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DOM Molecular Weight Fractionation and Fluorescence Quantum Yield Assessment Using a Coupled In-Line SEC Optical Property System

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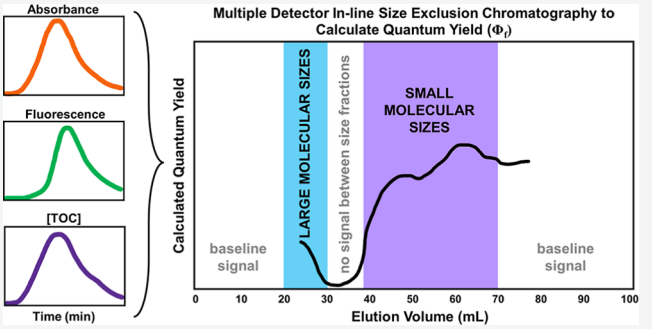
ABSTRACT: Size exclusion chromatography (SEC) in combination with optical measurements has become a popular form of analysis to characterize dissolved organic matter (DOM) as a function of molecular size. Here, SEC coupled with in-line absorbance scans and fluorescence emission scans was utilized to derive apparent fluorescence quantum yield (Φ_f) as a function of molecular weight (MW) for DOM. Individual instrument-specific SEC-fluorescence detector correction factors were developed by comparison of an SEC-based excitation emission matrix (EEM) to an EEM generated by a calibrated benchtop fluorometer. The method was then applied to several sample sets to demonstrate how to measure the Φ_f of unknown DOM samples and to observe changes to Φ_f following a processing mechanism (ozonation). The Φ_f of riverine water samples and DOM fulvic acid isolates from Suwannee River and Pony Lake increased from < 0.5% to a maximum of ~2.5−3% across the medium- to low-MW range. Following ozonation of PLFA, Φ_f increased most notably in the large-MW fractions (elution volumes < 40 mL). Overall, this method provides a means by which highly fluorescent size fractions of DOM can be identified for more detailed analyses of chemical composition and its changes through different processing mechanisms.



KEYWORDS: *dissolved organic matter, size exclusion chromatography, fluorescence, quantum yield, ozone, optical properties*

1. INTRODUCTION

Dissolved organic matter (DOM) is composed of a diverse mixture of compounds originating from the molecular remnants of plants, animal materials, and microbial exudates. DOM represents a major part of the global carbon cycle and is an important factor in numerous chemical and physical processes in natural and engineered systems.[1,2] For example, DOM serves as a substrate for microbial growth and can complex with metals and organic pollutants, impacting their fate in natural waters.[3,4] Additionally, DOM impacts water treatment processes, including reactions with chlorine, resulting in the formation of disinfection byproducts, some of which are harmful to humans if consumed.[5,6] However, due to the complex chemical composition of DOM, determination of its characteristics relies on the development and application of numerous analytical methods.[7]

One property that has received considerable attention in the study of DOM is its average molecular weight (MW) and the overall size distribution of sub-components. Although MW can be assessed using different techniques (e.g., vapor pressure osmometry, field flow fractionation, and high-resolution mass spectrometry[8−12]), most assessments are based on the use of size exclusion chromatography (SEC).[13−15] SEC can be used to determine the apparent MW (AMW) distribution of DOM. Determination of the AMW (in contrast to absolute molecular weight) is based on the fact that the separation is not strictly due to molecular weight but instead based on hydrodynamic size, which is affected by solution chemistry and non-ideal interactions within the SEC column.[16] Applications of SEC for the study of DOM include systems where quantification is based on carbon, nitrogen, or optical properties, therefore offering different qualitative and quantitative information about the samples.[13]

The application of fluorescence spectroscopy for the study of DOM has gained significant attention over the past 30 years.[17−20] Three-dimensional fluorescence excitation emission matrices (EEMs) are popularly used to distinguish source

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origin and inform physicochemical properties of DOM.[20−23] While fluorescence offers the possibility to collect signals with high sensitivity and relative simplicity,[24−26] the specific chemical components responsible for DOM fluorescence have yet to be identified.[27] Understanding the chemical characteristics of the main types of fluorophores within DOM would help to address deficiencies in fluorescence analysis, such as spectral overlap between fluorophores and the impacts of local environments on fluorescence signals (see Section S3 in the Supporting Information for an expanded discussion on expected chemical groups responsible for absorbance and fluorescence of DOM). Insights into fluorophores highlight fluorescence properties that are sensitive to differences in DOM source and composition and inform how they can be applied, such as the use of DOM fluorescence as a surrogate for wastewater impact.[28]

One fluorescence-based metric, the fluorescence quantum yield (Φ_f), describes the fraction of photons reemitted via fluorescence relative to the number of absorbed photons.[29,30] Φ_f is an intrinsic parameter (i.e., independent of concentration) and has been used to characterize the optical properties of DOM in different environments.[28,31−35] For example, Φ_f differentiated between effluent organic matter (EffOM) and naturally occurring DOM in wastewater blends with greater statistical power than other optical metrics.[28] Differentiation was ultimately possible because different fluorophores and chromophores existed at different relative abundances in each type of DOM.

While Φ_f is a sensitive measure used to quantify the unique fluorescence efficiencies of compounds, only the apparent Φ_f value of DOM can be determined for bulk-water samples by traditional fluorescence spectroscopy. This is because DOM represents a mixture of absorbing and fluorescing compounds summed by one apparent Φ_f value, where typical bulk values are in the order of 1−3% and are suppressed by nonfluorescing chromophores.[26,31−33,36] Therefore, to use Φ_f to further characterize the DOM mixture, it would be useful to fractionate bulk-water DOM from which varying Φ_f intensities can be observed for a single sample. It was reported previously that fluorescence to absorbance ratios are MW-dependent and that this ratio is the greatest for smaller-MW fractions.[37−39] Boyle and co-workers also found that among several DOM samples, the Φ_f increased with decreasing sample MW.[40] From these studies, it can be seen that (i) Φ_f varies between fractions of a given DOM sample and (ii) Φ_f is likely correlated to DOM MW. It should be noted that throughout the rest of this text, "Φ_f" refers to "apparent fluorescence quantum yield".

This study presents an SEC system in which Φ_f is calculated in-line as a function of AMW, while in-line total organic carbon (TOC) concentration measurements are used to identify the presence of spectroscopically undetectable DOM. To do this, dissolved organic carbon concentration (DOC), absorbance, and fluorescence were combined with a SEC system so that each signal was essentially collected simultaneously as a function of AMW during analysis. To demonstrate the application of the SEC system to characterize the Φ_f distribution within DOM, data are presented on the analysis of several DOM samples, consisting of riverine samples and ozonated DOM isolates. The goal of using this system was to better understand the fundamental properties of fluorescence in DOM, while also allowing the investigation of changes to fluorophores across a processing mechanism in natural and engineered systems.

2. MATERIALS AND METHODS

2.1. Instrumentation. The SEC system was composed of an Agilent 1260 high-performance liquid chromatography (HPLC) setup that included an Agilent 1200 Series Vacuum Degasser, an Agilent 1200 Series G1310A Isocratic Pump, an Agilent 1260 Infinity Series G1315D Diode Array Detector (DAD), an Agilent 1260 Infinity II Series G7121B Fluorescence Detectors (FLD), and a Sievers M9 TOC Analyzer. Absorbance and fluorescence signals were recorded directly by the Agilent OpenLab software (Rev. C.01.09). An Agilent Universal Interface Box II was utilized to transfer data from the TOC analyzer to the Agilent software in voltage units, which were later converted to DOC concentration ($mg_C\ L^{-1}$) (see the Supporting Information, Text S1.2.3, for a detailed description of conversion). Note that because samples were filtered through 0.45 μm polyethersulfone (PES) filters, analysis results from the TOC analyzer can be considered DOC. A schematic of the instrumental setup for the SEC system is shown in Figure 1.

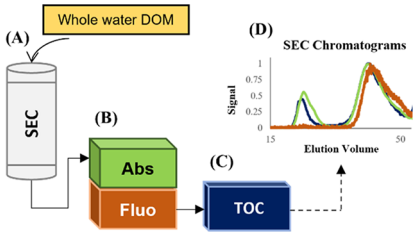

Figure 1. Schematic of the SEC system. Bulk water samples are injected into the SEC column (A). After eluting from the column, the sample passes through the absorbance (Abs) and fluorescence (Fluo) detectors (B) and then travel to the Sievers M9 TOC analyzer (C). The in-line coupled system allows for the determination of multiple optical metrics of the DOM based on apparent molecular weights (AMW), including Φ_f (D).

The size-based separations were achieved using a Toyopearl HW-50S column (internal diameter 20 mm × 25 cm, 92 mL total volume). Samples were injected via an Agilent Technologies 1100 Series G1328B Manual Injector Assembly with a Rheodyne 7725i Injection Valve and a 2 mL injection loop. The mobile phase consisted of phosphate buffer (0.0016 M Na_2HPO_4, 0.0024 M NaH_2PO_4, and 0.031 M Na_2SO_4, pH 6.8, ionic strength 0.1 M, see the Supporting Information, Table S1, for a full list of chemicals used and their sources) that was pumped at a flow rate of 1 mL min^{-1}. This mobile phase composition aimed to reduce unwanted column interactions and follows the methods of Her and co-workers.[14,41] The Agilent DAD was set to scan from 200 to 700 nm in 2 nm increments, and the Agilent FLD was operated in the multi-emission scan mode at λ_{ex} = 350 nm, λ_{em} = 350−700 nm at 5 nm increments. These settings were required for accurate spectral corrections (e.g., inner-filter effect corrections) and calculation of Φ_f for the different MW fractions of the DOM.

To properly align the different detector signals, salicylic acid (SA) (a single compound with well-described absorbance and fluorescence spectra)[21,29,31,32,42] was injected at a concentration of 5 mg L^{-1} and peak elution volumes were then used to account for inter-detector volume between absorbance, fluorescence, and DOC detectors. On average, the volumetric difference between the absorbance and fluorescence detectors

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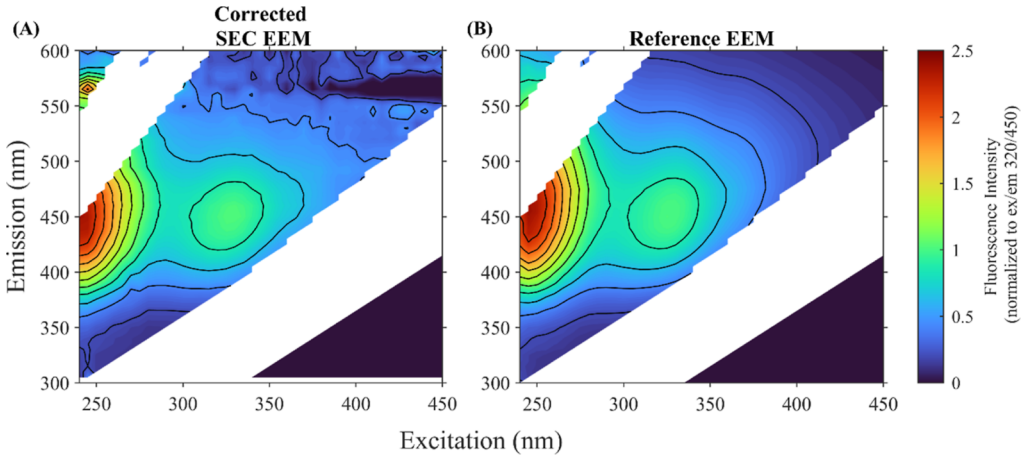

Figure 2. Corrected SRFA sample EEMs from the inline SEC−FLD (A) and the off-line benchtop fluorometer (B). Excitation wavelengths are plotted on the *x*-axis, and emission wavelengths are plotted on the *y*-axis. Both EEM FIs were normalized to excitation 320 and emission 450 nm for a spectral comparison.

was approximately 0.05 mL and 2.8 mL between the absorbance and TOC detectors.

All SEC analyses lasted 150 min (a total elution volume of 150 mL). Although all the compounds should theoretically have eluted well before 150 min (the void volume and bed volumes for the system were approximately 23 and 75 mL, respectively), compounds can experience non-ideal interactions causing them to elute after the bed volume.[13,14,43,44] Thus, extra time was utilized to ensure that all detectors returned to baseline. Data from the beginning of the run (i.e., before the elution of any compounds) as well as the end of the run (i.e., after the elution of all compounds and all detectors had returned to baseline) were treated as blanks to apply baseline corrections. Following Her, 2003, the SEC column was initially calibrated with polyethylene glycols (PEGs) to ensure that the results were comparable to previous studies (data not shown).[41] However, discrete MW values or cutoffs were not provided because of the relative nature of SEC. That is, molecular separation is dependent on hydrodynamic size and is affected by solution chemistry and non-ideal interactions within the SEC column, resulting in differing AMW estimations and AMW distributions.[14,16,43−46] In addition, commonly used calibration standards (e.g., polystyrene sulfonates and PEGs)[14,43] are uniform compounds, while DOM is a complex mixture of chemically diverse compounds.[45] Therefore, in this study, chromatographic results were presented in terms of elution volume and interpreted qualitatively with respect to AMW (i.e., small-, medium-, and large-AMW regions).

Bulk-water characteristics were measured for all samples using a spectrophotometer (Hach DR 6000; Hach, Company, CO, USA), a spectrofluorometer (Horiba Jobin Yvon Fluoromax-4; Horiba, Japan), and a DOC analyzer (Sievers M5310C TOC analyzer; Suez Water Technologies, CO, USA). A full description of the analysis methods is included in the Supporting Information, Table S3.

2.2. Samples. A total of nine natural water samples were collected from a subsection of Boulder Creek that flows through the City of Boulder, Colorado, and the suburban land surrounding the city as well as South Boulder Creek near the junction with Boulder Creek (see the Supporting Information, Figure S1, for exact sampling locations). Samples were collected in 250 mL pre-washed and combusted glass bottles, wrapped in foil to exclude light, stored in coolers on ice, and immediately transported to the University of Colorado Boulder. All samples were passed through pre-washed 0.45 μm pore size PES filters and transferred into pre-washed and combusted 40 mL amber vials for storage at 4 °C in the dark until analysis. Prior to analysis, 15 mL aliquots of each sample were spiked with ∼1 mL of a concentrated mobile phase solution (0.016 M Na_2HPO_4, 0.024 M NaH_2PO_4, and 0.031 M Na_2SO_4), added dropwise, to match the ionic strength and pH of the mobile phase of the column. In this way, samples are essentially constituted in the mobile phase and non-ideal interactions are suppressed as samples exchange into the mobile phase while entering the column after injection.

DOM fulvic acid isolates were obtained from the International Humic Substances Society (St. Paul, MN, USA). Suwannee River fulvic acid (SRFA, 2S101F) was used as the sample to verify method accuracy, and Pony Lake fulvic acid (PLFA, 1R109F) was used for ozonation experiments. Stock solutions of ∼100 $mg_C\ L^{-1}$ were prepared in 100 mM phosphate buffer (pH 6.8) for each isolate. The solutions were stirred continuously for 24 h and then filtered with ultrapure water pre-washed 0.45 μm (PES) filters. The exact carbon concentration was measured using a Sievers M5310C TOC analyzer.

For the ozonation experiments, pure oxygen was fed to an ozone (O_3) generator model TG-40 (Ozone Solutions), and the obtained O_3/oxygen gas mixture was bubbled into a 2 °C water jacketed 2 L glass reactor filled with ultrapure water. The obtained (O_3) stock solution had a concentration of ≈45 $mg_{O3}\ L^{-1}$, which was measured spectrophotometrically using a 0.2 cm pathlength quartz cell with an absorbance value of 3200 $M^{-1}\ cm^{-1}$ at λ = 260 nm.[47] Appropriate amounts of the O_3 stock solution were added to 5$mg_C\ L^{-1}$ PLFA samples to create various specific ozone doses (0.05, 0.1, and 0.2 $mmol_{O3}\ mmol_C^{-1}$), similar to ozonation steps in drinking or wastewater treatment (0.36−1.16 $mg_{O3}\ mg_C^{-1}$).

2.3. Method Development. *2.3.1. Development of Correction Factors for the FLD.* Before utilizing the data from the FLD to calculate Φ_f, the spectral bias of monochromators and charge-coupled device detectors had to be considered by applying correction factors. Typically, correction factors are generated by comparing National Institute of Standards and Technology (NIST) certified data

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of fluorescence standards, such as NIST SRM2942-4 or Rhodamine-B, to the uncorrected fluorescence spectra.[29,48] However, such standards are most commonly solid blocks or come pre-filled into sealed cuvettes and are incompatible with HPLC detector cells with non-standard dimensions and low volumes. Therefore, this study utilized a method whereby a sample EEM is measured by the FLD without prior separation (i.e., the analytical column was removed from the system) at a very low flow rate (0.025 mL min^{-1}). The low flow rate allows enough time to collect measurements for a single fraction across multiple excitation wavelengths (while entire emission spectra are measured) using the Agilent 1260 Infinity FLD. The obtained spectra were then compiled into an SEC-based EEM and compared against the EEM measured on a calibrated stand-alone fluorometer. In our study, the Agilent 1260 Infinity FLD data were compared to the calibrated Horiba Jobin Yvon Fluoromax-4 using SRFA (2S101F) as the standard. The Supporting Information, Figure S6, shows the obtained correction factors.

2.3.2. Verification of In-Line Fluorescence Data. Comparisons of the corrected SRFA EEM measured using the in-line method to the corrected SRFA EEM measured on the reference benchtop fluorometer were made to verify the adequacy of the correction factors (Figure 2). Corrected fluorescence spectra were highly similar at wavelengths with strong emission fluorescence intensities (FIs) (i.e., λ_{ex} = 250−400 nm and λ_{em} = 350−500 nm), while in low emission intensity regions < 350 nm and > 500 nm, SEC-based EEM signals were relatively noisy. Because the noise occurs in regions where the fluorescence signal is typically weak (λ_{ex} > 400 nm and λ_{em} > 550 nm) and not in the wavelengths used for Φ_f calculations, the calculated Φ_f are not significantly impacted.

Additionally, correction factors were applied to a second reference standard analyzed by the SEC system, quinine sulfate (QS), for which its fluorescence spectrum is well defined. QS has a fluorescence excitation/emission maximum at 347 and 455 nm, respectively, and well-characterized emission in the range of 400−530 nm.[48] The fluorescence spectrum of QS overlaps strongly with fluorescence emission of DOM, especially at λ_{ex} = 350 nm, which was the excitation wavelength chosen for this study. For these reasons, QS is a good reference standard for DOM research and commonly used in the field.[21,29] QS was prepared at a concentration of 10 mM in 0.1 N H_2SO_4 and analyzed by the SEC absorbance and fluorescence detectors using 0.1 N H_2SO_4 as the mobile phase. Because the SEC column is limited to a pH range of 2−13, this analysis was conducted with the column removed from the system. The results are displayed in Figure 3, where the emission spectrum of QS is closely replicated, with all data points falling within the error range of the reference spectrum.

2.3.3. Verification of In-Line Φ_f Calculation. As previously stated, Φ_f is defined as the ratio of the number of photons emitted via fluorescence to the number of absorbed photons. The value of Φ_f for a compound is calculated by comparison to a standard for which the absolute Φ_f is known.[29,30] Standards are typically pure compounds for which the Φ_f yield does not vary with excitation wavelength. While QS dissolved in H_2SO_4 is often used for this purpose,[28,30−32,42] this solution is not compatible with the SEC column (see Section 2.3.2). In this study, SA was used as a Φ_f standard as it is well characterized,[31,49] can be readily dissolved in the SEC mobile phase, and thus can be analyzed under the typical instrumental

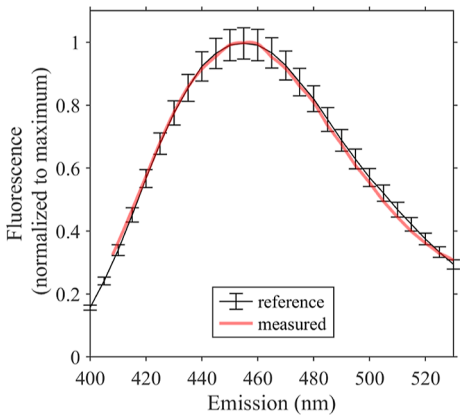

Figure 3. Comparison of the corrected SEC QS fluorescence emission spectrum (red) to the referenced spectrum (black). Emission spectra were obtained at λ_{ex} = 350 nm. FIs were normalized to the peak maximum to account for differences in concentration (*y*-axis).

conditions described in Section 2.1. The Φ_f values were calculated using eq. 1[42]

$$\frac{\Phi_{fDOM}}{\Phi_{fSA}} = \frac{\int_0^\infty I_{DOM}(\lambda_{ex})d\lambda_{em}}{A_{DOM}(\lambda_{ex})} \times \frac{A_{SA}(\lambda_{ex})}{\int_0^\infty I_{SA}(\lambda_{ex})d\lambda_{em}} \quad (1)$$

where $\Phi_{f,DOM}$ and $\Phi_{f,SA}$ are the Φ_f for DOM and SA, respectively; $A_{DOM}(\lambda_{ex})$ and $A_{SA}(\lambda_{ex})$ are the absorbance values of DOM and SA (at the fluorescence excitation wavelength), respectively; and $I_{DOM}(\lambda_{ex})$ and $I_{SA}(\lambda_{ex})$ indicate the FIs at the excitation wavelength and are integrated across the range of emission wavelengths ($d\lambda_{em}$). A 5 mg L^{-1} standard of SA was prepared in the mobile phase and analyzed by the SEC system under the same conditions described in Section 2.1, and the results were compared to the Φ_f reference value for SA.

The measured Φ_f for SA agrees well with a reference value of 36%,[31,49] with deviations less than 4.8% of the reference value (Figure 4). Notably, during data processing, Φ_f was calculated

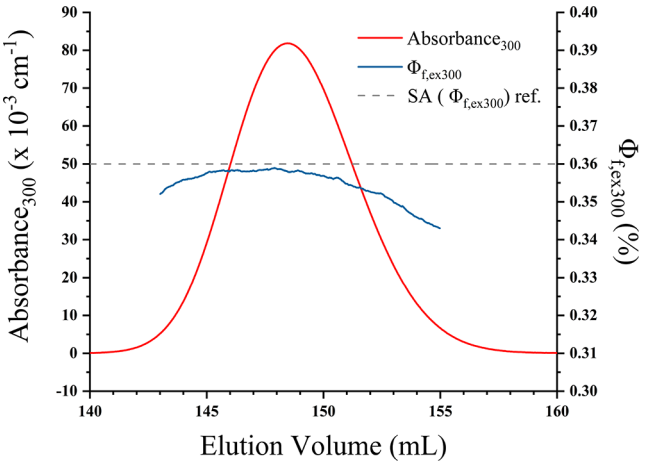

Figure 4. Absorbance and fluorescence quantum yield (Φ_f) SEC chromatograms for SA. Elution volume in mL is plotted on the *x*-axis; absorbance and percent fluorescence (Φ_f) values are plotted on the primary and secondary *y*-axes, respectively. The red line shows the chromatogram of absorbance at 300 nm, and the blue line shows Φ_f at λ_{Ex} = 300 nm. The reference value for $\Phi_{f,Ex=300}$ is 36%[49] and is shown by the gray dashed line.

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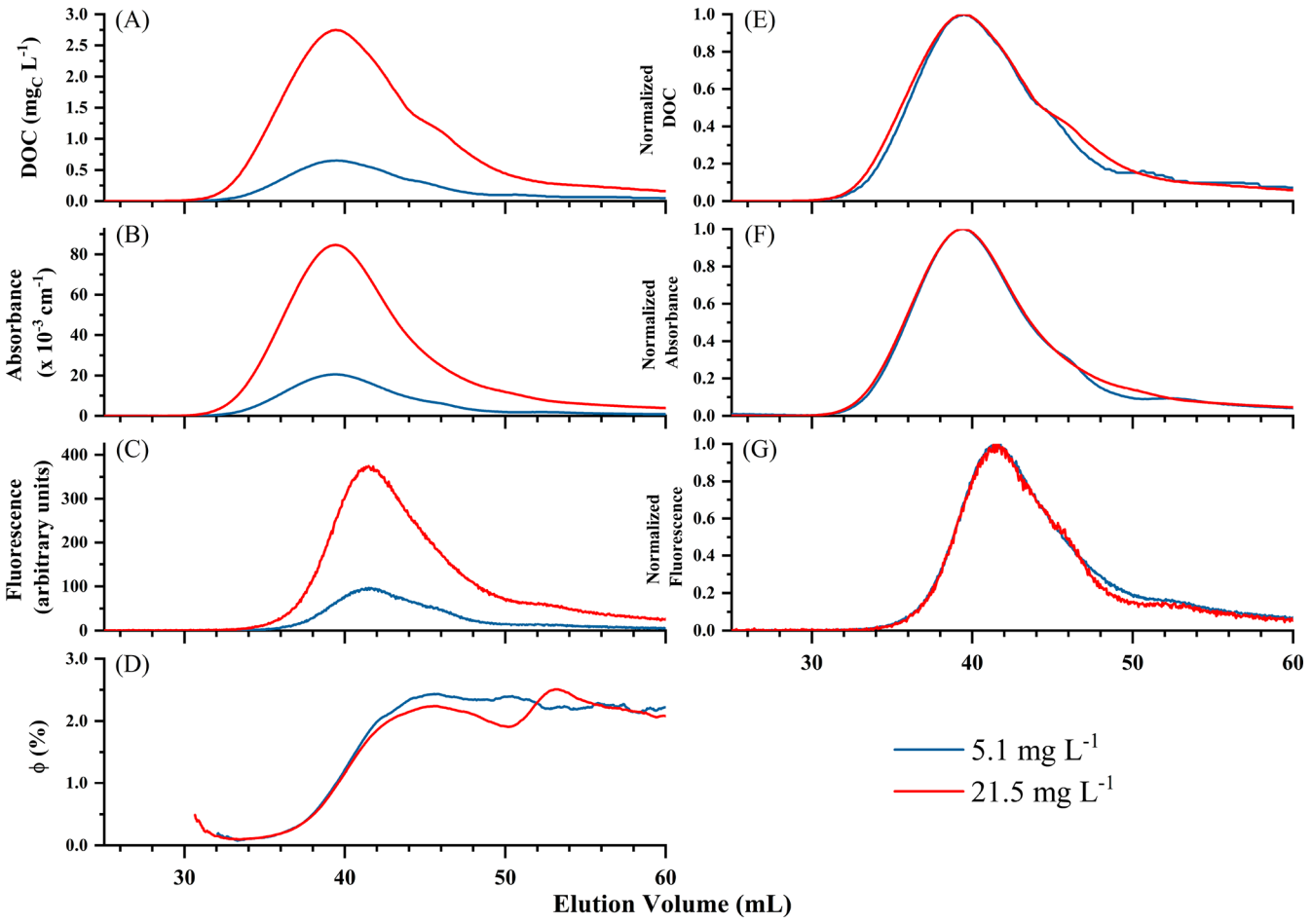

Figure 5. SEC chromatograms from the in-line system for SRFA (5.1 and 21.5 mg$_C$ L^{-1}). (A) DOC, (B) absorbance (λ = 350 nm), (C) fluorescence (λ_{ex} = 350 nm, λ_{em} = 390−700 nm), and (D) fluorescence quantum yield. (E−G) DOC, absorbance, and fluorescence chromatograms normalized to the emission peak maximum. Red chromatogram lines show SRFA (21.5 mg$_C$ L^{-1}), and blue chromatogram lines show SRFA (5.1 mg$_C$ L^{-1}). Absorbance was obtained at 350 nm; fluorescence and Φ_f were obtained at λ_{Ex} = 350 nm.

only when absorbances were above 0.5 × 10^{-3} cm^{-1}; below this threshold, data were noisy and Φ_f was unreliable. This was an important limitation for analyzing samples with very low concentrations (i.e., natural water samples, as shown below).

2.3.4. Verification of Method Accuracy. SRFA was analyzed at two concentrations (5.1 and 21.5 mg$_C$ L^{-1}) to verify method accuracy. Specifically, it was verified that (i) SEC chromatographic profiles of the same material are invariant with concentration (i.e., elution volume remains constant); (ii) DOC, absorbance, and fluorescence signals are proportional to concentration for the same sample at different concentrations;[25,50] and (iii) Φ_f is independent of concentration[28] (Figure 5). Tucker congruence coefficients (TCCs) were calculated to compare the normalized chromatograms of the 5.1 mg$_C$ L^{-1} sample to that of the 21.5 mg$_C$ L^{-1} for each signal. These TCC values were determined to be 0.998, 0.993, and 0.999 for DOC, absorbance, and fluorescence, respectively, indicating excellent agreement (TCC > 0.95 indicates that two components can be considered equal)[51] between normalized chromatograms of the two concentrations (Figure 5E−G) (refer to Supporting Information Text S2.4 for TCC calculations). The chromatographic peak maximum ratios of DOC, absorbance, and fluorescence (ratios of SRFA chromatographic maximums of two concentrations) for SRFA concentrations are 0.237, 0.242, and 0.257, respectively, representing errors of 3.5, 1.4, and 4.8% (see Supporting Information S2.5 for percent error calculations). The Φ_f profiles for the different SRFA concentrations overlay each other, indicating that the same Φ_f values were calculated for elution volumes of ∼32−42 mL. However, in Figure 5D, at ∼42 mL, Φ_f began to differ between the two concentrations. This results from improved resolution and accuracy of the fluorescence and absorbance signals at a higher sample concentration and not to a change in Φ_f, which is an intrinsic property. Thus, for the 5.1 mg$_C$ L^{-1} standard, the Φ_f signal increased to ∼2.5% (at ∼45 mL), where it remained (for elution volumes > 45 mL), although the signal variance increased. For the 21.5 mg$_C$ L^{-1} standard, two distinct Φ_f peaks were seen at ∼45 and ∼52 mL before the signal variance increased.

3. RESULTS AND DISCUSSION

3.1. Applications of the Coupled SEC System for the Quantification of Φ_f Distribution. *3.1.1. DOM from Boulder Creek.* The SEC-Φ_f method was applied to assess the Φ_f distribution for aqueous samples collected from Boulder creek. Figure 6 shows the DOC, absorbance, fluorescence, and Φ_f as a function of AMW for a subset of three Boulder Creek samples (SEC data for the additional Boulder Creek and South Boulder Creek samples are provided in Supporting Informa-

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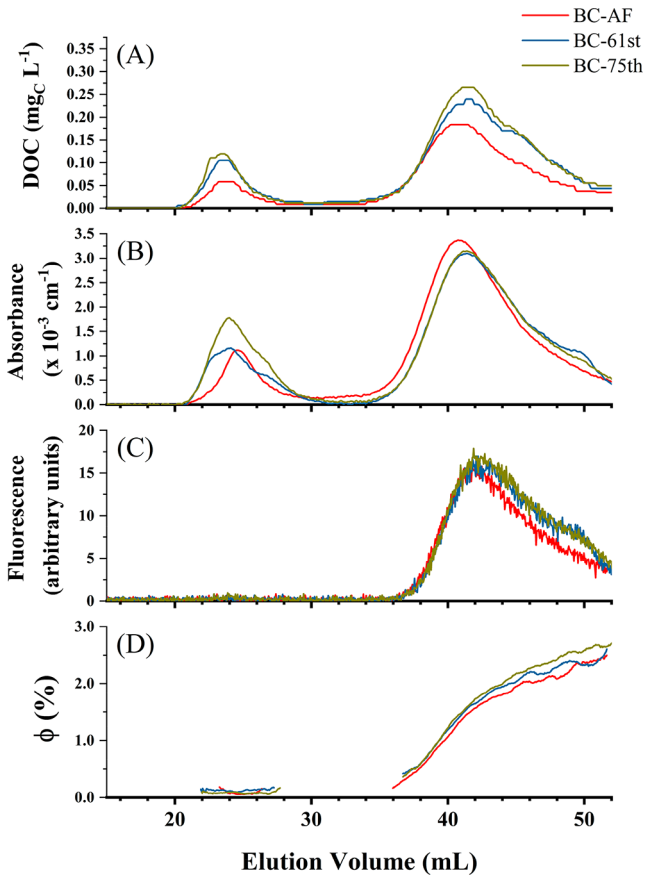

Figure 6. SEC chromatograms for Boulder Creek water samples. (A) DOC, (B) absorbance (λ = 350 nm), (C) fluorescence (λ_{ex} = 350 nm, λ_{em} = 390−700 nm), and (D) fluorescence quantum yield (Φ_f). Φ_f was not calculated when the absorbance was below 0.5 cm^{-1} 10^{-3}. Red lines show the sample from Boulder Creek at 61st Street (BC-61st), blue lines show the sample from Boulder Creek at Arapahoe Avenue (BC-AF), and yellow lines show the sample from Boulder Creek at 75th Street (BC-75th). Absorbance was measured at 350 nm; fluorescence and Φ_f were measured at λ_{Ex} = 350 nm.

tion Figure S7A,B, and bulk water data for all Boulder Creek and South Boulder Creek samples are provided in Supporting Information Figure S2 and Tables S2−S3). The DOC chromatograms showed two distinct peaks occurring in elution volume ranges of ∼20−30 mL and ∼35−50 mL. At sample locations further downstream (the streamflow direction is from BC-AF to BC-75th), the DOC concentration of both peaks (and thus the overall DOC concentration) increased (Figure 6). The stream section where BC-AF, BC-61st, and BC-75th samples were taken flows through an urban corridor of the city of Boulder; therefore, it is likely that a complex combination of anthropogenic inputs are responsible for the observed increases in DOC concentrations downstream.[52,53]

Absorbance chromatograms also displayed two distinct peaks within 20−30 and 35−50 mL, while fluorescence chromatograms show one peak within 35−50 mL. For the remainder of the discussion, the absorbance peaks within 20−30 mL and 35−50 mL will be referred to as "large AMW" and "medium to small AMW" peaks, respectively. Thus, chromophoric compounds (absorbing at 350 nm) contributed to both peaks, while fluorophores (excited at 350 nm) were constrained to the medium to small AMW peak. It has been reported elsewhere that upon fractionation by AMW, a distinction is observed between large AMW fractions with high absorbance (i.e., the fluorescence/absorbance ratio is small) and small AMW fractions with intense fluorescence (i.e., the fluorescence/absorbance ratio is large).[37,38] Interestingly, in the medium to small AMW peak, where absorbance and fluorescence signals are the greatest, the absorbance and fluorescence peaks vary much less between samples than the DOC, indicating that the differences between DOC chromatograms were largely due to nonchromophoric DOM (i.e., spectroscopically invisible).

The Φ_f results for Boulder Creek samples are shown in Figure 6D. The Φ_f was calculated in the elution volume range in which the absorbance intensities were above 0.5 × 10^{-3} cm^{-1}. Across elution volumes ∼35−53 mL, Φ_f increased from < 0.5 to ∼2.5% for smaller AMW fractions (later elution volumes) relative to larger AMW fractions (earlier elution volumes), where bulk water Φ_f values for the same samples were determined to be 0.97−1.39% (Supporting Information, Figure S5). These data indicate that although most absorbance and fluorescence (as a fraction of the overall DOM absorbance and fluorescence) occurred between 38 and 46 mL (where

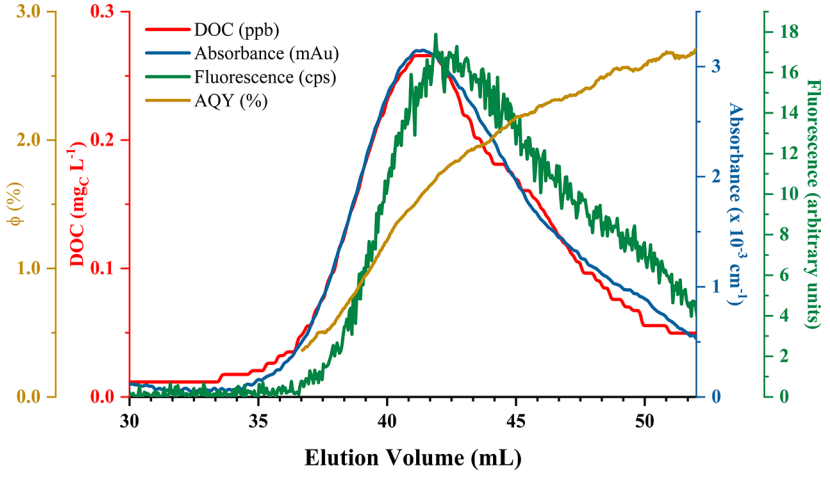

Figure 7. SEC chromatograms for Boulder Creek sample BC-75th in the medium to low AMW range. DOC, absorbance (λ = 350 nm), fluorescence (λ_{ex} = 350 nm, λ_{em} = 390−700 nm), and Φ_f are plotted on the red, blue, green, and yellow *y*-axes, respectively. Absorbance was measured at 350 nm; fluorescence and Φ_f were measured at λ_{Ex} = 350 nm.

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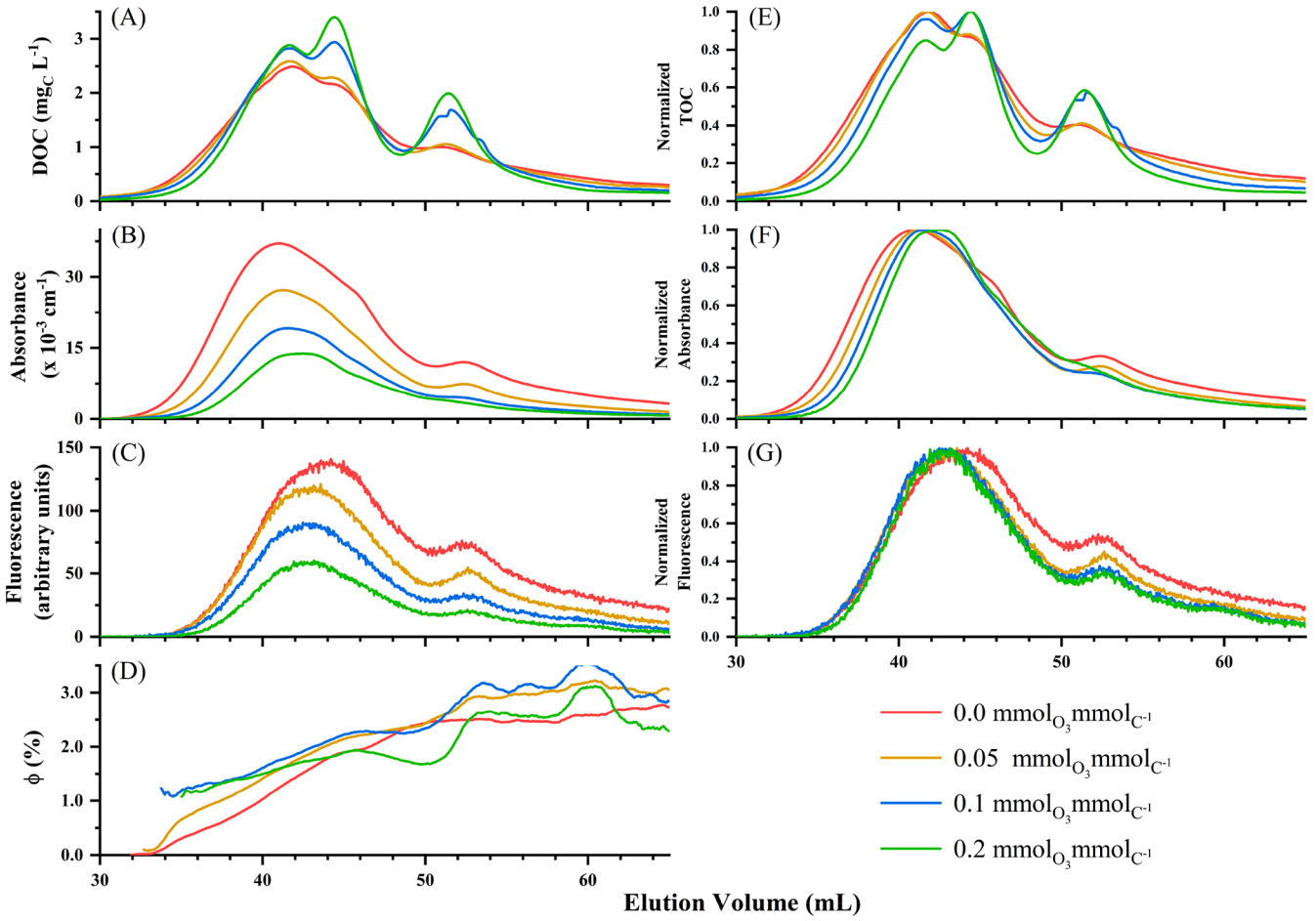

Figure 8. Left: DOC, absorbance (λ = 350 nm), fluorescence (λ_{ex} = 350 nm, λ_{em} = 390−700 nm), and Φ_f chromatograms for PLFA (5 mg$_C$ L^{-1}) samples treated with ozone. (A) DOC (mg$_C$ L^{-1}), (B) absorbance, (C) fluorescence, and (D) fluorescence quantum yield chromatograms. Right: normalized absorbance and fluorescence chromatograms for PLFA treated with ozone. (E) DOC chromatograms normalized to the peak maximum. (F) Absorbance chromatograms normalized to the chromatogram peak maximum (i.e., normalized to 1). (G) Fluorescence chromatograms normalized to the chromatogram peak maximum. All plots: the red line shows untreated PLFA, the yellow line shows PLFA ozonated at a dose of 0.05 mmol$_{O3}$ mmol$_C^{-1}$, the blue line shows PLFA ozonated at a dose of 0.1 mmol$_{O3}$ mmol$_C^{-1}$, and the green line shows PLFA ozonated at a dose of 0.2 mmol$_{O3}$ mmol$_C^{-1}$. Chromatograms were plotted as a function of elution volume (mL).

signal intensities increased to a chromatographic maximum at ∼40−42 mL before decreasing with increasing elution volumes), the Φ_f values continued to increase with increasing elution volumes of medium to small AMW fractions.

Prior research has been dedicated to understanding the structural properties of chromophores and fluorophores within DOM.[18,50,54,55] Although some correlations on the structural identities of these optically active species (phenols, quinones, etc.) have been made,[22,56−59] their distribution within the DOM molecular size continuum is not well understood.[31,60] The data presented here provide the first direct evidence of a clear separation between weakly fluorescing species present at higher concentrations (thus observed with relatively higher fluorescence and lower Φ_f signal intensities) eluting between 40 and 44 mL, as opposed to highly fluorescing species which dominate the lower AMW fractions, although their overall mass contributions are smaller (observed with lower fluorescence and higher Φ_f signal intensities). This de-coupling between numerous weakly fluorescent fractions with relatively larger AMW, and fewer highly fluorescent fractions with relatively lower AMW, matches well with other work where the Φ_f MW distribution was assessed.[37,38,40] It should be noted that this study analyzed the AMW distribution of Φ_f only at λ_{ex} = 350 nm. Future studies may benefit from exploring the relationship at other relevant λ_{ex}.

Figure 7 displays SEC-based DOC, absorbance, fluorescence, and Φ_f chromatograms for one Boulder Creek sample (BC-75th) to help understand the qualitative DOM behavior observed for Φ_f. While the absorbance trace closely mirrored the DOC in both shape and elution volume, the fluorescence material with smaller AMWs eluted with a similar but slightly offset size distribution. This suggests that within the medium to small AMW range, as the AMW decreased, DOM fluorescence increased relative to absorbance at λ_{ex} = 350 nm, resulting in increasing Φ_f values. This observation highlights the ability of SEC measurements to provide a more in-depth understanding of the complex composition of DOM, with respect to Φ_f.

3.1.2. Impact of Ozonation on PLFA. Section 3.1.1 presents an application of this method along a biogeochemical gradient. In this section, we describe the impact of a chemical process (ozonation) on DOM properties and Φ_f. Solutions of PLFA (5 mg$_C$ L^{-1}) were ozonated at ozone doses of 0.05, 0.1, and 0.2 mmol$_{O3}$ mmol$_C^{-1}$. Previous research indicates that ozonation of PLFA induces a decrease in absorbance and fluorescence but an increase in Φ_f.[61,62] Upon ozonation, bulk water DOC

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changes only minimally,[63] but low AMW products are formed, such as formaldehyde, acetaldehyde, or oxalic acid,[64] which should be observable by the SEC−DOC detector. The fact that DOC, absorbance, fluorescence, and Φ_f all change as a result of ozonation suggests that SEC coupled with DOC, absorbance, and fluorescence detection would prove a valuable tool to follow the changes induced by ozonation.

With increasing ozone doses, a decrease in absorbance and fluorescence in PLFA was observed (Figure 8B−C). The DOC chromatograms indicate that there was a reduction in large AMW compounds (< ∼40 mL) and a simultaneous increase in smaller AMW compounds (∼40−53 mL) with the formation of two distinct lower AMW peaks at ∼45 and ∼52 mL (Figure 8A,E). Additionally, the normalized (to the maximum) absorbance and fluorescence chromatograms are presented in Figure 8F,G. Interestingly, while both absorbance and fluorescence values across the associated chromatograms decreased, the normalized data revealed that with increasing ozone dose, the absorbance trace shifted to lower AMW, while the fluorescence trace remained roughly distributed over the same AMW range. As a result, the SEC-Φ_f showed a larger increase for large AMW molecules (∼33−40 mL), while the increase was less significant for smaller AMW (> ∼40 mL) (Figure 8D). Previous research observed increasing bulk Φ_f with increasing ozone doses.[61] This observation is confirmed here in more detail, in which the increase is particularly marked for the high AMW fraction (< ∼40 mL).

Ozonation of phenols leads to the formation of ring-opening products, indicating that carbon−carbon bonds can be broken by ozonation.[65] The DOC chromatograms indicate that ozonation induces a fragmentation of DOM molecules, an observation that concords with the breaking of carbon−carbon bonds and the aforementioned appearance of low AMW products such as formaldehyde, acetaldehyde, and oxalic acid.[64] The remaining fluorescence after ozone treatment is indicative of functional groups that are not as reactive with ozone and could include terpeniods or phenols with a high pK_a (the deprotonated form of phenol being more reactive by ≈ 4−6 orders of magnitude toward ozone). An example of such a phenol is SA, which has a pK_a for the phenolic moieties of 13.4.[47]

3.2. Further Potential Applications. Although the focus of this work was on calculating the Φ_f for AMW fractions from SEC analysis, the system as developed could be used to calculate a variety of additional optical parameters. Examples that have previously been used in the investigation of bulk water DOM include $SUVA_{254}$, spectral slopes, specific fluorescence, fluorescence indices, and fluorescence peak ratios.[54,66−71] Coupling these metrics with SEC analysis would lead to a more complete understanding of physiochemical properties of DOM as a function of MW. Additionally, recent work by Ulliman et al. (2020) proposed a methodology to evaluate the potential for several parameters (e.g., Φ_f, fluorescence peak ratios A/C and C/T, fluorescence peak T intensity, and fluorescence index) to differentiate natural DOM from EffOM using several paired samples.[28] A similar methodology can be applied to the same parameters coupled with SEC. Because SEC fractionates samples by size, it reduces the complexity of DOM with respect to bulk water analysis. We suggest that future work using this system could investigate whether this reduced complexity extends to other freshwater, marine, and soil porewaters, leading to a greater ability to differentiate DOM qualitative changes and DOM sources. Furthermore, this method provides a means by which highly fluorescent size fractions of DOM can be identified for more detailed analyses of chemical composition and its changes through different processing mechanisms. This system was specifically developed to capture different fractions for further off-line biological and chemical analysis at the molecular level using other analytical techniques (e.g., high-resolution mass spectrometry and nuclear magnetic resonance spectroscopy).

4. CONCLUSIONS

This study developed a novel in-line method for the determination of Φ_f as a function of AMW using a SEC system coupled with DOC, absorbance, and fluorescence. This method provides useful and important information regarding DOM characterization, especially regarding fluorescence properties, something that still is considered to be a black box in the DOM characterization community. The development and validation of instrument-specific correction factors for SEC−FLD were needed to produce accurate fluorescence emission spectra. We calculated the Φ_f with the help of an SA standard, confirmed method accuracy by varying concentrations, and monitored chemical processing effects of ozonation for different AMW DOM fractions. Φ_f of the DOM in natural water and fulvic isolate samples followed a characteristic profile whereby Φ_f increased with decreasing AMW. However, the profile of PLFA DOM changed following ozonation, suggesting that SEC-based Φ_f tracks important fundamental changes to DOM composition.

For all sample sets, a close investigation of all chromatographic results (fluorescence, absorbance, and DOC) individually is especially useful in the qualitative understanding of sample composition and chromatographic behavior. For example, the natural water samples and the isolates analyzed in this study showed that larger AMW fractions with lower Φ_f correspond with higher DOC concentrations, while smaller AMW fractions with higher Φ_f correspond with lower concentrations. While DOM components with higher Φ_f will contribute more to observed bulk fluorescence than components with lower Φ_f relative to their abundances, bulk water Φ_f values are weighted more heavily to lower SEC-based Φ_f (< 1.5%) due to higher abundances (i.e., concentration). Additionally, by comparing the SEC−DOC to SEC-absorbance and SEC−DOC to SEC-fluorescence signals, it can be understood which fractions contain DOM, that is, chromophoric and fluorophoric, and which fractions do not, providing more detail than is detected by bulk water absorbance and fluorescence analysis alone. Finally, it is proposed that future studies could utilize this method to differentiate between sources of OM (e.g., natural organic matter from diverse ecosystems and EffOM) and to identify highly fluorescent components for isolation and further detailed investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00318.

Additional materials and methods including a table of chemicals used, sample collection information, and map of sample locations; additional results and discussion including bulk water analysis results; additional sample results not presented in the main text; details of statistical analysis; and weighted integration of online

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SEC-based fluorescence quantum yield from the ozone experiment (PDF)

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Notes

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