Influence of pH on Fluorescent Dissolved Organic Matter Photo-degradation

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Abstract

A novel semi-continuous excitation emission matrix (EEM) fluorescence and absorbance monitoring system has been developed. Full EEMs were collected simultaneously with absorbance spectra every 20 minutes during 24 hour solar-simulated irradiation experiments, and the kinetic change of fluorescence of Suwannee River natural organic matter IHSS standard material (SRNOM) at various pH values was investigated. Parallel factor analysis (PARAFAC) was then used to isolate the photo-labile and pH-influenced fluorescent components of SRNOM. Kinetic analysis showed increasing rates of fluorescence loss with increasing pH. This has significant implications for the photo-degradation of dissolved natural organic matter during estuarine mixing, when large increases of pH are common. The influence of pH on fluorescence and photo-degradation kinetics emphasizes the need for pH to be monitored and accurately controlled during laboratory experiments. It is also highly recommended that when constructing PARAFAC models or monitoring changes in fluorescence data between samples of different origins, that the pH be held constant to remove any potential artifacts or misinterpretation of data.

Keywords: dissolved organic matter, excitation emission matrix fluorescence, pH dependency, absorbance, photochemistry, PARAFAC

1. Introduction

Natural dissolved organic matter (DOM) plays a key role in the global carbon cycle with an estimated 620 gigatonnes (Gt) dissolved in the World's Oceans (Hansell et al. 2009) and an estimated river transport of terrestrially-derived organic matter to the ocean of 0.9 Gt annually (Tranvik et al. 2009). A global estimate of DOM present in land-locked freshwater systems has

not yet been fully established, but it is clear that these systems play an important part in global carbon cycling (Bastviken et al. 2011, Cole et al. 2007). This DOM is the most dynamic component of the global carbon cycle and its reactivity is not fully understood due to its extreme complexity, but photochemical reactions are believed to be the major sink of terrestrially-derived DOM (Blough and del Vecchio 2002) and refs. within). Hence, a fundamental understanding of photochemical degradation pathways is important.

The photo-degradation of DOM and in particular of the light absorbing chromophoric DOM (CDOM) fraction produces carbon monoxide (Conrad et al. 1982, Gao and Zepp 1998, Valentine and Zepp 1993), dissolved inorganic carbon (DIC) (Gao and Zepp 1998, Miller and Zepp 1995), low molecular weight organic molecules (Backlund 1992, Gonsior et al. 2014, Kieber et al. 1989, Kieber et al. 1990, Kulovaara 1996, Mopper et al. 1991), and complex aromatic structures (Stubbins et al. 2010). Absorbance losses of CDOM due to photo-irradiation tend to be highest at the wavelength of irradiation (Del Vecchio and Blough 2002, 2004), with solar-simulated polychromatic irradiation leading to preferential loss at long (visible) wavelengths, or “photobleaching” (Del Vecchio and Blough 2002, Grebel et al. 2009, Shank et al. 2010). CDOM plays a key role in aquatic environments as the primary absorber of light, sensitizing production of reactive species (Cooper et al. 1989) and providing UV protection for aquatic organisms (Zepp et al. 2008). Photobleaching of CDOM both affects these processes and regulates the input of terrestrial CDOM to the oceans (Blough et al. 1993, Niu et al. 2014).

During estuarine mixing, fresh waters often undergo large increases in pH. Increasing pH increases the absorbance of DOM due to deprotonation of carboxyl and phenolic groups, and/or conformational changes (Dryer et al. 2008, Pace et al. 2012). DOM is now believed to be made up of a supra-molecular assembly of smaller molecules (Sutton and Sposito 2005); changing the

charge of molecules through protonation/deprotonation affects the interactions of these molecules. Increases in size with increased pH have been observed via dynamic light scattering, scanning electron microscopy, and atomic force microscopy (Pace et al. 2012). This increase could be due to expansion/contraction of the supra-molecular structure, or incorporation of new molecules into the aggregate at high pH. Changes in pH were also shown to have significant effects on DOM apparent molecular weight and fluorescence (Romera-Castillo et al. 2014). Deprotonation affects not only absorption properties, but also reactivity of phenolic groups. McNally *et al.* (2005) found that model lignin phenols reacted preferentially with excited state sensitizers when protonated (neutral), but with singlet oxygen when deprotonated (McNally et al. 2005). Changing solution pH could therefore have a significant impact on the rate of photo-degradation of DOM.

Previous studies have shown differing effects of pH on CDOM photo-degradation. While several studies have shown that increasing pH decreased the production of DIC (Anesio and Graneli 2003, Anesio and Granéli 2004, Gennings et al. 2001), Gao and Zepp (1998) showed increased DIC production with increased pH, an effect that was removed with the addition of fluoride as an iron chelator. Molot *et al.* (2005) showed decreased removal of dissolved organic carbon (DOC) with increasing pH, a trend that was reversed with the addition of 50 mM potassium iodide (Molot et al. 2005). While this change was attributed to quenching of hydroxyl radicals by iodide, iodide is also a strong quencher of triplet excited-state species (Ayatollahi 2013, Jammoul et al. 2009), which are believed to play a key role in DOM photo-degradation (Canonica et al. 1995, McNally et al. 2005). Brinkmann, *et al.* (2003) showed greater loss of DOC in lake water irradiated at pH 8 than pH 4, but did not find a clear relationship between loss of absorbance and pH (Brinkmann et al. 2003). Finally, Pace, *et al.* (2012) found higher rates of

photobleaching (defined as loss of absorbance at 440 nm) in solutions of higher pH (Pace et al. 2012). These studies used natural waters from various sources containing a large range of DOC and dissolved iron concentrations as well as DOM composition. Hence, a clear picture of a pH effect on DOM photo-degradation has not yet been established.

To gain further insight into the relationship between pH and DOM photo-degradation under controlled conditions, this study investigated the time-resolved changes in fluorescence of a standard reference material, Suwannee River natural organic matter (SRNOM), during solar-simulated irradiation. Fluorescence excitation emission matrices (EEMs) have become an increasingly popular tool to measure the quantity and quality of fluorescent dissolved organic matter (FDOM) in natural and engineered systems (Andrade-Eiroa et al. 2013, Ishii and Boyer 2012, Jaffé et al. 2014). Previous studies of the effect of photo-irradiation on EEMs, however, have taken a ‘before-and-after’ approach, or only measured a minimum number of time points throughout a photochemical experiment (Cory et al. 2007, Shank et al. 2010, Stedmon et al. 2007). In this study, we present a method allowing for semi-continuous monitoring of EEMs throughout a photochemical experiment combined with exact pH control. The simultaneous collection of absorption and EEM spectra enables the correction for inner filter effects as well as the determination of changes in all optical properties. The pH of each sample was carefully controlled to examine the effects of pH during photo-irradiation experiments. To the best of our knowledge, this is the first experimental design to document the changes in EEMs and absorbance of DOM with such high temporal resolution and pH control during photo-irradiation experiments.

2. Methods

2.1 Reagents

SRNOM (2R101N) was acquired from the International Humic Substances Society (IHSS). Details about the reverse osmosis, salt removal, and freeze-drying procedure can be found elsewhere (http://www.humicsubstances.org/ro_nom.html). Sodium hydroxide was purchased from Fisher Scientific, and high-purity HCl from Sigma Aldrich. All solutions were prepared in 18.2 MΩ NanoPure™ water (Thermo Scientific™).

2.2 Photochemical experiments

A detailed schematic of the experimental setup is shown in Supporting Information Figure S1. Irradiations were carried out with an Oriel® Sol2A Class ABA solar simulator (Newport Corporation, Irvine, CA). The 1000 W Xe arc lamp was equipped with an AM 1.5 filter to best match the total (direct and diffuse) solar spectrum when the sun is at a zenith angle of 48.2 °. Lamp power was measured with a Newport 91150V Reference Cell and adjusted to 1000 W m⁻² before each experiment. Lamp power was then kept constant during irradiation periods with a Newport 68951 Digital Exposure Controller. Variation in the light intensity across the working surface was consistently <3 %. Samples were irradiated in a custom-built flow cell (Supporting Information, Fig. S2) made from SCHOTT Borofloat® borosilicate glass (Hellma Analytics). The flowpath was a 2 mm wide by 1 mm deep channel within the cell that followed an Archimedean spiral with a total surface area of 101 cm². The irradiation cell was submerged in a temperature-controlled (25 °C) NanoPure water bath. During an irradiation experiment, a sample was pumped with a Teflon-lined solenoid pump through the irradiation cell and into a 2 mL vial with 750 µL of volume to allow equilibration with air and to avoid oxygen starvation during experiments. From the equilibration vial, the sample was then drawn through a 1.5 mm–

pathlength flow cell placed in an Aqualog spectrofluorometer (Horiba Instruments), and recirculated back into the irradiation cell.

Experiments were conducted using 100 mg L⁻¹ SRNOM solutions; this corresponds to about 50 mg C L⁻¹, a relevant value as the headwaters of the Suwannee River typically range from 25-75 mg C L⁻¹ (www.humicsubstances.org). After dissolution of SRNOM in NanoPure water, all samples were filtered through pre-washed 0.2 µm Whatman GD/X syringe filters immediately before use. The pH of the sample was monitored with an Orion 8220BNWP microelectrode and kept constant during irradiations using a J-Kem Infinity II reaction controller coupled with a dual syringe pump, which injected sub-microliter amounts of 0.1 M HCl or NaOH to keep the pH within 0.05 pH units of the individual set points (pH = 4, 5, 6, 7, or 8). Full EEMs and absorbance spectra were collected every 20 minutes for 24 hours, resulting in 73 EEMs and absorption spectra per experiment. Excitation scans, and therefore absorbance spectra, were conducted from 600 nm to 220 nm in 2 nm steps, while the emission wavelengths collected by the CCD detector ranged from 211.5 nm to 617.7 nm in ~3 nm increments. Integration times were set at 0.4 seconds.

Actinometry with *p*-nitroanisole/pyridine (Dulin and Mill 1982) showed that the average light flux from 300-400 nm, 33 W m⁻², was 76 % of the light intensity measured at the irradiation cell with the Reference Cell. This was expected, as it was approximately the ratio of sample volume within the irradiation cell to the volume of the entire system.

2.3 Data Analysis

The effect of Rayleigh and Raman scattering was removed from the raw fluorescence data using the Aqualog software. Fluorescence intensities were converted to Raman Units (RU) by dividing

measured intensities by the area under the water Raman peak at 350 nm excitation (emission range 383–442 nm). The Aqualog simultaneously collects fluorescence and absorbance data, allowing for inner filter effect corrections (McKnight et al. 2001). The short pathlength of the flow cell (1.5 mm) was sufficient so that the absorbance of the 100 mg SRNOM L⁻¹ solution was below 0.3 raw absorbance, ensuring that the correction was effective (Miller et al. 2010). Baseline drift in the absorbance spectra were corrected by subtracting the recorded absorbance at 600 nm (the highest wavelength of the Aqualog absorbance detector). Absorption coefficients were calculated using the equation:

$$a(\lambda) = 2.303 * \text{abs}(\lambda) / L \quad (1)$$

where $\text{abs}(\lambda)$ is the raw absorbance at wavelength λ , and L is the cell pathlength in meters (0.0015 m). Spectral slopes from 275–295 and 350–400 nm ($S_{275-295}$ and $S_{350-400}$, respectively) were calculated by performing linear regressions of the natural log-transformed absorbance spectra, and the slope ratio (S_R) calculated as the ratio of $S_{275-295} / S_{350-400}$ (Helms et al. 2008). E2:E3 ratio was calculated as the ratio of absorbance at 250 nm to 365 nm (De Haan and De Boer 1987). Both S_R and E2:E3 are established proxies for apparent molecular weight via ultrafiltration and/or gel permeation chromatography. Fluorescence peak intensities were determined by finding the maxima within pre-defined peak boundaries for the conventional humic-like A peak (ex/em <260/400–460 nm), and the humic-like C peak (ex/em 320–360/420–460 nm) (Coble et al. 1998). Each peak maximum was averaged with the surrounding eight data points, effectively creating a 6x9 nm ‘box’ centered around the maximum. This significantly reduced the effect of instrument noise, especially for ex<250 nm, where signal to noise ratios are much lower than at longer wavelengths. In order to describe the highly variable shape of the C peak, two peak metrics were used. The full width at half maximum (FWHM) was calculated for

the emission spectra at the excitation wavelength containing the peak maximum. To capture the shape of the elongated tail of the C peak with increasing wavelength, the elongation half width at half maximum (EHW) was calculated. Full widths could not be calculated due to interference from the A peak.

Parallel factor analysis (PARAFAC) statistically deconvolutes EEMs into distinct components. PARAFAC modeling and validation was performed by using the DOMFluor Toolbox (Stedmon and Bro 2008). Each EEM was divided by its total integrated fluorescence (RU) to reduce sample bias when fitting the PARAFAC model (Murphy et al. 2013). After fitting the model, results were multiplied by the original total fluorescence to reverse the normalization prior to kinetic analysis. Spectral loadings were evaluated according to previously published guidelines (Murphy et al. 2013, Stedmon and Bro 2008). Split-half validation was performed, as well as random initialization modeling and residuals analyses for model validation.

DOM is often characterized operationally as ‘labile,’ ‘semi-labile,’ or ‘refractory’ based on biological or photo-chemical reactivity (Nelson and Siegel 2013). The loss of fluorescence intensity over time for each experiment was modeled with a double exponential decay function (Eq. 2) using IGOR Pro (WaveMetrics, Inc) (Del Vecchio and Blough 2002, Sleighter et al. 2014):

$$F_t = F_L e^{-k_L t} + F_{SL} e^{-k_{SL} t} + F_R \quad (2)$$

where the fluorescence (F) at time t is comprised of photo-labile (L) and photo-semilabile (SL) fractions degrading at rates k_L and k_{SL} , respectively, and a photo-refractory fraction (R). Each photo-degradation experiment was modeled individually, with either the PARAFAC component

intensity or the fluorescence intensity at the wavelength couple of maximum degradation (ex/em= 346/460 nm) plotted against irradiation time.

3. Results

3.1 pH dependence of optical properties

The fluorescence of Suwannee River natural organic matter was highly pH dependent (Mobed et al. 1996). The peak intensities increased with pH in a similar fashion as previously reported with DOM from the Amazon River (Patel-Sorrentino et al. 2002). The fluorescence intensity of the A peak increased by 16 % when titrated from pH 4 to 8 in both un-irradiated and 24 hr-irradiated samples, while the maximum intensity increase of the C peak was 7.6 % from pH 4 to 6 in the un-irradiated samples and 18% from pH 4 to 8 in the 24 hr-irradiated samples (Fig. S3). When the pH was increased from 4 to 10, two peaks grew on the shoulders of the traditional A and C peaks at ex/em 246/480 nm and 390/480 nm (Figure 1). The peak on the shoulder of the C peak contributed as much as 35 % to the fluorescence intensity, and dramatically changed the shape of this peak. The elongation half width (EHW) increased by 57%, while the FWHM did not change significantly, underlining the very strong pH effect on the shape of the fluorescent C peak.

Solution pH also played an important role in the absorption properties of SRNOM. The absorption spectra showed a pH-dependent increase in absorbance centered around 350 nm (Dryer et al. 2008). When titrated from pH 4 to 10, the spectral slope ratio, S_R , increased by 10%, while the E2:E3 ratio decreased by 19 %. Both of these metrics have been inversely correlated with apparent molecular weight, and were expected to show similar, if not proportional, changes. This pH effect on commonly used optical properties needs to be

considered when these metrics are used to estimate apparent molecular weight or other properties of DOM (Poulin et al. 2014).

3.2 PARAFAC Analysis

A PARAFAC model was fitted from the EEMs collected during the photo-irradiation experiments. A visual inspection of the initial 1095 EEMs was conducted to identify scans with interferences such as bubbles. After removing these outliers, the data set contained a total of 1056 EEMs. High relative leverages were observed for excitation wavelengths <250 nm, most likely due to instrument noise (Stedmon and Bro 2008), so these wavelengths were removed from the dataset before fitting the model. Normalization was essential to reduce the leverages of samples at the beginning of irradiations, which had up to twice as much total fluorescence when compared to irradiated samples. The resulting model (>99.8% explained variance) was split-half validated, and contained three components (Fig. 2): C1 (ex/em <250, 300/418), C2 (ex/em <250, 338/454), C3 (ex/em 272, 382/504). C1 was the most abundant component, with 38-62 % of total fluorescence, and was therefore deemed the main SRNOM peak, while C2 and C3 comprised 25-37 % and 13-27 %, respectively. The location of C2 matched the regions of greatest fluorescence loss, and was therefore characterized as the “photo-labile” component; however, both C1 and C3 showed photo-lability (see below). C3 matched the region of fluorescence increase due to titration from pH 4 to pH 10 (Fig. 1), and was labeled the “pH-influenced” component.

The complete (1056 EEM) dataset was also divided into individual datasets containing only samples of the same pH. PARAFAC analysis of these five datasets yielded the same three component model, each of which had >99.8% explained variance and was split-half validated. These results further supported the validity of model created from the full dataset. The pH-

influenced component, C3, was likely separated out due to the difference in degradation kinetics compared to C2 (see below), allowing it to be distinguished without including samples from multiple pH values into the model. The results show that in future studies, a single pH value can be used to fit a PARAFAC model from EEMs collected throughout a photo-irradiation. Sub-datasets were also created with EEMs from only the first five hours of irradiation (all pH values) and the last five hours of irradiation (all pH values). PARAFAC analysis of these two sub-datasets likewise produced the same three component model (>99.8% explained variance, split-half validated). This demonstrates that the method can be applied to both “fresh” and photo-bleached samples, and that no new major fluorophores were produced during irradiation.

3.3 Photo-degradation kinetics

When exposed to simulated sunlight, SRNOM showed significant loss of fluorescence (Fig. 3). All samples showed rapid fluorescence loss in the first five hours of irradiation, followed by slower decay, consistent with previous studies (Schmitt-Kopplin et al. 1998). The areas of greatest loss were centered at ex/em 224/480 nm and 346/460 nm, matching the shape and location of C2. While C2 showed greatest total fluorescence loss, the pH-influenced component, C3, showed greater relative loss and faster degradation than C2.

Fluorescence loss showed a strong pH dependency. With increasing pH, the total fluorescence loss and overall degradation rate also increased (Fig. 4). The fluorescence loss of Component 2 after 24 hours irradiation increased significantly ($p < 0.05$) for each pH value, while the loss of Component 3 after 24 hours was not significantly different between pH 4 and pH 5 as well as between pH 6 and pH 7 (Fig. 4). C1 fluorescence intensity did not significantly change when irradiated at pH 4, while a decrease of 9.3 ± 5.6 %, 13 ± 5 %, 14 ± 1 % and 14 ± 2 % was

observed at pH 5, 6, 7, and 8, respectively. There was a significant effect of pH on the rate constants k_L and k_{SL} for both C2 ($p < 0.05$) and C3 ($p < 0.005$) (Table 1), and all rate constants were significantly different for pH 4 and pH 8 ($p < 0.05$; Fig. S4). Rate constants from the PARAFAC analysis (k_{C2-L} and k_{C2-SL}) and obtained via the peak-picking approach at ex/em 346/460 nm (k_{P-L} and k_{P-SL}) at each pH were not significantly different using standard t -tests and underlined that the pH dependency during photo-degradation experiments was independent of the technique used to determine the rate constants. The kinetic loss of fluorescence was independent of concentration from 25- 100 mg SRNOM L⁻¹ (Fig. S5). Dark controls showed no significant change in EEMs or absorbance spectra over 24 hours. The C peak became narrower during irradiation, with decreases in both EHW and FWHM. EHW did not show a strong pH dependence, in contrast to the FWHM widths, which decreased by 6.4 %, 9.9 %, and 13 % for pH 4, 6, and 8, respectively during 24 hr irradiation.

Rates of absorbance loss did not show a pH dependence. Losses of absorbance at 254 and 300 nm decreased on average by 14 % and 21 %, respectively over 24 hours. Increased long-wavelength absorbance (>400 nm) at high pH led to increased loss of absorbance at these wavelengths (Fig. 5). When irradiated at pH 4, absorbance loss was greatest at 350 nm during the first hour of irradiation, followed by preferential loss at shorter wavelengths. With increasing pH, a feature around 275 nm became apparent in the differential absorption spectra at longer irradiation times (Fig. 5). Carboxylic acids present in DOM are known to absorb in this region (Dryer et al. 2008); this feature may therefore be the result of relative photo-stability of these carboxylic acids compared to other absorbing DOM components (e.g. polyphenols) or the photochemical production of carboxyl groups (Xie et al. 2004).

4. Discussion

Semi-continuous excitation-emission matrix fluorescence monitoring has the capability of collecting kinetic fluorescent data with an unprecedented level of detail. Collecting full EEMs allowed the evaluation of peak shapes and location as well as fluorescence intensities during photochemical experiments. The short pathlengths used in the irradiation cell (1 mm) and the fluorometer flow cell (1.5 mm) allowed for experiments involving high concentrations of DOM with relatively high absorption coefficients without introducing unpredictable inner filtering effects (Sharpless et al. 2014). While DOC was not directly measured before and after the exposure to simulated sunlight, the loss of only 14% of absorbance at 254 nm during the irradiation experiments strongly supported the assumption that DOC values remained within the tested region (12.5-50 mg C L⁻¹). The observed concentration independence (over the relevant range in this study) on fluorescence degradation kinetics was important for a number of reasons. First, it verified that inner filter effects in the irradiation cell were minimal, and that these effects were properly corrected in the fluorescence measurements. It also validated that the kinetic results, namely the rapid loss of fluorescence in the first five hours followed by slower decay, were a result of changes in the DOM quality, and not due to changes in DOM concentration via photo-mineralization. This demonstrates the versatility of the system, allowing for the study of a large range of samples, from DOM-rich natural waters to DOM extracts and isolates. For natural water samples with lower DOM concentrations, longer pathlength (up to 1 cm) cells may be used in the fluorometer to measure the corresponding lower absorbance, and integration times increased to capture lower fluorescence intensities.

The loss of fluorescence was strongly tied to pH (Figs. 3, 4). As pH increased from 4 to 8, the degradation of C2 and C3 fluorescence increased, with the rate constants of the more labile fractions (k_{C2-L} and k_{C3-L}) increasing by 50 % and the rate constants of the more semi-labile

fractions (k_{C2-SL} and k_{C3-SL}) nearly doubling. Small increases in the rate constants between individual pH units were not always significantly different (Fig. S4), yet the resulting fluorescence loss of the most photo-labile component, C2, increased significantly (Fig. 4). This highlights the need to control pH during photo-irradiation experiments, as small changes in the kinetics can have a significant impact on the system, especially during longer exposure times to simulated sunlight. C1 fluorescence did not significantly change during irradiation at pH 4, but then decreased at pH 5-8. In an experiment where pH was not controlled, the pH dropped from 7.3 to 5.9 during 24 hours of irradiation (Supporting Information, Fig. S6). This large pH change affected not only the fluorescence of the organic matter, but also the decay kinetics. Photo-irradiation experiments of water samples or DOM isolates where pH is not controlled could therefore lead to errors in the kinetic analysis (Brinkmann et al. 2003, Chen et al. 2014, Cory et al. 2007, Schmitt-Kopplin et al. 1998).

The kinetic analysis validated the use of PARAFAC analysis as a tool for defining the photo-labile fractions of DOM during irradiation experiments. The decay rates at the locations of maximum fluorescence loss matched the rates of loss of the photo-labile component, C2 (Table 1). pH titrations of samples that were irradiated at pH 4 (Fig. 1) confirmed that the fluorescence of C3 is strongly controlled by pH. The 56 % loss of C3 fluorescence intensity during the irradiation matched the change in differential fluorescence intensity via peak-picking (58 %) when titrated from pH 4 to 10. The fluorescence signature of Suwannee River FDOM is relatively simple compared to other systems with large autochthonous inputs. In such systems, FDOM components with overlapping fluorescent signatures could prevent accurate analysis via the peak-picking method. Incorporating PARAFAC allows for not only the separation and more

accurate quantification of these signals, but also comparison with FDOM signals from different geographic origins.

The photo-labile components, C2 and C3, in this study showed similar spectral characteristics to components 7 and 5, respectively, in the model of Cory and McKnight (2005). These components were the most photo-labile during irradiations of Arctic DOM (Cory et al. 2007). Photo-irradiation experiments of boreal lake DOM samples showed greatest loss in fluorescence occurred in a pattern similar to C2 fluorescence (Gonsior et al. 2013). Interestingly, Stedmon, *et al* (2007) found that their component 3, whose spectra matched C3 in this study, was photo-refractory at one sampling site in the Baltic Sea, while at two additional sampling sites, the component increased in intensity at the 6 hour time point, and then decreased throughout the rest of the 48 hour experiments. The authors suggested that their component 3 might be an intermediate produced by the photo-degradation of terrestrial organic matter- if this was the case, the precursors were either not present in SRNOM, or the degradation rate of C3 was too fast for any production to be noticed.

Photo-irradiation has been shown to destroy the electron donating capacity of DOM, but have limited effect on the electron accepting capacity (Sharpless et al. 2014). The electron donors are suspected to be substituted (poly)phenols, key players in the charge transfer (CT) interactions that lead to long-wavelength absorption and fluorescence (Del Vecchio and Blough 2004), while the electron acceptors are believed to be quinones (Aeschbacher et al. 2012). Irradiations of natural waters showed preferential photo-degradation of the lignin phenol fraction of CDOM (Benner and Kaiser 2011), while a study of lignin model compounds found the phenolic ring to be the main reaction site (McNally et al. 2005). The rapid loss of C3 fluorescence suggested that

the species responsible for this fluorescence, either directly or through CT interactions, could be phenolic.

Increased photo-degradation at high pH was likely driven by increased reactivity of deprotonated phenolic groups (McNally et al. 2005). The non-linear increase in reaction rate constants inferred a grouping of compound classes that represent different ranges of pK_a values. The relative increase of the decay rate constants for the semi-labile fractions was much larger than for the labile fractions, refuting an absorbance-based mechanism, where rate constants would increase proportionally. Deprotonation of carboxyl groups was less likely to be a significant driver, as carboxyl groups have lower pK_a values, absorb at lower wavelengths (Dryer et al. 2008) and would be both degraded and generated during photo-irradiation (Xie et al. 2004). A distinct feature present in the differential absorbance spectra at 275 nm during irradiation experiments at high pH (Fig. 5) supported the preservation and possible production of carboxyl groups, which absorb in this region.

Conformational changes in the supra-molecular DOM structure due to pH may have played a role in reactivity changes as well. If size increases with pH are due to simple expansion of the structure (Pace et al. 2012), this could limit the electron- and energy transfer mediated degradation pathways, while introduction of new molecules into the structure due to increased bonding may increase these pathways (Romera-Castillo et al. 2014). Increasing the surface area of a DOM aggregate could make it more susceptible to attacks by reactive oxygen species, as well as exposing additional chromophoric moieties and thus allowing them to participate in direct- and indirect photolytic pathways (Pace et al. 2012). Further research into the nature of pH-driven conformational changes is needed in order to assess the contribution to the increased photo-reactivity at high pH observed in this study.

In contrast to the strong pH dependency of FDOM photo-degradation, rates of absorbance loss during irradiation experiments showed no significant relationship to pH. FDOM represents only a small fraction of CDOM, which in turn is a subset of the total DOM pool. The fluorescent fraction of CDOM in the Suwannee River may be small enough that the destruction of these fluorophores did not noticeably affect the absorption spectra. Alternatively, charge transfer interactions may have been interrupted via partial oxidation/reduction, leading to a loss in fluorescence but little change in absorbance properties. Hence, care must be taken when using FDOM as a tracer for total DOM dynamics, as changes in pH may alter FDOM photo-degradation kinetics much more severely than total DOM changes.

While previous work has shown the photochemically-induced changes of PARAFAC components (Chen and Jaffé 2014, Cory et al. 2007, Stedmon et al. 2007), this was the first time that PARAFAC has been used as a tool to both deconvolute the photo-labile portion of natural organic matter, as well as identifying the pH-dependency on component intensity. This has considerable implications: a study investigating the fluorescence of a large range of samples could potentially introduce errors if the pH is not consistent between samples. For example, Yamashita, *et al.* (2008) reported the non-conservative behavior of a PARAFAC component (labeled C3) during estuarine mixing (Yamashita et al. 2008). This component, which showed very similar shape to C3 in this study, deviated most from the conservative mixing line mid-estuary. While the authors attributed the increased relative abundance of this component to dissolution of particulate organic matter, the increase could have been caused by the pH change along the salinity gradient. It is therefore recommended that when comparing EEMs or PARAFAC component intensities from different sources, the pH of samples is kept constant.

The pH dependency of EEMs also has the potential to be an indicative tool for characterizing DOM of different origin.

In this study, we have demonstrated the effects of pH on fluorescence, optical properties, and photo-degradation of Suwannee River natural organic matter, one of the most widely used DOM reference materials. Future work investigating natural waters and isolates from different sources in both fresh and marine systems will shed further light on the mechanism(s) behind the observed pH effects. The results of this study further emphasize the need to account for and control pH during irradiation experiments and PARAFAC analyses (Yang and Hur 2014). In order to best simulate environmental conditions, it is recommended that DOM extracts or isolates be held at a constant pH reflective of their source waters during irradiation experiments due to the effect on degradation kinetics. While EEMs and PARAFAC are useful for the characterization of organic matter from diverse aquatic systems, the pH of all samples must be kept constant to prevent bias.

5. Conclusions

- A novel photo-chemical system for the semi-continuous monitoring of excitation emission matrix fluorescence and absorption spectra has been developed. The short pathlengths used in the system allowed for experiments involving high concentrations of DOM with relatively high absorption coefficients without introducing unpredictable inner filtering effects.
- Excitation emission matrix fluorescence can be used to quantitatively measure photo-degradation kinetics of dissolved organic matter. PARAFAC analysis can be incorporated to separate the photo-labile and pH-influenced components of fluorescent DOM.

- Rates of photochemical fluorescent DOM loss increased with increasing pH. Controlling the pH is therefore essential during irradiation experiments to eliminate the pH bias. Due to pH effects on optical properties, pH must also be controlled during comparisons of EEMs and within PARAFAC studies.
- In contrast to fluorescence loss, pH effects were not seen on the rates of absorbance loss. Care must be taken when using fluorescence as an indicator of total DOM dynamics.

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Table 1: Rates of fluorescence loss of Suwannee River natural organic matter at various pH

Rate constant (h^{-1})	pH=4	pH=5	pH=6	pH=7	pH=8
k_{P-L}	0.77 ± 0.07	0.91 ± 0.08	0.87 ± 0.07	1.0 ± 0.1	1.2 ± 0.1
k_{P-SL}	0.049 ± 0.008	0.078 ± 0.005	0.075 ± 0.006	0.10 ± 0.01	0.10 ± 0.01
k_{C2-L}	0.83 ± 0.09	0.90 ± 0.07	0.90 ± 0.07	1.1 ± 0.1	1.2 ± 0.1
k_{C2-SL}	0.055 ± 0.007	0.079 ± 0.005	0.079 ± 0.006	0.10 ± 0.01	0.098 ± 0.007
k_{C3-L}	0.98 ± 0.12	1.0 ± 0.1	1.1 ± 0.2	1.4 ± 0.1	1.5 ± 0.1
k_{C3-SL}	0.065 ± 0.007	0.083 ± 0.004	0.094 ± 0.008	0.11 ± 0.01	0.12 ± 0.01

Values represent mean $\pm$ standard deviation from triplicate experiments

NOTE: k_P = rate of fluorescence loss via peak-picking method at ex/em = 346/460 nm for the labile (*L*) and semi-labile (*SL*) fractions; k_{C2} and k_{C3} denote rates for PARAFAC Components 2 and 3, respectively

Figure 1: Effect of pH titration on the fluorescence of Suwannee River natural organic matter before and after 24 hours of solar-simulated irradiation at pH 4. Top row: un-irradiated sample at pH 4 titrated to pH 10, and the corresponding fluorescence change. Bottom row: sample irradiated for 24 hours at pH 4, then titrated to pH 10, and the resulting fluorescence change from the titration

Figure 2: PARAFAC components for Suwannee River natural organic matter irradiated at various pH values. C1 is the main humic component, C2 is the photo-labile component, and C3 is the pH-influenced component.

Figure 3: Effect of 24 hour irradiation on the fluorescence of Suwannee River natural organic matter at pH 4 (top row) and pH 8 (bottom row).

Figure 4: Loss of C2 (ex/em <250, 338/454), and C3 (ex/em 272, 382/504) during 24 hour solar-simulated irradiation. Solid lines represent the average of triplicate experiments, while shaded areas represent one standard deviation. Values normalized to component intensity at $t=0$ hrs. Letters indicate results of Tukey-Kramer test on percent fluorescence loss: Loss of C2 was significantly different for each pH value, while of C3 loss was not significantly different for pH 4 and 5 as well as pH 6 and 7.

Figure 5: Effect of 24 hour irradiation on 100 mg L^{-1} Suwannee River natural organic matter absolute and differential absorbance spectra for pH 4 (top row) and pH 8 (bottom row).

Graphical Abstract