumulation in periphyton in artificial flow-through channels supplied with natural freshwater. Two uptake phases for intracellular cadmium internalization were observed: A fast initial uptake rate (first 71 minutes of exposure) and a slower subsequent rate approaching steady state. A first order kinetic uptake model was used to analyze the initial cadmium uptake rates. Under two cadmium exposure scenarios, 5 nM and 20 nM, two uptake rates were found, 0.05 and 0.18 nmol Cd $g^{-1}$ dry weight $min^{-1}$, respectively. This data showed that periphyton can bioaccumulate cadmium rapidly, even at low environmental concentrations. They conjectured that successive exposures in natural waters may maintain elevated metal concentrations in periphyton over time (Bradac et al., 2009). In a field study, conducted by Meylan et al. (2003) in a freshwater river system during rain events, intracellular copper and zinc contents in periphyton were found to escalate rapidly in response to increases in metal concentrations. They found that the increases in periphyton metal contents corresponded to the

exchangeable copper fraction and the free zinc ion concentrations of the river water. The exchangeable copper fraction was defined as the fraction of copper in free or weakly complexed form that exchanged with copper-catechol complexes at a catechol concentration of 1 mM (Meylan et al., 2003). Another study addressed the issue of linking cadmium and lead speciation to green microalgae uptake in waste water treatment plant effluents, specifically looking into the role of effluent organic matter. Interestingly, a decrease in cadmium uptake was observed with the presence of effluent colloidal matter due to complexation; however, an increase in intracellular lead concentrations were observed. Thus, it was found that effluent colloidal matter complexation did not decrease the bioavailability of lead to green microalgae as a speciation model may predict, but instead enhanced the intracellular contents. They conjectured that this result may have occurred due to the metal complexation differences with distinct organic matter size fractions. A high proportion of lead was found bound to the high molar mass fraction of colloidal isolates (> 100 kDa), while no cadmium complexation to these higher molar fractions was observed (Worms et al., 2010). This finding stresses the importance of tying metal speciation to organism uptake kinetics under various water quality and organic matter complexation conditions in order to gain a further understanding of metal bioavailability and toxicity.

The target of this study was to examine the uptake kinetics of copper in periphyton (total and intracellular) under controlled settings. Periphyton were exposed to relevant environmental copper concentrations using freshwater, wastewater effluent sources and freshwater: effluent mixtures. It was hypothesized that as the effluent percentage increased, the copper uptake to periphyton would decrease due to enhanced DOM complexation to strong, hydrophilic ligands and increased binding to inorganic compounds. The differences in surface bound copper and intracellular uptake kinetics under the source waters were observed and modeled by examining periphyton metal content, periphyton cellular binding parameters, and metal speciation based on organic matter characteristics. The conditional binding constants of the freshwater and effluent organic matter were measured. First-order rate kinetics were used to quantify the differences in uptake rates between the various exposure water scenarios.

# 2 Materials and Methods

## 2.1 Periphyton Colonization

The periphyton colonization methodology was adapted from procedures used by Serra et al. (2009b). Approximately 100 frosted slides were preloaded onto glass racks and suspended in 38-liter colonization aquariums for three to five weeks to allow for mature biofilm communities to develop. The tops of the glass slides were positioned approximately 10 centimeters below the water surface. At the start of colonization, periphyton inoculum was introduced into the aquariums through the introduction of a rock collected from the Fenton River, Connecticut, or later by transferring slides from other aquariums. The aquariums were filled with approximately 23-liters of water from the Fenton River and exchanged on a weekly basis. The river water has low background copper concentrations (~ 1.0 µg/L) and no major wastewater or stormwater inputs leading to low nutrient concentrations. The aquariums were spiked with nutrients every other day with approximately 15 µg/L phosphorus (sodium phosphate dibasic) and 0.2 mg/L nitrogen (ammonium chloride and sodium nitrate) to encourage faster growth. Each aquarium contained two submersible pumps to simulate flow and contained an aeration system to supply sufficient dissolved oxygen and carbonate concentrations. Illumination was provided by fluorescent lights situated perpendicular to the growing surfaces of the frosted slides. The photoperiod was 14:10 hours light:dark and the average light irradiance was 300 µmol $m^{-2}$ $s^{-1}$.

## 2.2 Periphyton Composition Analysis

The periphyton matrix composition was analyzed prior to any exposure scenarios using the autotrophic index (AI). The AI is calculated by obtaining the dry weight, ash-free weight, and chlorophyll-*a* concentrations of a periphyton sample. The AI is a means of determining the trophic nature of a periphyton community and was calculated as follows (APHA., 2005)

$$AI = \frac{Biomass\ (ash - free\ weight), mg/cm^2}{Chlorophyll - a, mg/cm^2} \qquad \text{(Eqn 1)}$$

It is valuable to calculate the AI prior to conducting exposure experiments to assess differences in the biofilm communities and to assess any large shifts in the proportion of heterotrophic and autotrophic species. The dry and ash-free weight of the periphyton samples

were determined following Method 10300 C from *Standard Methods for the Examination of Water and Wastewater* (APHA, 2005). Chlorophyll-a was determined following EPA Method 445.0 and was conducted at the Center for Environmental Science and Engineering.

Species composition was assessed approximately every other month throughout the study. A species level analysis was completed by EnviroScience Inc., Ohio closely following the laboratory procedures described in Section 9.0 of the "National Rivers and Streams Assessment – Laboratory Operations Manual" for periphyton analyses (USEPA, 2014).

## 2.3 Trickle Apparatus – Copper Exposure Scenarios

Periphyton communities were examined under controlled conditions of flow on an indoor experimental trickle apparatus. The trickle apparatus was constructed to mimic the colonization systems used by Stroud Water Research Center in Avondale, Pennsylvania. Figure 1 shows the experimental trickle apparatus used for this study. Additional photos of various angles can be seen in the supplemental information.

Exposure experiments were completed using the constructed trickle apparatus and the stable isotopes, $^{63}$Cu or $^{65}$Cu, from August 2016 through April 2017. The natural abundance of $^{63}$Cu and $^{65}$Cu is approximately 69% and 31% respectively (NIDC, 2015). Four days prior to running an exposure scenario, approximately 190 liters of source water was collected from either the Fenton River and/or the University of Connecticut's Wastewater Treatment Plant and added to a 200-liter polyethylene barrel. Care was taken not to collect river water immediately following storm events. The U.S. Geological Survey streamflow gage 01121330 (Fenton River at Mansfield, CT) was monitored if collection was needed following rain or snow melt events (USGS, 2017). Copper isotopes were added to the collected volumes of exposure waters in order to favor a single isotope to more clearly observe uptake kinetics. Generally, $^{65}$Cu was selected as the copper isotope addition for exposure waters containing a high percentage of Fenton River water. This was largely due to the fact that the periphyton were grown in water collected from the Fenton River, which contained low levels of $^{65}$Cu. For exposure periods with high effluent percentages, $^{63}$Cu was favored due to the higher initial copper background concentrations. Various levels of copper isotope concentrations were investigated during the fourteen exposure scenarios ranging from 2.73 µg/L $^{63}$Cu to 17.82 µg/L $^{65}$Cu. Exposure waters consisted of 100% Fenton River, 100% WWTP effluent, and mixtures of both (10%, 30%, and 50% WWTP

effluent). The 100% exposure waters were used as baselines and the mixtures were used to represent the conditions of urbanized rivers experiencing effluent discharges. The solutions sat for four days to come to equilibrium. The temperature of the laboratory was set at 21°C.

Following the four-day equilibrium period, the exposure water was pumped from the 200-liter polyethylene barrel using a peristaltic pump into a 100-cm long trough. An overflow was constructed in the trough creating a 30-mm head before overflowing back into the barrel. A fixed head of 30-mm allowed for constant flow over the slides during the exposure period. Fifteen to 24 periphyton slides (76 x 25 mm) were placed on a 90-cm long Plexiglas board for each exposure scenario and positioned at a fixed angle to allow for uniform flow over the slides. The number of initial slides placed on the trickle apparatus was based on the amount of biomass.

*Figure 1: Experimental trickle apparatus used to run various exposure scenarios using mixtures of two source waters, Fenton River water and UConn WWTP effluent, and two copper isotopes, $^{63}Cu$ and $^{65}Cu$.*

The peristaltic pump was run for 10 minutes prior to adding the periphyton slides. Periphyton slides were exposed to the copper isotope solution for 2 – 7 hours and sampled three times within the first hour to examine initial uptake and twice throughout the rest of the exposure

period. Water samples were taken at the same interval as periphyton slide analysis in order to confirm that the exposure water characteristics were remaining constant over time.

### 2.3.1 Clean Trace Metal Handling

To avoid metal contamination of samples and equipment during experimental procedures, plastic gloves were used. Additionally, all experimental equipment, analysis vials, bottles, tubes, beakers, syringes, and filtration units were acid-washed in 5% $HNO_3$ for at least 24 h, rinsed with deionized water, and when necessary, sealed in plastic bags.

### 2.3.2 Periphyton Experimental Sampling and Processing

During each sampling period, three to five periphyton slides (depending on biomass growth) were sampled randomly to assess biomass copper concentrations. Periphyton were scraped from the slides using an acid washed microscope slide and rinsed thoroughly with experimental source water into a 50-mL centrifuge tube. To obtain homogeneous periphyton suspensions, the solution was mixed using a vortex mixer for 20 seconds. The periphyton suspension was then split into three to four centrifuge vials. The first vial was used to create two replicates to study the total periphyton copper concentration (a combination of surface bound and intracellular metal). The remaining two to three vials were used to study intracellular copper concentrations through washing with ethylenediaminetetraacetic acid (EDTA). These periphyton solutions were treated for 10 minutes with 4.0-mM EDTA to remove copper species adsorbed to the cell wall and extracellular matrix (Meylan et al., 2003; Bradac et al., 2009). The difference between the total and intracellular copper content will define the surface bound copper adsorbed to the cells' surface and extracellular matrix.

The EDTA treated periphyton and non-EDTA treated periphyton solutions were filtered (cellulose nitrate 0.45 μm) and dried at 70°C overnight to obtain the dry weight of each sample. The filters were then digested imitating EPA Standard Method 3050B. The filters were heated at 95°C in 1 mL of trace metal grade (TMG) concentrated nitric acid in a 15-mL digestion tube for 50 minutes. Additional TMG nitric acid was added in 250 μL volumes until no brown fumes were emitted. Hydrogen peroxide (30%, suprapure) was added stepwise until the effervescence was minimal or until the general sample appearance was unchanged. The maximum volume

added was 200 µL. The digested sample was then removed from the heat source and brought to 10-mL volume with deionized water. Subsequently, the digestion sample was diluted five-fold with deionized water for inductively coupled plasma mass spectrometry (ICP-MS) analysis.

### 2.3.3 Water Chemistry and Elemental Analyses

Water samples (total and dissolved) were collected in 50-mL centrifuge vials to analyze TOC, copper isotope concentrations, and pH each time periphyton slides were collected for analysis. Total copper isotope concentrations were determined by digesting 5 mL of acidified water samples (2% with $HNO_3$ (70% trace metal grade)) at 90°C for 30 minutes, followed by ICP-MS analysis. The ICP-MS was used to measure $^{63}Cu$ and $^{65}Cu$ concentrations. External standards, diluted from single-element stock, were used to create calibration curves. To check for instrument changes in sensitivity, a quality control sample was measured every 10 samples and standards and blanks were reanalyzed every 25 samples. QC samples were within 10 percent or better of expected values.

The TOC content was measured using an Apollo 9000 Combustion TOC Analyzer. Quality control standards were prepared from potassium hydrogen phthalate (Ricca Chemical, Arlington, TX). The pH was measured using a calibrated electrode.

For each exposure scenario investigated, a one liter composite water sample was obtained from the 200-L polyethylene barrel. These one liter samples were placed in the freezer to await further analyses. These samples were used to examine alkalinity, anion/cation concentrations (nitrate, ammonium, sulfate, and chloride), and organic matter binding constants. Alkalinity was measured using EPA Method 310.1 and anion concentrations were measured on a Dionex Ion Chromatography System (ICS)-1100 (Thermo Fisher Scientific, Waltham, MA).

### 2.3.4 Organic Matter Binding Constants

Binding constants for organic matter and copper were measured using a competitive ligand exchange-solid phase extraction (CLE-SPE) method resembling the procedure used by Craven (2012). Supelclean ENVI-18 (Supelco) was used as the solid phase exchange resin to separate the organic matter complexes into their hydrophobic and hydrophilic fractions. The hydrophobic fraction of dissolved organic matter sorbs to the resin and is retained, whereas the hydrophilic fraction does not interact with the resin and will pass through the column.

Competing ligands with well-characterized binding strengths were used to determine the binding strength of copper with the hydrophobic and hydrophilic fractions of the dissolved organic matter. The addition of hydrophobic competing ligands decreases the amount of copper that passes through the resin. The addition of hydrophilic competing ligands increases the amount of copper passing through the resin. The competing ligands that were used to effectively compete with dissolved organic matter for copper binding were benzoylactone (Bz) and nitrilotriacetic acid (NTA). Specific binding constants can be calculated for a given source water's organic matter by analyzing the concentrations of copper that pass through the resin with and without the competing ligands. For specific details on experimental procedures and calculations refer to Craven (2012) or Luan (2016).

## 2.4 Modeling Metal Surface Binding to Periphyton

Upon approaching the periphyton surface, metal species will encounter a protective hydrophobic, phospholipidic bilayer membrane speckled with proteins. These proteins may traverse the lipid bilayer and exist as transport proteins or ion channels that transport metal ions into the cell interior (Campbell, 1995). These proteins in the cell's membrane contain a number of functional groups, such as carboxylic, amino, phosphatic, sulfhydryl, thio, and hydroxo groups, that have a high affinity for metal ion binding (Xue et al., 1988; Knauer et al., 1997). The binding sites can generally be divided into two categories: inert sites, where metal ions can be adsorbed to the surface and active sites, where the metal ions can be adsorbed and internalized into the cell (Xue et al., 1988; Campbell, 1995; Sunda & Huntsman, 1998).

To become adsorbed to the cell surface, a complexed metal ion can exchange from its coordinated organic matter ligand to a site on the cell membrane. In other words, the metal ion dissociates from the organic matter ligand to become a free ion and associates to the binding site on a cell membrane protein. This process is described by (Campbell, 1995; Hudson, 1998; Worms et al., 2006):

$$ML \leftrightharpoons M^{z+} + L \qquad \text{Dissociation from OM Ligand} \qquad \text{(Eqn 2)}$$

$$M^{z+} + X_{cell} \leftrightharpoons MX_{cell} \qquad \text{Binding to Cell Membrane Protein Site}$$

Under equilibrium control, the rates of association and dissociation of metal ions to the cell surface binding sites are assumed equal and much faster than the intracellular internalization rate. This model also assumes that the reactions of metal ions and ligands in the bulk solution are essentially at equilibrium (Hudson, 1998). Therefore, the metal transport across the biological membrane is the rate-limiting step and the overall process can be simplified to a thermodynamic equilibrium among free metals ions in the bulk solution and those bound to sites on the cell surface (Worms et al., 2006). Under these assumptions, the equilibrium controlled metal uptake concentration can be expressed as a single, saturable function of the metal ion concentrations, irrespective of the strength or the concentration of the dissolved ligands (Hudson, 1998, Worms et al., 2006). At steady state the intracellular metal concentration, $C_{int}$, can be described by the saturation Michaelis-Menten type equation:

$$C_{int} = \frac{C_{max}\, K_m\, [M]}{1 + K_m[M]} \qquad \text{(Eqn 3)}$$

where $C_{max}$ is the maximum concentration achieved at metal saturation of the transport sites in μg Cu - g of dry weight$^{-1}$, $K_m$ is the affinity of the transport binding site for a free metal ion in L - μg Cu$^{-1}$, and M is the concentration of exchangeable or free metal ions in μg Cu - g dry weight$^{-1}$ (Pasciak & Gavis, 1974; Hudson, 1998; Sunda & Huntsman, 1998; Ploug et al., 1999; Worms et al., 2006).

Similarly, the total periphyton metal concentration (intracellular and surface bound), $C_{total,}$ can be described by a similar steady state equation assuming that metal ions adsorb to two types of binding sites, but only one is followed by internalization (Lamelas et al., 2008):

$$\mathrm{M}+(\mathrm{R_T}+\mathrm{R_S}) \overset{\mathrm{K_{M\text{-}S}}}{\Longleftrightarrow} \mathrm{M}\text{-}(\mathrm{R_T}+\mathrm{R_S}) \qquad \text{(Eqn 4)}$$

where $R_T$ is the concentration of active sites where internalization can occur and $R_S$ is the concentration of inert sites that only allow surface binding. The Michaelis-Menten type equation for total cellular metal concentrations can therefore be written as

$$C_{total} = \frac{C_{max,cell}\, K_{M-A}\, [M]}{1 + K_{M-A}[M]} \qquad \text{(Eqn 5)}$$

where $C_{max,cell}$ is the maximum cellular metal concentration achieved at metal saturation of both the inert and active transport sites and $K_{M\text{-}A}$ is the effective average binding constant of metal to algae. This represents the metal affinity for both surface binding and internalization in the periphyton matrix.

### 2.4.1 Periphyton Binding Parameters

Copper binding constants ($K_M$ and $K_{M-A}$) for active transport sites and inert surface binding sites were determined using short-term (35 minutes) uptake experiments using filtered Fenton River water with varying copper concentrations ranging from 0 to 320 µg/L. Two periphyton slides were suspended in 1-L polyethylene exposure bottles. After 35 minutes of exposure, total and intracellular copper concentrations were measured using the procedure described above. $C_{max,int}$ and $K_M$ were calculated from linearly fitting equation 3 to the measured results. $C_{max,cell}$ and $K_{M-A}$ were calculated from linearly fitting equation 5. Maximum binding sites could be obtained from the slope of the linearly fitted lines and binding constants were calculated from the y-intercepts (Lehninger et al., 1993).

## 2.5 Periphyton Uptake – Kinetics Modeling

The uptake and efflux rates of copper can be estimated using the following differential equation (Landrum et al., 1992; Komjarova & Blust, 2009):

$$\frac{dC_t}{dt} = (k_u C_w) - (k_e C_t) \qquad \text{(Eqn 6)}$$

which stipulates that the change in copper concentration in the periphyton, $C_t$, over time, t, is a function of uptake minus loss. The uptake rate from the surrounding bulk solution is defined by a conditional uptake constant, $k_u$, in units of µg Cu $g^{-1}$ tissue $hr^{-1}$ per µg Cu $L_{water}^{-1}$ (simplified to $L_{water}$ per g tissue hr) multiplied by the concentration in water, $C_w$, in ug per $L_{water}$. The efflux rate is defined by a rate constant of loss, $k_e$ (in $hr^{-1}$), multiplied by the concentration in the periphyton.

When the efflux process is negligible, the uptake rate becomes directly proportional to the metal concentration in water, resulting in the following derivation (Komjarova & Blust, 2009):

$$C_t = k_u C_w t + C_{t0} \qquad \text{(Eqn 7)}$$

When the elimination term is significant and $C_w$ held constants, the derivation of equation 6 results in the following solution (Landrum et al., 1992; Komjarova & Blust, 2009):

$$C_t = C_w \frac{k_u}{k_e}(1 - e^{-k_e t}) + C_{t0} \quad \text{(Eqn 8)}$$

Growth rates of the periphyton for modeling purposes are neglected due to the short duration of exposure (2 – 7 hours).

Based on the analysis of the various uptake curves, it was assumed that efflux was not significant during the first 30 – 60 minutes of exposure. Thus, the uptake rate constants, $k_u$, for the internalization of copper isotope to the periphyton were calculated by taking the slope of the linear regression (Equation 7).

## 2.6 Exchangeable Copper Concentrations – Equilibrium Ion Exchange Technique

The procedure outlined by Worms and Wilkinson (2008) was used to investigate the copper ion concentrations of the various periphyton exposure waters. Dowex 50W-X8 resin columns were constructed with polyethylene tubing. Approximately fifty milligrams of dry resin were packed into the columns and drawn with DI water. Four columns were run at one time using a four-channel peristaltic pump. Filtered (0.45 μm) Fenton River water, UConn WWTP effluent and mixtures of river and effluent water were tested with the resin columns.

The following procedure was performed for each experiment: the resin columns were flushed with DI water for 5 minutes at 5 mL/min and then exposed for 5 minutes to 1.5M optima nitric acid at 5 mL/min; 10 second rinse with DI water at maximum pump speed followed by an air gap; conversion of the resin to its sodium form by rinsing for 5 minutes with 0.1M NaOH at 5 mL/min; Rinse with DI water until pH equilibrium is reached followed by an air gap; pre-equilibration of the resin with an electrolyte solution with the same pH, [Na], [Mg], and [Ca] of the sample for 5 minutes at 5 mL/min; equilibration of the sample with the resin; rinse with DI water for 10 seconds at maximum pump speed followed by an air gap; elution of the metal bound to the resin at 0.5 mL/min with 1.5M optima $HNO_3$ into pre-weighed 15-mL centrifuge vials. Elution samples were diluted and analyzed for copper concentrations using an ICP-MS.

The amount of copper bound to the resin, {Cu-R} (mol/g), can be calculated through knowing the mass of resin in the column ($m_{res}$), the volume of acid elute ($V_{el}$), and the concentration of copper in the elute ($[Cu]_{el}$) through the following equation

$$\{Cu - R\} = \frac{V_{el} \times [Cu]_{el}}{m_{res}} \quad \text{(Eqn 9)}$$

The concentration of the exchangeable free copper ion concentration can be calculated by the following

$$[Cu^{2+}]_{ex} = \frac{V_{el} \times [Cu]_{el}}{m_{res} \times \lambda'} \quad \text{(Eqn 10)}$$

where λ' (L/g) is the partition coefficient between the free copper ion in solution and the copper bound to the resin. It is determined by calibrating the resin with known solutions of $Cu^{2+}$ with the same ionic compositions (ie., [Na], [Mg], [Ca]), pH, and temperature of the samples.

$$[\lambda'] = \frac{\{Cu - R\}}{[Cu^{2+}]} \quad \text{(Eqn 11)}$$

In this manner, the partition coefficient was found through running 1-L calibration samples through the Dowex resin. Due to the ionic compositions of the samples, the full 1-L calibration samples had to be run through the resin in order for equilibrium to be reached. Equilibrium for the Fenton River, WWTP effluent, and Fenton: Effluent mixtures were reached in 30 to 60 minutes (150 mL – 300 mL).

## 2.7 Visual MINTEQ Speciation Modeling

Visual MINTEQ was used to complete chemical equilibrium calculations for each exposure scenario. The exposure waters' pH, alkalinity, anion, and cation concentration measurements were input to the modeling program. New chemical parameters and thermodynamics inputs were created for the organic matter through modifications to the model's ligand and equilibria database. Two organic matter species were added, hydrophilic and hydrophobic fractions, and separate characteristics were input for each. In the procedure adopted from Craven (2012), conditional binding constants and ligand concentrations were found separately for the hydrophilic and hydrophobic fractions. These experimentally established values were utilized in the chemical equilibrium model to represent conditional copper-DOM binding. The model's output files were used to define the copper speciation for the fourteen exposure scenarios. The water quality Visual MINTEQ input data can be viewed in the supplemental section.

## 2.8 Statistics

For all of the periphyton replicates measured during trickle apparatus exposure experiments, standard deviations were calculated and plotted as error bars. For the periphyton replicates measured during the first 30 – 60 minutes of exposure, Excel regressions were developed in order to assess the uptake rates ($r_t$, $r_u$) and standard errors were calculated using Excel's statistics package. ANOVA tests (CI = 95%) were completed on the uptake rate constants, $k_u$, found for the trickle apparatus exposure experiments using Excel's statistics package.

# 3 Results and Discussion

## 3.1 Trickle Apparatus Exposure Experiments

### 3.1.1 Periphyton Composition

The autotrophic indexes (AI) of the periphyton were obtained by measuring dry masses, ash-free weights, and chlorophyll-a of a representative set of slides and ranged from 130 to 940 (Table 1). Normal AI's, as defined by the *Standard Methods for the Examination of Water and Wastewater* (APHA, 2005), range from 50 to 200. Larger values indicate heterotrophic associations. It was difficult to maintain steady AI between different growth aquariums and over time in the same aquarium. Typically, three dynamic trickle apparatus experiments were run out of a single growth aquarium. Despite the slides growing in the same aquarium, sometimes an extra two weeks of biomass growth could change the AI measured. However, since different slides were used to define the ash-free weight and the chlorophyll-a values, some discrepancies may exist in the AI calculation. The AI calculation is normalized by the slide area. It is likely that there were slight differences in the amounts of biomass used for the ash-free weight and chlorophyll-a analyses, which would impact the AI result. Based on the AI values found, the periphyton used for the various dynamic trickle apparatus analyses were largely heterotrophic based.

*Table 1: Autotrophic Indexes of the periphyton used in the trickle apparatus exposure experiments*

| Exposure Water | Exp. | Date | Autotrophic Index |
|---|---|---|---|
| **100% Fenton River** | 1 | 10/13/16 | 470 |
| | 2 | 10/6/16 | 230 |
| | 3 | 8/10/16 | - |
| | 4 | 9/1/16 | 320 |
| | 5 | 8/22/16 | 260 |
| | 6 | 4/6/17 | 430 |
| **100% WWTP Effluent** | 7 | 9/12/16 | 130 |
| | 8 | 12/8/16 | 940 |
| | 9 | 12/16/16 | 560 |
| **10% Effluent: 90% River** | 10 | 3/16/17 | 150 |
| **30% Effluent: 70% River** | 11 | 4/13/17 | 350 |
| | 12 | 2/22/17 | 830 |
| | 13 | 3/30/17 | 580 |
| **50% Effluent: 50% River** | 14 | 3/9/17 | 430 |

Microscopic, species-level and family-level analyses performed by EnviroScience, Inc. gave further insight into the composition of the periphyton (Table 2). Despite growing the periphyton in the lab under controlled temperature and nutrient conditions, consistency in the periphyton species was not obtained. This did not, however, seem to impact the uptake rates investigated (as discussed in later sections). It can be seen that a large percentage of the periphyton samples analyzed were predominately autotrophic (i.e., larger percentages of cyanobacteria and bacillariophyta-diatoms). Except for the sample sent to the lab in December, which indicated a very large percentage of green algae, *Chlorophyta,* it appears lab growth favored cyanobacteria species.

*Table 2: Summarization of periphyton species level analysis conducted by EnviroScience, Inc.*

| Sampling Date | % of Total Sample | | |
|---|---|---|---|
| | ***Bacillariophyta*** | ***Chlorophyta*** | ***Cyanobacteria*** |
| 9/21/16 | 4.6 | 16.6 | 78.8 |
| 12/1/16 | 2.8 | 96.3 | 0.9 |
| 2/2/17 | 26.7 | 29.2 | 43.9 |
| 4/10/17 | 0.7 | 16.4 | 82.9 |

It is not entirely clear why the shift in species abundance occurred in December. One possibility is that the distribution shift occurred due to small increases in the total copper concentrations in the growth water during the month prior. During November, batch experiments were run using one growth aquarium to test efflux potential (data not shown). This growth aquarium was exposed to low levels of $^{65}Cu$ (< 4.0 μg/L) for three weeks. During this time of year, when leaf litter was high in the Fenton River, previous aquarium slides were used as inoculum in new growth aquariums. It is likely that slides from the aquarium exposed to low levels of $^{65}Cu$ were used as inoculum for the next growth aquarium, which would align with the slides analyzed in December. Soldo and Behra (2000) performed 12-week long exposure scenarios with periphyton under different copper concentrations. Under the highest copper treatment (5.0 μM) they found a significant shift in the distribution of algal classes from a community dominated by Cyanophyceae to one dominated by Chlorophyta. While significant differences were only found at the highest copper treatment investigated, species' distribution shifts were also seen at the lowest copper concentration investigated (0.05 μM). Even though copper exposures used in our study were lower (< 0.04 μM) than the lowest copper concentration studied by Soldo and Behra and occurred for a shorter duration (3 weeks), it is possible that our shift from Cyanophyceae to Chlorophyta occurred because of the inoculum's previous exposure to copper. Even though the new aquarium was not exposed to $^{65}Cu$ and was exposed to natural periphyton from the Fenton River through weekly water exchanges, the aquarium slide inoculum could have favored Chlorophyta growth for the sample sent to EnviroScience Inc. in December. Two trickle apparatus uptake experiments were completed using the periphyton species with a higher Chlorophyta percentage (Experiments 8 and 9). The next fresh, growth aquarium that followed the growth aquarium used in December utilized inoculum directly from the Fenton River and not from a previous growth aquarium.

The structure of a periphyton community plays an important role in defining trophic interactions in dynamic environments. Specific growth rates vary among species and can largely depend on individual's sizes. Growth rates of specific species may also be enhanced by enriching elements or can be reduced by competitive exclusion between organisms or through contamination of toxicants (Morin et al, 2008). The laboratory experimental growth set-ups used in this study were designed to be as similar as possible to the freshwater environment they were mimicking. Yet, some technical limitations of laboratory growth could have influenced the periphyton species' distributions. Nutrient supply in the laboratory was different than what would be experienced in the field (continuous inputs). In the laboratory, macronutrient additions (N, P) were supplied every other day to compensate for depletion and micronutrient depletion was countered with whole aquarium water exchanges on a weekly basis. Under these pulsed nutrient-unlimited conditions, specific species may have been able to outcompete others (Morin, 2008). Light irradiance is another factor that had the potential to influence species distributions. The lab grown periphyton were exposed to a constant irradiance for their 14-hour day period (average of 300 μmol $m^{-2}$ $s^{-1}$) with no shading effects as may be experienced in a natural environment from clouds and biota. Furthermore, sloughing or grazing activities could have impacted growth in the aquariums. Since the aquariums were supplied with freshwater from the Fenton River on a weekly basis, it is natural that macroinvertebrates could have been collected and introduced into the aquariums as well. Without constant addition of fresh inoculum from an outside source, the grazing of preferred algal species by protozoa or daphnia could have allowed for other species to outcompete. While it is likely that grazing did not have as significant of an impact, it is still worth consideration.

A few previous studies investigating the effects of metal contamination on periphyton have reported species abundance. In a study by Bradac et al. (2009), periphyton were colonized in indoor artificial flow-through channels supplied consistently with freshwater from the Chriesbach stream (Dübendorf, Switzerland). Semiquantitative microscopal analysis after three weeks of growth showed that the periphyton were mostly composed of diatoms with the dominant species being *Nitzschia palea*. Water temperature was around 9.5°C and pH approximately 7.9. Another study using the Chriesbach stream as a growth source in indoor channels reported diatom dominance as well with the major species consisting of *Achnanthes*, *Navicula*, and *Nitzschia*. Species dominance was reported during the summer experiments where

water temperatures averaged 17.9°C and pH averaged 7.74 (Navarro et al., 2007). In the study by Soldo and Behra (2000), periphyton were colonized in outdoor flow-through glass aquaria supplied with water from the Glatt River (pH = 8.0 – 8.3). Control sample species composition consisted of approximately 24% Chlorophyta, 14% Bacillariophyceae, and 62% Cyanophyceae. All three of these studies had greater access to continuous freshwater replenishment by stationing their experiments so close to a freshwater source. Additionally, these studies reported initial/control species abundance for a single experiment. Our study investigated uptake over a nine-month period and required the growth of multiple sets of periphyton slides. Comparing our study's species distribution to those of other studies is difficult due to the many factors that can influence species abundance.

### 3.1.2 Exposure Water Characteristics

Periphyton exposure scenarios fell over a nine-month long period. Naturally, this lead to water quality variations in the Fenton River and UConn's WWTP effluent over time. Since natural freshwater and effluent sources were utilized for the exposures, variations were allowed for total alkalinity, total organic carbon (TOC), pH, initial copper concentrations, copper speciation, and background anions and cations (Table 3 and Table 4) and Six exposure scenarios using water collected only from the Fenton River, three using water collected solely from UConn's WWTP, and five using different mixtures of both source waters were analyzed.

The total alkalinity as well as the concentrations of major anions and cations were fairly consistent for each exposure water type throughout the year (Table 3). The Fenton River freshwater average total alkalinity was 15.4 ± 6.3 mg $CaCO_3$/L and the average total alkalinity for the WWTP effluent samples was 99.2 ± 13.4 mg $CaCO_3$/L. For the mixtures, as the percentage of effluent increased, the total alkalinity increased as well. Similar to total alkalinity, as the percentage of effluent increased in the exposure water, so did the concentration of anions and cations. The pH of the exposure waters varied slightly between the different sources. The pH of the WWTP effluent was fairly consistent at each collection period with an average of 8.3 ± 0.1. The average pH of the collected Fenton River water was 7.3 ± 0.25 and the average pH for the 10%, 30%, and 50% effluent mixtures were 7.2, 7.3 ± 0.1, and 7.5, respectively (Table 4).

TOC concentrations varied from 1.9 to 14.4 mg C/L in all source waters and likely varied due to the time of year water was collected. While all effluent samples were collected while students were on campus, the UConn WWTP effluent does not undergo a chlorinated disinfection process from the end of November through early April. The chlorination process could impact the organic matter concentration and well as its characteristics. Average TOC concentrations for the Fenton River and UConn's WWTP effluent was 5.2 ± 3.0 and 9.2 ± 3.0 mg C/L, respectively. Average TOC concentrations for the 10%, 30%, and 50% effluent mixtures were 3.5, 4.3 ± 0.25, and 5.1 ppm C respectively (Table 4).

Average background total copper concentrations of the Fenton River were low upon initial collection (0.66 ± 0.5 μg/L). Average background total copper concentrations of the UConn WWTP effluent were 5.57 ± 0.7 μg/L. Copper isotopes were added to the collected exposure waters following measurement of the background copper concentration. Fourteen exposure experiments were completed with the constructed trickle apparatus. Six experiments were run using water collected from the Fenton River. Five of these experiments were run using low to intermediate concentrations of copper isotope (2.4 – 4.5 μg/L). The sixth contained a high copper isotope concentration (15.9 μg/L) approximately three times higher than the U.S. EPA aquatic life freshwater final acute value (USEPA, 2007). Three experiments were run using effluent water collected from the UConn WWTP. One effluent collection occurred during the plant's chlorinated disinfection period and the other two occurred during the period when the plant does not conduct disinfection. All three effluent exposure waters were run using intermediate concentrations of copper isotope (3.0 – 5.7 μg/L). Four effluent: river water mixtures were analyzed on the trickle apparatus. One experiment was conducted using approximately 10% UConn WWTP effluent and 90% Fenton River water ($^{65}$Cu ~ 5.1 μg/L) and one experiment was conducted using 50% UConn WWTP effluent and 50% Fenton River water ($^{65}$Cu ~ 5.0 μg/L). Three mixture experiments were completed using 30% effluent and 70% river water. Two 30:70 experiments were run using intermediate copper isotope concentrations (3.5 and 5.0 μg/L) and one was run using a high copper isotope concentration (16.3 μg/L) (Table 4).

The copper isotope ($^{63}$Cu versus $^{65}$Cu) selection used in the different exposure scenarios generally followed the percentage of WWTP effluent. For exposures that contained a high percentage of freshwater (>50%), generally $^{65}$Cu was used as the isotope of importance. Only

two experiments deviate from this trend. Two 100% Fenton River exposure scenarios utilized $^{63}Cu$ rather than $^{65}Cu$. This occurred since the periphyton for these exposure scenarios had been exposed to slightly higher initial $^{65}Cu$ concentrations (~ 2.0 μg/L). It was not expected that the slight differences in the copper isotope's molecular weight ($^{63}Cu$ ~ 62.93 g/mol; $^{65}Cu$ ~ 64.93 g/mol) and atomic diameters had a significant impact on periphyton uptake. While differences in molecular weight and diameter can impact diffusive flux of free ions to the periphyton cells, it is expected that water quality characteristics (pH, alkalinity, TOC, background anion concentrations, DOM characteristics) and total copper isotope concentrations had a much greater influence on uptake due to copper's high affinity for complexation to inorganic and organic ligands.

*Table 3: Water quality characteristics of the exposure water during periphyton analyses on the trickle apparatus including total alkalinity, ammonium, nitrate, chloride, sulfate, sodium, calcium, and magnesium.*

| Exposure Water | Exp. | Total ALK mg $CaCO_3$/L | $NH_4^+$ µg/L | $NO_3^-$ mg/L | $Cl^-$ mg/L | $SO_4^{2-}$ mg/L | $Na^+$ mg/L | $Ca^{2+}$ mg/L | $Mg^{2+}$ mg/L |
|---|---|---|---|---|---|---|---|---|---|
| **100% Fenton River** | 1<br>2<br>3<br>4<br>5<br>6 | 15.4 ± 6.3 | 4.5 ± 5.3 | 1.0 ± 0.6 | 35.7 ± 3.2 | 7.0 ± 0.8 | 22.6 ± 14.4 | 10.9 ± 5.1 | 3.0 ± 0.7 |
| **100% WWTP Effluent** | 7[1]<br>8<br>9 | 99.2 ± 13.4 | 61.0 ± 49.5 | 27.5 ± 9.3 | 134.6 ±16.2 | 43.3 ± 7.6 | 101.3 ± 8.1 | 25.9 ± 4.6 | 14.6 ± 2.4 |
| **10% Effluent: 90% River** | 10 | 16.0 | 26.6 | 5.7 | 83.5 | 11.3 | 40.0 | 11.3 | 4.7 |
| **30% Effluent: 70% River** | 11[1]<br>12<br>13 | 22.7 ± 3.1 | 39.6 ± 28.1 | 14.3 ± 3.2 | 112.0 ± 8.8 | 18.0 ± 3.2 | 66.9 ± 5.7 | 16.7 ± 1.0 | 6.5 ± 0.2 |
| **50% Effluent: 50% River** | 14 | 40.6 | 71.0 | 17.4 | 145.4 | 20.7 | 82.1 | 17.8 | 9.0 |

[1] UConn waste water treatment plant effluent was collected during the chlorination disinfection period

*Table 4: Water quality characteristics of the exposure water during periphyton analyses on the trickle apparatus including total copper isotope concentrations, total organic carbon, and pH.*

| Exposure Water | Exp. | $^{63}$Cu ppb | $^{65}$Cu ppb | Total Cu ppb | TOC ppm C | pH |
|---|---|---|---|---|---|---|
| 100% Fenton River | 1 | 2.37 ± 0.08 | 0.41 ± 0.04 | 2.78 ± 0.13 | 4.2 | 7.3 ± 0.1 |
| | 2 | 2.53 ± 0.08 | 0.32 ± 0.01 | 2.86 ± 0.10 | 1.9 | 7.3 ± 0.1 |
| | 3 | 0.40 ± 0.07 | 3.00 ± 0.11 | 3.40 ± 0.15 | 4.2 ± 0.1 | 7.6 ± 0.0 |
| | 4 | 0.31 ± 0.13 | 4.15 ± 0.13 | 4.46 ± 0.23 | 5.6 ± 0.1 | 7.3 ± 0.0 |
| | 5 | 0.77 ± 0.03 | 4.45 ± 0.07 | 5.22 ± 0.08 | 11.5 ± 0.2 | 7.5 ± 0.1 |
| | 6 | 0.03 ± 0.05 | 15.70 ± 0.41 | 15.73 ± 0.37 | 3.9 ± 0.1 | 6.8 ± 0.0 |
| 100% WWTP Effluent | 7[1] | 2.57 ± 0.06 | 3.02 ± 0.07 | 5.59 ± 0.12 | 14.4 ± 0.1 | 8.4 ± 0.2 |
| | 8 | 5.36 ± 0.07 | 2.35 ± 0.09 | 7.71 ± 0.15 | 7.1 ± 0.1 | 8.3 ± 0.3 |
| | 9 | 5.70 ± 0.11 | 2.48 ± 0.04 | 8.17 ± 0.15 | 6.3 ± 0.5 | 8.2 ± 0.1 |
| 10% Effluent: 90% River | 10 | 0.27 ± 0.02 | 5.12 ± 0.09 | 5.39 ± 0.09 | 3.5 ± 0.1 | 7.2 ± 0.0 |
| 30% Effluent: 70% River | 11[1] | 1.30 ± 0.13 | 3.52 ± 0.15 | 4.82 ± 0.27 | 4.7 ± 0.0 | 7.2 ± 0.0 |
| | 12 | 1.22 ± 0.08 | 5.03 ± 0.30 | 6.25 ± 0.38 | 4.6 ± 0.1 | 7.4 ± 0.0 |
| | 13 | 1.49 ± 0.02 | 16.33 ± 0.20 | 17.82 ± 0.22 | 4.2 ± 0.2 | 7.2 ± 0.0 |
| 50% Effluent: 50% River | 14 | 1.60 ± 0.04 | 5.01 ± 0.09 | 6.61 ± 0.11 | 5.1 ± 0.1 | 7.5 ± 0.0 |

[1] UConn waste water treatment plant effluent was collected during the chlorination disinfection period

By allowing for inconstancies in water quality parameters not only between different categories (100% freshwater, 100% WWTP effluent, mixtures), but also within the same category (i.e., six different 100% freshwater scenarios), this adds another level of complexity. There are many potential water quality parameters in natural systems that can impact metal speciation and complexation. These include pH, temperature, hardness ($Ca^{2+}$, $Mg^{2+}$), alkalinity ($HCO_3^-$, $CO_3^{2-}$), total organic carbon (TOC) concentrations and organic matter properties. While this study aimed to investigate the differences in periphyton uptake due to differences in freshwater and effluent organic matter, it also strived to examine the differences in exposure waters as a whole.

The pH influences metal ion complexation with organic matter. There exist two major functional binding sites in DOM humic substances, carboxylic – (pKa ~ 4.5) and phenolic –

(pKa~ 10) types. For copper ions, studies have shown Cu-DOM complexation to be significantly pH dependent over the entire range of the pH scale. This significant pH dependency indicates that phenolic sites account for the majority of copper ion complexation to humic substances through Cu – H exchange at ligand sites (Lu & Allen, 2002; Matar et al., 2015). Thus, as pH increases, copper complexation to DOM is strongly enhanced through less competition with protons for phenolic sites. Lu and Allen found that Cu-DOM complexation increases approximately 10-fold per pH unit, even at relatively high pH (>8) (2002). Only at very high copper concentrations or very low pH would copper ions favor carboxylic sites, which have a much weaker affinity for copper ion binding (Lu & Allen, 2002). In this study, pH ranged from 6.8 (100% freshwater) to 8.4 (100% WWTP effluent), which indicates that pH dependent complexation has the potential to impact periphyton uptake kinetics by influencing the concentration of free and labile copper ions. Additionally, for the high copper exposure scenarios (> 15 μg/L), complexation of copper ions with carboxylic sites is likely and should be considered when contemplating the different organic matter properties (freshwater vs. effluent).

Not only will protons compete with metal ions for DOM binding, but so will other cations in natural waters. Water hardness ($Ca^{2+}$, $Mg^{2+}$) has the capacity to influence DOM binding. While the binding affinity of $Ca^{2+}$ can be weaker than other metal ions, namely $Cu^{2+}$, the effect of $Ca^{2+}$ is important to consider when distinguishing complexation with OM due to the high concentrations of $Ca^{2+}$ that can be found in natural waters and WWTP effluent (Lu & Allen, 2002). Chen et al. (2013) conducted a complexation analysis of copper with natural organic matter (NOM) at $Ca^{2+}$ concentrations of 0, 50, 100, and 150 mg/L to assess competitive binding effects (pH = 6; [Cu] = <100μM). The calcium concentration ranges of 0 – 150 mg/L represents the natural abundance range that can be found in most freshwaters. It was found that $\log K^{c}_{CuL}$ values at 150 ppm $Ca^{2+}$ were significantly smaller than those at 0 – 100 mg/L indicating that at high concentrations calcium ions effectively compete with copper for binding ligands. It was postulated that high calcium concentration compensates for a lower binding affinity to OM ligands and results in a significant competitive effect (Chen et al., 2013). Additionally, Lu and Allen (2002) found a lack of competition between $Ca^{2+}$ or $Mg^{2+}$ with $Cu^{2+}$ at concentrations ranging from $10^{-6}$ to $10^{-3}$ M (pH = 6 & 7; [Cu] = 2 or 5 μM). Through analyzing the pH dependency of Ca-DOM complexation they found that although a portion of calcium ions may be bound by phenolic sites at low concentrations or at a high pH, the majority of calcium ion

binding on DOM is at carboxyl sites. A similar finding was found for $Mg^{2+}$. Therefore, it is proposed that the lack of competition between calcium and copper ions is largely due to the fact that they bind to different ligand types (Lu & Allen, 2002). In this study, the 100% WWTP effluent samples had the highest $Ca^{2+}$ and $Mg^{2+}$ concentrations averaging 25.9 ± 4.6 and 14.6 ± 2.4 mg/L respectively. While water hardness may have some impact on Cu-DOM complexation, based on past findings (Lu & Allen, 2002; Chen et al., 2013), it is likely that in this study water hardness may not have had as substantial of an impact on controlling metal complexation to DOM.

While water quality characteristics have been shown to influence Cu-DOM complexation, a considerable amount of research has also been devoted to analyzing how DOM characteristics between sources can impact copper complexation. As urbanization expands, more natural water bodies are receiving effluent discharges from WWTPs. Since source can notably affect the make-up of organic matter (i.e. the percentage of humic substances), it is important to understand the relative differences in organic matter properties between source waters and how this can influence metal ion binding. In a study by Sarathy and Allen (2005), there were clear indications of strong binding ligands present in effluent DOM that were not present in NOM obtained from two freshwater resources. These strong binding ligands did not appear to be humic substances, but rather non-humic, sulfide materials that produced lower free $Cu^{2+}$ concentrations (Sarathy & Allen, 2005). This discovery led to additional investigations of effluent DOM. Matar et al. (2015) collected effluent DOM from a WWTP and compared the characteristics of the effluent DOM to DOM from the Seine River. They employed various physicochemical tools, namely UV-visible adsorption and polarity fractionation using resin columns to specify the DOM characteristics (hydrophobic, transphilic, and hydrophilic ligands). Trace metal complexation was modeled using a bimodal distribution for the freshwater DOM and a trimodal binding site distribution for effluent DOM. The trimodel distribution for effluent DOM, with its inclusion of a very high-affinity binding sites, was proven to be more effective than using a bimodal distribution with the typical low- (carboxyl) and high-affinity (phenolic) sites. Matar et al. found that the proportion of hydrophilic substances in effluent DOM was approximately 48%. For the freshwater DOM, the hydrophilic fraction ranged from 27 – 44% (2015). In other studies, the hydrophilic fraction for effluent DOM ranged from 41 – 80% (Pernet-coudrier et al., 2008; Quaranta, 2011). It is suggested that the higher percentage of hydrophilic fractions in effluent

DOM likely correlates with very high-affinity binding sites. These very high-affinity binding sites are largely composed of protein structures, amines, and amide groups containing high contents of hydrogen, nitrogen, and sulfur. According to speciation computations, $Cu^{2+}$ is almost entirely bound to very high-affinity binding sites (hydrophilic binding sites) in effluent DOM (Matar et al., 2015). With a greater percentage of high-affinity, hydrophilic binding ligands found in effluent DOM, the complexation of copper ions in natural environments exposed to WWTP discharges could be notably impacted. Thus, metal speciation and toxicity has the potential to vary substantially between aquatic resources that are influenced by effluent discharges and those that are not.

#### *3.1.2.1 Exposure Water Organic Matter Binding Properties – Conditional Binding Constants*

The source of a water can impact metal binding properties by influencing the percentages and binding strength of hydrophilic, hydrophobic, and non-humic ligands. When calculating the conditional binding constants of the organic matter of the exposure waters used in this study, ionic strength, pH, and copper concentrations were taken into account (Table 5). The hydrophilic DOM binding constants, $\log^{c}K$, for Fenton River water ranged from 7.5 - 9.6. The hydrophobic DOM binding constants for Fenton River water ranged from 8.6 - 10.6. For the UConn WWTP effluent, the hydrophilic and hydrophobic DOM binding constants ranged from 11.5 - 13.1 and 11.9 - 15.1 respectively. Generally, the WWTP effluent: Fenton River water mixtures had binding constants that fell between the 100% solutions. As expected, the exposure waters containing the highest copper concentrations (>15 µg/L) had the lowest conditional DOM binding constants since it is expected to see conditional binding constants decrease as the Cu:DOM ratio increases (Craven, 2012).

*Table 5: Trickle apparatus exposure water dissolved organic matter (DOM) conditional binding constants with copper. Binding constant ranges were determined through competitive ligand exchange-solid phase extraction and are summarized for the hydrophilic and hydrophobic DOM fractions.*

| Exposure Water | Exp. | Average Total Cu | TOC | pH | Ionic Strength[2] | $\log^{c}K$ (Cu-DOM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ppb | ppm C | | mM | Hydrophilic DOM Ligands Range ($M^{-1}$) | Percentage Cu Bound | Hydrophobic DOM Ligands Range ($M^{-1}$) | Percentage Cu Bound |
| **100% Fenton River** | 2 | 2.86 | 1.9 | 7.3 | 2.18 | 9.3 – 9.6 | 59% | 10.1 – 10.6 | 41% |
| | 6 | 15.73 | 3.9 | 6.8 | 1.71 | 7.5 – 8.0 | 61% | 8.6 – 9.1 | 39% |
| **100% WWTP Effluent** | 7[1] | 5.59 | 14.4 | 8.4 | 9.85 | 11.9 – 12.2 | 82% | 12.0 – 13.5 | 18% |
| | 8 | 7.71 | 7.1 | 8.3 | 9.72 | 12.3 – 13.1 | 62% | 15.1 | 38% |
| | 9 | 8.17 | 6.3 | 8.2 | 10.11 | 11.5 – 12.2 | 53% | 11.9 – 13.4 | 47% |
| **10% Effluent: 90% River** | 10 | 5.39 | 3.5 | 7.2 | 3.58 | 9.0 – 9.3 | 65% | 9.9 – 10.7 | 35% |
| **30% Effluent: 70% River** | 11[1] | 4.82 | 4.7 | 7.2 | 5.07 | 9.9 – 10.2 | 70% | 10.1 – 10.9 | 30% |
| | 12 | 6.25 | 4.6 | 7.4 | 5.84 | 9.8 – 10.2 | 63% | 10.6 – 11.7 | 37% |
| | 13 | 17.82 | 4.2 | 7.2 | 5.49 | 8.9 – 9.2 | 70% | 9.6 – 10.5 | 30% |
| **50% Effluent: 50% River** | 14 | 6.61 | 5.1 | 7.5 | 6.90 | 9.8 – 10.2 | 65% | 11.0 – 12.5 | 35% |

[1] UConn waste water treatment plant effluent was collected during the chlorination disinfection period
[2] Ionic strength calculated from the concentration of major anions and cations

Average conditional binding constants for the WWTP effluent hydrophilic DOM ligands were 3.5 to 3.7 log units higher than those for the freshwater Fenton River. Average conditional binding constants for the WWTP effluent hydrophobic DOM ligands were 2.6 to 4.1 log units higher. While differences in pH are expected to cause variation in binding constant values due to the protonation of ligand binding sites and increased competition of protons with copper (Breault et al, 1996), the differences in pH between WWTP effluent and Fenton River freshwater water alone do not explain the large differences seen in the conditional binding constants presented. The difference of conditional stability constants between the WWTP effluent and Fenton River freshwater DOM indicates that the WWTP effluent DOM has a stronger binding capacity for copper ions. The conditional stability constants for this study generally fell into the range for those of natural DOM isolates from the Suwannee River (Florida, U.S.A.) at pH 6.6 and 0.01 M ionic strength ($\log K = 10.4 – 13.5\ M^{-1}$) (Craven, 2012).

Despite the hydrophobic fraction of DOM for both the Fenton River water and WWTP effluent having higher conditional stability constants than the hydrophilic fraction, larger percentages of Cu were found bound to the hydrophilic fraction. In the 100% Fenton River exposure scenarios on average 60% of the copper was bound to the hydrophilic fraction. For the three 100% effluent experiments, 53% - 82% of copper was found complexed with the hydrophilic DOM ligands. Increased percentages of hydrophilic DOM binding were also observed for the freshwater: WWTP effluent mixtures. 63% - 70% of copper was found bound to the hydrophilic DOM fraction. Higher hydrophilic DOM binding indicates the possible presence of stronger binding sites with higher Cu binding affinity (Matar et al.,2015), which may not directly be quantified in the conditional binding constant measurements.

### 3.1.3 Periphyton Total (Surface Bound & Intracellular) and Intracellular Copper Concentrations

Each of the fourteen exposures on the trickle apparatus were used to analyze initial periphyton uptake kinetics and compare the differences in uptake kinetics under different water quality conditions. Periphyton exposure durations on the trickle apparatus ranged from 90 to 380 minutes (dependent on biomass growth). Periphyton slides were sampled over time throughout the exposure duration in order to investigate copper isotope surface binding and cell internalization. Figure 2 through Figure 4 display the total periphyton copper isotope

concentrations (surface bound and intracellular) and the periphyton intracellular copper isotope concentrations as a function of exposure duration. While equilibrium was not reached during the exposure period in all experiments (for periphyton copper isotope surface binding and intracellular uptake), it can be seen qualitatively that larger total and intracellular copper concentrations were obtained for the periphyton exposed to 100% Fenton River water over similar exposure durations. Periphyton exposed to WWTP effluent or mixtures containing WWTP effluent appear to experience reduced copper isotope uptake and surface binding. An example of this phenomenon can be seen in Figure 5. Experiment 6, which consisted of a 100% Fenton River exposure at high copper concentrations ([$^{65}$Cu] = 15.7 μg/L) is compared to the results from experiment 13, which consisted of a 30% WWTP effluent: 70% Fenton River exposure also at high copper concentrations ([$^{65}$Cu] = 16.3 μg/L). Both exposure waters had similar TOC concentrations of approximately 4.0 ppm C. By comparing the periphyton copper concentrations over time between the two experiments, it can be seen that the periphyton exposed to a 30: 70 effluent, freshwater mixture had diminished copper isotope cell surface binding and intracellular uptake. Through this realization, it was determined that uptake kinetic modeling and speciation modeling should be completed for each experiment to more efficiently compare the water quality influences on periphyton kinetics. The modeling results are discussed in Section 3.3.

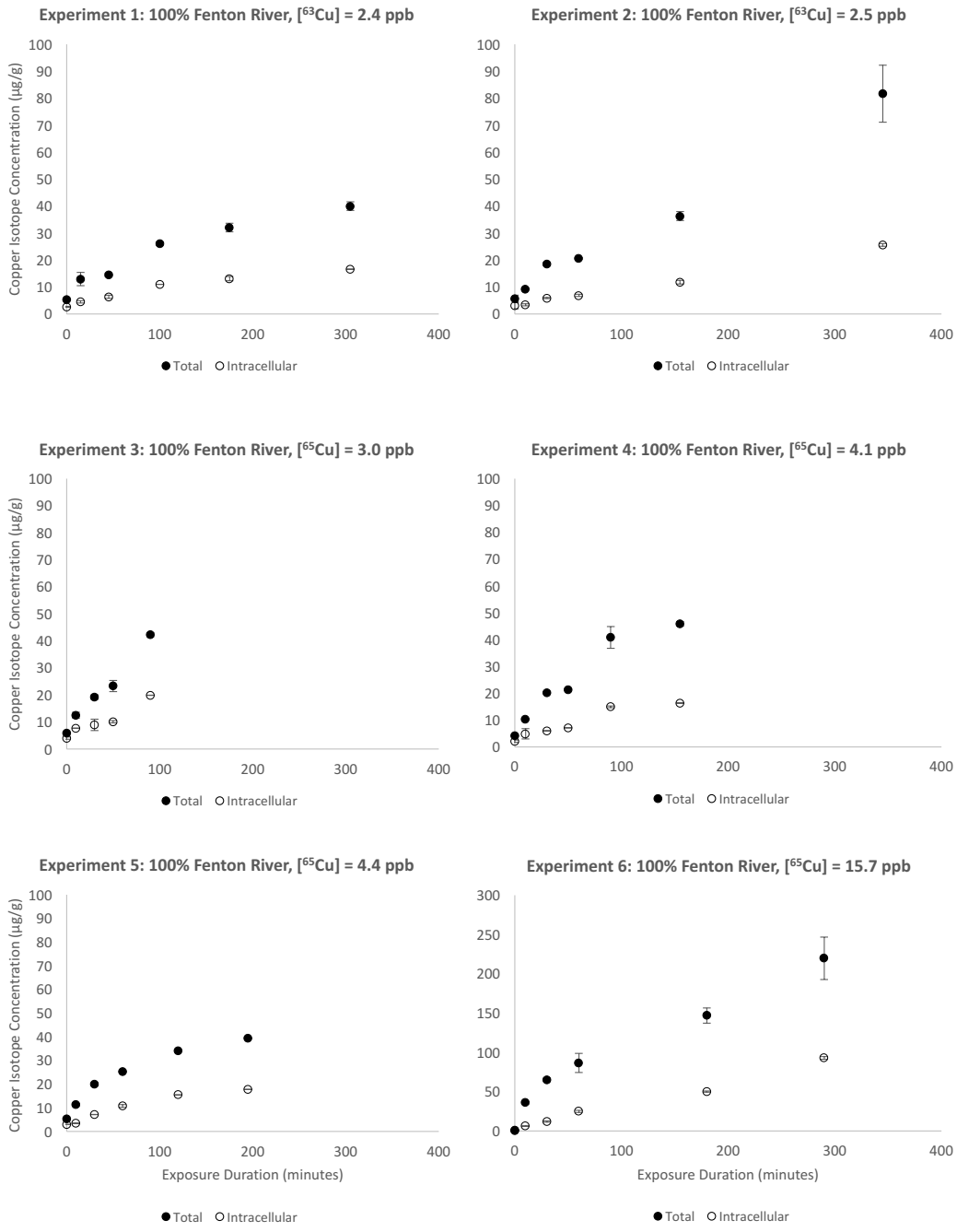


*Figure 2: Total (●) and intracellular (o) copper isotope concentrations (µg Cu/g of dry weight) in periphyton as a function of exposure time (minutes) to 100% Fenton River water on the trickle apparatus. Error bars represent ± 1 SD.*

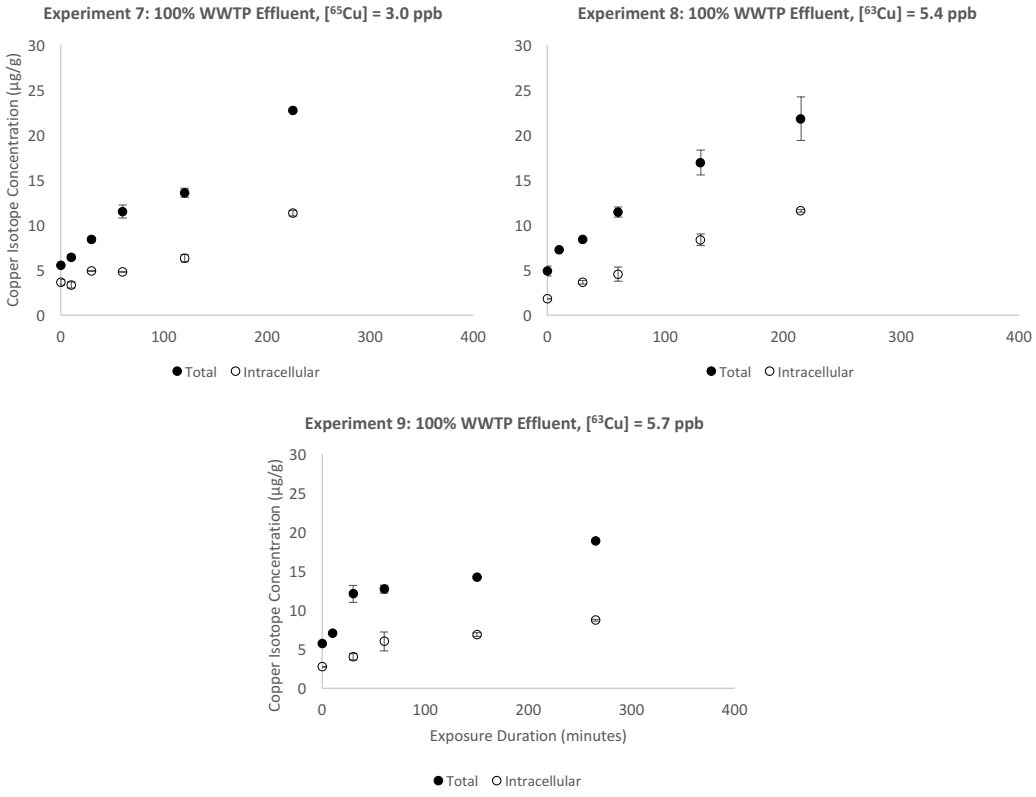


*Figure 3: Total (●) and intracellular (o) copper isotope concentrations (µg Cu/g of dry weight) in periphyton as a function of exposure time (minutes) to 100% UConn WWTP effluent on the trickle apparatus. Error bars represent ± 1 SD.*

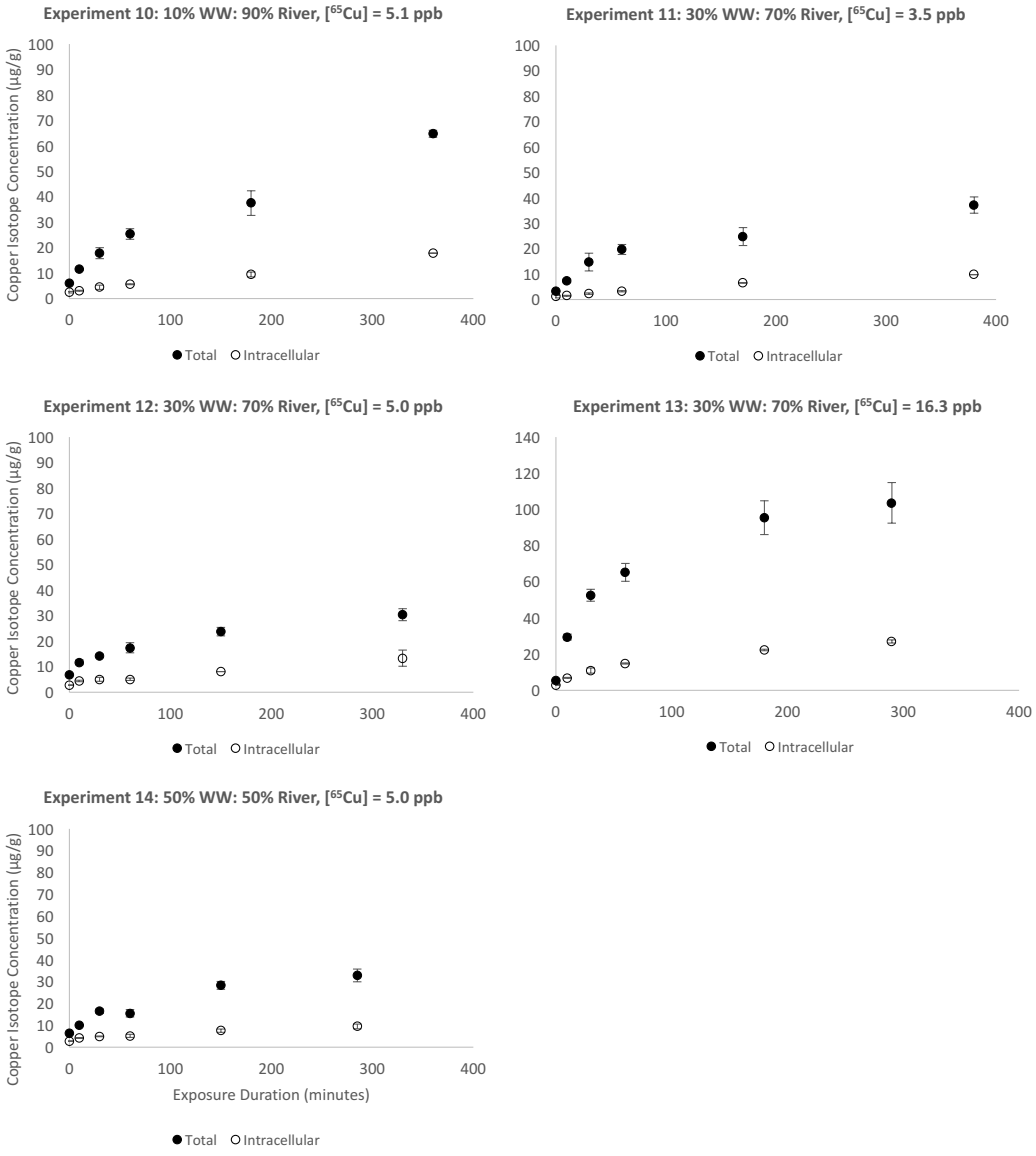


*Figure 4: Total (●) and intracellular (o) copper isotope concentrations (µg Cu/g of dry weight) in periphyton as a function of exposure time (minutes) to mixtures of Fenton River water and UConn WWTP effluent on the trickle apparatus. Error bars represent ± 1 SD.*

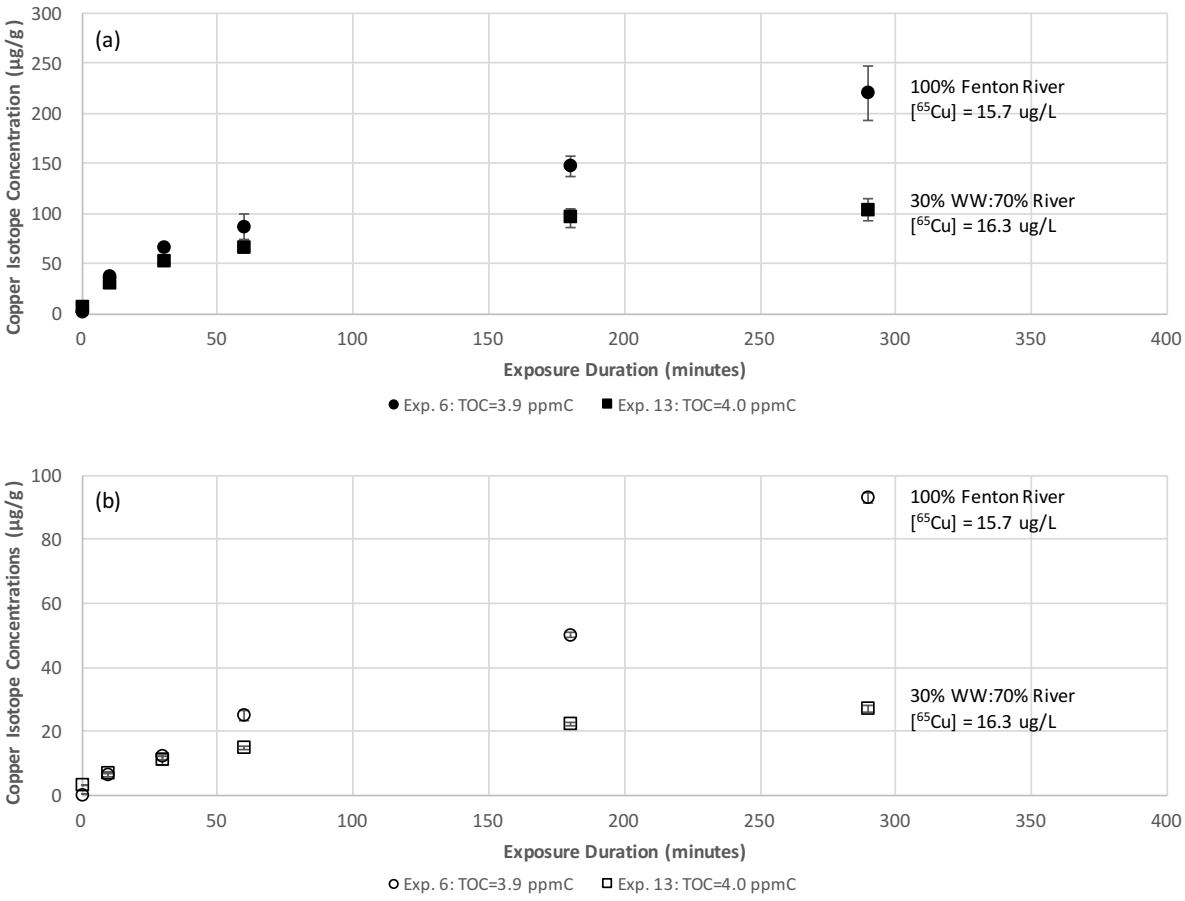


*Figure 5: Plots comparing (a) total and (b) intracellular periphyton copper isotope uptake over time between exposure waters containing 100% Fenton River (●,o) and 30% WWTP effluent (■,□). Reduced copper isotope uptake can be observed for the periphyton exposed to source water containing WWTP effluent. Error bars represent ± 1 SD.*

## 3.2 Periphyton Binding Constants

Periphyton slides were exposed to varying copper concentrations in filtered (0.45 µm) Fenton River water to determine cellular surface binding constants for active transport sites and inert binding ligands. Total and intracellular copper concentrations of the periphyton were measured for each copper solution (Table 6).

*Table 6: Total, intracellular, and surface bound copper concentrations after 35 minutes of exposure to various concentrations of copper spiked in filtered (0.45 µm) Fenton River water. Fitting this data to linearized Michealis-Menten-type saturation equations can provide details on periphyton surface ligand binding affinities.*

| **Exposure Water** | **Periphyton Cu Concentrations (µg/g)** | | |
|---|---|---|---|
| **Total Cu (µg/L)** | **$C_{total}$** | **$C_{intracellular}$** | **$C_{surface}$** |
| 0.0 | 0.0 | 0.0 | 0.0 |
| 9.2 | 7.3 | 0.9 | 6.3 |
| 23.1 | 11.9 | 2.5 | 9.4 |
| 48.2 | 23.6 | -[1] | - |
| 96.5 | 36.7 | 6.2 | 30.5 |
| 200.1 | 94.9 | 20.0 | 74.9 |
| 322.0 | 143.9 | 29.5 | 114.5 |

[1] Data point discarded due to percent difference between replicates being greater than 40%

By fitting the data presented in Table 6 to the linearized Michealis-Menton type equation (equation 12), the affinity constant for the binding of copper to active transport sites on the periphyton, $K_M$, and the affinity constant for the binding of copper to active and inert binding sites, $K_{M\text{-}A}$, can be determined (Lehninger et al., 1993).

$$\frac{1}{C_{int}} = \frac{1}{C_{max,int}} + \frac{1}{C_{max,int}K_M} \times \frac{1}{[Cu]} \qquad \text{(Eqn 12)}$$

By plotting the inverse of the periphyton concentrations against the inverse of the water total copper concentrations, the maximum copper concentrations at ligand saturation ($C_{max,int}$, $C_{max,cell}$) can be found by linearly fitting the data and analyzing the y-intercept. The affinity constants ($K_M$, $K_{M\text{-}A}$) can be calculated from analyzing the slopes (Figure 6).

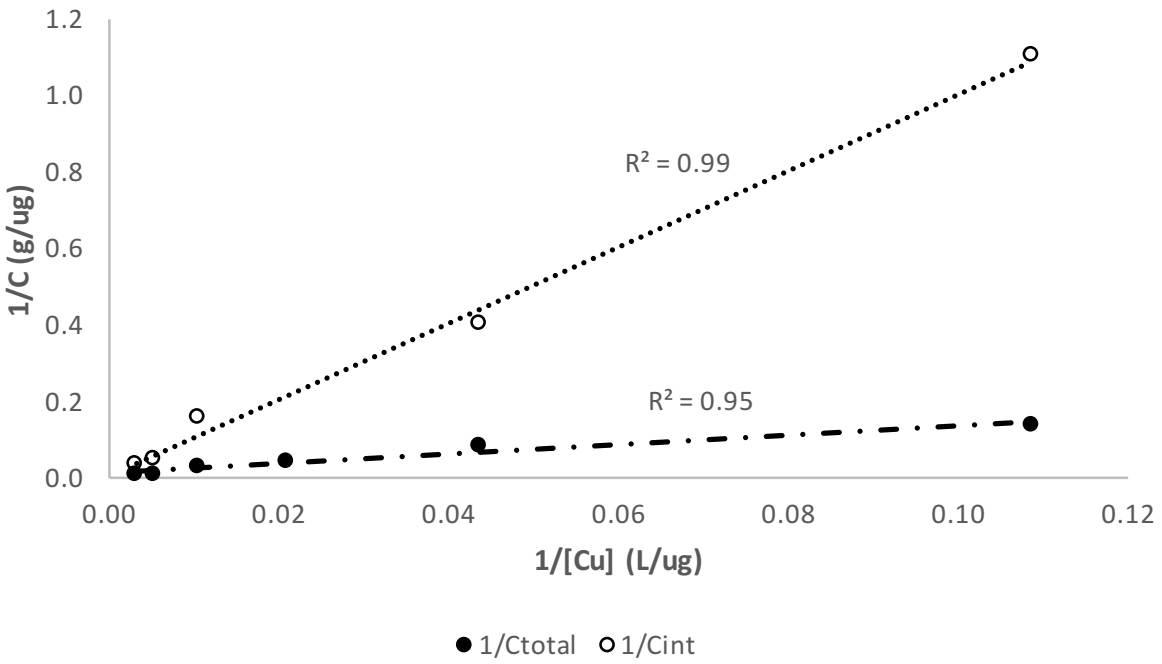


*Figure 6: Inverse of periphyton copper concentrations (total (•) and intracellular (o)), in grams of dry weight per µg Cu, plotted against the inverse of the water total copper concentrations. Linearly fitted trend lines allow for the calculation of Michaelis-Menten parameters.*

Analysis of the intracellular copper concentrations indicate that the periphyton have a $C_{max,int}$ approximately equal to 1.32 µmol Cu per gram of dry weight (84.03 ug/g) and a $\log K_M$ approximately equal to 4.88 L per mole Cu. Analysis of the total copper concentrations reveals that the periphyton have a $C_{max,cell}$ and a $K_{M-A}$ approximately equal to 1.27 µmol/g (80.65 ug/g) and 5.81 $M^{-1}$ respectively. The algal binding parameters found in this study are comparable to the values found for green algae (*Pseudokirchneriella subcapitata)* in a study by Luan (2016). Green algae samples were found to have $\log K_M$ and $C_{max,int}$ parameters equal to 6.60 $M^{-1}$ and 1.34 µmol/g respectively. $\text{Log}K_{M-A}$ was found to be 6.10 $M^{-1}$ and $C_{max,cell}$ was approximately 5.03 µmol/g (Luan, 2016).

The affinity constants for the periphyton binding ligands were several orders of magnitude lower than the both the hydrophilic and hydrophobic conditional binding constants found for the Fenton River and WWTP effluent organic matter ligands. This indicates that free copper ions should have a higher binding affinity for organic matter than the ligands on the periphyton cell surfaces.

## 3.3 Kinetics and Speciation Modeling

Total and intracellular copper isotope concentrations in periphyton increased linearly during the first 30 to 60 minutes of exposure. The consecutive total and intracellular copper isotope concentrations continued to increase steadily during the rest of the exposure period (Figure 2 - Figure 4), but at a slower rate than initial uptake. The rapid increases in total and intracellular copper concentrations that occurred during the first 30 – 60 minutes were fitted linearly using a form of equation 7,

$$C_t = k_u C_w t + C_{t0} = r_u t + C_{t0} \quad \text{(Eqn 13)}$$

where $r_u$ is the uptake rate in μg Cu per g tissue hr and is the product of the conditional uptake rate constant, $k_u$, and the copper concentration of the exposure water, $C_W$. This compartmental model, dependent only on water concentration and time, was deemed valid for the initial linear uptake phase where efflux is not substantial. The water represents the source compartment and the periphyton represent the toxicant sink compartment. The model holds the underlying assumption that the rate constants and clearances remain constant over time (Landrum et al., 1992). The initial total uptake rates (surface binding and intracellular uptake) and the initial intracellular uptake rates are presented in Table 7. The statistical results of all intracellular uptake replicates are also shown.

When comparing across different exposure waters with similar copper isotope concentrations (i.e., experiments 5, 8, 10, 12, and 14), as the percentage of effluent increases, in general, the total uptake rate and internalization rates decrease. Referring back to the example presented in Section 3.1.3 and Figure 5, which compares experiment 6 (100% Fenton River) to experiment 13 (30% WWTP Effluent, 70% Fenton River), both experiments have similar high copper isotope exposure concentrations (16.0 ± 0.31 μg/L) and approximately the same TOC concentration (3.95 ± 0.05 ppm C). However, the uptake rates between them notably vary. Experiment 6 displayed a periphyton total uptake rate of approximately 136.1μg Cu/g-hr, while experiment 13 experienced a periphyton total uptake rate of around 99.4 μg Cu/g-hr. The internalization rates of experiment 6 and 13 were approximately 24.6 and 16.7 μg Cu/g-hr. The observed differences in total uptake and internalization rates between the different exposure waters that have similar total copper isotope concentrations and TOC values indicates that total copper concentrations in the water are not controlling the uptakes rates. Instead, water quality

parameters (e.g., pH, alkalinity, organic matter properties, anion concentrations) are influencing the bioavailable copper through complexation and thus impacting the periphyton uptake rates.

In order to assess if this affirmation is accurate, we decided to calculate conditional rate constants, $k_u$ and $k_t$, for intracellular uptake and total uptake by changing the $C_W$ term in equation 13. Three trials were conducted by changing $C_w$ to the total copper isotope ($Cu_{total}$), the free copper isotope ($Cu^{2+}$) and the exchangeable copper isotope ($Cu_{ex}$) concentrations of the water. Total copper isotope concentrations are as listed in Table 7. $Cu^{2+}$ was found through speciation modeling using Visual MINTEQ and $Cu_{ex}$ was found through equilibrium ion exchange techniques using Dowex 50W-X8 resin and the method proposed by Worms and Wilkinson (2008).

*Table 7: Initial total and intracellular copper isotope uptake rates for the dynamic trickle apparatus experiments. Standard error statistical analyses are summarized for all intracellular replicates.*

| Exposure Water | Exp. | Average Total Cu Isotope μg/L | Periphyton Rate Constants | | Standard Error Statistics (Internalization Rates) | | |
|---|---|---|---|---|---|---|---|
| | | | Total Rate, $r_T$ μg Cu/hr-g | Internalization Rate, $r_U$ μg Cu/hr-g | R Squared | Standard Error | P-value |
| **100% Fenton River** | 1 | 2.37 | 13.96 | 4.57 | 0.91 | 0.011 | 0.001 |
| | 2 | 2.53 | 25.05 | 5.34 | 0.73 | 0.031 | 0.031 |
| | 3 | 3.00 | 28.02 | 11.01 | 0.64 | 0.067 | 0.105 |
| | 4 | 4.15 | 32.37 | 8.63 | 0.70 | 0.039 | 0.038 |
| | 5 | 4.45 | 30.13 | 7.83 | 0.95 | 0.017 | 0.001 |
| | 6 | 15.70 | 136.12 | 24.65 | 0.95 | 0.045 | 0.001 |
| **100% WWTP Effluent** | 7[1] | 3.02 | 5.80 | 2.81 | 0.75 | 0.022 | 0.026 |
| | 8 | 5.36 | 7.77 | 2.89 | 0.82 | 0.011 | 0.012 |
| | 9 | 5.70 | 12.37 | 3.14 | 0.72 | 0.014 | 0.007 |
| **10% Effluent: 90% River** | 10 | 5.12 | 24.52 | 4.51 | 0.68 | 0.020 | 0.023 |
| **30% Effluent: 70% River** | 11[1] | 3.52 | 23.02 | 2.36 | 0.72 | 0.011 | 0.016 |
| | 12 | 5.03 | 16.20 | 5.08 | 0.66 | 0.025 | 0.050 |
| | 13 | 16.33 | 99.45 | 16.66 | 0.91 | 0.041 | 0.003 |
| **50% Effluent: 50% River** | 14 | 5.01 | 20.47 | 4.76 | 0.84 | 0.013 | 0.011 |

[1] UConn waste water treatment plant effluent was collected during the chlorination disinfection period

### 3.3.1 Free Copper Ion Isotope Concentrations – MINTEQ Speciation Modeling

The speciation model, Visual MINTEQ, was utilized to find the free copper ion concentrations of the various periphyton exposure waters. The water quality parameters of the exposure waters were input into the program including pH, alkalinity, and various major anion and cation concentrations. New model species were added to the database for the hydrophobic and hydrophilic DOM. The experimentally determined DOM conditional binding constants and ligand concentrations were input into the thermodynamic files for Cu-DOM hydrophilic and hydrophobic complexation. With the provided water quality information, Visual MINTEQ calculated the concentrations of complexed (inorganic and organic) copper and free copper ions (Table 8).

*Table 8: Visual MINTEQ derived free copper ion concentrations in the trickle apparatus exposure waters.*

| Exposure Water | Exp. | Average $Cu_{total}$ µg/L | Average $Cu^{2+}$ µg/L | Average[1] $^{63}Cu^{2+}$ µg/L | Average[1] $^{65}Cu^{2+}$ µg/L |
|---|---|---|---|---|---|
| 100% Fenton River | 1 | 2.78 | 0.52 | 0.45 | 0.08 |
| | 2 | 2.86 | 0.51 | 0.45 | 0.06 |
| | 3 | 3.40 | 0.47 | 0.06 | 0.41 |
| | 4 | 4.46 | 1.13 | 0.08 | 1.05 |
| | 5 | 5.22 | 1.06 | 0.16 | 0.91 |
| | 6 | 15.73 | 5.98 | 0.01 | 5.56 |
| 100% WWTP Effluent | 7 | 5.59 | 0.04 | 0.02 | 0.02 |
| | 8 | 7.71 | 0.11 | 0.08 | 0.03 |
| | 9 | 8.17 | 0.13 | 0.09 | 0.04 |
| 10% Effluent: 90% River | 10 | 5.39 | 1.26 | 0.06 | 1.20 |
| 30% Effluent: 70% River | 11 | 4.82 | 1.42 | 0.38 | 1.04 |
| | 12 | 6.25 | 1.15 | 0.23 | 0.93 |
| | 13 | 17.82 | 4.40 | 0.37 | 4.03 |
| 50% Effluent: 50% River | 14 | 6.61 | 0.78 | 0.19 | 0.59 |

[1]Based on percentages of total copper isotope water concentrations

Despite the WWTP effluent exposure waters having higher intermediate total copper isotope concentrations (3.0 – 5.7 μg/L) than the Fenton River water (2.4 – 4.4 μg/L), a much smaller fraction is free copper ions. For the WWTP samples 0.02 – 0.09 μg/L were present as free isotope ions according to the MINTEQ modeling software. The Fenton River exposure waters had 0.41 – 1.05 μg/L free copper isotope ion concentrations for the low and intermediate copper exposure scenarios. Enhanced complexation was observed for the WWTP samples due to the higher DOM conditional stability constants, higher alkalinity concentrations (increased carbonate inorganic binding), and decreased proton competition (higher pH).

Enhanced complexation was also observed for the WWTP effluent: Fenton River freshwater mixtures. Referring back to our previous high copper example with experiments 6 (100% Fenton River) and 13 (30% WWTP effluent), the reduction in the percentage free copper ions with increased effluent can be observed. For experiment 6, the total copper isotope concentration was 15.70 μg/L and the MINTEQ derived free copper isotope ion concentration was 5.56 μg/L. Thus, approximately 35% of the total copper is present as free copper ions in this 100% Fenton River exposure source water. For experiment 13, the total copper isotope concentration was 16.3 μg/L and the MINTEQ derived free copper isotope concentration is 4.03 μg/L. Therefore, approximately 27% of the total copper is present as free copper ions in the 30% effluent, 70% freshwater solution. Even in the scenario of high total copper concentrations where ligand saturation would be expected, the 30% effluent reduced the free ion concentration through increased inorganic and organic complexation.

### 3.3.2 Exchangeable Copper Concentrations – Equilibrium Ion Exchange Techniques

Exchangeable copper concentrations were determined experimentally for representative source water samples (Table 9). Exchangeable copper represents the free copper ions in solution as well as the weakly complexed copper to inorganic and organic ligands that would become available as free metal ions during transport to the cells. Two to four replicates of each source water type were analyzed using the equilibrium ion exchange technique proposed by Worms and Wilkinson (2008). Initial total copper and TOC concentrations were noted for the representative samples in order to extrapolate the results to the fourteen trickle apparatus exposure waters. Total copper: TOC ratios were used to weigh the results from the ion exchange experiment to those of the trickle apparatus. For example, a total Cu: TOC ratio and exchangeable copper concentration

for the 10% effluent: 90% river water of 2.56 µg Cu/mg C and 0.19 µg/L respectively were used to find the exchangeable copper fraction of experiment 10. Thus, experiment 10, with a total Cu: TOC ratio of 1.54 µg Cu/mg C, has an exchangeable copper fraction of approximately 0.11 µg/L. It was assumed that a Cu: TOC ratio would reliably weight the exchangeable fraction for the trickle apparatus exposure solutions from the samples run with ion exchange. This assumption was made due to the fact that besides total copper concentrations and TOC concentrations, most of the other water quality parameters did not vary to the same extent within each category. Alkalinity, pH, and anion and cation concentrations did not vary by more than 20% in each category for the exposure waters containing WWTP effluent (100% and mixtures). The 100% Fenton River exposures had increased variation, but this was mostly due to higher background anion/cation concentrations observed in experiment 5. DOM binding properties were also relatable for the different source water types. The weighted exchangeable copper concentrations for the fourteen exposure experiments can be seen in Table 10. It was preferred to use the original trickle apparatus samples with the equilibrium ion exchange experimental technique; however, too much volume was required in order to use the original drum samples.

*Table 9: Copper exchangeable results from representative source water samples found experimentally using an equilibrium ion exchange technique with Dowex 50W-X8 resin columns.*

| Source Water | Total Cu Concentration | TOC Concentration | Cu Exchangeable Concentration | Source Water Average Cu Exchangeable |
|---|---|---|---|---|
| | µg/L | mg/L C | µg/L | µg/L |
| 100% Fenton River | 4.23 | 4.58 ± 0.30 | 0.16 | 0.13 ± 0.02 |
| | 4.74 | | 0.11 | |
| | 5.34 | | 0.13 | |
| | 5.31 | | 0.11 | |
| 100% WWTP Effluent | 9.00 | 2.38 ± 0.02 | 0.37 | 0.46 ± 0.23 |
| | 9.26 | | 0.24 | |
| | 9.01 | | 0.79 | |
| 10% Effluent: 90% River | 5.10 | 1.99 | 0.22 | 0.19 ± 0.03 |
| | 5.10 | | 0.16 | |
| 30% Effluent: 70% River | 5.02 | 4.22 | 0.15 | 0.18 ± 0.02 |
| | 5.52 | | 0.21 | |
| 50% Effluent: 50% River | 4.65 | 4.42 | 0.14 | 0.18 ± 0.05 |
| | 5.14 | | 0.25 | |

*Table 10: Exchangeable copper concentrations for the trickle apparatus exposure waters weighed with the results obtained from an equilibrium ion exchange technique.*

| Exposure Water | Exp. | Total Cu: TOC ratio<br>µg Cu/mg C | Average $Cu_{ex}$<br>µg/L | Average[1] $^{63}Cu_{ex}$<br>µg/L | Average[1] $^{65}Cu_{ex}$<br>µg/L |
|---|---|---|---|---|---|
| **100% Fenton River** | 1 | 0.66 | 0.08 | 0.07 | 0.01 |
| | 2 | 1.51 | 0.18 | 0.15 | 0.02 |
| | 3 | 0.81 | 0.09 | 0.01 | 0.08 |
| | 4 | 0.80 | 0.09 | 0.01 | 0.09 |
| | 5 | 0.45 | 0.05 | 0.01 | 0.05 |
| | 6 | 4.03 | 0.47 | 0.00 | 0.47 |
| **100% WWTP Effluent** | 7 | 0.39 | 0.05 | 0.02 | 0.02 |
| | 8 | 1.09 | 0.13 | 0.09 | 0.04 |
| | 9 | 1.30 | 0.16 | 0.11 | 0.05 |
| **10% Effluent: 90% River** | 10 | 1.54 | 0.11 | 0.01 | 0.11 |
| **30% Effluent: 70% River** | 11 | 1.03 | 0.15 | 0.04 | 0.11 |
| | 12 | 1.36 | 0.20 | 0.04 | 0.16 |
| | 13 | 4.24 | 0.61 | 0.05 | 0.56 |
| **50% Effluent: 50% River** | 14 | 1.30 | 0.21 | 0.05 | 0.16 |

[1]Based on percentages of total copper isotope water concentrations

A problem is encountered when comparing the exchangeable copper fraction found through the ion exchange technique to the free copper ions found with Visual MINTEQ. The relative copper concentrations of the exposure waters should display as follows, $Cu_{total} > Cu_{ex} > Cu^{2+}$. While the 100% WWTP effluent exposure waters display this behavior, any exposure water containing Fenton River water does not. The 100% Fenton River exposures and the mixtures were found to have higher free ion concentrations than the exchangeable fraction. There are a number of possible reasons why this may have occurred. First, for all of the exposure waters, it may not have been entirely accurate to use a Cu: TOC weigh factor to extrapolate the exchangeable copper fraction from the reference solutions. Specifically, the exposure waters with the highest total copper concentrations (> 15 µg/L) may experience a different type of behavior. Additionally, the waste water reference sample used in the ion exchange procedure had a lower TOC concentration than what was observed for the 100% WWTP effluent exposures.

With a lower TOC concentration, less strong binding ligands may have been available for copper complexation biasing a higher exchangeable fraction. Nevertheless, reference solutions and Cu:TOC ratios were required since enough sample from the original drums was not available to run an ion exchange procedure for each trickle solution. Secondly, the ion exchange procedure was difficult to replicate. In order to calculate the concentration of the exchangeable fraction, DI solutions with identical [Na], [Mg], and [Ca] to the solutions of interest must also be run through a resin column with a known [$Cu^{2+}$] (Equation 11). These solutions are used to calculate the partition coefficient between the free copper ions and the copper bound to the resin. It was difficult to get these solutions to reach equilibrium with the ion exchange resin. Between replicates, despite having the same cation concentrations, the solutions required different elute volumes to reach equilibrium. If a solution did not reach equilibrium after 1-L of elute, the experiment had to be run again. Modifications may be needed for the procedure in the future for more precise results. Lastly, the hydrophilic and hydrophobic DOM conditional stability constants were used to represent Cu-DOM complexation in Visual MINTEQ. Conditional stability constants provide an average binding constant over a wide range of binding ligands with different binding affinities for copper. It is likely that stronger binding ligands exist in the freshwater DOM that were not represented in the conditional binding constants and bias towards lower exchangeable values at lower copper concentrations. It is possible that stronger binding ligands may have been present for the samples not directly measured. Additionally, DOM conditional stability constants were not available for all the 100% Fenton River exposures. The DOM results from experiment 2 were applied to experiments 1, 3, 4, and 5 in Visual MINTEQ to calculate the free copper ion concentrations. Due to slight differences in the copper isotope concentrations, the conditional binding constants and ligand concentrations found for experiment 2 may have not been accurate to use for the other 100% Fenton River exposures.

It was decided to use the data as is and to not make any adjustments. Even though the free copper ion concentrations for the samples with the Fenton River were slightly higher than the exchangeable, use of the calculated values would provide a range of the possible bioavailable fraction that may be experienced.

### 3.3.3 Conditional Uptake Rate Constants

Through finding the total, free ion, and exchangeable copper isotope concentrations experimentally or through speciation modeling in the exposure waters from the trickle apparatus, conditional uptake rate constants could be investigated. Using equation 13, where $r_u = k_u C_w$, and the periphyton uptake rates (Table 7) found experimentally using a trickle apparatus, three conditional uptake rate constants could be found for each water concentration, $Cu_{toal}$, $Cu^{2+}$, and $Cu_{ex}$. Conditional uptake rate constants were found for total (surface bound and intracellular) (Table 11) and intracellular (Table 12) periphyton copper uptake rates.

While we observed a decrease in total and intracellular uptake rates ($r_t$, $r_u$) with an increase in the percentage of WWTP effluent, a different relationship was expected for the uptake rate constants ($k_t$, $k_u$). We predicted that a constant uptake rate constant would be found across the different exposure types when the bioavailable copper concentration was found. Specifically, the three copper concentrations of the water were used with the kinetic modeling equations to find when the $k_u$ constants across the exposure water types became similar.

*Table 11: Conditional uptake rate constants for total (surface bound and intracellular) periphyton copper isotope concentrations based on three copper water concentrations ($Cu_{total}$, $Cu^{2+}$, $Cu_{ex}$). All calculations are based on copper isotope of interest.*

| Exposure Water | Exp. | Total Rate, rt | $k_t$, $Cu_{total}$ (L/g-hr) | | | $k_t$, $Cu^{2+}$ (L/g-hr) | | | $k_t$, $Cu_{ex}$ (L/g-hr) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | µg/g-hr | Value | Average | RSD | Value | Average | RSD | Value | Average | RSD |
| 100% Fenton River | 1 | 13.96 | 5.89 | 8.06 | 0.17 | 31.31 | 40.16 | 0.39 | 204.69 | 349.74 | 0.51 |
| | 2 | 25.05 | 9.90 | | | 55.20 | | | 157.32 | | |
| | 3 | 28.02 | 9.34 | | | 67.57 | | | 352.84 | | |
| | 4 | 32.37 | 7.80 | | | 30.79 | | | 386.53 | | |
| | 5 | 30.13 | 6.77 | | | 33.28 | | | 706.87 | | |
| | 6 | 136.12 | 8.67 | | | 22.82 | | | 290.17 | | |
| 100% WWTP Effluent | 7 | 5.80 | 1.92 | 1.85 | 0.16 | 243.99 | 158.67 | 0.38 | 214.72 | 137.17 | 0.41 |
| | 8 | 7.77 | 1.45 | | | 100.69 | | | 85.97 | | |
| | 9 | 12.37 | 2.17 | | | 131.34 | | | 110.81 | | |
| 10% Effluent: 90% River | 10 | 24.52 | 4.79 | 4.79 | - | 20.45 | 20.45 | - | 234.66 | 234.66 | - |
| 30% Effluent: 70% River | 11 | 23.02 | 6.54 | 5.28 | 0.28 | 22.23 | 21.44 | 0.14 | 210.14 | 162.90 | 0.28 |
| | 12 | 16.20 | 3.22 | | | 17.44 | | | 100.65 | | |
| | 13 | 99.45 | 6.09 | | | 24.66 | | | 177.91 | | |
| 50% Effluent: 50% River | 14 | 20.49 | 4.09 | 4.09 | - | 34.44 | 34.44 | - | 128.73 | 128.73 | - |

*Table 12: Conditional uptake rate constants for intracellular periphyton copper isotope concentrations based on three copper water concentrations ($Cu_{total}$, $Cu^{2+}$, $Cu_{ex}$). All calculations are based on copper isotope of interest.*

| Exposure Water | Exp. | Internalization Rate, $r_u$ | $k_u$, $Cu_{total}$ (L/g-hr) | | | $k_u$, $Cu^{2+}$ (L/g-hr) | | | $k_u$, $Cu_{ex}$ (L/g-hr) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | µg/g-hr | Value | Average | RSD | Value | Average | RSD | Value | Average | RSD |
| 100% Fenton River | 1 | 4.57 | 1.93 | 2.19 | 0.31 | 10.25 | 11.59 | 0.61 | 67.01 | 96.41 | 0.54 |
| | 2 | 5.34 | 2.11 | | | 11.77 | | | 33.54 | | |
| | 3 | 11.01 | 3.67 | | | 26.55 | | | 138.64 | | |
| | 4 | 8.63 | 2.08 | | | 8.21 | | | 103.05 | | |
| | 5 | 7.83 | 1.76 | | | 8.65 | | | 183.70 | | |
| | 6 | 24.65 | 1.57 | | | 4.13 | | | 52.55 | | |
| 100% WWTP Effluent | 7 | 2.81 | 0.93 | 0.67 | 0.27 | 118.21 | 63.00 | 0.62 | 104.03 | 54.71 | 0.64 |
| | 8 | 2.89 | 0.54 | | | 37.45 | | | 31.98 | | |
| | 9 | 3.14 | 0.55 | | | 33.34 | | | 28.13 | | |
| 10% Effluent: 90% River | 10 | 4.51 | 0.88 | 0.88 | - | 3.76 | 3.76 | - | 43.16 | 43.16 | - |
| 30% Effluent: 70% River | 11 | 2.36 | 0.67 | 0.90 | 0.18 | 2.28 | 3.96 | 0.33 | 21.54 | 27.64 | 0.16 |
| | 12 | 5.08 | 1.01 | | | 5.47 | | | 31.56 | | |
| | 13 | 16.66 | 1.02 | | | 4.13 | | | 29.80 | | |
| 50% Effluent: 50% River | 14 | 4.76 | 0.95 | 0.95 | - | 8.00 | 8.00 | - | 29.91 | 29.91 | - |

Conjectures can be made about the uptake rate constants presented in Table 12. It is unlikely that the free copper ions are controlling internalization. The intracellular uptake rate constants ($k_u$) are much higher for the 100% WWTP effluent exposures than for any of the other exposure types. The $k_{u,WW}$ average is approximately 5.4 times higher than the $k_{u,Fenton}$ average. This does not correlate with the observations made based on periphyton copper concentrations and uptake rates. Intracellular uptake rates were smaller and the total periphyton copper contents experienced over the entire exposure period were appreciably lower for the 100% WWTP effluent exposure runs than for the other exposures. Therefore, having a higher uptake rate constant for the WWTP effluent periphyton exposures is inconsistent with the previous observed data. Additionally, an ANOVA statistics analysis of the $k_{u,Fenton}$ and $k_{u,WW}$ replicates rejected the null hypothesis suggesting that the means of the two populations were not equal (Table 15 – Supplemental Information). It is also unlikely that total copper in the water is controlling internalization. It is well established that copper has a high affinity for complexation. Furthermore, if total copper controlled periphyton uptake, we would not have seen the observed trends in periphyton copper content and uptake rates between the exposure waters (reduced copper uptake with increased percentage of effluent). The $k_{u,Fenton}$ average was approximately 3.3 times larger than the $k_{u,WW}$ average for the $Cu_{total}$ scenario and an ANOVA statistics analysis of the $k_{u,Fenton}$ and $k_{u,WW}$ replicates rejected the null hypothesis indicating that the means of the two populations were not equal (Table 16 – Supplemental Information). The results of using the exchangeable copper fraction water concentrations were more promising. Our data shows that the $k_{u,Fenton}$ average is approximately 1.8 times larger than the $k_{u,WW}$ average under the $Cu_{ex}$ scenario. The ANOVA statistics test between the $k_{u,Fenton}$ and $k_{u,WW}$ replicates did not reject the null hypothesis suggesting that the means of the two populations were similar (Table 17 – Supplemental Information). Since an ANOVA test using the 100% exposure uptake rate constants showed similarity between the means, another ANOVA test was included analyzing the $k_{u,Fenton}$, $k_{u,WW}$, and $k_{u,\ 30:70}$ replicates. The results of this ANOVA showed that the means of the three populations are within the 95% confidence interval (Table 18 – Supplemental Information). The statistical analyses indicate that the concentrations found for $Cu_{ex}$ of the water may be the bioavailable fraction. However, since representative samples were used to extrapolate

$Cu_{ex}$ concentrations to the fourteen trickle apparatus exposure waters, further research will be needed to make any further conclusions about the bioavailable copper fraction to periphyton.

Variations in the uptake rate constants between the experiments might be attributed to which step in the transport process is the rate controlling step. The general model of algal uptake can be attributed to four main steps: (1) diffusion or advection of metal species from the bulk solution to the biological surface through the diffusive boundary layer, (2) dissociation of metal species from inorganic/organic ligands, (3) sorption/complexation of the metal at binding sites on the cell, and (4) uptake of the metal into the cell through transport across the plasma membrane (Campbell, 1995; Campbell et al., 2002). At such minor molecular weight and diameter differences between free copper and complexed copper (inorganic and DOM), it was not expected that diffusion from the bulk solution impacted the uptake rate constants; however, the heterogeneity of biomass thickness across the slides may have impacted transport from the bulk solution to some extent. The error bars across periphyton replicates were small though, indicating that periphyton thickness would not have had a significant impact. Additionally, it is possible that the internalization rate was not limiting and instead the dissociation of the exchangeable copper fraction from inorganic and organic ligands was the rate limiting step. The flux of metal to the periphyton biological interfaces results from the coupled interactions of diffusion and the kinetics of copper association and dissociation with various species in a system. The lability of metal species will depend on the metal-to-ligand ratio, pH, and the degree of binding ligand heterogeneity (Van Leeuwen et al., 2005). In the case of copper, where a very small percentage is found as free metal ions and it can be complexed strongly with humic substances in natural systems, depletion of the small percentage of free copper ions through intracellular uptake could provoke the dissociation of the complexed species. If dissociation from humic substances is slow, this would make the mass transport step not negligible with respect to biouptake (Van Leeuwen, 1999). Slow DOM dissociation would cause uptake rate constants to vary between source waters since dissociation properties would vary between the two different organic matter sources. Using stripping chronopotentiometry at scanned deposition potential (SSCP) to measure the lability of cadmium and copper with complexes of fulvic and humic acids, cadmium was found to be generally labile while copper was found to be considerably less labile under similar experimental conditions. Copper complexation has also been observed to be more heterogeneous than lead complexation and a marked loss of lability at higher humic

concentrations was noted. SSCP wave analysis also hinted that there is a greater range of sites available for copper complexation that do not form complexes of significant stability with other metals (Town & Van Leeuwen, 2004). With a higher degree of heterogeneous ligand binding to humic substances and a loss of lability when humic substances are present, there may be times when dissociation becomes the rate limiting step. Further research comparing internalization rates to organic matter dissociation rates under varying conditions will be needed to make further assertions on this area.

Variations in the uptake rate constants could have also been impacted by the distribution of periphyton species. However, it is not believed that changes to the species distribution had as large of an impact. Various growth aquariums were utilized across the different exposure water types. The exposure water categories that had multiple trials (100% Fenton River, 100% WWTP effluent, 30:70 mixture) used periphyton slides that were grown in different growth aquariums with diverse species distributions. Despite diverse species being used for the various trials, the uptake rates across the different exposure water sources followed consistent trends based on the percentages of effluent and the total copper concentrations. In other words, relationships were present within the same exposure category (increased $[Cu_{total}]$ → increased uptake rate at similar magnitude) and across different exposure categories at similar $[Cu_{total}]$ (increased percentage of effluent → notable decreased uptake rate). Since the uptake rate constants are a function of water concentration it is more likely that the bioavailable copper fraction had an impact on the total and intracellular uptake rate constants. Furthermore, a study by Mylon et al. (2003) found that the normalized total uptake rates (surface bound and intracellular) for the diatom, *Stephanodiscus hantzschii*, and the chlorophyte, *Chlorella vulgaris*, were statistically indistinguishable between the two species for both copper and cadmium uptake. They conjectured that the similarities in metal uptake rates between the two, different species suggests that cellular biological factors play a less important role in determining uptake rates than ambient chemistry. On the other hand, a study by Quigg et al. (2006), which analyzed short-term uptake rates of copper to seven algal species in seawater, did find differences in uptake rates between the species. *Synechococcus* sp., part of the family Cyanophyceae, was found to have a 2 - 3 orders of magnitude higher copper accumulation rate (μmol Cu mol $C^{-1}$ $min^{-1}$) than those measured for diatoms, chlorophytes, dinoflagellates, and coccolithophores. It was conjectured that the higher uptake rates could be attributed to the large cell surface-to-volume ratio of the

species and also to the marked copper sensitivity of the group. The growth and evolution of eukaryotic algae in copper rich oceans may have provided copper-tolerance mechanisms such as a less reactive cell surface to explain the reduce copper sensitivity. Additionally, the greater cell size of eukaryotes relative to prokaryotes may provide an additional selective advantage against toxic metal accumulation (Quigg et al., 2006). Nevertheless, this experiment was run for algal species in saltwater, which may experience different binding strengths of their active sites due to competition for metal ions with seawater cations. Therefore, while it is important to note that differences in copper uptake rates were observed between the algal species it may not have a direct comparison to this study due to different water quality parameters coming into play and influencing uptake. Few studies specifically investigate copper uptake rates to different algal species. In the future, it will be important to analyze uptake rate differences across different periphyton species as well as looking at the influences of water quality parameters and source water on bioavailability in order to predict copper toxicity impacts.

## 3.4 Environmental Relevance

The data from this study shows that wastewater effluent has the potential to impact copper surface binding and intracellular uptake rates to periphyton. Reduced periphyton surface binding and intracellular uptake rates were observed with an increase in the percentage of WWTP effluent in the exposure solutions. With enhanced dissolved organic matter binding potential through higher ligand conditional binding constants and increased inorganic complexation due to higher alkalinity and higher background anion concentrations, wastewater effluent has the potential to impact the bioavailable fraction of copper. This study indicates that in terms of copper uptake kinetics, WWTP effluent could play a positive role in reducing copper toxicity to periphyton. However, WWTP effluent also tends to contribute a higher load of copper to the system, and therefore, the balance between ligands controlling bioavailability and concentration dependent uptake must be considered. As urbanized areas continue to expand and WWTP discharges continue to increase, understanding the role of wastewater in metal speciation and bioavailability will be pertinent to predicting impacts to primary producers in aquatic environments. In the future, when considering copper regulations, scientists and regulators will need to incorporate WWTP effluent organic matter properties and inorganic complexation into their studies in order to better understand bioavailability. Constant copper concentration

exposures were used in this study to analyze copper uptake kinetics. It will be important in the future to study the impacts of dynamic concentration changes over time to further comprehend how the flux of copper to the periphyton may differ between various source waters.

# 4 References


Al-Reasi, H.A., Wood, C.M. & Smith, D.S. (2013). Characterization of freshwater nautral dissolved organic matter (DOM): Mechanistic explanations for protective effects against metal toxicity and direct effects on organisms. *Environment International.* 59, pp. 201 – 207.

Allen, H.E. & Hansen, D.J. (1996). The importance of trace metal speciation to water quality criteria. *Water Environment Research*. 68, No. 1, pp. 42-54.

American Public Health Association (APHA) (2005). Standard methods for the examination of water and wastewater. 21st Ed., Washington, DC: American Public Health Association, American Water Works Association., & Water Environment Federation.

Breault, R.F., Colman, J.A., Aiken, G.R., & McKnight, D. (1996). Copper speciation and binding by organic matter in copper-contaminated streamwater. *Environmental Science and Technology.* 30, pp. 3477 – 3486.

Bradac, P., Navarro, E., Odzak, N., Behra, R., & Sigg, L. (2009). Kinetics of cadmium accumulation in periphyton under freshwater conditions. *Environmental Toxicology and Chemistry.* 28(10), pp. 2108 – 2116.

Campbell, P.G.C. (1995). Interactions between trace metals and aquatic organisms: A critique of the free-ion activity model. *Metal Speciation and Bioavailability in Aquatic Systems*. Edited by Tessier, A. and Turner, D.R. pp. 45 – 102.

Campbell, P.G.C., Erre´calde, O., Fortin, C., Hiriart-Baer, V.P., & Vigneault, B. (2002). Metal bioavailability to phytoplankton – applicability of the biotic ligand model. *Comparative Biochemistry and Physiology* Part C, 133, pp. 189 – 206.

Chen, W.B., Smith, D.S., & Guéguen, C. (2013). Influence of water chemistry and dissolved organic matter (DOM) molecular size on copper and mercury binding determined by multiresponse fluorescence quenching. *Chemosphere.* 92, pp. 351 – 359.

Craven, A.M. (2012). The importance of dissolved organic matter to the binding of copper and the release of trace elements from coal ash (Doctoral dissertation). Retrieved from ProQuest Dissertations & Theses Global. (Order no. 3549176).

Davis, B. & Birch, G. (2010). Comparison of heavy metal loads in stormwater runoff from major and minor urban roads using pollutant yield rating curves. *Environmental Pollution*. 158, pp. 2541 – 2545.

European Commission (2008) Priority Substances and Certain Other Pollutants According to Annex II of Directive 2008/105/EC.

Huber, M., Welker, A., & Helmreich, B. (2016). Critical review of heavy metal pollution of traffic area runoff: Occurance, influencing factors, and partitioning. *Science of the Total Environment*. 541, pp. 895 – 919.

Hudson, RJ.M. (1998). Which aqueous species control the rates of trace metal uptake by aquatic biota? Observations and predictions of non-equilibrium effects. *The Science of the Total Environment*. 219. pp. 95 – 115.

Komjarova I. & Blust R. (2009). Application of a stable isotope technique to determine the simultaneous uptake of cadmium, copper, nickel, lead, and zinc by the water flea daphnia magna from water and the green algae *Pseudokirchneriella subcapitata.* Environmental Toxicology and Chemistry. 28 (8), pp. 1739 – 1748.

Knauer, K., Behra, R., & Sigg, L. (1997). Adsorption and uptake of copper by the green alga Scenedesmus subspicatus (chlorophyta). *J. Phycol.* 33. pp. 596 – 601.

Landrum, P.F., Lee II, H., & Lydy, M.J. (1992). Toxicokinetics in aquatic systems: Model comparisons and use in hazard assessment. *Environmental Toxicology and Chemistry.* 11, pp. 1709 – 1725.

Lehninger, A.L., Nelson, D.L., & Cox, M.M. (1993). Principles of biochemistry 2nd ed. Richard L. Schowen. *Journal of Chemical Education.* 70 (8), A223. DOI: 10.1021/ed070pA223.1.

Lu, Y. & Allen, H.E. (2002). Characterization of copper complexation with natural dissolved organic matter (DOM) – link to acidic moieties of DOM and competition by Ca and Mg. *Water Research.* 36, pp. 5083 – 5101.

Luan, H. (2016). Impacts of effluent and stormwater runoff sources on metal lability and bioavailability in developed streams (Doctoral Dissertation). Retrieved from the University of Connecticut.

Matar, Z., Soares Pereira, C., Chebbo, G., Uher, E., Troupel, M., Boudahmane, L., Saad, M., Gourlay-France, C., Rocher, V. & Varrault, G. (2015). Influence of effluent organic matter on copper speciation and bioavailability in rivers under strong urban pressure. *Environmental Science Pollution Research.* 22, pp. 19461 – 19472.

Meyer, J.L., Paul, M.J., & Taulbee, W.K. (2005). Stream ecosystem function in urbanizing landscapes. *Journal of the North American Benthological Society.* 24(3), pp. 602 – 612.

Meylan, S., Behra, R., & Sigg, L. (2003). Accumulation of copper and zinc in periphyton in response to dynamic variations of metal speciation in freshwater. *Environmental Science and Technology.* 37, pp. 5204 – 5212.

Meylan, S., Behra, R., & Sigg, L. (2004). Influence of metal speciation in natural freshwater on bioaccumulation of copper and zinc in periphyton: A microcosm study. *Environmental Science and Technology.* 38, pp. 3104 – 3111.

Muller, F.L.L. (1995). Interactions of copper, lead, and cadmium with the dissolved, colloidal, and particulate components of estuarine and coastal waters. *Marine Chemistry.* 52, pp. 245 – 268.

Mylon, S.E., Twining, B.S., Fisher, N.S. & Benoit, G. (2003). Relating the speciation of Cd, Cu, and Pb in two Connecticut rivers with their uptake in algae. *Environmental Science and Technology.* 37, pp. 1261 – 1267.

National Isotope Development Center (NIDC). (2015). Copper. https://www.isotopes.gov.

Pasciak, W.J. & Gavis, J. (1974). Transport limitation of nutrient uptake in phytoplankton. *Limnology and Oceanography.* 19(6). pp. 881 – 888.

Pernet-coudrier, B., Clouzot, L., Varrault , G., Tusseau-vuillemin, M.H., Verger, A., & Mouchel, J.M. (2008). Dissolved organic matter from treated effluent of a major wastewater treatment plant: Characterization and influence on copper toxicity. *Chemosphere.* 73, pp. 593 – 599.

Pinto, J., Sigaud-Kutner, T.C.S., Leitão, M.A.S., Okamoto, O.K., Morse, D., & Colepicolo, P. (2003). Heavy metal-induced oxidative stress in algae. *Journal of Phycology.* 39, pp. 1008 – 1018.

Ploug, H., Stolte, W., & Jørgensen, B.B. (1999). Diffusive boundary layers of the colony-forming plankton alga Phaeocystis sp.— Implications for nutrient uptake and cellular growth. *American Society of Limnology and Oceanography.* 44(8), pp. 1959 – 1967.

Quaranta, M.L. (2011). Comprehensive analysis of effluent organic matter from five wastewater treatment plants in Connecticut and comparison to natural organic matter. Master's Thesis, University of Connecticut.

Quigg, A., Reinfelder, J.R., & Fisher, N.S. (2006). Copper uptake kinetics in diverse marine phytoplankton. *Limnology and Oceanography.* 51(2), pp. 893 – 899.

Sarathy, V. & Allen, H.E. (2005). Copper complexation by dissolved organic matter from surface water and wastewater effluent. *Ecotoxicology and Environmental Safety.* 61, pp. 337 – 344.

Serra, A., Corcoll, N., & Guasch, H. (2009a). Copper accumulation and toxicity in fluvial periphyton: The influence of exposure history. Chemosphere. 74, pp. 633 – 641.

Serra, A., Guasch, H., Martí E., & Geiszinger, A. (2009b). Measuring in-stream retention of copper by means of constant-rate additions. *Science of the Total Environment.* 407. pp. 3847-3854.

Slaveykova, V.I. & Wilkinson, K.J. (2005). Predicting the bioavailability of metals and metal complexes: Critical review of the biotic ligand model. *Environmental Chemistry.* 2, pp. 9 – 24.

Soldo, D. & Behra, R. (2000). Long-term effects of copper on the structure of freshwater periphyton communities and their tolerance to copper, zinc, nickel and silver. *Aquatic Toxicology.* 47, pp. 181 – 189.

Stevenson, R.J., Bothwell, M.L. & Lowe, R.L. (1996). Algal ecology – Freshwater benthic ecosystems. A volume in aquatic ecology. Front Matter. Elsevier, Inc. pp. 1 – 3.

Sunda, W.G. & Huntsman, S.A. (1998). Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems. *Science of the Total Environment.* 219. pp. 165 – 181.

Thurman, E.M. (1985). Geochemistry of natural waters. *Kluwer Academic Publishers Group*.

Tonietto, A.E., Lombardi, A.T., Choueri, R.B., & Vierira, A.A.H. (2015) Chemical behavior of Cu, Zn, Cd, and Pb in a eutrophic reservoir: speciation and complexation capacity. *Environ Sci Pollut Res.* 22, pp. 15920 – 15930.

Town, R.M. & Van Leeuwen, H.P. (2004). Dynamic speciation analysis of heterogeneous metal complexes with natural ligands by stripping chronopotentiometry at scanned deposition potential (SSCP). *Aust. J. Chem.* 57, pp. 983 – 992.

U.S. Environmental Protection Agency (USEPA) (2002). National Recommended Water Quality Criteria. Washington, DC.

U.S. Environmental Protection Agency (USEPA) (2007). Aquatic life ambient freshwater quality criteria – copper. 2007 Revision. Washington, DC. CAS Registry No. 7440-50-8.

U.S. Environmental Protection Agency (USEPA) (2014). National Rivers and Streams

Assessment 2013 – 2014 Laboratory Operations Manual. Section 9.0 – Periphyton.

U.S. Environmental Protection Agency (USEPA), Implementing Clean Water Act Section 303(d): Impaired Waters and Total Maximum Daily Loads (TMDLs), http://www.epa.gov/tmdl.

U.S. Geological Survey (USGS) (2017). USGS current water data for the nation, https://waterdata.usgs.gov/nwis/rt.

Van Leeuwen (1999). Metal speciation dynamics and bioavailability: inert and labile complexes. *Environmental Science and Technology.* 33, pp. 3743 – 3748.

Van Leeuwen, H.P., Town, R.M., Buffle, J., Cleven, R.F.M.J., Davison, W., Pay, J., Van Riemsdijk, W.H., & Sigg, L. (2005). Dynamic speciation analysis and bioavailability of metals in aquatic systems. *Environmental Science and Technology.* 39, pp. 8545 – 8556.

Worms, I., Simon, D.F., Hassler, C.S., & Wilkinson, K.J. (2006). Bioavailability of trace metals to aquatic microorganisms: importance of chemical, biological and physical processes on biouptake. *Biochimie.* 88, pp. 1721-1731.

Worms, I. & Wilkinson, K. (2008). Determination of $Ni^{2+}$ using an equilibrium ion exchange technique: Important chemical factors and applicability to environmental samples. *Analytica Chimica ACTA.* 616. pp. 95-102.

Worms, I., Traber, J., Kistler, D., Sigg, L. & Slaveykova, V.I. (2010) Uptake of Cd(II) and Pb(II) by microalgae in presence of colloidal organic matter from wastewater treatment plant effluents. *Environmental Pollution.* 158. pp. 369 – 374.

Xue, H., Stumm, W., & Sigg, L. (1988). Binding of heavy metals to algal surfaces. Wat. Res. 22(7). pp. 917 – 926.

# 5 Supplemental Information

Presented in Figures 7 through 9 are additional views of the trickle apparatus that was constructed to perform various periphyton exposure scenarios.

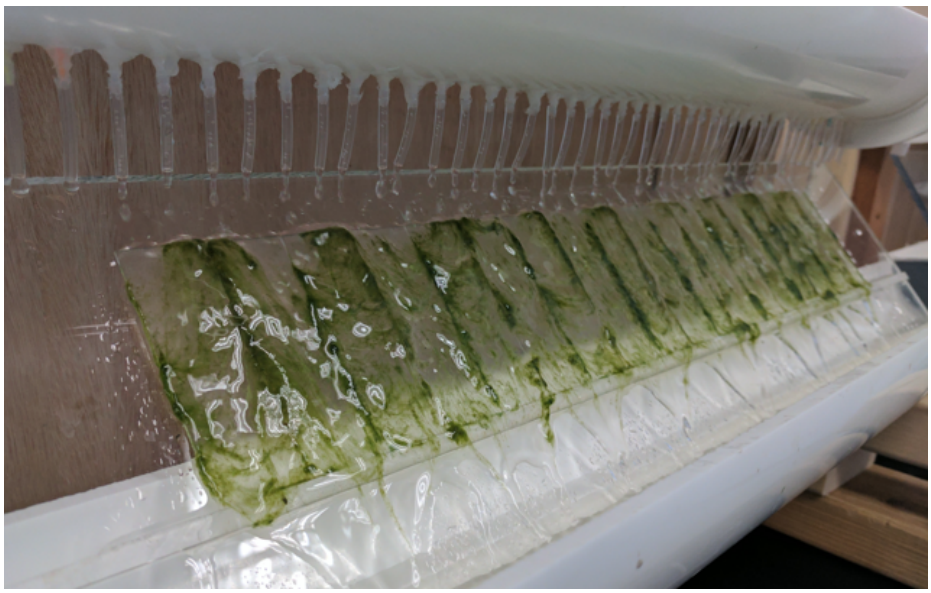

*Figure 7: Trickle apparatus view 1 – close-up of periphyton slide exposure to the source water (Fenton River freshwater, UConn WWTP effluent, freshwater: WWTP effluent mixtures)*

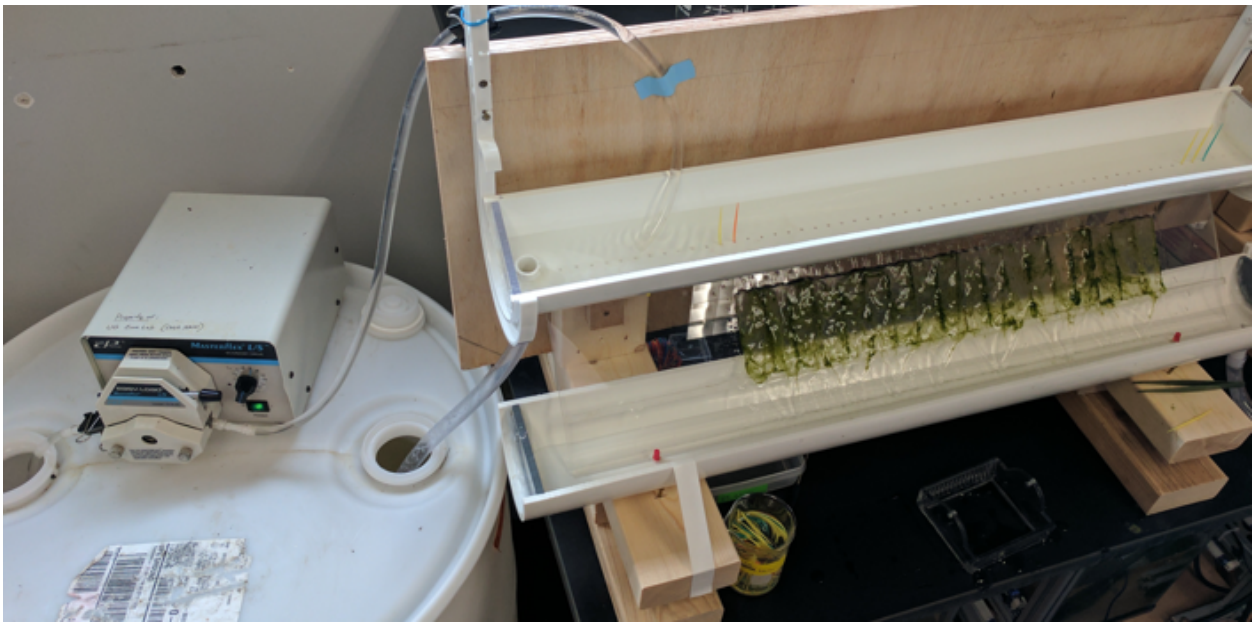

*Figure 8: Trickle apparatus view 2 – full set-up of apparatus including the 200-liter polyethylene barrel holding the exposure water and the peristaltic pump used to introduce the water into the trickle apparatus upper trough.*

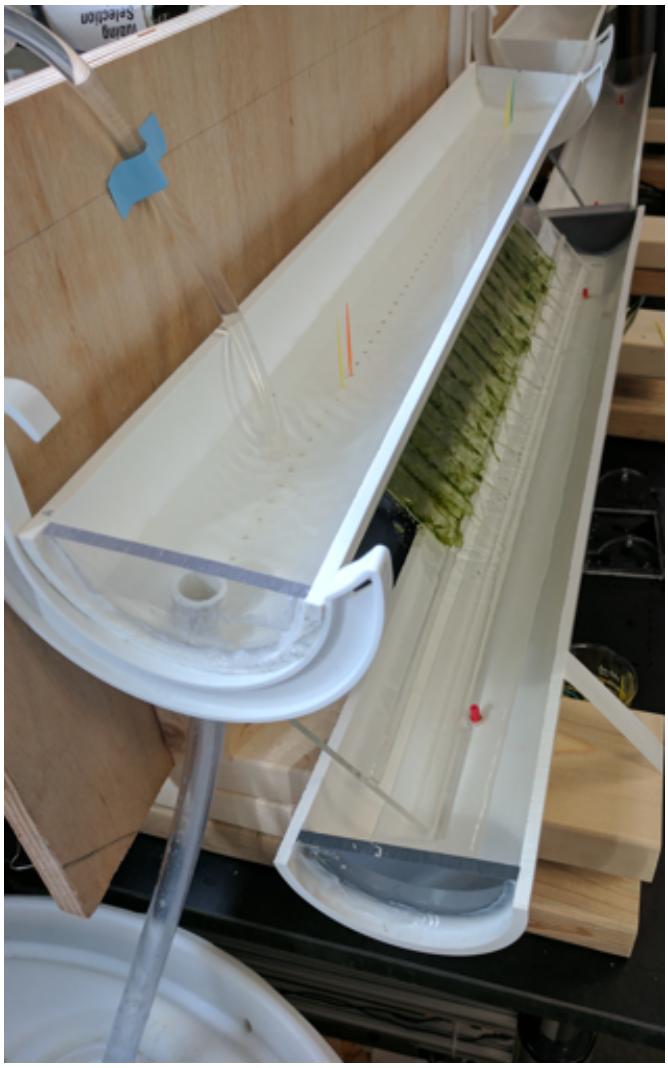

*Figure 9: Trickle apparatus view 3 – side view showing the top trough with the overflow pipe leading to the 200-liter barrel. The overflow pipe maintained a constant head during the entire exposure duration allowing for controlled flow over the periphyton slides.*

*Table 13: Visual MINTEQ model inputs for total alkalinity, pH, and the major anions and cations in solution*

| Exposure Water | Exp. | Total ALK mg $CaCO_3$/L | pH | $NO_3^-$ mg/L | $Cl^-$ mg/L | $SO_4^{2-}$ mg/L | $Na^+$ mg/L | $Ca^{2+}$ mg/L | $Mg^{2+}$ mg/L | Total Cu µg/L |
|---|---|---|---|---|---|---|---|---|---|---|
| 100% Fenton River | 1 | 18.6 | 7.3 | 0.61 | 40.2 | 8.1 | 22.6 | 10.9 | 3.0 | 2.78 |
| | 2 | 21.0 | 7.3 | 0.52 | 33.2 | 6.4 | 16.6 | 10.5 | 2.8 | 2.86 |
| | 3 | 15.4[1] | 7.6 | 1.0[1] | 35.7[1] | 7.0[1] | 12.3 | 9.0 | 2.8 | 3.40 |
| | 4 | 15.4[1] | 7.3 | 1.0[1] | 35.7[1] | 7.0[1] | 12.8 | 8.7 | 2.6 | 4.46 |
| | 5 | 15.4[1] | 7.5 | 1.0[1] | 35.7[1] | 7.0[1] | 50.6 | 20.7 | 4.3 | 5.22 |
| | 6 | 6.6 | 6.8 | 1.8 | 33.8 | 6.4 | 21.2 | 5.7 | 2.3 | 15.73 |
| 100% WWTP Effluent | 7 | 118.0 | 8.4 | 14.6 | 116.8 | 53.8 | 93.3 | 17.8 | 19.7 | 5.59 |
| | 8 | 88.0 | 8.3 | 31.7 | 131.0 | 36.3 | 97.8 | 30.8 | 12.2 | 7.71 |
| | 9 | 91.6 | 8.2 | 36.3 | 155.9 | 39.8 | 112.2 | 27.2 | 13.8 | 8.17 |
| 10% Effluent: 90% River | 10 | 16.0 | 7.2 | 5.7 | 83.5 | 11.3 | 40.0 | 11.3 | 4.7 | 5.39 |
| 30% Effluent: 70% River | 11 | 20.0 | 7.2 | 14.0 | 111.8 | 22.5 | 70.2 | 15.6 | 6.7 | 4.82 |
| | 12 | 27.0 | 7.4 | 10.4 | 122.8 | 16.3 | 71.4 | 17.9 | 6.4 | 6.25 |
| | 13 | 21.0 | 7.2 | 18.3 | 101.3 | 15.1 | 58.9 | 16.6 | 6.2 | 17.82 |
| 50% Effluent: 50% River | 14 | 40.6 | 7.5 | 17.4 | 145.4 | 20.7 | 82.1 | 17.8 | 9.0 | 6.61 |

[1] Measurements not available, category averages used for water quality data

*Table 14: Visual MINTEQ DOM inputs. New DOM species were created in the database files for the hydrophobic and hydrophilic fractions (DOM ligand molecular weight = 12 g/mol).*

| Exposure Water | Exp. | Hydrophilic DOM Ligands | | Hydrophobic DOM Ligands | |
|---|---|---|---|---|---|
| | | Average Log $^{c}K$ ($M^{-1}$) | Concentration (nM) | Average Log $^{c}K$ ($M^{-1}$) | Concentration (nM) |
| **100% Fenton River** | 1 | 9.5[1] | 11.0[1] | 10.3[1] | 1.4[1] |
| | 2 | 9.5 | 11.0 | 10.3 | 1.4 |
| | 3 | 9.5[1] | 11.0[1] | 10.3[1] | 1.4[1] |
| | 4 | 9.5[1] | 11.0[1] | 10.3[1] | 1.4[1] |
| | 5 | 9.5[1] | 11.0[1] | 10.3[1] | 1.4[1] |
| | 6 | 7.7 | 75.0 | 8.9 | 60.0 |
| **100% WWTP Effluent** | 7 | 12.1 | 8.6 | 12.7 | 7.2 |
| | 8 | 12.7 | 4.6 | 15.0 | 0.1 |
| | 9 | 11.9 | 10.2 | 12.6 | 1.8 |
| **10% Effluent: 90% River** | 10 | 9.2 | 27.2 | 10.3 | 10.1 |
| **30% Effluent: 70% River** | 11 | 10.1 | 9.8 | 10.5 | 7.3 |
| | 12 | 10.0 | 18.5 | 11.1 | 3.9 |
| | 13 | 9.0 | 70.2 | 10.0 | 24.2 |
| **50% Effluent: 50% River** | 14 | 10.0 | 22.5 | 11.7 | 1.9 |

[1] Measurements not available, results obtained from experiment 2 used as approximations

*Table 15: ANOVA statistics results for the 100% Fenton River and 100% WWTP effluent exposure intracellular uptake rate constants, $k_u$, when $C_w = [Cu_{total}]$. Null Hypothesis is rejected when $F > F_{crit}$ and p-value < 0.05.*

| **Anova: Single Factor - $k_u$, $Cu_{total}$ (CI = 95%)** | | | | |
|---|---|---|---|---|
| SUMMARY | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
| Fenton | 6 | 13.12 | 2.19 | 0.57 |
| WWTP | 3 | 2.02 | 0.67 | 0.05 |

ANOVA: Ho: $\mu_{ku,fenton} = \mu_{ku,WW}$ Hi: $\mu_{ku,fenton} \neq \mu_{ku,WW}$

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 4.577 | 1 | 4.577 | 10.88 | 0.01 | 5.59 |
| Within Groups | 2.946 | 7 | 0.421 | | | |
| Total | 7.523 | 8 | | | | |

*Table 16: ANOVA statistics results for the 100% Fenton River and 100% WWTP effluent exposure intracellular uptake rate constants, $k_u$, when $C_w = [Cu^{2+}]$. Null Hypothesis is rejected when $F > F_{crit}$ and p-value < 0.05.*

| **Anova: Single Factor - $k_u$, $Cu^{2+}$ (CI 95%)** | | | | |
|---|---|---|---|---|
| SUMMARY | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
| Fenton | 6 | 69.55 | 11.59 | 60.26 |
| WWTP | 3 | 189.00 | 63.00 | 2290.42 |

ANOVA: Ho: $\mu_{ku,fenton} = \mu_{ku,WW}$ Hi: $\mu_{ku,fenton} \neq \mu_{ku,WW}$

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 5285.507 | 1 | 5285.507 | 7.58 | 0.03 | 5.59 |
| Within Groups | 4882.139 | 7 | 697.448 | | | |
| Total | 10167.646 | 8 | | | | |

*Table 17: ANOVA statistics results for the 100% Fenton River and 100% WWTP effluent exposure intracellular uptake rate constants, $k_u$, when $C_w = [Cu_{ex}]$. Null Hypothesis is rejected when $F > F_{crit}$ and p-value < 0.05.*

| **Anova: Single Factor - $k_u$, $Cu_{ex}$ (CI = 95%)** | | | | |
|---|---|---|---|---|
| SUMMARY | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
| Fenton | 6 | 578.48 | 96.41 | 3237.70 |
| WWTP | 3 | 164.13 | 54.71 | 1827.68 |

ANOVA: Ho: $\mu_{ku,fenton} = \mu_{ku,WW}$ Hi: $\mu_{ku,fenton} \neq \mu_{ku,WW}$

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 3478.31 | 1 | 3478.31 | 1.23 | 0.30 | 5.59 |
| Within Groups | 19843.84 | 7 | 2834.83 | | | |
| Total | 23322.14 | 8 | | | | |

*Table 18: ANOVA statistics results for the 100% Fenton River, 100% WWTP effluent, and 30:70 WW:Freshwater exposure intracellular uptake rate constants, $k_u$, when $C_w = [Cu_{ex}]$. Null Hypothesis is rejected when $F > F_{crit}$ and p-value < 0.05.*

| **Anova: Single Factor- $k_u$, $Cu_{ex}$ (CI = 95%)** | | | | |
|---|---|---|---|---|
| SUMMARY | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
| Fenton | 6 | 578.48 | 96.41 | 3237.70 |
| WWTP | 3 | 164.13 | 54.71 | 1827.68 |
| 30:70 | 3 | 82.91 | 27.64 | 28.61 |

ANOVA: Ho: $\mu_{ku,fenton} = \mu_{ku,WW}$ Hi: $\mu_{ku,fenton} \neq \mu_{ku,WW}$

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 10254.12 | 2 | 5127.06 | 2.32 | 0.15 | 4.26 |
| Within Groups | 19901.05 | 9 | 2211.23 | | | |
| Total | 30155.18 | 11 | | | | |