

Tracking Changes in Organic-Copper Speciation during Wastewater Treatment Using LC-ICPMS-ESIMS

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Laurinda Nyarko, Christian Dewey, Jeffrey A. Nason, and Rene M. Boiteau*



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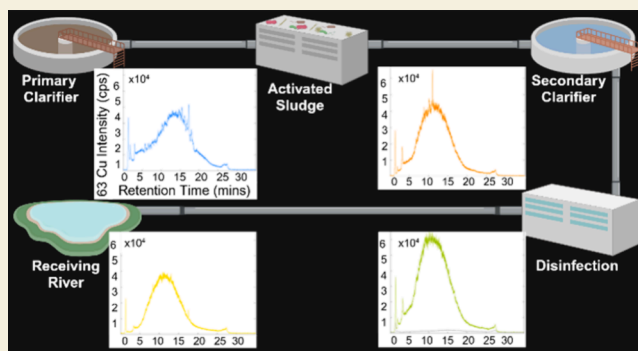
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ABSTRACT: Wastewater is a significant source of copper to freshwater environments, which can severely harm aquatic life. The bioavailability and toxicity of copper in water are influenced by its complexation with dissolved organic matter (DOM). Speciation models, like the biotic ligand model (BLM) that guides Cu regulations, assume DOM is dominated by humic substances. Research suggests that anthropogenic compounds in wastewater discharge may be important copper binding ligands, although their identities remain largely unknown. To address this knowledge gap, we identified and quantified organic copper species isolated from 24 h composite wastewater samples by solid phase extraction (SPE) using liquid chromatography (LC) with inductively coupled plasma mass spectrometry (ICPMS) and electrospray ionization mass spectrometry (ESIMS). Analyses of samples across different stages of treatment revealed the net removal of Cu (73%) and DOC (66%). LC-ICPMS showed that certain complexes were selectively removed, while others evaded removal or were generated during treatment. Relatively hydrophobic complexes decreased in abundance from the initial to the secondary treatment stage. In contrast, more hydrophilic organic Cu complexes, likely formed during treatment, showed a significant increase from the secondary to the tertiary stage. The molecular mass and formula of seven discrete chromatographically resolved complexes were identified by LC-Orbitrap MS. Six were detected only in wastewater, and one was detected in all the wastewater and river samples. Identification of these compounds provides additional evidence for the formation of new copper-binding ligands during treatment and confirms the presence of nitrogen- and sulfur-containing compounds with copper-chelating properties in the wastewater. These findings demonstrate the utility of LCMS approaches for identifying and quantifying distinct organic-copper species in wastewater, as well as tracking their changes and removal during the treatment process.

KEYWORDS: Copper, Wastewater, Organic matter, Liquid chromatography, Mass spectrometry



1. INTRODUCTION

High concentrations of dissolved copper (Cu^{2+}) have been measured in wastewater effluent,^{1–3} exceeding the US environmental protection agency's (USEPA) final acute value for the most sensitive species ($4.7 \mu\text{g/L}$).⁴ Thus, these waste streams are considered significant contributors to copper (Cu) impairment of freshwater systems, posing a risk to the environment and aquatic life.^{5–7} However, the reactivity and bioavailability of Cu in aquatic environments are influenced by its chemical form (speciation), which is primarily determined by its complexation with strong organic ligands.^{8–11} Therefore, an understanding of organic-Cu speciation in these anthropogenic source waters is needed to effectively assess the risk of copper toxicity and develop strategies for Cu removal.

While it is widely recognized that dissolved copper primarily exists as complexes with dissolved organic matter (DOM) in wastewater,^{2,12–14} Cu chemical speciation remains poorly

characterized. Free Cu^{2+} is generally considered toxic to aquatic life, and predictions of free Cu^{2+} concentrations rely on estimates of their binding with organic ligands. For example, the biotic ligand model (BLM) is used to determine the aquatic life freshwater quality criteria for copper.⁴ Speciation models like the Windermere humic aqueous model (WHAM) that simulates Cu-organic complexation in the BLM generally incorporate generic parameters for the Cu-complexing DOM representative of naturally occurring organic matter in pristine systems.^{15–17} However, the BLM has been found to deviate from experimental

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results in the presence of wastewater DOM. For example, the BLM was found to overpredict free copper concentrations, and thus bioavailability and toxicity in wastewater effluent and wastewater impacted receiving waters at ambient total copper concentration.^{11,18} This suggests that the chemical properties of organic ligands in wastewater may differ significantly from those in natural waters.

Previous studies have shown that DOM from wastewater effluent (EfOM) and natural environments (NOM) can have distinct compositions.^{19–22} Analysis of DOM using UV–visible adsorption spectroscopy and fluorescence emission–excitation spectroscopy suggests differences in the aromaticity, hydrophobicity, and protein content present in wastewater effluent in comparison with natural waters. NOM is reported to be composed of mostly humic-like substances with higher hydrophobicity and aromaticity, while EfOM is observed to be more proteinaceous, have lower aromaticity, and have higher hydrophilicity.^{19,20,23} Furthermore, the composition of wastewater DOM can change during the microbial treatment and disinfection stages. Maizel and Remucal²¹ and Verkh et al.²⁴ reported a loss in highly saturated molecular formulas of wastewater DOM after secondary treatment. Verkh et al. also reported the removal of 67% of molecular features and the simultaneous formation of 24% of new features during secondary treatment, while 34% of new molecular features were detected after tertiary treatment.²⁴ These results suggest that Cu binding ligands in wastewater may either be composed of organic ligands that escape removal during treatment or be formed during the treatment steps. Relating the alterations of DOM across treatment steps to Cu-binding properties requires additional analytical approaches to characterize organically complexed Cu.

Untargeted molecular approaches that combine liquid chromatography (LC) with online inductively coupled plasma mass spectrometry (ICPMS) and LC coupled to electrospray ionization mass spectrometry (ESIMS) enable the separation, quantification, and identification of distinct Cu-organic complexes.^{25,26} With this approach, a portion of the Cu-organic complexes in a sample is first extracted by solid phase extraction, and then these complexes are separated by liquid chromatography. The metal ion component of a given complex is detected by online ICPMS or as an intact complex on the ESIMS. The calibrated ICPMS signal is used to quantify chromatographically resolved Cu-organic species, which can include discrete well-resolved complexes that occur as a sharp chromatographic peak as well as polydisperse Cu-species appearing as a chromatographically unresolved complex mixture generated by the decomposition of organic matter. To identify discrete metal complexes, computational algorithms are used to identify Cu complexes by detecting peaks that match the diagnostic isotope pattern of ⁶³Cu/⁶⁵Cu, and to which a Cu-containing molecular formula can be assigned. These tools hold promise for constraining the composition of Cu-binding ligands in wastewater as well as the changes in Cu speciation that accompany treatment steps that remove and alter DOM and Cu.

Here, we employ this novel combination of liquid chromatography with ICP-MS and high-resolution ESIMS (HR-ESIMS) to examine the composition of Cu ligands during wastewater treatment. The main objectives were to (1) identify differences in solid phase extracted Cu-organic species before and after wastewater treatment, (2) quantify the Cu complexes at three treatment stages, and (3) identify the organic compounds in the Cu complexes. Wastewater composite

samples were collected at three locations along the treatment train of a single facility and compared with a sample collected from the river receiving the wastewater discharge. The results from this study demonstrate the applicability of LCMS techniques to obtain molecular information on ligands that complex Cu in waste streams, a major step toward improving the understanding of metal–organic speciation in different aqueous environments.

2. MATERIALS AND METHODS

2.1. Collection of Water Samples

Composite wastewater samples (8 L) were collected with automatic samplers (Teledyne ISCO) over 24 h at the Rock Creek Wastewater Treatment Plant (WWTP) in Hillsboro Oregon, USA. The samples were collected in the fall on October 27, 2021. Unit operations at this plant include coarse screening, primary clarification, activated sludge with enhanced biological nutrient removal, secondary clarification, tertiary treatment for the removal of phosphorus and metals (Actiflo coagulation and settling followed by filtration), and disinfection. Samples were collected from the primary clarifier effluent (PE), secondary clarifier effluent (SE), and final effluent (FE) prior to discharge. The sample collection times were staggered based on the retention times of the treatment stages, which are 8 h for secondary treatment and 2.5 h for tertiary treatment and disinfection. This approach was employed to capture the changes occurring in a volume of water that entered the treatment plant at the time of sampling. An 8 L grab sample of river water was also collected from the Tualatin River, one mile upstream of the WWTP outfall (UPR). The composite samples and grab samples were split into triplicate samples for further processing. All samples were collected in acid-washed (10% HCl) polypropylene bottles, transported on ice, and stored at 4 °C until analyses. The pH and conductivity of each sample were measured after transport to the laboratory, and the results are in Table S1.

2.2. Preparation of Samples for LCMS Analyses

Samples were filtered, conditioned, and concentrated prior to liquid chromatography mass spectrometry (LCMS) analysis, as shown in Figure S1. Briefly, a 47 mm filter paper holder was stacked with a 1.2 μm glass microfiber filter (Whatman, grade GF/C circles, 47 mm) and a 0.22 μm polyethersulfone (Millipore Express PLUS, PES discs, 47 mm) filter and connected to the SPE columns (Figure S1). The filtered samples were sequentially extracted using two SPE columns in series: first, a 1 g Agilent Bond Elut-ENV (styrene-divinylbenzene polymer, SDVB) and then a 0.5 g Supelclean ENVI-Carb (graphitized carbon) column with a peristaltic pump (Masterflex) and acid-washed platinum cured silicone tubing at a flow rate of 20 mL/min. Each sample was extracted in triplicate, and the sample volumes and concentration factors that were used to correct for the concentration in the quantitative analyses are shown in Table S1. The SPE columns were primed with 5 mL of methanol (Fisher LCMS grade) and then rinsed with ultrapure 18.2 MΩ cm water (qH₂O). The columns were rinsed with qH₂O after the extraction to remove salts and stored frozen at −20 °C prior to elution. SPE blanks were prepared for each resin by extracting 2 L of qH₂O in lieu of a wastewater sample.

Prior to analysis, the columns were eluted with LCMS grade methanol (5 mL/g of resin). The methanol was dried down to 0.1 mL residual solvent by vacuum centrifugation, and qH₂O was added to bring the final volume of the ENV extracts to 2 mL and the ENVI-Carb extracts to 1 mL. Aliquots of the ENV extracts were spiked with additional copper (423 μg/L) to determine if there were excess binding ligands in the isolates. These Cu addition samples were shaken at 160 rpm for 24 h to allow equilibration. Before loading samples onto the LCMS, they were centrifuged for 5 min at 3200 rpm and filtered through 0.45 μm hydrophilic polytetrafluoroethylene (4 mm, Millex-LH hydrophilic polytetrafluoroethylene) syringe filters. Cyanocobalamin was added to each sample at a concentration of 1 μM as an internal standard to ensure that the chromatography was reproducible across both LC-ICPMS and LC-Orbitrap MS analyses.

2.3. Metal and Carbon Concentrations

The concentrations of metals (Cu, Co, Pb, Cd, Fe, and Zn) and dissolved organic carbon (DOC) were measured for the 0.22 μm PES filtered samples before and after SPE. For metal quantification using an inductively coupled plasma mass spectrometer (Thermo Scientific iCAP RQ ICP-MS), samples were acidified by adding 70% nitric acid (trace metal grade) to obtain a solution of 2% nitric acid and spiked to 0.5 ppb indium as an internal standard. DOC measurements were obtained using the combustion catalytic oxidation method on a Shimadzu TOC-VCSH Combustion Analyzer.

2.4. Spectroscopic Measurements

Ultraviolet–visible absorption at 254 nm (Thermo Scientific Orion Aquamate 8000) and 3D-fluorescence excitation emission matrices (EEMs) (Varian Cary Eclipse) were used to probe the bulk character of the organic matter prior to SPE as a complement to mass spectrometry. EEMs were collected over excitation wavelengths 200–400 nm and emission wavelengths 300–600 at 5 nm increments, with 10 nm slit width and a scan rate of 600 nm/min. Raleigh and Raman scattering peaks in the EEMs data were corrected using the procedure developed by Zepp et al.²⁷ The specific ultraviolet absorbance at wavelength 254 nm (SUVA_{254}), used to estimate aromaticity and predominant origin of dissolved organic matter (DOM), was calculated using Equation S1 following established methods.^{28,29}

2.5. LC Operating Conditions

Reverse phase chromatographic separations were performed with a bioinert ultraperformance liquid chromatography (UPLC) system (Thermo Scientific Dionex UltiMate 3000 RSLCnano) fitted with polyetheretherketone (PEEK) and titanium tubing and connections. The stationary phase was an ACQUITY Premier CSH Phenyl-Hexyl column (1.7 μm particle size, 2.1 \times 100 mm). Samples were injected in 50 μL volumes during analysis with ICPMS or 15 μL volumes during analyses with Orbitrap MS. The mobile phase solvents were qH_2O buffered with 5 mM ammonium formate (A) and LCMS grade methanol buffered with 5 mM ammonium formate (B). The entire separation was achieved in 36 min at a flow rate of 0.2 mL/min. The first step is a 0.15 min isocratic elution at 95% A, then a 19.85 min linear gradient from 95% A to 95% B, a 5 min isocratic elution in 95% B, followed by a linear gradient from 95% B to 95% A, and held at 95% A for a 10 min re-equilibration to 95% A. The temperature of the column was controlled at 40 $^\circ\text{C}$. Other instrument operating conditions and settings are listed in Table S2.

2.6. ICPMS Conditions

The ICPMS (Thermo Scientific iCAP RQ) was coupled to UPLC via PEEK tubing. Samples were injected in a full loop mode (50 μL). An internal standard (4 ppb indium in 1% nitric acid) was pumped at a constant flow rate of 300 $\mu\text{L}/\text{min}$ to monitor signal intensities throughout the run. A compensation gradient comprised of 63.3% A and 33.3% B was also introduced after the LC column to ensure that the solvent phase remained constant in the ICPMS. Both the indium and compensation gradient solvents were mixed with the eluents from the column with a high-pressure PEEK Tee assembly before going to the ICPMS. Additional details of the instrumentation, operating conditions, and settings are listed in Table S2.

2.7. Orbitrap MS Conditions

For ESIMS, a tribrid orbitrap mass spectrometer (Thermo Scientific IQ-X) with a heated electrospray ionization source was coupled to the same UPLC system during separate analytical runs from the LC-ICPMS analyses. MS1 scans were collected in both positive and negative ionization modes in 500 K resolution mode over the 100–1000 m/z mass range. Other instrument operating conditions and settings are listed in Table S2.

2.8. Data Processing and Analysis

Copper concentrations in the SPE extracts were quantified by analyzing the LC-ICPMS data using a Python-based application described by Dewey et al.³⁰ Briefly, a calibration curve for the metal of interest is prepared with standards (0–200 ppb) that are directly injected into the

ICPMS at the same volume, flow rate, and mobile phase composition used for the sample separation. A linear regression is derived by integrating the peak areas that correspond to the signal response for each metal, as shown in Figure S2. The resulting output is a calibration file that is loaded into the program to determine the total concentration of the metal–organic complexes extracted, by integrating their corresponding chromatographic peak areas. Signal drifts that impact metal concentration are accounted for by applying a correction factor calculated from the signal intensity of 115 In. All concentrations determined for Cu recovered from SPE were well above the limit of detection (0.0005 μM) reported by Dewey et al.³⁰

Compounds containing Cu were characterized by using two complementary computational approaches. First, features in a modified version of the algorithm described by Boiteau et al. were implemented to curate a list of compounds that have an isotope pattern that matches Cu.²⁶ To use this algorithm, the LC-ESIMS datafiles were converted into mzML format (MSconvert, proteowizard) and aligned with the LC-ICPMS data based on the retention time of the cyanocobalamin peak as $[\text{M} + \text{H}]^{2+}$ ($m/z = 678.2910$) in the LC-ESIMS and ⁵⁹Co peak in LC-ICPMS. The algorithm searches the ESIMS scans and produces a list of peaks that have an isotope pattern of copper with the expected mass difference (1.9982 ± 0.002 m/z) and abundance ratios (0.34–0.58) of ⁶⁵Cu vs ⁶³Cu and which were chromatographically correlated ($r^2 > 0.7$). The result includes an extracted ion chromatogram (EIC) with a unique m/z , retention time, and peak intensity for each compound, aligned with their corresponding peak in the LC-ICPMS chromatogram.

Second, Cu-organic complexes detected in orbitrap mass spectra were assigned a molecular formula using CoreMS and Chemcalc.^{31,32} Data were internally calibrated using the CoreMS platform, and molecular formulas were assigned within a mass error of ± 2 ppm. Details on the elements that were included in the formula assignments and other constraints that were applied in CoreMS are listed in Table S3.

3. RESULTS AND DISCUSSION

3.1. Evaluation of Bulk Dissolved Copper and Dissolved Organic Carbon Removal during Wastewater Treatment

Conventional wastewater treatment is intended to remove dissolved organic carbon and heavy metals below regulatory thresholds for discharge into the environment. The extent of removal during each treatment step was first evaluated. The results shown in Figure 1 revealed that the Cu concentration in wastewater decreased by $\sim 73\%$ and DOC by $\sim 66\%$ in the final effluent relative to the primary effluent. The majority of Cu was removed during the secondary treatment with additional significant removal occurring during the final treatment stage (p -value = 0.0098, Welch two sample t test). This is consistent with other studies that have documented major removal of dissolved Cu in wastewater during the secondary stage of treatment primarily due to mechanisms like biosorption to organics.^{33,34} Others have also documented additional removal of Cu after tertiary treatment.¹³ DOC was primarily removed during secondary treatment (no statistical difference between secondary and final; p -value = 0.3634) which was not surprising since secondary biological treatments are designed to break down organic carbon. The results show that organic carbon was only removed during secondary treatment, and any alterations to organic matter during the tertiary treatment stages did not reduce organic carbon concentration.

Despite the net removal of copper and dissolved organic carbon (DOC) during primary treatment, effluent concentrations remained higher than those in the upstream river water (Figure 1). This suggests that discharging treated wastewater slightly elevates the environmental copper and DOC levels. Furthermore, it is likely that the secondary and tertiary

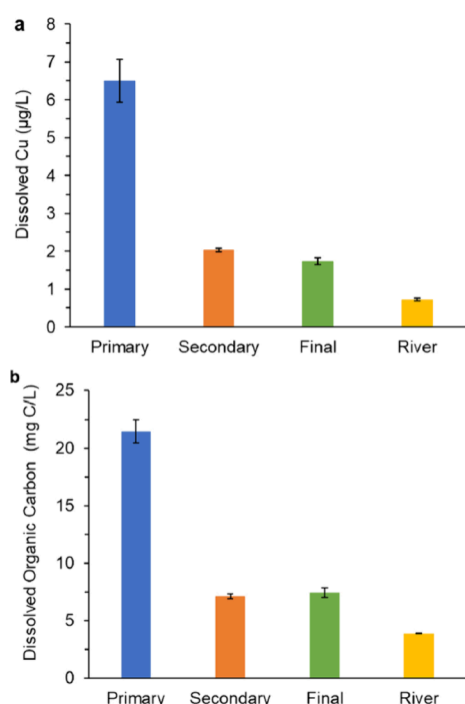


Figure 1. Dissolved copper (a) and dissolved organic carbon (b) measured at the different stages of treatment (primary, secondary, and final) and in the receiving river upstream of wastewater discharge.

wastewater treatment processes selectively removed certain components of both DOC and Cu. In a study by Buzier et al., the researchers observed a 57% decrease in inert Cu (strongly complexed Cu), while the labile fraction increased by 30% after tertiary treatment, highlighting that wastewater treatment does impact copper speciation.¹³ To better understand the chemical forms of copper that remain in the treated wastewater and their potential sources, we next characterized the chemical composition of DOM and copper-organic species across the treatment stages.

3.2. Treatment Increases Organic Matter Aromaticity while Decreasing Microbially Derived Organics

UV absorbance and fluorescence spectroscopy were used to assess changes in the bulk DOM composition due to treatment. $SUVA_{254}$, which reflects the aromaticity of the DOM, was significantly lower in the primary effluent compared to the secondary and final effluent (Table 1). These values are consistent with other reports that show microbial degradation increases wastewater $SUVA_{254}$.^{11,20,21} The increase in DOM aromaticity during treatment may result from the preferential degradation of nonaromatic carbon.^{21,35} Alternatively, it could

be due to the generation of aromatic byproducts, like soluble microbial products, during the activated sludge treatment.^{36,37} Interestingly, $SUVA_{254}$ decreased after the final treatment. Maizel and Remucal also observed a decrease in $SUVA_{254}$ value for the final effluent in their study and concluded that UV disinfection could lead to subtle changes in DOM character.²¹ Addition of coagulants during the tertiary stage of treatment in our study could also alter the composition of DOM, leading to a measured difference in aromaticity.

Fluorescence analyses also revealed a shift to nonprotein/nonmicrobial/humic-like components during treatment. The EEMs spectra in Figure 2 were divided into five regions based on previous classifications.³⁸ DOM in the primary effluent showed the highest activity in aromatic protein regions (I and II) and soluble microbial byproduct region (IV) (Table 1), which are associated with aromatic amino acids and recondensation products.³⁸ Normalizing the fluorescence intensity at the peak center for each region by the DOC concentrations (Table 1) highlighted a decrease in the protein-like and soluble microbial byproduct-like fluorescent DOM components (I, II, and IV) during each stage of treatment. In contrast, the relative contribution of the humic- and fulvic-like components (III and V) increased from the primary effluent to the secondary effluent and to a lesser extent from the secondary to final effluent. This provides additional evidence that while the wastewater treatment process ultimately reduced DOC concentrations the composition of the remaining DOM was altered, with new condensed aromatic humic-like components being formed. Since aromatic functional groups may include phenolic or other ligand binding sites that may complex with Cu,³⁹ it is possible that these OM transformations also lead to differences in organic Cu speciation. However, these bulk organic analyses do not necessarily reflect the chemistry of the minor subset that binds to Cu, and further analyses are needed to directly detect and characterize differences in organic-Cu speciation.

The DOM composition of the final effluent was compared to that of the river water to evaluate how the character of the organic matter in the wastewater discharge may influence the composition of the river organic matter. The river sample had the highest $SUVA_{254}$ value of 4.24 which is within the typical range (4–5) that has been observed for other river samples.²¹ Other studies have also observed the trend toward more humic-like fluorescent DOM in freshwater sources compared to treated wastewater.^{38,40} However, the DOC normalized fluorescent intensities for all five components and, in particular, the humic-like component, were lower in the river water compared to the final effluent. These results qualitatively indicate that the composition of colored and fluorescent DOM in the wastewater

Table 1. Copper and Organic Carbon Concentrations and Measurement of Spectroscopic Properties for Wastewater and Upstream River Samples ($n = 3$)

Sample	UV_{254} (cm^{-1})	$SUVA_{254}$ ($cm^{-1}/L/mg\ C$)	Fluorescence Peak Intensity/DOC				
			Aromatic Protein I (I)	Aromatic Protein II (II)	Fulvic Acid-like (III)	Soluble Microbial Byproduct-like (IV)	Humic Acid-like (V)
Primary Effluent	0.261	1.22 ± 0.06	18.10	39.97	12.13	11.57	10.82
Secondary Effluent	0.188	2.64 ± 0.08	10.38	22.67	25.57	10.18	26.41
Final Effluent	0.170	2.30 ± 0.13	6.78	16.06	25.43	8.41	32.74
Upstream River	0.164	4.24 ± 0.09	6.43	12.90	21.02	1.80	15.94

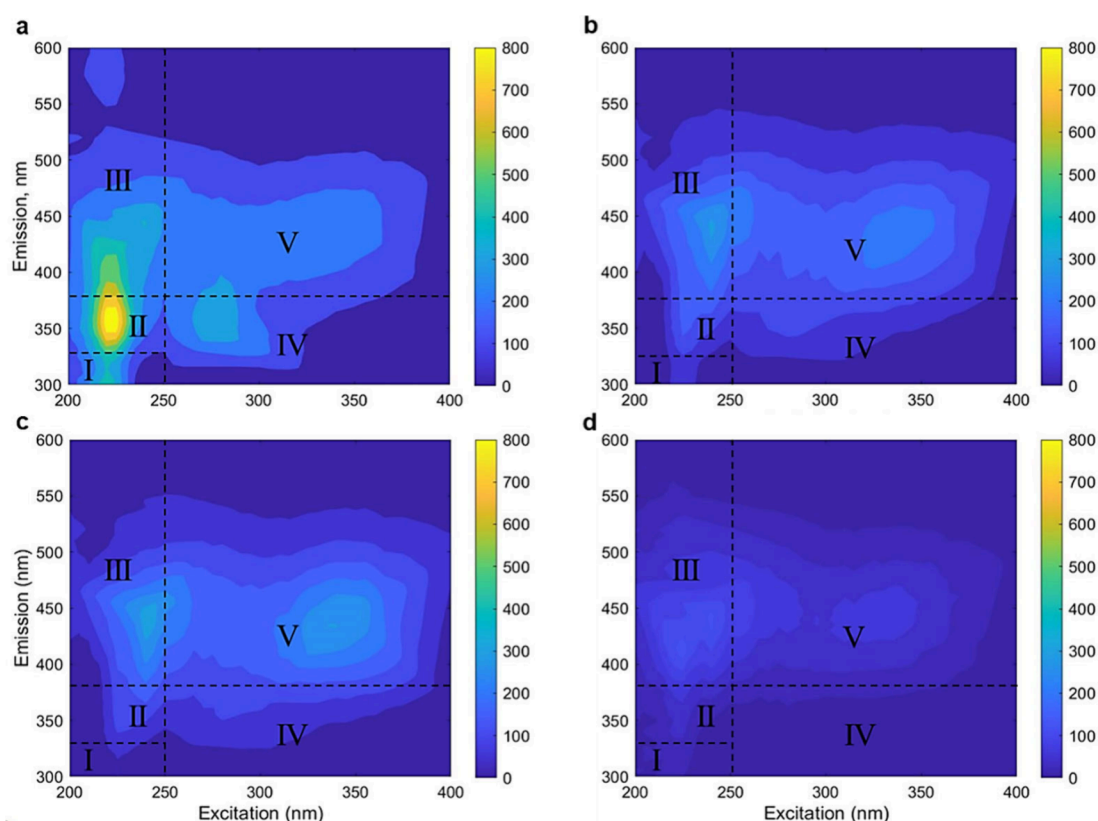


Figure 2. 3D fluorescence excitation–emission matrices for (a) primary effluent, (b) secondary effluent, (c) final effluent, and (d) upstream river. Regions I and II represent aromatic proteins; III are fulvic acid-like; IV are soluble microbial byproduct-like; and V are humic acid-like. Regions are as defined by Chen et al. (2003).³⁸

Table 2. Concentration ($\mu\text{g/L}$) and Percent Composition of SPE-Cu Measured via LC-ICPMS^a

Sample	SPE Cu by Column ($\mu\text{g/L}$)		Percent Composition by Column (%)		Total SPE Cu Ligands ($\mu\text{g/L}$)
	ENV	ENVI-Carb	ENV	ENVI-Carb	
Primary Effluent	0.291 ± 0.085	0.022 ± 0.002	93	7	0.629 ± 0.062
Secondary Effluent	0.189 ± 0.018	0.009 ± 0.000	95	5	0.579 ± 0.034
Final Effluent	0.302 ± 0.020	0.014 ± 0.002	96	4	0.722 ± 0.057
Upstream River	0.164 ± 0.010	0.003 ± 0.000	98	2	0.529 ± 0.003

^aSPE-Cu refers to the mass concentration of copper in the original sample that was recovered from the SPE extractions. Total SPE Cu ligands refers to copper measured after adding $423 \mu\text{g/L}$ additional copper to saturate the ligands in the ENV extracts.

effluent differs from that of the river and hence demonstrates that Cu-organic speciation may differ as well.

3.3. Wastewater Treatment Increases the Hydrophilicity of Copper Binding Ligands

To evaluate how the changes in OM composition during wastewater treatment could alter organic copper speciation, samples were analyzed by LC-ICPMS. Prior to LC-ICPMS, organically complexed copper was isolated and concentrated from the samples via SPE. Mass balance analyses of the extracts from both ENV and ENVI-Carb columns showed that 78–95% of the copper loaded onto the SPE column did not elute with methanol, nor did it elute with acetonitrile or isopropanol. Improving the recovery of Cu from solid phase extraction thus remains a challenge for a comprehensive Cu speciation analysis. Nevertheless, LC-ICPMS enabled the characterization of the composition of copper speciation within the recovered fraction and evaluation of differences between the treatment stages. Over 90% of the recovered copper was present in the ENV SPE extract

rather than the porous graphite SPE extracts (Table 2); thus, subsequent analyses focused on the ENV extracts.

Chromatograms of ENV extracts for the three wastewater samples (Figure 3a) reveal chromatographic profiles that consist of discrete peaks (A–J) superimposed on a broad baseline of unresolved Cu species. The unresolved chromatographic features (the broad baseline) represent complex mixtures of polydisperse Cu-organic species like those that are observed for humic and fulvic acids.³⁰ The resolved peaks correspond to distinct Cu-organic species with specific molecular composition that can be characterized by LC-Orbitrap MS (section 3.4.). Although the noisy baseline from the unresolved complex mixture precluded accurate quantitation of these individual species, their presence and absence could be determined. The separation in reverse phase LC is driven by hydrophobic interactions with the nonpolar stationary phase, and thus retention time relates directly to compound hydrophobicity. The occurrence of these resolved species and their retention times across samples were compared to evaluate their removal

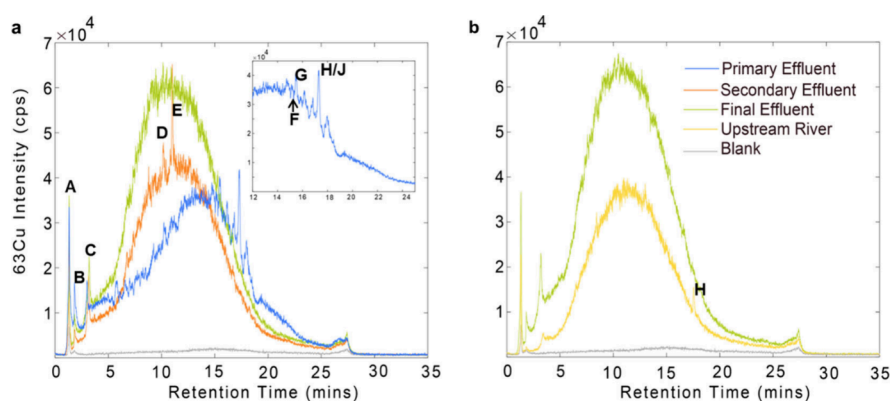


Figure 3. LC-ICPMS chromatogram comparing copper complexes in (a) the three wastewater samples and (b) the final wastewater effluent and river sample upstream of the wastewater discharge. The broad peak represents unresolved complexes, and the lettered peaks are chromatographically resolved organic copper species. **A** is a highly hydrophilic organic or inorganic copper complex not retained by the chromatography column; **B** and **C** are highly hydrophilic organic Cu complexes that were not removed during treatment; **D** and **E** are hydrophilic organic complexes produced during secondary treatment; **F**, **G**, and **J** are hydrophobic organic complexes removed after secondary treatment; and **H** is a hydrophobic copper complex found in wastewater and the river.

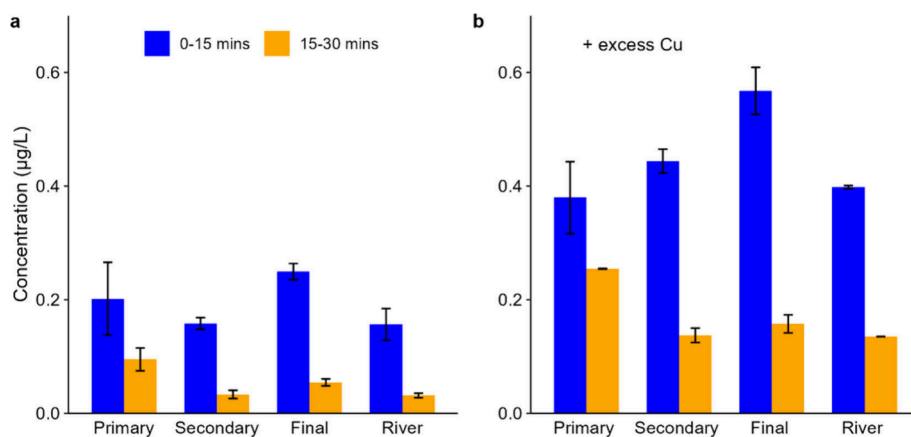


Figure 4. Concentrations of the hydrophilic (0–15 min) and relatively hydrophobic (15–30 min) fractions of SPE Cu complexes (a) and total SPE Cu ligands (b) in wastewater after three stages of treatment (primary, secondary, and final) and the receiving river upstream of the plant.

during different stages of treatment. Peaks **F**, **G**, and **J** were detected in only the primary effluent, which suggests that these Cu complexes were either removed or degraded during secondary treatment. Surprisingly, two discrete species **D** and **E** superimposed on the chromatogram for the secondary effluent were absent from the primary effluent, suggesting that they were produced transiently during secondary treatment. The loss of relatively hydrophobic ligands like **F**, **G**, and **J** from primary to secondary effluent and the subsequent formation of relatively hydrophilic complexes like **D** and **E** are consistent with the oxidation of biodegradable carbon that occurs during the secondary treatment. The addition of oxygen during the activated sludge treatment process likely converts aliphatic and aromatic reduced carbon to oxidized and polar moieties, which would result in an increase in the hydrophilicity of the resulting products.

While some Cu species were present only at specific treatment stages, others (**A**, **B**, and **C**) were present throughout the treatment process, highlighting selective removal of certain copper species. Peak **A** is representative of highly hydrophilic organic or inorganic copper species that are not retained by the hexyl-phenyl chromatography column. Peaks **B** and **C** indicate recalcitrant organic copper complexes that were not removed at any stage of treatment. These results also show that certain

hydrophobic Cu organic species are efficiently removed or transformed during secondary treatment, while other hydrophilic forms escape treatment or are formed during the secondary treatment process.

The trends regarding selective removal of certain hydrophobic Cu-organic species and the generation of new hydrophilic Cu-organic species during treatment were further highlighted by quantifying compounds eluting between 0 and 15 min as hydrophilic and those eluting between 15 and 30 min as hydrophobic (Figure 4). Initial analyses considered 5 min time fractions, but all fractions between 0 and 15 min and 15–30 min exhibited similar trends. Based on the quantification results in Table 2, the overall amount of SPE extracted Cu organic species decreased from the primary to secondary treatment but increased during the final treatment. This is reflected in a decrease in hydrophobic organic copper species in the primary effluent compared to the secondary effluent, as shown in Figure 4a. Meanwhile, hydrophilic Cu complexes significantly increased from the secondary treatment to the final treatment (p-value 0.012, Tukey HSD). The abundance of these complexes could be due to the formation of new unresolved hydrophilic ligands that stabilize dissolved Cu within the wastewater. Alternatively, the degradation of certain complexes

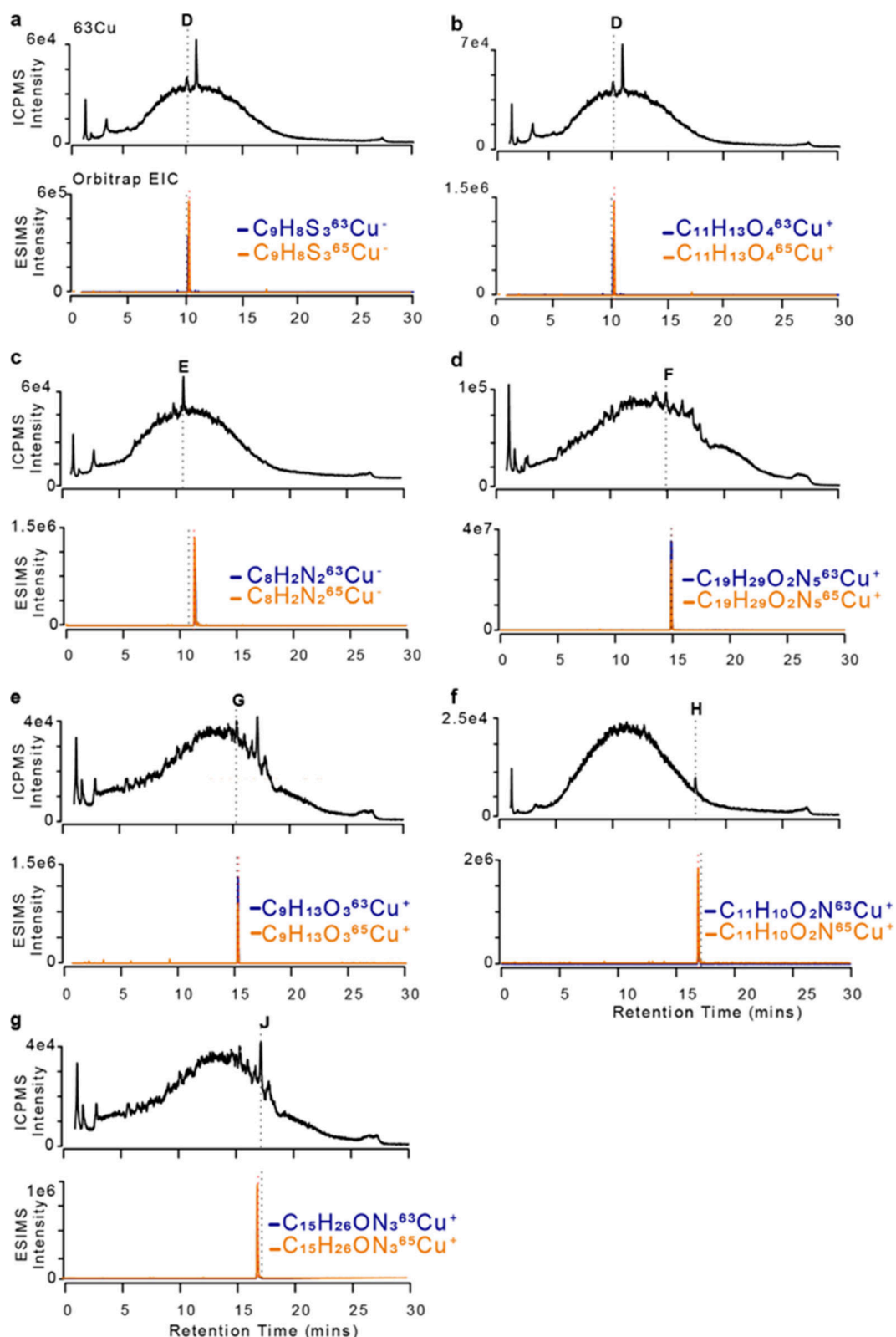


Figure 5. Aligned LC-ICPMS peak (top) and orbitrap EIC (bottom) for resolved Cu complexes from secondary effluent (peaks A–C), primary effluent + excess Cu (peak D), primary effluent (peaks E and G), and upstream river sample (peak H). Molecular formulas were assigned based on detected mass in either positive (peaks B, D–G) or negative (peaks A and C) ion mode.

could result in the release of Cu to saturate existing unbound ligands.

To determine whether the increased abundance of hydrophilic Cu-species was due to the generation of new hydrophilic

ligands or saturation of existing ligands, additional copper (423 $\mu\text{g/L}$) was added to aliquots of the extracts from the ENV column and reanalyzed. The addition of copper saturated excess ligands, enabling quantification of the total SPE extracted Cu

Table 3. Molecular Formula of Distinct Cu Species with the Cu Redox State and m/z Error of Measured Mass and Formula Mass

Peak	Retention Time (min)	Predicted Molecular Formula	Cu redox state	Calibrated m/z	Theoretical m/z	m/z Error (ppm)	Occurrence
D	10.2	$[\text{C}_9\text{H}_8\text{S}_3\text{Cu}]^-$	1	274.9091	274.9090	0.4	Secondary Effluent
D	10.2	$[\text{C}_{11}\text{H}_{13}\text{O}_4\text{Cu}]^+$	2	272.0101	272.0104	−1.1	Secondary Effluent
E	11.3	$[\text{C}_8\text{H}_2\text{N}_2\text{Cu}]^-$	1	188.9516	188.9519	−1.8	Secondary Effluent
F	15.1	$[\text{C}_{19}\text{H}_{30}\text{O}_2\text{N}_3\text{Cu}]^+$	2	423.1694	423.1690	1.05	Primary Effluent
G	15.4	$[\text{C}_9\text{H}_{14}\text{O}_3\text{Cu}]^+$	1	233.0232	233.0233	−0.6	Primary Effluent
H	17.0	$[\text{C}_{11}\text{H}_{10}\text{O}_2\text{NCu}]^+$	2	251.0001	251.0002	−0.2	All samples
J	17.3	$[\text{C}_{15}\text{H}_{26}\text{ON}_3\text{Cu}]^+$	2	327.1370	327.1366	1.3	Primary Effluent

ligand concentration for all samples (Table 2, Figure 4b, and Figures S3–6). Interestingly, the intensity of the EICs of the distinct complexes that were identified (peaks D, E, F, G, H, and J) remained unchanged with the added Cu. This suggests that those ligands were saturated, and the excess ligands that bound the added Cu were mostly unresolvable and heterogeneous. Based on the LC-ICPMS analysis of the spiked samples (Figure 4b), hydrophobic ligands that eluted between 15 and 30 min were significantly more abundant in the primary effluent compared to the secondary effluent (p-value 0.007, Tukey HSD) and final effluent (p-value 0.042, Tukey HSD). This included a series of distinct species that were detected only in the primary effluent (F, G, and J; Figure S3) superimposed on the broad unresolved complex mixture. Hydrophilic ligands eluting under 15 min, however, increased from the primary to the secondary and final effluents and more significantly from the secondary treatment to final treatment (p-value 0.004, Tukey HSD). This confirms that the increased abundance of hydrophilic ligands in the secondary and final effluents was likely not due to the saturation of existing ligands but the formation of hydrophilic Cu ligands during secondary and final treatment. These hydrophilic ligands could be formed by the addition of disinfectants during the final stage of treatment.⁴¹ Additionally, the biodegradation and oxidation of organic matter that occurs during the activated sludge process⁴² could degrade reduced and aliphatic ligands and potentially form new oxidized ligands with increased polarity that complex Cu.

The increase in the hydrophilicity of SPE extracted complexes moving from secondary to final treatment coincided with the increased fulvic acid-like fluorescence intensity (Table 1), suggesting that these ligands may be generated by the same processes and may even represent the same molecular components. Indeed, other studies have shown that carboxylates associated with fulvic acids tend to be the active Cu binding sites of NOM.^{16,43} Previous studies have suggested that fulvic-like DOM components may represent an important fraction of DOM based on similar correlations between humic- and fulvic-like fluorescence and DOM copper complexing ligand concentrations.⁴⁴ Nonetheless, the organic class of fulvic acids represents a large group of molecules, and new molecular characterization techniques like the one discussed in this study are necessary to determine the physiochemical properties of specific ligands that control Cu complexation in different aquatic systems.

We also observed higher concentrations of SPE extracted Cu and the total SPE Cu ligands in the final effluent compared with the upstream river (Table 2). In particular, hydrophilic ligands that eluted between 0 and 15 min were significantly greater in the final effluent compared to the upstream river sample (p-value 6.21×10^{-5} , Tukey HSD). These differences highlight that the ligand pool responsible for copper binding may be more hydrophilic in an anthropogenic water source like wastewater

compared to freshwater. This is consistent with results from a previous study that found that the hydrophilic fractions of effluent organic matter exhibited higher Cu binding affinities and capacities compared to that of NOM.⁴⁵

3.4. Molecular Formula Assignment Reveals Distinct Nitrogen and Sulfur Containing Cu Ligands in Wastewater

To characterize discrete molecules detected in wastewater, the samples were analyzed by LC-Orbitrap MS, and isotope pattern matching algorithms were used to identify the parent molecular ion. These algorithms identify ion peak pairs that match the natural abundance mass difference (1.9982 ± 0.002 m/z) and relative abundance of ^{63}Cu vs ^{65}Cu isotopologues. Molecular formulas were determined for seven complexes (Figure 5, Table 3) that were detected in the wastewater and river extracts. A formula was accepted as a good match if the EICs of the isotopologues were chromatographically correlated as shown in Figure 5 ($r^2 > 0.7$ within a 30 s time interval centered at the peak), and the m/z error was <2 ppm. Four of the 5 formulas detected in positive mode implied that Cu was present in the 2+ redox state when assuming common valencies for C, H, O, N, and S (Table 3). Two copper complexes were detected in negative mode, for which the Cu redox state was 1+ (Table 3). This redox state may be an artifact of negative mode ionization, which has been shown to preferentially generate ions of reduced Cu(I) complexes in other studies.⁴⁶ Two different complexes, one detected in negative mode ($[\text{C}_9\text{H}_8\text{S}_3^{63}\text{Cu}]^-$) and the other in positive mode ($[\text{C}_{11}\text{H}_{13}\text{O}_4\text{Cu}]^+$), were determined as good matches for peak D (Figure 3a) in the secondary effluent, suggesting coeluting complexes that were not separated chromatographically. The molecular formula composition of these seven complexes identified in our study indicates that Cu ligands in wastewater have a range of complexation chemistries.

Previous studies have reported indirect evidence of both nitrogen and sulfur as major chemical moieties that complex copper in wastewater.^{11,23,45} In the study by Yoo et al., the researchers calculated a higher N/C ratio (0.04–0.09) for the strong copper binding hydrophilic fraction of sewage EfOM compared to SRNOM (N/C = 0.02).⁴⁵ Matar et al. also observed a third group of copper ligands with very high binding affinity (Log K = 12.9) that was absent in NOM and attributed that strong complexation to the high nitrogen and sulfur content of EfDOM.¹¹ In our study, four of the seven complexes were all assigned nitrogen containing molecular formulas (Table 3). Among these nitrogen compounds, those only present in the wastewater samples (E, F, and J) exhibited a higher N/C (>0.2 , Table S4) than the average bulk ratio that is commonly found in wastewater or NOM. Additionally, another complex that was only detected in wastewater was highly sulfur rich, $[\text{C}_9\text{H}_8\text{S}_3\text{Cu}]^-$. Although these compounds represent a small subset of the total Cu ligand pool, our findings provide direct molecular evidence for what others have inferred, that nitrogen-

and sulfur-containing compounds are strong chelators for Cu in wastewater.

The complexes identified also provided additional support that less polar, more reduced compounds were preferentially degraded or removed, while more oxidized ones were produced during secondary treatment. The nominal oxidation state of carbon (NOSC) values (Table S4) for the complexes that were treated from the primary effluent (F, G, and J) were more negative (< -0.58) compared to the complexes (D, E) that were produced during secondary treatment ($\text{NOSC} > -0.55$). Additionally, among the oxygen containing complexes, $[\text{C}_{11}\text{H}_{13}\text{O}_4\text{Cu}]^+$ formed during secondary treatment had a higher O/C value (0.36) compared to the ones that were degraded or transformed during secondary treatment (average O/C = 0.17). Interestingly, the EIC for relatively nonpolar copper complex H from the Orbitrap MS (Figure S7) revealed that this complex was present in all the wastewater samples as well as in the upstream river sample. The NOSC value calculated for this complex was also higher (-0.36) compared to those that were removed during treatment (< -0.58) which further suggests that more oxidized organic ligands that complex Cu are more likely to escape wastewater treatment. Future work that targets these molecules for fragmentation analysis is needed to gain additional insight into the structure and functional groups of these Cu ligand complexes. Such information may provide insight into why certain complexes elude treatment or are generated during treatment, with the potential to be released into the environment.

4. CONCLUSIONS AND IMPLICATIONS

Our results demonstrate that while wastewater treatment removes Cu, the remaining copper is bound to different types of ligands, whose hydrophilicities increase during secondary treatment. We discovered that the complexes that were removed or transformed tended to be more hydrophobic. Conversely, the transformation products were more hydrophilic, which was consistent with the oxidative degradation processes. These transformation products could be potentially released into the receiving water. Furthermore, the identification of distinct nitrogen- and sulfur-containing Cu complexes is consistent with previous suggestions that these elements may act as important Cu coordinating atoms. These complexes are valuable targets for future work to determine their chemical structures and Cu binding domains. Such information will be useful for determining the processes that generate Cu ligands during wastewater treatment. Further work is also needed to understand the Cu binding parameters of these ligands to improve Cu^{2+} prediction by existing speciation models.

Our study also highlights that most Cu ligands in wastewater are too polar to be retained by common solid phase extraction approaches using polystyrene divinylbenzene resin. While other SPE columns such as the graphitized carbon used in the study can effectively remove a greater fraction of organically complexed Cu from wastewater, recovery from the SPE column remains a challenge. This suggests that there are likely other important types of polar Cu ligands in wastewater that still need to be characterized. Future work should investigate different SPE columns or target new strategies, like reverse osmosis,⁴⁷ to isolate organic copper complexes from wastewater.

The bioavailability and toxicity of Cu are related to its complexation by organic ligands. To better understand and address the discrepancies between the BLM model predictions and measurements of DOM binding to Cu in wastewater,

additional information is needed regarding the composition and sources of Cu-binding ligands. These discrepancies suggest the presence of other ligands, such as microbially derived and anthropogenic compounds containing nitrogen or sulfur functionalities, which are not currently represented in the model.^{11,18} Our findings provide clear molecular insights that align with previous research and introduce a new analytical approach to advance the understanding of Cu ligands in wastewater. This is crucial for improving the current understanding of DOM binding to Cu in water and provides relevant information to address the discrepancies between BLM model predictions and experimental measurements. Such information contributes to improving Cu speciation models and mitigating the environmental impact of Cu pollution.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsenvironau.4c00114>.

Scheme of the sample filtration and solid phase extraction; Calibration curve for quantifying organic copper measured via LC-ICPMS; LC-ICPMS chromatograms for all samples showing ^{63}Cu intensity before and after the addition of excess copper; Extracted ion chromatogram for complex H detected in all samples; Equation for calculating SUVA_{254} ; Sample pH, conductivity, and volumes extracted; Additional information on the ICPMS and Orbitrap ESIMS operating conditions; Additional molecular properties of the individual complexes that were assigned molecular formulas (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Rene M. Boiteau – Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455, United States; orcid.org/0000-0002-4127-4417; Email: rboiteau@umn.edu

Authors

Laurinda Nyarko – School of Chemical, Biological, and Environmental Engineering, Oregon State University, Corvallis, Oregon 97331, United States

Christian Dewey – Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455, United States; orcid.org/0000-0003-1954-8298

Jeffrey A. Nason – School of Chemical, Biological, and Environmental Engineering, Oregon State University, Corvallis, Oregon 97331, United States

Complete contact information is available at: <https://pubs.acs.org/10.1021/acsenvironau.4c00114>

Author Contributions

CRediT: **Laurinda Nyarko** conceptualization, formal analysis, investigation, methodology, visualization, writing - original draft; **Christian Dewey** formal analysis, methodology, writing - review & editing; **Jeffrey A. Nason** conceptualization, formal analysis, supervision, writing - review & editing; **Rene M. Boiteau** conceptualization, formal analysis, investigation, methodology, supervision, writing - original draft.

Notes

The authors declare no competing financial interest.

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