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Effect of instrument-specific response on the analysis of fulvic acid fluorescence spectra

Rose M. Cory[1], Matthew P. Miller[1], Diane M. McKnight[1], Jennifer J. Guerard[2], and Penney L. Miller[3]*
[1]The Institute of Arctic & Alpine Research and Civil & Environmental Engineering, University of Colorado, Boulder, CO, USA
[2]School of Earth Sciences, The Ohio State University, Columbus, OH, USA
[3]Chemistry, Rose-Hulman Institute of Technology, Terre Haute, IN, USA

Abstract

Fluorescence spectroscopy has been extensively employed to characterize the source, age, and reactivity of aquatic dissolved organic matter (DOM). However, there is no consensus on the protocols for collecting and correcting DOM fluorescence spectra for the instrument-specific response associated with each component on a fluorometer involved in the excitation of DOM and the resulting detection of DOM emission. The central objective of this study was to evaluate the removal of instrument-specific response from DOM fluorescence spectra collected on three different fluorometers using manufacturer-provided emission and excitation correction files. We evaluated criteria and protocols for comparison of removal of instrument response, using quinine sulfate, a well-characterized fluorophore, as well as the International Humic Substance Society's microbially and terrestrially derived reference end-member fulvic acids: Pony Lake and Suwannee River fulvic acids, respectively. Our results demonstrate that sample spectra collected on different fluorometers differed significantly before correction. Although the effectiveness of manufacturer-provided correction factors in removal of instrument response from sample spectra varied by instrument, spectral overlap of the same sample on multiple instruments improved after correction. Our results suggest that conclusions based on analysis of trends within a dataset of DOM fluorescence spectra should be expected to be independent of the fluorometer employed.

Dissolved organic matter (DOM) is a complex, heterogeneous mixture resulting from the breakdown of higher plant and microbial organic matter in aquatic environments. Absorbance of light by DOM decreases with increasing wavelength in an approximately exponential fashion across the ultraviolet and visible portions of the spectrum (Stedmon and Markager 2001). DOM fluorescence spectra are characterized by broad, featureless emission curves that shift to the red (longer wavelength) and decrease in intensity with increasing excitation wavelength (Del Vecchio and Blough 2004). The quantum yield of DOM emission, which is the fraction of light emitted relative to the light absorbed for a pair of excitation and emission wavelengths, exhibits a maximum at 350 nm and then decreases with increasing excitation wavelength (Del Vecchio and Blough 2004).

*Corresponding author: E-mail: rmcory@unc.edu
Present address: Environmental Sciences & Engineering, University of North Carolina, Chapel Hill

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The excitation and emission properties of fluorophores are dependent on the chemical structure of the fluorophores as well as the local chemical environment around the fluorophores. In addition, the emission spectrum may depend on reactions that can occur in the excited state, such as charge transfer or hydrogen bonding that differs from the ground state. The fluorescence spectrum of a sample is also a function of the instrument employed for the analysis. Every component on a spectrofluorometer has a non-uniform response across the range of wavelengths useful in fluorescence spectroscopy (Lakowicz 1999; Fig. 1). The wavelength dependencies of spectrofluorometer instrument components are significant; consequently, the characteristics of a spectrofluorometer are superimposed on sample spectra. Thus, for accurate intensity or peak position comparison, sample spectra are commonly corrected for variation in lamp profile as a function of wavelength (Fig. 1A), lamp decay over time, as well as performance in the gratings or emission detector as a function of wavelength (Fig. 1B).

Fluorescence spectra of DOM have been studied extensively to characterize source, age, and reactivity of DOM from a variety of aquatic and marine sources (Coble et al. 1990; Coble et al. 1993; Coble 1996; Cory and McKnight 2005; McKnight et al.

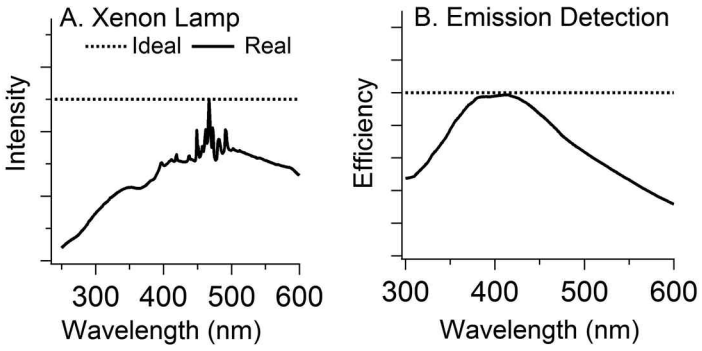

Fig. 1. Ideal versus real instrument response for A. lamp output (using a xenon lamp spectrum as the 'real' example) and B. wavelength selection plus emission detection. This figure was adapted from Lakowicz (1999).

2001; Murphy et al. 2008; Stedmon et al. 2003). For example, the relative contribution of microbial or terrestrial organic matter precursor material to a DOM sample can be determined by analyzing the fluorescence properties of the fulvic acid fraction of DOM, a major DOM fraction, and the dominant light-absorbing fraction in natural waters (McKnight and Aiken 1998). Based on the distinct fluorescence spectra of terrestrial and microbial "end-member" fulvic acids, McKnight et al. (2001) introduced the fluorescence index (FI) approach for characterization of the fulvic acid fraction of DOM. The FI characterizes the slope of an emission curve obtained at an excitation wavelength of 370 nm. In the McKnight et al. (2001) study, a high FI around 1.8-1.9, corresponding to a steeper slope and a narrow width of the emission curve, was correlated with microbially derived organic matter while a low FI (1.3-1.4) was correlated with terrestrially derived organic matter.

Procedures for correcting for the effect of the instrument-specific response on DOM sample spectra have been demonstrated (Coble et al. 1993; DeRose et al. 2007; Holbrook et al. 2006). However, still some studies correct DOM spectra (Boyd and Osburn 2004, for example) while others do not (Kelton et al. 2007, for instance). Thus, as of yet there is no consensus on how to evaluate and remove the instrument response, or on how to adapt analytical approaches for the different types of samples and research questions. For instance, McKnight et al. (2001) used an older model fluorometer that was not equipped with a manufacturer-generated emission correction factor. The authors noted that because their emission data were not corrected for the response of the instrument, the absolute values of the FI might not be comparable between studies. Indeed, FI values of 1.56 to 1.90 have been reported for a microbially-derived fulvic acid from Lake Fryxell in the McMurdo Dry Valleys of Antarctica (Fulton et al. 2004; McKnight et al. 2001; Schwede-Thomas et al. 2005), which is much higher than the instrument error (0.01, this study). Similarly, uncorrected FI values for Suwannee River fulvic acid, the terrestrial reference fulvic acid of the International Humic Substance Society (IHSS) have been reported to be from 1.15 to 1.40 (McKnight et al. 2001; Schwede-Thomas et al. 2005).

The latter studies have corroborated the hypothesis put forward by McKnight et al. (2001) that the FI patterns hold regardless of the absolute FI values measured for a set of samples. However, it remains to be investigated whether instrument-specific corrections can remove the variability in the FI values reported across studies, thereby improving comparisons between studies and over time.

The interpretation of DOM fluorescence with intensity ratios does not allow for full examination of the pairs of excitation and emission curves that comprise DOM fluorescence spectra. It is the pairs of excitation and emission curves, found in DOM excitation-emission matrices (EEMs), which provide chemical information about the fluorophores. Parallel factor analysis (PARAFAC), a statistical modeling approach, separates a dataset of EEMs into mathematically and chemically independent components (each representing a single fluorophore or a group of strongly co-varying fluorophores) multiplied by their excitation and emission spectra (representing either pure or combined spectra). The result is the reduction of complex, three-dimensional data (EEMs) into several two-dimensional spectra representing chemically independent components that describe the total EEM (Stedmon et al. 2003).

PARAFAC analysis of EEM spectra from DOM is well suited to the study of DOM fluorescence because this method of analysis can identify patterns in a dataset that would otherwise not be identified by visual inspection alone. For example, Cory and McKnight (2005) developed a PARAFAC model that identified 13 components representing fluorescing groups described by their characteristic excitation and emission spectra, some of which were associated with humic substances exhibiting consistent variation across environmental redox gradients. Identification of redox-active fluorescing constituents in DOM has enabled researchers to sensitively observe redox processes likely involving DOM in the environment. Miller and coworkers (2006) successfully used a conservative tracer approach combined with PARAFAC analysis of DOM fluorescence spectra in an alpine stream/wetland ecosystem to evaluate the effect of stream-hyporheic exchange on DOM redox reactivity.

When employing fluorescence-based analysis of DOM, it is valuable to obtain spectra that have been corrected to remove instrument response. This correction not only allows comparison of spectra from different fluorometers, but also is needed to examine the chemical nature and biogeochemical reactivity of the fluorophores identified by PARAFAC models. While the effects of instrument correction on specific features in DOM EEMs have been presented (Coble et al.1993; Holbrook et al. 2006), our main intent was to expand upon and broaden these studies to include an inter-instrument comparison of effects of correction on fluorescence index and PARAFAC component distribution among a wider range of fluorometers, using end-member fulvic acids representing the typical range of variation in DOM EEMs encountered particularly in freshwater studies.

The end-member fulvic acids used in this study were Pony Lake (PLFA) and Suwannee River (SRFA) fulvic acids, the International Humic Substance Society's fulvic acids representing microbially and terrestrially derived DOM reference end-members. We investigated the removal of instrument-specific response from sample spectra on three commonly used fluorometers using respective manufacturer-provided correction factors. We provided criteria to evaluate the instrument response based on comparison to the National Institute of Standards and Technology (NIST) reference spectrum of quinine sulfate (Velapoldi and Mielenz 1981), as well as comparison of spectral overlap for two reference fulvic acids on each instrument. Last, we fit the reference fulvic acids emission spectra collected on each fluorometer to the existing PARAFAC model developed by Cory and McKnight (2005) to investigate the effect of instrument response on model fit. If all instrument-specific response is removed, the PARAFAC model analysis of the same sample analyzed in different instruments should be the same within replicate-determined instrumental error.

Materials and procedures

Solution preparation—Pony Lake fulvic acid and Suwannee River fulvic acid, the International Humic Substance Society microbial and terrestrial reference 'end-member' fulvic acids, respectively, were obtained from the International Humic Substances Society. Chemical characteristics of these fulvic acids can be found at http://www.ihss.gatech.edu/. Whereas earlier studies focused on Lake Fryxell fulvic acid as the microbial end-member (McKnight et al. 2001), accessibility issues necessitated that Pony Lake, another Antarctic lake rich in organic matter (Brown et al. 2004), be the site for the large-scale isolation of the microbial end-member fulvic acid. Because many studies to date have referred to Lake Fryxell as the microbial end-member, we analyzed the fluorescence index of Lake Fryxell here to compare to Pony Lake. Chemical characterization and isolation details of the Lake Frxyell fulvic acid sample can be found in McKnight et al. (2001) and Fulton et al. (2004).

Fluorescence spectra were collected on instruments housed in three different laboratories. Dilute fulvic acid solutions (3-6 mg-C/L) were prepared in each laboratory by dissolving freeze-dried fulvic acid in MilliQ water in amber vials. After the solutions were stirred for 24 h, the pH was adjusted to achieve a pH of 6.0-7.0 using 0.1 N HCl or 0.1 N NaOH. All fulvic acid solutions in this study were analyzed at pH 6.0 to 7.0. Solutions were prepared to be fresh just before analysis, and thus the same solution was not analyzed on each fluorometer. Preparing fresh fulvic acid solutions separately in each laboratory led to small differences in fulvic acid concentration. Thus, for this study, we focus on comparison of fulvic acid excitation and emission peak positions and intensity ratios, rather than absolute intensity values.

Quinine sulfate was obtained from Sigma-Aldrich and was used without further purification. Concentrated sulfuric acid (certified ACS plus grade) was purchased from Fisher Scientific. A 6 µM solution of quinine sulfate in 0.05 N sulfuric acid was prepared for fluorescence scans. All spectra were collected from the solution at room temperature (22-25°C).

Instruments—Three instruments were used in this study in order to compare instrument response: a Fluoromax-3 fluorometer (Horiba, Jobin Yvon), a Cary Eclipse (Varian Instruments), and an LS50B (Perkin Elmer). The Fluoromax-3 employed continuous light from a 150 W ozone-free xenon arc lamp as the excitation source. The Flouromax fluorometer had excitation and emission monochromators to select desired wavelengths of light using all-reflective optics. The excitation and emission monochromators had continuously adjustable entrance and exit slits controlled by Datamax software. A photomultiplier tube (PMT) served as the sample detector. The reference detector (R), which monitors the xenon lamp to correct for its wavelength dependent output, was a UV-enhanced silicon photodiode. The Fluoromax-3 used in this study was manufactured in March 2004. The only component on the instrument that was replaced since its manufacture date was the xenon lamp. The xenon lamp on the Fluoromax-3 was routinely replaced after 1000 h of use following manufacturer recommendations to prevent damage to the instrument and ensure analysis integrity.

The signal to noise ratio for the Fluoromax-3 fluorometer was 16,118:1 (± 1031), for the water-Raman peak at an excitation wavelength of 350 nm. We calculated the signal to noise ratio as the difference in the peak intensity (at emission wavelength 397.5 nm) and background signal (at emission wavelength 450 nm), divided by the square root of the background signal (Jobin Yvon Horiba operation manual; http://www.isainc.com), at the scan parameters used in this study (presented below).

For the Fluoromax-3 fluorometer, it is possible to choose the scan mode, monitoring only the signal from the sample (S mode), or monitoring the signal from the sample normalized to the signal from the reference detector (R); S normalized to R is also called ratio mode (S/R). Emission scans collected at a single (constant) excitation wavelength, such as the fluorescence index scan, were analyzed in S mode. EEMs were analyzed in ratio mode (S/R).

The Cary Eclipse, manufactured in November 2000, was equipped with the original flash xenon lamp at 75 Hz as the excitation source, excitation and emission monochromators, and a photomultiplier tube detector. The Cary had a signal:noise ratio of 49:1 (± 15) for the water-Raman peak at an excitation of 350 nm, calculated as described above. The Cary Eclipse analyzed samples by default in ratio mode (S/R). The Cary Eclipse was used as received from manufacturer, no components were replaced.

The Perkin Elmer LS50B Luminescence Spectrometer, manufactured in 2000, employed a pulsed xenon discharge lamp as the excitation source (20 kW for 8 microsecond duration). The excitation source, swapped according to manufacturer guidelines before the start of this study, was the only component replaced. The signal:noise ratio for the LS50B was 35:1 (± 5) for

the water-Raman peak at an excitation of 350 nm, calculated as described above for the Fluoromax-3 fluorometer. The LS50B also analyzed samples by default in ratio mode (S/R).

Fluorescence data acquisition—All samples were run in 1 cm pathlength quartz cuvettes. Daily lamp and water-Raman checks were done for all instruments. The lamp check is an excitation scan and serves the purpose of calibrating for excitation by verifying that the maximum intensity is in the correct place for a xenon lamp (e.g., around 467 nm). The daily water-Raman scan serves to calibrate for emission wavelength. For the Fluoromax-3 fluorometer, daily checks showed that the xenon lamp peak maximum was at 467 ± 0.5 nm, while the water Raman peak maximum occurred at 397 ± 0.5 nm. For the Cary Eclipse, the daily xenon peak was found daily at 461 ± 0.5 nm and the water-Raman peak maximum occurred at 397 ± 0.3 nm. The most intense xenon lamp peak occurred at 567 nm regularly for the LS50B fluorometer, however there was a peak at 467 ± 1 nm. The water Raman peak maximum was at 397 ± 1.5 nm for the LS50B.

For all constant excitation wavelength emission scans (fluorescence index and quinine sulfate scans), the integration time was 0.25 s. For the fluorescence index scan, the excitation wavelength was 370 nm and emission data were collected over 2 nm increments from 380 to 550 nm. For the quinine-sulfate emission scan, the excitation wavelength was 350 nm and the emission scan was collected from 380-600 nm with a 2 nm increment. Fulvic acid EEMs were collected in ratio mode (S/R) with an excitation increment of 5 nm over the range of 240-400 nm. The emission range was 350-550 nm with a 2 nm increment. The integration time was 0.25 s.

Fluorescence data correction—For an in-depth treatment of fluorescence data correction, we refer the reader to *Principles of Fluorescence Spectroscopy* (Lakowicz 1999). Here we summarize elements of the latter work that we believe are most important for obtaining accurate DOM EEMs.

Excitation-emission matrices (EEMs) contain a set of emission spectra collected over a range of excitation wavelengths. Because the excitation wavelength is not constant, the light intensity varies as a function of the output of the excitation source (e.g., the lamp), which increases with wavelength across the range of excitation wavelengths commonly used to generate EEMs of DOM (~240-450 nm). Thus, when collecting emission data for DOM EEMs, it is necessary to operate in ratio mode (S/R), which corrects the sample signal for the non-uniform output of the lamp over the excitation range by normalizing the sample signal to the response from the reference detector monitoring the lamp output (DeRose et al. 2007). DOM EEMs collected in ratio mode depend less on variation in the lamp output (Fig. 1A), however the response of the reference detector may also be dependent on wavelength (DeRose et al. 2007). Some instruments collect spectra by default in ratio mode, whereas on other instruments, ratio mode is a user-specified scan mode (Table 1). Consequently, for any instrument used, it is important to know the mode in which an EEM is collected.

Additional inefficiencies in excitation are commonly corrected for using manufacturer-generated excitation correction factors as a function of wavelength, which correct for wavelength-dependent transmission inefficiency of the excitation monochromators, for example. Added to the factors affecting the excitation of the sample, emission spectra are also influenced by efficiency of the emission monochromator and photomultiplier tubes as a function of wavelength. These inefficiencies are also commonly corrected by application of manufacturer-generated emission correction factors as a function of wavelength. Fluorometer users may also generate excitation and emission correction factors (DeRose et al. 2007; Lakowicz 1999).

Each EEM is a data matrix in the format shown in Eq. 1 where Ex = excitation wavelength and Em = emission wavelength, and $I_{i,j}$ is the intensity at the i^{th} emission wavelength and j^{th} excitation wavelength:

	Ex_1	Ex_2	Ex_3	Ex_j	(1)
Em_1	I_{11}	I_{12}	I_{13}	I_{ij}	
Em_2	I_{21}	I_{22}	I_{23}	I_{2j}	
Em_3	I_{31}	I_{32}	I_{33}	I_{3j}	
Em_i	I_{i1}	I_{i2}	I_{i3}	I_{ij}	

Plotting data down a column shows an emission spectrum for a given excitation wavelength. Plotting the data across the rows shows an excitation spectrum for a given emission wavelength. It follows that emission correction factors should be applied down the columns (emission axis) and excitation corrections should be applied across the rows (excitation axis). For most fluorometers using a xenon lamp as the excitation source, the excitation correction factors should increase the intensities observed at lower wavelengths relative to higher wavelengths where the output of the lamp is highest (Fig. 1A). Emission correction factors generally serve to increase emission intensities at wavelengths where the efficiency of the gratings and PMT decline (generally at > 400 nm; see Fig. 1B and "Supplementary Information"). Manufacturers define their emission and excitation factors differently, so that instrument users must sometimes multiply and sometime divide their uncorrected spectra by the correction factors to obtain properly corrected spectra (Table 1).

For the Fluoromax-3 fluorometer, intensities in DOM EEMs should be multiplied by the emission correction factor and divided by the excitation correction factors, according to Eq. 2 where X_D is the diagonal matrix of the excitation correction column vector, M_D refers to the diagonal matrix of the emission correction row vector (Table 1), and the prime indicates the matrix transposed.

$$EEM_{corrected} = ([EEM \div X_D]' \times M_D)' \quad (2)$$

For the Cary Eclipse fluorometer, intensities should be multiplied by both the excitation and emission correction factors (Table 1):

$$EEM_{corrected} = ([EEM \times X_D]' \times M_D)' \quad (3)$$

Table 1. Key instrument-specific parameters affecting DOM EEMs.

Fluorometer	Scan mode options	Excitation correction operation	Emission correction operation
Horiba Scientific Fluoromax-3	ratio mode by user specification	divide	multiply
Varian Cary Eclipse	ratio mode by default	multiply	multiply
Perkin Elmer LS50B	ratio mode by default	multiply	divide

For the LS50B fluorometer, intensities should be multiplied by the excitation correction factor and divided by the emission correction factor (Table 1):

$$EEM_{corrected} = ([EEM \times X_D]' \div M_D)' \quad (4)$$

For the LS50B fluorometer, the excitation correction factors are applied automatically (by default when collecting an EEM). Correction factors as a function of wavelength for each instrument are included in the "Supplementary Information."

Ratio mode and excitation correction factors serve to increase intensities where the lamp output is lowest. A DOM EEM that has been collected in ratio-mode and properly excitation-corrected will almost always exhibit the most intense emission curve at the lowest excitation wavelength used (typically 240-250 nm) because the absorbance of light by DOM is highest at lower wavelengths. DOM EEMs that have not been collected in ratio mode and/or have had an incorrect application of the excitation correction factor can look more like the output of the xenon lamp (Fig. 1A), exhibiting the most intense emission curves at higher excitation wavelengths (e.g., ≥ 260 nm; see "Supplementary Information").

A check on the proper application of the manufacturer-generated emission correction factors can be done by examining the variation in the emission correction factors as a function of wavelength. Efficiencies in emission detection generally decrease with increasing wavelength, thus the emission correction factors should serve to increase the emission intensity at longer wavelengths (see emission correction factors in "Supplementary Information").

In summary, all EEMs in this study were collected in ratio mode and corrected for excitation and emission according to Eq. 2, 3, or 4 depending on the instrument used. Constant excitation wavelength emission scans (quinine sulfate or fluorescence index scans) collected on every fluorometer were corrected by multiplying or dividing by the emission correction factor as a function of wavelength depending on the format of the manufacturer-generated emission correction file (Table 1) according to Eq 5:

$$S_{i,corrected} = S_i \times M_i \text{ or } S_{i,corrected} = S_i \div M_i \quad (5)$$

where S_i is the intensity of the sample signal at the i^{th} emission wavelength, and M_i is the corresponding emission correction factor at the i^{th} emission wavelength.

Milli-Q water blanks were collected daily and were corrected for excitation and emission. After sample and Milli-Q blank EEMs were corrected, Milli-Q blank EEMs were subtracted from each sample EEM. Sample EEMs were also corrected for the inner-filter effect (McKnight et al. 2001). To account for the variation of the lamp intensity over time, sample intensities were converted to Raman units (RU, nm^{-1}) by normalizing intensities to the area under the water-Raman curve at an excitation of wavelength 350 nm (Stedmon et al. 2003). All corrections to EEMs were done in Matlab (MATLAB 6.5.0, Mathworks).

Criteria for inter-instrument comparison—To evaluate removal of instrument-specific response from sample spectra, uncorrected and corrected spectra of quinine-sulfate, a well-studied fluorophore with a fluorescence quantum yield near unity, were compared with the reference spectrum of quinine sulfate (Velapoldi and Mielenz 1981) following the guidelines of Lakowicz (1999). Second, uncorrected and corrected FI spectra of the end-member fulvic acids collected on multiple instruments were compared. The FI values and average peak positions from these scans were compared before and after correction.

EEMs of DOM are typically dominated by conventionally labeled peak A and peak C (Coble 1996), which are thought to be associated with the humic fraction of DOM. Peaks A and C have excitation/emission wavelength maxima (Ex_{max}/Em_{max}) of 240-260/380-460 nm and 320-360/420-460 nm, respectively. A third peak region visible in many DOM EEMs contains one or more amino acid-like peaks (Coble 1996). We compared the peak positions and intensity ratios of peaks A and C in the fulvic acid EEMs before and after correction. We did not compare peak positions in the amino acid region, given that the terrestrial end-member, Suwannee River fulvic acid, does not exhibit visually apparent peaks in this region. If instrument-specific bias is removed, quinine sulfate spectra and peak positions, the FI values, and peak positions and intensities of the FI peaks as well as peaks A and C should overlap within instrument error for a given same sample collected on different instruments.

Because the peak-picking analysis compares only the three dominant peaks visible in the EEM, we fit EEMs of the end-member fulvic acids collected on different instruments to an existing PARAFAC model (Cory and McKnight 2005) as an additional tool to more broadly and quantitatively compare pairs of excitation and emission curves in a given EEM. The latter PARAFAC model was based on a dataset of 379 EEMs of chemically distinct DOM samples, including SRFA and PLFA, as well as other samples collected mainly from a wide range of freshwater systems. Model fits were evaluated visually comparing the measured, modeled and residual EEMs using the DOM Fluor Toolbox (Stedmon and Bro 2008). Again, if all instrument-specific response is removed, the distribution of

fluorophores as determined by the PARAFAC model fit of the same sample analyzed on different instruments should overlap within instrumental error.

Assessment

Quinine sulfate comparison—In Fig. 2, the NIST reference spectrum for quinine sulfate was compared with uncorrected and corrected spectra of quinine sulfate analyzed on each fluorometer. Before correction, none of the quinine sulfate spectra collected on the Fluoromax-3, Cary Eclipse, or the LS50B exhibited good agreement with the NIST reference spectrum across the whole wavelength range shown (370-550 nm). Specifically, none of the uncorrected spectra had an emission peak position centered at 450 nm as does the NIST spectrum. The Fluoromax-3 and the LS50B showed slightly blue-shifted peak positions whereas the Cary Eclipse shows an emission peak slightly to the red of 450 nm. Further, the overall shapes of the spectra did not agree with the NIST reference spectrum. For example, the Fluoromax-3 had an emission curve that is broader than the NIST spectrum at wavelengths shorter than the peak position (i.e., < 450 nm) and shallower than the NIST spectrum at longer wavelengths (> 450 nm).

After instrument-specific emission correction factors were applied (Eq. 5), the overlap between the spectra was considerably improved for the quinine sulfate spectrum collected on the Fluoromax-3 and LS50B (Fig. 2). The Fluoromax-3 showed near perfect overlap with the NIST reference spectrum across the whole wavelength region, sharing a peak position of 450 nm. For the LS50B, application of the emission correction factor yielded an improved overlap to the reference quinine sulfate scan across emission wavelengths of ~400-470 nm, however at wavelengths greater than 470 nm, the comparison was worse after correction. The Cary Eclipse spectrum did not change markedly after correction. The peak position was still red-shifted (456 nm compared to 450 nm), and broader in the portion of the curve at wavelengths longer than the peak maximum.

Inter-instrument comparison of fulvic acid emission spectra—Figure 3 provides an inter-instrument comparison of the fluorescence index (FI) emission spectra for PLFA and SRFA, the microbial and terrestrial end-member fulvic acids, respectively.

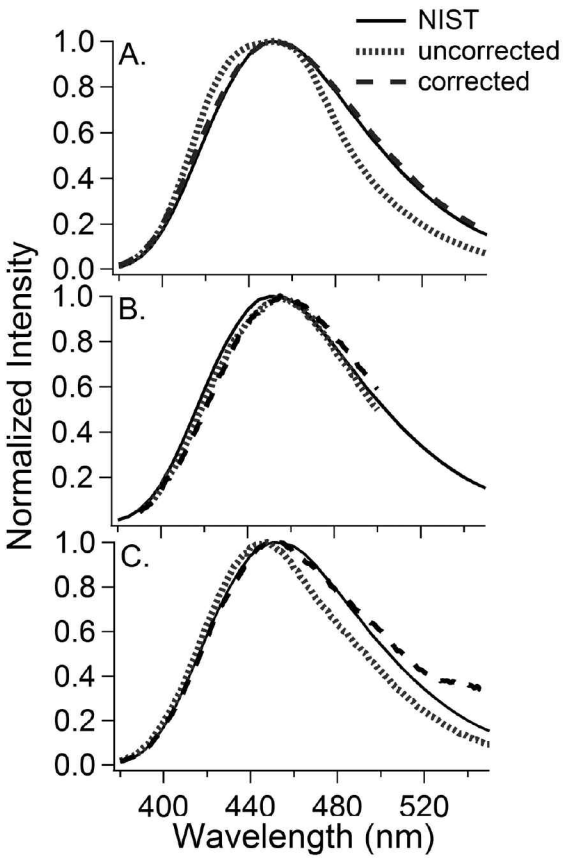

Fig. 2. NIST reference spectrum of quinine sulfate compared to uncorrected quinine sulfate and corrected quinine sulfate emission collected on A. Fluoromax-3 (Fmax), B. Cary Eclipse, and C. LS50B.

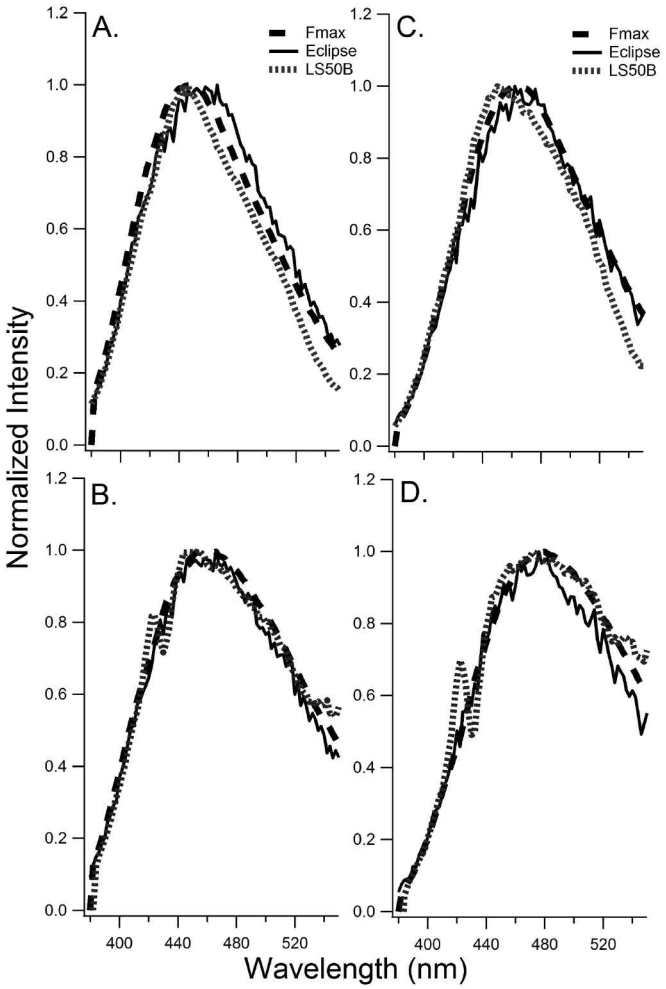

Fig. 3. Inter-instrument comparison of fluorescence index emission spectra (excitation wavelength = 370 nm). A. PLFA uncorrected, B. PLFA corrected, C. SRFA uncorrected, D. SRFA corrected.

Uncorrected PLFA analyzed on the Fluoromax-3 and the LS50B had similar peak maxima, but did not overlap well on the descending portion of the emission spectrum (Fig. 3A). Uncorrected PLFA on the Cary Eclipse had a red-shifted emission spectrum compared to the Fluoromax-3 and LS50B (Fig. 3A).

After correcting the PLFA spectra collected on each fluorometer, there was very good overlap across the emission wavelength region analyzed for the FI scan (Fig. 3C; Table 2). However, the FI peak positions for the corrected Fluoromax-3 and Cary Eclipse PLFA spectra remained significantly different than the LS50B after correction (Table 2).

Similar results were obtained for SRFA (Fig. 3; Table 3). Whereas the Fluoromax-3 and Cary Eclipse showed good overlap for uncorrected SRFA, the uncorrected SRFA spectrum on the LS50B was comparatively blue-shifted and did not show good overlap across the wavelength region analyzed. Uncorrected peak positions ranged from 453 nm (LS50B) to 468 nm (Cary Eclipse). The uncorrected FI values ranged from 1.17 to 1.37. After the emission correction factor was applied, there was improved overlap among all the SRFA spectra across the wavelength range. Like PLFA, the corrected SRFA spectra exhibited a red-shifted peak maximum position (Fig. 3; Table 3) compared with uncorrected SRFA on each instrument.

One effect of correction on the FI emission spectra was a red shift in the peak position compared with the uncorrected spectra in this study and in the McKnight et al. (2001) study (Tables 2 and 3). This trend was most pronounced for the PLFA collected on the Fluoromax-3 and the SRFA collected on all three fluorometers; with an average red shift of approximately 15 nm. In the McKnight et al. (2001) study, the fluorescence index was calculated as the ratio of intensities at 450 over 500 nm, corresponding to the slope of the observed (uncorrected) emission peak. Therefore, to evaluate the same slope as the McKnight et al. (2001) study, the fluorescence index obtained from corrected spectra from each fluorometer was shifted to the ratio of intensities at 470 over 520 nm.

Using the McKnight et al. (2001) FI calculation of the ratio of emission intensities at 450 nm divided by 500 nm for the uncorrected spectra resulted in FI values for PLFA ranging from 1.47 to 1.75, which is significantly greater than standard deviations of 0.01 to 0.01 for uncorrected PLFA analyzed in triplicate on each fluorometer (Table 2). Calculating the FI value on the corrected PLFA spectra as the ratio of intensities at 470 nm divided by 520 nm gave FI values for PLFA ranging from 1.45 to 1.52 (Table 2). While the latter difference in FI values obtained for PLFA was still greater than the standard deviation of PLFA analyzed in triplicate on each

Table 2. Peak locations and fluorescence index values for uncorrected and corrected Pony Lake Fulvic Acid fluorescence spectra.

	Uncorrected					Corrected				
Instrument	Peak Max A Ex, Em (nm)	Peak Max C Ex, Em (nm)	Intensity Ratio (A/C)	Peak Max FI Ex, Em (nm)	FI*	Peak Max A Ex, Em (nm)	Peak Max C Ex, Em (nm)	Intensity Ratio (A/C)	Peak Max FI Ex, Em (nm)	FI†
Fluoromax-3	240, 432 ± 0, 2	300, 426 ± 0, 2	1.41 ± 0.01	446 ± 3	1.63 ± 0.01	240, 448 ± 0, 2	330, 434 ± 5, 4	1.91 ± 0.01	460 ± 3	1.46 ± 0.01
Eclipse	240, 423 ± 0, 4	315, 424 ± 13, 2	1.82 ± 0.03	461 ± 3	1.47 ± 0.01	240, 442 ± 0, 2	312, 436 ± 6, 7	2.41 ± 0.02	463 ± 1	1.52 ± 0.01
LS50B	240, 437 ± 0, 2	328, 434 ± 13, 3	1.41 ± 0.01	445 ± 3	1.75 ± 0.01	240, 443 ± 0, 1	310, 443 ± 0, 1	1.75 ± 0.01	451 ± 5	1.45 ± 0.01

Peak max: average position of maximum intensity of humic peaks in EEM at excitation (Ex) and emission (Em) wavelengths ± standard deviation (n = 3).
*The FI for the uncorrected spectra was taken as the ratio of intensities at 450/500 nm at Ex = 370 nm
†The FI for the corrected spectra was taken as the ratio of intensities at 470/520 nm at Ex = 370 nm

Table 3. Peak locations and fluorescence index values for uncorrected and corrected Suwannee River fulvic acid fluorescence spectra.

	Uncorrected					Corrected				
Instrument	Peak Max A Ex, Em (nm)	Peak Max C Ex, Em (nm)	Intensity Ratio (A/C)	Peak Max FI Ex, Em (nm)	FI*	Peak Max A Ex, Em (nm)	Peak Max C Ex, Em (nm)	Intensity Ratio (A/C)	Peak Max FI Ex, Em (nm)	FI†
Fluoromax-3	260, 452 ± 5, 2	320, 442 ± 5, 2	1.49 ± 0.01	464 ± 3	1.23 ± 0.01	240, 454 ± 0, 2	325, 454 ± 5, 2	1.86 ± 0.01	480 ± 3	1.21 ± 0.01
Eclipse	240, 448 ± 0, 4	327, 451 ± 3, 6	1.86 ± 0.02	468 ± 4	1.17 ± 0.03	240, 450 ± 0, 1	325, 456 ± 5, 5	2.35 ± 0.08	477 ± 1	1.28 ± 0.01
LS50B	240, 438 ± 0, 2	317, 436 ± 3, 2	1.63 ± 0.01	453 ± 1	1.37 ± 0.03	240, 446 ± 0, 1	315, 444 ± 0, 1	1.82 ± 0.01	479 ± 2	1.22 ± 0.01

Peak max: average position of maximum intensity of humic peaks in EEM at excitation (Ex) and emission (Em) wavelengths ± standard deviation (n = 3).
*The FI for the uncorrected spectra was taken as the ratio of intensities at 450/500 nm at Ex = 370 nm
†The FI for the corrected spectra was taken as the ratio of intensities at 470/520 nm at Ex = 370 nm

instrument (0.01 Table 2), the difference was much smaller than the 0.28 difference observed among the uncorrected spectra. The improved agreement in FI values for the corrected PLFA spectra resulted from improved overlap in emission spectral shape and peak maximum position (Fig. 3).

Before correction, the FI values (calculated as the ratio of emission intensities at 450 over 500 nm) for SRFA ranged from 1.17 to 1.37. Similar to PLFA, there was also a smaller range of variation in the corrected FI values (calculated as the ratio of emission intensities of 470/520 nm) after correction with values ranging from 1.21 to 1.28.

In the McKnight et al. (2001) study, it was found that the FI, calculated on uncorrected emission spectra, ranged from 1.4 for terrestrially derived organic matter such as SRFA to 1.9 for microbially derived organic matter such as Lake Fryxell fulvic acid (Table 4). Analysis of the FI values of these two samples on the Fluoromax-3 fluorometer verified the trend and the general range presented by McKnight et al. (2001), although values obtained here were significantly different than those of McKnight et al. (2001) (Table 4), both before and after correction. As shown above, the corrected FI values for SRFA range from 1.21 to 1.28, which is lower than the McKnight et al. (2001) value of 1.4 (Tables 3-4). The corrected FI value for Lake Frxyell fulvic acid was 1.55 for spectra collected on the Fluoromax-3 fluorometer (Table 4). This value is about 0.2 lower than the value reported in the McKnight et al. (2001) study, and higher than FI values obtained for the Pony Lake fulvic acid (Table 2). This result suggests that even in lakes where DOM source is dominated by microbial inputs, there can be a range in observed FI values.

Inter-instrument comparison of fulvic acid EEMs—EEMs of PLFA collected on each of the three fluorometers are shown in Fig. 4. Visual comparison of the PLFA EEMs analyzed on the different fluorometers showed that the signal:noise ratio was highest for the Fluoromax-3 flourometer, followed by the Cary Eclipse, and lowest for the LS50B, consistent with the signal:noise ratio trends obtained from the water Raman scans on each instrument. Quantitative comparison of the similarity between fulvic acid EEMs collected on different instruments was evaluated by identifying the excitation and emission maxima associated with the dominant humic peaks A and C (Coble et al. 1990).

It was clear that EEMs of PLFA analyzed on different instruments did not match well before correction, particularly for peak C, which differed in excitation maximum position by 28 nm between instruments (Fig. 4; Table 2). There was also poor agreement in the emission position of peak A for PLFA analyzed on multiple instruments (Table 2), with a range of 423 to 437 nm. The intensity ratio, intensity at the emission maximum of peak A divided by the intensity at the emission maximum for peak C, agreed very well for uncorrected EEMs of PLFA analyzed on the Fluoromax-3 and LS50B fluorometers, and was higher for the Cary Eclipse (Table 2).

After excitation, emission, and intensity correction factors were applied to EEMs of PLFA, there was improvement in emission peak positions for both peaks A and C, and there was a smaller span in the observed excitation maxima for peak C (Table 2). However, agreement between the A/C intensity ratios did not improve. The Cary Eclipse still had a much higher A/C ratio relative to the Fluoromax-3 and LS50B intensity ratios from corrected PLFA (Table 2).

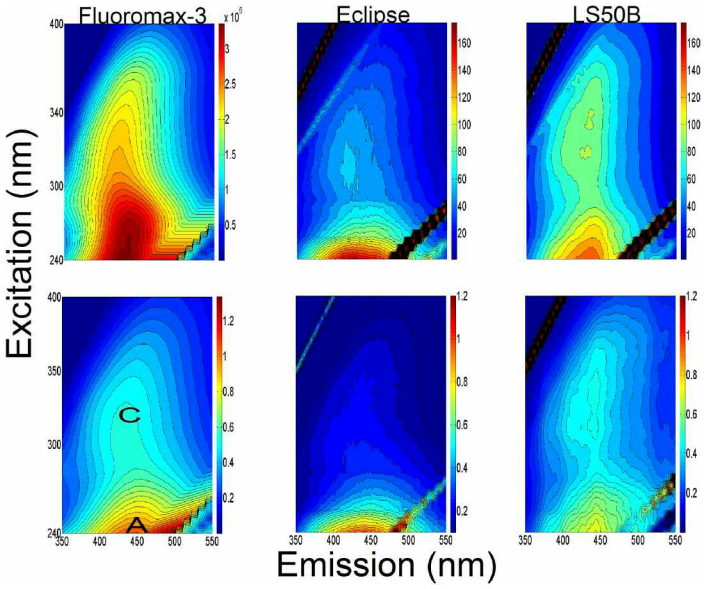

Fig. 4. Pony Lake fulvic acid EEMs before correction (top) and after correction (bottom). Intensities in relative units specific to each instrument before correction, and normalized to peak max after correction. The peak locations for humic peaks A and C are shown for all EEMs on the bottom left EEM.

Table 4. Current Fluorescence index (FI) values compared to McKnight et al. (2001) study

		Uncorrected				Corrected	
		McKnight et al. (2001)		JY Fluoromax-3		JY Fluoromax-3	
Sample	pH*	FI†	Peak (nm)	FI†	Peak (nm)	FI‡	Peak (nm)
Lake Fryxell FA (7.5 m)	6-7.5	1.9	448	1.68	449	1.55	457
Suwannee River FA	6-7.5	1.4	461	1.23	464	1.21	480

*Fluorescence scans collected in the McKnight et al. (2001) study were collected at pH 7.5. Scans collected on the Fluoromax-3-2 and Fluoromax-3 fluorometers were collected at pH 6.0-7.0.
†The FI for the uncorrected spectra was taken as the ration of intensities at 450/500 nm at Ex = 370 nm.
‡The FI for the corrected spectra was taken as the ration of intensities at 470/520 nm at Ex = 370 nm.

Inter-instrument comparison of SRFA EEMs showed poor agreement in excitation and emission peak positions for peaks A and C before correction (Fig. 4; Table 3). Additionally, there was poor agreement in the ratios of peak A/C between fluorometers before correction. After correction, there was improved overlap between SRFA EEMs analyzed on the Fluoromax-3 and Cary Eclipse fluorometers. The span of excitation and emission peak maxima for both peaks A and C decreased after correction (Table 3). For example, after correction, the emission maximum of peak C differed by 12 nm compared to 15 nm before correction (Table 3). After correction, there was good agreement in the intensity ratio of peak A/C between the Fluoromax-3 and Cary EEMs. Like PLFA, the A/C intensity ratio was highest for SRFA on the Cary Eclipse (Table 3).

Inter-instrument comparison: fulvic acid EEMs fitted to an existing PARAFAC model—For this study, we employed the PARAFAC approach as a tool to quantitatively evaluate agreement in PLFA and SRFA excitation and emission spectra across the entire wavelength region of an EEM. In the previous section, we compared how well excitation and emission peak positions agreed for PLFA and SRFA analyzed on different fluorometers. PARAFAC allows us to evaluate whether differences in peak position and intensity remaining after correction will lead to widely different distributions of components for a given sample. Table 5 gives the percent contribution (amount of each PARAFAC component normalized to the total modeled fluorescence) for the PLFA and SRFA samples analyzed on each fluorometer. Because the Cory and McKnight (2005) PARAFAC model included PLFA and SRFA samples and was generated with EEMs collected on the Fluoromax-3, Fluoromax-3 PARAFAC components are assumed to be the correct values for this study.

Triplicate analyses of PLFA and SRFA EEMs fit to the PARAFAC model resulted in less than or equal to two percent standard deviation for each component (Table 5), regardless of the fluorometer used. Although few components for either PLFA or SRFA agreed to within instrument-specific standard deviation, there was generally good agreement in component distribution for PLFA and SRFA analyzed on different instruments (Table 5).

Given that all instrument-specific response cannot be removed from an EEM after post-processing and correction, the question remains as to whether the same trend(s) may be observed in datasets of EEMs analyzed by PARAFAC, independent of the fluorometer employed in the study. Here we present fluorescence spectra of PLFA and SRFA, the microbial and terrestrial end-member reference fulvic acids, respectively. From previous EEM and PARAFAC studies, as well as complementary analyses of chemical characteristics of these fulvic acids, we know that PLFA should have a strong microbial signature in its EEM, whereas the fluorescence signature of SRFA should be dominated by components associated with soil and higher plant sources of organic matter.

Component three (C3) identified by Cory and McKnight (2005) is similar to peak M (Coble 1996), a fluorescing constituent strongly associated with autochthonous production in aquatic and marine systems (Table 5). The amino acid components (C8 and C13 in Cory and McKnight, Table 5) have also been associated with microbial production and degradation. Given that PLFA is derived entirely from microbial production and degradation and has roughly 10 times the nitrogen as SRFA by mass (Brown et al. 2004), we expect that PLFA should have a greater proportion of C3, C8, and C13 relative to SRFA. The percent contribution of C3 in PLFA agreed well across instruments, ranging from ~8% to 9%. In addition, there was much greater percentage of C3 in PLFA relative to SRFA, independent of the fluorometer used. Similarly, there was excellent agreement for the tryptophan-like component (C8) in PLFA across instruments, and for each fluoromter, there was more of this amino acid–like component in PLFA relative to SRFA. There was discrepancy between instruments for the contribution of the tyrosine-like component (C13). PLFA analyzed on the Fluoromax-3 was determined to have greater C13 than PLFA on the Cary Eclipse or LS50B (Table 5). Further, both the Cary Eclipse and the LS50B showed relatively higher C13 in SRFA than PLFA, opposite from the expected trend.

Last, C5 has been strongly associated with terrestrial organic matter inputs to the DOM pool (Cory and McKnight 2005), and is thus expected to contribute proportionately more to the SRFA EEM relative to PLFA, as exhibited by the ~18% versus 11% for C5 in SRFA and PLFA EEMs, respectively, as analyzed by the Fluoromax-3 (Table 5). The amount of C5 was also greater in SRFA compared with PLFA analyzed on the Cary Eclipse and LS50B, as expected. However, the difference in C5 between PLFA and SRFA measured on the Cary Eclipse and LS50B was not as great as measured on the Fluoromax-3.

Discussion

Results of this study show that significantly different spectra will be obtained for the same sample analyzed on different fluorometers. Further, removal of instrument-specific response from sample spectra varied by fluorometer, and differences in DOM excitation and emission spectra collected on different instruments did remain after correction. However, for the fluorescence index (FI) analysis of fulvic acid source (microbial versus terrestrial), the trend was found to be robust. Independent of instrument or application of the correction factor, the FI was always higher (greater than 1.4) for the microbial end-members PLFA and Lake Frxyell fulvic acid and always lower (less than 1.4) for terrestrially derived SRFA. Thus, our results provide support for studies that interpreted source of the fulvic acid on trends in the FI value, regardless of the absolute value of the FI.

The corrected quinine sulfate spectra collected on the Fluoromax-3 yielded the best comparison with the NIST reference spectrum of quinine sulfate. Consequently, we conclude that the FI values with the greatest accuracy are those calculated from the corrected spectra of the Fluoromax-3 fluorometers as the ratio of intensities at 470 nm over 520 nm. For the PLFA

Table 5. PARAFAC component distributions for PLFA and SRFA

			PLFA			SRFA		
Component	EEM Region*	Source Association†	Fluoromax-3	Eclipse	LS50B	Fluoromax-3	Eclipse	LS50B
C1	C	Terrestrial	12.0 ± 1.0	11.0 ± 1.0	13.4 ± 0.1	16.4 ± 0.4	17.2 ± 0.2	21.0 ± 1.0
C2	A	Microbial	12.0 ± 0.1	16.0 ±.2.0	11.0 ± 0.1	13.0 ± 1.0	15.5 ± 0.2	11.4 ± 0.1
C3	M‡	Microbial	8.9 ± 0.1	8.0 ± 1.0	9.3 ± 0.1	3.0 ± 0.3	1.5 ± 0.2	1.4 ± 0.1
C4	HQ§	Both	10.0 ± 1.0	9.6 ± 0.2	7.4 ± 0.4	13.0 ± 0.5	13.5 ± 0.1	9.0 ± 0.2
C5	FI	Terrestrial	11.0 ± 1.0	8.0 ± 1.0	13.7 ± 0.1	17.6 ± 0.3	9.7 ± 0.1	16.0 ± 1.0
C6	A	Microbial	5.2 ± 0.4	10.0 ± 1.0	0 ± 0	8.4 ± 0.2	18.6 ± 0.2	13.0 ± 1.0
C7	FI	Microbial	10.0 ± 1.0	10.5 ± 0.4	13.9 ± 0.1	10.0 ± 1.0	6.1 ± 0.1	8.0 ± 1.0
C8	amino acid: Trp	Microbial	4.0 ± 1.0	4 ± 1	4.6 ± 0.1	2.0 ± 1.0	0 ± 0	0 ± 0
C9	C	Microbial	6.5 ± 0.1	5.6 ± 0.4	9.5 ± 0.1	2.0 ± 1.0	0 ± 0	0.6 ± 0.4
C10	C	Terrestrial	6.4 ± 0.1	3.0 ± 1.0	8.0 ± 0.1	6.4 ± 0.3	3.3 ± 0.2	8.0 ± 0.2
C11	A	Terrestrial	5.4 ± 0.1	5.9 ± 0.4	4.5 ± 0.1	5.9 ± 0.2	8.6 ± 0.1	7.2 ± 0.1
C12	A	Microbial	4.8 ± 0.1	8.0 ± 1.0	4.8 ± 0.9	2.5 ± 0.2	4.1 ± 0.3	3.1 ± 0.1
C13	amino acid: Tyr	Microbial	3.0 ± 1.0	1.0 ± 1.0	0 ± 0	0 ± 0	2.0 ± 0.5	0.3 ± 0.3

Distribution of PARAFAC components in the end-member fulvic acids shown as mean percent (%) contribution of each component to the total modeled, ± standard deviation (n = 3).

*Each component contributes to one or more of the major peaks visible in DOM EEMs depending on its unique excitation and emission spectrum as identified in Cory and McKnight (2005).

†'Microbial' refers to components associated with microbially-derived organic matter; 'terrestrial' refers to components associated with terrestrially-derived organic matter in Cory and McKnight (2005).

‡Peak M, as designated by Coble (1996), is associated with biological (microbial) production.

§Component HQ was associated with reduced DOM in Cory and McKnight (2005), and is prevalent in EEMs of isolated humic substances from soil organic matter.

and SRFA end-member fulvic acids, this calculation gave FI values of 1.46 and 1.21, respectively. These end-member FI values are lower than the uncorrected values originally reported by McKnight et al. (2001) for microbially and terrestrially derived end-members.

It was evident that the magnitude of the difference between uncorrected and corrected FI values depended on the instrument as well as fulvic acid sample. For example, for Fluoromax-3 fluorometer, the magnitude of the emission correction factor is greater than one for the descending limb region of the emission curve characterized by the FI (excitation is 370 nm, emission 450-550 nm; see "Supplementary Information"). Inherent in the FI is a greater slope of the falling limb observed for microbially derived samples. Therefore, correction factors applied to the absolute values that make up the falling limb will result in a greater difference between the corrected and uncorrected FI values for microbially derived samples compared with terrestrially derived samples. Indeed, the differences between the corrected and uncorrected FI values of PLFA were greater than those for SRFA.

Comparison of PLFA and SRFA fluorescence signature obtained on different instruments via the EEM peak-picking method showed that there was better agreement in major EEM peak positions after correction procedures were carried out. However, DOM emission curves are typically very broad, and examination of the full FI emission scan before and after correction demonstrated that agreement in peak maximum position does not necessarily indicate a good overlap across the spectral range. The improved spectral overlap across the excitation and emission wavelength ranges, as exhibited by the FI emission curve for each PLFA and SRFA after correction, likely led to the general agreement in PARAFAC component distribution for each PLFA and SRFA analyzed on the three different instruments (Table 5). Because there was satisfactory agreement in the PARAFAC distribution for PLFA or SRFA analyzed on different instruments, the same conclusions can be made about the relative importance of microbial versus terrestrial precursor material in PLFA compared with SRFA, regardless of the fluorometer employed for the analysis. It is possible that a PARAFAC model with fewer components would have shown less discrepancy between the component distribution capturing the major variation in fluorescence in the A, C, and amino acid region of the EEMs.

Comments and recommendations

The effectiveness in removing instrument response from sample emission spectra varied with instrument. Not all instrument response was removed by the corrections used in this study using manufacturer-generated correction factors. Inter-instrument comparison post-correction was greater than the measured instrumental error. The Fluoromax-3 fluorometer showed the best overlap with the reference quinine spectrum after instrument specific correction factors were applied. Thus, for the instruments and the scan parameters used in this study, the Fluoromax-3 fluorometer yielded the most accurate spectra.

We recommend comparison of manufacturer-generated emission correction factors by comparing to the NIST spectrum of quinine sulfate as done in this study. Emission correction factors should be applied to sample spectra assuming improved overlap with the NIST spectrum after correction. If a poor overlap with the NIST quinine sulfate spectrum is observed due to inadequate or unavailable correction factors, it is still possible to analyze the dominant source of the fulvic acid by comparing trends in the FI values for a set of samples, as long as it is clearly stated that correction factors have not been applied, and the trends shown are relative.

When using a fluorometer with scanning capability, we recommend that the fluorescence index (FI) values be presented along with the position of the emission peak maximum (peak position). Solutions of end-member fulvic acids, PLFA and SRFA at pH 6.0 to 7.0, analyzed on all fluorometers had FI emission peak positions between 453-464 nm and 477-480 nm, respectively after correction, and it follows that most aquatic DOM samples should have an FI emission peak within this range. The FI peak position serves as one indication of the performance of the instrument. If the FI peak position is found to be within the range determined to be correct by this study, the FI should be calculated as the ratio of emission intensities at 470 over 520 nm. Likewise, if insensible FI values are obtained for a given sample, it may be because the sample lacks the combination of fluorescing constituents that give rise to a broad emission curve with a maximum between 450-480 nm at an excitation of 370 nm.

Given the similarity in fluorescing components identified with a PARAFAC analysis of EEM datasets collected across different freshwater and marine systems (Coble 1996; Cory and McKnight 2005; Fellman et al. 2009a; Fulton et al. 2004; Murphy et al. 2008; Stedmon et al. 2003), results from this study suggest that it would be helpful to compare independent models derived from replicate datasets analyzed on various instruments. This comparison may be a better test on whether or not a statistical model developed from data collected on one instrument are the same as those developed on another. Importantly, this kind of cross-comparison would help identify whether slight differences in component shapes across systems are due to instrument bias or variability, or due to the quality of the DOM.

The chemical character of DOM depends on the dynamic interplay between DOM sources and biochemical reactions, both of which may shift in response to changes in hydrologic regime or land cover (Fellman et al. 2009b; Jaffe et al. 2008). The fluorescence signature of DOM is increasingly used as a proxy measurement to assess shifts in DOM composition occurring in response to changes in the watershed (Huang and Chen 2009). To implement this approach most effectively, it is necessary to obtain fluorescence spectra of DOM that are independent of instrument, analyst, or laboratory. Fully corrected fluorescence spectra should be accurate, thus satisfying the latter criteria (DeRose et al. 2007; Lakowicz 1999). However, results from this study demonstrated that applying manufacturer-generated instrument correction factors did not fully account for instrument biases. We recommend continued evaluation of overlap between spectra obtained in different laboratories including an investigation of routinely updated user-generated correction factors (Holbrook et al. 2006).

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