

CONTRIBUTION OF BACTERIAL CELLS TO THE FLUORESCENCE SPECTRA OF
NATURAL ORGANIC MATTER IN FRESHWATERS

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ABSTRACT

Carrie Blomquist Doyle: Contribution of bacterial cells to the fluorescence spectra of Natural Organic Matter in freshwaters
(Under the direction of Rose M. Cory)

Aquatic Natural Organic Matter (NOM) fuels heterotrophic respiration. The fraction Dissolved Organic Matter (DOM) is operationally defined as material in water samples passing through 0.45 – 0.7 micron filters. Amino acid fluorescence of DOM serves as a proxy for DOM bioavailability. However, bacterial cells, only largely removed by filtration, also demonstrate amino acid fluorescence. The objective of this study was to determine contributions of bacterial cells to amino acid fluorescence in freshwaters. Unfiltered bacterial suspensions demonstrated amino acid fluorescence proportional to bacterial cell concentration; however, 0.22 micron filtration removed most fluorescence. For freshwaters, losses in amino acid fluorescence (up to 60%) with varying pore size filtration largely paralleled losses in particulate material. Using reported retention efficiencies for 0.7 micron filters, bacterial cells in natural waters may account for 5 – 50% of amino acid fluorescence in freshwaters, thereby potentially confounding the interpretation of amino acid fluorescence as a proxy for labile DOM.

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LIST OF ABBREVIATIONS

CDOM	Colored or Chromophoric Dissolved Organic Matter
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EEM	Excitation Emission Matrix
FDOM	Fluorescent Dissolved Organic Matter
NOM	Natural Organic Matter
POM	Particulate Organic Matter
SUVA	Specific Ultra Violet Absorbance
TOC	Total Organic Carbon
UV	Ultra Violet
WCC	White Clay Creek

BACKGROUND AND INTRODUCTION

Soils, sediments, freshwaters, and marine waters contain natural organic matter (NOM) – a complex, heterogeneous mixture of organic compounds exhibiting a broad range of chemical properties. Derived from all living things, this mixture includes as the byproducts of biomolecules: carbohydrates, amino acids and proteins, lipids, lignin, cellulose, and chitin. NOM is a critical component of natural waters, functioning as a control on photic zone depth and as C and energy fueling heterotrophic respiration. Because NOM's roles depends on its size, which spans a continuum from large particulate matter to operationally defined “dissolved” molecules (nm in size), filtration has been widely used to separate and classify fractions of NOM.

In the mid-nineteen hundreds, available glass fiber and silver filters were used to separate NOM into two fractions: Particulate Organic Matter (POM) and Dissolved Organic Matter (DOM); for this reason, an operational definition for DOM based on minimal filter pore sizes of 0.45 micron – 1.0 micron was established (Hedges, 2002). Small bacteria and viruses were known to be part of the dissolved fraction of organic matter; all other living matter was believed to be in the particulate fraction. The dissolved fraction included colloids as well as truly dissolved molecules; the upper limit of 1.0 micron precluded from the dissolved fraction substances prone to sink (Hedges, 2002). Today, the standard classification of dissolved constituents in natural waters is often 0.45 micron; however, analysis of organic matter typically requires preference for a binder-free glass fiber filter, of which 0.7 micron is the smallest

nominal pore size available. Therefore, the 0.45 micron or 0.7 micron filter fraction (Yamashita & Tanoue, 2004; Stedmon et al., 2003) of natural waters is often analyzed to characterize DOM – the complex, heterogeneous fraction of NOM important in the global C cycle due to its role in heterotrophic respiration.

As well as a major participant in the global carbon cycle (Fisher & Likens, 1973; Kling et al., 1991), DOM is an important determinant of water quality. DOM is a source of nutrients for aquatic organisms (Kaplan et al., 2008) and fosters bacterial regrowth in distribution systems (Page et al., 2002). DOM sorbs pollutants and influences their fate (Chin, 2003). Another aspect of water quality is color; as concentrations of dissolved organic carbon (DOC) increase, the more sunscreen there is to shade aquatic organisms from harmful UV sunlight.

Not all DOM (~20-70%, depending on the system) is colored, but the portions rich in aromatic rings and double bonds account for colored DOM (CDOM). CDOM absorbs UV radiation and attenuates light in the water column. Light absorption also can result in photolysis of DOM, altering its chemical composition and causing evolution of carbon dioxide (Moran & Covert, 2003; Coble, 2007). The UV-Vis absorbance spectrum for DOM is an exponentially decaying curve with increasing wavelength; there are no discernible peaks. Nevertheless, DOM can be partially characterized – by source and molecular weight, for example – simply by how it absorbs light. Ratios of the spectral slopes of different sections of the curve are used as proxies of DOM molecular weight and thereby source and diagenesis (Helms et al., 2008). The specific UV absorbance (SUVA, with units $L\ mg\ C^{-1}\ m^{-1}$) of CDOM, which is the absorbance coefficient at 245 nm divided by DOC concentration, is a measure of relative aromaticity of DOM (Weishaar et al., 2003). DOM derived from terrestrial sources is rich in aromatic, high

molecular weight lignin-like compounds; DOM derived from autochthonous sources is rich in low molecular weight amino-acid like compounds (Cory et al., 2007).

Some CDOM emits light, known as fluorescence; while this fluorescent DOM (FDOM) is a small percentage (~1%) of the DOM pool, it is representative of the bulk properties and source of DOM (Mopper & Schultz, 1993; McKnight et al., 2003; Cory et al., 2007). Therefore, CDOM and FDOM are often measured to characterize DOM in natural waters. A three dimensional contour plot of DOM fluorescence – with emission wavelength (nm) on the x-axis, excitation wavelength (nm) on the y-axis, and fluorescence intensity (raman units or RU) on the z-axis – is known as an Excitation-Emission Matrix (EEM). Peaks in different regions of an EEM are associated with different DOM components. For example, both peak A, in the EEM region 260nm/400-460nm (Ex/Em), and peak C, in the EEM region 320-360nm/420-460nm, are associated with fulvic acids. In contrast, peak T, in the EEM region 275nm/340nm is associated with tryptophan and amino acid-like DOM (Coble et al., 1998; McKnight et al., 2001). While fulvic acids are generally associated with more recalcitrant DOM and vegetative sources, amino-acid like material is associated with autochthonous production. The amino-acid rich DOM is considered to be more bioavailable to microorganisms in the water column. (Hudson et al., 2008; Fellman et al., 2009; Cory & Kaplan, 2012) Thus, EEMs of natural water samples are widely analyzed for the abundances of peaks A, C, and T especially to infer the source and lability of DOM.

Studying DOM quality includes a constant challenge in distinguishing the non-living organic matter from intimately associated bacteria and viruses (Minor & Nallathamby, 2004). Bacteria inhabit organic particles as they are also producing organic matter, creating an intricate relationship between OM and bacteria in both freshwater and marine waters. Bacteria degrade

POM to DOM, and simultaneously produce organic material from organic molecules that they consume (Azam et al., 1993; Pomeroy et al., 2007). POM converts to DOM by photolysis; concurrently, DOM converts to POM by mixing and settling, as well as by activity of bacterial cells. There is an arbitrary aspect to the operational definition of DOM in identifying the point at which the complex mixture is truly dissolved; there is also a difficulty in knowing when physically entwined substances have been separated from each other for analysis. Therefore, challenges arise when assuming that a 0.45 micron filter, or often in practice a 0.7 micron filter, separates bacteria from non-living DOM.

This same methodological issue received some attention in the marine science literature in the 1990s. Studying DOM fluorescence in seawater, Determann and fellow researchers expressed the problem of the arbitrary cut-off of 0.45 micron this way: “the so-called dissolved fraction includes bacteria, colloids, and other aggregates in addition to the true dissolved monomers and macromolecules (Determann et al., 1994, p. 659).” In the 1990s, fluorescence was attributed to the dissolved fraction, and because of concern over contamination from the filters themselves, samples analyzed for fluorescence were not always filtered (Determann et al., 1994; Mopper & Schultz, 1993). However, while identifying three fluorophores in near-surface samples of the eastern Atlantic Ocean, Determann et al. (1994) observed that the amino acid-like signals (tyrosine-like, and a more dominant tryptophan-like) were blue-shifted to shorter wavelengths from those of free amino acids, suggesting the amino acids were protein-bound in the particulate phase. This preliminary conclusion was confirmed (Determann et al., 1996) upon analyzing deep-water samples from the eastern Atlantic. The humic-like fluorescence was at a minimum in surface waters, where photo-bleaching was believed to be significant, and at a constant level in deeper waters (~4800m). In contrast, the tryptophan-like (and to a lesser extent,

the tyrosine-like) fluorescence was high in surface waters down to 50 m depth, and low in the deeper waters. It was concluded that the surface waters had greater bacterial biomass, and that the tryptophan was largely bound to bacteria and associated with the particulate fraction. Therefore, the amino acid-like fluorescence attributed to DOM was likely intimately associated with bacterial cells in the particulate phase.

Determann et al. (1998) further investigated the relationship between fluorescence of particulate matter and unfiltered cultures of algae and bacteria. Cultivated bacterial cells were cleaned and counted by epi-fluorescence microscopy. Similarly, the algae species were cultivated, not cleaned due to potential breakage, and then the algal particles were counted. Following these preparations, fluorescence spectra of the bacteria species, and fluorescence spectra of the algae species, were compared to fluorescence spectra of free tryptophan. Fluorescence intensities were standardized to the integrated water Raman scatter band, and for the algae samples spectra had to be corrected for scattering with a barium sulfate suspension. The bacteria fluorescence analysis revealed the following excitation/emission pairs in an average EEM: 230nm/325nm; 225nm/340nm; and, 280nm/340nm. Blue shifting of the emission spectra versus free dissolved tryptophan suggested protein-bound tryptophan, as previously. Then using only the 230 nm/325 nm peak, fluorescence was found to increase linearly with increasing total bacteria number (TBN). This relationship was true for each of the 16 bacteria species (22 cultures), and each species displayed a linear relationship with a unique slope. Similarly, though noting that bacteria might be attached to the phytoplankton, the algae fluorescence showed linear relationships for fluorescence vs. cell number. Based on these results, Determann et al. (1998) attributed amino acid-like UV fluorescence in the ocean photic zone not to DOM but to algae and bacteria.

Also during this time, other researchers considered the impact of inconsistent pore sizes in separating cells in sea water from dissolved materials. Lee et al. (1995) filtered bacterial cells from sea water and determined a large range in retention efficiencies for 0.7 micron glass fiber filters; the amount of cells passing through the filters ranged from 35% to 43% and included cells as large as 0.8 microns in diameter. By determining similar cell-size frequency distributions of both the material trapped by the filters and the filtrate, they discovered that the filters do not have a strict cut-off size. In other words, the glass fiber matrix in these and similar filters do not have consistently shaped pores, and the problem is aggravated by commonly used pre-combustion technique (Lee). Inconsistent pore sizes may contribute to the problem of separating DOM from POM and bacterial cells in natural waters.

Despite these concerns that bacterial cells may not be completely removed during filtration, amino acid-like fluorescence in filtered natural waters has become an accepted proxy for amino acid-like carbon within the DOM pool. In fresh waters (Balcarczyk et al., 2009; Lutz et al., 2012) as well as in marine waters, separating DOM from whole water samples using a 0.7 micron (GF/F) filter is an accepted method for isolating the DOM fraction.

The goal of this study is to investigate the concern that bacterial cells contribute to the peak T fluorescence of DOM in freshwater using two approaches:

- (1) First, the fluorescence properties of unfiltered bacterial cell suspensions were analyzed to confirm the linear relationship between cell concentration and peak T fluorescence for freshwater. Next, it was evaluated whether using 0.22 micron filter removed peak T fluorescence from cell suspensions, or whether residual cells or substantial cell lysis products resulted in significant peak T fluorescence for filtered cell samples.

(2) Secondly, a robust filtering scheme was used to separate freshwater samples into five filter fractions, in order to compare and contrast absorbance and fluorescence characteristics of the whole and filtered waters. The filter scheme covered a range of pore sizes from 3.0 micron to 0.22 micron to identify the changes in absorbance and fluorescence that occur along the particulate to operationally dissolved continuum.

Finally, peak T fluorescence was compared between bacterial cell suspensions and whole or filtered waters. This final step helped to constrain the concerns regarding the contributions of bacterial cell fluorescence to peak T fluorescence attributed to DOM, potentially interfering with this proxy of DOM bioavailability.

METHODS

Preparation of bacterial cell suspensions

Three different isolates of bacterial cell cultures were investigated: *Pseudomonas fluorescens* (P-17; species from the American Type Culture Collection) and two isolates of natural bacterial communities from the sediment of White Clay Creek in southeastern PA (WCC2 and WCC4). The watershed of White Clay Creek is predominantly agricultural lands (74%) with an intact riparian zone forest (23%). This third-order stream previously described in detail (Newbold et al., 1997), is enriched in nutrients from agriculture within the basin, with most N present as 3 to 5 mg nitrate-N L⁻¹ at baseflow conditions. Orthophosphate is present at 10 to 30 µg of orthophosphate-P L⁻¹. Seasonal average baseflow concentrations of DOC range from 1.3 mg C L⁻¹ in the winter to 1.7 mg C L⁻¹ in the summer (Hullar et al., 2006). Baseflow POC concentrations in White Clay Creek range from 200 µg C L⁻¹ to 300 µg C L⁻¹, and the density of cells in the streamwater range from 10⁵ to 10⁶ cells mL⁻¹. Estimated contribution of organic carbon from typical baseflow cell densities is approximately 20 µg C L⁻¹ (Kaplan, unpublished).

The three isolates were streaked on R2A plates to create the inoculum colonies for growing the cultures. The inocula were each grown in 100 mL of culture broth; upon reaching stationary phase, they were then used to inoculate 6 L volumes of broth. The batch cultures were grown for 48 hours at 28° C in a shaking water bath before harvesting by centrifugation. The cultures were harvested by centrifugation using the SLC 1500 head and 200 mL autoclaved

centrifuge bottles, spinning at 6000 RPM for 15 minutes. Each 6 L of culture was processed in 200 mL portions; when all 6 L of culture were processed, the resulting pellets were consolidated into one pellet and washed two times in sterile PBS buffer. After the final wash, the pellet was resuspended in 200 mL of PBS and then fixed with 10 mL of 37% formaldehyde; this suspension was then refrigerated overnight. Each of the three harvested suspensions was then washed several times with Nanopure water to reduce carbon background. The consolidated, resuspended pellet for each isolate was split into four equal volumes, each rinsed with about 150 mL of Nanopure water after centrifugation at 6000 RPM. A total of 15 washes were done, with carbon measurements taken at several points of the process. For each isolate, the carbon level remained constant after the 13th or 14th rinse step. The DOC final readings after the 15th rinse are as follows: 1.05 ppm for P-17; 2.51 ppm for WCC4; 1.95 ppm for WCC2. The pellets were again consolidated and resuspended in 150 mL Nanopure. Each suspension was sub-sampled for cell counts: 2×10^8 cells/mL for P-17; 2×10^{10} cells/mL for WCC2; and 1×10^{10} cells/mL for WCC4. The remaining volume of 147 mL was frozen and freeze-dried; the material from each freeze-dried culture was collected and placed into muffled glass vials with the following weights: 0.4275 g for P-17; 1.0822 g for WCC2; and 0.6864 g for WCC4. Relating cell count values in cells/mL to the volume (mL) and mass (g) of the freeze dried cultures, cell densities were calculated in cells/gram of freeze-dried powder to be 8×10^{10} cells/g, 3×10^{12} cells/g, and 2×10^{12} cells/g for P-17, WCC2, and WCC4, respectively.

Aqueous cell suspensions of each isolate were prepared by dissolving the freeze-dried bacterial powder in 30 mL of laboratory grade DI to yield a stock concentration of $\sim 4 \times 10^7$ cells/mL. Aliquots of each stock solution were used to generate a dilution series of resuspended bacterial cells in DI over a range of cell densities from 3×10^4 cells/mL to 1×10^7 cells/mL to

overlap with cell densities in White Clay Creek (10^5 to 10^6 cells/mL; Kaplan, unpublished), with similarly studied bacteria in marine systems (10^6 to 10^7 cells/mL, Determann, 1998) and freshwaters in general (10^4 to 10^6 cells/mL, Daley and Hobbie, 1975; 10^6 to 10^7 cells/mL, Berman et al., 2001). The pH of filtered and unfiltered cell suspensions ranged from 5.0 to 6.8.

Analysis of bacterial cell suspensions

The dilution series of each bacterial cell suspension was analyzed the same day the stock and dilution samples were prepared. Triplicate fluorescence spectra were collected from two treatments at each concentration of a cell suspension: (1) unfiltered (“as is”) cell suspensions and (2) filtered cell suspensions via 0.22 μm filter (Table 1). Unfiltered samples were gently shaken for 30-60 seconds to make sure cells were suspended in DI water; then, 3 mL aliquots of the sample were pipetted into a quartz cuvette for fluorescence analysis. Following analysis of the unfiltered treatment, the cuvette was rinsed with DI $\sim 20\times$ and prior to adding 3 mL of the filtered treatment.

Fluorescence spectra from all bacterial cell samples were measured as excitation-emission matrices (EEMs) using a Fluorolog-321 (Horiba Scientific) fluorometer equipped with a CCD detector over excitation wavelengths of 240-450 nm (in 5 nm increments) with emission detected from 300-600 nm. Integration times varied with the concentration of bacterial cells and ranged from 0.2 to 5.0 seconds, for a total analysis time of 8.4 seconds to 3.5 minutes per EEM. EEMs were corrected for instrument bias, and intensities were Raman normalized (Cory et al., 2010) using a user-generated rhodamine spectrum for excitation correction (DeRose et al., 2009) and a manufacturer-provided emission correction spectrum (Horiba Scientific). Similarly-analyzed blank EEMs, of laboratory-grade DI water and free of detectable fluorescence emission, were subtracted from sample EEMs to minimize the influence of water Raman peaks.

All EEM-post processing was done in Matlab (Version 7.12). Fluorescence intensities were reported as the mean \pm standard error (SE; N = 3).

Analysis of aquatic NOM

Site descriptions

Surface water was collected from various freshwaters in central North Carolina, USA, selected to cover a range of NOM concentration and source (Table 2).

Eno River – The Eno River is a 3rd order stream in the upper portion of the Neuse River drainage basin. The Eno River travels through mixed agricultural, forested, and urban areas from Cedar Grove to Falls Lake. The Eno River is managed by Orange County to control water availability and use at three small reservoirs, supplying water to Hillsborough Water System, the Orange-Alamance Water System, Piedmont Minerals, Durham Water System, and other smaller operations. The river is classified as normal to moderately-elevated in terms of nutrient status. Surface water was sampled at this stream at Few's Ford near Cox's Mountain in Orange County in October 2012 (Eno River Capacity Use Investigation Management Operations Plan, 1990; Lake and Reservoir Assessments: Neuse River Basin, 2011; Eno River Watershed Surface Water Quality Monitoring Project In and Around Hillsborough, NC, 2011).

Jordan Lake – Jordan Lake, formally known as B. Everett Jordan Reservoir, impounds a 5th order stream in the upper portion of the Cape Fear River drainage basin. Upstream of Jordan Lake are mixed agricultural, forested, and urban areas, including numerous wastewater treatment facilities. Designed originally for flood control, Jordan Lake serves as the primary drinking water source for the towns of Cary, Morrisville, Apex, as well as for Northern Chatham County; Jordan Lake also serves as a recreational site. The lake, with a watershed areas of 1689 mi², is

classified as eutrophic. Surface water was sampled at this lake at Seaforth Recreation Area in Chatham County in November 2012 (Lake and Reservoir Assessments Cape Fear River Basin, 2009; B. Everett Jordan Lake TMDL Watershed Development, 2003).

Haw River – The Haw River is a 5th order stream in the upper portion of the Cape Fear River drainage basin. The Haw River travels through mixed agricultural, forested, and urban areas from north of Burlington to Jordan Lake, receiving runoff and wastewater. The Haw River provides hydropower in Saxapahaw. The river is classified as nutrient-sensitive or impaired. Surface water was sampled at this stream at Saxapahaw in Alamance County, in January 2013 (Lake and Reservoir Assessments Cape Fear River Basin, 2009; B. Everett Jordan Reservoir Phase 1 Nutrient TMDL Final Report, 2007).

Lake Orange – Lake Orange impounds a 2nd order stream in the upper portion of the Neuse River drainage basin. Upstream of Lake Orange are historically agricultural and forested areas, today including a residential community. Designed as a privately managed drinking water source, Lake Orange provides additional drinking water to the town of Hillsborough; it is also a privately accessed recreation site. The lake with a watershed area of 10 mi² is classified as eutrophic. Surface water was sampled at this lake near the earthen dam in Orange County in January 2013 (Lake and Reservoir Assessments: Neuse River Basin, 2011).

Little Contentnea Creek – Little Contentnea Creek is a 3rd order stream in the central portion of the Neuse River drainage basin. Little Contentnea Creek travels through agricultural areas from the north to the south of Farmville, receiving the town's treated wastewater before joining Contentnea Creek. The creek is classified as an impaired swamp. Surface water was sampled at this stream at Ballard's Crossroad in Pitt County in February 2013 (Vähätalo et al.,

2005; Interbasin Transfer Petition: From the Tar River to Contentnea and Neuse River Subbasins, 2009).

Sample collection, storage and analysis

Surface water samples were collected in triplicate in amber HDPE bottles prepared for the field by acid and DI rinses in triplicate; the clean bottles were triple-rinsed with sample water before gathering. Water samples were transported on ice to the laboratory where splits of unfiltered “whole water” were set aside from each of the triplicate bottles and stored at 4 °C until filtration. Splits from each replicate bottle were filtered within 36 hours of collection in the field. There were five filter treatments: 3.0 µm, 1.2 µm, 0.7 µm, 0.45 µm, and 0.22 µm pore-size filters (Table 1). Immediately following sample collection and filtration, sample splits were analyzed for CDOM and FDOM by absorbance and fluorescence spectroscopy, respectively. The total time from sample collection to CDOM and FDOM analysis was at most 72 hours, during which the samples were kept in the dark and near 4 °C until warmed to room temperature (20-25 °C) just prior to analysis.

UV-Vis absorbance spectra of CDOM were collected using 1-cm path length quartz cuvettes on a HP 8452 Diode Array Spectrophotometer or on an Aqualog CDOM fluorometer. Sample absorption was measured against laboratory-grade water blanks. The spectral slope (S_R) ratio was calculated from the absorbance spectrum of each sample following Helms et al. (2008). Napierian absorption coefficients, a_λ , were calculated at 254 and 350 nm as follows:

$$a_\lambda = \frac{A_\lambda}{l} 2.303 \quad (1)$$

where A is the absorbance reading and l is the path length in meters. $SUVA_{254}$ ($L\ mg\ C^{-1}\ m^{-1}$) was calculated following Weishaar et al. (2003) where absorbance readings at 254 nm were divided by the cuvette pathlength (m) and then divided by the DOC concentration ($mg\ C\ L^{-1}$).

Excitation-emission matrices (EEMs) were measured on a Fluorolog-321 (Horiba Scientific) or on an Aqualog fluorometer following the same protocols as described for the Fluorolog-321 fluorometer (Cory et al. 2010). The fluorescence index (FI, McKnight et al., 2001) was calculated from the corrected EEM as the ratio of emission intensity at 470 nm over the emission intensity at 520 nm at an excitation wavelength of 370 nm (Cory et al., 2010). Filter blank EEMs were analyzed using a DI rinse of the syringe, filter housing + filter to identify potential contamination or background fluorescence leaching from the filtration components or sample bottles.

Splits of each treatment filtered were set aside in a borosilicate bottle for total organic carbon (TOC) analysis and subsequently spiked with 100 μ L of 6 N HCl. Splits for TOC were stored at 4 °C until analysis. TOC concentrations were analyzed as non-purgeable organic carbon (NPOC) on a Total Carbon Analyzer (Shimadzu Model 5000). Calibration curves were prepared using potassium hydrogen phthalate in DI water over concentration range of 0.5 – 10 $mg\ C\ L^{-1}$. Blanks (DI water) and check standards were analyzed every 6 and 12 samples, respectively.

RESULTS

Fluorescence characterization of bacterial cell suspensions

Bacterial cell suspensions shared a common peak characterized by maximum excitation and emission wavelengths of 280 nm and 330 nm, respectively (Fig. 1). Fluorescence in this peak region is labeled “peak T”, by convention, based on similar excitation and emission maxima to fluorescent amino acids tyrosine and tryptophan (Coble, 1990; Determann, 1998). Peak T emission intensity increased linearly with increasing cell density in unfiltered cell suspensions (Fig. 2) with slopes significantly different from zero ($p < 0.05$). In addition, P-17 bacterial strain had a significantly higher slope compared to the stream cell suspensions (WCC2 and WCC4, Fig. 2). Stream cell suspensions WCC2 and WCC4 had similar slopes for peak T intensity vs. cell density (no significant difference at $p < 0.05$). When cell suspensions were filtered (0.22 μm), peak T intensities were below the limit of detection for all samples except P-17 at the highest cell density tested (Fig. 2).

Absorbance and fluorescence characterization of NOM in surface freshwaters

All water samples exhibited an UV-Visible absorption spectrum characterized by an exponential decay of absorption coefficients with increasing wavelength. An exponentially decaying absorption spectrum in natural waters is attributed to chromophoric dissolved organic matter (CDOM), the main UV and visible light-absorbing constituent in natural waters (Bricaud et al., 1981; Green and Blough, 1994). CDOM absorption coefficients of unfiltered, whole

water at 305 nm, a_{305} , a measure of the concentration of CDOM, ranged from $24.00 \pm 0.18 \text{ m}^{-1}$ to $50.69 \pm 0.20 \text{ m}^{-1}$, depending on source water. CDOM concentrations were lowest in the Eno and Haw rivers (as indicated by the lowest absorption coefficients, Table 2), followed by the two lakes, while the black water swamp Little Contentnea Creek had the greatest concentration of CDOM (Table 2). The spectral slope ratio, S_R , a proxy for average DOM molecular weight (MW), was lowest for Eno River and Little Contentnea Creek, 0.74 ± 0.01 , and 0.80 ± 0.01 , respectively, indicating higher molecular weight associated with terrestrial sources of DOM. The highest S_R was observed in Jordan Lake (2.18 ± 0.03 , Table 2) suggesting that Jordan Lake DOM had the lowest molecular weight, associated with autochthonous sources of DOM or processing of DOM in the water column (Helms et al. 2008).

In contrast to the bacterial cell suspensions which exhibited fluorescence only in the peak T region, the natural water samples exhibited fluorescence in each of the three characteristic peak regions, T, A, C, common in streams, lakes, and marine waters (Coble, 1990; Stedmon et al., 2003; Cory & McKnight, 2005). Consistent with typical natural water samples, fluorescence intensities of the whole water samples in this study were usually highest for peak A which is associated with terrestrially-derived fulvic and humic acids rich in aromatic carbon (Fig. 3). The exception was Haw River, which had higher fluorescence for peak T than for peak A. Peak T intensities from whole water samples were on average approximately half of the intensities of peak A, and were on average greater than peak C intensities. However, the ratio of peak T to peak A intensity (T/A) varied among the whole water samples (Table 2). The lowest T/A ratios were observed in Little Contentnea Creek (0.139 ± 0.001), and Eno River (0.17 ± 0.01). Higher T/A ratios were observed in the larger, more anthropogenically-impacted waters receiving more

urbanized and agricultural run-off, including the fifth-order stream (Haw River, 1.240 ± 0.005) and the reservoir that impounds it (Jordan Lake, 0.565 ± 0.002).

Effect of filter pore size on NOM absorbance and fluorescence

Filtration of the whole water samples effected a significant reduction in a_{305} and the magnitude of the decrease in a_{305} varied by site and by the pore-size cut-off of the filter. Generally, there was a decrease in a_{305} relative to the whole water with decreasing pore-size. For example, in Eno River and Little Contentnea Creek, filtration of the whole water decreased a_{305} by 5-8% for the 3.0 μm filter and up to 44% for the 0.22 μm (Fig. 4). Filtration of Haw River, Jordan Lake and Lake Orange whole waters through 3.0 μm filter caused a greater decrease in a_{305} compared to Eno River and Little Contentnea Creek; however, unlike the Eno and Little Contentnea Creek, there was not always a successive decrease in a_{305} with decreasing pore size (Fig. 4). For example, both the Haw River and Lake Orange filtration through 3.0 μm resulted in greater loss a_{305} compared to filtration through the 1.2 μm . Jordan Lake differed from the other waters in that it exhibited the greatest loss of a_{305} upon filtration ($\sim 60\%$ decrease in a_{305}), and loss of a_{305} was nearly the same (losses of 58% to 64%) for all filters, and thus independent of pore-size cut off (Fig. 4).

Filtering whole water through different pore size filters decreased or did not change the spectral slope (S_R) of CDOM depending on the whole water and the pore-size cut-off of the filter (Fig. 4). For example, the greatest decrease in S_R with filtration was observed for Jordan Lake, which exhibited 35-52% decrease in S_R upon filtration of the whole water, with the 0.45 μm filter yielding the largest percent difference relative to the whole water. Filtration led to a smaller decrease in S_R (8-15%) for Eno River, Haw River, and Lake Orange; consistent with Jordan Lake, the largest decrease in S_R for these waters was observed by filtering the whole

water through the smallest pore size filters (0.45 μm or 0.22 μm). In contrast to the other whole waters, there was no significant change in the S_R of Little Contentnea Creek upon filtration of the whole water through any pore size (Fig. 4).

Filtering whole water samples caused a decrease in fluorescence intensity, with the magnitude of the decrease varying by pore size and by peak (A, C, T) and thus by carbon type. Humic peaks A and C exhibited smaller decreases in intensity (up to 20%) in response to filtration, compared to peak T intensities which decreased up to 60% (Fig. 4). For example, all whole waters except Jordan Lake had less than 10% decrease in peak A intensity and less than 15% decrease in peak C intensity upon filtration through any pore size. Jordan Lake was different from the other whole waters in that it exhibited up to 20% decrease in both peaks A and C intensities upon filtration. Filtration of the whole water from Jordan Lake exhibited the largest change in peak T intensity (50-60%), with the greatest loss observed between the whole water and the 0.45 μm filtrate (Fig. 4). Filtration of the other whole waters led to smaller differences in peak T intensities (4-53%), and like Jordan Lake, the largest difference was observed between the whole water and the smallest pore size (0.45 μm or 0.22 μm).

DISCUSSION

Peak T fluorescence of filtered natural waters has become a widely accepted proxy for amino acid-like carbon within the DOM pool, and for the labile carbon fraction of DOM fueling bacterial respiration (Yamashita & Tanoue, 2003; Stedmon et al., 2003; Cory & McKnight, 2005; Fellman et al., 2009; Balcarczyk et al., 2009; Cory & Kaplan, 2012). However, because bacterial cells may exhibit fluorescence in the peak T region (Determann 1998), an increase in peak T intensity may indicate increased bacterial abundance (Mladenov et al., 2011), thereby confounding the use of peak T as a marker for DOM substrates that feed bacterial respiration. The results confirmed that bacterial cells fluoresce in the peak T region (Fig. 1) in proportion to their abundance (Fig. 2), while the effects of filtration on cell suspensions and natural waters constrained the conditions in which bacterial cells may contribute to peak T fluorescence in aquatic ecosystems.

Peak T fluorescence intensity increased linearly with cell concentration in all unfiltered cell suspensions (Fig. 2), consistent with previous relationships between peak T fluorescence and bacterial cells in marine waters (Determann et al., 1998). In addition to bacterial cell concentration, bacterial cell type also affected levels of peak T fluorescence. *Pseudomonas fluorescens* cells emitted more peak T fluorescence than did native freshwater WCC2 and WCC4 cells, as indicated by the significantly higher slope of peak T intensity with increasing cell concentration for *P. fluorescens* compared to the WCC2 and WCC4 cells (Fig.2). Determann et al. (1998) also demonstrated that the slope of peak T fluorescence vs. cell abundance varied by

bacterial species; similarly, Leblanc and Dufour (2002) were able to identify bacteria at the genus, species, and strain level on the basis of cellular fluorescence, for which tryptophan residues were the primary contributors. Together, these studies confirm that while bacterial cells consistently fluoresce in the peak T region in proportion to their abundance, the nature of this relationship will depend strongly on the community of cells present.

The results also demonstrated that bacterial cells may contribute to peak T fluorescence in unfiltered natural waters. Peak T intensities of unfiltered cell suspensions ranged from detection limit (~ 0.005 RU) to ~ 1 RU, overlapping the range of peak T intensities in natural waters (Fig. 2; Table 2). Because the range of bacterial cell densities tested spanned the range of cell densities observed in freshwaters (Kaplan, unpublished; Daley and Hobbie, 1975; Berman et al., 2001), bacterial cells could account for peak T intensities observed in the unfiltered water samples in this study (Table 2; Fig. 3). These findings are consistent with a recent study demonstrating a linear relationship between bacterial cell abundance and total fluorescence measured in alpine lakes (Mladenov et al., 2011).

However, standard procedure for analysis of DOM in most studies is filtration of whole water samples, and thus the objective was to constrain the likelihood of bacterial cell contribution to operationally defined fluorescent DOM (FDOM). A $0.22 \mu\text{m}$ filter reduced peak T intensities from cell suspensions to below detection limit for all cells tested at all abundances except for *P. fluorescens* at the highest cell abundance (10^7 cells mL^{-1} , Fig. 2). Given that *P. fluorescens* was more fluorescent than other native bacterial cell communities in natural waters (Fig. 2 and Determann et al., 1998), these results suggest that bacterial cells likely cannot account for the peak T intensities observed in $0.22 \mu\text{m}$ filtered waters (Fig. 3). However, over the range of cell abundances tested, using a filter of larger pore sizes may have allowed more

bacterial cells, and associated peak T intensity, to remain in the filtrate. For example, Lee et al. (1995) found that 0.7 μm (e.g. GF/F) filtration of seawater removed 57-65% of the bacterial cells present in the whole water. The range in retention efficiencies was attributed to the irregularly shaped and sized pores of the glass fiber matrices, a problem aggravated by the commonly used pre-combustion technique intended to eliminate background concentrations (Lee et al., 1995).

Although we didn't test the effect of 0.45 or 0.7 μm (GF/F) filtration on retention of bacterial cells in this study, we estimated the contribution of bacterial cells to GF/F-filtered water based on the relationship between peak T intensity and cell abundance in Fig. 2. Assuming that up to 40% of natural cell communities from White Clay Creek cells can pass through GF/F pores based on the findings of Lee et al. (1995) with no change in fluorescence per cell, peak T intensity would be decreased by 60% compared to the unfiltered cell suspensions. In that case, peak T intensities would be above the detection limit for cell abundances greater than 10^6 cells mL^{-1} and range from 0.007 RU to 0.1 RU (Fig. 2). This range is on the low end of peak T intensities observed from filtered waters in this study and in other studies (Murphy et al., 2010; Cory & Kaplan, 2012), but nonetheless suggested that at least for the natural waters tested in this study, incomplete removal of bacterial cells during filtration could account for 5-50% of peak T intensities in GF/F filtered samples.

Another application of the results suggested that bacterial cells may contribute peak T fluorescence to natural waters disproportionately to their carbon concentration. Assuming a carbon content of 20 fg per bacterial cell (Troussellier et al., 1997), the estimated concentration of carbon in cell suspensions in this study ranged from 0.5 to 200 $\mu\text{g C L}^{-1}$, much lower than the DOC concentration in freshwaters in general and in this study ($\sim 5 \text{ mg C L}^{-1}$, Table 2). Despite a much lower concentration of C, bacterial cells exhibited fluorescence in the peak T region

spanning the same magnitude as natural water samples (Table 2, Fig. 2), indicating that the efficiency of fluorescence is higher for peak T emission from bacterial cells compared to the amino acid-like moieties in natural waters. Thus, while POM, including bacterial cells, may contribute only a small fraction of the carbon concentration relative to DOM in a GF/F filtered sample (< 10%, Meybeck, 1982), POM may nevertheless contribute a disproportionate amount of the measured peak T fluorescence signal. Therefore, while incomplete removal of cells during filtration does not influence the DOC concentration of a filtered water sample, incomplete filtration of cells may influence the fluorescence characterization of the operationally defined “DOM” pool. In this way, the peak T contribution of bacterial cells in natural waters would be greater on a per mass C basis than the corresponding contribution from DOM.

Further evidence in support of bacterial cell contribution to peak T intensities in natural waters was that the effect of filtration was largest on peak T intensities compared to peaks A and C (Fig. 4). That peak T decreased by 10-60% upon filtration of the whole water, depending on the pore size and water sample tested, strongly suggests a large fraction of amino acid-like FDOM associated with particulate organic matter. A large loss of amino acid-like fluorescence intensity from freshwaters has previously been observed upon removal of particulate matter larger than 1.2 and 0.45 μm (Baker et al., 2007). These findings are consistent with recent work showing that extracts of particulate organic matter along a riverine to estuary transect were enriched in peak T fluorescence compared to the corresponding water sample (Osburn et al., 2012). The latter study attributed amino acid-like FDOM in particulate organic matter to autochthonous production in the water column.

The variability in the removal of peak T intensity by filtration with pore size and natural water was likely due to differences in the chemical composition and size of the particulate

organic matter in the whole water sample. The freshwaters characterized in this study included third to fifth order streams, a small lake impounding a second order stream, and a large lake impounding a fifth order stream. All these waters had inputs from forested, agricultural, and residential catchments, with the largest order stream (Haw River) and the larger impounded lake (Jordan Lake) receiving more industrial and wastewater inputs (Table 2). As expected, optical proxies indicated a range of size and source of the CDOM and FDOM in the GF/F fraction – the fraction for which these proxies have been developed (e.g. McKnight et al., 2001; Helms et al., 2008). For example, the $SUVA_{254}$ ranged from $4.33 \pm 0.05 \text{ L mg C}^{-1} \text{ m}^{-1}$ in Little Contentnea Creek to $1.22 \pm 0.08 \text{ L mg C}^{-1} \text{ m}^{-1}$ in Jordan Lake, suggesting a range of sources to the DOM pool, from terrestrial soil organic matter enriched in aromatic C in Little Contentnea Creek to a predominance of autochthonous organic matter in Jordan Lake (McKnight et al., 2001; Jaffé et al. 2008). There was relatively small effect of filtration on peak T for Little Contentnea Creek (~30%, Fig. 4) compared to eutrophic Jordan Lake (~60%, Fig. 4). This difference was expected given that the sources of larger particulate matter enriched in peak T fluorescence – such as bacterial cells (this study, Determann, 1998; Cotner et al., 2004; Mladenov, 2011) or the degradation products from autochthonous production (Osburn et al., 2012) – are relatively low in a blackwater swamp compared to a eutrophic lake (Vähätalo et al., 2005). Surprisingly, the effect of filtration on peak T intensity was lowest in the Haw River, a river augmented by wastewater inputs (Table 2), which may be expected to result in proportionally greater fraction of particulate organic matter enriched in peak T fluorescence. However, given that each site was sampled only once and on different dates (Table 2), it was not possible to generalize how the effect of filtration on peak T fluorescence may vary across water types, except to hypothesize

that waters with high autochthonous production relative to terrestrial inputs from the catchment may likely exhibit high peak T fluorescence associated with larger particulate material.

In comparison to the effect of filtration on peak T fluorescence, the much smaller effect of filtration on peak A and C intensities confirmed that even in unfiltered waters, the majority of fluorescence associated with peak A and peak C is attributed to operationally defined dissolved humic and fulvic acids (Yoshioka et al., 2007). All waters tested except for Jordan Lake reduced peak A and C intensities by less than 10% or 15%, respectively (Fig. 4) consistent with a previous study showing no significant change in humic fluorescence observed upon filtration (Baker et al., 2007). The higher loss of peak A and peak C intensities (~20%, Fig. 4) in Jordan Lake was likely due to the eutrophic status of Jordan Lake, resulting in elevated bacterial abundances or particulates associated with the degrading autochthonous organic matter. While Lake Orange was also eutrophic, its smaller (three orders of magnitude) size relative to Jordan Lake may mean that Orange Lake is more strongly influenced by inputs of soil organic matter relative to Jordan Lake, and thus the organic matter signature was not as strongly dominated by autochthonous organic matter.

CONCLUSIONS

Bacterial cells exhibit peak T fluorescence in proportion to their abundance. For my “native” freshwater cells, filtration by 0.22 μm filters removed virtually all peak T fluorescence; however, caution is warranted given that the relationship between cells and peak T fluorescence varies by community. Also, for my natural water samples, filtration in general reduced peak T fluorescence more than peaks A or C, consistent with the fact that peak T fluorescence is associated with POM in natural waters. POM includes both cells and degraded OM, so for studies proposing in-situ monitoring of FDOM without pre-filtration, consideration should be given to the possibility that peak T could be dominated by cells. For studies relying on the traditional GF/F filter to remove particulates from DOM, peak T fluorescence may be significantly influenced by bacterial cells. My results suggest that the peak T fluorescence of filtered natural waters may not always be an accurate measure of amino acid-like DOM, depending on bacterial cell abundance and type, filter pore size and on the natural water source.

TABLES AND FIGURES

Table 1: Characteristics of filters used.

Pore size and name	Manufacturer	Diameter	Material
3.0 μm MF	Millipore Corp	47 mm	mixed cellulose esters
1.2 μm GF/C	Whatman	47 mm	glass microfiber - combusted
0.7 μm GF/F	Whatman	25 mm	glass microfiber - combusted
0.45 μm GDX	Whatman	13 mm	glass microfiber with membrane layer
0.22 μm Millex GV	Millipore Corp	13 mm	hydrophilic PVDF

Table 2: Characteristics of natural waters sampled (mean \pm SE, N=3).

	Eno River	Jordan Lake	Haw River	Lake Orange	Little Contentnea Creek
Date	October 14, 2012	November 3, 2012	January 11, 2013	January 25, 2013	February 2, 2013
Water Type	stream	lake	stream	lake	Blackwater stream or swamp
Nutrient Status	Normal to moderately elevated	eutrophic	Nutrient sensitive to impaired	eutrophic	Impaired
pH whole water	7.67 \pm 0.07	7.72 \pm 0.09	7.29 \pm 0.21	7.19 \pm 0.20	7.17 \pm 0.05
TOC (mgC L⁻¹)					
3.0 μ m	3.85 \pm 0.02	5.48 \pm 0.02	4.79 \pm 0.01	6.30 \pm 0.04	8.07 \pm 0.09
a₃₀₅ (m⁻¹)					
<i>whole water</i>	24.00 \pm 0.18	32.68 \pm 0.78	27.47 \pm 0.13	37.11 \pm 0.17	50.69 \pm 0.20
0.7 μ m	20.42 \pm 0.28	13.75 \pm 1.09	20.81 \pm 0.08	26.59 \pm 0.52	43.84 \pm 0.30
0.22 μ m	13.49 \pm 1.17	12.02 \pm 0.15	14.30 \pm 0.91	16.77 \pm 0.12	31.09 \pm 0.29
S_R					
<i>whole water</i>	0.74 \pm 0.01	2.18 \pm 0.03	0.94 \pm 0.02	1.20 \pm 0.02	0.80 \pm 0.01
0.7 μ m	0.65 \pm 0.02	1.40 \pm 0.21	0.838 \pm 0.003	1.035 \pm 0.005	0.789 \pm 0.001
0.22 μ m	0.64 \pm 0.03	1.16 \pm 0.06	0.88 \pm 0.01	1.00 \pm 0.01	0.818 \pm 0.002
FI					
<i>whole water</i>	1.64 \pm 0.01	1.517 \pm 0.004	1.81 \pm 0.01	1.55 \pm 0.01	1.487 \pm 0.004
0.7 μ m	1.58 \pm 0.02	1.52 \pm 0.02	1.800 \pm 0.004	1.54 \pm 0.01	1.48 \pm 0.01
0.22 μ m	1.52 \pm 0.01	1.51 \pm 0.01	1.78 \pm 0.01	1.53 \pm 0.01	1.49 \pm 0.01
peak A (RU)					
<i>whole water</i>	1.28 \pm 0.03	1.47 \pm 0.02	1.60 \pm 0.01	1.37 \pm 0.01	2.57 \pm 0.02
0.7 μ m	1.22 \pm 0.01	1.22 \pm 0.03	1.57 \pm 0.01	1.315 \pm 0.005	2.49 \pm 0.02
0.22 μ m	1.17 \pm 0.01	1.20 \pm 0.04	1.55 \pm 0.02	1.290 \pm 0.005	2.56 \pm 0.01
peak C (RU)					
<i>whole water</i>	0.468 \pm 0.003	0.399 \pm 0.002	0.698 \pm 0.002	0.564 \pm 0.001	1.187 \pm 0.003
0.7 μ m	0.448 \pm 0.003	0.34 \pm 0.01	0.689 \pm 0.001	0.540 \pm 0.001	1.164 \pm 0.003
0.22 μ m	0.426 \pm 0.003	0.324 \pm 0.004	0.674 \pm 0.003	0.537 \pm 0.004	1.178 \pm 0.003
peak T (RU)					
<i>whole water</i>	0.231 \pm 0.004	0.86 \pm 0.01	2.00 \pm 0.01	0.49 \pm 0.01	0.38 \pm 0.02
0.7 μ m	0.20 \pm 0.01	0.42 \pm 0.04	1.893 \pm 0.002	0.307 \pm 0.004	0.33 \pm 0.01
0.22 μ m	0.15 \pm 0.01	0.36 \pm 0.01	1.83 \pm 0.01	0.23 \pm 0.01	0.272 \pm 0.003
T/A whole water	0.17 \pm 0.01	0.565 \pm 0.002	1.240 \pm 0.005	0.36 \pm 0.01	0.139 \pm 0.001

Table 3: TOC and SUVA averages (mean \pm SE, N = 3) for the five filter treatments.

TOC averages (mg C L⁻¹)					
Treatment	Eno R	Jordan L	Haw R	Lake Orange	Little Cont. Cr
3.0 micron	3.89 \pm 0.02	5.46 \pm 0.04	4.79 \pm 0.01	6.38 \pm 0.08	8.20 \pm 0.03
1.2 micron	3.90 \pm 0.02	5.50 \pm 0.04	4.81 \pm 0.03	6.30 \pm 0.02	8.18 \pm 0.03
0.7 micron	3.81 \pm 0.02	5.42 \pm 0.03	4.76 \pm 0.01	6.39 \pm 0.03	8.14 \pm 0.06
0.45 micron	3.86 \pm 0.05	5.50 \pm 0.02	4.80 \pm 0.02	6.28 \pm 0.01	8.06 \pm 0.05
0.2 micron	3.81 \pm 0.05	5.54 \pm 0.03	4.76 \pm 0.04	6.17 \pm 0.05	7.74 \pm 0.04

SUVA averages (L mg C⁻¹m⁻¹)					
Treatment	Eno R	Jordan L	Haw R	Lake Orange	Little Cont. Cr
3.0 micron	2.636 \pm 0.005	1.22 \pm 0.02	3.56 \pm 0.05	3.59 \pm 0.08	4.58 \pm 0.03
1.2 micron	2.55 \pm 0.03	1.174 \pm 0.002	3.67 \pm 0.06	3.85 \pm 0.03	4.56 \pm 0.03
0.7 micron	2.49 \pm 0.03	1.22 \pm 0.08	3.53 \pm 0.02	3.49 \pm 0.06	4.33 \pm 0.05
0.45 micron	2.18 \pm 0.09	1.05 \pm 0.01	3.13 \pm 0.06	3.19 \pm 0.08	3.98 \pm 0.10
0.2 micron	1.68 \pm 0.13	1.06 \pm 0.01	2.72 \pm 0.13	2.66 \pm 0.04	3.52 \pm 0.02

Table 4. Relationships Between Peaks A, C, and T with a305.

Regression coefficients (R^2 for linear relationships where the slope was significantly different than zero ($p > 0.05$)).

	Peak A	Peak C	Peak T
Eno R	<i>NS</i>	0.9	1
Jordan L	1	1	1
Haw R	<i>NS</i>	<i>NS</i>	0.7
L Orange	0.8	0.8	1
Little Cont. Cr	<i>NS</i>	<i>NS</i>	0.9

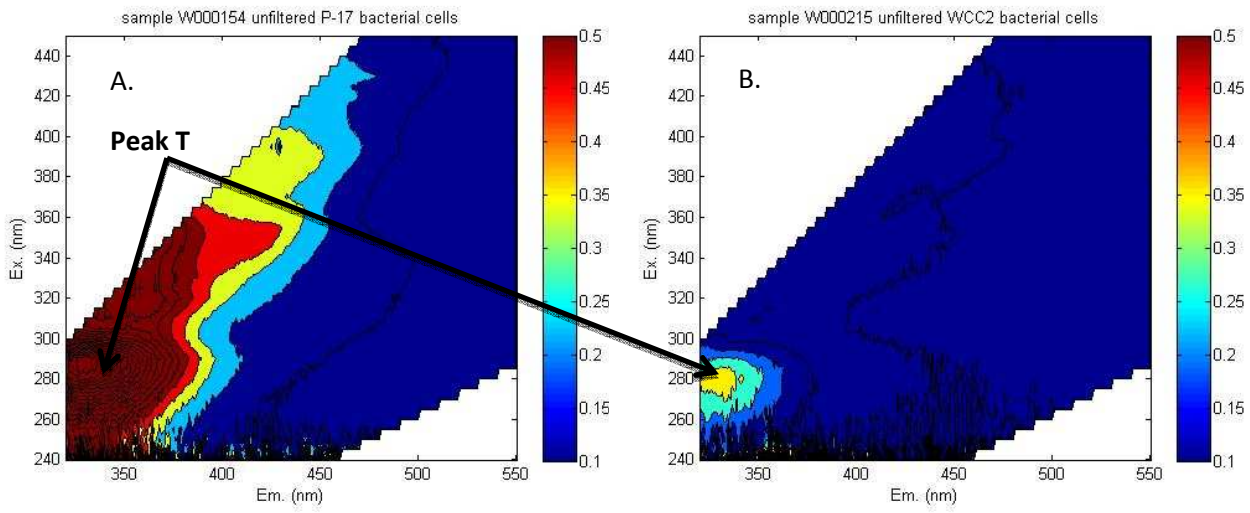


Fig. 1 Characteristic EEMs of bacterial cell suspensions (a) P-17 and (b) WCC2.

Bacterial cell suspensions shared a common peak characterized by maximum excitation and emission wavelength of 280 nm and 330 nm, respectively.

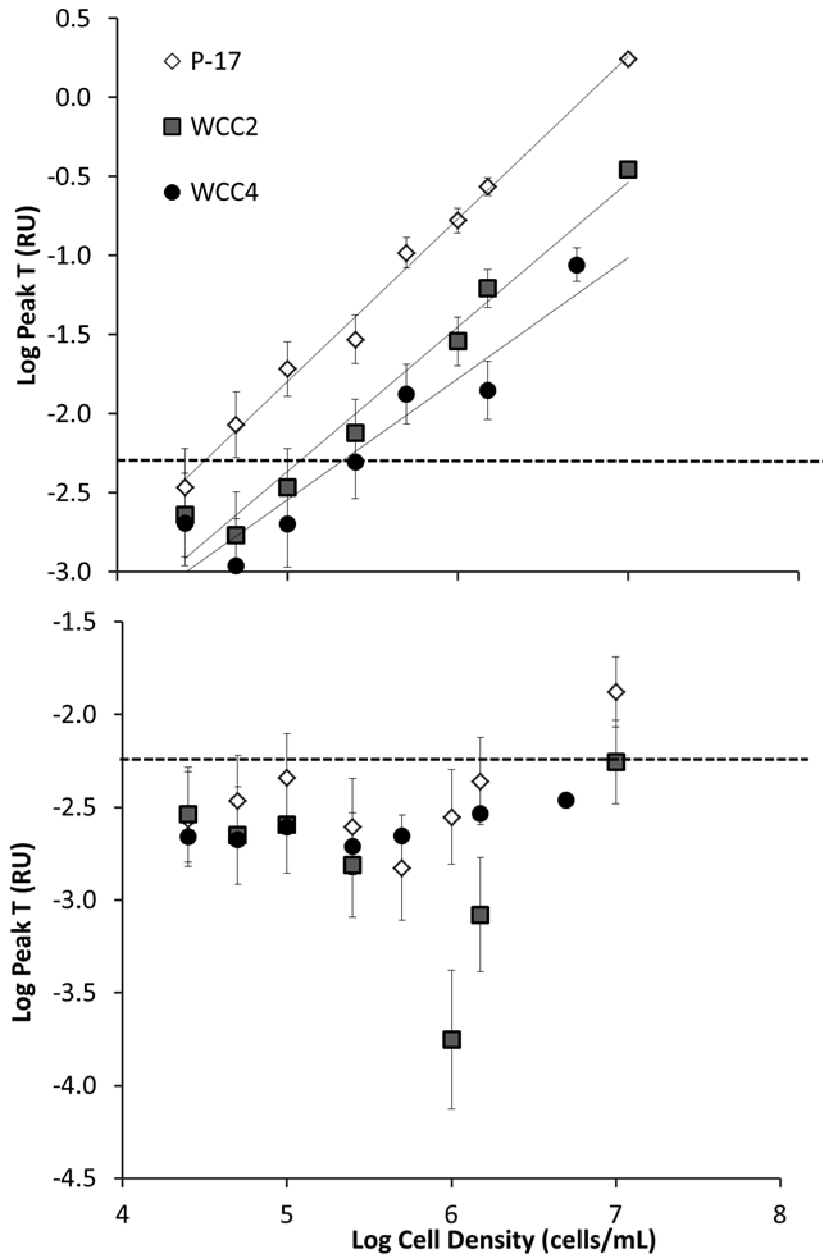


Fig. 2 Peak T intensity (RU) vs. cell density (cells/mL) in Log-Log scales for: (top) unfiltered bacterial cell suspensions; and (bottom) filtered cell suspensions. Dotted line marks the limit of detection.

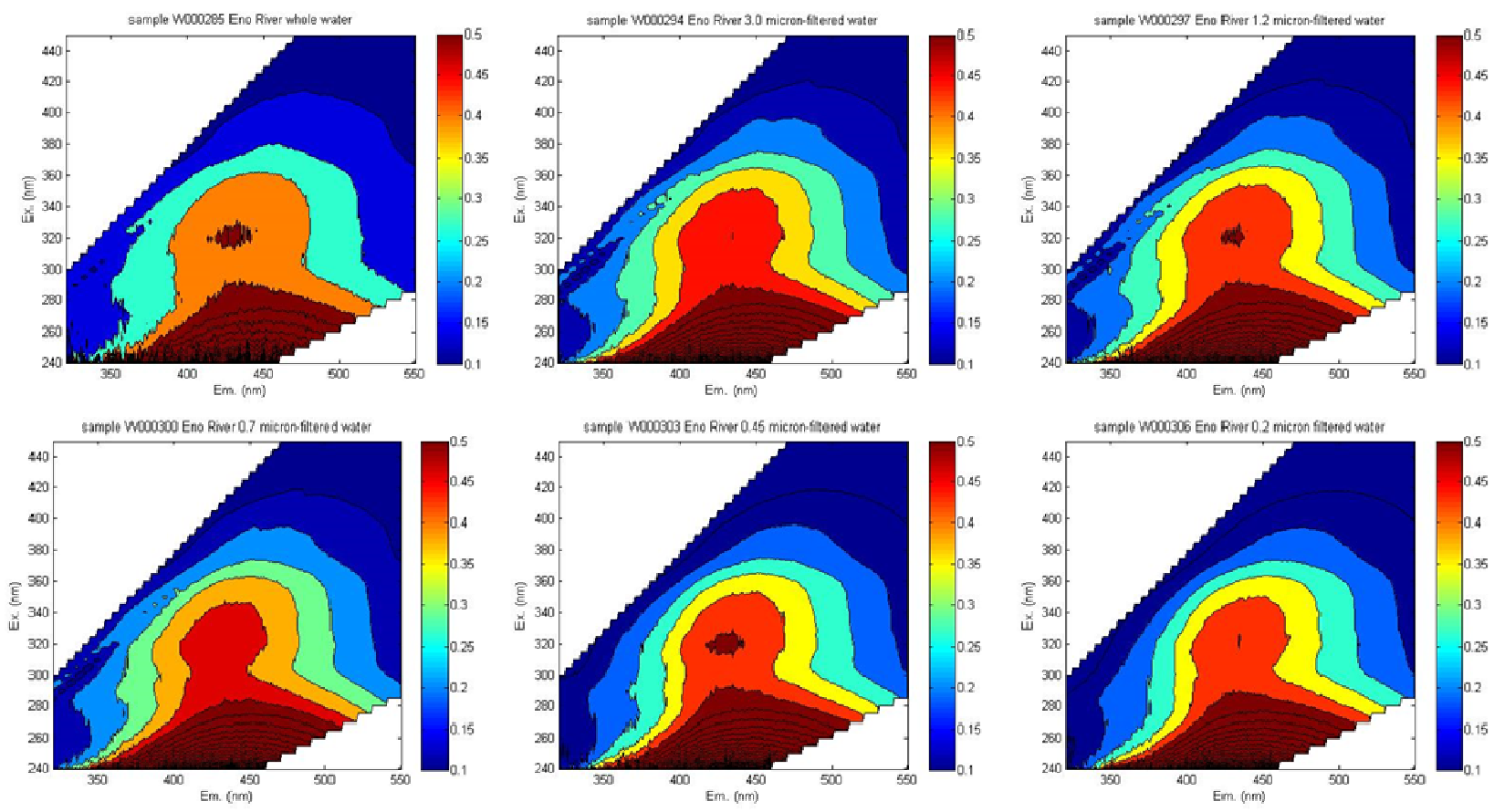


Fig. 3. Representative Natural Water EEMs (Eno River). Peak A region of EEMs are in 260nm/400-460nm (Ex/Em); Peak C region is in 320-360nm/420-460nm; Peak T is in 275nm/340nm.

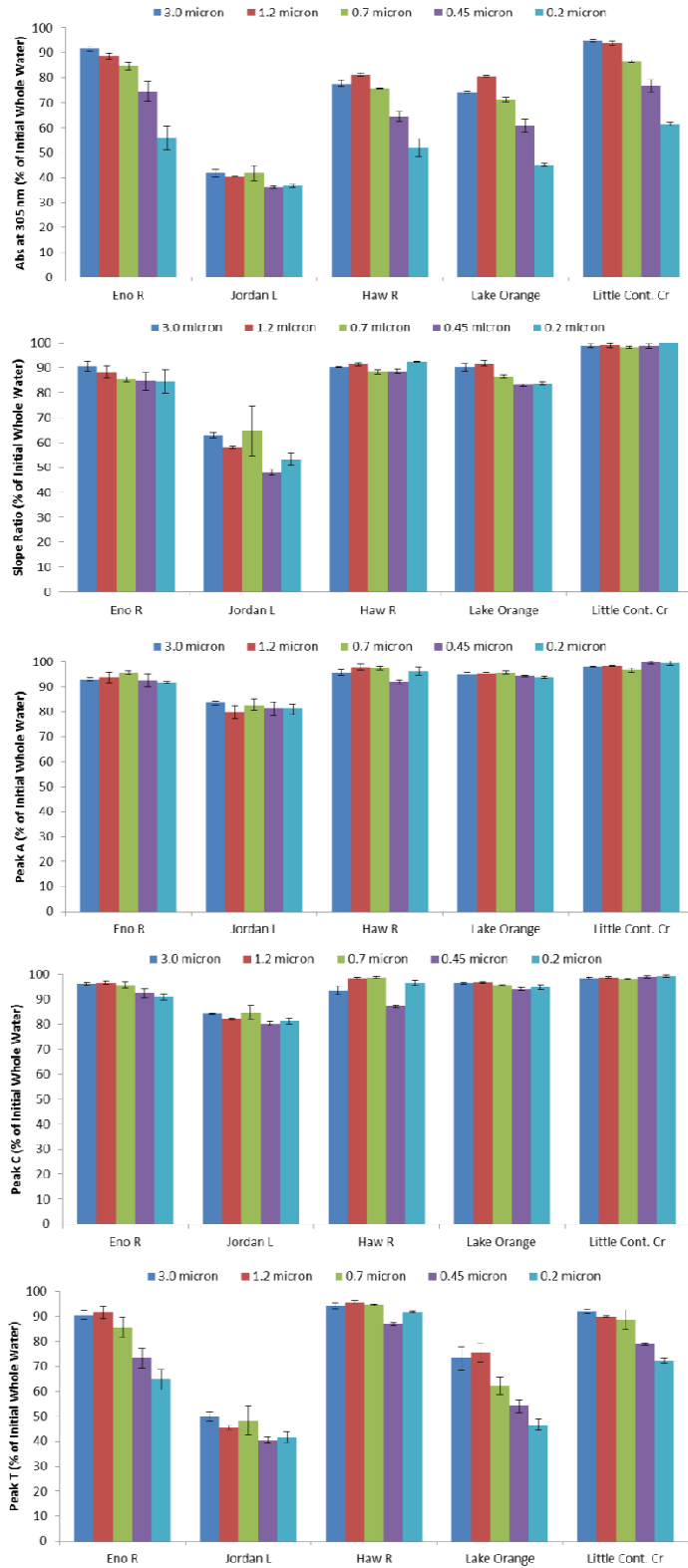


Fig. 4. Absorbance and fluorescence measures of filtrates by pore size and by site, as % of initial whole water; from top: (a) a_{305} (b) S_R (c) peak A (d) peak C (e) peak T

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