AN ABSTRACT OF THE THESIS OF

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Title: Quantification of Copper Speciation in Pulp and Paper Wastewater and Implications for the Biotic Ligand Model.

Abstract approved:

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Jeffrey A. Nason

The purpose of this research was to measure the copper-binding behavior of

Dissolved Organic Matter (DOM) contained in Pulp and Paper (P&P) wastewater

effluent and evaluate the Biotic Ligand Model's (BLM) ability to quantify copper

speciation in P&P influenced waters. A copper ion selective electrode was used to

measure copper activity in four P&P mill effluents and their receiving waters with the

purpose of quantifying metal binding behavior with a two-site ligand model. In

addition, USGS flow data and the optical properties of DOM were used to evaluate

the relative contribution of P&P organic material to each river system studied and the

ability of the specific UV absorbance at 254 nm (SUVA$_{254}$) to predict metal-binding

behavior. Experimentally measured copper-ligand affinity and site-density parameters were used to develop a site-specific copper-speciation model in the chemical equilibrium software program Visual MINTEQ for comparison to the BLM. Results indicate P&P effluent DOM has a greater strong-ligand site density and $SUVA_{254}$ in comparison to receiving waters, but the ability to detect the influence of these characteristics downstream was limited by the high dilution ratios and uncertainty of accounting for all inputs to the river system. While $SUVA_{254}$ was poorly correlated with metal-binding when analyzing data from all sites, there was a strong, positive correlation between the variables at two sites, and therefore more work is needed to determine if $SUVA_{254}$ can be used to predict metal-binding behavior. Regarding simulation results, the BLM and MINTEQ models were consistently within an order of magnitude, but the BLM regularly predicted less available copper at environmentally relevant concentrations. Diluted effluent sample simulations were in better agreement (within 2-3x) at environmentally relevant total copper concentrations (5-15 µg/L) than their corresponding upstream and downstream counterparts, an indication of more copper-binding potential in the effluent samples that isn't accounted for in the BLM. In contrast, the BLM predicted up to 5x more bioavailable copper at lower total copper concentrations in one fully concentrated (7.23 mg/L DOC) effluent sample, indicating the BLM may not properly quantify low-level copper availability in concentrated, P&P wastewater effluent. Thus, while the BLM is a useful tool for predicting copper speciation, the model may be improved by allowing researchers to specify more detailed binding characteristics of DOM, especially when evaluating P&P influenced waters.

Quantification of Copper Speciation in Pulp and Paper Wastewater and Implications

for the Biotic Ligand Model

by

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Joseph Mitzel, Author

ACKNOWLEDGEMENTS

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1. Introduction

Since the early 1980's, a body of research has been dedicated to advancing the science behind

the Biotic Ligand Model (BLM), a conceptual framework (and software program) linking several

mechanistic models that incorporate inorganic and organic metal speciation to predict copper

metal bioavailability and toxicity.[1–6] As of 2016, the EPA uses the Biotic Ligand Model for

setting freshwater criteria as outlined in the EPA's *Aquatic Life Ambient Freshwater Criteria –*

Copper 2007 Revision[7] publication. The Aquatic Life Criterion (ALC) can be defined as the

highest concentration of a toxic pollutant that does not significantly threaten most aquatic species

in a given water body.[8] These criteria are a subset of the broader Water Quality Criteria (WQC)

which the states are authorized to establish for water bodies with different designated uses

(drinking water, recreation, industrial, propagation of fish, etc.) (40 C.F.R § 131.10(a)).[9] The

EPA is also interested in implementing a BLM for aluminum, zinc, and lead.[6] As the BLM

becomes more widely used for permitting and regulatory decisions, it is important to understand

model behavior in different environmental settings. The purpose of this research is to better

understand the BLM's predictive capability and limitations when implemented in aquatic

systems that receive Pulp and Paper (P&P) wastewater.

1.1. Objectives and Hypothesis

To understand the BLM's predictive capabilities in P&P influenced surface waters, it is

important to evaluate the water quality parameters most likely to vary between natural and P&P

influenced aquatic systems. The BLM requires ten input parameters: pH, Dissolved Organic

Carbon[*] (DOC), Ca^{2+}, Mg^{2+}, Na^+, SO_4^{2-}, K^+, Cl^-, alkalinity, and temperature.[6] Among these parameters, the EPA has stated that DOC and pH are "very influential" in determining the Instantaneous Water Quality Criteria and Fixed Monitoring Benchmark.[10] The BLM utilizes the Windermere Humic Aqueous Model (WHAM)[11] for quantifying metal-organic (presently copper-DOC) interactions. This model was developed around the behavior of humic and fulvic acid species present in natural organic matter (NOM).[1] Humic and fulvic acids typically represent the largest fraction of NOM in natural aquatic systems[12], and play a key role in metal speciation. When using the BLM, the model developers recommend a default value of 10% humic acid content to be used when the distribution of humic/fulvics is not known.[1] In fact, the developers explicitly state a chemical-specific model may need to be used when there exists a significant amount of artificial organic carbon.[1]

> *"It should not be expected that DOC from artificial sources (e.g. acetate, sugar, MOPS, etc...) behave as natural organic matter. The organic matter*
>
> *binding model that the BLM uses is based on natural sources of organic matter, and*
>
> *inputs should therefore not include known quantities of artificial organic matter. If there*
>
> *is a significant amount of artificial organic carbon in the solution, then a speciation*
>
> *model that includes that chemical component may be necessary to accurately model it."*[1]

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[*] Dissolved Organic Carbon (DOC) is operationally defined as the organic carbon present in solution after passing through a 0.45 µm filter. When used in this research, Dissolved Organic Matter (DOM) is used in lieu of DOC when discussing metal-binding characteristics, since there may be non-carbon functional groups responsible for binding behavior. When citing other researchers work, terms such as, Natural Organic Matter (NOM) may also be used.

Therefore, the BLM may not accurately predict metal speciation in situations where artificial organic matter with unique metal-binding behavior is present.[1] Since the P&P industry likely has unique organic matter in wastewater effluent, it is important to understand how this might impact the BLM. Thus, the objective of this research is to characterize DOM from both P&P and natural water and evaluate the accuracy of BLM predictions. The objectives can be summarized as follows:

1) Characterize and compare copper binding behavior in P&P effluent and receiving waters.

2) Evaluate the Biotic Ligand Model's ability to accurately predict bioavailable copper in P&P effluent-impacted surface waters.

3) Explain variance in the BLM's prediction capability (if any) using optical properties of the DOM.

It is hypothesized that P&P DOM will complex copper differently than NOM and that those differences will be evident in comparisons of experimentally measured and BLM predicted free ion activities.

1.2. Methodology

To test the hypothesis that P&P DOC may cause the BLM to deviate from experimentally measured concentrations of bioavailable copper in P&P influenced surface waters, experiments were designed to evaluate whether 1) P&P DOC is measurably different than natural DOC and 2) the BLM predictions of copper bioavailability in P&P influenced waters are significantly different than the predicted (site specific) values. The first question was evaluated by comparing copper-binding affinity and ligand-site densities between river and effluent DOM as measured

with a Copper Ion Selective Electrode (CuISE) and copper titration. In addition, the Specific Ultraviolet Absorbance at 254 nm (SUVA$_{254}$) was measured and used as a proxy for the aromaticity of river and effluent DOM, a parameter commonly associated with metal binding. The second question was evaluated by performing Visual MINTEQ simulations using the experimentally determined binding parameters and comparing the results to the BLM's predictions of ionic copper concentration. The methodology can be summarized as follows:

1) Collect upstream, downstream, and effluent water samples from four P&P sites in the United States.

2) Measure SUVA$_{254}$ and % aromaticity in river water and P&P effluent samples.

3) Use a CuISE to directly measure copper activity in samples during standard-addition copper titrations.

4) Use measured copper titration data and a ligand-binding model to determine Cu-DOM conditional stability constants.

5) Use measured conditional stability constants to develop a site-specific copper speciation model (in Visual MINTEQ) for comparison to the BLM.

The rest of this document is organized as follows: Chapters 2 and 3 include a background discussion of topics relevant to P&P wastewater, the BLM, and methods used in this research for measuring copper speciation and evaluating the BLM. Chapters 4 and 5 report the results of copper-DOM titrations and model simulations with a discussion of the implications from the present research.

2. Background

2.1. Copper Bioavailability and Quantification in Aquatic Systems

While copper is an important nutrient in aquatic systems, chronic or acute exposure to elevated concentrations can cause numerous detrimental health effects and death to aquatic species.[13] Copper can enter water bodies from both natural (geologic formations, erosion, etc.) and anthropogenic (mining, agricultural, antifouling paints, and pesticides, stormwater runoff, etc.) sources.[13] Free ionic copper is widely regarded as the most bioavailable form responsible for toxicity to aquatic species. For this reason, decades of research have been dedicated to accurately predicting copper speciation in natural systems. Between 1980 and 1996, the EPA published several guidance documents providing recommendations for determining the copper ALC.[7] Traditionally, WQC for copper (and other metals) were derived from empirical regressions between water hardness and copper toxicity.[7,14,15] Hardness is the term commonly used to describe the concentration of divalent ions, generally Mg^{2+} and Ca^{2+} in natural waters. Although these ions compete with toxic metals and provide protection to aquatic species[16,17], the hardness-based standards were/are often over or under-protective depending on several other water quality parameters such as pH and DOC.[6] For example, in a recent study on rivers in the UK[18], researchers noted that while total copper concentrations did not vary significantly between seasons, the bioavailability of copper did. The researchers concluded that total copper concentration is less a predictor of bioavailability than other water quality parameters. For situations where water quality parameters differ between rivers/sites, it is useful to have a model that incorporates parameters unique to the water body under investigation, especially when setting water quality criteria and regulatory standards.

Enter the Biotic Ligand Model (BLM)[1–5], a conceptual framework leveraging several mechanistic models that incorporate inorganic and organic metal speciation to predict metal bioavailability and toxicity. In 2000, when Paquin *et. al*. published their description of the BLM, they highlighted the model's ability to provide "*an alternative means of developing site-specific permit limits and WQC*." [17] This was because the model accounted for much more than water hardness; notably, pH and DOC.[17] The latest version of the copper BLM[1] (v.3.16.2.41) utilizes the Chemical Equilibria in Soils and Solutions[19] (CHESS) model, the Windermere Humic Aqueous Model (WHAM) model[11], and competition of other ions to determine the amount of active metal inducing toxic effects at the biotic ligand, or gill. In this manner, the BLM merges inorganic and organic copper speciation models with quantification of binding with the biotic ligand and the associated toxic effects.

When it was originally published, the developers noted the BLM's ability to predict copper and silver LC50s (lethal doses for 50% of a population) for certain aquatic species within a factor of two, and this was consistent for LC50s that ranged over two orders of magnitude.[2–4] While the BLM is an easier, more economical method to determine site specific WQC than labor-intensive toxicity tests, it is important to understand the environmental conditions and model assumptions under which the BLM is valid. The BLM assumes chemical equilibrium and, as mentioned before, that organic matter is similar to well-characterized natural sources (*i.e.* humic/fulvic acids).

There have been several examples where the source of DOM influenced the speciation of copper and therefore the toxicity of copper to aquatic species. For example, Ryan *et. al.* investigated the influence of NOM source on copper toxicity to larval fathead minnows and

found that while the BLM predictions were agreeable with observed toxicity, the model should

not be expected to adequately predict toxicity without considering the source of organic carbon.[20]

Even so, the researchers also concluded that DOC and humic acid concentrations are better

attributed to LC50 variability than other more descriptive copper-binding characteristics. In two

separate studies, Luider *et. al.* and Schwartz *et.al.* found that high color (high optical

absorbance), allochthonous[†,12] NOM decreased copper accumulation on the gills of rainbow trout

more than low-color autochthonous NOM.[21,22] The former group therefore recommended the

copper BLM should incorporate a modifying factor, such as an optical measurement, to account

for "NOM Cu-binding quality."[21] Similarly, Schamphelaere *et. al.* performed toxicity tests on

Daphnia magna and modeling with the acute copper-BLM, demonstrating a linear correlation

between % active fulvic acid (an adjustable BLM parameter) and the DOM's UV absorbance

coefficient at 350 nm.[23] Using absorbance to adjust the BLM parameter, the researchers observed

90% of the predicted toxicity concentrations were within a factor of 1.3 from the measured value

(in comparison to a factor of 2 without adjustment).[23]

Even when comparing the BLM predictions to toxicity tests utilizing a solution matrix

with humic species present, the model can produce inconsistent results. For example, McGeer *et

al.* used different concentrations of humic acid (from sodium salt) to investigate its influence on

copper bioavailability to rainbow trout. While increasing the concentration of humic acid

alleviated both chronic and acute effects, the BLM predicted a higher level of protection. That is,

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[†] Allochthonous refers to organic carbon derived from terrestrial or vegetative sources, while Autochthonous refers to organic carbon derived from aquatic or microbial sources.

the measured toxicity concentrations were $70 \pm 5\%$ the values predicted by the BLM, although the authors note this could be because the experimental humic acid was not representative of the natural humic species upon which the BLM is based.[24] This behavior is consistent with other researchers' findings that the WHAM model (version V) under-predicts the amount of copper activity in natural waters.[25–28]

The BLM in demonstrably a useful tool for predicting toxicity concentrations and setting metal ALCs due to its relative accuracy and ease of use. While it clearly improves the effectiveness and efficiency of the regulatory process, a number of researchers have demonstrated that even regarding natural sources of organic matter, the BLM can continue to be improved through characterization/adjustment of organic matter properties.

2.2. Properties of Pulp and Paper Wastewater

In the present research, "Pulp and Paper (P&P) Industry" is a general term that encompasses all the processes necessary to produce paper. Some of the processes include debarking, pulping (can be chemical, thermal, mechanical or a combination[29]), bleaching (removes color and brightens pulp), washing (to separate bleaching chemicals and dissolved organics that contribute to color from the pulp), and final production of paper products.[30] The P&P industry is of global significance, both for its economic value and contribution to environmental pollution.[29–33] Wastewater from this industry contains trace elements that originate from the raw wood and bark material entering the P&P plant. Although these elements are generally at low concentrations, the mass loading to natural water bodies can be high due to the large amount of raw material processed each year.[34,35] In Norwegian P&P wastewater, Skipperud *et al*. reported average trace

metal concentrations of 67 µg/L copper, 1.88 µg/L cadmium, 1.7 µg/L lead, and 0.21 mg/L zinc, among others. With regard to copper, the researchers reported 26%, 41%, and 33% present as colloids (0.45 µm), low molecular weight (<10 kDalton), and particulate (>0.45 µm) fractions, respectively. Zinc and cadmium were primarily in the low molecular weight form, while 45% and 38% of lead was in the colloidal and low molecular weight fractions, respectively.[34]

Besides the trace metals found naturally in the raw materials, researchers have identified more than 250 chemicals and organic constituents in P&P wastewater.[30] These include lignins, sulfur compounds, tannins, resin acids, fatty acids, phenols, dioxins, furans, chlorinated organics, and many others.[29,33] Lignins, tannins, and resin acids are inherent in the wastewater since the P&P process involves the breakdown of wood containing these building blocks.[36] In a review of P&P wastewater treatment, Renata reported tannin and lignin concentrations of 2730 and 11000-25000 mg/L, respectively, in wastewater from the mechanical and chemical pulping steps.[33] Tannins are polar and phenolic compounds[37] that can contribute up to 50% of the Chemical Oxygen Demand (COD) in P&P wastewaters.[30,38] Polymerized tannins and lignin compounds contribute substantially to the color of P&P wastewater[39–41], especially during the pulping, bleaching, and washing phases.[30] Although accounting for a relatively small fraction of the plant's total wastewater, the washing phase can contribute up to 80% color, 30% BOD, and 60% COD as this phase is rich in lignin/tannin compounds.[30,42] These compounds' contribution to P&P wastewater color is notable, as it was previously demonstrated that high-color organic matter protected some aquatic species from copper toxicity more than low-color organic matter. However, while these compounds could indirectly reduce metal toxicity through binding, lignin and its derivatives are also known to be directly toxic and mutagenic to aquatic species. [30,43,44]

Therefore, P&P effluent organic material could simultaneously reduce toxicity through metal binding and increase toxicity/mutagenicity depending on the quantity and type of organic constituents.

In addition to the organics naturally present in wood material and byproducts of the various pulping/bleaching steps, there are also classes of organic compounds intentionally added during the mill process steps and wastewater treatment process. For example, coagulants/flocculants such as polyacrilamide, polyacrylic acid, and DADMAC are added to remove COD and color.[33,45] In addition, researchers have noted the marked increase of ethylenediaminetetraacetic acid (EDTA) use in Swedish P&P operations, as it helps regulate free metal concentrations in processes sensitive to these ions.[35] The researchers noted both the recalcitrant nature of EDTA and its ability to regulate metal bioavailability in wastewater effluent by acting as a "reservoir" for metals.[35] Simply put, EDTA holds metals in the dissolved fraction and releases them in equilibrium with the nutrient demands of aquatic species.[35] In Belgium and Germany, researchers measured micromolar concentrations of EDTA in urban-impacted waters and concluded it was partly responsible for increased metal-binding.[46] In a 1997 study of wastewater effluent and surface runoff discharges to South San Francisco Bay, researchers found that "activated sludge biopolymers and humic substances" were responsible for binding up to 50% of copper, while the remaining binding behavior was similar to that of synthetic chelators.[47]

P&P wastewater can therefore be unique due to the metals and organics found naturally in the raw materials, as well as from the chemical byproducts formed and additives used in

processing. While unique among industries, the individual characteristics of each P&P mill/site can also vary depending on the processes and wastewater treatment techniques used.

2.3. Copper-DOM Binding Models

NOM originates from a combination of allochthonous and autochthonous sources.[12] Because NOM is a mixture of chemical species with different binding characteristics, it can be difficult to describe its metal-binding behavior in a manner that is simplistic,[12] but consistent with the natural mechanisms of metal-ligand chemistry. While empirical models may accurately describe metal-binding for a given water body, these models are unappealing since they cannot be readily applied to other water bodies/data sets.[12,48] When constructing metal-NOM binding models, researchers' approaches typically vary when it comes to quantifying ligand-site concentration and binding affinity. Different approaches for quantifying metal-NOM binding typically include: 1) discrete site models that assign equilibrium constants and site densities to a discrete number of ligands[12], 2) an assumption that the metal-binding affinity of a mixture of ligands can be aggregately described with a continuous distribution function such as a normal Gaussian curve[12,49], and 3) the assumption that even if all ligand binding sites were intrinsically the same, the observed metal affinity associated with metal-binding behavior would be dependent on electrostatic effects.[12] As previously mentioned, the WHAM model[11] used in the BLM combines proton binding with both a discrete model and electrostatic model.[12]

This research utilizes a discrete binding model for organic-metal complexes that relies on a bimodal distribution of binding sites, commonly thought to represent carboxylic and phenolic groups.[50,51] Carboxylic acid and phenolic groups are important in the speciation of copper with

fulvic acid[52,53] and are common species found in NOM. A metal's partitioning between the free form and organic complex has been similarly described by Brezonik and Arnold[12] and others[54,55] with a multi-site Langmuir binding model:

$$M_T = M_f + \sum_{i=1}^{n} \frac{K_i L_i M_f}{1 + K_i M_f} \qquad (1)$$

Where M_T is the total amount of metal, M_f is the free metal ion, and the summation expression represents the amount of copper bound to organic ligands. The Langmuir expression consists of two terms that describe the organic matter's binding character: the binding site concentration (L_i) and the binding site affinity for copper (K_i). For two, discrete, mono-dentate binding sites, the formation reactions can be described as:

$$Cu^{2+} + L_1^- \leftrightarrow CuL_1^+ \qquad (2)$$

$$Cu^{2+} + L_2^- \leftrightarrow CuL_2^+ \qquad (3)$$
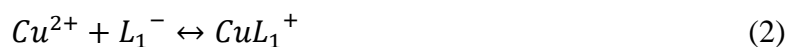
The stability constants associated with the formation reactions can be expressed as:

$$K_1^C = \frac{[CuL_1^+]}{\{Cu^{2+}\}[L_1^-]} \qquad (4)$$

$$K_2^C = \frac{[CuL_2^+]}{\{Cu^{2+}\}[L_2^-]} \qquad (5)$$

The "c" denotes "conditional", since these binding constants are typically determined at constant pH, temperature, and ionic strength and are therefore only valid in similar water matrixes. When comparing K_i and L_i across samples, it is desirable to normalize to the concentration of DOC. Therefore, the DOC-normalized expression for copper binding to two, discrete ligands is:

$$\frac{[CuL_T]}{DOC} = \frac{K_1^C [L_{1,T}]\{Cu^{2+}\}}{1 + K_1^C \{Cu^{2+}\}} + \frac{K_2^C [L_{2,T}]\{Cu^{2+}\}}{1 + K_2^C \{Cu^{2+}\}} \qquad (6)$$

Where K_i is unit-less and $L_{i,T}$ is in mmol sites/mg-C. This model is simplistic but consistent with the mechanisms of copper-ligand interactions. In practice, the stability constants can be determined from experimental data using a graphical method (Scatchard plot[56]) or non-linear least squares regression.[48] For copper titrations, a Scatchard plot of ([CuL]/[DOC])/{Cu^{2+}} vs [CuL]/[DOC] produces a hyperbolic curve indicating the presence of at least two ligands, whereby the slopes of the hyperbolic tails distinguish the strong and weak-site stability constants.[12] When determining stability constants with either method, it is important that titrations are performed at the same environmental conditions to facilitate parameter comparison across samples.

For natural systems, it is appropriate to fit experimental data to a 2-site model since it is consistent with carboxylic and phenolic binding sites, although some researchers have used three sites.[54] The 3-site model has been used most often when investigating waters impacted by urban activities. This is supported through the observation that the hydrophilic[‡] fraction in urban-influenced waters exhibits a stronger metal binding capability than (hydrophobic) humic substances.[46,50,57] Organic matter from wastewater effluent contributes more hydrophilic and low aromaticity compounds[58–60] than natural water systems that have a larger hydrophobic fraction.[61] Matar *et al*. reported a 3rd "very high-affinity" binding site present in municipal wastewater effluent DOM in Paris, France that consisted of approximately 80% non-humic substances (of total DOC).[62] Pernet-Courdrier *et al*. also showed that water receiving effluent wastewater had

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[‡] The hydrophilic fraction is an operational definition, based upon the separation of organic matter using ion exchange resins. Typically, humic and fulvic acids (natural NOM) are associated with the hydrophobic fraction.

low humic substance content and a potentially high affinity for metals.[59] They utilized a 3rd site

for modeling the hydrophilic fraction that was thought to represent high-affinity amide/amine

nitrogen groups.[50,59]

Whether a two or three site ligand model is used, the mechanistic consistency, non-empirical

nature, and accessibility make this model ideal for comparing metal-binding affinities from

different water bodies.

2.4. Copper-DOM Binding and SUVA$_{254}$

The type of ligands present in natural or effluent DOM is especially important for the

relationship between light absorbance, metal-binding affinity, and %aromaticity. Several

researchers have noted the positive relationship between Specific Ultraviolet Absorbance

(SUVA) and % aromaticity when measuring SUVA at wavelengths of 254, 272, and 280 nm.[63–65]

In addition, researchers have noted higher metal-binding behavior from high-color,

allochthonous sources rich in lignin whose degradation byproducts result in aromatic fulvic

acids.[21,66] Since specific absorbance is well correlated with aromaticity[63] and subsequent metal-

binding[22], the behavior of wastewater effluent DOM is unique. In wastewater effluent DOM, the

low-aromaticity (low light absorbing) hydrophilic fraction is thought to be responsible for the

high-affinity "3rd ligand". For example, researchers observed that a low UV-absorbing

hydrophilic fraction of effluent DOM complexed strongly with zinc.[58] Baken *et. al.* found UV-

absorbance was correlated to metal-binding (Cd, Cu, Ni, and Zn) affinity but not in the case of

urban-impacted waters.[46] Additionally, urban-impacted waters had higher metal-binding

affinities and contained synthetic ligands such as EDTA in micro molar concentrations. The

researchers concluded that anthropogenic discharges were responsible for increased metal binding that would not be predicted by humic-based models.[46]

Therefore, when quantifying copper speciation with two or three site ligand models, it is important to understand that while allochthonous-based material (more similar to the type of materials entering P&P mills) may absorb more light and bind more strongly to metals than its autochthonous counterparts, the high affinity ligand responsible for some of the binding may in fact be a low light-absorbing, hydrophilic compound.

3. Materials and Methods

3.1. Sample Collection, Preparation, and Analysis

River water and effluent samples were collected from rivers and P&P plants at four sites in the United States (**Figure 1**). Three samples were collected at each site: upstream, downstream, and effluent. Samples from Sites 1 and 2 were collected in October of 2017 while Sites 3 and 4 were collected in February and April of 2018, respectively. Site 2 was at a downstream location from Site 1, while Sites 3 and 4 were on different rivers. Samples were collected by mill personnel in polyethylene bottles and subsequently sent to ALS Environmental laboratories for anion, cation, DOC, and metals analysis. Approximately 2-L of each sample was stored on ice, left unfiltered, and sent to Oregon State University for copper titrations and optical analysis.

Cations (Cu^{2+}, Ca^{2+}, Mg^{2+}, Na^+, and K^+) were analyzed with ICP using EPA Method 200.7/8, anions (SO_4^{2-} and Cl^-) were analyzed with Ion Chromatography using EPA method 300.0, and DOC was analyzed with the Standard Methods 5310 B. High-Temperature Combustion Method.[67]

At the Oregon State Lab, samples were pre-filtered with a 1.0-1.2 µm glass microfiber pre-filter (Gelman A/E 61631 and Whatman GF/C Cat. No. 1822-042/047) if necessary, then filtered with a 0.45 µm polyethersulfone filter (PALL Supor-450 P/N 60173 or Quick Filter Mod. No. QF045) and subsequently stored at 4°C until analysis.

Effluent discharge flow rates were provided by P&P mill personnel for the day of sampling, while river flow data was obtained from the United States Geological Survey (USGS) National Water Information System[68]. The total upstream and downstream flows (*i.e.*, the sum of multiple tributary flows) as well as the reported effluent flow rates are tabulated in a later section.
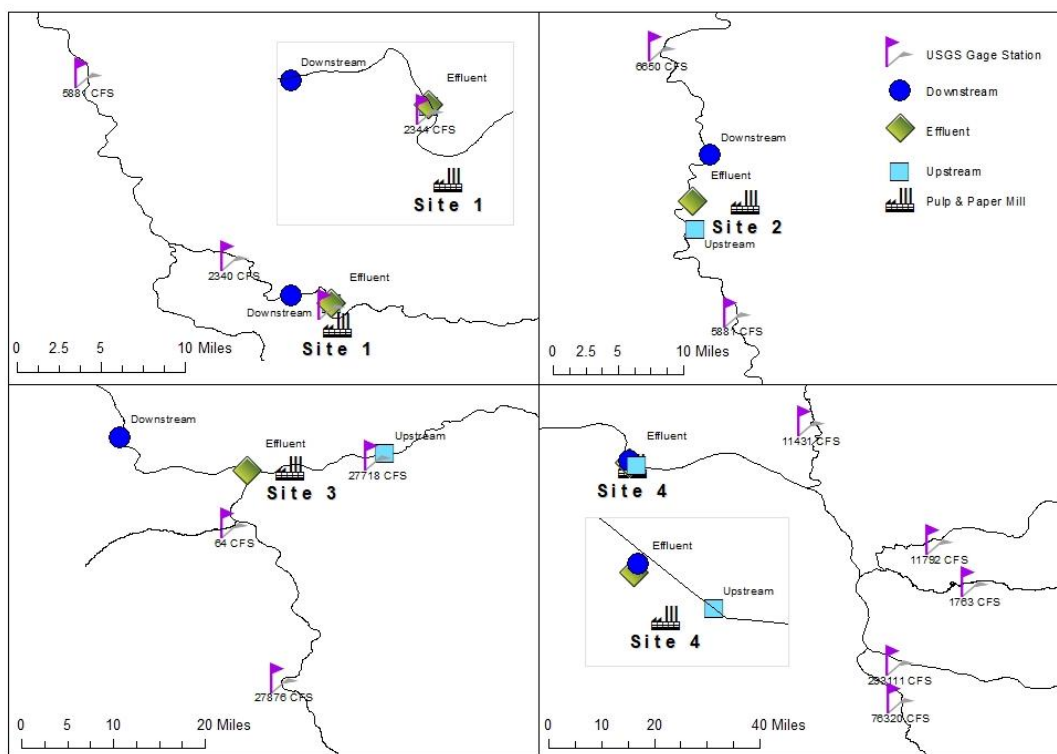
Figure 1: Schematic showing relative locations of upstream, effluent, and downstream samples for four Pulp and Paper Mills in the United States. Discharge (cfs) from effluent plants was provided by mill personnel and river flow data was obtained from the USGS National Water Information System database.

3.2. Specific Ultraviolet Absorbance & % Aromaticity

$SUVA_{254}$ was measured using an Orion Aquamate 8000 UV-VIS Spectrophotometer following

procedures outlined in EPA Method 415.3 and Standard Methods 5910 [67,69] and as used by other

researchers.[70,71] Briefly, absorbance was measured in triplicate and blank corrected using a 1-cm

quartz cuvette and a 200-700 nm scan at 1 nm increments.[72] Effluent samples were diluted to

DOC concentrations similar to the upstream/downstream samples so as to eliminate potential

inner-filter effects. In some instances, UV absorption was not measured within the method-

recommended 48 hour holding time, but samples had been filtered and were stored in a dark

refrigerator until analysis.

3.3. Calibration of CuISE

This research required the accurate detection of copper activity as low as 10^{-11} and therefore

required utilization of polishing and conditioning procedures designed to enhance CuISE

sensitivity at these concentrations. While some researchers and manufacturers have stated the

limitations of using CuISEs below copper concentrations of 10^{-6} $M^{[73]}$ and at environmentally

relevant copper/DOC ratios[74–76], other researchers reported a CuISE provided a linear Nernstian

response between 10^{-3} and 10^{-19} M Cu^{2+} when calibrated with ethylenediamine and pH titration at

25° C. Therefore, ionic copper was measured directly using a Copper Ion Selective Electrode

(CuISE) (Orion 9629BNWP) and calibrated using procedures similar to those outlined by

previous researchers.[48,77–79] A flowchart of this procedure is included in the appendix. pH

(Accumet 13-620-183A) and conductivity (Accumet SN 2253364) probes were calibrated daily

with pH 7 and pH 4 buffers and 0.01 M KCl, respectively. A pH calibration was accepted only if

the % slope was greater than 95%, (see appendix for details). Similar to other researchers[75,77,80], the CuISE was conditioned daily by 1) polishing the sensing surface with DDI water and polishing strips (Orion 948201) for 45 seconds, 2) filling the probe with Optimum Result D filling solution (Orion 900063), and 3) sequentially soaking the sensing surface in solutions of 10^{-8} M Cu^{2+}, 0.025 M H_2SO_4 (diluted Fisher Scientific SA218-1), and DDI for a minimum of 10 minutes each.

A CuISE calibration solution was prepared daily consisting of 0.1 M copper standard (Orion ionplus 942906), ethylenediamine (EN) (Sigma Aldrich ReagentPlus >99%, Cat. No. E26266), Low Ionic-Strength Adjuster (Low-ISA - 1:5 diluted Orion 94001), 70% trace-grade nitric acid (BDH Aristar-Plus Cat. No. 87003-259), and DDI water. A typical solution consisted of 180 µL copper standard, 1.4 mL Low-ISA, 86 µL 70% nitric acid, 55 µL EN, and DDI water to a total volume of 150 mL. The probe was then calibrated by adjusting the pH from approximately 6.8 to 4.8 through the addition of 10% trace-grade nitric acid and 0.1 M Sodium hydroxide (TraceSELECT Cat. No.01968-25G-F).

Visual MINTEQ was used to determine the theoretical copper activity with input variables of ion concentration, pH, and temperature, and subsequently recorded by an AccumetAR50 multi-meter. Five paired data points (mV, $\{Cu^{2+}\}$) were collected throughout the titration and a calibration curve of mV vs. $\log\{Cu^{2+}\}$ was generated. The calibration curve was accepted if the response was sufficiently close to the theoretical Nernstian response of -29.6 mV/pCu^{2+} at 25°C.[73] The average calibration curve (n = 28) was mV = (-29.23 ± 0.23) p$\{Cu^{2+}\}$ + (143.93 ± 1.56) with an average (n = 27) final conductivity of 2.69 ± 0.06 mS/cm (See appendix for calibration curve). Copper activities were read directly from the multi-meter in "ion mode" or by

converting mV to activity using the calibration curve (both values were recorded for comparison). Conductivity was measured at the end of the calibration.

3.4. Copper Titration with CuISE

Copper titrations were performed on 0.45 µm filtered samples using titration procedures similar to those described by others.[48,80–82] A flowchart of this procedure is included in the appendix. Briefly, 100 mL of each sample was measured in a 100 mL grade-A volumetric flask and transferred to a borosilicate jacketed beaker. For wastewater effluent samples, 5 mL or 10 mL aliquots were diluted to 100 mL (10x and 20x dilution) to obtain DOC concentrations similar to upstream and downstream samples. The samples were purged with N_2 for a minimum of 30 minutes before the first copper addition to ensure elimination of carbonate species. The pH and temperature were kept constant at 6.00 ± 0.05 and $25.0°C \pm 0.1$ through the addition of 0.1 M NaOH and 10% HNO_3 and by circulating water through the jacketed beaker. The CuISE sensing surface was conditioned following calibration and subsequent titrations by sequentially soaking in 0.025 M H_2SO_4 and DDI for a minimum of 10 minutes each. To ensure the titration was performed at a similar ionic strength as the calibration, the specific conductivity (used as a proxy for ionic strength) was adjusted with Low-ISA to be similar to the final conductivity of the calibration solution. Therefore, ionic strength varied slightly between titrations but was consistently calculated in the range of 0.02 and 0.024 M. Titrations were performed through incremental addition of 10^{-3} and 10^{-4} M Cu^{2+} solutions (diluted 0.1 M Orion 942906) until a minimum of 15 data points ($\{Cu^{2+}\}$, pH, mV) were measured. At least 5 minutes was allowed

between recording data points and the CuISE was allowed to stabilize to ± 0.1 mV/min to ensure equilibrium.

3.5. Determining Copper Binding-Affinity and Ligand Site Density

Experimental copper activities measured via CuISE were used to perform a mass balance and determine the amount of copper bound to organic ligands. Copper activity was converted to copper concentration using the ionic strength of major ions in solution and a Davies activity coefficient. Inorganic copper complexes (hydroxide, sulfate, chloride, and nitrate) were estimated using stability constants from MINTEQ. Carbonate complexes were assumed to be negligible since the titrations were performed with constant N_2 purging. Once the quantity of CuL_T was determined, a 2-ligand mathematical model was used to fit the equilibrium distribution of copper bound to organic matter. The model assumes two, mono-dentate ligands with conditional stability constants and is similar to those used by other researchers for determining NOM binding with zinc[58] and other metals.[83,84] In the present research, two sites were used and the amount of copper bound to ligands was normalized to the amount of DOC present in the sample:

$$\frac{[CuL_T]}{DOC} = \frac{[CuL_{1,T}] + [CuL_{2,T}]}{DOC} = \frac{K_1^C[L_{1,T}]\{Cu^{2+}\}}{1 + K_1^C\{Cu^{2+}\}} + \frac{K_2^C[L_{2,T}]\{Cu^{2+}\}}{1 + K_2^C\{Cu^{2+}\}} \tag{7}$$

Where $[CuL_T]$/DOC has units of mol/mg-C. The data was log transformed prior to fitting with Microsoft Excel's Solver tool to ensure equal weighting of data points over several orders of magnitude copper activity. The experimental data and model results were plotted and visually checked to ensure Solver produced a good fit. In addition, data points were evaluated on a Scatchard plot to ensure they were consistent with the hyperbolic shape expected for copper-

DOM titrations. Plots showing the results of the data fitting procedure and Scatchard analysis are included in the appendix.

The calibration/titration method had previously been tested by another researcher by subsequently determining copper-binding parameters in Suwannee River Fulvic Acid.[48] The results indicated the calibration/titration method produced binding parameters that were comparable to other researchers' work.[52,60]

3.6. Analytical Window and Data Treatment

The titration data used for the determination of model parameters was restricted to a CuT/DOC range of 0.002 to 0.11 (mg/mg) since other researchers[48,74] have indicated the CuT/DOC window under which data is analyzed can impact the resulting metal-binding parameters. This CuT/DOC cutoff range was established by comparing plots of CuT/DOC (included in appendix) across samples and was designed to include as much data from each titration as possible, minimize procedural error, and allow inter-site comparison of binding parameters. Therefore, titration data that was outside this range were not included in the model-fitting procedure. Individual titrations often terminated within the CuT/DOC window but did not span the entire range. In this case, the highest/lowest data points were within a factor of 2 from the cutoff. In addition, data points that did not follow normal Scatchard plot-behavior after correction for copper contamination (discussed below) were not included in the model-fitting procedure. A plot of the Scatchard plot analysis is included in the appendix.

3.7. Sources of Error and Interferents

At concentrations below 10^{-5} M, copper contamination or adsorption to beaker walls becomes significant when using a CuISE.[73] This source of error was minimized to the extent possible through a rigorous washing procedure where glassware was sequentially washed with Alconox soap, triple rinsed with 18MΩ DDI water, soaked in 10% HNO_3 for a minimum of 4 hours, triple rinsed with DDI, soaked in DDI for a minimum of 30 minutes, and finally triple rinsed with DDI and allowed to dry.

Chloride and bromide ions can cause the CuISE to malfunction,[73] but they begin to interfere only at certain concentrations of copper and chloride. For copper ion concentrations less than 10^{-6} M, the chloride concentration would need to be greater than 1.0 M to interfere. While bromide was not measured, the highest concentration of chloride was 3.3E-04 M, occurring in the diluted Site 3 effluent sample. Therefore, chloride concentrations were not large enough to cause interference.

Silver or mercury concentrations greater than 10^{-7} M and ferric iron concentrations greater than 1/10 the concentration of copper ions can interfere with the electrode's performance.[73] In the event of "poisoning" of the electrode, the manufacturer recommends standard polishing procedures described in the user manual. Although the concentrations of these metals were not measured, the conditioning procedure described in previous sections was repeated daily and should minimize electrode poisoning.

Iron, nitrate, nitrite, and bromide have been identified as interferents[63,69] in $SUVA_{254}$ measurements and therefore could be applicable in this research. It is expected that iron and nitrate would be found in relatively low concentrations in the river water samples, but the same

could not be assumed for P&P effluent. Therefore, effluent samples were diluted 10x and 20x prior to titrations and $SUVA_{254}$ analysis, thereby partially eliminating major interference from these ions. In addition, $SUVA_{254}$ measurements were taken at a pH range between 7.2 and 8.1 where little pH interference is expected.[63]

Due to miscommunications with 3rd party laboratory personnel regarding the proper analytical methods, several titrations at Sites 1-2 were performed before the correct copper and DOC concentrations were known and the data points were not logarithmically spaced as precisely along the CuT/DOC ratio range as were Sites 3-4 (see appendix). Therefore, additional titrations were performed at Sites 1-2 to "fill in" data gaps along the CuT/DOC spectrum. As previously mentioned, data analysis was therefore constricted to a CuT/DOC ratio of 0.002 to 0.11 mg/mg since the ratio is known to influence the calculated binding parameters.[85] The analytical window reduced procedural differences within titrations and across sites, but there remained variable data coverage at Sites 1-2 near the lower CuT/DOC cutoff range, potentially introducing error in the final model-fitted parameters. In contrast, Sites 3-4 had excellent data coverage and consistency.

3.8. Biotic Ligand Model and Visual MINTEQ Modeling

Using experimental conditions and background water quality data, the BLM was compared to a Visual MINTEQ[86] speciation model that incorporated laboratory-measured metal-binding characteristics. The models were compared over the total copper range of 0.1 to 180 µg/L. Both MINTEQ and BLM model runs were performed on effluent samples for two situations; 1) with diluted background water quality and DOC concentrations consistent with the titration

procedure, and 2) undiluted, "full strength" model runs that represent effluent wastewater before it mixes with the river.

3.8.1. Visual MINTEQ Speciation Modeling

Visual MINTEQ Model (v3.1) was used in conjunction with laboratory-measured stability constants and ligand concentrations to evaluate the BLM predictions. Water quality data for each sample (effluent dilution factors considered) were entered with the same environmental conditions under which the stability constants/ligand concentrations were determined: alkalinity = 0, temperature = 25.0 °C, pH = fixed at 6.00, and partial pressure of CO_2 = 1E-10 of atmospheric CO_2 (0.0038 atm). L_1 and L_2 (mmol/g-C) were converted to mmol/L using the concentration of DOC for each site and their chemical species added to the comp_2008.vba database with charges equivalent to −1. CuL_1 and CuL_2 were added in the aqueous component database, with a charge of +1, the atomic weight equivalent to that of copper, and values of "0" for Debye-Huckel "a" and "b" parameters. dHr was set to 0, since all titrations and models were performed at 25 °C and pH fixed at 6.00. For each model run, the specific ion concentrations were added to Visual MINTEQ and the comp_2008.vba database was updated with the LogK parameters associated with CuL1 and CuL2. The water quality data did not include the LowISA added during titrations since it was previously determined to be insignificant.[48]

3.8.2. BLM Speciation Modeling

The BLM (v3.16.2.41)[1] modeling was performed in freshwater and speciation mode with temperature = 25.0 °C, pH = fixed at 6.00, and the "*set inorganic carbon*" parameter set

to the "*closed system, input alkalinity.*" Alkalinity and sulfide values were set to the

recommended 1E-10 mg/L since titration parameters were determined with constant

purging of N_2 and sulfide information was not available for all sites. The DOC was

assumed to have a humic acid fraction of 10% as recommended in the user manual.

4. Results and Discussion

4.1. Water Quality Data

Water quality data for the three water samples at each site are reported in **Table 1** and indicate

P&P wastewater effluent had relatively high concentrations of BLM constituents relative to the

upstream and downstream samples. Chloride, sulfate, sodium, and DOC were in particularly

high concentrations, with the Site 3 effluent reaching 92 mg/L DOC. Effluent $SUVA_{254}$

measurements were also greater than the corresponding upstream and downstream samples and

will be discussed more precisely in a later section. Copper concentrations were relatively similar,

with the highest sample reaching 3.37 µg/L at the Site 2 effluent. pH was consistently between

7.3 to 8.1 and was consistent the expected range of natural waters.

Table 1: Water quality data for upstream, effluent, and downstream locations at four P&P mills in the United States.

Site Number	Date Collected	Position	Field pH	Chloride (mg/L)	Sulfate (mg/L)	Calcium (mg/L)	Magnesium (mg/L)	Potassium (mg/L)	Sodium (mg/L)	Diss. Copper (ug/L)	Copper Total (ug/L)	DOC (mg/L)	Alkalinity (as CaCO$_3$)	SUVA$_{254}$
Site 1	10/2/2017	Upstream	7.62	1.4	1.1	4.41	1.86	0.851	4.16	0.12	-	0.54	27	3.15
Site 1	10/2/2017	Effluent	7.3	5.9	565	64.3	2.88	17.9	228	1.35	2.22	7.23	97	5.26
Site 1	10/2/2017	Downstream	7.7	1.4	5.4	4.87	1.89	1.02	5.92	0.16	-	0.56	28	3.63
Site 2	10/2/2017	Upstream	7.55	1.5	2.8	5.27	1.83	0.836	4.44	0.26	0.27	0.86	28	3.60
Site 2	10/2/2017	Effluent	7.8	95.8	217	25.2	3.73	14.2	245	3.37	3.85	35.62	234	6.10
Site 2	10/2/2017	Downstream	7.56	2.0	3.3	5.38	1.9	0.916	5.32	0.36	-	1.11	28	3.57
Site 3	2/22/2018	Upstream	-	1.18	1.78	5.34	1.39	0.734	2.57	0.37	0.54	1.71	-	3.45
Site 3	2/22/2018	Effluent	7.55	234.0	419	44.7	3.79	16.5	384	0.98	1.51	92.23	-	4.32
Site 3	2/22/2018	Downstream	-	7.80	18.1	19	5.95	1.65	9.98	0.41	0.55	1.70	-	3.08
Site 4	4/11/2018	Upstream	8.12	3.72	8.81	12.7	4.16	1.07	5.56	0.59	1.05	2.4	50.3	1.89
Site 4	4/11/2018	Effluent	7.61	68.7	130	17.9	3.95	8.97	166	0.57	1.36	53.9	217	5.55
Site 4	4/11/2018	Downstream	8.13	3.40	8.22	11.7	3.75	1.0	5.06	0.61	1.16	1.8	49.5	2.59

4.2. Quantifying Wastewater Dilution and the Assumption of a Closed System

Before making statistical comparisons between upstream and downstream sites, an evaluation of wastewater dilution was performed to provide context to measured differences in DOM characteristics (SUVA$_{254}$, K_i, L_i), since significant differences may not be detected because of intense dilution or because the upstream-downstream sequence was not a closed system. As shown in **Table 2**, flow data were used with SUVA$_{254}$ and DOC measurements from to determine the relative volumetric, Ultraviolet Absorbance (UVA), and DOC contributions of P&P effluent at the mixing point. In addition, the expected downstream UVA and DOC values (assuming the downstream is a combination of upstream and effluent) were compared to the actual downstream values to determine if mass/absorbance was conserved.

In general, effluent discharges accounted for less than 1% the total volumetric flow in the river, indicating the samples were intensely diluted. However, except for Site 4, effluent wastewater contributed relatively modest amounts (3 to 17%) of DOC and UVA at the mixing point. Therefore, despite intense volumetric dilution, there was a measurable contribution of organic material. The difference between the expected downstream UVA (due to effluent + upstream mixing) versus the measured value was within ± 20%, although the 2% value of Site 4 was dominated by the large upstream flow and would therefore produce a similar result even with more extreme effluent DOC or SUVA values. Site 3 was unique, since the effluent sample occurred at the confluence of two rivers, only one of which was sampled for upstream DOC. Therefore, the second tributary's average DOC concentration was estimated to be 2.7 mg/L based on two years of historical data provided by the NCASI research team. SUVA was not measured for this tributary and therefore the UVA analysis was ignored for Site 3. This

estimation could explain the negative conservation of DOC downstream. Finally, DOC was generally "lost" between the upstream and downstream sites.

While in relatively good agreement for complex river systems, the values indicate there was generally a measurable contribution of P&P DOM to each river system but there may be additional inputs or unaccounted flows along the upstream-downstream sequence. Therefore, statistical observations made for rivers with variable uncertainty should be assessed with additional scrutiny.

Table 2: Cumulative flow rates for upstream, effluent, and downstream sites with differences between the expected downstream UVA and DOC based on dilution of wastewater effluent and the actual measured value. The value of % effluent at mixing point indicates the relative fraction of effluent flow, UVA, and DOC to the total loading at the point of mixing. The underlined flow rate was divided by two, since the P&P mill was on one side of an island-divided river. Bolded points indicate the downstream flow is the sum of the upstream and effluent flows, since some sites did not have a downstream gage. Two years of historical data was used to estimate the DOC concentration of the unmeasured Site 3 tributary.

| Site	Flow Data				% Effluent at Mixing Point			Actual vs. Expected Downstream	
	Q_{up} (cfs)	Q_{eff} (cfs)	Q_{down}(cfs)**	Flow $Q_{eff}/(Q_{eff}+Q_{up})$	UVA $UVA_{eff}/(UVA_{eff}+UVA_{up})$	DOC $DOC_{eff}/(DOC_{eff}+DOC_{up})$		UVA % Difference	DOC % Difference
Site 1	2344	18.1	2340	0.77%	15%	9%		2%	-7%
Site 2	5881	17.8	6650	0.30%	17%	11%		16%	23%
Site 3	55658	40.1	**55698**	0.07%	NA	3%		NA	-33%
Site 4	197209	21.7	**197230**	0.01%	1%	0%		2%	-34%

4.3. Specific Ultraviolet Absorbance (SUVA$_{254}$) and %Aromaticity

4.3.1. Intra-Site Comparison of SUVA$_{254}$

SUVA$_{254}$ results, shown in **Figure 2**, indicate wastewater effluent samples had consistently higher SUVA$_{254}$ (detailed statistics below) than both upstream and downstream samples. Since %aromaticity is positively correlated with SUVA$_{254}$[63], it naturally follows that effluent samples had higher %aromaticity than both upstream and downstream samples. This is consistent with observations by Shi *et. al.* who reported higher SUVA$_{254}$ values in P&P mill secondary effluent before coagulation treatment where hydrophobic humic species were the dominant fraction.[36] Even after coagulation treatment, the P&P effluent had significant amounts of hydrophobic acids and hydrophilic neutrals that exhibited complex fluorescent behavior as measured by excitation-emission fluorescence.[36]

Statistical tests, shown in **Table 3**, were used to determine if effluent-DOM SUVA$_{254}$ was different than the receiving waters and if the difference was observable in the mixed downstream sample. Thus, the relevant questions can be summarized as:

1) Is the effluent parameter different than the upstream parameter? (two-tailed heteroscedastic t-test, H$_o$: $\mu_{eff} = \mu_{up}$; H$_{alt}$ $\mu_{eff} \neq \mu_{up}$)

2) If yes, was the effluent parameter > upstream parameter? (one-tailed heteroscedastic t-test, H$_o$: $\mu_{eff} = \mu_{up}$; H$_{alt}$ $\mu_{eff} > \mu_{up}$)

3) If yes, was the downstream parameter > upstream parameter? (one-tailed heteroscedastic t-test, H$_o$: $\mu_{down} = \mu_{up}$; H$_{alt}$ $\mu_{down} > \mu_{up}$)
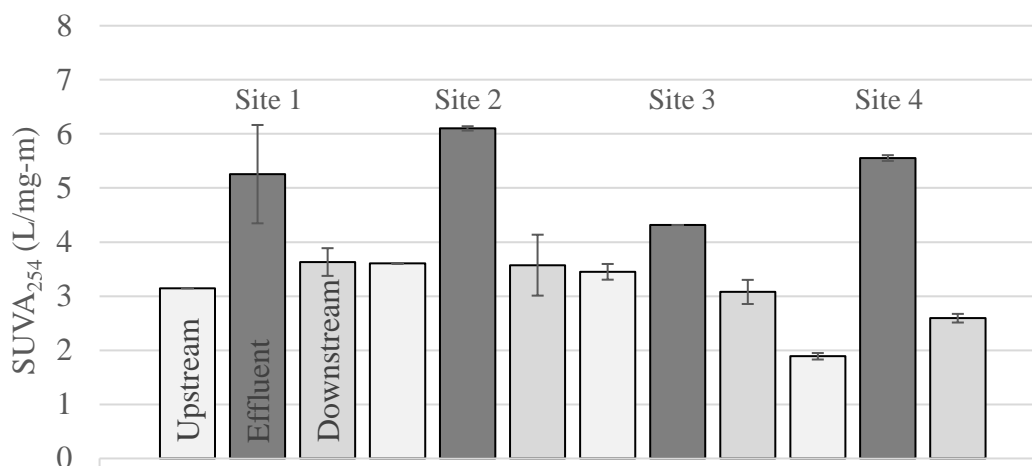
Figure 2: SUVA results for Sites 1-4. Data are presented as Upstream, Effluent, and Downstream for each site as shown in Site 1. Error bars represent the 95% t-distribution confidence interval.

All tests were performed with $\alpha = 0.05$ and with the assumption that the sample mean variances were not equivalent. For example, $\sigma_{eff}^2 \neq \sigma_{up}^2$. In the event the effluent parameter was less than the upstream parameter, the p-value was reported for the test in reverse.

For all four sites, the effluent $SUVA_{254}$ was significantly greater than the upstream sample ($\alpha = 0.05$) (**Table 3**). However, for only two of the four sites was the difference observed in the mixed downstream sample (Sites 1 and 4). The significance of the Site 4 downstream test was unexpected given that effluent DOM accounts for less than 1% the total DOC/UVA in the river.

The significant upstream-downstream finding of Site 1 is supported by the dilution data, since the effluent discharge contributed modest amounts of UVA and DOC (15% and 9%), the largest fraction of volumetric flow (0.77%), and the expected/measured downstream values were in good agreement. As previously discussed,

relatively large differences between the expected/measured downstream DOC/UVA values at Sites 2-3 indicate $SUVA_{254}$ differences may not have been detectable. In fact, the Site 3 upstream-$SUVA_{254}$ was greater than the downstream value, indicating there is significant uncertainty that all variables were accounted for in the upstream-downstream sequence.

In summary, $SUVA_{254}$ of effluent DOM was significantly greater than DOM in receiving waters, the difference was detected downstream in 2-out-of-4 samples, and the results at the remaining sites could be explained by a failure to control all variables in the upstream-downstream sequence.

Table 3: Results of statistical tests between upstream, effluent, and downstream samples with corresponding p-values. For the 3rd question of Sites 2-3, a negative t-statistic indicated the one-tailed test was in the opposite direction, and therefore the p-value is the significance in the opposite direction (i.e., is Up > Down?).

Was Effluent Different than Upstream? (two-tailed t-test, $\alpha = 0.05$, H_o: $\mu_{eff} = \mu_{up}$; H_{alt} $\mu_{eff} \neq \mu_{up}$)			
Parameter	Site 1	Site 2	Site 3	Site 4
$SUVA_{254}$	Yes (P = 0.0099)	Yes (P = 1.40E-05)	Yes (P = 0.00152)	Yes (P = 3.98E-09)
If Yes, was Effluent > Upstream? (one-tailed t-test, $\alpha = 0.05$, H_o: $\mu_{eff} = \mu_{up}$; H_{alt} $\mu_{eff} > \mu_{up}$)			
Parameter	Site 1	Site 2	Site 3	Site 4
$SUVA_{254}$	Yes (P = 0.00495)	Yes (P = 7.02E-06)	Yes (P = 7.60E-04)	Yes (P = 1.99E-09)
If Yes, was Downstream > Upstream? (one-tailed t-test, $\alpha = 0.05$, H_o: $\mu_{down} = \mu_{up}$; H_{alt} $\mu_{down} > \mu_{up}$)			
Parameter	Site 1	Site 2	Site 3	Site 4
$SUVA_{254}$	Yes (P = 0.00743)	No (Up > Down; P = 0.417)	No (Up > Down; P = 0.00462)	Yes (P = 3.49E-06)

4.3.2. Comparison of Effluent SUVA$_{254}$ Across P&P Mill Sites

A Single Factor Analysis of Variance (ANOVA) test was performed to compare the average SUVA$_{254}$ values from P&P mill effluents. This test essentially determines whether the effluent parameters were from the same population of DOM. A significant result suggests the parameter from at least one sample site is from a different population than the other sites. The results indicate that at least one P&P mill SUVA$_{254}$ measurement was significantly different than the others (P = 1.602E-06). To determine which P&P mills were significantly different than the rest, a post hoc Tukey test was performed with a simultaneous 95% confidence interval. The results indicated that Sites 1 and 4 were from the same group, but that Sites 2 and 3 were significantly different from Sites 1 and 4 and from one another. Therefore, P&P effluent DOM has significantly different optical properties when compared to the receiving waters and when compared to other P&P mill effluents. This suggests P&P DOM has variable aromaticity and consequently different metal-binding properties. The optical behavior is consistent with high color, allochthonous sources rich in lignin whose degradation byproducts result in aromatic fulvic acids.[21,66] In general, researchers should not assume P&P effluent DOM from different sites has similar optical characteristics or aromatic content.

4.4. Copper-NOM Binding Parameters

4.4.1. Copper Contamination

Contradicting the typical hyperbolic Scatchard plot associated with metal-binding experiments, concave-downward behavior was regularly observed during the course of

titrations and was attributed to copper contamination resulting in an inaccurate mass balance. Therefore, a series of contamination experiments and additional titrations were performed to quantify the extent of contamination that can be expected in any given titration. ICP-MS was used to quantify copper contamination in a "blank" DDI sample exposed to the same experimental procedures and again during titrations before the first addition of copper. After performing the regular calibration procedure and preparing a sample for titration, 5-mL aliquots were removed for copper analysis by ICP-MS (Thermo Elemental X-series II Quadrupole) and preserved with 70% trace grade HNO_3 (BDH Aristar-Plus Cat. #87003-259). The results are tabulated in the SI and indicated an average copper contamination of 0.21 ± 0.08 µg (n = 14). The average mass of copper contamination was subsequently added to the background copper of all previous titrations. A graphical representation of the Scatchard plot behavior before and after correction with contamination data is shown in the SI.

4.4.2. Intra-Site Comparison of Copper-NOM Binding Parameters

A 2-site binding model was used to fit experimental copper activity measurements and subsequently determine DOM-copper affinity (K_i) and ligand site densities ($L_{i,T}$) for the strong and weak ligands in each sample (**Figure 3**). After the copper-ligand binding parameters were determined, a series of statistical tests were performed to determine if binding parameters measured in P&P effluent DOM were significantly different than the binding parameters measured in the receiving water. The tests followed the same

procedure as described in the $SUVA_{254}$ analysis. The results are shown in **Table 4** with the corresponding p-values.

The measurement variability of each parameter ($LogK_2$, L_1, and L_2) in the upstream and downstream samples reduce the power of the statistical tests. However, Sites 3-4 had less variability across all parameters. In addition, the effluent parameters at all four sites had notably less variability than the receiving water samples.

<u>Site 1</u>: There was no significant difference between the copper-binding parameters of the effluent DOM and upstream DOM. However, the measurement variability in the upstream sample's $LogK_2$ and L_1 parameters were relatively large.

<u>Sites 2 & 3</u>: The strong ligand site-density (L_2) of the effluent DOM was significantly greater than the density of strong ligands in the upstream DOM. However, no difference was observed between the density of the strong ligands in the upstream and downstream samples. This could indicate the P&P effluent discharge did impact downstream copper binding or that the impact was undetectable as the difference between the measured and expected downstream DOC concentration was -33%, indicating a significant portion of DOC was unaccounted for.

<u>Site 4</u>: Three of the Site 4 effluent parameters ($LogK_1$, $LogK_2$, and L_2) were significantly greater than the upstream parameters, but the difference was observed in the mixed downstream sample for only two parameters: the strong and weak ligand binding-affinities ($LogK_1$ and $LogK_2$.). This finding tracks the $SUVA_{254}$ results but was unexpected due to the amount of dilution and the fact that the relative contribution of effluent DOC to the total carbon load in the river was essentially 0%.
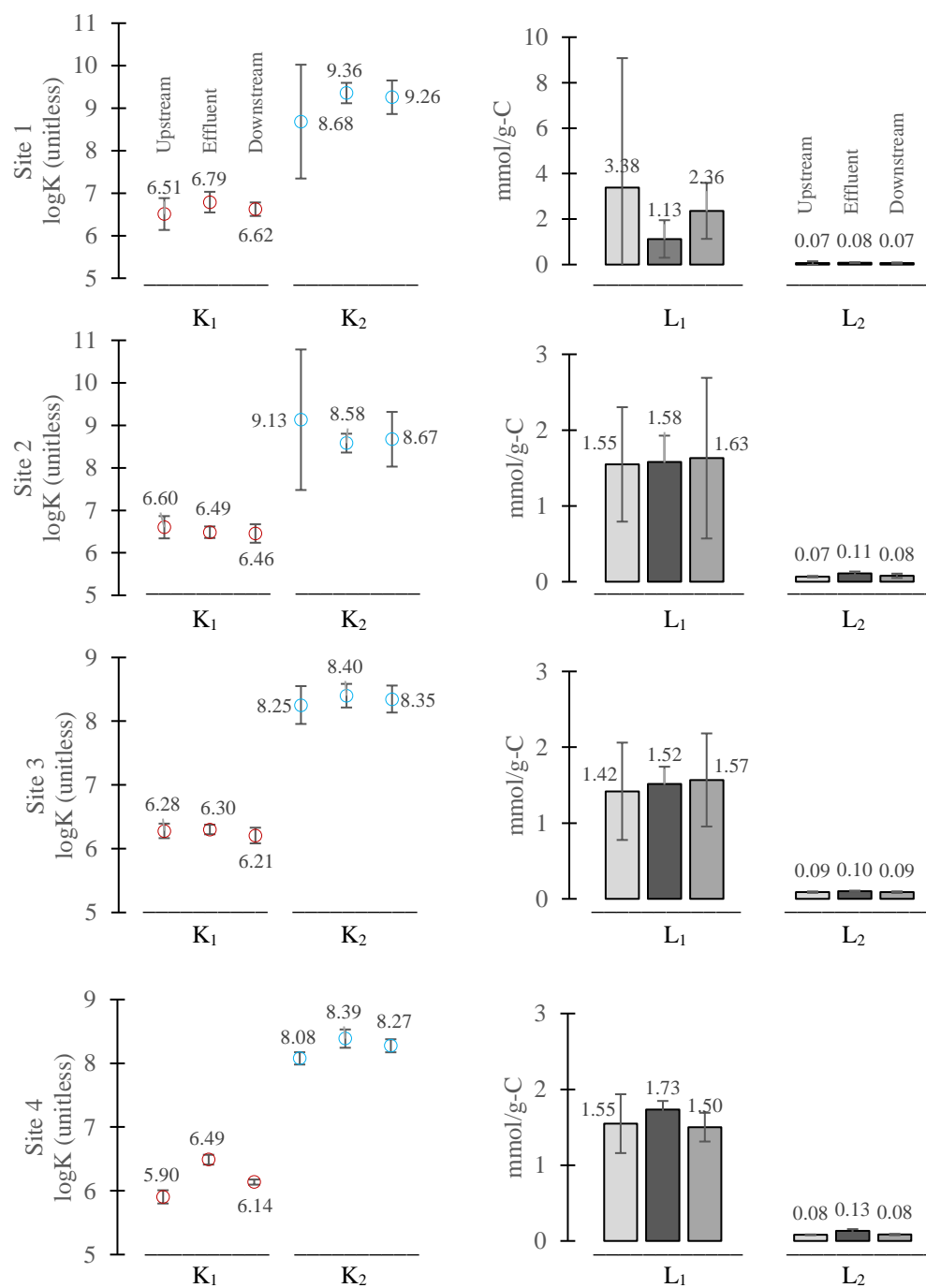
Figure 3: Copper-binding affinity and normalized ligand site-densities for Sites 1-4. Data are reported from left to right as upstream, effluent, and downstream. Error bars represent the 95% confidence interval as determined using a t-distribution.

In summary, the high-affinity site-density parameter (L_2) in effluent DOM was significantly greater than L_2 in upstream DOM in 3-out-of-4 sites. However, the binding-affinity and site-density differences were not observed at the downstream site except for the case of the binding-affinities of Site 4. The L_2 parameter is typically associated with phenolic functional groups[12], and as previously discussed, P&P DOM is rich in phenolic tannins and lignin. Therefore, although these experiments did not directly test the relationship of the L_2 parameter to a specific compound, the findings are consistent with the chemical makeup of P&P effluent DOM. The effect of statistically different binding parameters on the total amount of bound copper is especially noticeable when comparing Site 3 (1 significant parameter) and Site 4 (3 significant parameters) and is shown in **Figure 4**. While the aggregate and fractional copper binding behavior of upstream and effluent DOM is similar at Site 3, the DOM in the Site 4 effluent exhibited more copper binding and different ligand-partitioning than the upstream sample.

Table 4: Statistical test results for copper-binding parameters. The tests are sequential, where the second and third tests were performed only if the previous result was significant (labeled as "Yes"). If the t-statistic was negative in a one-tailed test, the reported p-value is for the question in reverse. For example, for the 3rd test of Site 3, the reported p-value tests Up > Down.

Was Effluent Different than Upstream? (two-tailed heteroscedastic t-test, $\alpha = 0.05$, H_o: $\mu_{eff} = \mu_{up}$; H_{alt} $\mu_{eff} \neq \mu_{up}$)				
Parameter	Site 1	Site 2	Site 3	Site 4
$LogK_1$	No (P = 0.0727)	No (P = 0.191)	No (P = 0.635)	Yes (P = 7.11E-06)
$LogK_2$	No (P = 0.171)	No (P = 0.294)	No (P = 0.240)	Yes (P = 0.00230)
L_1	No (P = 0.237)	No (P = 0.887)	No (P = 0.669)	No (P = 0.221)
L_2	No (P = 0.476)	Yes (P = 0.0139)	Yes (P = 0.0185)	Yes (P = .00846)
If Yes, then was Effluent > Upstream? (one-tailed heteroscedastic t-test, $\alpha = 0.05$, H_o: $\mu_{eff} = \mu_{up}$; H_{alt} $\mu_{eff} > \mu_{up}$)				
Parameter	Site 1	Site 2	Site 3	Site 4
$LogK_1$	-	-	-	Yes (P = 3.55E-06)
$LogK_2$	-	-	-	Yes (P = 0.00115)
L_1	-	-	-	-
L_2	-	Yes (P = .00694)	Yes (P = 0.00927)	Yes (P = 0.00423)
If Yes, then was Downstream > Upstream? (one-tailed heteroscedastic t-test, $\alpha = 0.05$, H_o: $\mu_{down} = \mu_{up}$; H_{alt} $\mu_{down} > \mu_{up}$)				
Parameter	Site 1	Site 2	Site 3	Site 4
$LogK_1$	-	-	-	Yes (P = 0.00134)
$LogK_2$	-	-	-	Yes (P = 0.00209)
L_1	-	-	-	-
L_2	-	No (P = 0.165)	No (Up > Down; P = 0.448)	No (P = 0.218)
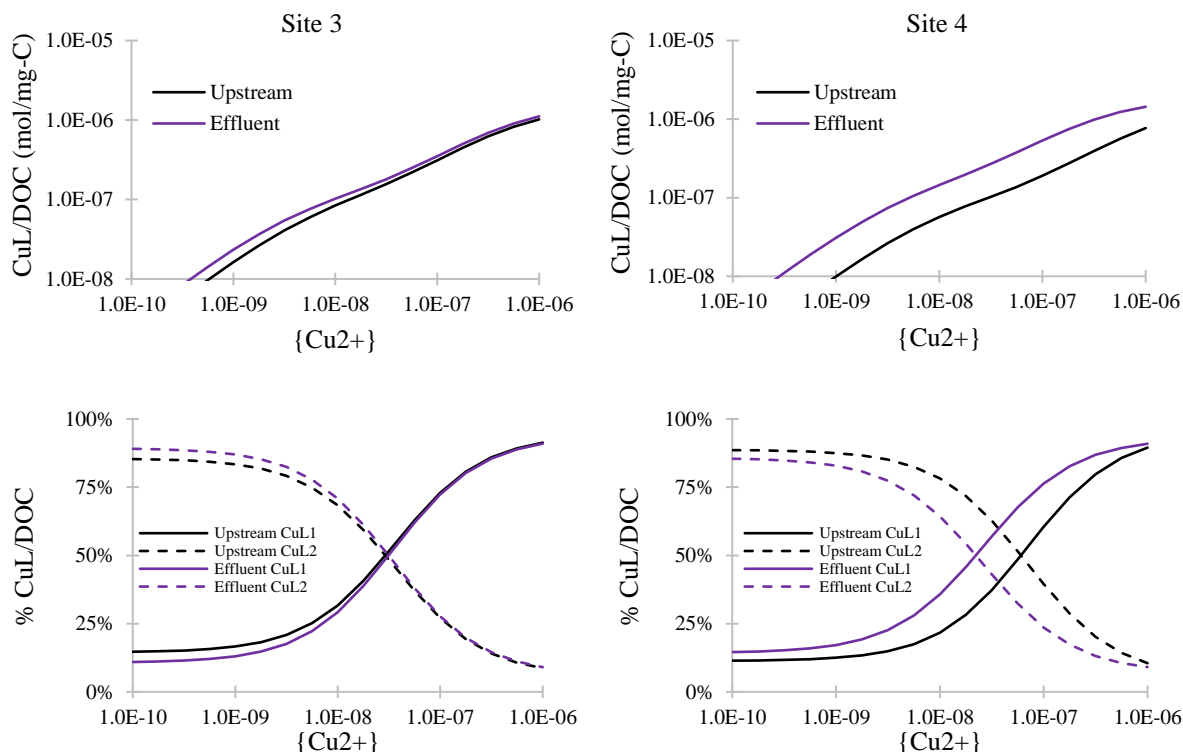
Figure 4: The upper plots show the amount of copper bound to organic ligands per mg of carbon (CuL/DOC) for upstream and effluent samples at Sites 3 and 4. The lower plots show the relative contribution of copper binding attributed to weak/strong ligand sites.

4.4.3. Comparison of P&P Effluent Copper-NOM Binding Parameters

Consistency in the P&P effluent parameters (**Figure 5**) allowed for a statistical test to determine whether P&P effluent DOC was significantly different among mills/plants with respect to copper binding parameters. As with SUVA$_{254}$ comparisons for P&P effluent DOM, a Single Factor Analysis of Variance (ANOVA) test was performed ($\alpha = 0.05$) and is reported in **Table 5**. A follow-up Tukey test was also performed with a simultaneous 95% confidence interval. The results indicate that the parameters LogK_1, LogK_2, and L_2 were significantly different between Sites 1-4. However, the test failed to reject the

hypothesis that the L_1 parameter was the same among sites. The post hoc Tukey test indicated Site 1 was responsible for the significant difference in K_1 and K_2 parameters and that based on the L_2 parameter, the sites could be divided into two groups (Site 4 vs. Site 1 and 3), with Site 2 a member of both.
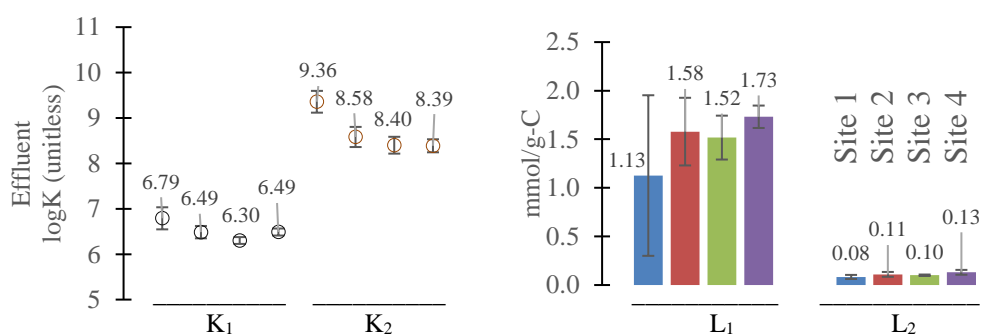
Figure 5: Ligand site density and copper-binding affinity parameters for four P&P effluent discharges. Each parameter is displayed by site from left to right: Site 1, Site 2, Site 3, and Site 4.

These findings suggest P&P effluent DOM has unique copper-binding characteristics among different mill sites, and therefore will bind varying amounts of copper. Taken with the SUVA$_{254}$ results, the site density of phenolic groups in different P&P effluents is likely unique. In addition, it suggests the weak ligand site-density commonly associated with the carboxylic functional group may be similar at the P&P sites evaluated.

The comparison of copper-binding parameters between upstream, effluent, and downstream sites was used to determine if effluent was significantly different than upstream receiving waters, and if so, if that difference was evident in downstream samples after P&P wastewater had mixed with upstream water. In addition, the comparisons sought to determine if P&P effluent copper-

binding behavior was essentially the same among P&P mill discharges. The resulting

conclusions can be summarized as follows:

1) The high-affinity site ligand-density (L_2) for P&P effluent was significantly greater than the receiving waters for 3-out-of-4 sites, indicating P&P DOM may contribute relatively more high-affinity sites than natural DOM. These high-affinity sites are consistent with phenolic functional groups associated with tannins and lignin found in P&P effluent.

2) The aforementioned observation of the effluent L_2 parameter did not translate to significant effects on the downstream mixed sample, since no downstream samples had greater L_2 parameters than their corresponding upstream sample. A likely explanation for this behavior is the significant dilution of the effluent wastewater in the downstream sample.

3) Only 1-out-of-4 sites (Site 4) had effluent $\text{Log}K_1$ or $\text{Log}K_2$ parameters significantly greater than the upstream sample. In addition, this was the only site where the effluent parameters' effect was observed in the downstream mixed sample.

4) Regarding the effluent samples from the four sites, only one parameter (L_1) was not different among the sites, indicating P&P DOM has variable binding characteristics at different sites and likely cannot be represented by a single set of copper binding parameters.

Table 5: Single Factor ANOVA results for pulp and paper wastewater effluent parameters.

| Parameter	F(α, df factor, df error)	F-critical	P-value	Significant?
$LogK_1$	F(0.05, 3, 12) = 18.61	3.49	8.28E-05	Yes
$LogK_2$	F(0.05, 3, 12) = 52.80	3.49	3.47E-07	Yes
L_1	F(0.05, 3, 12) = 3.09	3.49	6.76E-02	No
L_2	F(0.05, 3, 12) = 9.14	3.49	2.00E-03	Yes

4.5. Predicting Metal-Binding Behavior with $SUVA_{254}$

In a study of the optical properties of organic matter from wastewater effluent, river water, and reservoirs in Japan, Kikuchi *et al*. sought to use optical characteristics to predict dissolved metal concentrations. The researchers found a strong correlation between $SUVA_{254}$ and the ratio of CuT/DOC, a variable assumed to be equivalent to MeL/DOC, for mainstream and tributary waters (r = 0.68 and r = 0.69). They concluded aromaticity is an important variable in metal speciation and that trace metals were preferentially complexed by functional groups of aromatic humic species.[87] However, the researchers also found that $SUVA_{254}$ was not significantly correlated with metal binding affinity (CuT/DOC) for WWTP effluents.

To evaluate this finding in the context of P&P effluent DOM, a regression analysis was performed following the graphical method used by Louis *et. al*.[58], where $SUVA_{254}$ was plotted separately against K_1L_1 and K_2L_2 for all data. The results are shown in **Figure 6** with the K_1L_1 and K_2L_2 products representing the partitioning of copper to each ligand site at low free ion activities. In addition, a correlation calculation was performed producing Pearson values of 0.249 and 0.382 for K_1L_1 and K_2L_2 respectively.

At first glance, the results indicate $SUVA_{254}$ has a very weak, positive correlation with, and is a poor predictor of, ligand-binding behavior in P&P effluent and the subsequently impacted waters. This would be consistent with Baken *et. al.*, who observed a correlation between $SUVA_{254}$ and metal binding-affinity in natural waters but did not observe the same behavior in anthropogenically (locations close to WWTP and urban areas, higher ratio of sodium/chloride to DOC) impacted samples.[46] Therefore, a regression between $SUVA_{254}$ and binding-affinity for natural samples (without effluent samples) could be expected to provide a stronger correlation, but **Figure 6** provides no indication that this would occur.

The weak correlation between these variables is consistent with other work demonstrating $SUVA_{254}$ as a poor predictor of the extent of metal binding in waters impacted by wastewater effluent. For example, in speciation modeling of wastewater impacted surface waters, Matar *et al.* showed that upstream free copper concentrations were higher than the downstream samples by a factor of 2 to 4.[62] This would indicate the organic matter from effluent discharges contributed relatively more copper binding than did NOM. However, because the downstream and effluent organic material had relatively low UV absorbance and aromaticity values, the authors concluded that SUVA would not be useful in predicting copper speciation in waters under intense urban pressure.[62]
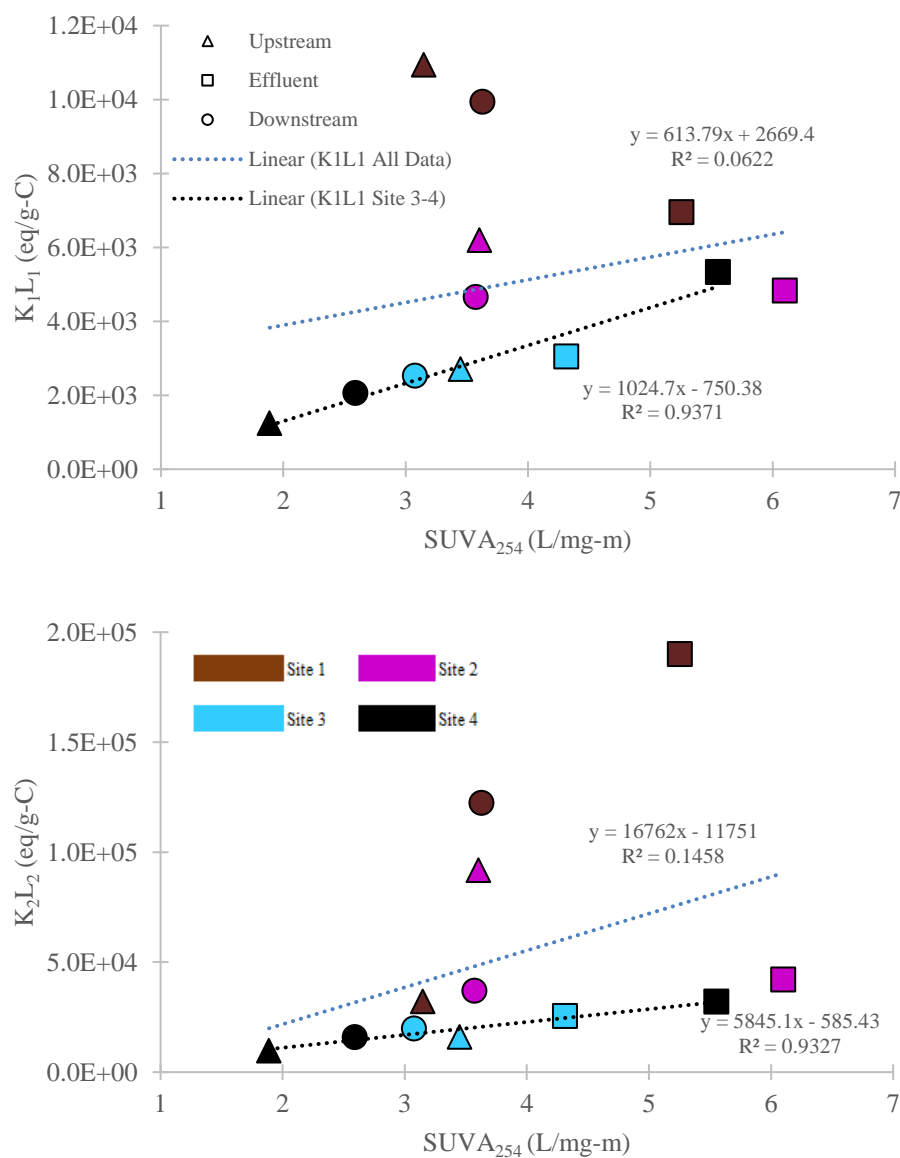
Figure 6: Linear regression between SUVA254 and the product of each ligand-site's binding parameters. Upstream, effluent, and downstream locations are plotted with different shapes while the site number is classified by color. A regression was performed for all data (dotted blue) and for Sites 3-4 (dotted black) separately.

However, when data from the present research was classified by site, it appeared there was a strong linear trend between $SUVA_{254}$ and the binding products at Sites 3 and 4. Indeed, a regression between these variables at Sites 3-4 (excluding Sites 1-2) produced adjusted R-squared values of 0.9371 and 0.9327 for K_1L_1 and K_2L_2 respectively. The researchers hypothesize this inconsistency was caused by differing binding-parameter variability when comparing Sites 1-2 to Sites 3-4. As previously mentioned, the titration data for Sites 1-2 (CuT/DOC ratio window, logarithmic spacing) was not as consistent as Sites 3-4 and therefore the determined binding parameters had more uncertainty.

It was previously shown that effluent samples at all four sites had greater $SUVA_{254}$ than receiving water samples and 3-out-of-4 effluent samples had greater strong-ligand site densities than the receiving waters. This could lead to the conclusion that metal-binding behavior in the P&P effluent can be predicted with $SUVA_{254}$, but the regression results indicate that, at least when data from all the sites is included, $SUVA_{254}$ is a poor quantitative predictor of the partitioning of copper to the ligand sites. Because of the inconsistent finding regarding the regression of data for Sites 3-4, this conclusion should be tested further to ensure the poor correlation is not caused from variability in the experimentally determined binding-parameters.

4.6. Biotic Ligand Model vs. Visual MINTEQ Model

Speciation modeling was performed with the BLM and Visual MINTEQ for a side-by-side comparison. The simulations were performed for two different scenarios:

1) Side-by-side comparison of upstream, downstream, and effluent samples with diluted effluent DOC and effluent water quality data equivalent to the conditions under which the

titrations were performed. That is, Sites 1-2 were diluted 10x and Sites 3-4 were diluted 20x (**Table 6**). This simulation ensures the data can be compared within the CuT/DOC model boundaries and represents the situation where P&P wastewater has been relatively mixed and is diluted. Since water quality parameters such as DOC would be in similar concentration ranges as the upstream and downstream sites, the effects of differences between the four binding parameters ($LogK_1$, $LogK_2$, L_1, and L_2) in the form of copper binding could be isolated and compared to the BLM. Importantly, the dilution does not affect the $LogK_1$/$LogK_2$ binding parameters, although the total concentration of ligand sites (L_1 and L_2) was reduced since the DOC normalized values were multiplied by the diluted DOC concentration before being entered into MINTEQ.

2) Side-by-side comparison of "full strength" effluent wastewater samples using undiluted background water quality data. This scenario represents the P&P effluent copper-binding as it is "in the pipe." In this simulation, the DOC and other water quality data were undiluted.

**Table 6**: DOC and site-density parameters used for MINTEQ and BLM modeling. Diluted (10x and 20x) DOC concentrations are shown for comparison between upstream, effluent, and downstream concentrations. L_1 and L_2 parameters were converted to units of mmol/L using the diluted concentration of DOC (underlined).

	Site 1			Site 2		
	Upstream	Effluent	Downstream	Upstream	Effluent	Downstream
DOC (mg/L)	0.54	<u>0.72</u>	0.56	0.86	<u>3.56</u>	1.11
L_1 (mmol/L)	1.83E-03	8.15E-04	1.32E-03	1.33E-03	5.62E-03	1.81E-03
L_2 (mmol/L)	3.58E-05	6.04E-05	3.78E-05	5.83E-05	3.91E-04	8.70E-05
	Site 3			Site 4		
	Upstream	Effluent	Downstream	Upstream	Effluent	Downstream
DOC (mg/L)	1.71	<u>4.61</u>	1.7	2.4	<u>2.70</u>	1.8
L_1 (mmol/L)	2.43E-03	7.00E-03	2.67E-03	3.71E-03	4.67E-03	2.70E-03
L_2 (mmol/L)	1.52E-04	4.65E-04	1.50E-04	1.96E-04	3.53E-04	1.51E-04

The results of the simulations are shown in **Figure 7** and **Figure 8**, while a plot showing the ratio of the concentration of available copper predicted by MINTEQ to the concentration predicted by the BLM is shown in **Figure 9**. The plots include vertical lines representing the CuT/DOC window (0.002 to 0.11 mg/mg) under which the four copper-binding parameters were determined. Since other researchers[74] have indicated the CuT/DOC ratio can affect the resulting binding parameters, care should be taken when evaluating the MINTEQ model outside these boundaries. Results for each site are summarized below:
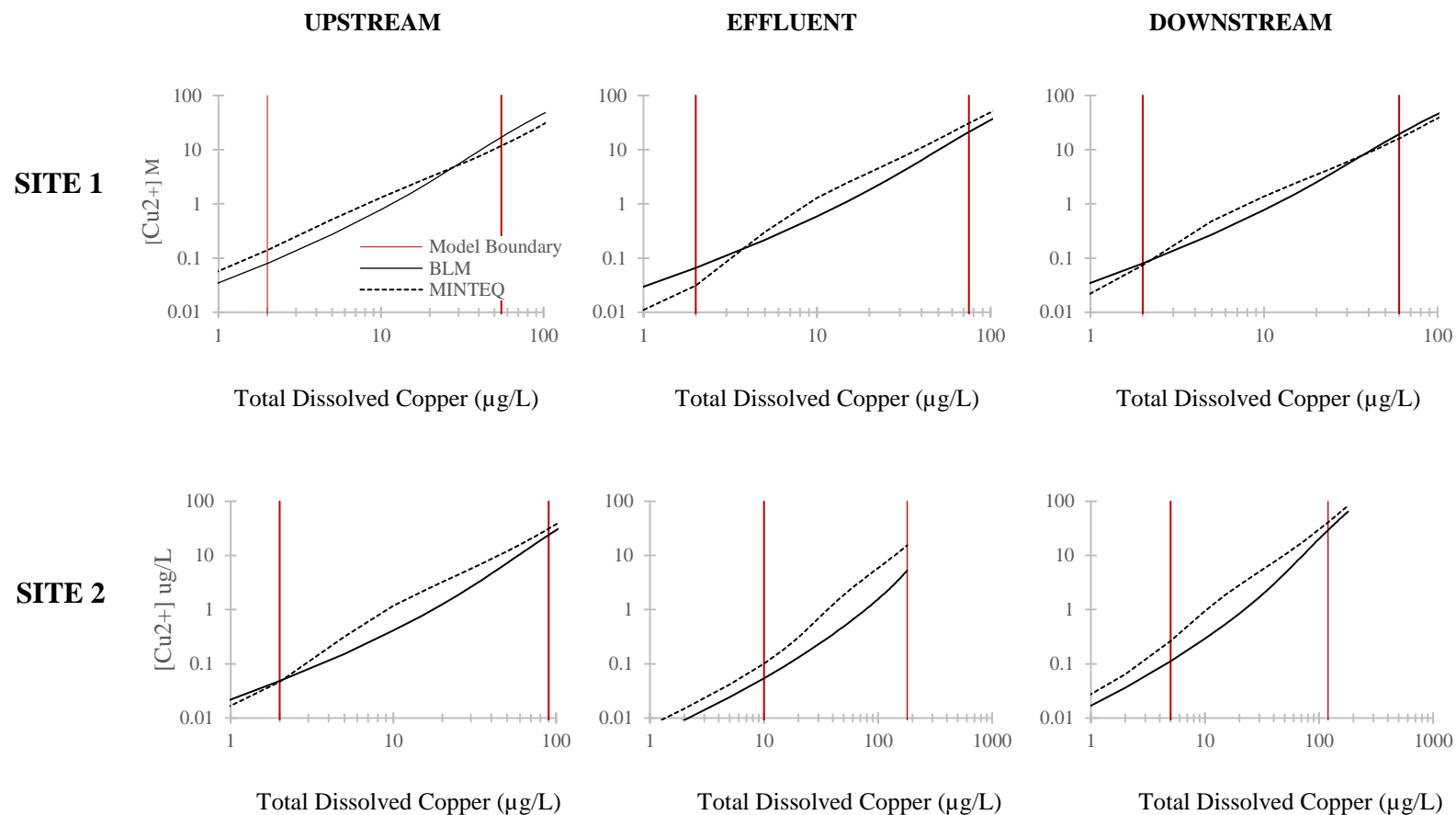
Figure 7: Plots comparing the Biotic Ligand Model and a MINTEQ speciation model for Sites 1-2 using laboratory measured binding parameters. Vertical red lines represent model boundary conditions since binding parameters were determined within a certain CuT/DOC window.
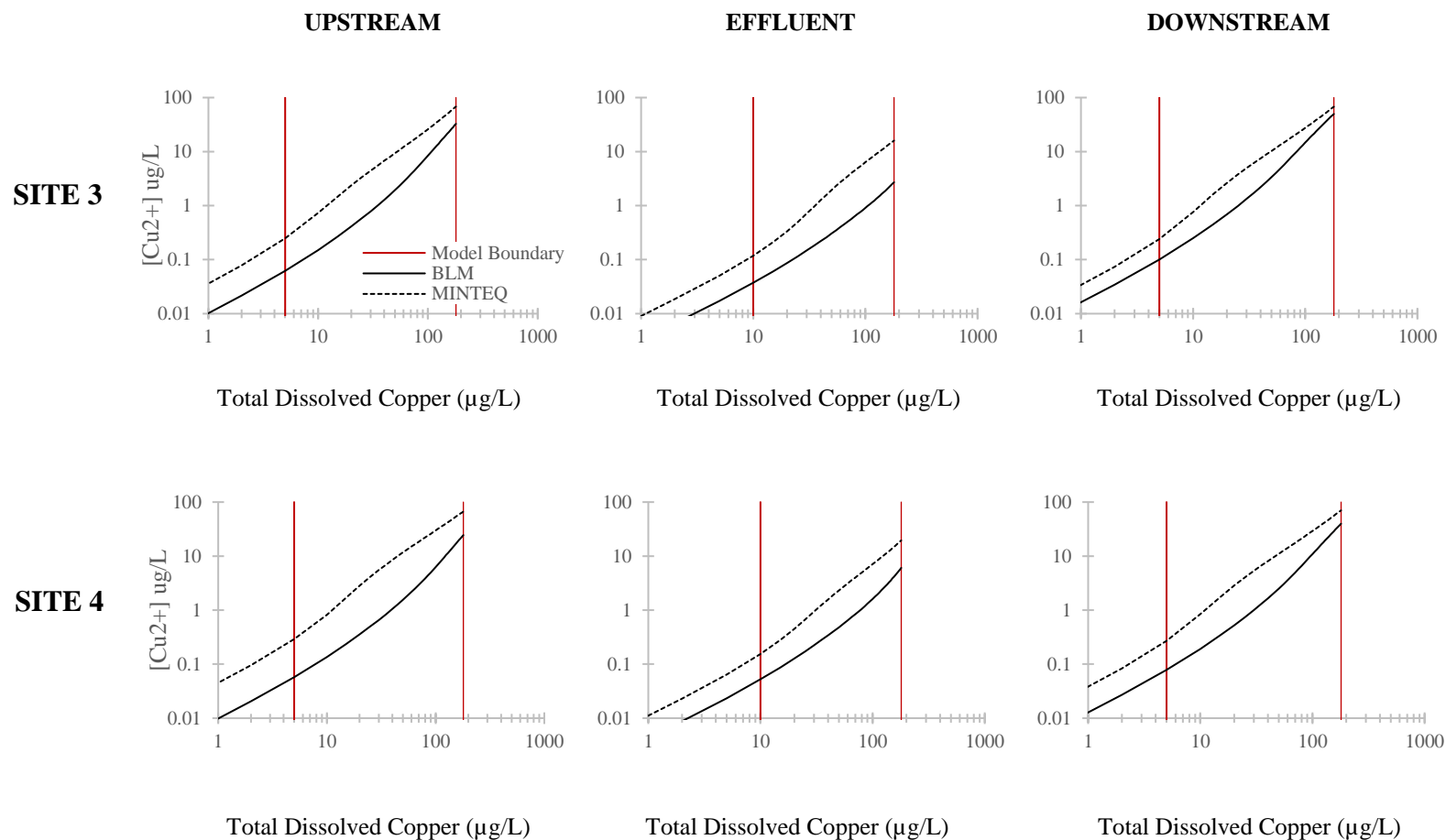
Figure 8: Plots comparing the Biotic Ligand Model and a MINTEQ speciation model for Sites 3-4 using laboratory measured binding parameters. Vertical red lines represent model boundary conditions since binding parameters were determined within a certain CuT/DOC window.
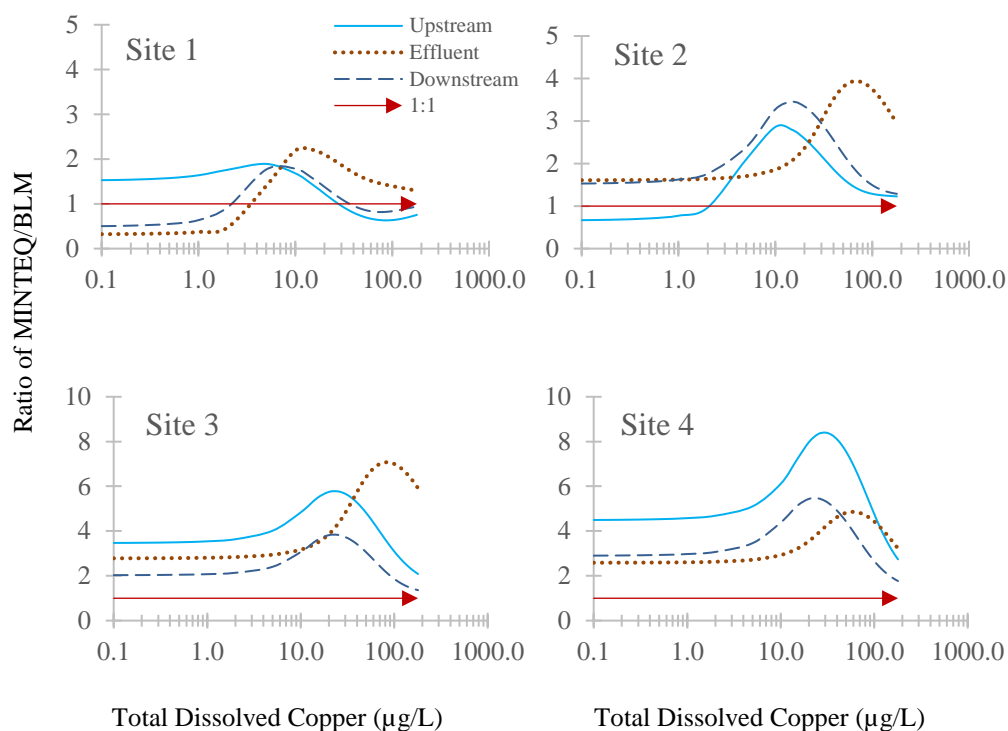
Figure 9: Plots showing the ratio of the MINTEQ model to the BLM for upstream, effluent, and downstream samples. A 1:1 ratio line is plotted for reference. Effluent model simulations were performed at the same dilution concentration as the titrations; Sites 1-2 = 10x and Sites 3-4 = 20x.

<u>Site 1</u>: When compared within the CuT/DOC model boundaries, the MINTEQ and BLM models were in good agreement at environmentally relevant CuT concentrations (2-15 ug/L); the upstream and downstream MINTEQ models were approximately within a factor of 2. The effluent MINTEQ model predicted ½ the bioavailable copper of the BLM at a CuT concentration of 2 µg/L, but then consistently predicted more by a factor of 1-2. In referencing **Table 6**, the DOC concentrations used in each location's simulation were similar (up = 0.54, eff = 0.72, down = 0.56 mg/L). When converted to L_1 and L_2 binding sites, the effluent sample had less L_1 sites and more L_2 sites than both upstream and downstream samples. This, along with the larger K_2 value, likely explains why the

MINTEQ model predicted less bioavailable copper at low concentrations in the effluent than the BLM.

Site 2: When compared near the lower CuT/DOC model boundary, the MINTEQ and BLM models were in relatively good agreement between 5-15 µg/L CuT. The upstream and downstream MINTEQ simulations predicted more bioavailable copper with factors between 2-3.5X, while the effluent simulations were between 1.7-2X. However, as CuT increased, the effluent MINTEQ simulation predicted up to 4X more copper than the BLM. Similar to Site 1, the effluent ligand concentrations were relatively greater than the other upstream and downstream locations.

Site 3: When compared near the lower CuT/DOC model boundary at 5-15 µg/L CuT, the MINTEQ models consistently predicted more free copper by factors between 2-5.5X the BLM predictions. Similar to Sites 1 and 2, the largest model ratios (BLM predicting less free copper) occurred in the effluent samples at higher CuT concentrations. Although the ligand and DOC concentrations at the upstream and downstream sites were nearly identical, the downstream simulations were in better agreement than the upstream simulations. The downstream BLM model also predicted significantly more free copper than the upstream and effluent. Although it is unclear why, the downstream sample had significantly higher concentrations of calcium and sulfate than the upstream sample.

Site 4: When compared near the lower CuT/DOC model boundary at CuT concentrations between 5-15 µg/L, the MINTEQ model consistently predicted more available copper

than the BLM. The effluent simulations agreed most closely, varying by a factor of 3 while the upstream and downstream sites varied by factors up to 7 and 5 respectively. Again, the concentration of ligand sites in the effluent sample were greater than the upstream and downstream samples even though the DOC concentrations were relatively similar.

These results demonstrate that the MINTEQ and BLM predictions were within an order of magnitude, with MINTEQ consistently predicting more bioavailable copper. Surprisingly, the effluent simulations at Sites 1, 2, and 4 were in better agreement than their corresponding upstream and downstream simulations at the environmentally relevant concentration of 5 µg/L. Assuming the MINTEQ model is more representative of actual metal-binding conditions (parameters were experimentally measured), the data suggests that at low levels of total copper, the BLM describes the behavior of effluent-DOM-impacted waters more accurately than it does natural DOM in the receiving waters. This trend is reversed, with the exception of Site 4, as the total copper concentration increases to what would be considered relatively high environmental concentrations of copper (40-100 µg/L).

It was previously shown at several P&P sites that effluent DOM exhibited significantly greater aromaticity and strong-ligand site densities in comparison to natural DOM, variables indicative of increased metal-binding (although poorly predicted by SUVA). The results suggest the BLM is generally in better agreement with the MINTEQ model when simulating diluted P&P effluent but is likely predicting less bioavailable copper than should be expected in natural waters. Therefore, it is reasonable to expect the

BLM to predict free copper within an order of magnitude (and generally within 5X) when modeling effluent impacted waters. While the upstream, effluent, and downstream models were relatively similar, the BLM systematically predicted less bioavailable copper than the MINTEQ models. This systematic trend presumably occurred because, unlike the MINTEQ model, the BLM did not utilize site-specific binding parameters and treated all DOC in the same manner.

In view of these results, an additional BLM simulation of the Site 4 upstream sample (**Figure 10**) was performed with 1% Humic Acid (HA) to determine if the % HA parameter could be sufficiently adjusted to force the models to agree. This improved the model ratios to within a factor of 2-3X at environmentally relevant concentrations, but the 1% HA value is outside the WHAM calibrated 10-60% HA range[1] and was arbitrarily determined. That is, there was no systematic way of determining % HA for all simulations. As previously mentioned, other researchers have used UV absorbance to determine the amount of fulvic acid,[23] but inconsistent $SUVA_{254}$ regression results in the present research did not indicate a similar method could be followed. Even if UV absorbance could be used to fine-tune the % HA parameter, it is not clear this would shift the parameter towards the value needed for model agreement. Namely, adjusting with UV absorbance could result in % HA values higher than the default 10% used in the present simulations and subsequently cause reduced model agreement. Ultimately, more work is needed to determine if the % HA parameter in the BLM is sufficient to characterize the metal-binding behavior of DOM and whether this parameter can be fine-tuned with UV absorbance data.
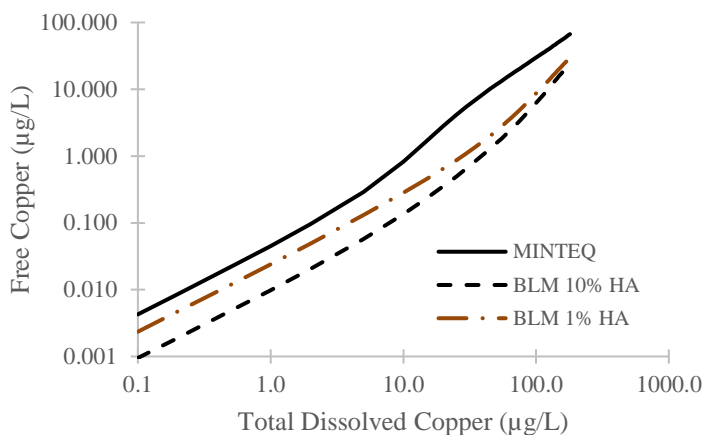
Figure 10: Plot showing the results of a 1% HA BLM simulation for the Site 4 upstream location. The MINTEQ and 10% HA simulations are included for comparison.

4.7. Full Strength Effluent Biotic Ligand Model and Visual MINTEQ Model

As shown in **Figure 11**, full strength effluent simulations at 3 of the four sites were relatively

constricted by the CuT/DOC model boundaries, since several effluent samples had high

concentrations of DOC. At Site 3, for example, the entire 0.1 to 180 µg/L simulation range was

completely outside the CuT/DOC window used for parameter determination. A plot of the

MINTEQ/BLM model ratios is shown in Figure 12. In the small model window for Sites 2 and 4,

the MINTEQ/BLM model ratios were close to unity (high/low = 1.17/0.96 and 1.26/1.24). The

effluent model with the largest comparative window and environmentally relevant copper and

DOC concentrations was Site 1 (DOC = 7.23 mg/L). This model showed a reversal in model

predictions; the BLM predicted more available copper at lower total dissolved copper

concentrations and relatively less at higher concentrations. The ratio of MINTEQ/BLM for the

Site 1 effluent simulations was high/low = 1.33/0.22, indicating the BLM predicted

approximately 5x more available copper at lower total dissolved copper concentrations but

slightly less at higher concentrations. This result provides an extreme "in the pipe" scenario that can be compared to the diluted effluent simulations. While only 1 pair of simulations could be compared at an environmentally relevant range, the BLM appears to overestimate the amount of bioavailable copper in concentrated, anthropogenic wastewater.
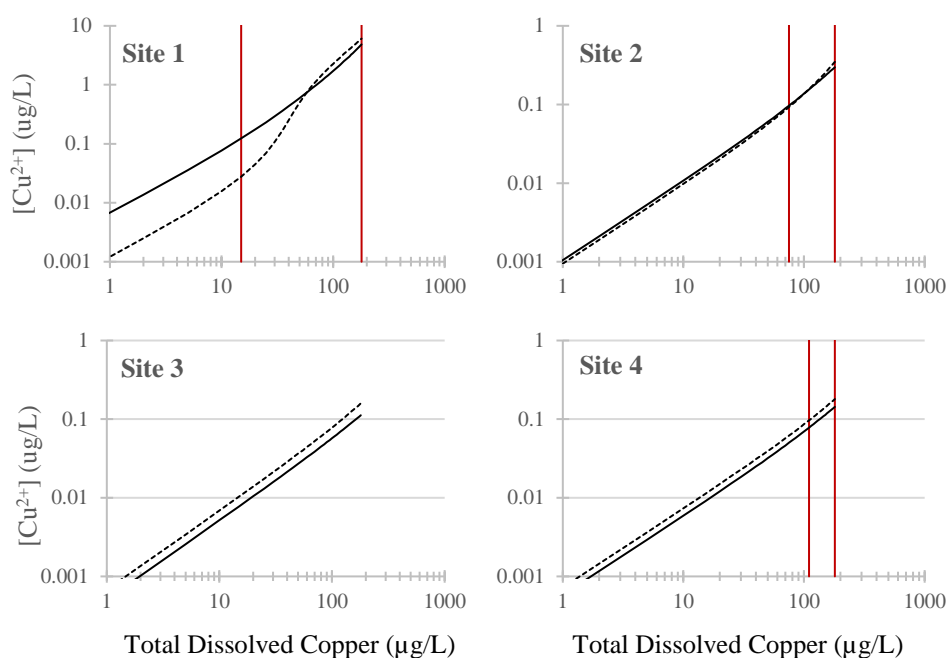
Figure 11: Plots comparing the Biotic Ligand Model and MINTEQ speciation model in an undiluted DOC concentration scenario for Site 1; DOC = 7.23 mg/L, Site 2; DOC = 35.62 mg/L, Site 3; DOC = 92.23 mg/L, and Site 4; DOC = 53.9 mg/L. Vertical lines represent CuT/DOC model boundary conditions. Because of the relatively large amounts of DOC in effluent samples, the 0.1 to 180 ug/L copper range used for speciation modeling is not within the CuT/DOC boundary and the window for comparison is narrower.
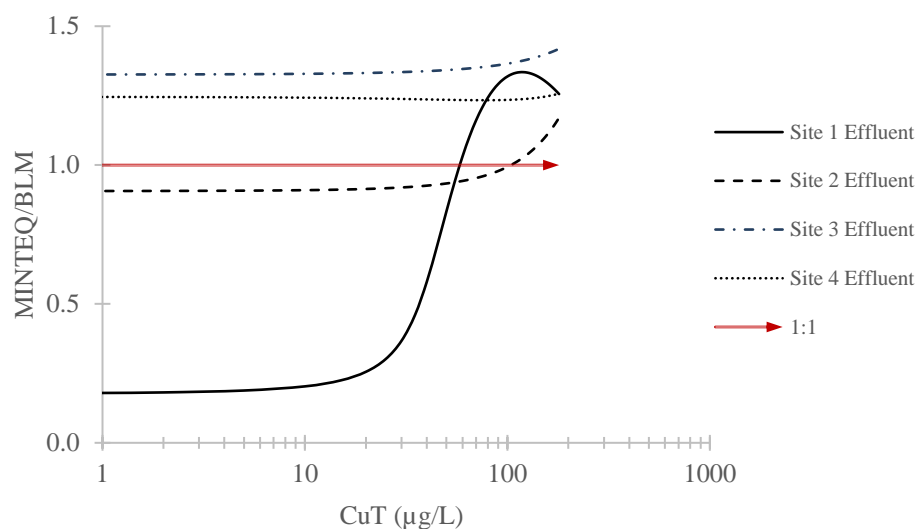
Figure 12: Plot showing the ratio of the MINTEQ speciation model to the BLM for an undiluted P&P effluent simulation. The effluent concentrations for Sites 1, 2, 3, and 4 are 7.23, 35.62, 92.23, and 53.9 mg/L respectively

5. Conclusion

The purpose of this research was to determine if P&P effluent DOM exhibits different copper-binding characteristics than NOM and whether any differences influence the BLM's ability to accurately predict bioavailable copper in P&P influenced waters. The contributions of effluent DOC, UVA, and volumetric discharges were quantified at each P&P site to determine the relative amount of anthropogenic influence in each river system and assess the ability to detect copper-binding properties downstream. Copper-binding characteristics of upstream, effluent, and downstream DOM were determined by fitting experimentally measured copper activity to a 2-site ligand model and compared within each river system. In addition, the optical behavior (SUVA$_{254}$)

of DOM was measured and used to directly compare DOM sources and assess the ability of SUVA$_{254}$ to predict copper-binding behavior.

Measurements of effluent DOC, UVA, and volumetric flow indicated that while P&P effluent represented less than 1% the total flow in the river, it contributed measurable amounts of light absorbing DOC. However, variation in the expected/measured values of downstream DOC and UVA indicated that, at least in some cases, the river was not a sufficiently closed system so as to detect downstream effects of P&P copper-binding behavior.

The copper-binding results suggest P&P effluent DOM has a greater high-affinity ligand site-density than DOM in receiving waters as this behavior was observed in effluent samples at 3-out-of-4 sites. When comparing effluent DOM amongst P&P mills, the data also suggests P&P DOM binding characteristics and optical absorbance vary from site to site. While SUVA$_{254}$ was consistently greater in P&P effluent DOM than in receiving waters, SUVA$_{254}$ was a poor predictor of copper partitioning at individual ligand sites. The lack of correlation between the high SUVA$_{254}$ and the K_2L_2 partitioning expression could indicate there were low-UV absorbing compounds present in the effluent DOM (and consequently the downstream samples). However, strong trends between SUVA$_{254}$ and binding products when using data from Sites 3-4 alone indicate that the poor correlations could be due to variability in the binding parameters of Sites 1-2. Therefore, more measurements would be needed to determine if the poor correlation is a property of P&P impacted waters or simply due to variability in the experimentally determined binding parameters.

While this research did not try to chemically characterize or isolate such compounds, other researchers have noted the presence of synthetic chelators and proteinaceous ligands in

effluent impacted waters. Matar *et. al.* reported the presence of a diversity of nitrogen functional groups in effluent organic matter[88] and attributed the increased metal-affinity of the hydrophilic fraction to these protein-like groups.[62] Shi *et. al.* reported P&P effluent containing a large amount of low UV absorbing "carbohydrates and aliphatic compounds"[36], and Baken *et. al.* found urban-impacted waters had higher metal-binding affinities and contained synthetic ligands such as EDTA in micro molar concentrations. They concluded that anthropogenic discharges were responsible for increased metal binding that would not be predicted by humic-based models.[46] Therefore, the difference in binding-affinities and strong-ligand site density among P&P sites could be due to the type of material processed, the pulp and paper processes used, and the wastewater treatment practices/chemicals employed.

The MINTEQ and BLM model predictions of bioavailable copper were consistently within an order of magnitude, with the MINTEQ model consistently predicting more available copper than the BLM. At environmentally relevant concentrations (5 µg/L), the diluted effluent simulations were consistently more agreeable (within a factor of 3) than their upstream and downstream counterparts and this was likely caused by the contribution of copper-binding by P&P DOM. In other words, the BLM consistently predicted relatively less copper availability in upstream and downstream waters than in diluted effluent when compared within each upstream-downstream sequence. In contrast, the BLM predicted 5x more bioavailable copper in a full strength effluent sample at lower concentrations of CuT. This indicates that, at least in this single case, the BLM predicts more bioavailable copper than should be expected in concentrated effluent samples. These conclusions are consistent with other researchers' findings that the BLM predicts less bioavailable copper than should be expected in natural waters. As previously

mentioned, McGeer *et al*. found that increasing humic acid concentrations alleviated toxic affects to rainbow trout, but the BLM predicted a higher level of bioavailable of copper necessary to induce toxicity than was observed.[24] In addition, other researchers have found that the WHAM model (version V) under-predicts the amount of copper activity in natural waters.[25–28] Thus, these findings support the original hypothesis that P&P DOM binds to copper differently than NOM, but the difference appears to cause the BLM to more accurately predict bioavailable copper in P&P DOM-influenced waters rather than natural waters.

Therefore, P&P effluent DOM should be 1) considered unique across different P&P mill sites, 2) expected to have a relatively greater density of high-affinity binding sites than DOM present in receiving waters, and 3) engage in binding behavior that is systematically over predicted by the BLM. More generally, the BLM appears to systematically over-predict copper-binding behavior, irrespective the presence of P&P DOM.

In the present research, there were significant limitations in comparing upstream and downstream samples, primarily due to uncertainty in the measurements of organic matter and flow in the complex river systems. Future research should better control the upstream-downstream sequence; for example, by performing a parallel tracer test in P&P effluent with which to normalize downstream organic matter concentrations. In addition, it would be useful to chemically isolate compounds in P&P effluent and distinguish their copper-binding and UV absorbance behavior from the relatively large fraction of aromatic humic species. Due to the relative ease with which optical scans can be performed, perhaps observing absorbance at additional wavelengths could be used to evaluate the convolution of absorbance from different classes of ligands. Finally, further investigation of the BLM's treatment of DOM is warranted to

better understand its systematic over-prediction of copper-DOM binding. Besides adjusting the "% humic acid" parameter in the BLM, perhaps the developers could incorporate a "% anthropogenic" parameter that accounts for the class of high affinity/site-density ligands. This research would help regulatory bodies and industrial parties determine what type and quantity of anthropogenic carbon loading from P&P mills would result in bioavailable copper levels not adequately predicted by the BLM.

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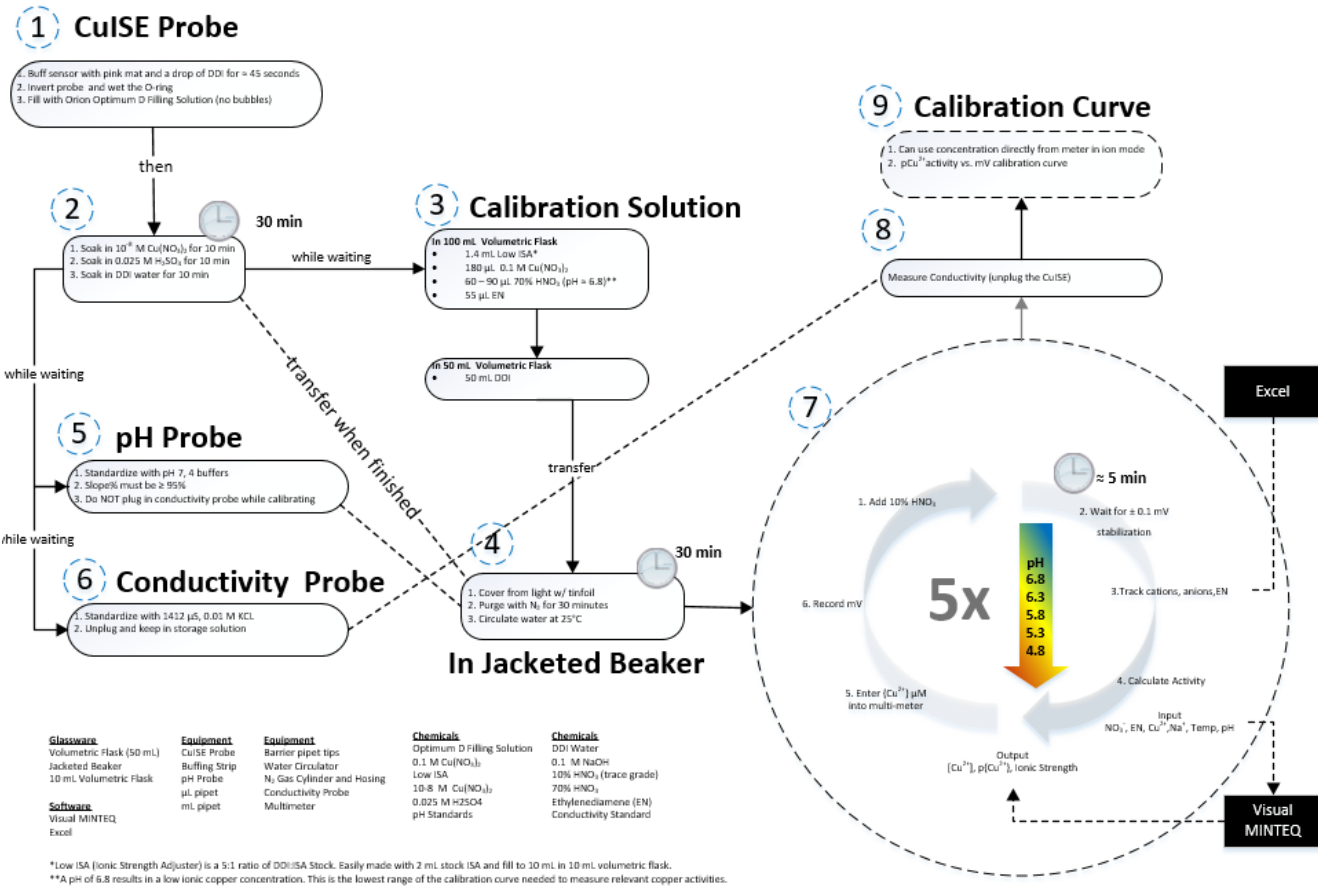
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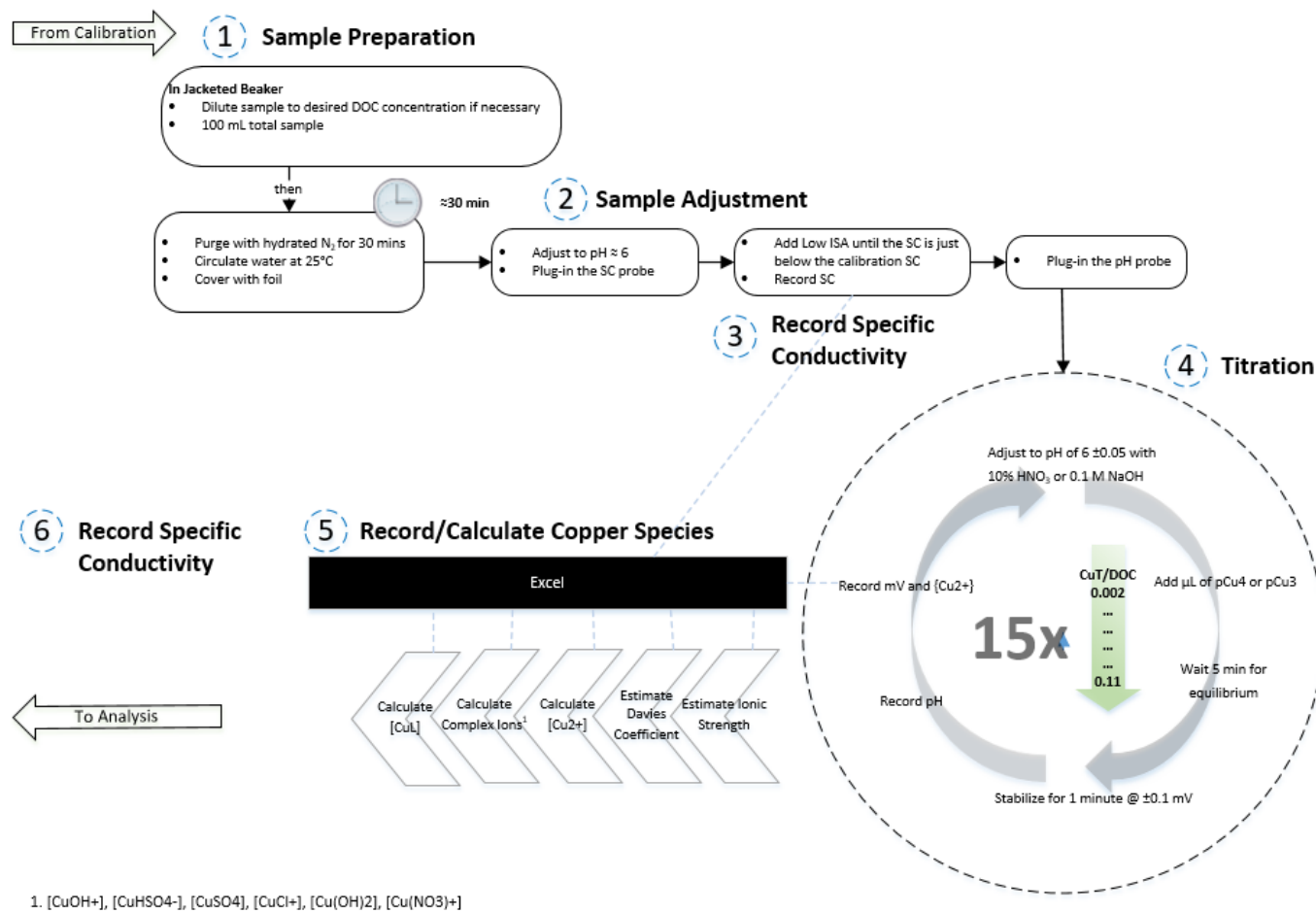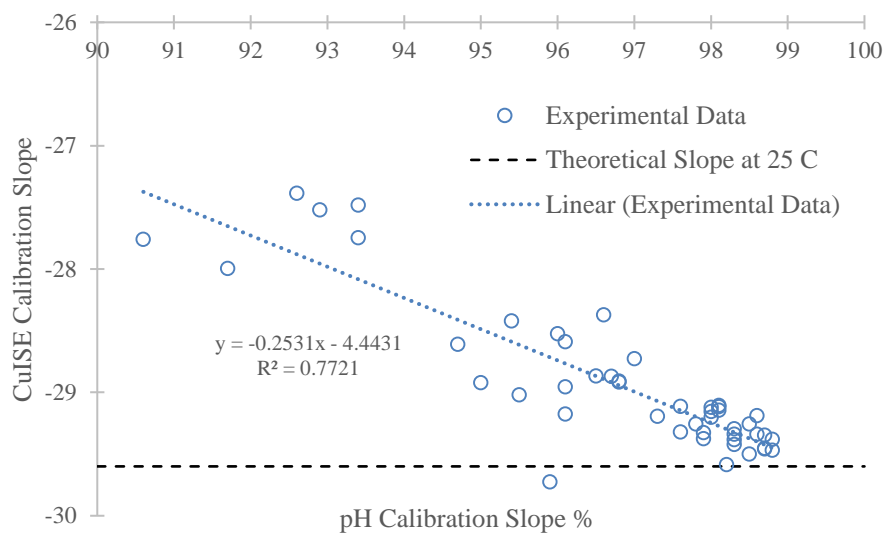
APPENDIX A: Supporting Figures

Figure 13: Calibration procedure for low-level copper. Procedure adapted from Mosbrucker (2016) detailed methods.

Figure 14: Titration procedure for natural and effluent DOM samples. Procedure adapted from Mosbrucker (2016) detailed methods.

Figure 15: The relationship between the pH calibration slope and the resulting CuISE calibration slope. Dashed line represents the theoretical Nernstian slope of -29.6 pCu/mV @ 25°C. The relationship suggests the pH probe should be calibrated to a relatively high slope% before proceeding with CuISE calibration.
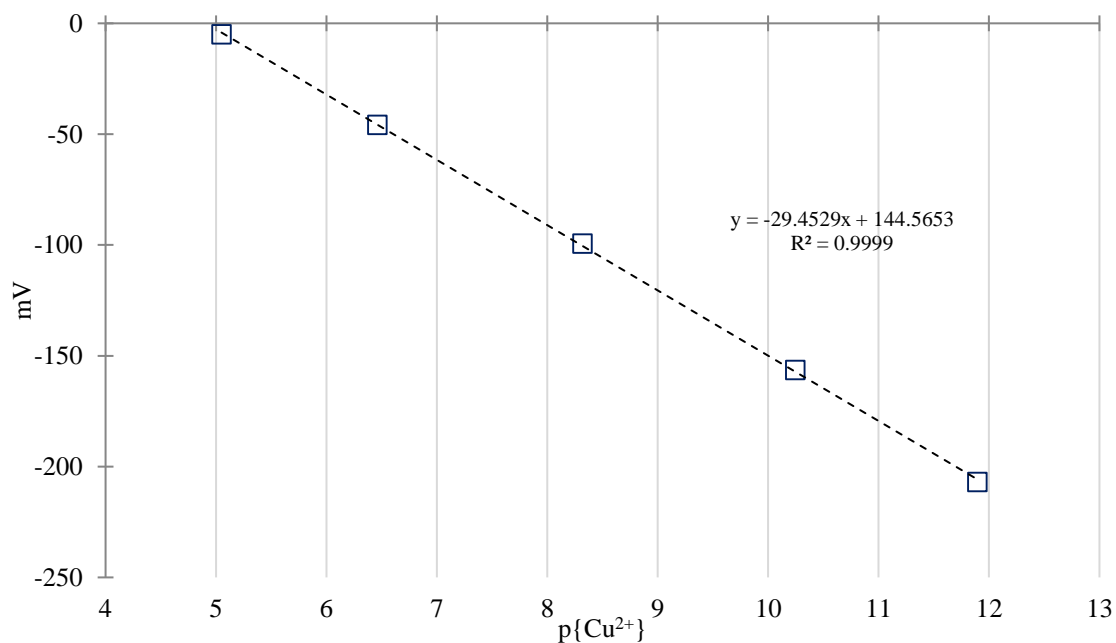
Figure 16: Example showing a calibration curve from 06/01/2018 with regression slope near the theoretical value of -29.6.
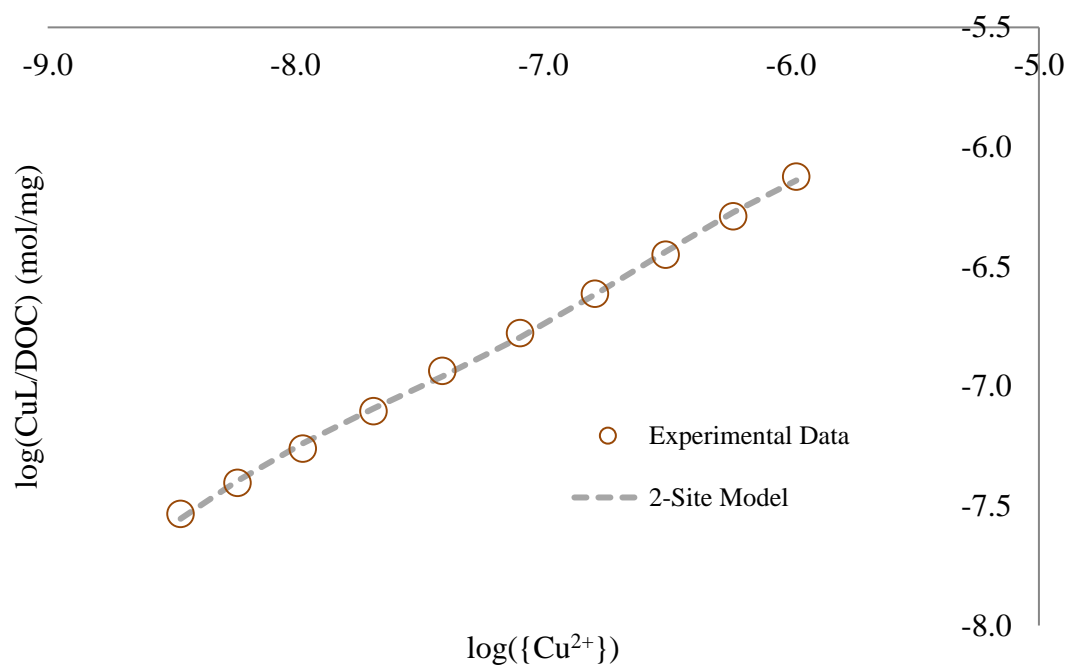
Figure 17: Graphical depiction of log-transformed experimental data and model fitting procedure using Excel Solver. This example is from a titration of the Site 4 Upstream sample.
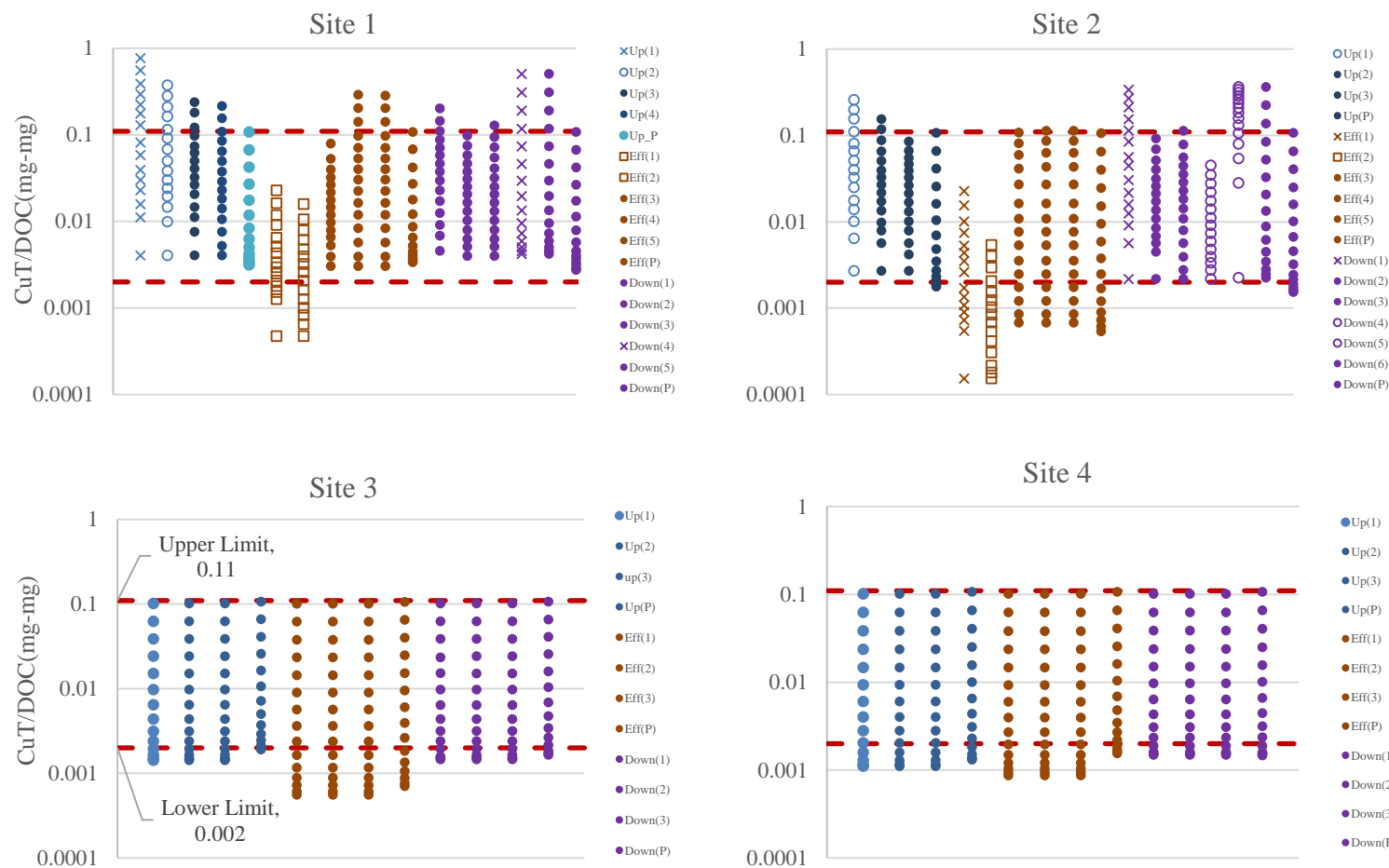
Figure 18: Plots showing the CuT/DOC window used for data analysis and titrations that could not be used for procedural issues (O, □) such as different ionic strengths or non-diluted samples. Plots with an (X) represent titrations that appeared to have been noticeable contamination. The dashed lines represent the upper and lower CuT/DOC model boundaries.

APPENDIX B: Contamination Experiments

Concave-downward Scatchard plot behavior was regularly observed during the course of titrations. This was unexpected, since copper titrations[76,83] (and metal-binding more generally[12,89]) typically result in hyperbolic shaped graphs that allow the researcher to distinguish one or two ligand binding sites. This behavior was investigated in the literature to determine if the Scatchard plot behavior was due to actual NOM-copper interactions similar to protein-ligand binding[90–92], kinetic behavior[93] that contradicts the assumption of equilibrium, adsorption of copper to instruments[94] and glassware[73], or copper contamination more generally. Ultimately, the most reasonable and simplest explanation was copper contamination resulting in an inaccurate mass balance and consequently a concave-downward Scatchard plot.

Three contamination experiments were performed to quantify the aggregate amount of copper contamination that could occur from carry-over between experiments and adsorption to lab-ware. The experiments were performed on DDI water with the same experimental set-up as a titration. The CuISE was calibrated as previously described, followed by purging the DDI water with N_2, pH adjustment, and ionic strength adjustment. Two, 5-mL aliquots were then removed for copper analysis by ICP-MS (Thermo Elemental X-series II Quadrupole) and preserved with 70% trace grade HNO_3 (BDH Aristar-Plus Cat. #87003-259). The experiments indicated contamination between 0.047 and 0.206 µg, the latter occurring after a calibration and 2 titrations. Consequently, these experiments were followed by 12 parallel "pristine titration" and contamination experiments. That is, an additional titration was performed on each sample (3 locations x 4 sites = 12) where a 5-mL aliquot was removed after stabilization (pH adjust, ionic strength, etc.) but prior to copper addition. The 12 pristine titration experiments and 2/3 of the

initial contamination tests were representative of the contamination that could occur after

calibrations and titrations (n = 14). These experiments, summarized in **Table 7**, resulted in an

average copper contamination estimate of 0.21 ± 0.08 µg. The average mass of copper

contamination was subsequently added to the background copper of all previous titrations. A

graphical representation of the Scatchard plot behavior before and after correction with

contamination data is shown in **Figure 19**. As shown in **Figure 20**, data points that resulting in a

concave-downward trend, even after contamination correction, were not included in the model

fitting procedure.

Table 7: Results of the contamination experiments and calculated average. Contam 2 was not included since it was performed immediately after Contam 1 and therefore the equipment was not exposed to levels of copper common in the calibration/titration experiments. The term "Pristine" indicates the titration was performed with a sample removed for ICP-MS. This allowed for mass reconciliation and decreased uncertainty when calculating the value of CuL.

Experiment	Type	Site Identity	Copper Contamination (moles)	Copper Contamination (ug)
Contam 1	Test	NA	1.14E-09	0.073
Contam 2*	Don't Use	NA		
Contam 3	Test	NA	3.24E-09	0.206
6/1/2018	Pristine Titration	Site 3 Upstream	4.51E-09	0.287
6/1/2018	Pristine Titration	Site 3 Downstream	3.75E-09	0.238
6/2/2018	Pristine Titration	Site 4 Downstream	3.19E-09	0.203
6/2/2018	Pristine Titration	Site 4 Upstream	4.03E-09	0.256
6/4/2018	Pristine Titration	Site 3 Effluent	4.22E-09	0.268
6/4/2018	Pristine Titration	Site 4 Effluent	6.06E-09	0.385
6/5/2018	Pristine Titration	Site 1 Upstream	2.47E-09	0.157
6/5/2018	Pristine Titration	Site 2 Downstream	2.11E-09	0.134
6/5/2018	Pristine Titration	Site 2 Upstream	1.99E-09	0.127
6/6/2018	Pristine Titration	Site 1 Downstream	2.15E-09	0.137
6/6/2018	Pristine Titration	Site 2 Effluent	2.74E-09	0.174
6/6/2018	Pristine Titration	Site 1 Effluent	3.73E-09	0.237
Average			3.24E-09	0.21
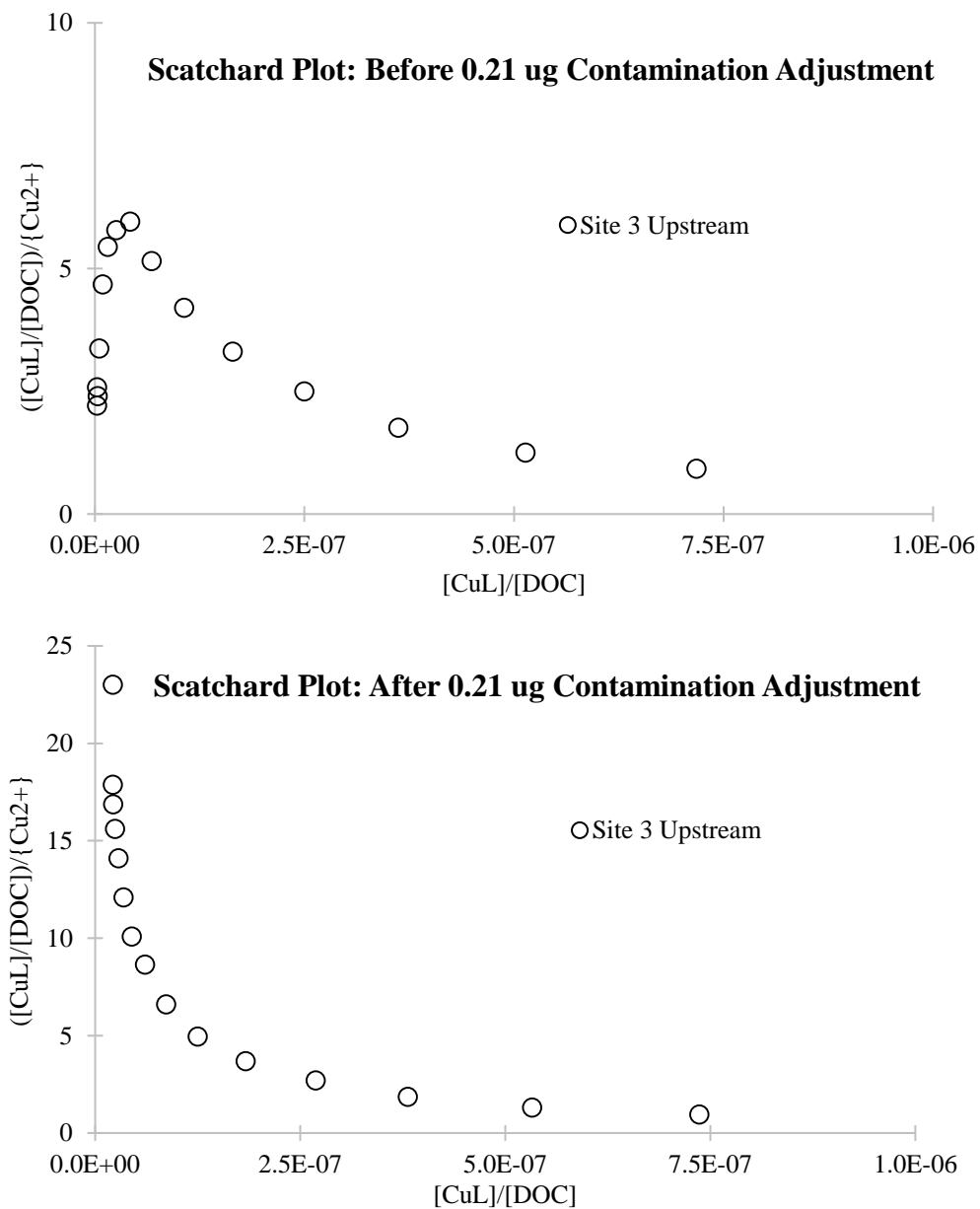
Figure 19: Scatchard plot behavior before and after experimental data were corrected for background copper contamination. After correction with 0.21 ug of copper contamination, the Scatchard plot resembles the asymptotic plots commonly associated with metal-binding titrations.
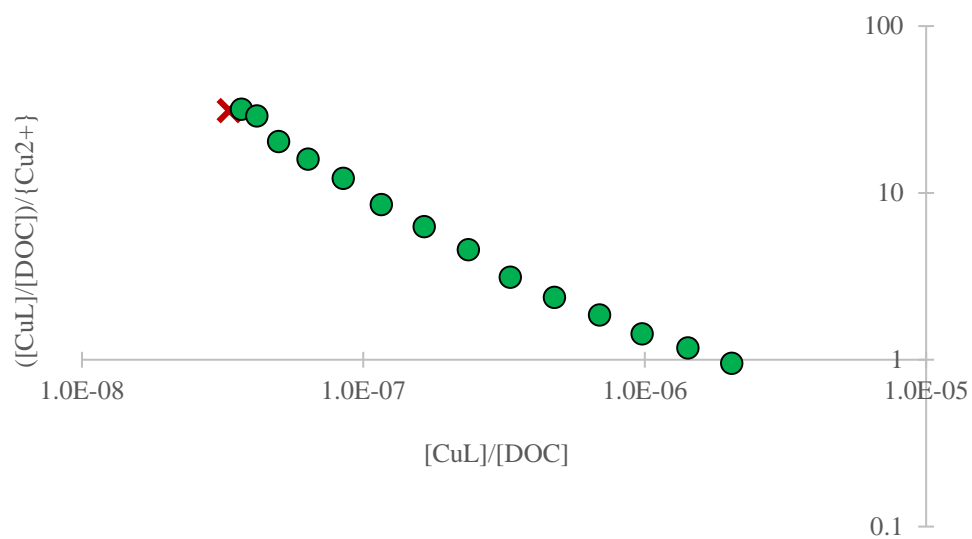
Figure 20: Log-transformed (for emphasis) Scatchard plot from a Site 2 Downstream titration showing the first data point (X) not used in the data fitting procedure since it exhibited the concave-downward behavior.