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UNIVERSITY OF CALIFORNIA, IRVINE

Photochemistry of Dissolved Organic Matter:
Reactivity and application in constructed treatment wetlands

DISSERTATION

submitted in partial satisfaction of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in Engineering, with a concentration in Environmental Engineering

by

Stephen Andrew Timko

Dissertation Committee:
Dr. William J. Cooper
Dr. Sergey Nizkorodov
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2015

For Molly
My light at the end of the tunnel

No water, no life. No blue, no green.

Sylvia Earle

Water, water, water, water

Sesame Street

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ACKNOWLEDGEMENTS

This dissertation would not have been possible without the generous help and support of Michael Gonsior. I can never thank you enough.

Thank you to my adviser, Dr. Bill Cooper, for guiding me and doing everything possible to ensure I had the tools necessary to complete this work.

Thank you to Dr. Pat Neale at the Smithsonian Environmental Research Center for a great summer with the Beast.

No work can be done alone, and I give many thanks to my many collaborators: Cristina Romero-Castillo, Rudolf Jaffé, Anastasia Maydanov, Maureen Conte, Boris Koch, and Philippe Schmitt-Kopplin.

Thank you to those who inspired me in science, research, and life throughout the years: my parents, Dr. Joe Thorstenson (always Dr. T.), Dr. Scott Goode, Dr. Claudia Benitez-Nelson, Dr. Tammi Richardson, Dr. Ron Benner, Walter Liniger, Dr. Ed Wirth, Dr. Tim Shaw, and my mentor, Dr. John Ferry.

Finally, thank you to my labmates, Barbara, Alice, Laura, Jenna, and Graham, for their helpful assistance, discussions, coffee, and pints.

Research funding was provided by National Science Foundation (NSF) Grant No. CBET-1034555, Graduate Research Fellowship Program, Grant No. DGE-1321846, and the Graduate Research Internship Program. Partial funding also provided by the Maryland Sea Grant REU program and the Oceanic Flux Program (OFP) time-series (NSF OCE 0927098 and OCE 1234294; Chapter 2), as well as NSF Grant No. DBI 0220409 (Chapter 3).

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Cottrell, B.A., Gonsior, M., **Timko, S.A.**, Simpson, A.J., Cooper, W.J. and van der Veer, W. (2014) Photochemistry of marine and fresh waters: A role for copper-dissolved organic matter ligands. *Marine Chemistry* **162** pp 77-88

Timko, S.A., Romero-Castillo, C., Jaffe, R., and Cooper, W. J. (2014) Photo-reactivity of natural dissolved organic matter from fresh to marine waters in the Florida Everglades, USA. *Environmental Science: Processes & Impacts* **16** (4) pp 866-878

Cottrell, B.A., **Timko, S.A.**, Devera, L. Robinson, A.K., Gonsior, M., Vezenor, A.E., Simpson, A., and Cooper, W.J. (2013) Photochemistry of excited-state species in natural waters: A role for particulate organic matter. *Water Research* **47** pp 5189-5199

ABSTRACT OF THE DISSERTATION

Photochemistry of Dissolved Organic Matter:
Reactivity and application in constructed treatment wetlands

By

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Doctor of Philosophy in Engineering, with a concentration in Environmental Engineering

University of California, Irvine, 2015

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Constructed wetlands have the potential to provide low-energy treatment of pharmaceuticals and other micropollutants via photolysis. Natural dissolved organic matter (DOM) reacts with sunlight to produce reactive species such as singlet oxygen and excited triplet states of DOM that react quickly and efficiently with contaminants. To study the photo-chemistry of DOM, a unique solar-simulation system was developed, allowing for semi-continuous monitoring of absorbance and fluorescence spectra throughout the irradiation. This system was utilized to investigate the effect of solution pH on the fluorescence properties and degradation of DOM. For the first time, parallel factor (PARAFAC) analysis was utilized to deconvolute photo-labile, photo-stable, and pH- dependent fluorescent components during irradiation. Fluorescence is highly pH dependent, and as pH increases, the total amount of fluorescence loss, and the rate at which it is lost, increases significantly. This has important implications in treatment systems, as the photo-reactivity of DOM will change when pH gradients or fluctuations are observed. The photo-degradation of organic matter collected from a depth profile (0-4,500 m) the Sargasso Sea showed increased reactivity with increasing depth. Ultra-high resolution mass spectrometry (FT-

MS) was used to characterize the samples, and correlation analyses between the fluorescence data and mass spectra revealed that high molecular weight, aromatic, and possibly polyphenolic compounds are contributing the most to the fluorescence properties that show the most reactivity in marine samples.

Production of singlet oxygen, hydroxyl radical, and triplet excited states of DOM along two riverine transects in the Everglades were measured. The abundance of fluorescence PARAFAC components was compared to the reactive species production, and suggested that terrestrial organic matter may be more efficient producers of reactive species than microbial- or seagrass-derived organic matter.

Finally, the relationship between optical properties of DOM samples collected around the world and their reactivity were investigated. Photo-irradiations and pH titrations were performed on all samples, and mass spectra collected via FT-MS. Humification indices, the biological index (BIX), and spectral slope from 275-295 nm were found to be most closely tied to changes in the mass spectra of the samples, and showed strong relationship to DOM source.

INTRODUCTION

Micropollutants are a class of anthropogenic organic compounds that enter the environment primarily through discharge of treated municipal and industrial waste. These compounds, which are comprised mainly of pharmaceuticals and personal care products (PPCPs), vary in stability and toxicity. Once in the environment, the parent compounds and their transformation products can be removed from water through a number of mechanisms: 1) adsorption to soil, 2) uptake by plants and animals, 3) transformation/ degradation by bacteria and other microbes, and 4) photolysis, or degradation by light. While the first two processes remove micropollutants from water, the pollutants often remain intact, and can still be harmful to the ecosystem. Compounds that have entered the environment after standard biological wastewater treatment have been exposed to high concentrations of bacteria, which remove the most readily biodegradable compounds during treatment. This leaves PPCPs which are more resistant to biodegradation, although some degradation does occur in the environment. Photolysis utilizes sunlight to break down PPCPs through direct and/or indirect processes. Direct photolysis occurs when a compound absorbs light, enters an excited state, and then undergoes a chemical change, usually the breaking of bonds. For indirect photolysis, molecules known as sensitizers absorb light, and then those sensitizers undergo chemical reactions that degrade the PPCP, either through direct reactions with the PPCP or the production of reactive intermediate species. The most important photosensitizer in natural waters, and the subject of this dissertation, is natural organic matter (NOM).

NOM is an extremely complex mixture of organic molecules found in all natural waters. NOM originates primarily from decaying plant material and plant- and microbial exudates. The

“fresh” organic matter undergoes biotic and abiotic transformations, resulting in tens to hundreds of thousands of unique compounds (Hertkorn *et al.* 2008).

The first studies of natural organic matter in water came from descriptions of “gelbstoff,” literally meaning “yellow substance.” Naturally, this preliminary work on colored or chromophoric dissolved organic matter (CDOM) led to characterization of its absorption properties using spectrophotometry. The UV-visible spectrum of dissolved organic matter (DOM) is broad and typically featureless, with an exponential increase in absorption with decreasing wavelength. In an effort to compare DOM from different sources/locations, indices were developed to remove the effect of concentration and relate optical properties to chemical properties such as aromaticity and molecular weight. Specific UV absorbance, SUVA, divides the absorbance at a specific wavelength by the dissolved organic carbon (DOC) concentration. Mathematical fits of the exponential slope of the absorbance spectra, deemed the “spectral slope,” S , are also used to describe DOM quality. Helms *et al.* (2008) found that examining smaller ranges of the spectral slope, 275-295 and 350-400 nm, and then taking the ratio of the two, deemed the slope ratio or S_R , allows for more detail to be ascertained about the apparent molecular weight and photochemical/biological history.

When excited with UV (and near-visible) light, DOM also emits fluorescence (Kalle 1949). Early studies of DOM fluorescence typically used a single excitation/emission wavelength couple with a large bandpass (Willey 1984). Later, emission scans at a single excitation wavelength became popular, followed by synchronous scan fluorescence, where excitation and emission wavelengths are scanned simultaneously (Cabaniss and Shuman 1987, Chen and Bada 1992, Smart *et al.* 1976). Finally, with advances in hardware and data handling software, excitation emission matrices (EEMs) have become the standard for measuring and reporting

fluorescence (Andrade-Eiroa *et al.* 2013a, b). An EEM is a contour plot or ‘heat map’ of emission spectra at multiple excitation wavelengths, with color indicating fluorescence intensity. EEMs provide the most data about DOM quantity and quality of any optical technique, and it has quickly become the method of choice for studies of DOM.

The most recent, and most intricate, development in the study of DOM optical properties is the use of parallel factor (PARAFAC) analysis (Murphy *et al.* 2013, Stedmon *et al.* 2003). PARAFAC uses large datasets of EEMs to statistically deconvolute independent fluorescent components. This allows not only for more detailed information about fluorescent DOM in one study, but also for the comparison of fluorescent components on large geographic and temporal scales. Models range from a single drinking water treatment plant (Sanchez *et al.* 2014), to the 6,200 km² Everglades (Chen *et al.* 2010), to the World’s Oceans (Catalá *et al.* 2015, Jørgensen *et al.* 2011). OpenFluor (openfluor.org; (Murphy *et al.* 2014)) is an online database that compiles published PARAFAC models, allowing for new comparisons of components between such models in an effort to better understand the nature and drivers of DOM fluorescence.

In addition to optical properties, the chemical properties of DOM can be studied in a multitude of ways, including infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (Minor *et al.* 2014). FT-IR and NMR provide information about functional groups and structural features within the DOM. Mass spectrometry, on the other hand, gives detailed information on the masses present within the DOM, but provides little insight on structure. This dissertation will explore the relationships between optical properties and photochemistry of DOM, and Chapters 2 and 4 will couple these studies with ultra-high resolution mass spectrometry to gain further insight into the chemistry of DOM.

Photochemistry of Natural Organic Matter

The photo-degradation of DOM and in particular of the light absorbing 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.* 2014a, 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, 2004a), 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.* 2010b). 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).

One of the greatest impacts of DOM on water chemistry is its role as a major source and sink of reactive photo-produced species in surface waters (Cooper *et al.* 1989). Singlet molecular oxygen ($^1\text{O}_2$) (Haag *et al.* 1984b, Shao *et al.* 1994, Zepp *et al.* 1977) and hydroxyl radical ($\bullet\text{OH}$) (Zafiriou 1974) have long been understood to react with DOM (Scully *et al.* 2003, Westerhoff *et al.* 2007) and organic contaminants (Andreozzi *et al.* 2003, Boreen *et al.* 2003) in sunlit surface waters. More recently, the contribution of triplet excited states of DOM ($^3\text{DOM}^*$) to either enhance or inhibit the degradation of different classes of contaminants have been the subject of extensive study (Canonica *et al.* 2006, Canonica *et al.* 2000, Canonica *et al.* 1995, Canonica and Laubscher 2008, Luo *et al.* 2012, Santoke *et al.* 2012, Wang *et al.* 2012, Wenk and Canonica

2012, Wenk *et al.* 2011, Xu *et al.* 2011). Steady-state concentrations of $^1\text{O}_2$ and $^3\text{DOM}^*$ typically range on the order of $\sim 10^{-15}$ - 10^{-13} M in sunlit surface waters (Zepp *et al.* 1985). In estuarine and marine systems, the highly reactive, but much less abundant $\bullet\text{OH}$ (10^{-19} - 10^{-16} M) reacts with bicarbonate and carbonate to form longer-lived carbonate radicals, which can be present at up to two orders of magnitude higher than $\bullet\text{OH}$ (Canonica *et al.* 2005, Sulzberger *et al.* 1997).

The rate of degradation of a PPCP by reactive species is therefore defined as:

$$\frac{d\text{PPCP}}{dt} = \left(k_{^1\text{O}_2} [^1\text{O}_2]_{ss} + k_{\bullet\text{OH}} [\bullet\text{OH}]_{ss} + k_{^3\text{DOM}^*} [^3\text{DOM}^*]_{ss} + k_{\text{CO}_3^{\bullet-}} [\text{CO}_3^{\bullet-}]_{ss} + \dots \right) [\text{PPCP}] \quad (\text{i})$$

where k_{RS} is the reaction rate constant between the PPCP and the reactive species and $[\text{RS}]_{ss}$ is that species' steady-state concentration. While other reactive species such as $\text{O}_2^{\bullet-}$ and H_2O_2 are important in $\bullet\text{OH}$ cycling, their reaction rate constants with PPCPs tend to be low compared to the other reactive species. Hydrated electrons have fast reaction rates, comparable to $\bullet\text{OH}$, but are quickly quenched by oxygen in aerated waters. The four reactive species in Equation (i) are therefore the main focus of much of the research in the field as the most relevant for the degradation of PPCPs in engineered systems.

Constructed Treatment Wetlands

Constructed treatment wetlands have been traditionally used for the removal of nutrients such as nitrate and phosphate from surface waters and treated wastewater. These wetlands have the potential, however, to be utilized for PPCP treatment. Constructed wetlands often have a large surface area; while this can make them difficult to implement in many urban settings, it does allow for a large amount of light penetration and therefore photolysis of PPCPs. The unit process model has been proposed as the most effective for achieving both nutrient and PPCP removal in constructed wetlands (Jasper and Sedlak 2013). In this model, each wetland pond or

“cell” is dedicated to a specific function. Clear, shallow, open water cells can be used to effectively degrade compounds that undergo direct photolysis. Organic matter can then be added in successive cells to initiate indirect photolysis processes. The application of this organic matter via plant or algae exudates as opposed to decaying plant material or peat could have profound effects on the amount and the reactivity of organic matter that is added. Only by understanding the fundamental photochemistry of DOM can these systems be optimized to provide the highest level of treatment. The effects of organic matter source, molecular makeup, and water quality parameters such as pH on DOM reactivity must be investigated so that these parameters can be optimized. This dissertation will focus on the photochemistry of DOM, and the factors that affect the production of reactive species for the treatment of micropollutants in constructed wetlands.

CHAPTER 1:

Influence of pH on Fluorescent Dissolved Organic Matter Photo-degradation

1.1 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.

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 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 dissolved inorganic carbon (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.* 2003b). 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.* 2013b, 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.* 2010b, 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.

1.2 Methods

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 M Ω NanoPure™ water (Thermo Scientific™).

Photochemical experiments

A detailed schematic of the experimental setup is shown in Appendix A, Figure A.1. 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 (Fig. A.2) 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 μ L 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.

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 $100 \text{ mg SRNOM L}^{-1}$ 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 (base e) were calculated using the equation:

$$a(\lambda) = 2.303 * \text{abs}(\lambda) / L \quad (1.1)$$

where $\text{abs}(\lambda)$ is the raw absorbance (base 10) 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 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = <260/400-460$ nm), and the humic-like C peak ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 320-360/420-460$ 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 $\lambda_{\text{ex}} < 250$ 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 (in 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):

$$F_t = F_L e^{-k_L t} + F_{SL} e^{-k_{SL} t} + F_R \quad (1.2)$$

where the fluorescence (F) at time t is comprised of photo-labile (L) and semi-labile (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 ($\lambda_{ex}/\lambda_{em} = 346/460$ nm) plotted against irradiation time.

1.3 Results

pH dependence of optical properties

The fluorescence of Suwannee River natural organic matter was highly pH dependent, as reported previously (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. A.3). When the pH was increased from 4 to 10, two peaks grew on the shoulders of the traditional A and C peaks at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 246/480$ nm and 390/480 nm (Fig. 1.1). 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). Further discussion on the effect of pH on optical properties is presented in Chapter 4.

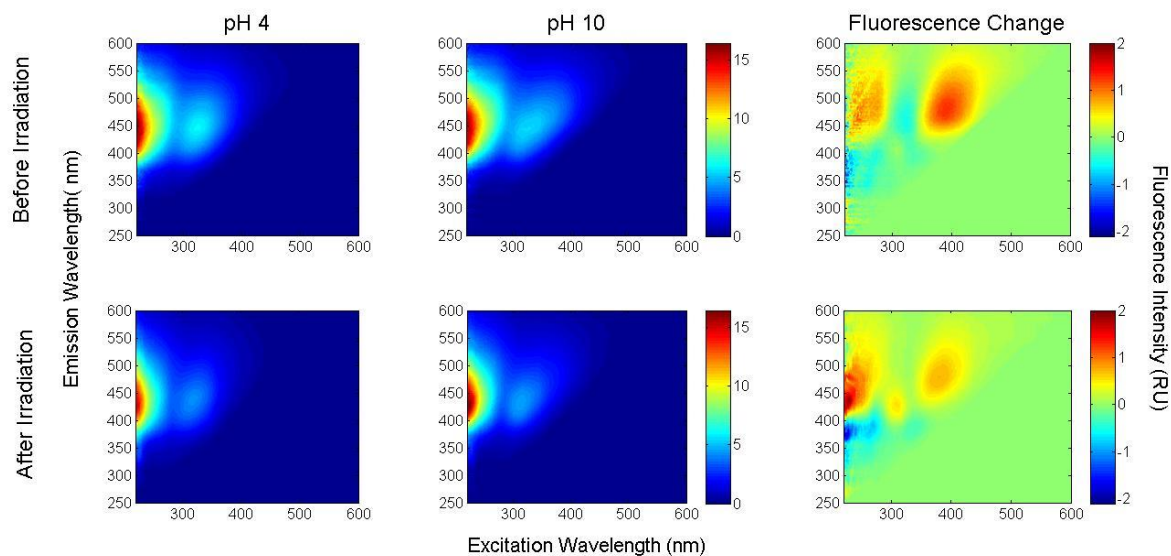


Figure 1.1: Effect of pH titration on the fluorescence of Suwannee River natural organic matter before and after 24 hours of solar-simulated irradiation at pH 4. Top row: un-irradiated sample at pH 4 titrated to pH 10, and the corresponding fluorescence change. Bottom row: sample irradiated for 24 hours at pH 4, then titrated to pH 10, and the fluorescence change

PARAFAC Analysis

A PARAFAC model was fit 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. 1.2): C1 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = <250, 300/418$), C2 ($<250, 338/454$), C3 (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. 1.1), and was labeled the “pH-influenced” component.

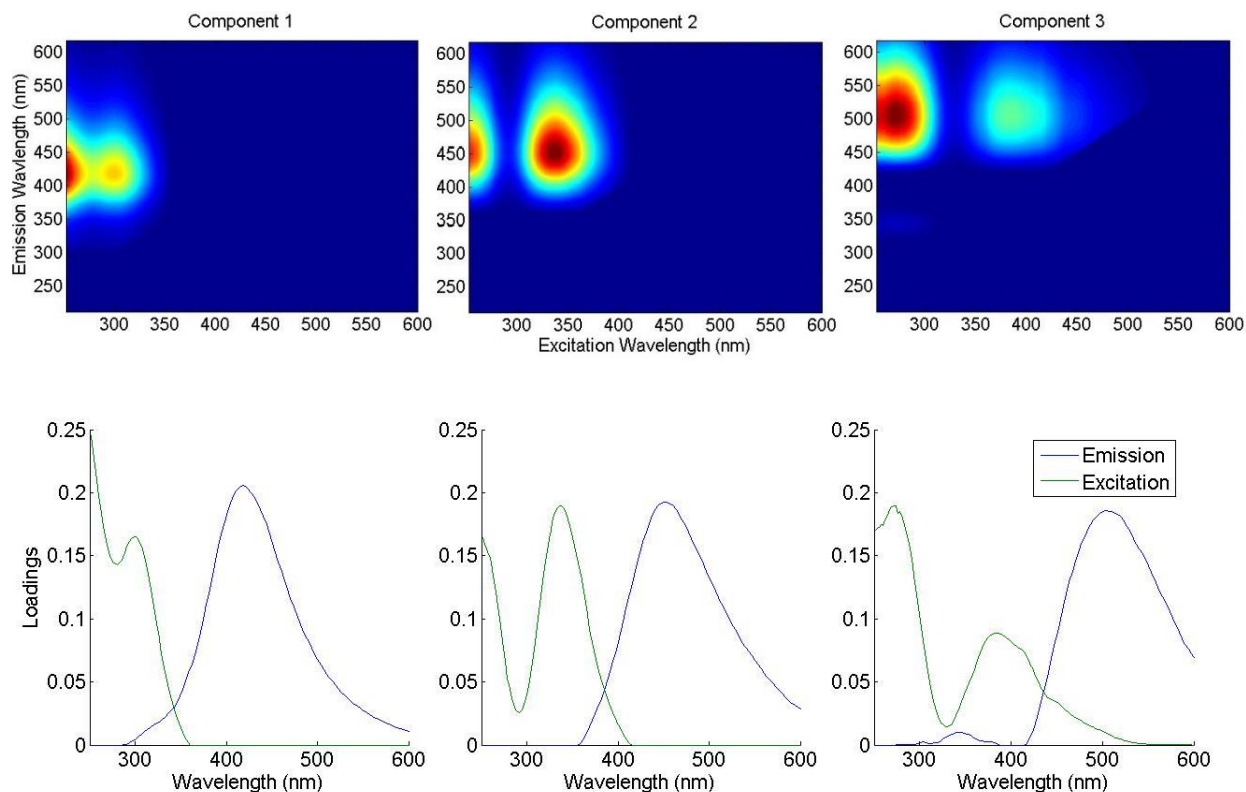


Figure 1.2: PARAFAC components for Suwannee River natural organic matter irradiated at various pH values. C1 is the main humic component, C2 is the photo-labile component, and C3 is the pH-influenced component.

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.

Photo-degradation kinetics

When exposed to simulated sunlight, SRNOM showed significant loss of fluorescence (Fig. 1.3). 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 $\lambda_{\text{ex}}/\lambda_{\text{em}} = 224/480$ 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.

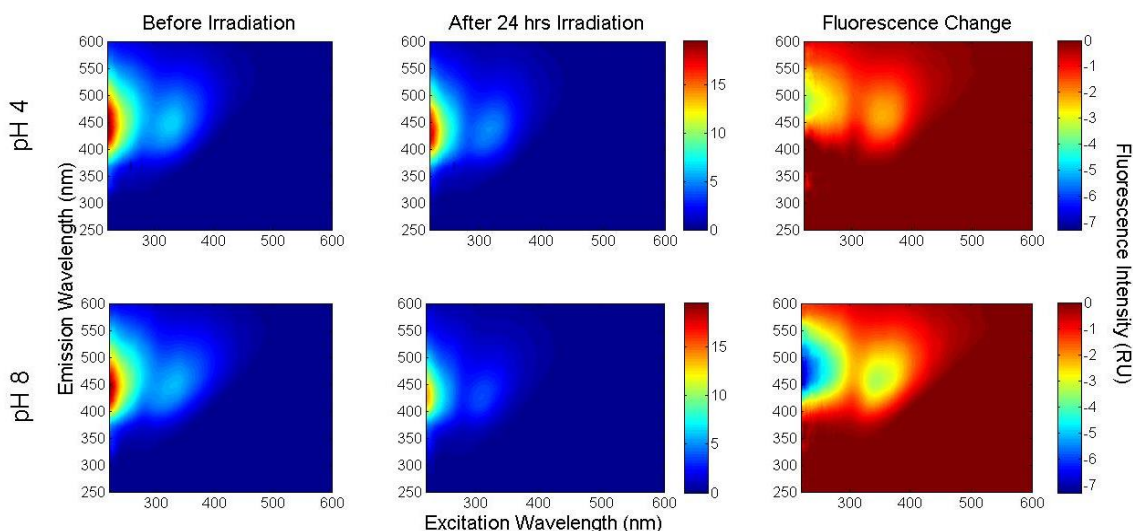


Figure 1.3: Effect of 24 hour irradiation on the fluorescence of Suwannee River natural organic matter at pH 4 (top row) and pH 8 (bottom row).

Fluorescence loss showed a strong pH dependence. With increasing pH, the total fluorescence loss and overall degradation rate also increased (Fig. 1.4). 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. 1.4). 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 1.1), and all rate constants were significantly different for pH 4 and pH 8 ($p < 0.05$; Fig. A.4). Rate constants from the PARAFAC analysis (k_{C2-L} and k_{C2-SL}) and obtained via the peak-picking approach at $\lambda_{ex}/\lambda_{em} = 346/460$ 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. A.5). 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.

Table 1.1: 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 $\lambda_{\text{ex}}/\lambda_{\text{em}} = 346/460$ nm for the labile (L) and semi-labile (SL) fractions; k_{C2} and k_{C3} denote rates for PARAFAC Components 2 and 3, respectively

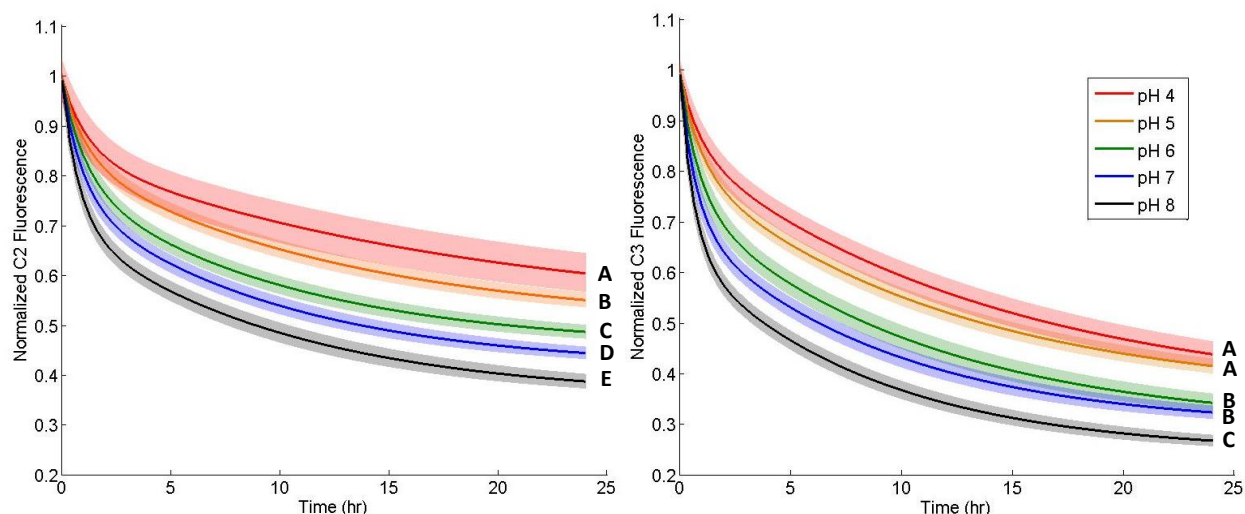


Figure 1.4: Loss of C2 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = <250, 338/454$), and C3 (272, 382/504) during 24 hour solar-simulated irradiation. Solid lines represent the average of triplicate experiments, while shaded areas represent one standard deviation. Values normalized to component intensity 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.

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. 1.5). 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. 1.5). 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).

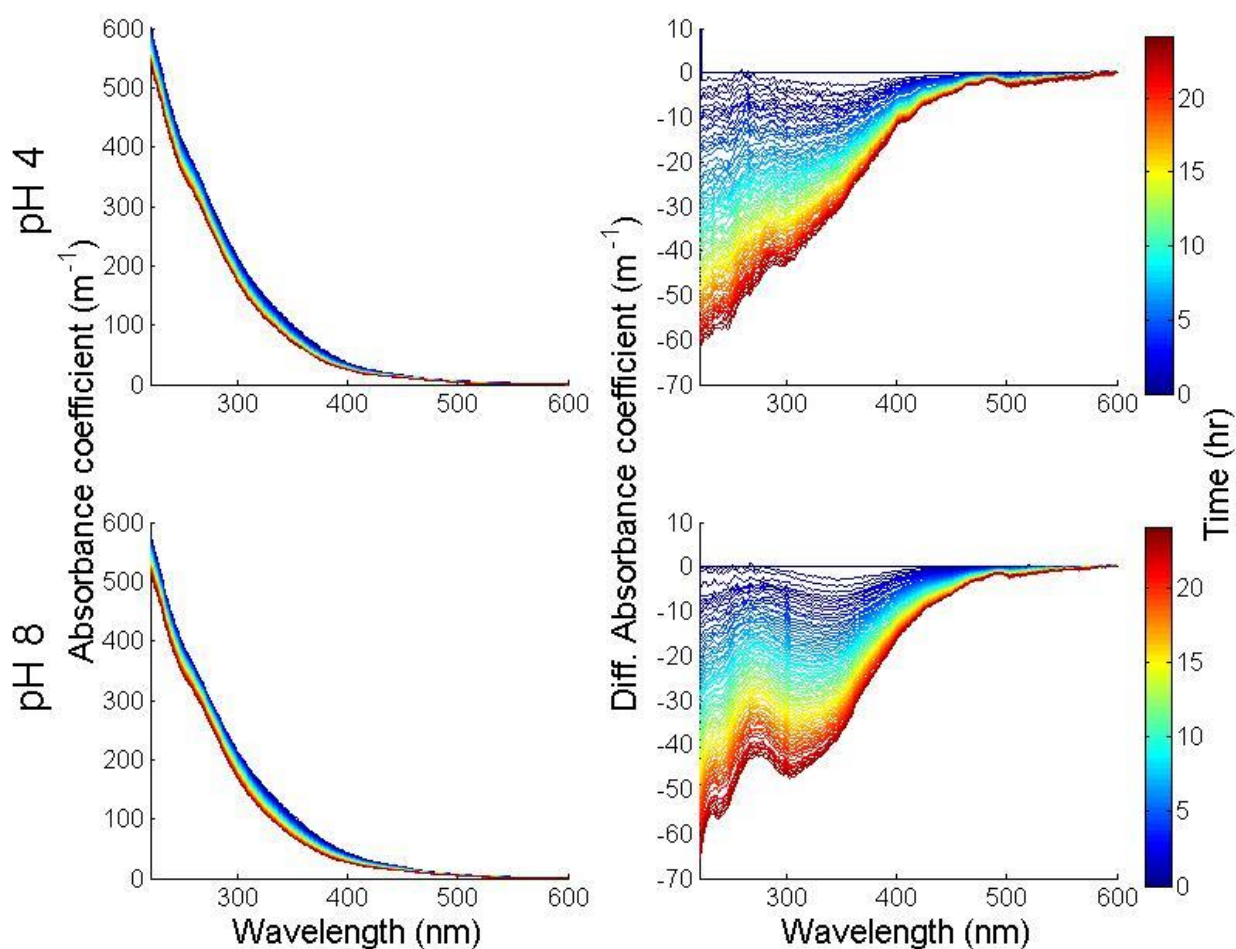


Figure 1.5: Effect of 24 hour irradiation on 100 mg L⁻¹ Suwannee River natural organic matter absolute and differential absorbance spectra for pH 4 (top row) and pH 8 (bottom row).

1.4 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. 1.3, 1.4). 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. A.4), yet the resulting fluorescence loss of the most photo-labile component, C2, increased significantly (Fig. 1.4). 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 (Fig. A.6). 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.* 2003b, Chen *et al.* 2014a, 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 1.1). pH titrations of samples that were irradiated at pH 4 (Fig. 1.1) 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 2004a), 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. 1.5) 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.

CHAPTER 2:

Depth-dependent Photo-degradation of Marine Dissolved Organic Matter

2.1 Introduction

Marine dissolved organic matter (DOM) is one of the largest carbon reservoirs on Earth. At 662 Pg of carbon, it contains over 200 times the amount of carbon stored as marine biomass (Hansell *et al.* 2009). Consequently, the composition and reactivity of marine DOM is of utmost importance in understanding the role that this material plays in the marine carbon cycle.

Traditionally, chromophoric DOM (CDOM) in the ocean has been attributed to terrestrial sources, namely lignin and other higher plant material, while autochthonous production was only recognized on local- and short temporal scales (Andrew *et al.* 2013, Coble 2007). More recently, the microbial carbon pump model for microbial production has been found to be an important oceanic source of biologically recalcitrant CDOM in the oceans (Jiao *et al.* 2010), and it has been suggested that autochthonous biological production may surpass terrestrial input of FDOM to the oceans (Jørgensen *et al.* 2011, Nelson and Siegel 2013, Yamashita and Tanoue 2008).

Photo-bleaching has been recognized as the most important sink for CDOM in the ocean, both through the direct mineralization as well as potential transformation of CDOM to smaller and more bio-available forms (Gonsior *et al.* 2014a). In the open ocean, surface waters are usually CDOM- and FDOM depleted and concentrations increase with depth (Catalá *et al.* 2015, Jørgensen *et al.* 2011). The light history and the biogeochemical origin of the CDOM affect its lability; for example, areas of upwelling, high biological productivity, and decreased irradiance have higher CDOM and FDOM content (Nelson and Siegel 2013). Additionally, deep water DOM that has not been exposed to light for extended periods of time may be more

photosensitive, degrading faster than DOM that has already undergone some photobleaching (Gonsior *et al.* 2013).

Excitation emission matrix (EEM) fluorescence is the most common method of investigating FDOM in the ocean. Fluorescence detection is much more sensitive than absorbance, making it more practical for analyzing low levels of FDOM (Coble 2007). EEM spectroscopy in particular is sensitive enough to analyze low concentrations of DOM in bulk seawater and fast enough to investigate small-scale variation in composition (Coble 1996). While direct comparisons of EEMs is an important qualitative (and semi-quantitative) tool, parallel factor (PARAFAC) analysis, which compares large datasets of EEMs and deconvolutes them into statistically independent components, has emerged as the leading method for quantification of fluorescence signatures (Murphy *et al.* 2013).

Ultrahigh resolution electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) has been used to characterize marine DOM (Chen *et al.* 2014b, D'Andrilli *et al.* 2010, Flerus *et al.* 2012, Gonsior *et al.* 2014a, Gonsior *et al.* 2011, Hertkorn *et al.* 2006, Koch *et al.* 2005, Lechtenfeld *et al.* 2014); however, the relationship between molecular composition and optical properties remains unclear. Herzsprung *et al.* (2012) compared humic-like fluorescence intensities of water from a German drinking water reservoir catchment area with mass peaks identified using FT-ICR-MS, and found strong correlations between unsaturated, highly oxidized compounds and this fluorescence. Studies of DOM-rich boreal rivers and lakes likewise compared EEM-PARAFAC components with molecular formulas derived from FT-ICR-MS, identifying similar relationships between longer wavelength, 'humic-like' fluorescent components and oxidized aromatic compounds (Kellerman *et al.* 2015, Stubbins *et al.* 2014).

In order to better understand the processing of FDOM in the ocean, we investigated depth-related changes in abundance and photochemical reactivity of FDOM in the North Atlantic Ocean. The molecular composition of DOM from a detailed depth profile was analyzed by FT-ICR-MS and then correlated to EEM-PARAFAC components.

2.2 Materials and Methods

Sample sites

Samples were collected on three separate cruises in the Atlantic Ocean. Surface seawater samples were collected during a cruise of the R/V *Polarstern*, Nov. 2008, along a transect from 50.2° N to 31.4° S in the eastern Atlantic Ocean. Samples were collected using a 24 Niskin bottle rosette CTD profiler at the surface and 200 m depth. Depth profiles (0-4525 m) of seawater were also collected at the Bermuda Atlantic Time Series (BATS) site in the northern Sargasso Sea southeast of Bermuda during cruises of the R/V *Atlantic Explorer* in June 2012 (0, 110, 750, 1500, 3000, and 4527 m) and July 2013 (every 200 m from 0 m to 4400m, and 4525 m).

All samples were solid phase extracted using a previously described method (Dittmar *et al.* 2007). Briefly, 5-20 L of filtered samples (0.7 µm Whatman GF/F) were acidified with hydrochloric acid (p.a. grade) to pH 2. Solid-phase extraction cartridges (Agilent Bond Elut PPL) packed with 1 g resin were conditioned with methanol, rinsed with acidified (pH 2) ultra-pure water, and the samples were gravity-fed through each cartridge. Cartridges were then rinsed with additional acidified water, dried, and the samples eluted with 5 or 10 mL methanol. Methanolic extracts were stored at -18°C prior to further analyses. Previous work has shown that for open ocean samples, DOC extraction efficiency is ~ 42 % (Dittmar *et al.* 2008, Lechtenfeld *et al.* 2014), and that while CDOM and FDOM extraction with this method is incomplete, extracts are

representative, showing similar relative changes in absorbance and fluorescence as whole water samples (Roettgers and Koch 2012).

Photo-irradiation experiments

Photo-irradiation experiments were conducted on extracts collected at BATS in June 2012. The volume of extract used for each sample was proportional to the volume of seawater extracted in order to keep the concentration factor constant. Methanol extracts were dried under N₂, dissolved in 25 mL NanoPure water, sonicated for five minutes, and filtered through pre-washed 0.2 µm Whatman GD/X syringe filters immediately before use. Each sample was then irradiated for 24 hours with the photo-irradiation system described in detail in Chapter 1.

Absorbance and EEM spectra were collected simultaneously at twenty minute intervals throughout the photo-irradiation experiments. Excitation scans were recorded from 600 nm to 220 nm in 2 nm steps. Emission spectra were collected in ~3 nm steps from 211.5 nm to 617.7 nm, with integration times of 1 second. Spectra were converted to Quinine Sulfate Units (QSU) by dividing fluorescence intensities by the fluorescence intensity of 1 mg L⁻¹ standard reference quinine sulfate or to Raman Units (RU) by dividing intensities by the area under the water Raman peak at 350 nm excitation (emission range 383-442 nm). Rayleigh- and Raman scattering and inner filter effects were corrected for by the Aqualog software and in MATLAB with the methods described by Zepp *et al.* (2004). Methanol extracts of cruise samples not used in photo-irradiation experiments were dried under N₂, dissolved in NanoPure water, and fluorescence spectra recorded as above.

Photo-degradation of PARAFAC Components C2 and C4 (see below) were modeled using a double exponential function using IGOR Pro (WaveMetrics, Inc) with Equation 1.2. Samples were irradiated without pH adjustment or control. The pH of samples increased slightly (0.09-

0.65 pH units, 0.32 pH units average) during 24 hours of irradiation, with the maximum change of 0.65 pH units occurring with the 3000 m depth sample. As shown in Chapter 1, rates of FDOM loss during irradiations of Suwannee River natural organic matter were shown to be not significantly different when the pH was varied by 1 pH unit; we therefore assumed that the small pH changes during irradiations in this study had no significant impact on the reaction kinetics.

PARAFAC modeling

PARAFAC modeling was conducted using the drEEM toolbox (Murphy *et al.* 2013). A total of 611 EEMs, of which 173 were from cruise samples and 438 from photo-irradiation experiments, were visually investigated, and outliers (scans with bubbles, spectral errors, etc.) removed. Samples were normalized to unit variance to reduce concentration effects during modeling, and the normalization reversed after model completion. Excitation wavelengths < 250 nm showed high leverages (deviation from average distribution), most likely due to higher relative values and/or the lower signal to noise ratio at such wavelengths (Stedmon and Bro 2008); removal of these wavelengths improved the modeling, namely the spectral loadings of longer-wavelength components. Two separate models were fit: the East Atlantic transect (surface and 200 m depth), and the Sargasso Sea samples (including spectra collected during photo-irradiation experiments). Evaluation of spectral loadings, split-half validation, random initialization analysis, and residuals analyses were performed as described elsewhere (Murphy *et al.* 2013, Stedmon and Bro 2008). Criteria for final model assignment were 1) split-half validation 2) residuals analysis and 3) spectral loadings.

Ultrahigh resolution mass spectrometry

The solid-phase extracted samples from the July 2013 depth profile at BATS (26 samples total) were analyzed using the electrospray ionization (ESI) source of the Bruker Apex QE 12

Tesla FT-ICR-MS located at the Helmholtz Center for Environmental Health, Munich, Germany. Samples were diluted with methanol 1:20 before injection to avoid overloading the ion cyclotron resonance ion trap which could lead to peak splitting and inconsistent calibration across the desired mass range (147-2000 Dalton). To avoid any cross-contamination between samples, an automated cleaning procedure was implemented that used 600 μL (80% methanol, 20% pure water) at a flow rate of 300 $\mu\text{L min}^{-1}$ between samples. The sample flow rate was set to 3 $\mu\text{L min}^{-1}$, and ESI was run in negative mode at -3.6 kV. Five hundred spectra were averaged to obtain a mass accuracy better than 0.2 ppm and an average mass resolution of about 500,000 at m/z 350-400. The MS system was pre-calibrated using arginine and then post-calibrated by using known DOM m/z ions ($255.08741 = \text{C}_{12}\text{H}_{15}\text{O}_6^-$, $297.09798 = \text{C}_{14}\text{H}_{17}\text{O}_7^-$, $367.13984 = \text{C}_{18}\text{H}_{23}\text{O}_8^-$, $491.15589 = \text{C}_{24}\text{H}_{27}\text{O}_{11}^-$, $553.1562 = \text{C}_{25}\text{H}_{29}\text{O}_{14}^-$, $611.19814 = \text{C}_{28}\text{H}_{35}\text{O}_{15}^-$ and $707.25566 = \text{C}_{34}\text{H}_{43}\text{O}_{16}^-$) throughout the entire mass range of interest (200-800 Da). The signal to noise ratio was set to 10 and mass lists were subsequently generated.

The mass resolution and accuracy of the FT-ICR-MS system allowed molecular formula assignments to ions up to m/z 800. Not all m/z ions could be assigned, indicating that some m/z peaks were outside of the used atomic combination of $^{12}\text{C}_{0-\infty}$, $^1\text{H}_{0-\infty}$, $^{16}\text{O}_{0-\infty}$, $^{14}\text{N}_{0-3}$ and $^{32}\text{S}_{0-2}$, as well as the following isotopologues ^{13}C , and ^{34}S . Visualization of complex mass spectrometric data have been achieved by elemental or van Krevelen diagrams (van Krevelen 1950). A modified Kendrick plot was also used to visualize homologues series that are spaced only by CH_2 groups (Shakeri Yekta *et al.* 2012).

Multivariate Statistical Analysis

All data were normalized via autoscaling by subtracting the average of a variable from the data and then dividing by the standard deviation, allowing for multivariate statistical analysis

(Bro and Smilde 2014). Generated mass lists of all FT-ICR-MS samples were then combined in a large matrix. The generation of the matrix was undertaken by searching m/z ions within an error of 0.2 ppm to be able to assign one m/z value to all intensities of all spectra. If a m/z was found in one sample but not in others, the missing intensity value was replaced by 0. This approach resulted in a matrix where all m/z ions represented the variables and the m/z intensities the data. Principal component analyses (PCA) were undertaken on the normalized FT-ICR-MS and EEM-PARAFAC data sets, respectively. The normalized fluorescence data was then used to create a resemblance matrix using Spearman Rank Correlations. This EEM-PARAFAC resemblance matrix was then used for the canonical analysis on principal coordinates (CAP) of the FT-ICR-MS data matrix. CAP analysis allowed to discriminate between sets of variables, in this case, between the EEM-PARAFAC Components and m/z molecular ions and their intensities.

2.3 Results

PARAFAC modeling

PARAFAC analysis of the Sargasso Sea dataset (492 EEMs) produced a five component model (split-half validated, >99.8 % explained variance; Fig. 2.1). The components contained ‘humic-like’ components characterized by fluorescence in the visible region, as well as components in the UVA portion of the spectrum. This UVA fluorescence is characteristic of small aromatic molecules, including the amino acids tryptophan and tyrosine. The model components were labeled according to conventional terminology as ‘marine-like’ C1 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = <250$ (306)/ 404 nm), ‘terrestrial-like’ C2 (<250 (360)/447), UVA component C3 (<250 (282)/332), ‘terrestrial-like’ C4 (276(399)/497) and UVA component C5 (276/304) (Coble 2007, Stedmon and Nelson 2015). To examine the robustness of the model, a five component model was fit to only the East Atlantic cruise data (119 EEMs), with 99.7 % explained variance.

Models with more than five components could not be split-half validated. Models with less than five components showed significant peaks and troughs throughout the residuals, indicative of a poor model fit, while the residuals of the five component models only showed minor peaks along the Raman and Rayleigh scattering lines (Murphy *et al.* 2013). The components in both the East Atlantic and the Sargasso Sea models were very similar with regards to peak location and shape, especially for the visible fluorescent components (Fig. 2.2). This similarity provides a strong validation that the modeling of the EEMs collected during photo-irradiation experiments are indicative of fluorescence changes occurring in the natural environment, even on large geographical scales. Hence, the observed results on the photochemical changes in marine FDOM are not limited to the Sargasso Sea, but can presumably generalized, at least to the greater Atlantic Ocean.

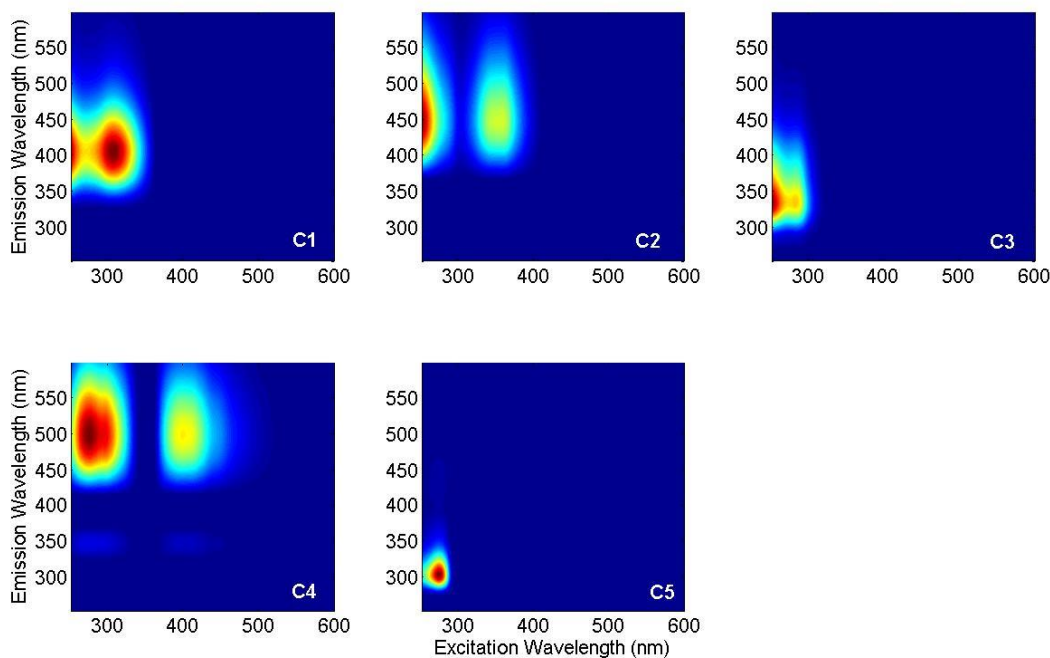


Figure 2.1: PARAFAC components in Sargasso Sea FDOM: marine-like C1, terrestrial-like C2, UVA C3, terrestrial-like C4, and UVA C5.

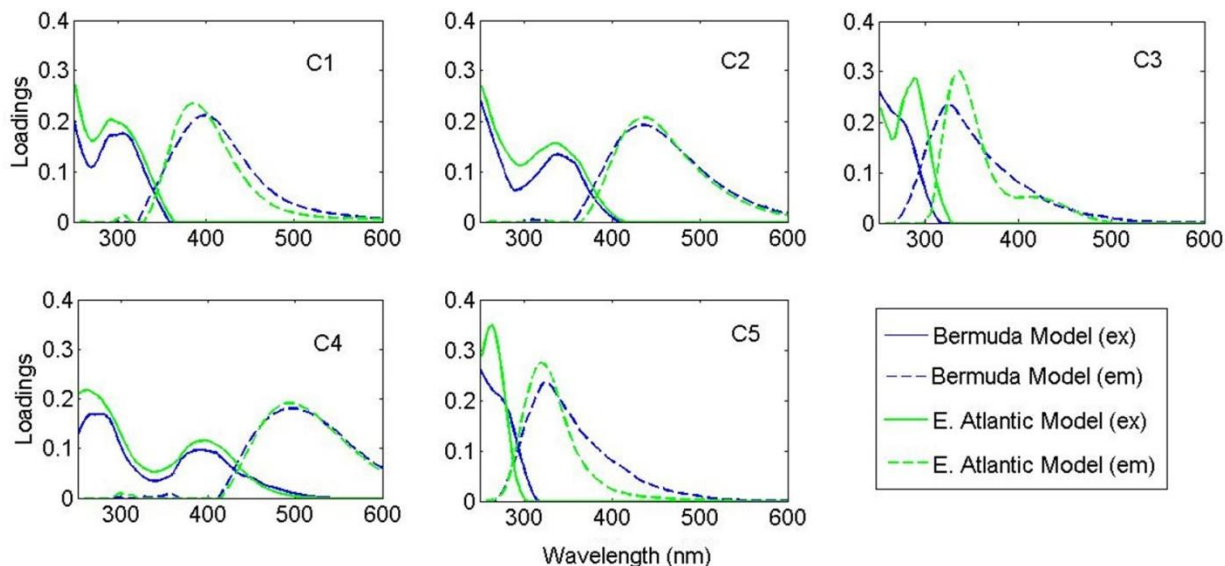


Figure 2.2: Spectral loadings of the PARAFAC components in the Bermuda model (blue) and East Atlantic model (green)

Depth dependence of FDOM fluorescence intensity at BATS

All three visible fluorescence components (C1, C2, and C4) were significantly depleted in surface waters and increased with depth (Fig. 2.3). The intensity of marine-like C1 was on average 3.4 times higher below 1000 m than at the surface. The intensity of ‘terrestrial-like’ components C2 and C4 were also higher below 1000 m (5.9 and 5.6 times, respectively) than at the surface. In contrast, UVA component C5 was maximal at the surface and decreased with depth, albeit less dramatically. On average, C5 intensity was 1.5 times higher at the surface than in waters >1000m depth. UVA component C3 showed no strong depth-dependence. No strong correlations between PARAFAC component intensity and apparent oxygen utilization (AOU) were seen, although the small dataset (two depth profiles) prevented detailed interpretation of these results.

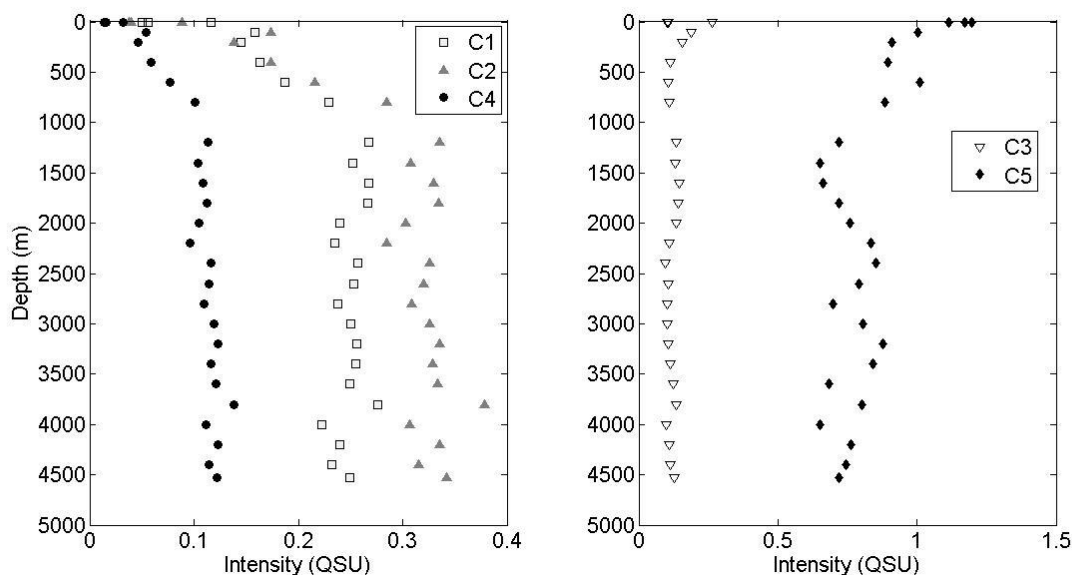


Figure 2.3: Depth profiles of visible fluorescence components C1, C2, and C4 and UVA components C3 and C5

Photo-degradation kinetics of FDOM at BATS

EEMs before and after irradiation, and differential plots, are shown in Figure 2.4. Terrestrial-like components C2 and C4 showed significant photo-lability at all depths. The long-wavelength component C4 showed greater relative fluorescence loss (%), whereas component C2 showed greater total loss in fluorescence (Fig. 2.5). The rate of loss of the semi-labile fraction, k_{SL} , showed no depth-dependent trend, with an average $0.063 \pm 0.008 \text{ hr}^{-1}$ for C2 and $0.069 \pm 0.008 \text{ hr}^{-1}$ for C4. The rate of loss of the labile fraction, k_L , was faster in the surface and 110 m samples ($2.2 \pm 0.2 \text{ hr}^{-1}$ and $1.4 \pm 0.2 \text{ hr}^{-1}$ for C2 and C4, respectively) than in the deep samples ($1.1 \pm 0.1 \text{ hr}^{-1}$ and $0.89 \pm 0.08 \text{ hr}^{-1}$ for C2 and C4, respectively). Loss of C2 over the 24 hr irradiations was lowest in the surface sample (38 %), followed by the 110 m sample (42 %), while samples at depth showed extremely similar C2 loss ($50 \pm 1 \%$). Loss of C4 showed a similar trend, with the least amount of fluorescence lost in the surface sample (51 %), while the samples at greater depth all showed similar loss ($60 \pm 3 \%$).

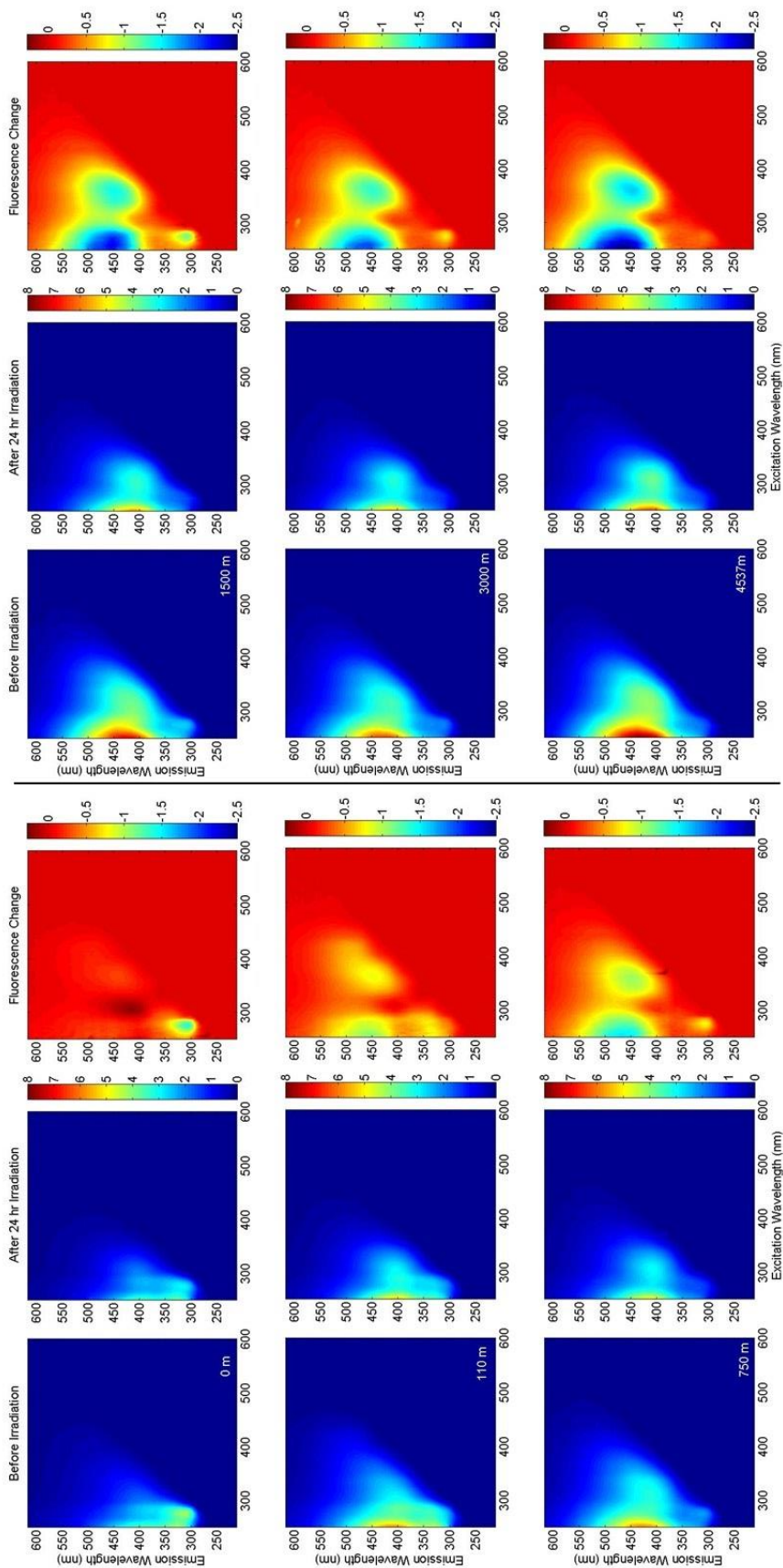


Figure 2.4: EEM fluorescence of BATS samples at various depths before and after irradiation, and the fluorescence change.
All fluorescence intensities are in Raman Units

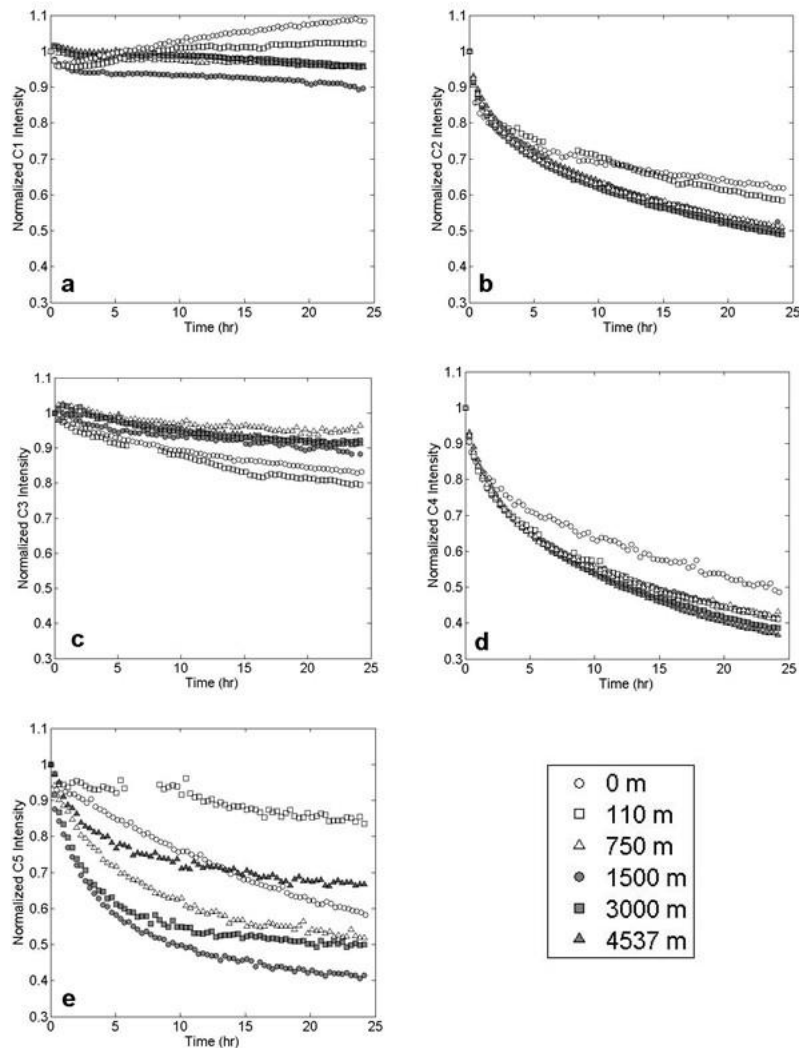


Figure 2.5: Photo-degradation of PARAFAC components (a) marine-like C1, (b) terrestrial-like C2, (c) UVA C3, (d) terrestrial-like C4, and (e) UVA C5 at various depths in the Sargasso Sea.

Marine-like component C1 showed variable, but more limited, photo-reactivity than either C2 or C4 (Fig. 2.5). At the surface, C1 fluorescence decreased by 4 % in the first 20 minutes of irradiation and then increased to a final value which was 8 % higher than the initial fluorescence. Similarly, C1 fluorescence at 110m depth (the chlorophyll fluorescence maximum) initially decreased by 3% and then increased to a final value that was 2% greater than the initial fluorescence. In deep waters, C1 fluorescence decreased over the 24 h irradiation period by 10% at 1500 m depth and by an average of 5% at other depths.

UVA fluorescent components C3 and C5 showed variable photo-lability (Fig. 2.5c,e). Maximum decreases in C3 fluorescence over the 24 hr irradiation period were observed at the surface (15%) and at the 110 m chlorophyll fluorescence maximum (20%). The minimum decrease in C3 fluorescence was observed at 750 m depth (< 4 %). Samples from 1500, 3000, and 4537 m depths showed intermediate decreases in fluorescence (12, 8, and 9 %, respectively). Component C5 fluorescence intensity decreases ranged from 16-58 % with no discernible depth trend.

Ultrahigh resolution mass spectrometry

The majority of m/z peaks were shared between all samples in the 0-4525 m depth profile at the BATS site. However, direct comparison between averaged surface samples collected at BATS in 2013 and the 4525 m sample (35 m above bottom) revealed distinct characteristic and unique signatures (Fig. 2.6). In surface waters, there were 165 unique CHO molecular ions at a signal to noise ratio of 10, representing largely aliphatic compounds with hydrogen to carbon ratios (H/C) between 1.5-2.0 and oxygen to carbon ratios (O/C) between 0.2-0.7, as well as 90 nitrogen-containing ions (CHNO). These occupied the same aliphatic area within the chemical space indicative of presumably labile compounds, with the exception of a distinct higher abundant group centered on H/C of 1.2 and O/C of 0.5. Interestingly, the largest unique signature was the presence of 230 high abundant sulfur-containing ions that again were localized in the aliphatic region of the van Krevelen diagram.

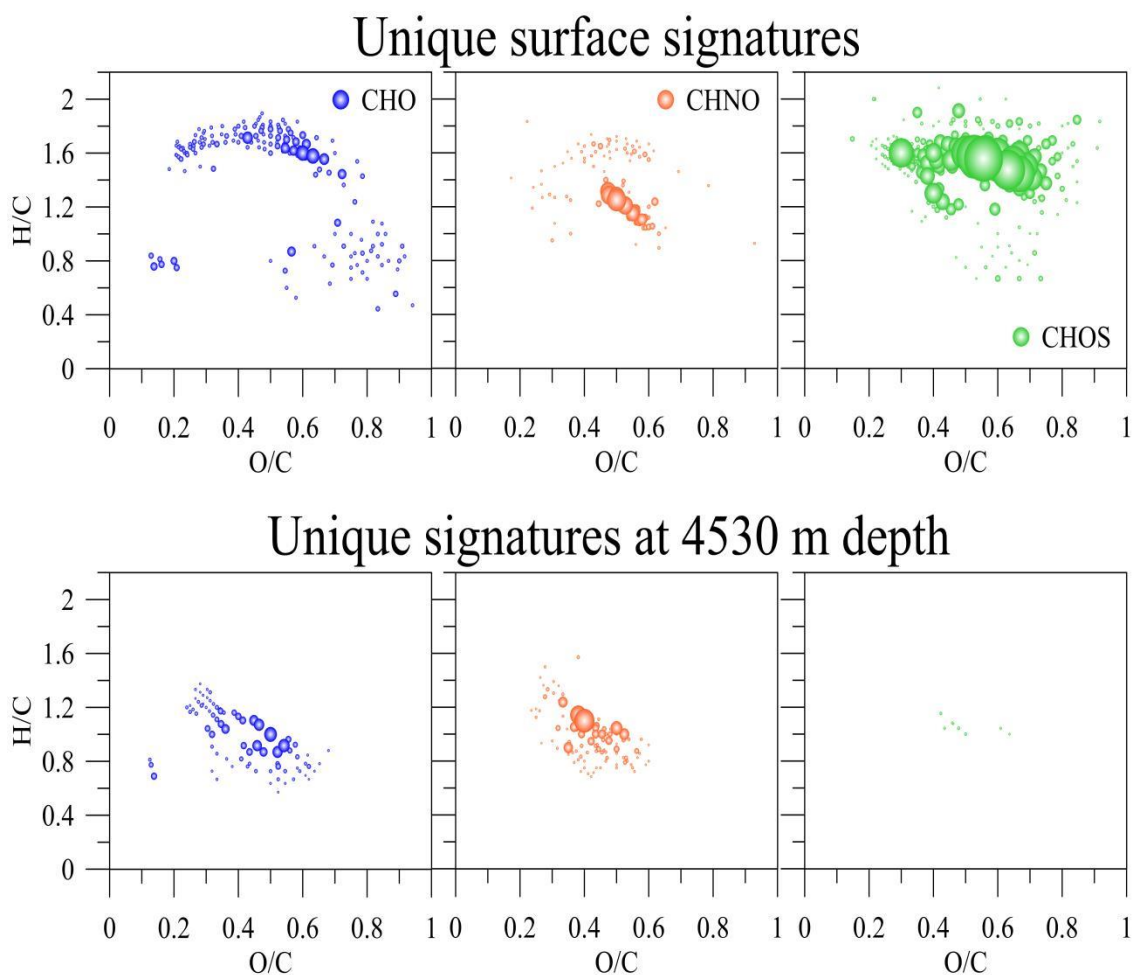


Figure 2.6: Unique assigned molecular formulas of the SPE-DOM at 5 m and 4530 m depth collected at BATS in the Sargasso Sea, 2013.

In contrast to surface waters, waters at 4525 m depth contained unique low abundant, hydrogen-deficient molecular CHO ($n=78$) and CHNO ($n=112$) ions that showed H/C of 0.6-1.2 and O/C of 0.2-0.6 but very few unique CHOS ions. Hence, aliphatic compounds were enriched at the surface and more aromatic compounds were present at depths. This finding agrees with the increase of FDOM with depth and the biological, and potentially photochemical, production of labile aliphatic compounds at the surface.

Statistical Evaluation of FT-MS and EEM PARAFAC Data

The PCA results from the FT-ICR-MS data clearly differentiated molecular variations in surface, mixed layer, and waters from > 800 m depth (Fig. 2.7B). A Spearman Rank correlation of the variables (m/z) and the Principal Component 1 (indicative of depth) revealed similar patterns in the van Krevelen diagram (Fig. 2.8) when compared to the unique signatures (Fig. 2.6): aliphatic CHO and CHNO ions correlated well with the surface while more hydrogen-deficient ions correlated with depth. However, the region of the van Krevelen with O/C ratios between 0.5-0.8 and H/C ratios between 0.5-1.2 was much more occupied (Fig. 2.8, CHO plot). This region in the van Krevelen diagram has been previously suggested to be highly correlated with CDOM that can be flocculated by aluminum sulfate in a drinking water treatment plant (Gonsior *et al.* 2014b).

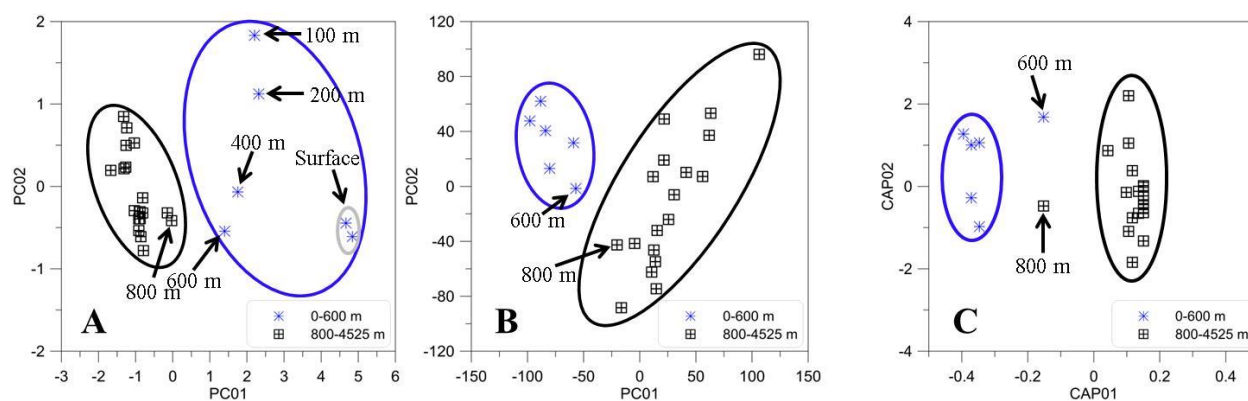


Figure 2.7: Statistical analyses of BATS depth profile (2013) **A**: Principal Components 1 and 2 for all PARAFAC component intensities **B**: Principal Components 1 and 2 for all FT-ICR-MS m/z molecular ions and their intensities for the same samples. **C**: Canonical analysis on principal coordinates (CAP) of the normalized EEM resemblance matrix and the FT-ICR-MS data.

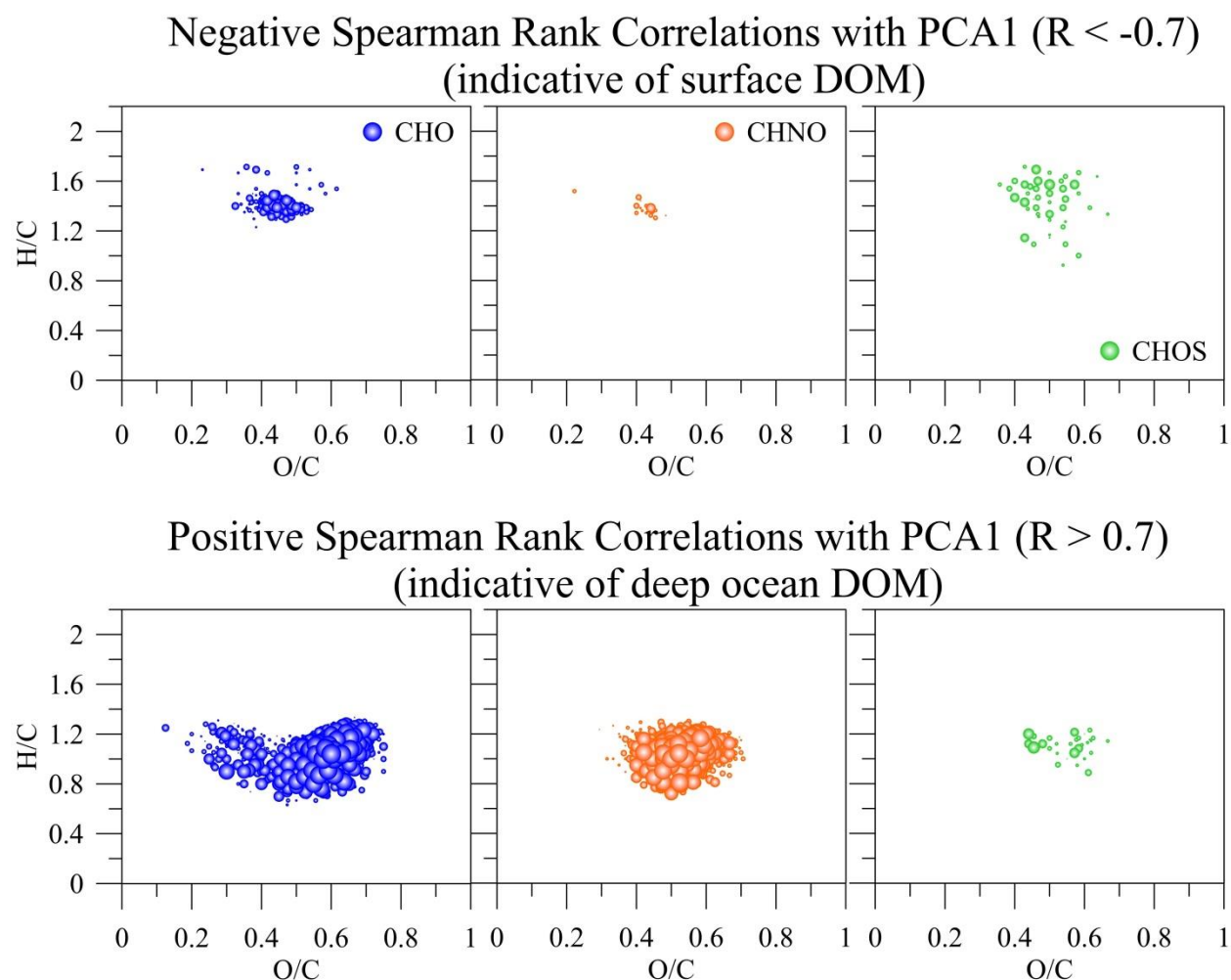


Figure 2.8: Positive (indicative of surface DOM) and negative (indicative of deep ocean DOM) Spearman Rank correlations greater $R = 0.7$ and lower -0.7 , respectively of all common m/z ions, their intensities, and assigned molecular formulas with the first principal component (PCA1) derived from SPE-DOM samples collected at BATS in 2013 and analyzed by FT-ICR-MS. Note: The bubble size corresponds to R values between 0.7 and 1 and -0.7 and -1 , respectively.

PCA was also carried out on the intensities of the five PARAFAC components (Fig. 2.7A). Results showed a similar separation between surface and deep waters as for the FT-ICR-MS data (Fig. 2.7B). Lastly, Canonical Analysis on Principal coordinates (CAP) (Anderson and Willis 2003) was used to correlate the two independent FT-ICR-MS and EEM-PARAFAC datasets (Fig. 2.7C). In this method, ordination can be constrained by using any dissimilarity measures while also incorporating the correlation structures of variables. Results of the CAP analysis of the EEM-PARAFAC data resemblance matrix (Spearman Rank correlations) with the FT-ICR-MS data also showed a clear separation between surface, mixed layer and >800 m samples (Fig. 2.7C) along CAP Component 1 (CAP1). Eigenvector values of CAP1 were then used to distinguish between m/z ions and associated molecular formulas that were indicative of surface and mixed layer (negative values) and of the deep ocean (positive values). Results again indicate enrichment in aliphatic compounds (CHO, CHNO) and substantial sulfur-containing molecules (CHOS) in the surface waters and enrichment in more unsaturated/aromatic compounds and polyphenolic-like composition at depth (Fig. 2.9).

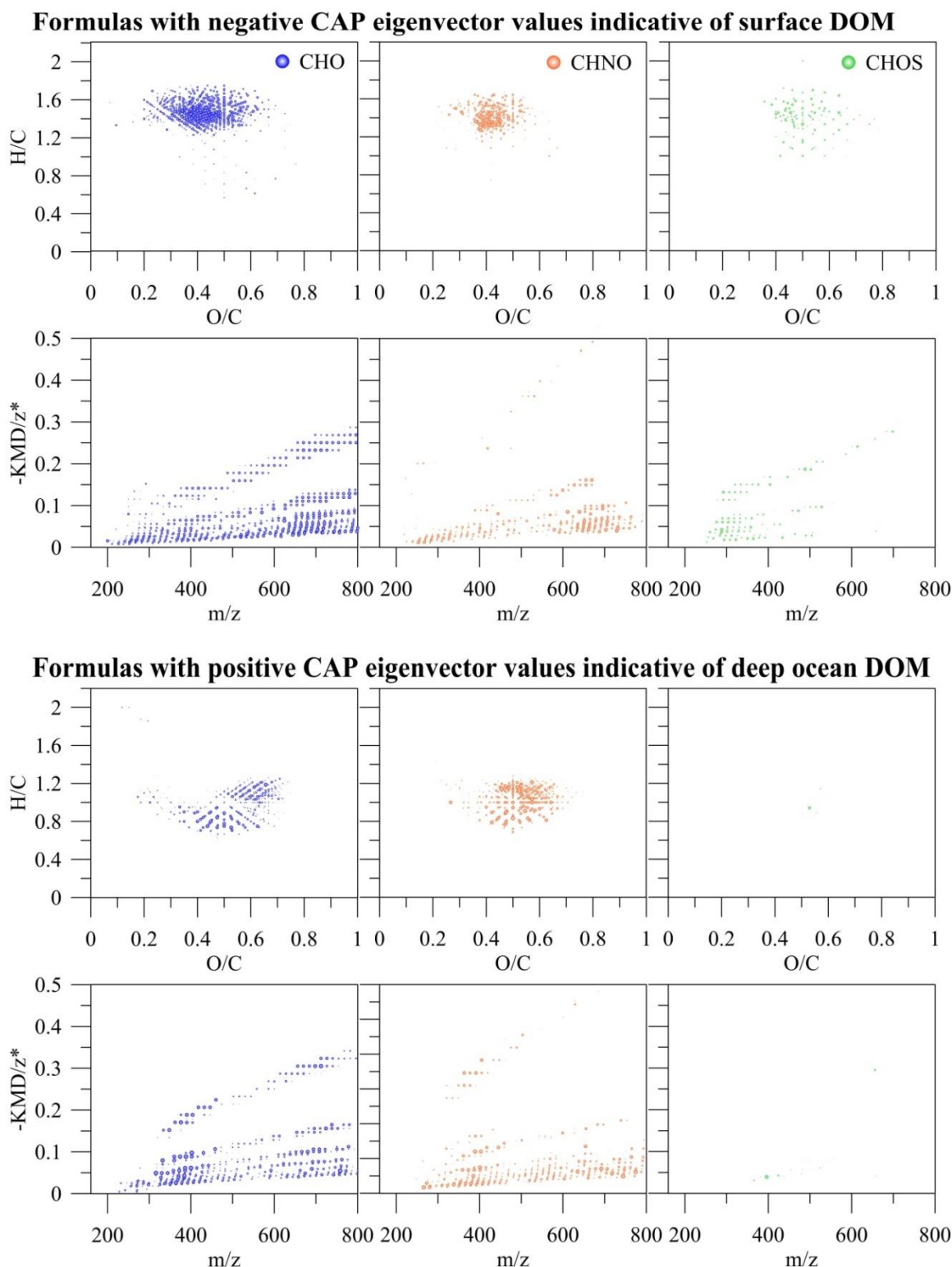


Figure 2.9: m/z ions and associated molecular formulas with negative and positive eigenvector values of the canonical analysis on principal coordinate 1 (CAP1) of the EEM resemblance matrix and the FT-ICR-MS data of all SPE-DOM samples collected at BATS in 2013 (see also Fig. 2.7C)

2.4 Discussion

Photochemical loss of fluorescence in Sargasso Sea waters was dominated by terrestrial-like components C2 and C4. The photo-reactivity of these components explains why these components were found to be depleted at the ocean surface (Jørgensen *et al.* 2011). The lesser degradation in the surface samples was expected, as the most photo-labile structures were already bleached from the surface water. Interestingly, the kinetic analysis showed no depth-dependent trends in the rate of loss of the semi-labile fraction of each component, k_{SL} . This suggests that the groups of fluorophores responsible for this fraction of the fluorescence are compositionally similar at all depths, while the labile fraction, k_L , may consist of different classes of compounds that contain similar fluorescent moieties. Photo-degradation of terrestrial-like components C2 and C4 showed similar relative kinetics to Suwannee River Natural Organic Matter (IHSS standard), with C4 losing a greater percentage of its initial fluorescence, but C2 losing greater total fluorescence (Chapter 1). The fluorophores responsible for C4 fluorescence are believed to be high molecular weight (poly)phenolic compounds, which are some of the most reactive sites in CDOM (Boyle *et al.* 2009, Kellerman *et al.* 2015, McNally *et al.* 2005). The abundance and photo-lability of terrestrial-like C2 and C4 at depth could be due to the prevalence of terrestrial organic matter in Arctic waters, where this DOM is subducted before it can be extensively photo-bleached (Benner *et al.* 2005). Alternatively, there is growing evidence that these ‘terrestrial’ signals may be produced *in situ* microbially (Jørgensen *et al.* 2014, Shimotori *et al.* 2012), and/or excreted by macro brown algae (Shank *et al.* 2010a), zooplankton, and *Trichodesmium Sp.* (Steinberg *et al.* 2004).

In contrast to the terrestrial-like FDOM signals, marine-like C1 showed limited photo-degradation, and even slight production in surface waters. Marine-like C1 production may have

only been seen in the surface and 110 m samples due to lack of precursor material in deeper waters. Such materials may include tryptophan, tyrosine, and other low molecular weight aromatic structures, which have been shown to produce CDOM and ‘humic-like’ FDOM photochemically (Bianco *et al.* 2014, Biers *et al.* 2007, De Laurentiis *et al.* 2013). UVA component C5 was significantly enriched in the surface and 110 m samples, and while typically are classified as “protein-like,” may also contain low molecular weight aromatics. Input of these precursors was likely from primary producers in the photic zone (Jørgensen *et al.* 2011), as well as CDOM exuded by the brown macroalgae *Sargassum natans* (Shank *et al.* 2010a), which is prevalent in the Sargasso Sea in July, when sampling occurred. Despite its lack of photo-degradation and even slight photo-production, C1 was found to be depleted in the surface ocean, which has been shown previously (Heller *et al.* 2013). The observed photo-products may not be photo-stable over long time periods, as photo-degradation of marine FDOM has been shown during longer irradiation experiments (Helms *et al.* 2013). Dried PPL extracts were reconstituted in deionized water for the photo-irradiation experiments, removing the potential impact of reactive halogen species on FDOM loss. Previous work showed that while halides had little effect on the photo-bleaching of terrestrial FDOM, an algal exudate showed enhanced FDOM loss in the C1 region when halides were present (Grebel *et al.* 2009). Additionally, Romera-Castillo *et al.* reported loss of fluorescence in the C1 region during microbial incubations (Romera-Castillo *et al.* 2011). Photo-bleaching via reactive halogen species or microbial degradation (all samples in this study were filter sterilized) may therefore further account for the depletion of C1 fluorescence in the surface ocean.

Protein-like compounds undergo both sunlight-induced direct photolysis and indirect photolysis via reactive intermediate species. The location of reactive sites such as tryptophan and

tyrosine within the structure are a critical factor in reaction rates, with steric hindrances affecting reactions with singlet oxygen and proximity to DOM sensitizers/quenchers (Janssen *et al.* 2014, Lundeen *et al.* 2014). The kinetics of UVA fluorescence loss (C3 and C5) are likewise complicated by the fact that protein-like fluorescence undergoes variable quenching depending on the location of fluorescent amino acids in (or free from) the protein structure (Kronman and Holmes 1971, Lakowicz 2006). Additionally, protein-like fluorescence has been shown to be quenched by humic substances (Wang *et al.* 2015). The effects of quenching on the UVA fluorescence intensity could not be determined due to the changes in quantity and quality of the proteinaceous and humic-like materials during irradiation. Therefore, while these components showed photo-lability, alternate techniques would be necessary to accurately quantify changes in proteinaceous material. Nevertheless, the photo-lability of these components is likely offset by high primary production in the surface ocean.

Ultrahigh resolution mass spectrometry showed unique aliphatic signatures in the surface waters, including a diverse group of aliphatic sulfur-containing molecular ions which may be generated by photoautotrophs or communities depending on primary production (Fig. 2.6). The principal component analysis using the m/z ions and their intensities (Fig. 2.8), as well as the correlation between the mass peaks and the fluorescence (Fig. 2.9) associated aliphatic compounds with surface waters and hydrogen-deficient compounds with deep water samples. The enrichment of aliphatic compounds in marine surface waters have been previously documented and is in good agreement with our data (Flerus *et al.* 2012). In 57 day irradiations of Congo River water, the majority of photo-resistant peaks and photo-products identified by FT-ICR-MS were aliphatic, while aromatic compounds were the most photo-reactive (Stubbins *et al.* 2010). NMR and FTIR spectroscopy have similarly identified aromatic sites as the most photo-

labile (Helms *et al.* 2014, Thorn *et al.* 2010). These depth-dependent trends match those of the North Pacific, including an apparent homogeneity in the FT-ICR peaks below 1,000 m depth (Medeiros *et al.* 2015). This apparent homogeneity is notable because it matched exactly the trends observed in EEM-PARAFAC components that are indicative for conjugated aromatic compounds such as polyphenols.

Fluorescence in the marine- and terrestrial-like regions of the EEM have been shown to be produced by microbes (Biers *et al.* 2007, Rochelle-Newall and Fisher 2002), and correlated well with apparent oxygen utilization (AOU) at depth (Catalá *et al.* 2015, Jørgensen *et al.* 2011, Kowalczyk *et al.* 2013, Lønborg *et al.* 2015, Yamashita and Tanoue 2008). The microbial carbon pump is believed to produce (semi)refractory molecules, including CDOM and FDOM, in the mesopelagic zone, which are then transported to the deep ocean (Flerus *et al.* 2012, Hansell 2013, Jiao *et al.* 2010). The fast photo-kinetics shown in this study as well as the enrichment in hydrogen-deficient molecular ions at depth and depletion of these molecules at the surface highly suggests that deep-sea FDOM is very unlikely to survive overturning circulation and hence cannot be responsible for the DOM component that contributes to the very old apparent ^{14}C age of deep sea DOM of 3,700-6,000 years (Bauer *et al.* 1992).

CHAPTER 3:

Photo-reactivity of Natural Dissolved Organic Matter From Fresh to Marine Waters in the Florida Everglades, USA

3.1. Introduction

The Everglades is one of the largest sub-tropical wetlands in the world (*ca.* 6,200 km²), and includes extensive freshwater marsh and estuarine areas, which are characterized by open prairies and fringe mangrove swamps, respectively. Dissolved organic matter (DOM) dynamics in the Everglades are to a large extent controlled by regional soil and vegetation patterns and seasonal hydrology. Clear spatial patterns have been observed for DOM optical properties throughout the systems (Chen *et al.* 2013, Yamashita *et al.* 2010), seasonally (Chen *et al.* 2010, Maie *et al.* 2012), and along salinity transects in the fringe mangrove rivers (Cawley *et al.* 2013, Jaffe *et al.* 2004, Maie *et al.* 2005). As such, optical properties of DOM in the Everglades have been well defined and related to both physical (hydroperiod, water discharge, tidal pumping, and saltwater intrusions) and biological (primary productivity) drivers. While several studies reported on the potential effects of photo-exposure on the mineralization and degradation of chromophoric and fluorescent DOM (CDOM and FDOM, respectively) in the Shark River (Clark *et al.* 2004, Clark *et al.* 2002, Zanardi-Lamardo *et al.* 2002, Zanardi-Lamardo *et al.* 2004), the photo-dissolution of particulate organic matter in the Everglades and Florida Bay (Pisani *et al.* 2011, Shank *et al.* 2011), and differences in optical properties between surface and groundwater in the Everglades as a potential result of light exposure (Chen *et al.* 2010), little is known about the photo-reactivity of DOM in this system and its relationship to the production of reactive species.

The effects of reactive species on the photodegradation of DOM are different depending on the reactant. Singlet oxygen, while reacting with fulvic acids on the order of $10^5 \text{ M-C}^{-1}\text{s}^{-1}$, does not change the DOC concentration or optical properties (Cory *et al.* 2009), but can lead to partial oxidation and an increase in oxygen content (Cory *et al.* 2010a). $^3\text{DOM}^*$ has been proposed as a major source of DOM photo-oxidation, although the mechanisms are not well understood (Loiselle *et al.* 2012). Hydroxyl radical, on the other hand, is not suspected to be a major contributor to the photodegradation of DOM due to its low formation rates (Loiselle *et al.* 2012), although high levels of $\bullet\text{OH}$ formation, such as in waters with a high nitrate/DOC ratio, could lead to photomineralization (Brinkmann *et al.* 2003a, Goldstone *et al.* 2002, Vione *et al.* 2006). Other reactive species, such as halide radicals, could contribute significantly to the photodegradation of DOM as well (Grebel *et al.* 2009). Understanding the quantity and speciation of photo-produced reactive species is therefore essential in predicting the potential for photochemical processing of organic matter, as well as contaminants, in surface waters.

There have been several studies that have looked at the changes in molecular character of DOM from fresh to marine systems (Gonsior *et al.* 2009, Gonsior *et al.* 2011, Moran *et al.* 2000, Sleighter and Hatcher 2008, Stubbins *et al.* 2010). Photolytic effects can result in a change in the overall aromatic character of the DOM (Stubbins *et al.* 2012) as well as the formation of lower molecular weight compounds (Mopper *et al.* 1991, Schmitt-Kopplin *et al.* 1998, Zanardi-Lamardo *et al.* 2002, Zanardi-Lamardo *et al.* 2004), but the effect of these structural changes on reactive species photo-production are not well understood. A study of the plumes of the Mississippi and Atchafalaya Rivers in the Gulf of Mexico showed that $^1\text{O}_2$ production did not vary across the salinity gradient, although total free radicals decreased (Sandvik *et al.* 2000). Due to the low DOC concentrations and detection methods, samples were ultrafiltered and

concentrated through freeze-drying in order to measure reactive species production. In contrast, the organic-rich mangrove estuaries of the Everglades provide ideal study sites for conducting a detailed investigation into DOM-produced reactive species in whole waters (no preconcentration steps required) across estuarine systems, and they have been well characterized with regards to DOM dynamics, quality, and quantity over spatial and temporal scales (Cawley *et al.* 2013, Chen *et al.* 2013, Maie *et al.* 2012, Maie *et al.* 2005).

In recent years, excitation emission matrix fluorescence spectroscopy combined with parallel factor analysis modeling (EEM-PARAFAC) has been widely applied in the assessment of DOM dynamics in aquatic ecosystems. For the Everglades, a PARAFAC model has been established and applied to a variety of studies including spatial (Yamashita *et al.* 2013, Yamashita *et al.* 2010), and seasonal (Chen *et al.* 2013, Maie *et al.* 2012) DOM source assessments, as well as for the estimation of source changes in the Shark River estuary (Cawley *et al.* 2013). The model consists of four terrestrial humic-like, two microbial humic-like and two protein-like PARAFAC components (Chen *et al.* 2010, Yamashita *et al.* 2010). Consequently, PARAFAC components in this system are well characterized and ideally suited to be applied in the development of reactivity proxies for DOM as evidenced by prior reports on potential use of two Everglades PARAFAC components as indicators of DOM light exposure (Cawley *et al.* 2012, Chen *et al.* 2010). This study attempted to correlate organic matter quantity and quality using reactive species generation as the measure of photoreactivity. Formation rates of singlet oxygen, $^3\text{DOM}^*$, and hydroxyl radical were measured, and their relation to optical properties and PARAFAC components in two Everglades estuaries. Steady-state concentrations of these species as well as those calculated for carbonate radical are reported across the two systems.

3.2 Methods and Materials

Sample Sites

Surface water samples for this study were collected in two different estuarine regions in Everglades National Park, Florida, USA: the Shark River Slough (SRS) and Taylor Slough (TS). Estuarine inundation characteristics are quite different for these two main drainage systems for the Everglades, as the SRS is tidally influenced through the Gulf of Mexico, while the TS features no significant tidal action due to the dampening effects of the multiple mud banks throughout Florida Bay. Consequently, the mangrove swamps of the TS estuary feature longer inundation periods compared to those of the SRS, resulting in differences in mangrove forest structure, soil type (peat vs. marl) and organic matter accumulation (Rivera-Monroy *et al.* 2011). Throughout the estuary, the freshwater slough environment is replaced by mangrove channels, tidal creeks and rivers such as the Shark and Harney rivers (for SRS) and Taylor River (for TS). Surface water samples were collected in late April 2013 (early wet season) from the Harney River and Taylor River/Florida Bay, covering a salinity range from the oligohaline ecotone to the respective marine end-member (Fig. 3.1). The Harney River samples consisted of a salinity transect (six samples) covering the estuarine section between Tarpon Bay (oligo/meso-haline zone) and Ponce de Leon Bay (coastal end-member; Fig. 3.1a). The Taylor River samples consisted of a salinity transect along the lower Taylor River estuary (four samples) extended into Florida Bay (two samples; Fig. 3.1b). While low salinity samples were obtained for the upper Harney River estuary, the lowest salinity sample for the Taylor River was higher at 11.2. During the late dry season (early April), saltwater intrusions from NE Florida Bay reach up into the Taylor River due to reduced freshwater head; this effect was still evident at the time of sampling.

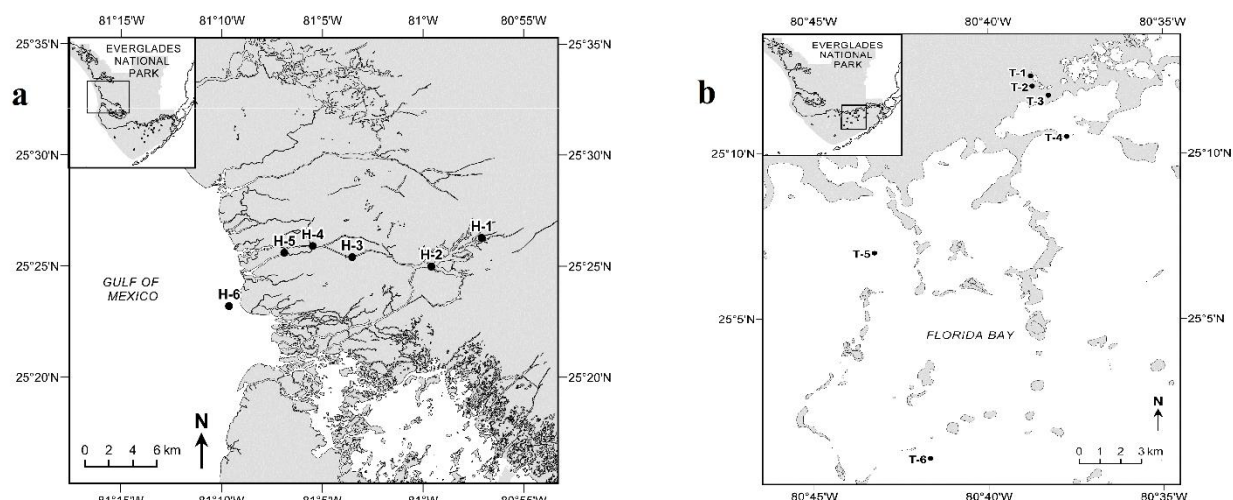


Figure 3.1: Sample sites in the (a) Harney River and (b) Taylor River/Florida Bay

Sample Collection

Whole water samples for reactive species analysis were collected in 1 L glass jars, stored on ice and in the dark during transport to the lab, and then stored at 4 °C until use. Samples for DOM analysis were collected in 2 L pre-washed (soaked in 0.1 M HCl and 0.1 M NaOH for 24 h each) brown Nalgene[®] polyethylene bottles and stored on ice until return to the lab where they were filtered through pre-combusted GF/F fiber filters prior to analysis. Salinity, temperature and pH were determined on site using a 600XL YSI probe (Xylem Inc., Yellow Springs, OH).

Reagents

Furfuryl alcohol (FFA), terephthalic acid (TPA), and sorbic acid (SA), were purchased from Sigma Aldrich (St. Louis, MO) at the highest purities available. HPLC solvents and additional reagents were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA).

DOC Measurement

DOC concentrations were measured by high temperature combustion with a Shimadzu TOC-5000 analyzer. Each sample (4 mL) was acidified with 10 µL of concentrated HCl and sparged

for 5 min with nitrogen to remove inorganic carbon. The mean of 3–6 injections (coefficient of variation < 2 %) was reported for each sample. The system was standardized daily with a potassium hydrogen phthalate standard.

Optical Properties

UV-Vis analyses were performed using a Varian Cary 50 Bio spectrophotometer with a 1 cm quartz cuvette, scanning from 250 nm to 800 nm. Absorption coefficients were calculated with Equation 1.1. Spectral slope (S) was obtained by fitting the absorbance spectra to the equation:

$$a_{(\lambda)} = a_{(\lambda_0)} \cdot e^{[-S(\lambda_0 - \lambda)]} + K \quad (3.1)$$

where λ_0 is 250 nm and K is a background constant due to residual scattering by fine particle fractions or micro-air bubbles that allow for any baseline shift. Spectral slope ratio (S_R) was calculated following Helms *et al.* (2008) as the ratio of $S_{(275-295)}/S_{(350-400)}$. Both spectral slopes were obtained using the linear regression of the narrow wavelength ranges, $S_{(275-295)}$ and $S_{(350-400)}$, of the natural log-transformed $a_{(\lambda)}$ spectra. The carbon-specific absorption coefficient at 254 nm, $SUVA_{254}$ (expressed in $L\ m^{-1}\ mg\ C^{-1}$), was calculated by dividing the decadic a_{254} by DOC concentration (Weishaar *et al.* 2003). UV-Vis analyses were also used to correct the inner filter effects from the fluorescence measurements.

Fluorescence and PARAFAC Analysis

Excitation-emission matrix fluorescence spectra (EEMs) were measured using a Horiba Jovin Yvon SPEX Fluoromax-3 spectrofluorometer equipped with a 150 W continuous output Xe arc lamp. Slits were set at 5.7 nm for excitation and 2 nm for emission. Forty-four emission spectral scans were acquired in a 1 cm quartz cell at excitation wavelengths (λ_{ex}) between 240 and 455 nm at 5 nm intervals. The emission wavelengths (λ_{em}) were scanned from 250 nm to 705 nm in 2

nm steps. Fluorescence signals were acquired in signal over reference ratio mode (S/R) to eliminate potential errors from fluctuations of the Xe lamp. More detailed information of post-acquisition steps for correction (inner filter effects, instrumental bias) and unit conversion to quinine sulphate units (QSU) can be found elsewhere (Chen *et al.* 2010). PARAFAC modeling applied here was achieved by fitting the EEMs of all the samples to an already established PARAFAC model for surface water from the Everglades (Chen *et al.* 2010, Yamashita *et al.* 2010). Briefly, the eight components are ($\lambda_{\text{ex}}/\lambda_{\text{em}}$): C1 (<260(345)/462) ubiquitous humic-like; C2 (<260/454) terrestrial humic-like possibly photo-refractory; C3 (<260(305)/416) terrestrial humic-like, fulvic acid-type; C4 (<260(305)/376) microbial humic-like; C5 (275(405)/>500) terrestrial humic-like, humic acid-type; C6 (325/406) ubiquitous humic-like, possibly generated during biodegradation, photo-labile and agricultural land use derived; C7 (275/326) and C8 (300/342) protein-like. PARAFAC component spectral characteristics and split-half validation can be found elsewhere (Chen *et al.* 2010). The analysis was carried out in MATLAB 7.0.4. (Mathworks, Natick, MA) with the DOMFluor toolbox (Stedmon and Bro 2008).

Irradiation Experiments

Irradiation experiments were carried out in a Luzchem SolSim solar simulator (Ottawa, Canada). The output of the 300W ceramic Xe lamp was adjusted with a 1/8" Esco optical glass filter and dimmer to best match the AM 1.5 solar spectrum with irradiation from 300 nm- 900 nm. Lamp output was measured daily with the Reliability Direct (League City, Texas) AR823 power meter that comes standard with the system. The sample chamber was well-ventilated, keeping the samples at a constant 20°C. Samples were placed in sealed quartz cells (1 cm path length) on a rotating sample holder to ensure even irradiation. Aliquots were taken at intervals

ranging from 5-30 minutes, and the samples analyzed by HPLC with on-line UV or fluorescence detection.

Hydroxyl and Carbonate Radicals

Hydroxyl radical formation and steady-state concentrations were monitored using terephthalic acid (TPA) (Page *et al.* 2010). To measure the rate of •OH production, each sample was adjusted to pH<2 with HCl and bubbled with air to strip off any (bi)carbonate in the system. The samples were raised back to the original pH with NaOH and spiked with TPA (6 µM final concentration). Formation of 2-hydroxyterephthalic acid, 2HTPA, is described by Equation 3:

$$\frac{d[2HTPA]}{dt} = k_{\text{OH,TPA}} Y [\text{TPA}] [\bullet\text{OH}]_{\text{ss}}^* \quad (3.2)$$

where $k_{\text{OH,TPA}} = 4.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, the yield $Y = 0.35$, and $[\bullet\text{OH}]_{\text{ss}}^*$ is the steady-state concentration of •OH in carbonate-free solutions. Formation rates of •OH, R_{OH} , were calculated by dividing the rate of 2HTPA formation by the reaction yield. While 2HTPA is subject to photolysis by light below 360 nm, no loss of 2HTPA was seen in irradiations <100 min, so in order to keep the light source constant between experiments, no additional filters were added to the lamp for •OH measurements. Whole water $[\bullet\text{OH}]_{\text{ss}}$ were then determined with Equation 4:

$$[\bullet\text{OH}]_{\text{ss}} = \frac{R_{\text{OH}}}{k_{\text{OH,HCO}_3^-} [\text{HCO}_3^-] + k_{\text{OH,CO}_3^{2-}} [\text{CO}_3^{2-}] + k_{\text{OH,DOM}} [\text{DOM}]} \quad (3.3)$$

with the reaction rates between hydroxyl radical and bicarbonate and carbonate ($k_{\text{OH,HCO}_3^-} = 8.50 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, and $k_{\text{OH,CO}_3^{2-}} = 3.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, respectively) (Buxton *et al.* 1988), and the reaction rate with DOM, $k_{\text{OH,DOM}}$, calculated as the average of the values for organic matter determined by Westerhoff *et al.* (2007) (excluding effluent organic matter), $(1.4 \pm 0.2) \times 10^4 \text{ L}$

(mg C)⁻¹ s⁻¹. Bicarbonate and carbonate concentrations were determined with a Metrohm 855 Robotic Titrosampler (Riverview, FL). Carbonate radical concentrations were calculated with the following equation:

$$[\text{CO}_3^{\cdot-}]_{\text{ss}} = \frac{[k_{\text{OH},\text{HCO}_3^-} [\text{HCO}_3^-] + k_{\text{OH},\text{CO}_3^{2-}} [\text{CO}_3^{2-}]] [\bullet\text{OH}]_{\text{ss}}^*}{k_{\text{CO}_3^{\cdot-},\text{DOM}} [\text{DOM}]} \quad (3.4)$$

where $k_{\text{CO}_3^{\cdot-},\text{DOM}} = 280 \pm 90 \text{ L (mg C)}^{-1} \text{ s}^{-1}$ (Canonica *et al.* 2005). Carbonate radicals are also produced by the reaction between carbonate ions with triplet excited state DOM, but the reaction rate ($1 \times 10^5 \text{ M s}^{-1}$) (Canonica *et al.* 2005) is such that the contribution of ³DOM* to carbonate radical formation was negligible. Due to long-term monitoring data that has consistently shown low (<0.1 mg/L) concentrations of nitrate and nitrite (Childers and Troxler 2013a, b), these species were not measured. Dissolved iron concentrations were not determined, but have been reported to be low in the Everglades (0-0.03 mg/L). In addition, total Fe concentrations in Everglades' soils and sediments have been reported to be low (<http://fcelter.fiu.edu/>), and sulfate reducing conditions in the ecotonal fringe mangrove sediments further limit iron solubility due to the formation of insoluble sulfides (Osborne *et al.* 2011). All •OH production was therefore attributed to DOM.

Singlet Oxygen

Steady-state concentrations of singlet oxygen were determined using furfuryl alcohol (FFA) as a probe (Haag *et al.* 1984a). Water samples were spiked with FFA (1.5 mM final concentration), placed in the solar simulator, and aliquots taken every 10 minutes. The concentration of FFA was plotted versus time to determine the initial rate of FFA loss, R_{FFA} . The rate of ¹O₂ production was then determined by incorporating the deactivation of ¹O₂ due to

solvent effects, $k_d = 2.5 \times 10^5 \text{ s}^{-1}$ (Rodgers and Snowden 1982), with Equation 6 (Minella *et al.* 2011):

$$R_{^1\text{O}_2} = R_{\text{FFA}} \frac{k_{\text{FFA}, ^1\text{O}_2} \text{FFA}_0 + k_d}{k_{\text{FFA}, ^1\text{O}_2} \text{FFA}_0} \quad (3.5)$$

where FFA_0 is the initial FFA concentration and $k_{\text{FFA}, ^1\text{O}_2}$ is the reaction rate between FFA and $^1\text{O}_2$, $1.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Haag *et al.* 1984a). While there is potential for error due to reaction with hydroxyl radical ($k_{\text{FFA}, \cdot\text{OH}} = 1.2 \times 10^{10}$) (Buxton *et al.* 1988), $R_{\cdot\text{OH}} \ll R_{^1\text{O}_2}$, making the contribution of hydroxyl radical negligible. Steady-state concentrations in the absence of the probe were determined by dividing the formation rate by k_d :

$$[^1\text{O}_2]_{\text{ss}} = \frac{R_{^1\text{O}_2}}{k_d} \quad (3.6)$$

Triplet Excited State of DOM ($^3\text{DOM}^*$)

$^3\text{DOM}^*$ formation was measured with sorbic acid as described in Grebel *et al.* (2011). 2,4,6-trimethylphenol (TMP) was not used as a probe due to changes in its reaction rate with $^3\text{DOM}^*$ at varying ionic strengths (Parker *et al.* 2013). Formation rates of the cis-trans isomer of sorbic acid were divided by the yield, 0.18, to obtain the removal rate of $^3\text{DOM}^*$ by sorbic acid, R_{SA} . The concentration of the probe, $[\text{SA}]$, divided by R_{SA} was plotted against the concentration of the probe:

$$\frac{[\text{SA}]}{R_{\text{SA}}} = \frac{[\text{SA}]}{R_{^3\text{DOM}^*}} + \frac{k'_s}{R_{^3\text{DOM}^*} k_{\text{SA}, ^3\text{DOM}^*}} \quad (3.7)$$

where $R_{^3\text{DOM}^*}$ is the formation rate of triplets, k'_s is the reaction rate of solution scavengers with the triplets (inverse of triplet lifetimes), and $k_{\text{SA}, ^3\text{DOM}^*}$ is the reaction rate between sorbic acid and $^3\text{DOM}^*$, calculated as the average of reported values between sorbic acid and various

organic matters, $(3.35 \pm 1.00) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Cottrell *et al.* 2013). Formation rates of triplets were therefore calculated as the inverse of the slope, while k'_s and steady-state concentrations, $[^3\text{DOM}^*]_{ss}$, (in the absence of probe) were determined as follows:

$$k'_s = k_{SA, ^3\text{DOM}^*} \cdot \frac{\text{intercept}}{\text{slope}} \quad (3.8)$$

$$[^3\text{DOM}^*]_{ss} = \frac{R_{^3\text{DOM}^*}}{k'_s} \quad (3.9)$$

Analytical Methods

Probe compounds were quantified using an Agilent 1200 Series HPLC with UV detection at wavelengths 254 nm for TPA and SA and 219 nm for FFA. Formation of 2HTPA was monitored using on-line fluorescence, $\lambda_{ex} = 240 \text{ nm}$, $\lambda_{em} = 425 \text{ nm}$. The isocratic mobile phase for TPA/2HTPA detection was 50:50 0.08% H_3PO_4 :methanol. The remaining compounds used 30 mM sodium acetate buffer at pH=4.75 with ratios of 90:10 acetate:methanol for FFA, and 85:15 acetate:acetonitrile for SA. FFA was monitored using a Phenomenex Gemini $3\mu\text{m}$ C18 column (50 x 4.6 mm i.d.), while TPA and SA were monitored using a Phenomenex Gemini $5\mu\text{m}$ C18 column (250 x 4.6 mm i.d.).

3.3 Results

DOC distribution

Samples along the Harney River and the Taylor Slough showed decreasing DOC with increasing salinity (Table 3.1), which was expected due to the dilution of freshwater marsh-derived DOC with the marine end-members. The observed decrease in the Harney River shows non-conservative mixing indicative of DOC contributions from the fringe mangroves as previously reported (Cawley *et al.* 2013). The Taylor Slough, with minimal tidal activity and

significantly lower freshwater discharge compared to the Harney, did not show this same trend. However, as mentioned above, low-salinity samples of the Taylor River were not obtained due to the low-discharge conditions during time of sampling, and the two high salinity end-member points of the Taylor Slough sample set were taken in Florida Bay (Fig. 3.1b). Since these samples are not part of a spatial, riverine salinity transect, the DOC origin in the TS sample set cannot be exclusively linked to the Taylor River, and the apparent conservative mixing trend cannot be confirmed. The changes in DOC concentration along the salinity gradient in these two systems were nonetheless quite similar, allowing for a comparison of formation rates and steady-state concentrations of reactive species between the Harney River and Taylor River/Florida Bay estuaries.

Table 3.1: Site information and water quality analysis

Station	Latitude °N	Longitude °W	Salinity	pH	DOC (mg L⁻¹)	Total Alkalinity (mg CaCO₃ L⁻¹)
H1	25.43798	80.9513	2.30	7.11	19.34	278.2
H2	25.41641	80.99297	8.03	7.51	17.80	295.4
H3	25.42352	81.05861	17.19	7.47	16.36	308.2
H4	25.43184	81.09126	26.63	7.40	13.87	293.8
H5	25.42671	81.1148	33.89	7.68	8.98	244.4
H6	25.38675	81.16042	37.96	7.94	6.37	217.6
T1	25.19582	80.63785	11.15	7.80	14.43	217.3
T2	25.20554	80.64632	19.82	8.00	12.26	210.2
T3	25.20044	80.64553	23.87	8.09	11.45	207.9
T4	25.17505	80.6292	27.17	8.15	8.86	192.7
T5	25.11645	80.72102	35.25	8.09	9.26	180.2
T6	25.01301	80.69453	37.37	8.15	5.74	171.3

Optical properties and PARAFAC analysis

Optical properties for the twelve sample sites are summarized in Table 3.2. The distribution of CDOM as indicated by a_{254} was in general agreement with the DOC concentrations in both systems. The Harney River was enriched significantly with CDOM compared to the Taylor River

sample set. This is, in part, due to the fact that the DOM loadings to the Taylor River are enriched in microbial sources (Chen *et al.* 2013), particularly during the end of the dry season, when intrusions of Florida Bay waters upriver are prominent. Waters from Florida Bay are enriched in seagrass-derived DOM and therefore feature higher abundances in carbohydrates and proteins (protein-like fluorescence) compared to the humic-like materials and lignins found in the SRS mangrove rivers such as the Harney River (Maie *et al.* 2012, Maie *et al.* 2005). The higher SUVA₂₅₄ values and PARAFAC component fluorescence confirm these differences in DOM source and character between the two systems (Table 3.2). Fluorescent PARAFAC components showed a similar behavior to DOC, with a decrease of the fluorescent intensity with the increase of salinity. Humic-like components C1, C3, C4, C5 and C6 and the protein-like C7 presented a non-conservative behavior, showing clear DOM contributions from the fringe mangrove swamps (values above the theoretical conservative mixing line) (Cawley *et al.* 2013); however, humic-like C2 and protein-like C8 showed a nearly conservative behavior. In all the cases, fluorescent intensity values of Harney River were higher than that of Taylor Slough. In terms of abundance (percent total fluorescence), the Harney was enriched in terrestrial humic-like components C1 ($p < 0.005$), C3 ($p < 0.01$), and C5 ($p < 0.05$), while the Taylor was enriched in microbial humic-like C4 ($p < 0.05$), and protein-like C7 ($p < 0.005$) and C8 ($p < 0.001$). DOC and PARAFAC results from the Harney River agree with those previously observed for the same season (Cawley *et al.* 2012). Spectral slope ratios (S_R), an established proxy for DOM molecular weight (MW) (Helms *et al.* 2008), showed a general trend of decreasing MW (increasing S_R) with increasing salinity (Table 2). This is expected due to the formation of lower MW DOM compounds due to photobleaching (Dalzell *et al.* 2009) as well as the predominance of lower molecular weight autochthonous material at the marine end-member sites. Sample H1, the

uppermost and most freshwater-influenced sample of the Harney River, showed lower MW compared to the oligohaline zone samples due to contributions of periphyton-derived DOM (lower MW) from the freshwater marshes (Chen *et al.* 2013). DOM from the most saline site of the Taylor Slough, T6, showed a slightly higher MW (lower S_R) than the preceding estuarine sites. This site is located in a region of the bay that has low water exchange and consequently high residence times. It is also in a zone of high seagrass mortality and associated detritus. Bio- and photo-polymerization reactions could therefore be responsible for the MW increase seen in this region.

Table 3.2: Optical properties of water samples and PARAFAC component fluorescence

Site	S_R	$a_{CDOM}(254)$ (m^{-1})	SUVA ₂₅₄ ($L \cdot m^{-1} \cdot mg \ C^{-1}$)	C1 (QSU)	C2 (QSU)	C3 (QSU)	C4 (QSU)	C5 (QSU)	C6 (QSU)	C7 (QSU)	C8 (QSU)	Total Fluor (QSU)
H1	5.04	138.65	3.11	224.62	84.12	105.84	85.92	102.81	57.28	24.37	24.37	709.32
H2	4.95	135.69	3.31	189.59	70.98	75.17	72.83	87.58	53.05	21.22	22.02	592.43
H3	4.61	144.38	3.83	192.04	59.10	72.17	66.85	92.07	50.52	20.62	18.87	572.24
H4	4.57	118.41	3.71	153.07	33.06	56.89	50.86	74.53	39.18	14.77	13.95	436.31
H5	4.92	70.47	3.41	80.81	15.55	28.71	30.21	38.95	23.24	10.61	9.17	237.25
H6	6.52	34.26	2.33	30.39	10.55	11.11	15.18	14.77	9.74	6.40	5.22	103.36
T1	6.60	80.69	2.43	66.25	20.45	30.40	31.51	31.93	17.94	21.20	12.79	232.47
T2	7.43	56.84	2.01	39.22	16.35	14.47	21.23	21.24	12.99	14.57	8.29	148.37
T3	9.46	40.90	1.55	25.29	14.59	6.26	15.35	12.30	9.81	11.73	6.30	101.64
T4	13.77	26.81	1.31	13.88	10.26	1.57	9.64	6.67	6.37	10.51	4.69	63.58
T5	13.65	28.32	1.33	15.21	10.12	2.49	11.25	6.01	7.54	13.54	5.89	72.06
T6	8.94	13.19	1.00	7.25	3.37	0.65	4.40	3.09	3.60	7.89	2.77	33.02

Reactive Species

Normalized formation rates and steady-state concentrations for the four photo-produced reactive species are shown in Figures 3.2 and 3.3. Formation rates and steady-state concentrations of singlet oxygen (normalized to DOC concentration) decreased ($p < 0.01$) in the Harney River by only 13 % across the salinity gradient, compared to the Taylor Slough sites, which decreased ($p < 0.01$) by 56 % (Fig. 3.2a, 3.3a). Formation rates and steady-state concentrations of $^3DOM^*$ normalized to DOC did not change significantly along the two transects (Fig. 3.2b, 3.3b). The average normalized formation rates of $^3DOM^*$ for the Harney

River and Taylor Slough were 4.80×10^{-9} and $3.38 \times 10^{-9} \text{ M s}^{-1} (\text{mg C/L})^{-1}$, respectively, while the average normalized steady-state concentrations were 2.55×10^{-14} and $1.26 \times 10^{-14} \text{ M (mg C/L)}^{-1}$, respectively. The decrease in normalized formation rates of $\bullet\text{OH}$ (Fig. 3.2c) was much greater than the decrease in singlet oxygen, and was similar in the two systems: 90 % in the Harney River and 87 % in the Taylor Slough. The normalized steady-state concentrations decreased by 75 % and 71 %, respectively (Fig. 3.3c). This trend is likely due to both the loss of DOM as well as decreasing bicarbonate and carbonate concentrations along transects (Table 3.2), resulting in less scavenging and therefore higher relative steady-state concentrations. The soils of the Everglades contain significant amounts of calcareous periphyton remains. Dissolution of these carbonates results in the fresher waters having higher bicarbonate and carbonate (total alkalinity) concentrations than the marine end-member, particularly for the Harney River. The gradient was less steep for the Taylor system as the marine end-member, Florida Bay, is characterized by calcareous mud sediments. Normalized carbonate radical formation rates therefore decreased by 91 % and 88 % and normalized steady-state concentrations decreased by 72 % and 70 % and along the transects of the Harney and Taylor Rivers, respectively, as the major reactants decreased.

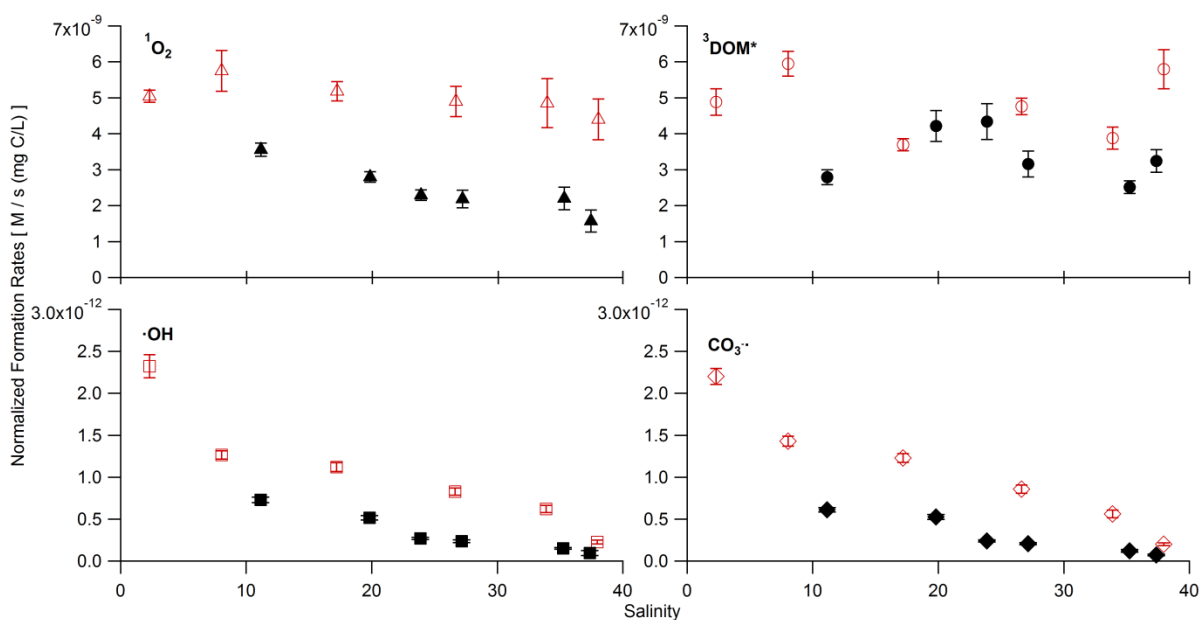


Figure 3.2: Normalized formation rates of (a) $^1\text{O}_2$ (triangles), (b) $^3\text{DOM}^*$ (circles), (c) $\bullet\text{OH}$ (squares) and (d) $\text{CO}_3^{\bullet-}$ (diamonds) across transects of the Harney River (red, open) and Taylor River/Florida Bay (black, filled). Error bars represent standard deviations of triplicate experiments for $^1\text{O}_2$, $\bullet\text{OH}$, and $\text{CO}_3^{\bullet-}$. $^3\text{DOM}^*$ error bars are calculated from standard deviations of the regression analysis (Equation 3.7).

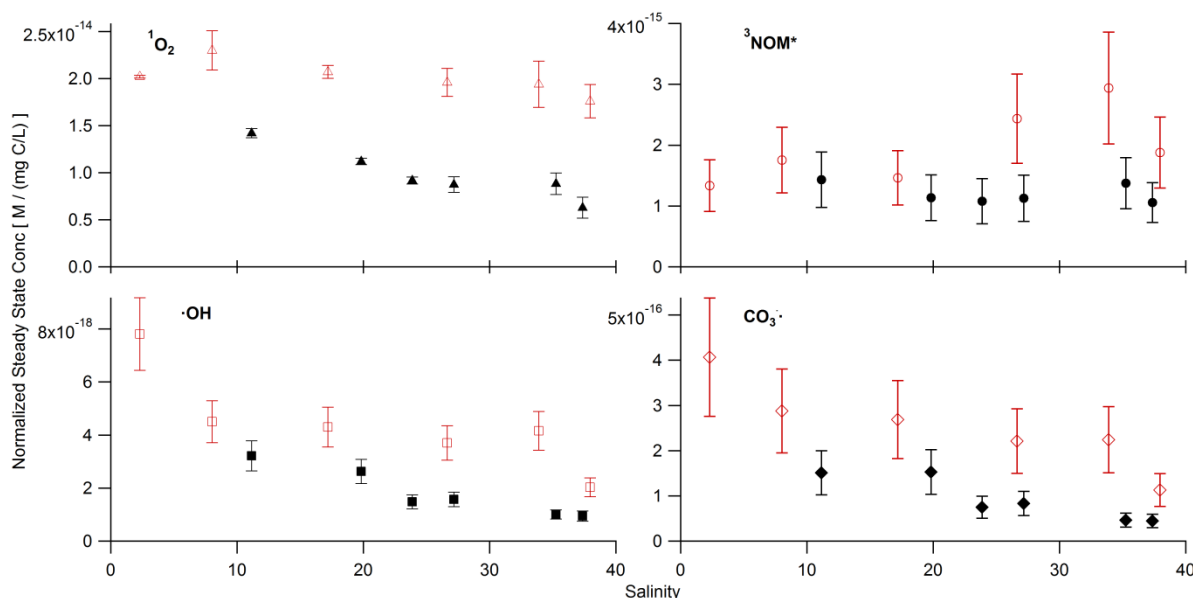


Figure 3.3: Normalized steady-state concentrations of (a) $^1\text{O}_2$ (triangles), (b) $^3\text{DOM}^*$ (circles), (c) $\bullet\text{OH}$ (squares) and (d) $\text{CO}_3^{\bullet-}$ (diamonds) across transects of the Harney River (red, open) and Taylor River/Florida Bay (black, filled). Error bars represent standard deviations of triplicate experiments for $^1\text{O}_2$, $\bullet\text{OH}$, and $\text{CO}_3^{\bullet-}$. $^3\text{DOM}^*$ error bars are calculated from standard deviations of the regression analysis (Equation 3.7).

3.4. Discussion

Reactive species

Parker *et al.* (2013) reported an increase in $^3\text{DOM}^*$ lifetimes and therefore steady-state concentrations of $^3\text{DOM}^*$ with increasing ionic strength. These results are supported in the present study, as normalized $^3\text{DOM}^*$ lifetimes increased in the Harney river until the marine end-member, where a significant increase in the abundance of protein-like C7 and C8 and microbial humic-like C4 and decrease in terrestrial humic-like C1 and C5 was observed (Appendix B, Fig. B.1). The Taylor River did not show a significant change in normalized $[\text{}^3\text{DOM}^*]_{\text{ss}}$ across the salinity gradient, possibly due to a balance between the loss of $^3\text{DOM}^*$ due to marine end-member dilution with an increase of $^3\text{DOM}^*$ lifetimes. It should be noted that the high uncertainties in the steady-state concentrations ($\sim 30\%$) are due mainly to the uncertainty associated with the estimation of the rate constant between SA and $^3\text{DOM}^*$ (Equations 3.8, 3.9). Experimental error in determining the formation rates were much lower ($<10\%$), as evident in Table 3.3.

Table 3.3: Reactive species formation rates (R) and steady-state concentrations ($[\text{SS}]$)

Site	$^1\text{O}_2$		$^3\text{DOM}^*$		$\text{CO}_3^{\cdot-}$		$\cdot\text{OH}$	
	R ($\times 10^8 \text{ M s}^{-1}$)	$[\text{SS}]$ ($\times 10^{14} \text{ M}$)	R ($\times 10^8 \text{ M s}^{-1}$)	$[\text{SS}]$ ($\times 10^{15} \text{ M}$)	R ($\times 10^{12} \text{ M s}^{-1}$)	$[\text{SS}]$ ($\times 10^{16} \text{ M}$)	R ($\times 10^{12} \text{ M s}^{-1}$)	$[\text{SS}]$ ($\times 10^{18} \text{ M}$)
H1	9.76 ± 0.07	39.0 ± 0.3	9.45 ± 0.64	25.8 ± 8.2	42.6 ± 1.2	78.7 ± 25.4	44.9 ± 2.2	151 ± 27
H2	10.2 ± 0.9	41.0 ± 3.7	10.6 ± 0.5	25.1 ± 14.5	25.5 ± 0.3	51.2 ± 16.5	22.5 ± 0.3	80.1 ± 14.1
H3	8.48 ± 0.28	33.9 ± 1.1	6.05 ± 0.14	23.9 ± 7.3	20.1 ± 0.4	41.9 ± 13.5	18.3 ± 0.5	70.3 ± 12.5
H4	6.79 ± 0.51	27.2 ± 2.0	6.60 ± 0.14	33.8 ± 10.2	11.9 ± 0.5	26.2 ± 8.5	11.5 ± 0.3	51.3 ± 9.1
H5	4.36 ± 0.56	17.4 ± 2.2	3.48 ± 0.18	26.4 ± 8.3	5.06 ± 0.26	12.4 ± 4.0	5.58 ± 0.16	37.3 ± 6.6
H6	2.80 ± 0.28	11.2 ± 1.1	3.69 ± 0.18	12.0 ± 3.7	1.28 ± 0.02	4.61 ± 1.49	1.44 ± 0.10	12.9 ± 2.4
T1	5.13 ± 0.18	20.5 ± 0.7	4.02 ± 0.25	20.7 ± 6.6	8.82 ± 0.15	38.1 ± 12.3	10.5 ± 0.2	46.4 ± 8.2
T2	3.43 ± 0.10	13.7 ± 0.4	5.17 ± 0.47	13.9 ± 4.6	6.44 ± 0.25	30.5 ± 9.9	6.31 ± 0.14	32.2 ± 5.7
T3	2.62 ± 0.11	10.5 ± 0.4	4.96 ± 0.52	12.4 ± 4.3	2.76 ± 0.08	6.15 ± 1.99	3.10 ± 0.05	16.9 ± 3.0
T4	1.94 ± 0.18	7.74 ± 0.73	2.80 ± 0.27	10.0 ± 3.4	1.82 ± 0.06	5.70 ± 1.84	2.09 ± 0.09	13.9 ± 2.5
T5	2.04 ± 0.26	8.16 ± 1.06	2.33 ± 0.08	12.7 ± 3.9	1.11 ± 0.12	3.39 ± 1.15	1.41 ± 0.02	9.31 ± 1.64
T6	0.90 ± 0.16	3.61 ± 0.63	1.86 ± 0.10	6.07 ± 1.87	0.41 ± 0.04	1.72 ± 0.58	0.55 ± 0.16	5.42 ± 1.80

Previous studies showed that increases in ionic strength do not affect $[^1\text{O}_2]_{\text{ss}}$ (Parker *et al.* 2013, Sandvik *et al.* 2000), suggesting that changes in $^1\text{O}_2$ production along a salinity transect and between the two sites were due to changes in the quality of the DOM. The ratios of formation rates of $^1\text{O}_2$ and $^3\text{DOM}^*$, $R_{^1\text{O}_2}/R_{^3\text{DOM}^*}$, ranged from 0.76-1.40 in the Harney River, and from 0.48-1.28 in the Taylor Slough. While it may seem counter-intuitive that at some sites $^1\text{O}_2$ is forming faster than its precursor, $^3\text{DOM}^*$, the $^3\text{DOM}^*$ formation reported only accounts for $^3\text{DOM}^*$ with triplet energies $\geq 250 \text{ kJ mol}^{-1}$ necessary to react with sorbic acid, while the energy required to excite ground-state molecular oxygen to $^1\text{O}_2$ is only 94 kJ mol^{-1} . These high-energy triplets- on average, 35 % of total triplets- possess the range of energies required for triplets to react with many contaminants, making the reported formation rates and steady-state concentrations relevant for calculating environmental fate of contaminants (Zepp *et al.* 1985). The decrease in $^1\text{O}_2$ formation across the transects could be due to the loss of either oxygen in the system or $^3\text{DOM}^*$ with triplet energies between 94 and 250 kJ mol^{-1} , as $R_{^3\text{DOM}^*}$ did not vary significantly across either system. While the solubility of oxygen decreases with increasing salinity, if the loss of O_2 was the cause of the decrease in $^1\text{O}_2$, the Harney River would theoretically show greater $^1\text{O}_2$ decrease than the Taylor Slough as the salinity range was greater. As this was not the case- decrease in the Harney was only 13 %, compared to 56 % in the TS- this mechanism is unlikely. No evidence of significant bio- or photo-degradation of the DOM was found in the optical properties or PARAFAC analysis, in support of the latter mechanism. Therefore, the most likely cause for changes in $^1\text{O}_2$ is the change in DOM quality resulting from increased contributions of DOM from the marine end-member. This is evident from the data shown in Table 3.2, where optical properties indicative of higher abundance of humic-like substances and aromaticity such as a_{254} , SUVA_{254} and the relative abundance of the terrestrial

humic-like PARAFAC components (C1, C3 and C5) were enriched in the Harney compared to the Taylor estuarine samples, and also changed along the salinity transect with lower a_{254} and humic-like fluorescence and higher protein-like fluorescence at higher salinities. Grandbois *et al.* (2008) showed that a microbial-derived DOM isolate (Pony Lake Fulvic Acid) has lower formation rates of $^1\text{O}_2$ than a terrestrial-derived DOM isolate (Suwannee River Humic Acid), and that the ratio of steady-state concentrations constrained in the DOM micelles to that in the bulk solution were higher in microbial-derived DOM by over a factor of seven. These results support the hypothesis that changes in DOM source from terrestrial to microbial were the main drivers behind the measured changes in $^1\text{O}_2$ formation.

While the effects of ionic strength on $^3\text{DOM}^*$ and $^1\text{O}_2$ production were shown to be non-halide-specific (Parker *et al.* 2013), at constant ionic strength, chloride and bromide concentrations do affect the production of these species as well as $\bullet\text{OH}$ (Glover and Rosario-Ortiz 2013). The influence of ionic strength on the production of $\bullet\text{OH}$ is not known, although halides are known quenchers of $\bullet\text{OH}$. While the loss of $\bullet\text{OH}$ to halides, namely bromide, could have influenced the measured formation rates in this study, it is believed that these effects were minimal due to the high solution pH; however, the reduction in quantum yield of production of $\bullet\text{OH}$ by halides may have contributed to the decreased formation rates measured (Glover and Rosario-Ortiz 2013). More research is needed to determine the mechanism of $\bullet\text{OH}$ formation so that the effects of ionic strength as well as halides can be better understood.

Relationships between formation rates of reactive species and optical properties and PARAFAC results were investigated in place of steady-state concentrations as formation of reactive species is dependent on DOM composition, while steady-state concentrations are heavily dependent on the presence of solution scavengers. No attempt was made to correlate

formation rates of carbonate radical with optical properties or PARAFAC components as the majority of formation results from reactions with the hydroxyl radical and is not directly formed as a result of DOM photochemistry.

Optical properties and reactive species

Photo-degradation of DOM has previously been shown to decrease not only the average molecular weight of the DOM (Dalzell *et al.* 2009), but also the reactive species production (Loiselle *et al.* 2012). In this dataset, as the spectral slope ratios increased (higher S_R values are indicative of lower molecular weights), nonlinear decreases in reactive species formation rates were observed when considering the entire, combined dataset (Fig. B.2). However, when considering the individual data for the two systems, no clear trend was observed. Multiple studies have shown that the smaller size fractions of DOM are more efficient at producing reactive species (Cavani *et al.* 2009, Lee *et al.* 2013, Minella *et al.* 2011, Richard *et al.* 2004, Sandvik *et al.* 2000). These results come from the size fractionation of individual DOM sources through filtration or size exclusion chromatography. While the smallest size fractions of a single DOM sample may be the most photo-reactive, changes in S_R in this study more likely represent bulk MW changes across the sample sites than photo-degradation products. They are a result of changing DOM quality from enriched soil/terrestrial sources at the freshwater end-member to microbial/autochthonous source enriched DOM at the marine end-member. The decreased reactive species formation rates with increasing S_R are consequently caused by a gradual shift (estuarine mixing) of more humic-like, higher MW DOM to less humic-like, lower MW DOM along the salinity gradient. This source change and associated DOM quality change is clearly reflected in its reactivity.

The absorbance at 254 nm, a_{254} , had a positive linear relationship ($R^2=0.959$) with singlet oxygen production (Fig. 3.4a), which is in agreement with previous studies (Mostafa and Rosario-Ortiz 2013). $SUVA_{254}$, an established proxy for aromaticity (Weishaar *et al.* 2003), increased linearly with formation rates of 1O_2 in the Taylor Slough ($R^2=0.980$), but was only modestly correlated to the formation rates of 1O_2 in the Harney River ($R=0.499$) (Fig 3.5a) possibly due to the DOM quality variations resulting from mangrove swamp contributions (non-conservative behavior) for the mid-salinity range. Formation rates of $^3DOM^*$ showed a strong positive correlation to a_{245} ($R=0.855$) and modest positive correlation to $SUVA_{254}$ ($R=0.659$) (Fig. 3.4b, 3.5b). While it may seem surprising that the correlation to $SUVA_{254}$, and by association aromaticity, was not stronger as aromatic ketones (Canonica *et al.* 2006, Canonica *et al.* 2000, Canonica *et al.* 1995, Golanoski *et al.* 2012, Sharpless 2012) and quinones (Golanoski *et al.* 2012, Maurino *et al.* 2011, Sharpless 2012) are thought to be major sources of triplet excited states found in DOM, quinones may play a lesser role in DOM optical properties (Ma *et al.* 2010) and therefore, the contributions of quinones to $^3DOM^*$ formation would not necessarily be captured by the $SUVA_{254}$ measurement. Formation rates of hydroxyl radical in the Taylor Slough sample set showed a very strong correlation to a_{245} ($R=0.995$), while the Harney River rates showed a less strong correlation ($R=0.740$) (Fig. 3.4c). Taylor Slough showed a very strong correlation between R_{OH} and $SUVA_{254}$ ($R=0.984$), while the Harney River showed a very weak correlation ($R=0.171$) (Fig. 3.5c). This suggests that aromaticity may be more important for hydroxyl radical generation in microbial and seagrass-derived DOM than in terrestrial humic-like DOM, a result that merits further study, as the mechanisms of hydroxyl radical photo-production by DOM is poorly understood (Vione *et al.* 2006). Seagrass-derived DOM has been

reported to be enriched in non-lignin polyphenols (Maie *et al.* 2005) which may be important in this process.

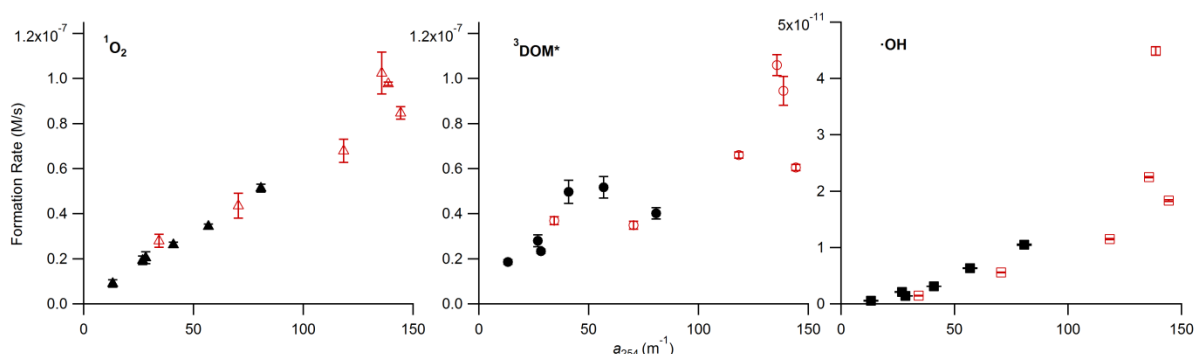


Figure 3.4: Formation rates of (a) $^1\text{O}_2$ (triangles) and (b) $^3\text{DOM}^*$ (circles), and (c) $\bullet\text{OH}$ (squares) in relation to the absorbance at 254 nm, a_{254} , of the Harney River (red, open) and Taylor River/Florida Bay (black, filled). Error bars represent standard deviations of triplicate experiments for $^1\text{O}_2$, $\bullet\text{OH}$, and $\text{CO}_3^{\bullet-}$. $^3\text{DOM}^*$ error bars are calculated from standard deviations of the regression analysis (Equation 3.7).

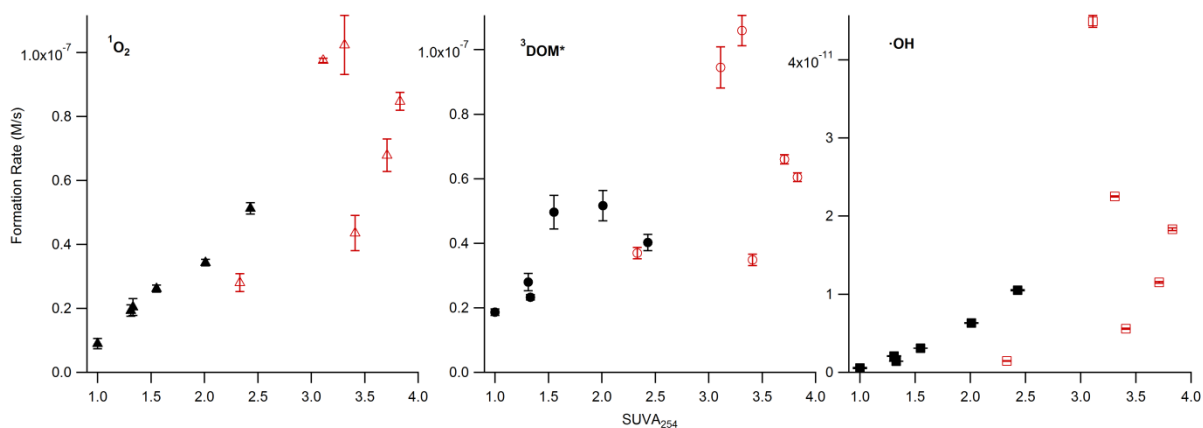


Figure 3.5: Formation rates of (a) $^1\text{O}_2$ (triangles) and (b) $^3\text{DOM}^*$ (circles), (c) $\bullet\text{OH}$ (squares) and (d) $\text{CO}_3^{\bullet-}$ (diamonds) in relation to SUVA_{254} of the Harney River (red, open) and Taylor River/Florida Bay (black, filled). Error bars represent standard deviations of triplicate experiments for $^1\text{O}_2$, $\bullet\text{OH}$, and $\text{CO}_3^{\bullet-}$. $^3\text{DOM}^*$ error bars are calculated from standard deviations of the regression analysis (Equation 3.7).

PARAFAC and reactive species

The relationships between reactive species formation rates and the fluorescence intensity of the PARAFAC components were investigated to examine the potential for use of fluorescence as a proxy for reactive species formation. Excellent linear correlations between $^1\text{O}_2$ and PARAFAC components were observed ($R^2 = 0.916\text{--}0.976$) for both Harney and Taylor rivers and for all

PARAFAC components (Fig. 3.6), suggesting that FDOM is a comparable proxy for $^1\text{O}_2$ formation to a_{254} ($R^2 = 0.959$) and superior to SUVA_{254} ($R^2 = 0.701$). For the Harney River, the correlation with C2 does not visually seem linear. While the reason for this difference is not clear, this component has been proposed to be photo-stable or a product of photo-degradation, (Chen *et al.* 2010, Yamashita *et al.* 2013), which may affect its potential to generate reactive species. Similarly, excellent linear correlations were observed between $\bullet\text{OH}$ formation rates and PARAFAC components (other than C2) in the Taylor River ($R^2 = 0.854\text{--}0.991$; Fig. B.3), while the relationship appeared nonlinear in the Harney River. Formation rates of $^3\text{DOM}^*$ and PARAFAC component fluorescence were less correlated ($R^2 = 0.558\text{--}0.839$; Fig. B.4).

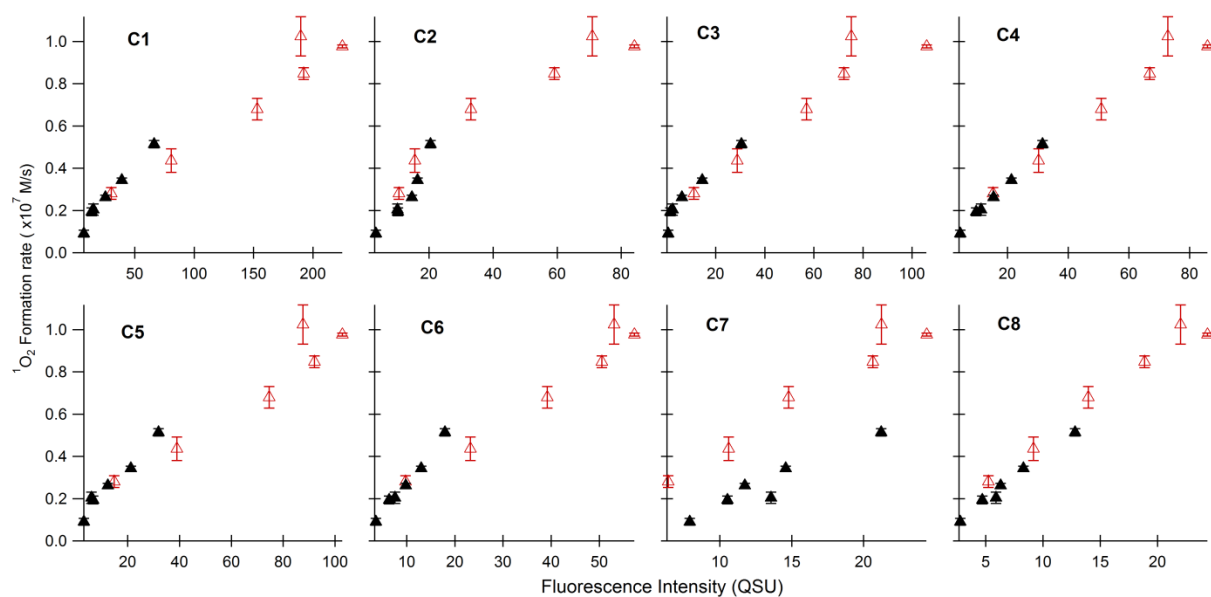


Figure 3.6: Formation rates of $^1\text{O}_2$ in the Harney River (red, open) and Taylor River/Florida Bay (black, filled) as related to fluorescence intensity (QSU) of the different DOM components as identified by PARAFAC analysis. Error bars represent standard deviations of triplicate experiments

The relationships between the DOC-normalized formation rates of reactive species with respect to the relative abundance of the PARAFAC components (% FDOM) were examined as well. In general, $^1\text{O}_2$ showed positive relationship to the relative abundance of terrestrial humic-like PARAFAC components C1, C3, and C5 and a negative relationship to percent of protein-like components C7 and C8 and microbial humic-like C4 (and C6 for Taylor; Fig 3.7). These data suggest that moieties present in the terrestrial humic substances are the main source of $^1\text{O}_2$ in the bulk waters, with other fractions of the DOM playing a smaller role. The change in $[\text{}^1\text{O}_2]_{\text{ss}}$ with the abundance of terrestrial or microbial PARAFAC components in the Harney River was significantly lower than in the Taylor Slough, probably as a result of a significantly stronger DOM quality gradient in the latter system. The influence of microbial and seagrass-derived DOM in the Taylor samples is clearly reflected by a reduction in its photochemical reactivity compared to the DOM in the Harney. While $\bullet\text{OH}$ formation showed a similar relationship with PARAFAC components relative abundance (Fig. B.5), $^3\text{DOM}^*$ formation did not show a strong correlation to the abundance of any individual PARAFAC component (Fig. B.6). This is not unusual, as there was not a significant change in $^3\text{DOM}^*$ formation rates across the two systems. The strongest correlations were modest negative correlations with the two protein-like components, C7 ($R = -0.646$) and C8 (-0.617), followed by modest positive correlations to terrestrial humic-like C5 ($R = 0.560$), C1 ($R = 0.553$) and C3 ($R = 0.530$).

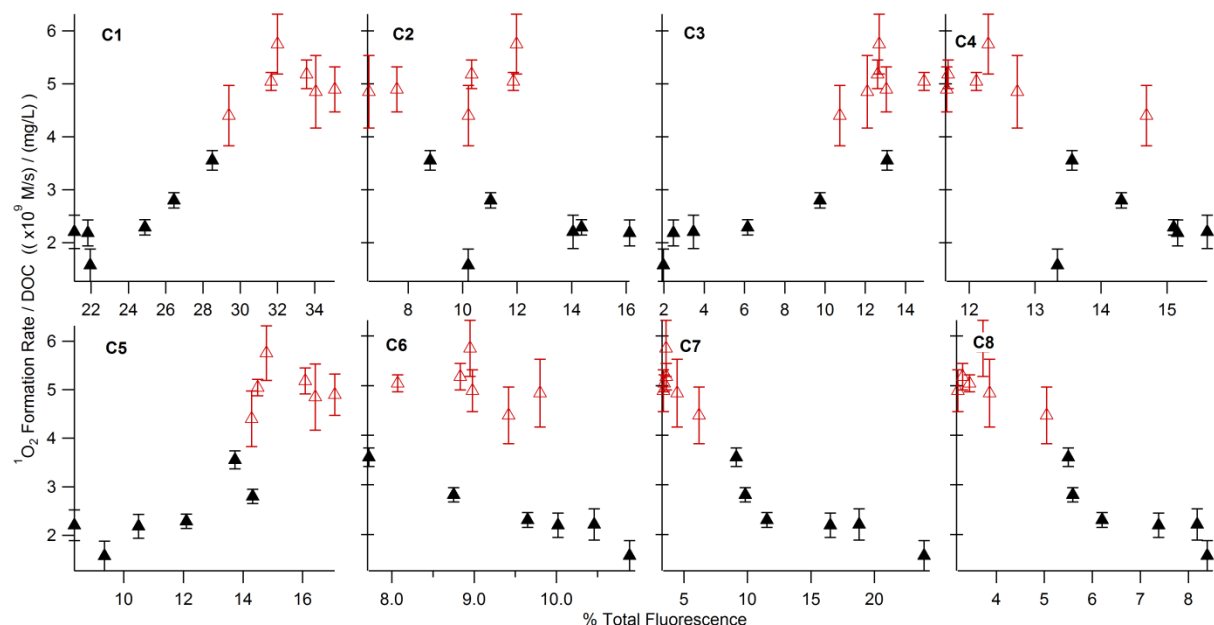


Figure 3.7: Normalized formation rates of $^1\text{O}_2$ in the Harney River (red, open) and Taylor River/Florida Bay (black, filled) as related to percent total fluorescence intensity of the different DOM components as identified by PARAFAC analysis. Error bars represent standard deviations of triplicate experiments

As mentioned previously, the mechanisms of hydroxyl radical photoproduction by DOM are poorly understood (Vione *et al.* 2006). Similar to $^1\text{O}_2$, absolute formation rates of the hydroxyl radical showed positive correlation to the absolute fluorescence of each of the PARAFAC components, while the normalized formation rates showed positive correlation to abundance of terrestrial humic-like components C1, C3, and C5, and negative correlation to microbial humic-like C4, and C6, and the protein-like C7 and C8 (Fig. B.4). These relationships highlight the importance of caution when attempting to correlate quantitative and qualitative PARAFAC component information with photoreactivity of DOM: for example, simply looking at the relationship between absolute formation rates of $\bullet\text{OH}$ and C8 fluorescence would give the impression that the two have a strong positive correlation, while examining the normalized formation rate versus the abundance of C8 shows an exponential decrease in $\bullet\text{OH}$ formation with higher C8 abundance. Thus, while the abundance of FDOM is clearly a driver for reactive

species formation rates, it is ultimately the DOM quality (allochthonous vs. autochthonous) which is critical in this formation process. As such, the relative abundance of terrestrial, humic-like PARAFAC components could serve as a proxy for the potential for reactive species generation, particularly for $^1\text{O}_2$.

This study demonstrates the strength of combining reactive species measurements with DOM optical properties as a means to assess environmental drivers and molecular controls on DOM reactivity in aquatic environments. PARAFAC analysis has been demonstrated as a useful tool to qualitatively assess the contributions of different organic matter components to the photo-production of reactive species. The correlations between FDOM and reactive species production are to be expected: fluorescent emissions are generated by excited singlet states of DOM, which are the precursors of $^3\text{DOM}^*$, the main source of $^1\text{O}_2$ in sunlit natural waters. Additional research is needed on greater spatial and temporal scales to be able to use PARAFAC analyses to not only determine reactive species source, but also as quantitative proxies for the estimation of reactive species potential as a measure of DOM photoreactivity.

CHAPTER 4:

Linking the Chemical and Optical Properties of Natural Organic Matter

4.1 Introduction

The methods with which to study natural dissolved organic matter (DOM) are almost as numerous and complex as DOM itself. These techniques range from simple absorbance measurements in the ultraviolet (UV) and visible (Vis) range, which can be performed quickly and easily in the field, to FT-ICR-MS and 2D-NMR, which require advanced instruments and expertise that are only found at a few laboratories in the world. There is an ever-present desire, therefore, to relate the most simple of measurements, such as optical properties, to the complex chemistry of DOM. These properties then can become ‘surrogate measures’ for expensive and difficult methodologies, to be used at the (in)discretion of scientists and engineers that do not have access to other highly sophisticated equipment.

As mentioned previously, the first studies of DOM relied heavily on UV-Vis absorbance measurements. Such measurements led to the development of the first optical property- simple UV-Vis absorbance at a pre-defined wavelength. Researchers found that within a given system, absorbance at 254 nm (a strong emission line of Hg lamps) is an excellent indicator of organic carbon content (Dobbs *et al.* 1972). When investigating similar geographical aquatic environments (i.e. river systems), absorbance at 254 nm has been shown to correlate very well with DOC. When investigating systems of greater variability, however, such as marine vs. terrestrial systems, the correlation between DOC and absorbance is weaker. In addition, the complexation of metals, namely iron, affects the absorbance and fluorescence spectra of DOM, and therefore can lead to erroneous correlations if not corrected for (Weishaar *et al.* 2003, Willey

1984). Because absorbance at a single wavelength is closely tied to concentration, the utilization of multiple measurements, and their ratios, must be used to improve information related to DOM quality.

To move past concentration and onto quality of organic matter, ratios of absorbance and fluorescence measurements are used. Absorbance ratios are again based on the emission bands of Hg lamps, with E2:E3 (254 nm/365 nm) and E4:E6 (465 nm/ 665 nm) the most common. Changes in these ratios reflect changes in the spectral slope (S) of the absorbance spectrum. In general, a steeper S indicates smaller molecular weight (MW) and more autochthonous material, while shallower S reflects larger MW DOM and more terrestrial, allochthonous material. S is found from various methods of fitting an exponential curve to the absorbance spectrum (often measured from 200-700 nm) of DOM, which appears exponential, but can often deviate from a simple exponential curve (Coble 2007, Del Vecchio and Blough 2004b). Helms *et al.* (2008) therefore focused on narrower ranges of the spectrum, and found that 275-295 nm and 350-400 nm ranges were the most consistent and indicative of changes in DOM quality, and that the ratio of these two slopes, S_R , was inversely proportional to MW. The absorbance of DOM at a specific wavelength can also be normalized to the DOC, called specific UV absorbance (SUVA). SUVA can be measured for any wavelength, but the most common is SUVA measured at 254 nm, $SUVA_{254}$, which has been correlated to % aromaticity in the DOM using NMR (Weishaar *et al.* 2003).

With the advent of smaller, cheaper spectrofluorometers, fluorescence indices have surpassed absorbance indices in popularity. The humification index (HIX) was first introduced by Zsolnay as a metric to assess the degree of humification in soil organic matter (Zsolnay *et al.* 1999). The index is defined as the ratio of the “upper quarter” (435-480 nm) to the “lower quarter” (300-345

nm) of the fluorescence emission spectrum at 254 nm excitation. Later, Ohno proposed a normalized index of the 435-480 nm range divided by the sum of the 300-345 nm and 435-480 ranges, arguing that this accounted better for inner filtering effects (Ohno 2002a), although this has been debated (Ohno 2002b, Zsolnay 2002). Regardless, both humification indices are commonly used in the literature, with seldom explanation as to the choice of why a specific index was used. One of the most common indices is the fluorescence index (FI). Originally defined as the ratio of emission at 450/500 nm at 370 nm excitation (McKnight *et al.* 2001), the index was later moved to 470/520 nm to reduce noise and variability between instruments (Cory *et al.* 2010b). This index, which was inversely correlated with % aromaticity of a diverse set of DOM samples, is often used as an indication of terrestrial vs. marine/microbial DOM source. Most recently, the biological index (BIX; also known as the $\beta:\alpha$ ratio (Parlanti *et al.* 2000)) was introduced as a replacement for FI (Huguet *et al.* 2009). By measuring the ratio of emission at 380/430 nm at 310 nm excitation, the marine-like or ‘M’ fluorophore is directly targeted, and is therefore argued to be a more accurate representation of recent microbial input to the FDOM pool.

The most common optical properties, their calculations, and uses are shown in Table 4.1. To investigate the relationships between these properties and the chemistry of DOM, a set of samples from around the globe was collected. The absorbance and fluorescence spectra of these samples were measured, and the effects of pH titration and solar-simulated irradiation investigated. Mass spectra were collected using FT-ICR-MS, and the relationships between the optical properties and mass spectra investigated.

Table 4.1: Common optical properties and their uses

Optical Property	Definition	Use	Reference
E2:E3 ratio	Absorbance at 250 nm / 365 nm	Inversely correlated to molecular weight (MW)	(De Haan and De Boer 1987)
E4:E6 ratio	Absorbance at 465 nm / 665 nm	Inversely correlated to MW	(Kononova 1966)
SUVA ₂₅₄	Absorbance at 254 nm / DOC (mg C L ⁻¹)	Correlated to % aromaticity	(Weishaar <i>et al.</i> 2003)
Spectral Slope, S	Slope of exponential fit of DOM absorbance spectra	Indicative of DOM source	(Del Vecchio and Blough 2002)
S ₂₇₅₋₂₉₅	Slope of the log-normalized absorbance spectrum 275-295 nm	Inversely correlated to MW	(Helms <i>et al.</i> 2008)
S ₃₅₀₋₄₀₀	Slope of the log-normalized absorbance spectrum 350-400 nm	Inversely correlated to MW	(Helms <i>et al.</i> 2008)
Slope Ratio, S _R	Ratio of S ₂₇₅₋₂₉₅ / S ₃₅₀₋₄₀₀	Inversely correlated to MW	(Helms <i>et al.</i> 2008)
Fluorescence Index, FI	Ratio of fluorescence emission at 470 nm / 520 nm at 370 nm excitation	Indicative of DOM source	(Cory <i>et al.</i> 2010b, McKnight <i>et al.</i> 2001)
Humification Index (HIX)	Integrated emission from 435-480 nm / 300-345 nm at 254 nm excitation	Indicative of DOM source and processing	(Zsolnay <i>et al.</i> 1999)
normalized HIX (nHIX)	Integrated emission from 435-480 / (300-345 + 435-480) nm at 254 nm excitation	Indicative of DOM source and processing	(Ohno 2002a)
Biological Index (BIX)	Ratio of the fluorescence intensity at 380 nm to 430 nm at 310 nm excitation	Indication of recent microbial	(Huguet <i>et al.</i> 2009)

4.2 Methods

Water samples were collected between 2011 and 2014, and DOM was solid phase extracted using the PPL method described in Chapter 2 (Dittmar *et al.* 2008). Sample sites, dates, and descriptions are given in Table 4.2.

Table 4.2: Sample site locations

Sample name	Description	Sample Date	Latitude	Longitude
<i>Terrestrial</i>				
Lillsjön Bottom	Boreal lake bottom water	6/2011	58.659	16.144
Lillsjön Surface	Boreal lake surface water	6/2011	58.659	16.144
Rio Madeira	Amazon tributary with high sediment load	5/2013	-8.497	-63.485
Rio Negro	Black water river in the Amazon basin	4/2013	-2.613	-60.942
Rio Tapajos	Clear water river in the Amazon basin	4/2013	-2.351	-54.769
Suwannee River	Black water river, site of SRNOM reference material collection by IHSS	5/2012	30.804	-82.418
Svartån River	Boreal river	2/2012	58.440	15.457
<i>Coastal</i>				
Baltic Sea	Coastal surface water	10/2011	58.809	17.608
Brazil Lagoon	Impacted by seawater and groundwater	5/2011	-22.162	-41.300
Delaware Bay	Mouth of the Delaware Bay	3/2014	39.839	-75.354
Susquehanna River	Tributary of Chesapeake Bay	2/2013	39.657	-76.175
<i>Marine</i>				
Bering Strait	Surface water	5/2013	65.718	-168.878
Fiji Depth	Seawater at 100 m depth	3/2012	-16.570	-179.843
Fiji Surface	Surface seawater	3/2012	-16.570	-179.843
N. Atlantic Depth	North Atlantic seawater 200 m depth	1/2011	29.930	-65.420
N. Atlantic Surface	North Atlantic surface seawater	1/2011	28.833	-65.702
Sargasso Deep	Bermuda Atlantic Time Series (BATS) station, 4,500 m depth	8/2012	31.556	-64.167
Sargasso Surface	BATS station, surface water	8/2012	31.556	-64.167

Methanolic extracts were dried under a gentle stream of ultra-high purity nitrogen gas, and then dissolved in NanoPure water. Extracts were diluted to have absorbance values at 220 or 240 nm of ~ 0.2 to stay within the range where inner-filter corrections are known to be accurate (Miller *et al.* 2010). Samples were irradiated using the experimental system described in Chapter 1. Irradiation experiments were conducted over a period of 20 hours, with EEMs and absorbance spectra collected every 20 minutes. A 3 mm pathlength flow cell was used in an Aqualog spectrofluorometer. Scans were conducted from 600 to 240 nm in 3 nm steps, with 1 sec

integration times. The pH was adjusted to 8.0 before irradiation, and held constant throughout the irradiation with the Infinity II reaction controller system.

All pH titration experiments were performed using the Infinity II system and optical properties were monitored using the Aqualog spectrofluorometer. A 1 cm pathlength flow cell in the Aqualog was filled with sample, and 1.8 mL of sample was placed in a Teflon vial with a stir bar. The sample was then pumped continuously from the Teflon vial and through the flow cell. The pH was controlled in the vial using the same pH electrode and reaction controller as in the irradiation experiments. Sample pH was brought to 4.0, and EEMs/absorbance spectra collected. The titrations were then performed in 0.5 unit steps, until reaching pH 10, with EEMs/absorbance spectra collected at every 0.5 unit step.

Absorbance spectra were converted to absorption coefficients via Equation 1.1. S_R , $S_{275-295}$, $S_{350-400}$, and E2:E3 ratios were determined as described in Chapter 1. FI was calculated as the ratio of the fluorescence emission at 470 nm to 520 nm at 370 nm excitation (Cory *et al.* 2010b). The humification index (HIX) was calculated as the ratio of the area under the fluorescence emission spectra from 435-480 nm divided by the area from 300-345 nm at 254 nm excitation (Zsolnay *et al.* 1999). The normalized humification index (nHIX) was calculated as the area from 435-480 nm divided by the sum of the area from 300-345 nm and 435-480 nm at 254 nm excitation (Ohno 2002a). BIX was calculated as the ratio of the fluorescence intensity at 380 nm to 430 nm at 310 nm excitation (Huguet *et al.* 2009).

Ultra-high resolution mass spectrometry was conducted at the Helmholtz Center for Environmental Health in Munich, Germany, as described in Chapter 2. Samples were diluted in methanol to approximately the same concentration based on absorbance spectra collected during titrations.

Additional irradiation experiments were performed with a custom-designed system at the Smithsonian Environmental Research Center (SERC) for singlet oxygen measurements. In this system, the light from a 2.5 kW Xenon arc lamp was focused on a mirror which re-directed the beam vertically. Samples were placed in one of eight water-cooled aluminum blocks, each of which can be equipped with a long-pass wavelength filter. Absolute irradiance in each sample cell was measured with a spectroradiometer for apparent quantum yield calculations. Full details on the design and calibration of the custom spectroradiometer can be found elsewhere (Neale and Fritz 2002). Samples were diluted to approximately the same concentration based on absorbance spectra, and spiked with furfuryl alcohol (FFA) to ~1.5 mM final concentration. Aliquots were taken every 10 minutes for 50 minutes of irradiation time, and the loss of FFA was monitored using an Agilent HPLC as described in Chapter 3. Sample blocks were outfitted with 295 nm long-pass filters, and FFA loss due to direct photolysis was negligible.

Rates of singlet oxygen production were calculated using Equation 3.5. The light screening factor, $S(\lambda)$, was calculated via Equation 4.1 (Schwarzenbach *et al.* 2005):

$$S(\lambda) = \frac{[1 - 10^{-D \cdot a(\lambda) \cdot z}]}{2.3 \cdot D \cdot z \cdot a(\lambda)} \quad (4.1)$$

where $a(\lambda)$ is the sample absorbance calculated using Equation 1.1, z is the pathlength in meters (0.025), and D is the light distribution coefficient (1.2) combining direct and diffuse radiation (Schwarzenbach *et al.* 2005, Zepp and Cline 1977). The rate of light absorbance, k_a , by the sample was defined as:

$$k_a = \sum \frac{S(\lambda)W(\lambda)(1 - 10^{-a(\lambda) \cdot z})}{z} \quad (4.2)$$

where $W(\lambda)$ is the irradiance in the sample holder as measured by the spectroradiometer.

Apparent quantum yields of singlet oxygen production are then calculated with Equation 4.3:

$$\Phi_{^1O_2} = R_{^1O_2} / k_a \quad (4.3)$$

4.3 Results

Optical properties at pH 8 and $\Phi_{^1O_2}$ are shown in Table 4.3, as are the rate constants of fluorescence loss of the labile and semi-labile (k_L and k_{SL} , respectively) fractions at $\lambda_{ex}/\lambda_{em} = 340/450$ nm.

Table 4.3: Optical properties, degradation kinetics, and singlet oxygen production of DOM

Sample	HIX	nHIX	BIX	FI	E2:E3	S ₂₇₅₋₂₉₅	S _R	k _L	k _{SL}	Φ^{1O_2}
Baltic	5.30	0.841	0.717	1.50	8.56	0.0192	1.51	1.1 ± 0.1	0.071 ± 0.002	0.040 ± 0.013
Bering	1.51	0.601	0.832	1.55	9.14	0.0233	1.23	2.7 ± 0.1	0.092 ± 0.003	0.035 ± 0.006
Brazil Lagoon	8.23	0.892	0.690	1.53	36.3	0.0296	0.106	1.2 ± 0.1	0.089 ± 0.003	0.086 ± 0.009
Delaware Bay	4.74	0.826	0.679	1.60	6.11	0.0148	0.541	0.88 ± 0.04	0.100 ± 0.003	0.035 ± 0.006
Fiji Depth	1.27	0.560	0.949	1.53	11.8	0.0269	1.46	1.6 ± 0.1	0.099 ± 0.002	0.028 ± 0.005
Fiji Surface	0.543	0.352	0.864	1.41	15.4	0.0304	3.21	2.3 ± 0.1	0.057 ± 0.003	0.025 ± 0.005
Lillsjön Bottom	22.6	0.958	0.434	1.38	4.50	0.0120	0.597	1.6 ± 0.1	0.116 ± 0.004	0.021 ± 0.003
Lillsjön Surface	20.1	0.953	0.434	1.35	4.83	0.0127	0.572	1.11 ± 0.04	0.101 ± 0.003	0.019 ± 0.003
N. Atl. Depth	1.54	0.606	0.856	1.63	10.2	0.0281	2.26	1.4 ± 0.1	0.113 ± 0.003	0.041 ± 0.007
N. Atl. Surf	0.712	0.416	0.966	1.56	10.2	0.293	2.87	2.0 ± 0.2	0.13 ± 0.01	0.032 ± 0.004
Rio Madeira	17.9	0.947	0.561	1.54	5.78	0.0136	0.644	0.85 ± 0.04	0.088 ± 0.004	0.042 ± 0.006
Rio Negro	24.4	0.961	0.404	1.33	4.18	0.0113	0.621	1.08 ± 0.05	0.12 ± 0.01	0.023 ± 0.002
Rio Tapajos	23.9	0.960	0.490	1.43	5.29	0.0134	0.624	0.87 ± 0.03	0.095 ± 0.003	0.032 ± 0.005
Sargasso Deep	3.81	0.792	0.726	1.62	9.62	0.0242	1.22	1.7 ± 0.1	0.121 ± 0.004	0.036 ± 0.004
Susquehanna River	6.99	0.875	0.645	1.57	6.86	0.0158	0.749	1.1 ± 0.1	0.099 ± 0.003	0.035 ± 0.006
Suwannee River	29.5	0.967	0.423	1.36	5.11	0.0126	0.636	1.5 ± 0.1	0.116 ± 0.004	0.030 ± 0.004
Sargasso Surface	0.838	0.456	0.973	1.60	29.0	0.0398	1.74	0.87 ± 0.1	0.11 ± 0.01	0.024 ± 0.006
Svartån River	14.7	0.936	0.560	1.48	6.42	0.0151	0.567	0.99 ± 0.03	0.088 ± 0.002	0.033 ± 0.007

Photo-chemical loss of fluorescence

The photo-chemical losses of fluorescence over 20 hours of solar-simulated irradiation for all of the samples are shown in Figures 4.1-4.3. All terrestrial and coastal samples showed similar patterns in loss of fluorescence (Fig. 4.1-4.2). Greatest fluorescence losses were seen at ~250 nm excitation and 425-475 nm emission. These were coupled with losses in the 'C' region of the spectrum with loss maxima at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 325\text{-}350/445\text{-}460$ nm. Marine samples, which contain more UVA fluorescence, showed significant losses in this region, as well as in the 'A' region at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 250/425\text{-}450$ nm and in the 'C' region $320\text{-}350/415\text{-}440$ nm. The location of the maximum loss in the 'C' region for each experiment is shown in Fig. 4.4. Terrestrial samples showed greatest loss at longer excitation and emission wavelengths, with coastal and marine samples losing the most fluorescence at shorter wavelengths. The increased wavelengths of loss for terrestrial samples were likely due to the presence of higher molecular weight material, both in the 'C' region and in the longer-wavelength, pH-dependent region, likely dominated by large polyphenolic compounds (see Chapter 1).

As the location of maximum fluorescence loss in the 'C' region may be influenced by loss of longer-wavelength fluorescent components, degradation kinetics were calculated at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 340/450$ nm for all samples. This is the location of the peak maximum of the photo-labile component of SRNOM (Chapter 1). The loss rate of the fluorescence at this point did not show specific source-dependent trends (Table 4.3). However, the marine surface samples (other than the Sargasso Sea surface) showed the fastest photo-degradation rates of the photo-labile fraction, k_L . The marine deep waters and coastal systems were similar to terrestrial rates of loss both in the labile and semi-labile fractions.

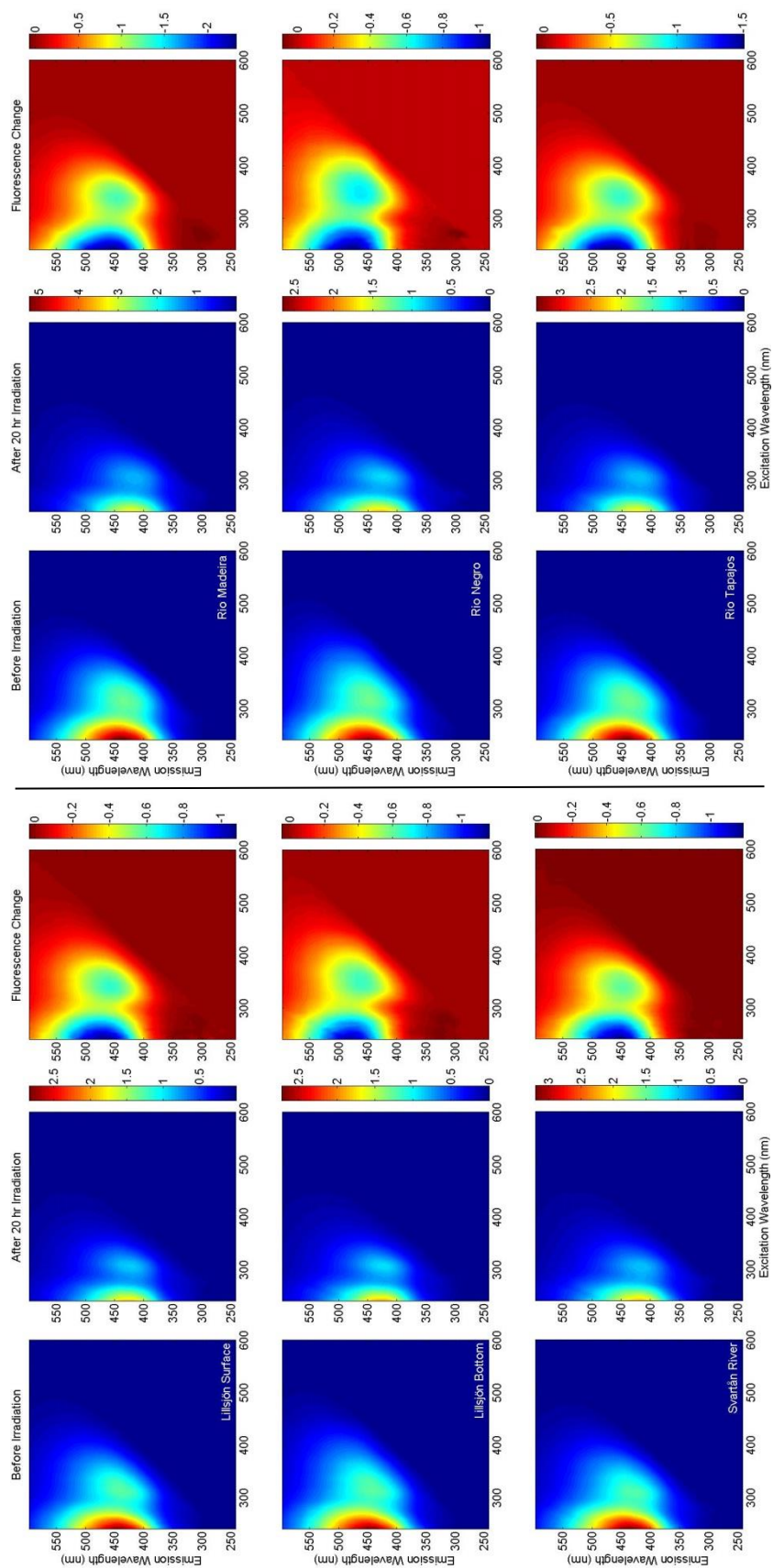


Figure 4.1: Fluorescence spectra of terrestrial samples before and after 20 hours of irradiation, and the fluorescence change. All fluorescence intensities are in Raman Units (RU)

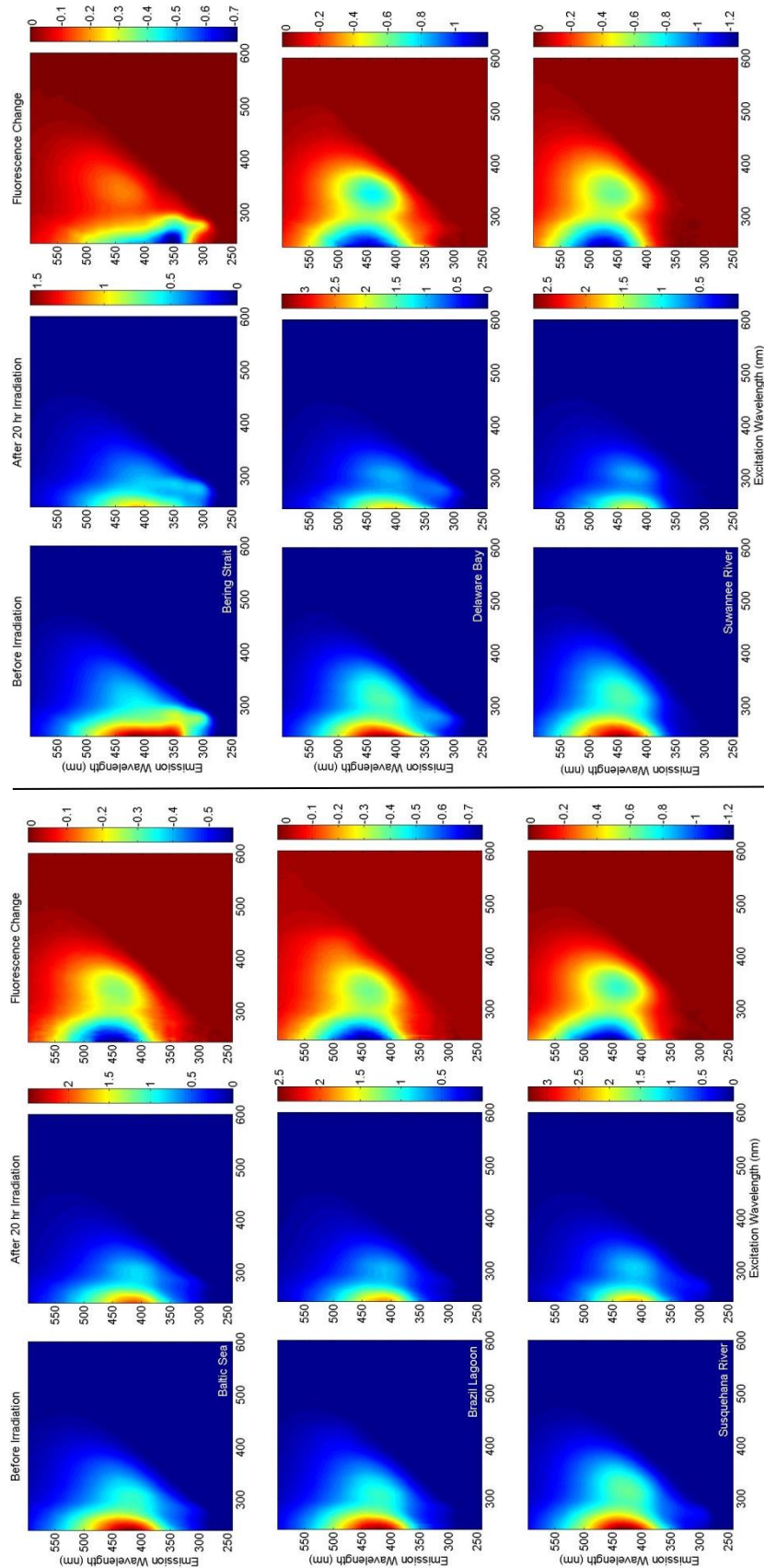


Figure 4.2: Fluorescence spectra of samples before and after 20 hours of irradiation, and the fluorescence change. All fluorescence intensities are in Raman Units (RU)

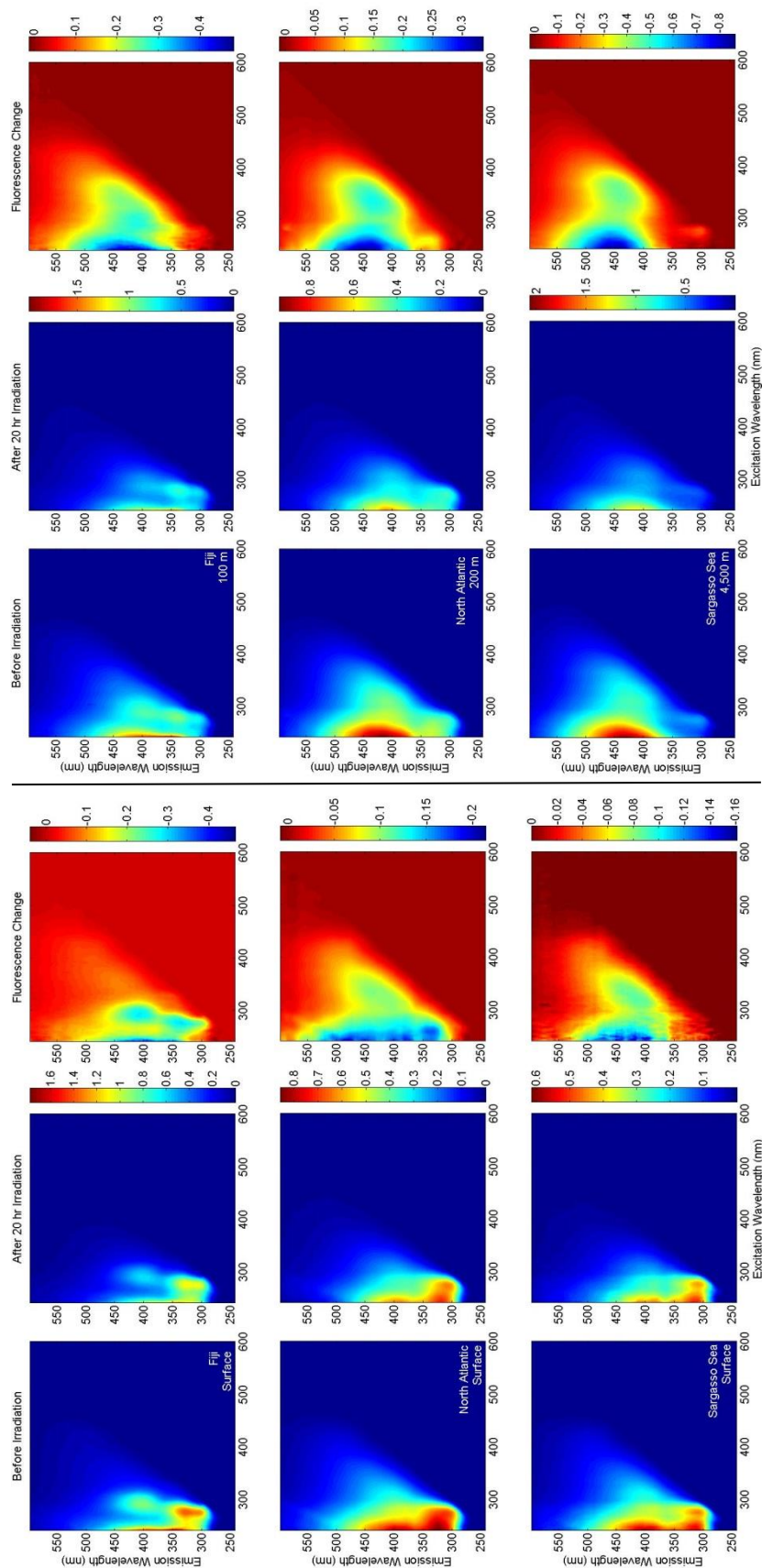


Figure 4.3: Fluorescence spectra of marine samples before and after 20 hours of irradiation, and the fluorescence change. All fluorescence intensities are in Raman Units (RU)

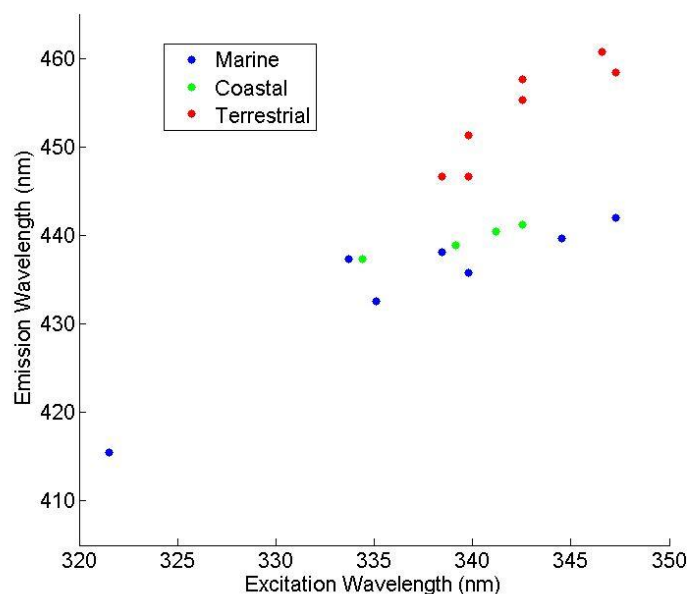


Figure 4.4: Wavelength couple of greatest fluorescence loss during irradiation in the ‘C’ region of the EEM spectra

Effect of pH titration on EEMs

Solution pH had a strong effect on the fluorescence of DOM. When titrated from pH 4 to pH 10, the most prominent feature in terrestrial systems was the increase of a peak at $\lambda_{ex}/\lambda_{em} = 390/490$ nm. This peak was most prominent in terrestrial systems. To reduce concentration effects on the peak intensity and relative increase during titration, a seven component PARAFAC model was fit to the EEM spectra collected at pH 10. This model explained 99.8% of the variability, but had a very low core consistency (0.88) and significant peaks and troughs in the residuals. While this model was not adequate for comparisons of all components and their intensities, the long wavelength, pH-dependent peak was well resolved. The abundance of this component, defined as the percent of the total fluorescence (sum of all component intensities), was much higher in terrestrial samples than coastal and marine samples. The percent abundance of the pH-dependent peak correlated very well with the humification indices, and cluster analysis showed >85% similarity in the variation of the pH peak abundance and the humification indices.

In Chapter 1, it was posited that the fluorophores responsible for this fluorescence are likely high molecular weight polyphenolic compounds. These compounds are believed to be mainly lignin-derived, explaining their prevalence in terrestrial systems and depletion in marine waters.

The most prominent features in titrations of marine DOM samples were decreases in the UVA portion of the spectrum, centered around $\lambda_{\text{ex}}/\lambda_{\text{em}} = 250/350$ nm. The fluorophores in this region of the spectrum, as discussed in Chapter 2, are comprised mainly of proteinaceous material and low molecular weight aromatic compounds. The decrease in fluorescence with increasing pH may be due to changes in the folding or orientation of proteinaceous material, altering the quenching and intra-molecular interactions of this material (Kronman and Holmes 1971).

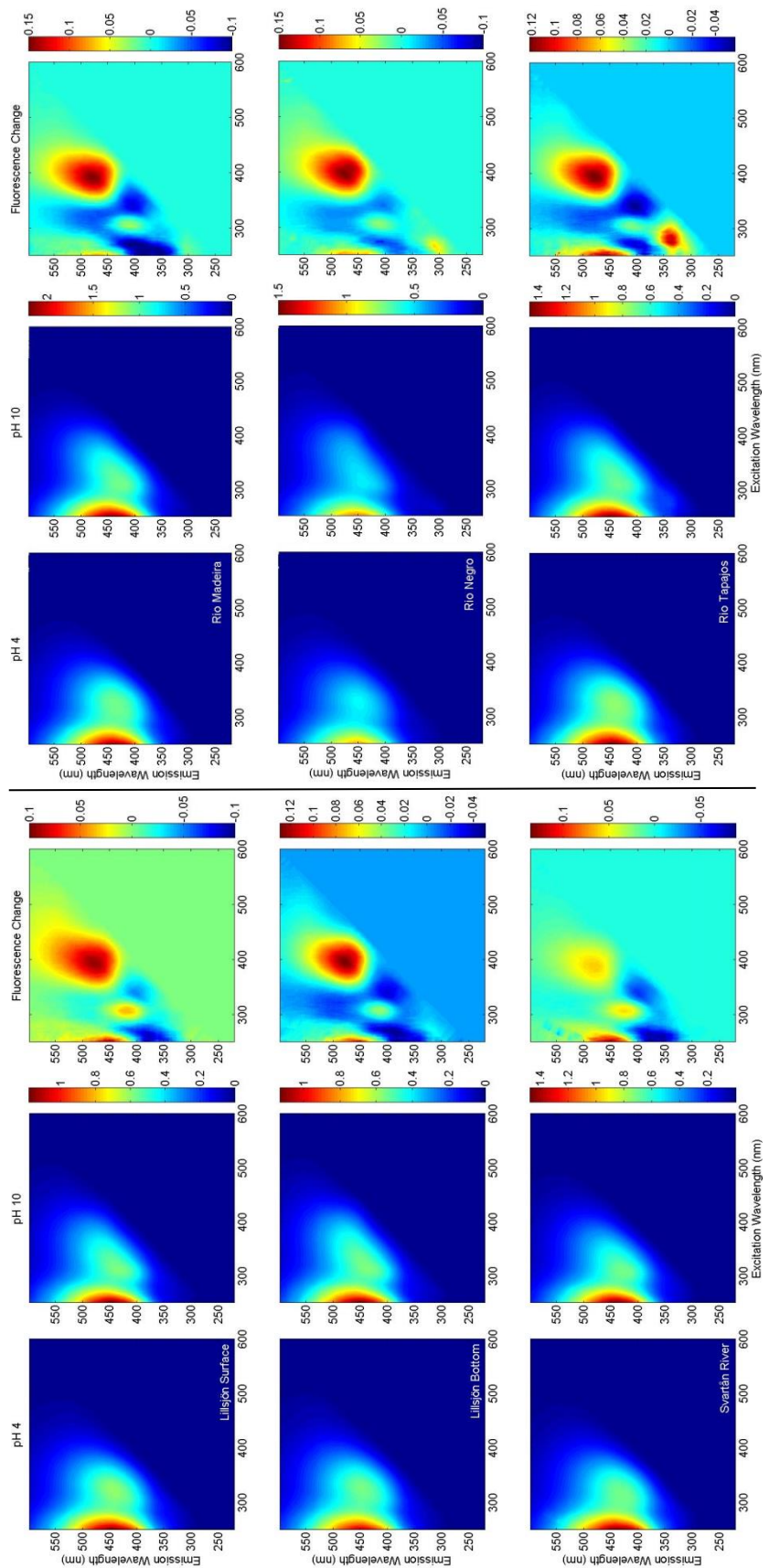


Figure 4.5: Fluorescence spectra of terrestrial samples at pH 4, pH 10, and the fluorescence change. All fluorescence intensities are in Raman Units (RU)

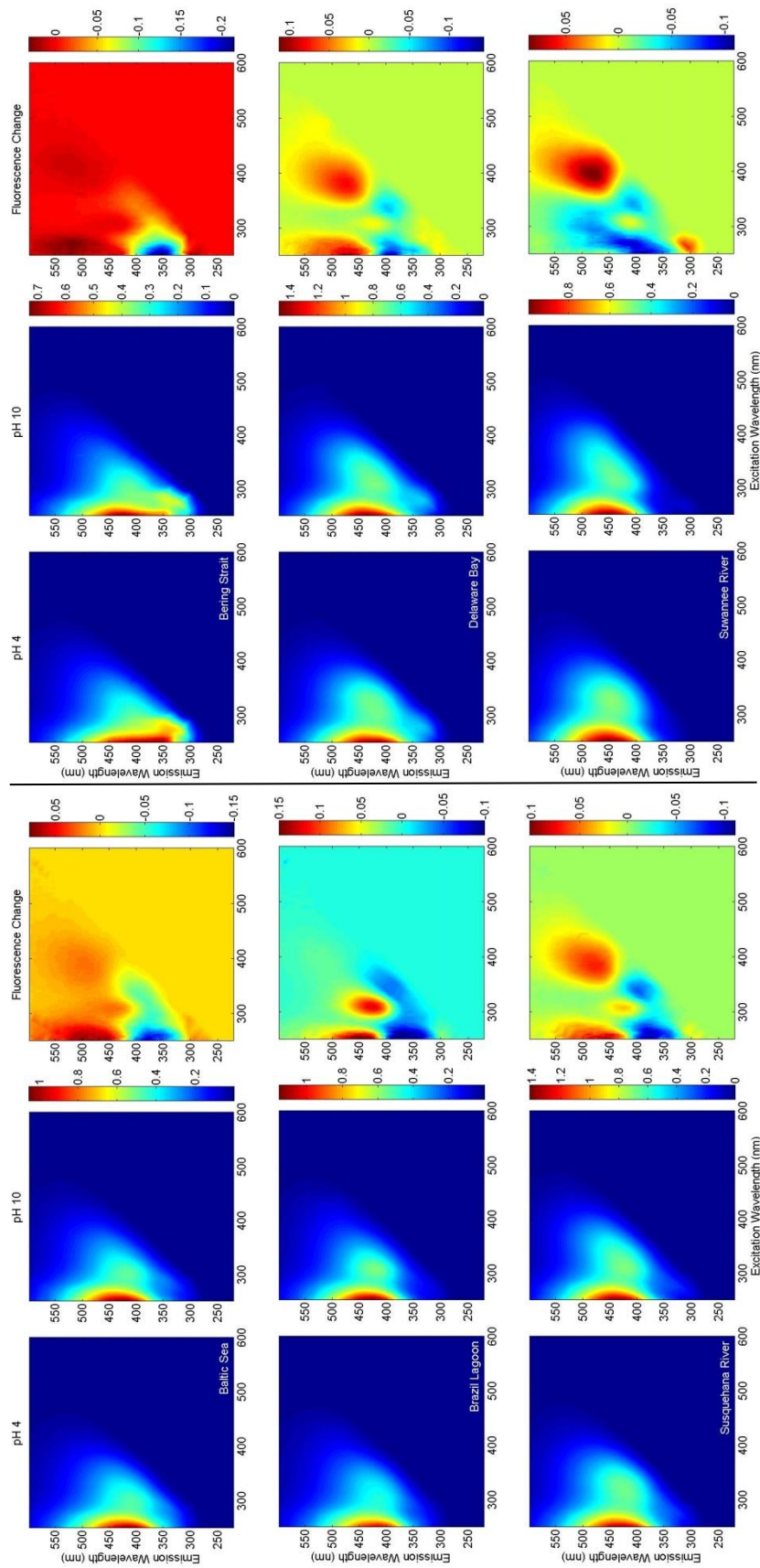


Figure 4.6: Fluorescence spectra of samples at pH 4, pH 10, and the fluorescence change. All fluorescence intensities are in Raman Units (RU)

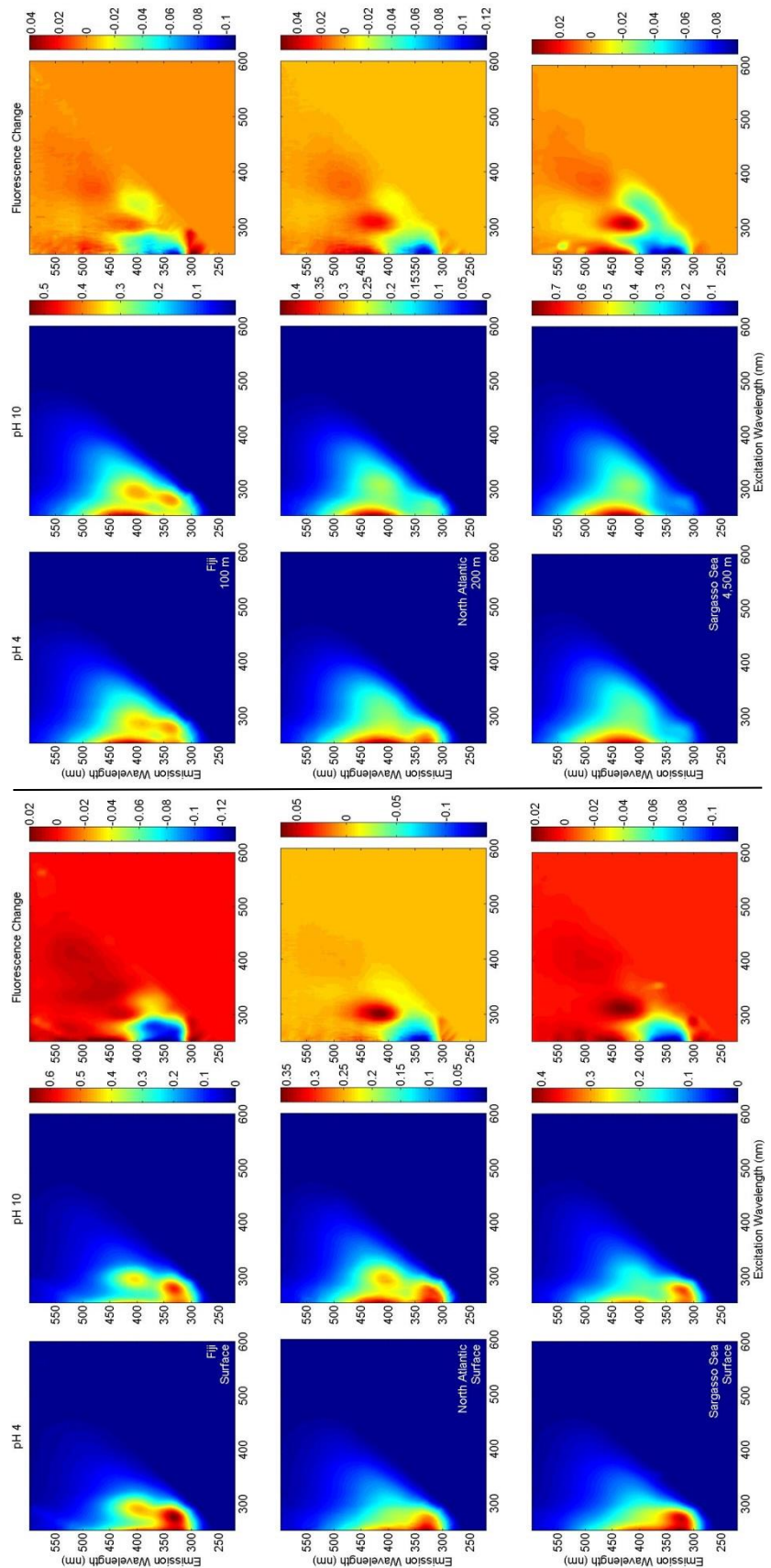


Figure 4.7: Fluorescence spectra of marine samples at pH 4, pH 10, and the fluorescence change. All fluorescence intensities are in Raman Units (RU)

Humification Indices

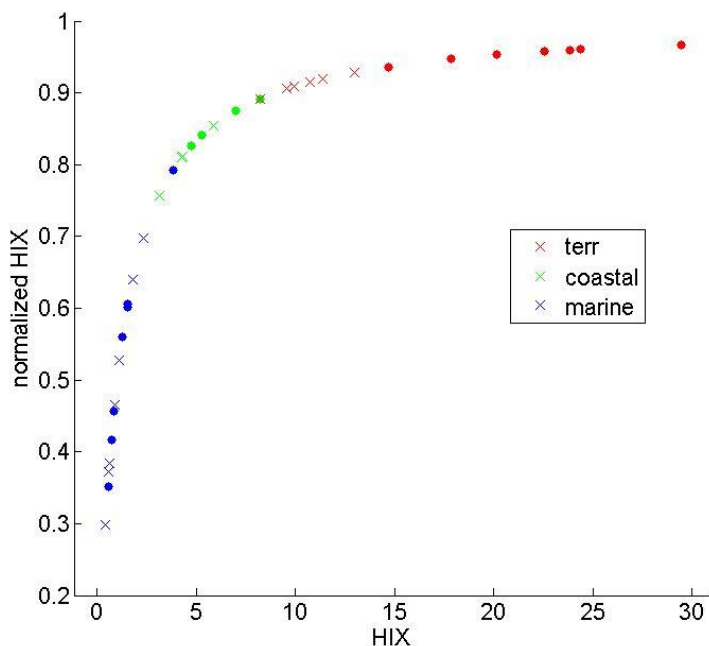


Figure 4.8: Humification Index (HIX) and normalized index (nHIX) for samples before (o) and after (x) irradiation experiments

Fig 4.8 shows the relationship between the original humification index, HIX, and the normalized index, nHIX. There was a large variation in the HIX values in terrestrial samples, ranging from ~14 to 29. The marine samples, however, showed the largest relative change when using the nHIX metric, ranging from 0.35 to 0.79. The coastal samples fell in between the two end members. These data suggest that the traditional HIX could be best utilized to distinguish between terrestrial samples, while nHIX could be best used to evaluate marine samples. Irradiation decreased both HIX and nHIX from their original values, yielding values for terrestrial samples closer to those of the coastal samples, and the irradiated coastal samples had values closer to those of marine samples. The decrease in the value of these indices is due to the preferential loss of long wavelength fluorescence (Fig. 4.9b), and while indices of irradiated

samples looked more autochthonous than the starting materials, this should not be interpreted as evidence that all marine DOM fluorescence arises from photobleached terrestrial DOM.

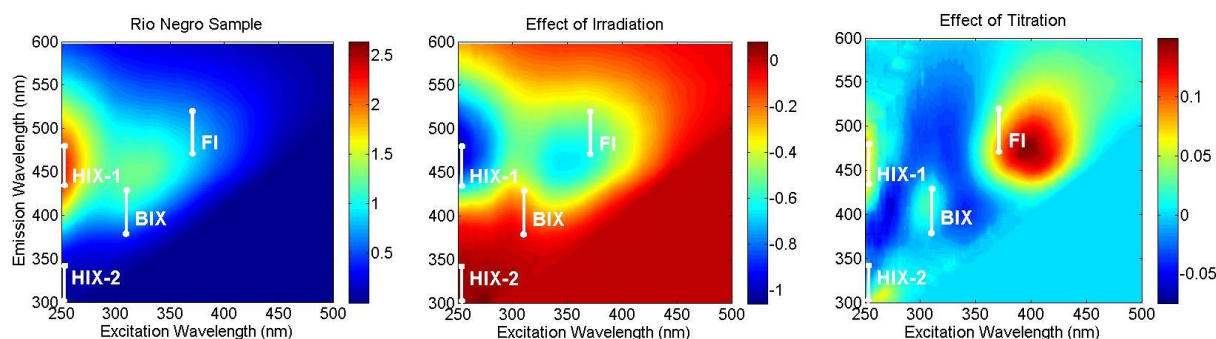


Figure 4.9: Location of measurements for fluorescence index (FI), biological index (BIX), and humification index (HIX) in EEM, differential EEM after 20 hr irradiation, and differential EEM from titration from pH 4 to pH 10. Note the color scale (intensity) differences between the subplots.

Solution pH also had a strong effect on HIX and nHIX values. HIX values increased by up to 56% when samples were titrated from pH 4 to pH 10 (Fig 4.10). These increases showed no relation to DOM source, unlike nHIX, which showed significant relation to source. Terrestrial samples increased only up to 3%, while the nHIX of coastal samples increased up to 6% and marine samples up to 20%. The changes in these indices are expected when looking at the differential fluorescence plots from the pH titrations (Fig. 4.9c). With increasing pH, long wavelength fluorescence increases, and often, fluorescence losses were seen centered around 350 nm, decreasing the denominator in both indices. Rio Tapajos and Rio Negro both showed small decreases in nHIX and HIX when titrated from pH 4 to pH 10. By examining the differential titration plots (Fig. 4.6), the driver appeared to be the introduction of peaks in the proteinaceous region of the EEM. These increases may have been due to changes in protein-like fluorescence, or the introduction of protein-like contamination during the titration experiment.

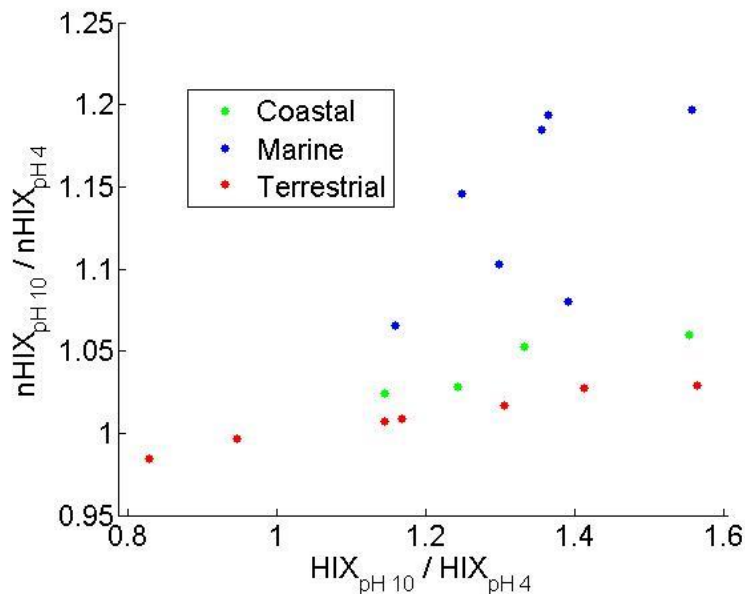


Figure 4.10: Effect of pH on humification index (HIX) and normalized humification index (nHIX)

Given these results, it appears that nHIX may be best suited for comparison of marine samples ($nHIX < 0.9$), while HIX may be best for comparison of terrestrial samples ($HIX > 10$). The strong effect of pH on these values must be taken into consideration, and HIX or nHIX values only compared between samples of the same pH. The effect of proteinaceous contamination must also be considered.

Fluorescence and Biological Indices

FI was affected by pH, but only by $\pm 7\%$. BIX, however, decreased by up to 25% when titrated from pH 4 to pH 10. While FI values have been reported in the range of 1.8-2.8 for microbial samples, the highest FI in this dataset was 1.7. Previous work has shown that FI is strongly influenced by the location of long-wavelength emission peaks (Korak *et al.* 2014). The PPL extraction process may preferentially select for longer-wavelength fluorescence, lowering the FI values. Alternatively, the increased concentration of DOM in the samples via the extraction process could have changed the peak shapes, altering the FI values from those

collected on natural waters. BIX values, however, were much more indicative of source (Fig. 4.11). During irradiation, FI decreased in every sample. Such a decrease would suggest that photo-degradation led to more ‘terrestrial-like’ DOM, which was contrary to every other optical metric, including BIX, which increased during irradiation in every sample except for the Fiji Surface and Sargasso Sea Surface samples, where BIX stayed relatively constant. The relatively low FI values for marine samples and the decrease during irradiation suggested that FI might not be a suitable index to for use with PPL extracts.

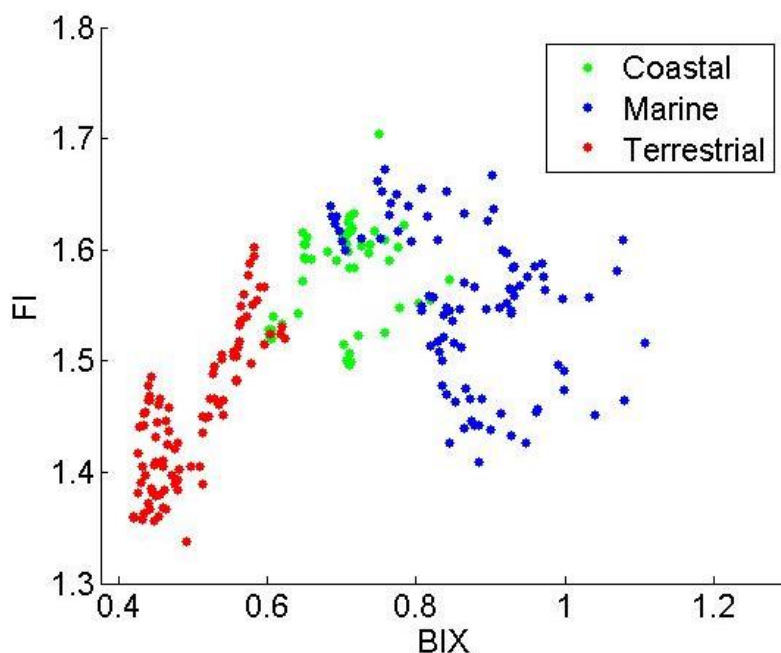


Figure 4.11: Fluorescence index (FI) and biological index (BIX) values for all 18 global DOM samples at 13 pH values

Ultra-high resolution mass spectrometry

Mass spectra from each sample were collected in duplicate. Mass peaks not present in each of the duplicate runs were discarded. Further, mass peaks not present in at least two of the 18 samples were removed. This reduced the number of mass peaks to ~24,000. Prior to statistical analyses, molecular formulae were assigned to the mass peaks according to previously published guidelines (Shakeri Yekta *et al.* 2012), and peaks with no formula assignments discarded, further

reducing the number of m/z peaks to $\sim 7,800$. The intensities of these m/z peaks were then autoscaled (see Chapter 2) and analyzed via hierarchical clustering, using both the sample locations as well as the individual mass peaks as variables. The results of the clustering on the sample locations are shown in Figure 4.12. The entire dataset contained 28% similarity, above which there was a clear divide between terrestrial/coastal and marine samples. Marine samples together had 66% similarity, with samples from individual depth profiles clustering at up to 92% similarity. Coastal samples clustered poorly with both marine and terrestrial samples, but the Susquehanna River clustered most closely with the Delaware Bay. Terrestrial samples showed 76% similarity, with the boreal lake samples clustering at $>90\%$ similarity.

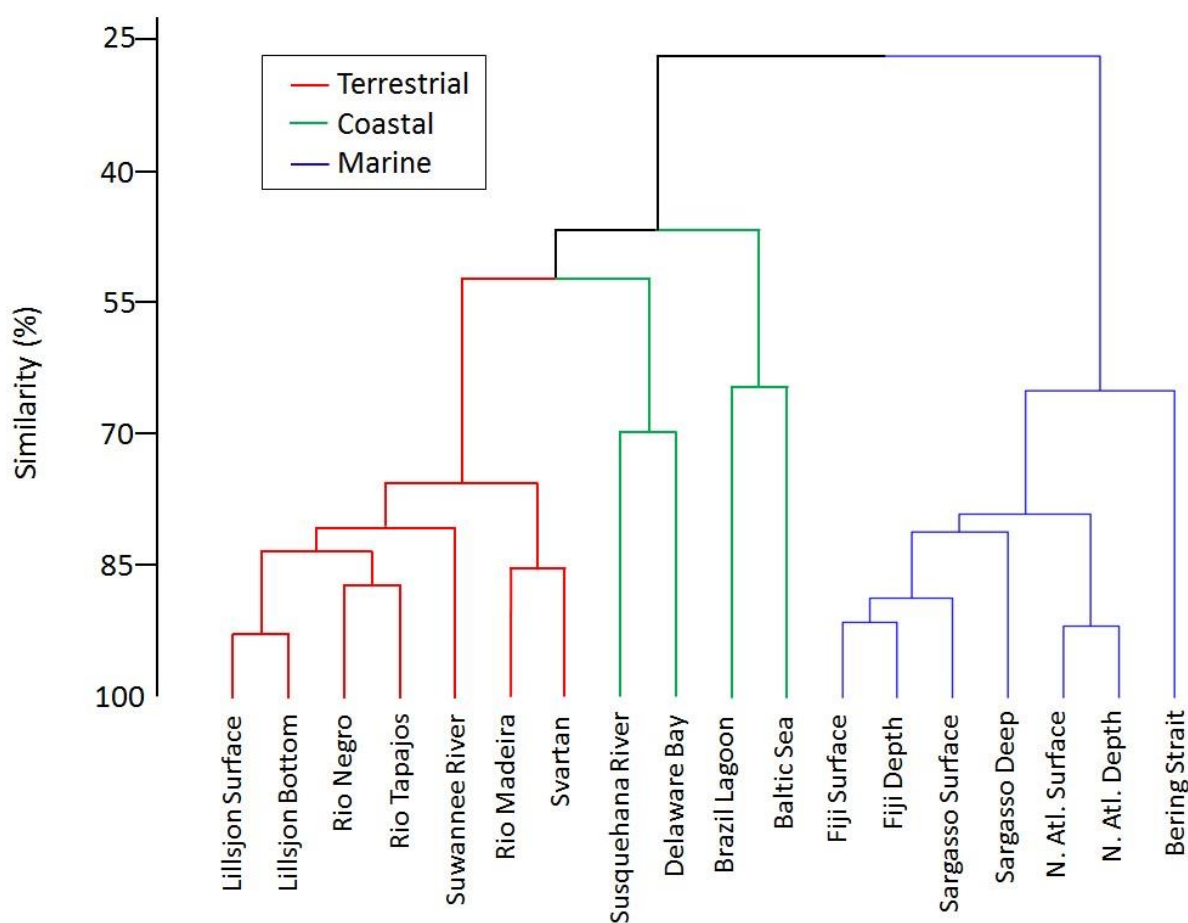


Figure 4.12: Hierarchical cluster of mass spectra by sample location

Hierarchical clustering of the mass peaks showed two major clusters that were indicative of terrestrial and marine samples, as shown in Figure 4.13. Masses that were indicative of terrestrial samples were mainly located in the bottom right corner of the van Krevelen diagram, a region that is occupied by aromatic, oxygen-rich compounds. These masses included mainly CHO and CHNO formulas, with only 2 sulfur-containing compounds present. In contrast, m/z ions indicative of marine samples were much more unsaturated, located in the aliphatic and oxygen-poor region of the van Krevelen diagram, and contained 375 sulfur-containing formulas. The masses indicative of marine samples also contained more CHNO formulas than CHO formulas. The high abundance of nitrogen- and sulfur containing compounds is likely due to the significant contribution of recent primary production to the marine DOM pool, while terrestrial organic matter is mainly comprised of degrading lignin/tannin structures and other higher plant materials that are relatively depleted in heteroatoms.

The measured optical properties were autoscaled and added into the matrix of m/z ions. The masses that clustered at >85% similarity with the humification indices (both HIX and nHIX) and with BIX are shown in Figure 4.14. Both humification indices clustered with mass peaks that were present in group of peaks indicative of terrestrial systems, and contained formulas that were aromatic and oxygen-rich. The masses that clustered with BIX were within the group indicative of marine samples, with more aliphatic and oxygen-poor structures. This is a strong indication that these optical indices are actually indicative of the chemical properties that they are meant to represent. In addition to m/z peaks indicative of marine systems, variations in BIX showed >85% similarity to $S_{275-295}$.

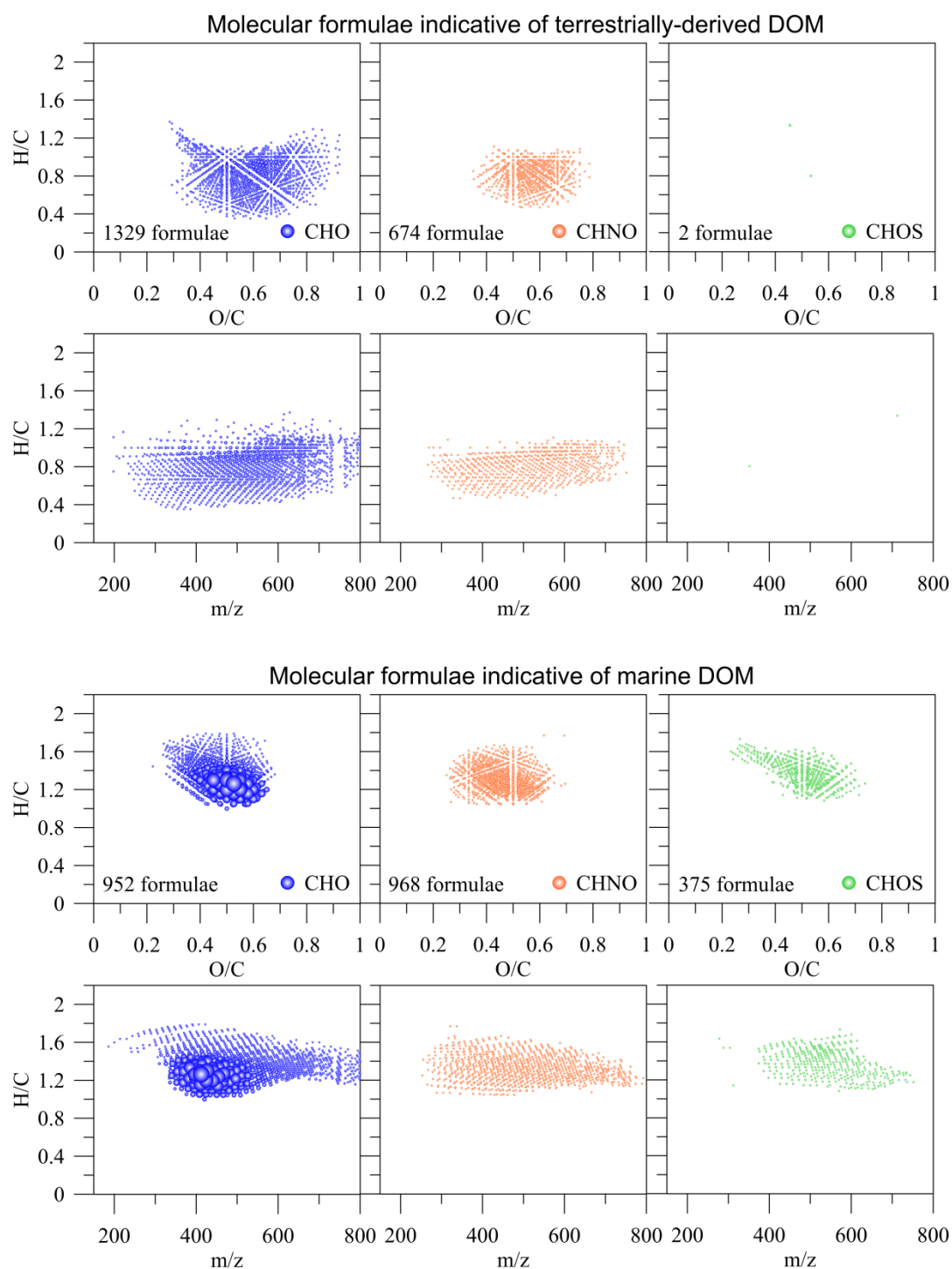


Figure 4.13: van Krevelen diagram (H/C vs O/C) and H/C vs m/z plots of assigned molecular formulae indicative of terrestrial and marine DOM pools, as determined by hierarchical clustering. Bubble size represents relative abundance.

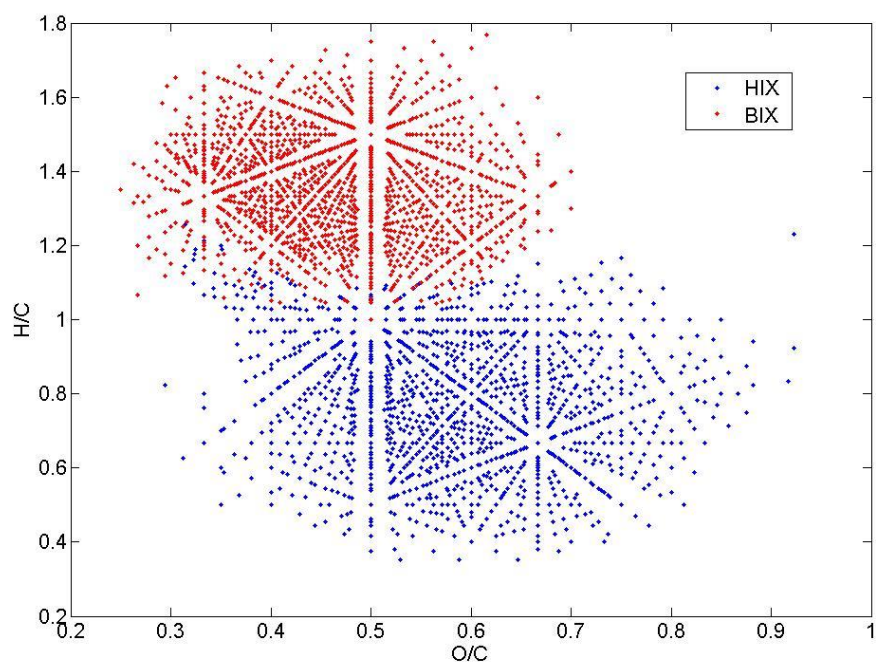


Fig. 4.14: m/z ions and their assigned molecular formulae that cluster with HIX (traditional and normalized) and BIX at > 0.85 similarity

Singlet oxygen production

Apparent quantum yields of singlet oxygen production are shown in Table 4.3. Neither a clear source-dependent trend was seen, nor any strong correlations to optical properties or m/z ions found. Apparent quantum yields ranged from 0.019 -0.086, with an average value of 0.034 and a median value of 0.033. The Brazil Lagoon had the largest apparent quantum yield at 0.086, more than double the next highest sample, the North Atlantic 200 m sample.

4.4 Discussion

Optical properties of DOM samples from a wide variety of sources were examined, and the effects of pH and photo-chemical irradiation on these properties determined. When exposed to solar-simulated irradiation, the BIX values increased. This increase made the fluorescence signature of the DOM more similar to microbial DOM, and less similar to terrestrial sources. Both humification indices (HIX and nHIX) decreased with irradiation, again making the

fluorescence signature less similar to terrestrially-derived material. FI, however, decreased during irradiation experiments, yielding FI values that approached those of terrestrial organic matter. These results suggest that BIX may be a more suitable index for determining impact of source on DOM fluorescence than FI (Helms *et al.* 2013). BIX also correlated strongly with aliphatic compounds that were indicative of marine waters, which has been shown previously (Kellerman *et al.* 2015). The m/z ions that correlated with BIX, or any optical property, should not be interpreted as the chromophores/fluorophores directly responsible for the optical properties observed, but rather as representative of the greater DOM pool indicative of the source of the chromophores/fluorophores. For example, while ~1,900 mass peaks correlated with BIX, only a fraction of these peaks may contain the fluorophores responsible for the changes observed in BIX, and the remaining pool represents compounds that presumably originated from the same DOM source, but may not necessarily contain specific fluorophores. That being said, the fact that FI did not correlate well with the masses indicative of marine sources further suggested that this metric might not be as suitable as BIX as a proxy for DOM sources. One of the spectral slopes, $S_{275-295}$, did correlate with BIX and the mass peaks indicative of marine sources. This portion of the spectral slope has previously been inversely correlated with molecular weight, shown to increase with photo-bleaching, and be a strong indicator of autochthonous sources (Helms *et al.* 2013, Helms *et al.* 2008). In addition to BIX, $S_{275-295}$ is therefore recommended as a suitable proxy for determining the source of DOM.

Both the traditional (HIX) and normalized (nHIX) humification indices correlated strongly with m/z peaks assigned to unsaturated, aromatic, and oxygen-rich formulas (Kellerman *et al.* 2015). While these indices appeared to be good predictors of terrestrially-sourced material, the traditional HIX may be best for comparison only of terrestrial samples ($HIX > 10$), while nHIX

may be best suited for comparison of marine samples ($nHIX < 0.9$). These indices are also strongly affected by pH and proteinaceous contamination, both of which must be considered when comparing samples using only these indices.

It should be stressed that all experiments were carried out on desalted, concentrated extracts. While this method allowed for direct comparison between the DOM optical properties and chemistry, matrix effects must be accounted for when studying natural waters. Metals and other ions have been shown to significantly alter the fluorescence and absorbance properties of DOM (Gao *et al.* 2015, Poulin *et al.* 2014, Willey 1984), and pH effects have been shown in this study. Changes in ionic strength, metal complexation, and concentration must be taken into account when using optical properties as surrogate measures or proxies for DOM source, molecular weight, or other properties.

The Brazil Lagoon had by far the largest apparent quantum yield of singlet oxygen production at 0.084. The E2:E3 ratio was also significantly larger than the rest of the samples at 36.3. E2:E3 has been inversely correlated with molecular weight, suggesting that this sample contained relatively smaller MW compounds than the other samples. The smallest fractions of DOM have been shown to have the highest apparent quantum yields of singlet oxygen formation (Mostafa and Rosario-Ortiz 2013), which could explain why the singlet oxygen production was so high. The mass spectrum of the Brazil Lagoon sample did not appear to have particularly high abundances of low MW compounds, but this cannot be used to discount the information from the E2:E3 ratio. The FT-ICR-MS was optimized for analysis of compounds from 150-2000 Da, and intensities of compounds at the lower m/z range are often reduced due to bias towards larger masses. Additionally, these smaller compounds may not ionize as efficiently, resulting in lower signal intensities even if the compounds responsible for the m/z peaks are in higher abundance.

Apparent quantum yields of singlet oxygen production did not show any significant relationship to DOM source. Previous work on Lake Superior showed that singlet oxygen production was most closely tied to DOM concentration, with DOC and absorbance at 300 nm the most accurate predictors for singlet oxygen production (Peterson *et al.* 2012). The lack in variation of the measured apparent quantum yields may therefore stem from the fact that extracts were diluted to approximately the same absorbance in the UVC region of the spectrum, thereby removing the absorbance/concentration- based variation. This would suggest that the chromophores responsible for singlet oxygen production are present in all DOM samples in similar relative proportions. Alternatively, singlet oxygen production has been shown to be highly wavelength-dependent, with decreasing quantum yields at increasing excitation wavelengths (Marchisio *et al.* 2015, Sharpless 2012). Triplicate samples were irradiated in three unique sample positions within the experimental setup to reduce bias based on which sample holder was utilized. This resulted in standard deviations of ~15%, likely due to spectral variations between the sample positions. While these spectral variations are accounted for in the apparent quantum yield calculations, wavelength-dependent quantum yields would have a significant impact on these apparent quantum yields, granting bias to longer wavelengths by assuming constant efficiencies over the range measured (290-400 nm). When the wavelength range was extended to 290-500 nm, standard deviations in the apparent quantum yield increased to >20 %, suggesting that this mechanism may have played a role in the variation observed between samples. Assessing the wavelength dependence of the quantum yield of formation of singlet oxygen in a diverse sample set such as the one used in this study may reduce the standard deviations, allowing for more subtle variations to be seen between samples of different origins.

CONCLUSIONS

A novel photo-chemical system incorporating the semi-continuous monitoring of excitation emission matrix (EEM) fluorescence and UV-Vis 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. With this system, EEMs can be used to quantitatively measure photo-degradation kinetics of dissolved organic matter. PARAFAC analysis can additionally be incorporated to separate the photo-labile and pH-influenced components of fluorescent DOM. This has significant implications for the use of PARAFAC as a quantitative tool, providing more detailed information on which fluorescent components are responsible for bulk changes seen in EEMs during irradiations and titrations.

Rates of photo-chemical 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.

The photo-reactivity of marine DOM increased with depth. This is due in part to the recent photo-bleaching of surface waters relative to those below the photic zone. Additionally, clear molecular differences were seen below the mixing zone, with more aromatic, oxygen-rich compounds present at depth. These compounds were among those believed to be the largest contributors to the fluorescence properties of DOM, and were highly correlated with long wavelength fluorescent PARAFAC components.

Formation rates and steady-state concentrations of singlet oxygen, triplet excited-state DOM, and hydroxyl radical were determined for two different estuarine transects of the Florida Everglades. Both formation rates and steady-state concentrations were directly related to CDOM, SUVA₂₅₄ and FDOM, but were clearly driven by the relative abundance of terrestrial humic-like DOM components, while they correlated negatively with the microbial humic-like and protein-like components of DOM. Singlet oxygen formation rates and steady-state concentrations (normalized to DOC) decreased slightly in the Harney River and more so in the Taylor Slough along the salinity gradient, most likely due to changes in the abundance of humic-like substances along the salinity gradient, and due to differences in dissolved organic matter quality (microbial vs. terrestrial) between the two estuarine systems. Normalized formation rates and steady-state concentrations of ³DOM* were found not to change significantly along the salinity gradients. Hydroxyl radical formation (normalized to DOC) decreased by >87 % across the transects of both systems, and showed similar relationships to DOM as singlet oxygen, with higher production in waters with a higher abundance of terrestrial humic-like components and decreased production in waters with higher abundance of microbial humic-like and protein-like DOM. A strong correlation between •OH formation and SUVA₂₄₅, an established proxy for aromaticity, was seen in the Taylor River/Florida Bay samples, but not in the Harney River, suggesting that aromaticity may be more important in •OH formation from microbial/autochthonous DOM than terrestrial-derived DOM.

In engineered systems, it is critical to be able to monitor water quality in real time in order to assess performance and adjust controls. Fluorescence and UV-Vis absorbance measurements are very fast and inexpensive, making them ideal candidates for monitoring in a constructed treatment wetland. Of the optical properties tested, the humification indices (HIX and nHIX)

were found to be closely tied to the abundance of terrestrially-derived organic matter, while BIX and $S_{275-295}$ were most indicative of microbially-sourced DOM. Measuring these properties in a wetland system could be useful to assess the contribution of microbial/algal sources in a pond to the overall DOM pool. Singlet oxygen apparent quantum yields were not found to be tied to source in the global DOM sample set, while in the Everglades, terrestrially-derived DOM produced more reactive species than microbial/seagrass DOM. This increase in production is likely because of increased absorbance of the terrestrially-derived DOM; DOM from the two sources may have similar $\Phi_{^1O_2}$, but increased absorbance of terrestrial DOM versus marine DOM would result in higher production rates of singlet oxygen. Further research into the nature of reactive species production is needed to assess how optical properties can be used to properly predict formation rates and quantum yields, and in turn, predict contaminant degradation rates.

Understanding the factors that affect DOM reactivity is key to constructing predictive models of micropollutant photolysis in constructed treatment wetlands. Current photochemical models include GCSolar (Zepp and Cline 1977) and APEX (Bohrato and Vione 2014). These models incorporate changes in light conditions based on time of day and geographical location, and use databases of kinetic rate constants between micropollutants and reactive species to calculate degradation rates of specific contaminants. The production rates of reactive species, however, are either estimated or input by the user. These models will be greatly enhanced when they can incorporate the effects of pH and DOM quality on reactive species production.

Improved modeling of photolysis kinetics in wetland systems will allow for the better construction and optimization of treatment wetlands. This research has shown that the most photo-reactive organic matter may be derived from terrestrial sources, and includes high molecular weight, aromatic, oxygen-rich compounds. It is therefore recommended that when

introducing DOM to a treatment system for use as a photo-sensitizer, that terrestrial source material is utilized instead of microbially derived DOM.

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APPENDIX A

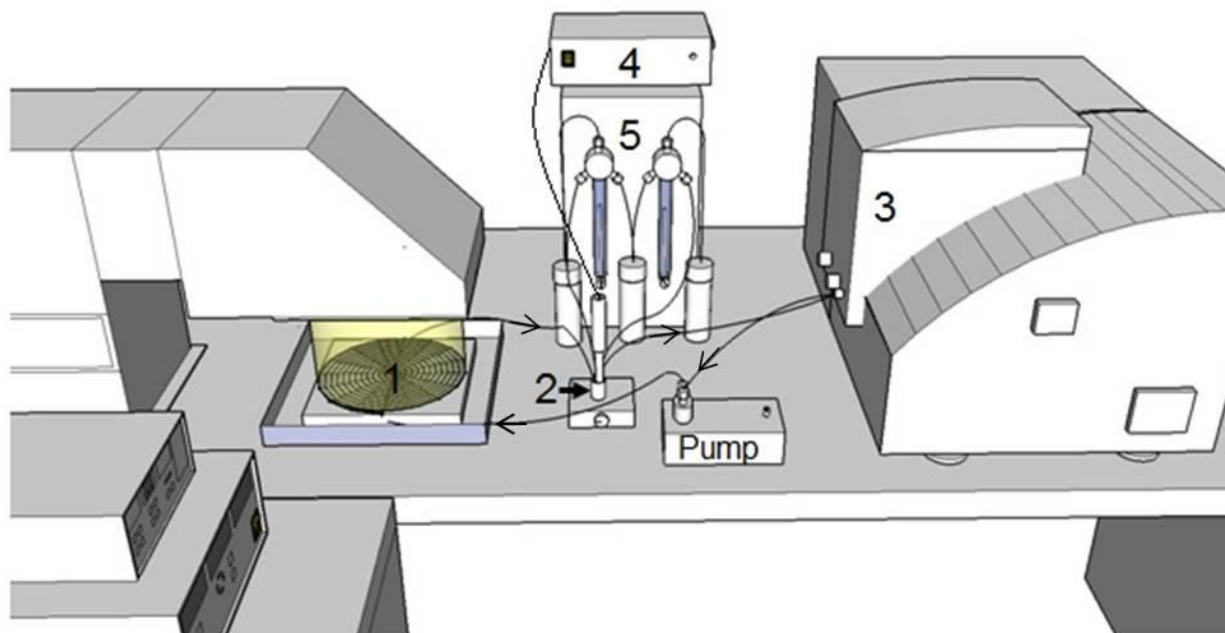


Figure A.1: Experimental layout. Sample is pumped through irradiation cell (1) into equilibrator (2), then drawn through the Horiba Aqualog fluorometer (3). pH is monitored in the equilibrator with the reaction controller (4), and adjusted with sub-microliter injections of acid or base with the dual syringe pump (5).

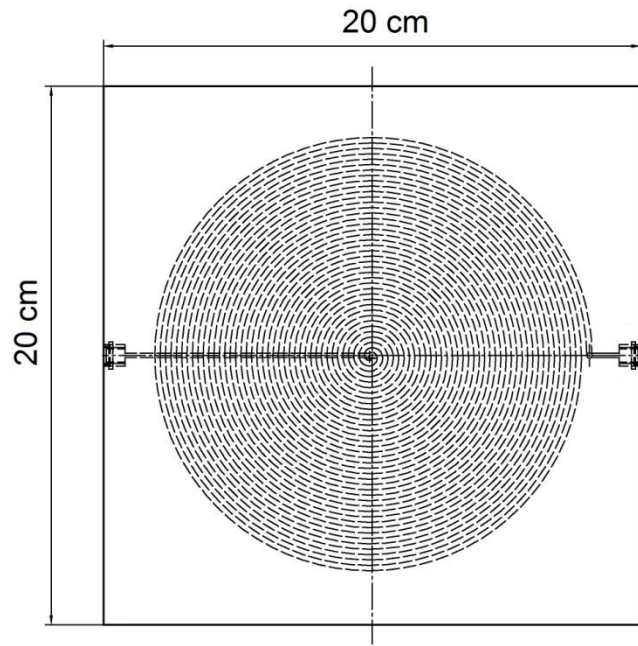


Figure A.2: Irradiation cell design

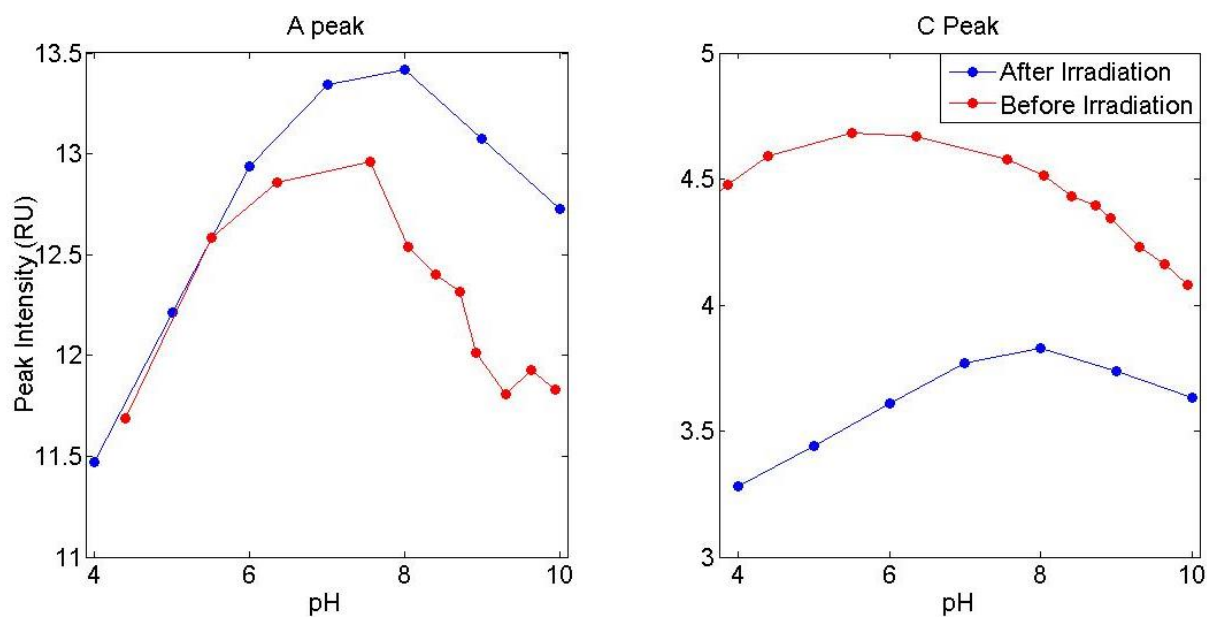


Figure A.3: Influence of pH on A and C peak intensities before and after 24 hour irradiation at pH 4.

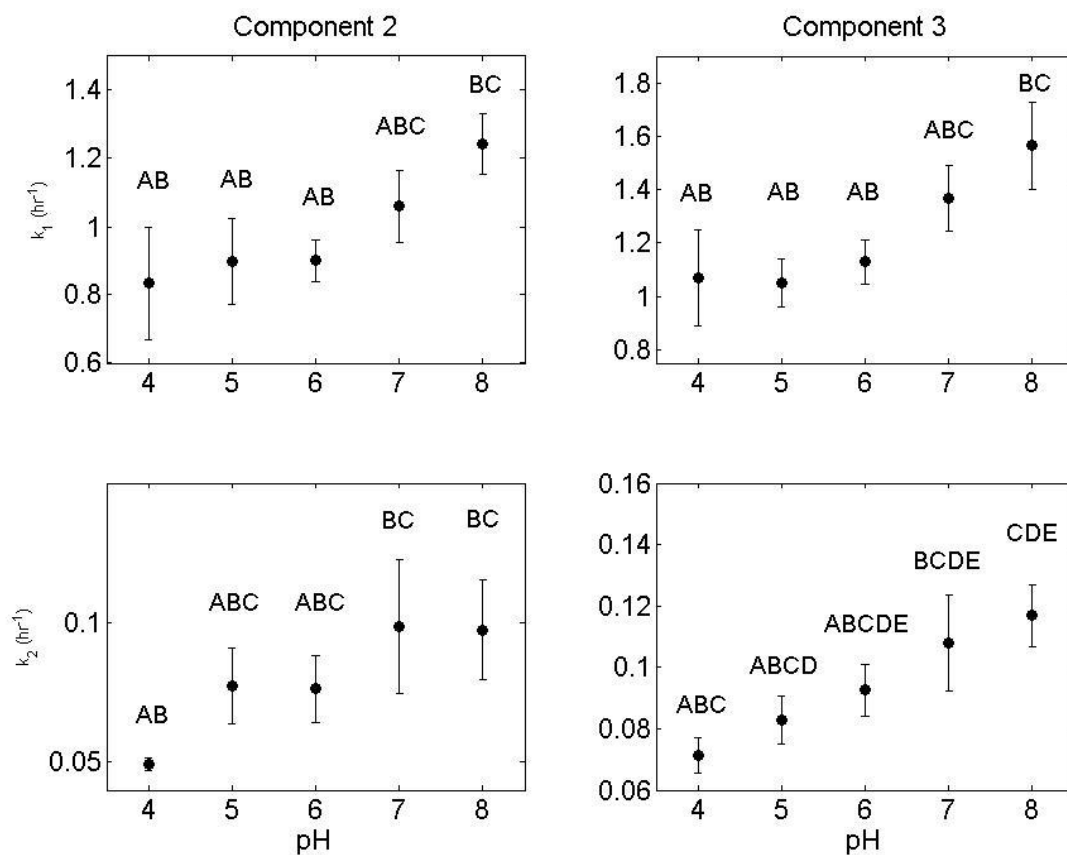


Figure A.4: Rate constants of loss of Components 2 and 3 during 24 hours irradiation. Letters indicate results of Tukey-Kramer test. pH values during irradiation were controlled to ± 0.05 units from the setpoints

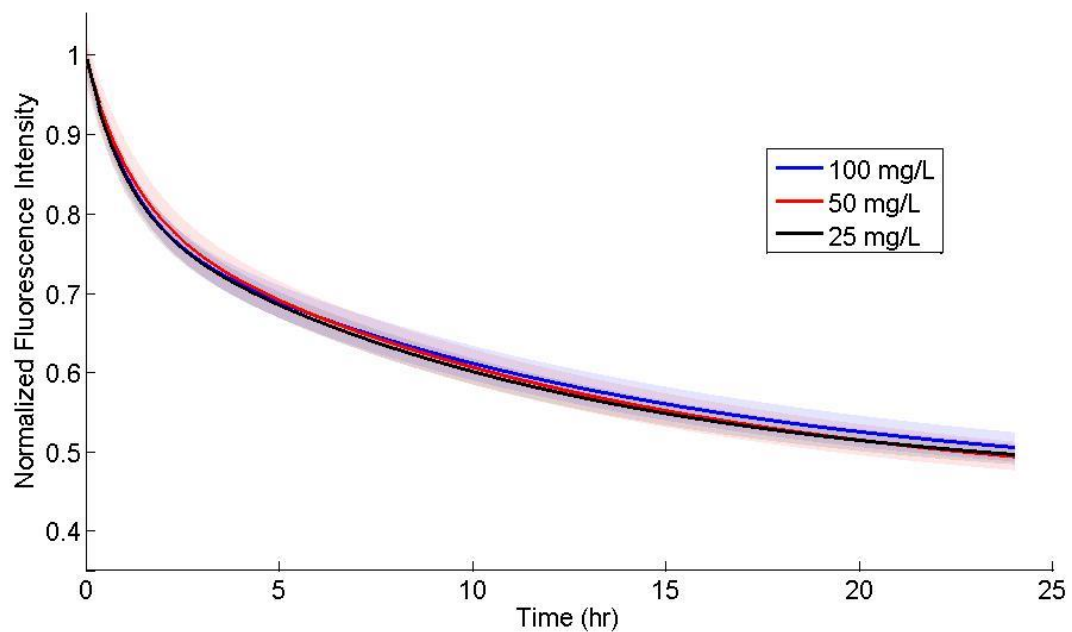


Figure A.5: Loss of C2 at peak maximum at 100, 50, and 25 mg SRNOM L⁻¹. 24 hour irradiations were conducted at pH 6. Solid lines represent average of triplicate experiments, while shaded areas indicate one standard deviation

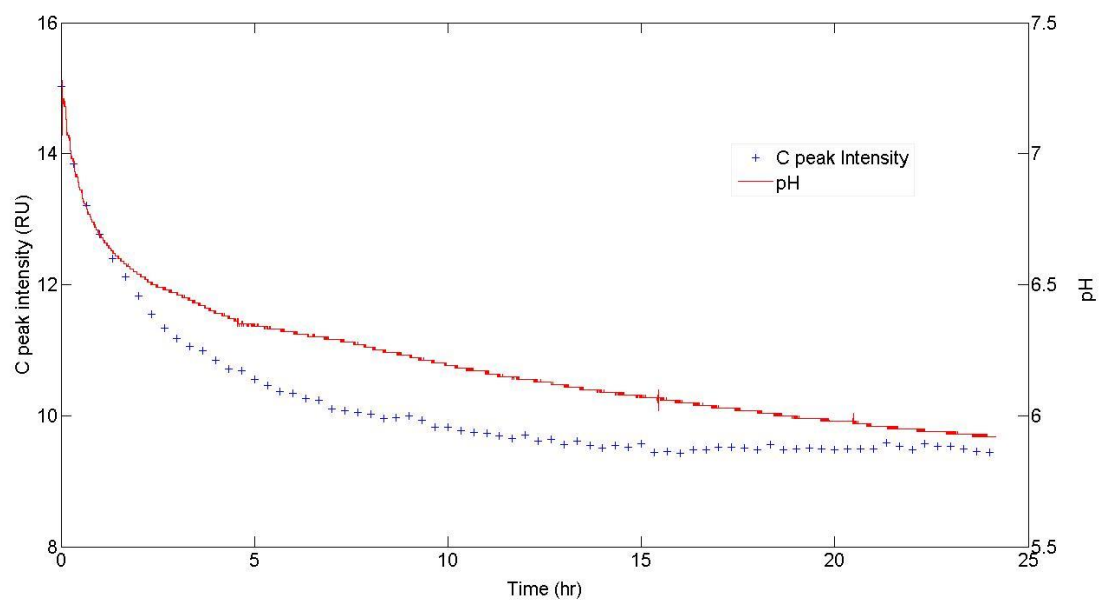


Figure A.6: pH change in irradiation experiment where pH was not controlled (50 mg C L^{-1}).

APPENDIX B

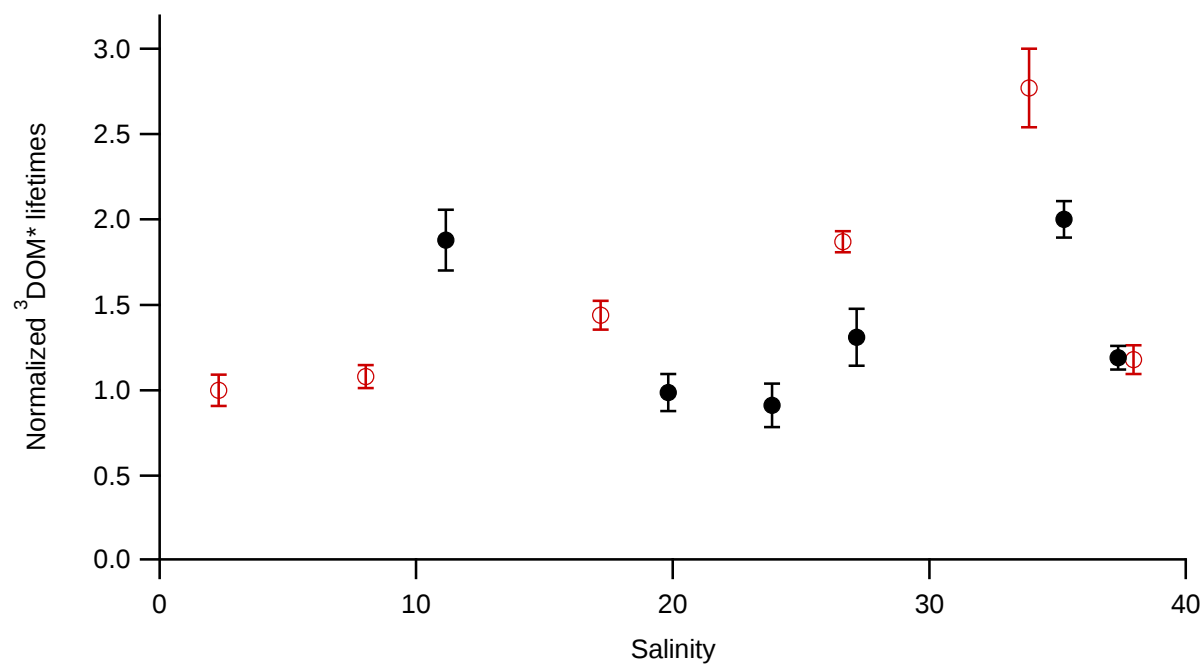


Figure B.1: $^3\text{DOM}^*$ lifetimes in the Harney River (red, open) and Taylor Slough (black, filled) samples normalized to the $^3\text{DOM}^*$ lifetime of the freshwater sample of the Harney River. Error bars are calculated from standard deviations of the regression analysis (Equation 3.7).

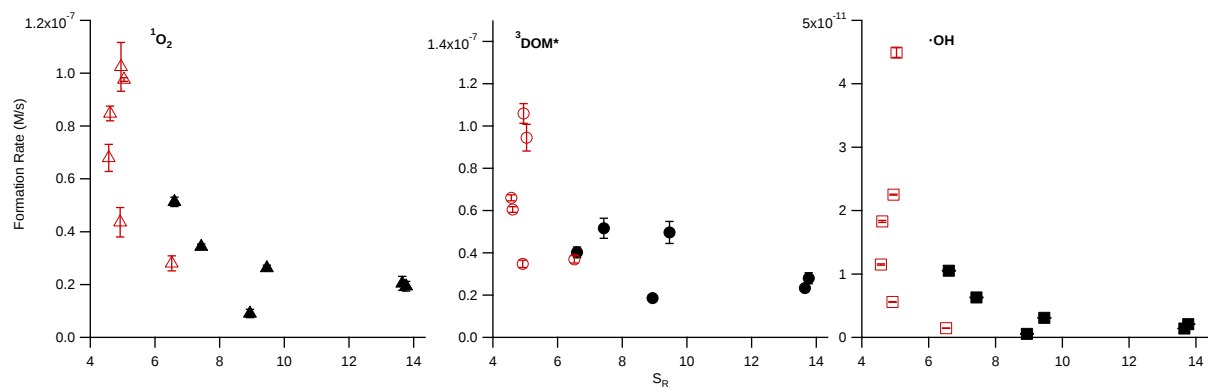


Figure B.2: Formation rates of (a) 1O_2 (triangles) and (b) $^3DOM^*$ (circles), and (c) $\bullet OH$ (squares) in relation to the spectral slope ratio, S_R of the Harney River (red, open) and Taylor Slough (black, filled). Error bars represent standard deviations of triplicate experiments for 1O_2 , $\bullet OH$, and $CO_3^{\bullet -}$. $^3DOM^*$ error bars are calculated from standard deviations of the regression analysis (Equation 3.7).

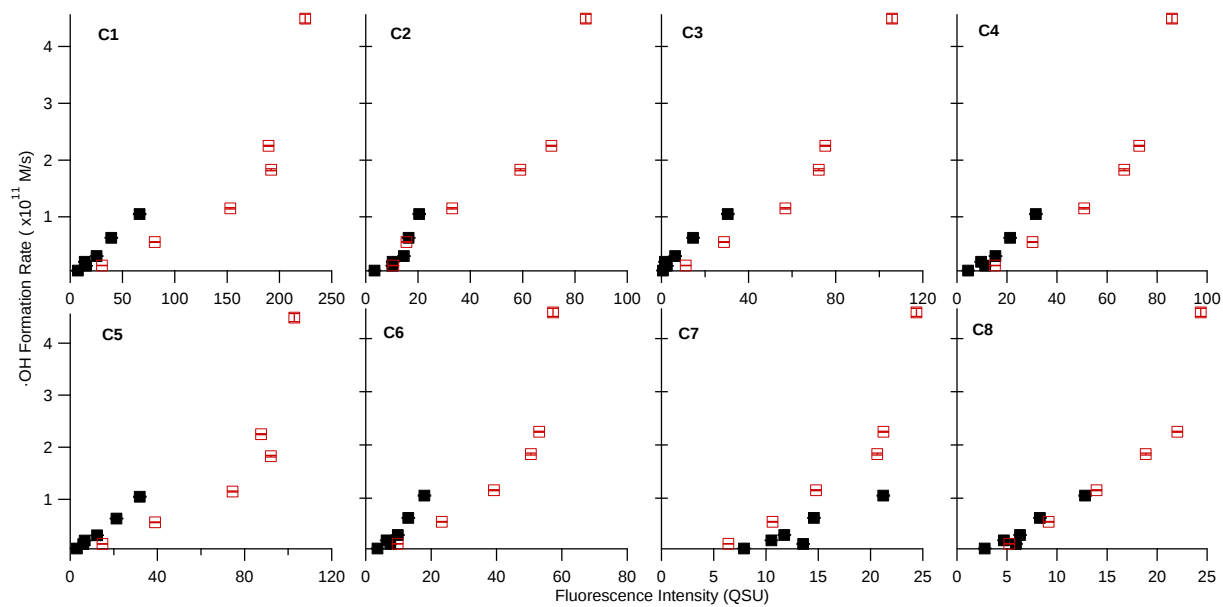


Figure B.3: Formation rates of $\bullet\text{OH}$ in the Harney River (red, open) and Taylor Slough (black, filled) as related to fluorescence intensity (QSU) of the different DOM components as identified by PARAFAC analysis. Error bars represent standard deviations of triplicate experiments.

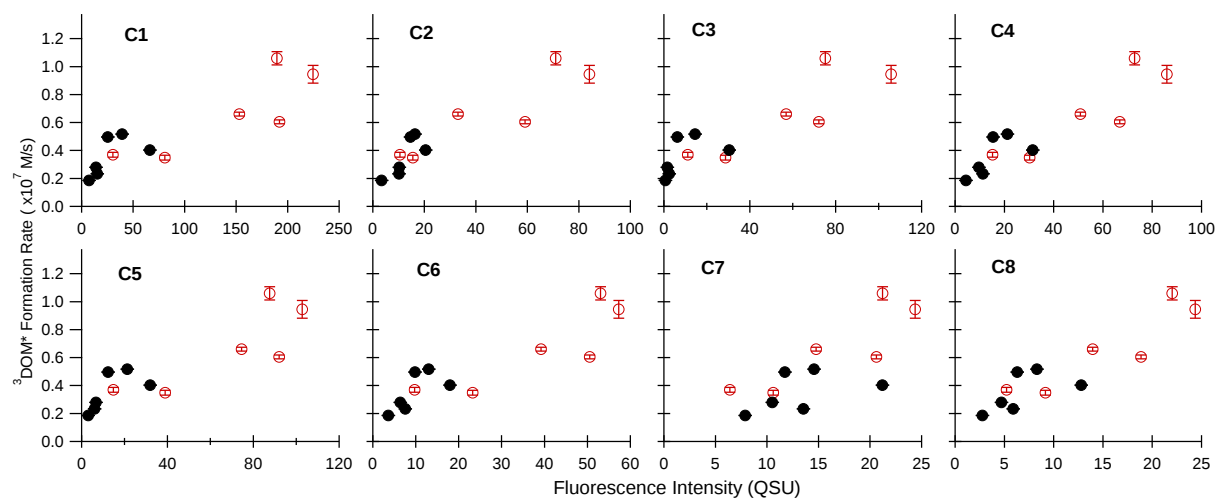


Figure B.4: Formation rates of $^3\text{DOM}^*$ in the Harney River (red, open) and Taylor Slough (black, filled) as related to fluorescence intensity (QSU) of the different DOM components as identified by PARAFAC analysis. Error bars are calculated from standard deviations of the regression analysis (Equation 3.7).

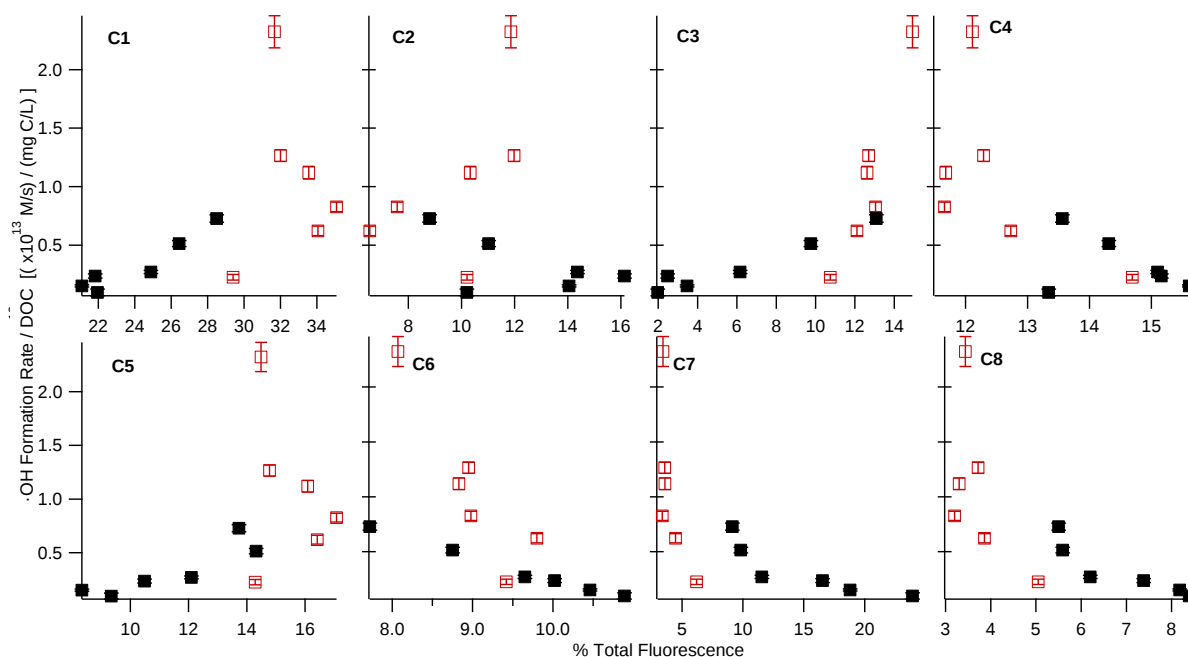


Figure B.5: Normalized formation rates of $\bullet\text{OH}$ in the Harney River (red, open) and Taylor Slough (black, filled) as related to percent total fluorescence intensity of the different DOM components as identified by PARAFAC analysis. Error bars represent standard deviations of triplicate experiments

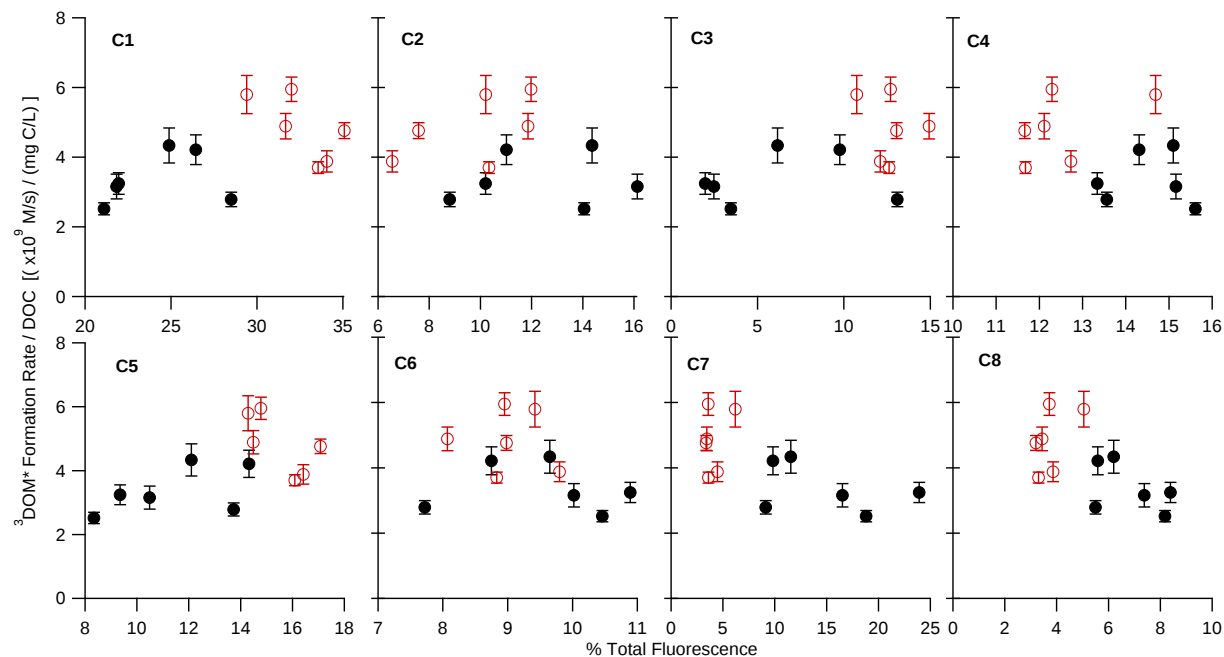


Figure B.6: Normalized formation rates of $^3\text{DOM}^*$ in the Harney River (red, open) and Taylor Slough (black, filled) as related to percent total fluorescence intensity of the different DOM components as identified by PARAFAC analysis. Error bars are calculated from standard deviations of the regression analysis (Equation 3.7).