

New light on a dark subject: comment

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Abstract In their article, “New light on a dark subject: On the use of fluorescence data to deduce redox states of natural organic matter,” Macalady and Walton-Day (2009) subjected natural organic matter (NOM) samples to oxidation, reduction, and photochemical transformation. Fluorescence spectra were obtained on samples, which were diluted “to bring maximum uv-visible absorbance values below 1.0.” The spectra were fit to the Cory and McKnight (2005) parallel factor analysis (PARAFAC) model, and consistent variation in the redox state of quinone-like moieties was not detected. Based on these results they concluded that fitting fluorescence spectra to the Cory and McKnight (2005) PARAFAC model to obtain information about the redox state of quinone-like moieties in NOM is problematic. Recognizing that collection and correction of fluorescence spectra requires consideration of many factors, we investigated the potential for inner-filter effects to obscure the ability of fluorescence spectroscopy to quantify the redox state of quinone-like moieties. We

collected fluorescence spectra on Pony Lake and Suwannee River fulvic acid standards that were diluted to cover a range of absorbance wavelengths, and fit these spectra to the Cory and McKnight (2005) PARAFAC model. Our results suggest that, in order for the commonly used inner-filter correction to effectively remove inner-filter effects, samples should be diluted such that the absorbance at 254 nm is less than 0.3 prior to the collection of fluorescence spectra. This finding indicates that inner-filter effects may have obscured changes in the redox signature of fluorescence spectra of the highly absorbing samples studied by Macalady and Walton-Day (2009).

Keywords Inner-filter effect · PARAFAC · Quinone · Redox · Fluorescence

Introduction

In recent years, there have been rapid advances in the use of fluorescence techniques to gain insights into the complex chemical character of natural organic matter (NOM) in environmental samples (e.g. McKnight et al. 2001; Stedmon et al. 2003; Yamashita and Tanoue 2008). One challenge is that collection and correction of fluorescence spectra of water samples requires accounting for many factors that influence the fluorescence signal. In their article, “New light on a dark subject: On the use of fluorescence data to deduce redox states of natural organic matter,” Macalady and Walton-Day (2009) report the results of a study designed to detect changes in the fluorescence signal of samples due to oxidation, reduction, and photochemical transformation of NOM. The authors employed analytical methods that were based on those presented by Cory and McKnight (2005), obtaining

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fluorescence spectra followed by parallel factor analysis (PARAFAC), to characterize the NOM in their samples. The authors conclude that using this method to learn about the redox state of quinone-like moieties in NOM is problematic. Our own analytical work, past and ongoing, as well as the work of others is in disagreement with the results of this study, and suggests that fluorescence spectroscopy and PARAFAC are indeed useful tools that can provide insights into the redox state of quinone-like moieties in NOM (Cory and McKnight 2005; Miller et al. 2006; Mladenov et al. 2008).

Here, we present evidence that one possible reason for the discrepancy among these studies is that Macalady and Walton-Day (2009) obtained fluorescence spectra on samples that were too dark for the spectra to be adequately corrected for the inner-filter effect, which would likely have obscured a change in redox signature and influenced their conclusions. Specifically, their spectra were collected on samples that were diluted only “to bring maximum UV–visible absorbance values below 1.0”. We agree that further studies of this nature are needed to determine the applicability of fluorescence and PARAFAC methods and their potential to characterize NOM in many fields of environmental science. However, the analytical issues in this study related to inner-filter effects warrant further evaluation and comment.

Characterization of natural organic matter using fluorescence

Fluorescence spectroscopy has proven to be a useful tool for investigating the chemical character of NOM, including redox state, in field and laboratory settings. Using fluorescence spectroscopy, chromatographic separation, and isotopic analyses, Hood et al. (2005) showed that there was a seasonal shift in the chemical composition of dissolved organic matter (DOM) in subalpine and alpine reaches of a catchment in the Colorado Front Range. Specifically, it was demonstrated that alpine lakes in the catchment act as sources of microbially derived DOM in the summer months. Reporting on a field study that took place in the alpine reaches of the same catchment studied by Hood et al. (2005), Miller et al. (2006) reported the results of a stream tracer experiment, where a conservative solute was injected into the stream to characterize exchange between the stream and an adjacent wetland. They were the first to develop the fluorescence/PARAFAC based redox index (RI) to investigate concomitant production/decay of DOM and changes in DOM redox state associated with hydrologic exchange across the large redox gradients of the stream and its hyporheic zone. More recently, Fimmen et al. (2007) used fluorescence and electrochemical

reduction to examine the relationship between redox state and fluorescence spectra of different DOM isolates.

While fluorescence spectroscopy has become a widely used and effective tool for characterizing NOM, there are multiple factors that should be considered and accounted for when collecting and analyzing fluorescence spectra. The inner-filter effect has been shown to be an important consideration when interpreting data from fluorescence spectra (Tucker et al. 1992). The inner-filter effect is the attenuation of light by the solution prior to detection by the fluorometer. The primary inner-filter effect occurs due to absorption of the excitation light prior to reaching the fluorescent molecule, whereas the secondary inner-filter effect refers to absorption of the light emitted from the fluorescent molecule prior to detection by the fluorometer. Therefore, the inner-filter effect is most pronounced in highly absorbing samples, and has the potential to influence the fluorescence signal. In order to obtain a NOM fluorescence signal that is not influenced by the inner-filter effect, it is critical to correct most fluorescence spectra with an inner-filter correction (e.g. Mobed et al. 1996; McKnight et al. 2001). The most commonly used inner-filter correction (McKnight et al. 2001) assumes that the emission spectrometer views only a small illuminated volume in the center of the 1 cm cell and that the effective pathlength is 0.5 cm for both the excitation light (to the center of the cell) and the emitted light (to the emission monochromator). However, application of this inner-filter correction alone is not sufficient for samples with high absorbance values because the simplification of using a 0.5 cm effective pathlength does not adequately represent the absorbance of excited and emitted light. Thus, highly absorbing samples must be diluted prior to collection of fluorescence spectra for this correction to be applied properly. For example, Ohno (2002) showed that, when calculating humification index (HIX) values from fluorescence spectra, the inner-filter correction did not remove the influence of inner-filter effects in samples with absorbance values at 254 nm greater than 0.3.

Materials and methods

To investigate the ability of this commonly used inner-filter correction to remove the influence of the inner-filter effect from two-dimensional fluorescence spectra, and three-dimensional fluorescence spectra fit to an existing PARAFAC model, we collected fluorescence spectra on Pony Lake fulvic acid (PLFA) and Suwannee River fulvic acid (SRFA) standards that were diluted with Milli-Q water to cover a range of absorbance values at 254 nm (A_{254}) from 0.05 to 1.0. Here we report absorbance (A_{254}) as the

dimensionless value obtained from the spectrophotometer using a 1 cm pathlength cuvette according to:

$$A_{254} = \log_{10}(1/T_{254}) \quad (1)$$

where T_{254} is the fraction of light transmitted through the sample at a wavelength of 254 nm. PLFA and SRFA are commercially available end-member fulvic acids supplied by the International Humic Substances Society (IHSS). PLFA represents microbially-derived NOM and SRFA represents terrestrially-derived NOM. Standard solutions were adjusted to pH 4 or pH 7 with 0.1 N NaOH and/or 0.1 N HCl. Absorbance and fluorescence spectra were collected and corrected using the same methods that were used by Macalady and Walton-Day (2009) and are described in detail in Cory and McKnight (2005). Fluorescence scans were collected in ratio mode and a Milli-Q water blank was subtracted from each scan. In addition, scans were corrected for excitation and emission, and normalized to the area under the Raman peak. A commonly used inner-filter correction that assumes an effective pathlength of 0.5 cm for both excitation and emitted light was applied to all scans.

For a given sample, in the absence of inner-filter effects, two-dimensional fluorescence spectra at a given excitation wavelength are expected to have similar shapes regardless of the A_{254} of the sample. To investigate the ability of the inner-filter correction to remove inner-filter effects at high A_{254} values from non-PARAFAC fluorescence results, we compared corrected two-dimensional fluorescence spectra of PLFA at pH 4, at an excitation wavelength of 370 nm and covering a range of emission wavelengths, at various A_{254} values. The excitation wavelength of 370 nm was chosen because it is the wavelength used to calculate the commonly reported fluorescence index (FI; McKnight et al. 2001). The peak intensities of the spectra occurred at 460 ± 2 nm for each A_{254} , and these peak intensities were normalized to a value of 1 to allow for easier comparison of spectral shape.

Corrected three-dimensional fluorescence spectra were fit to the PARAFAC model of Cory and McKnight (2005), which resolves the three-dimensional fluorescence scans into 13-components. Model fit was investigated by comparing the absolute values of the intensities of the residual excitation–emission matrices (EEMs), calculated as the difference between the measured and PARAFAC modeled EEMs, with intensities in the measured EEMs. In the absence of inner-filter effects, measured F_{\max} values (in Raman units) for a given PARAFAC component are expected to increase linearly as absorbance increases. To predict F_{\max} values in the absence of any inner-filter effects (herein referred to as “predicted values”), a linear line-of-best-fit was determined based on the measured F_{\max} values for each component covering a range of A_{254} from 0.05 to

0.3, where the inner-filter effect is least pronounced and a linear relationship between A_{254} and F_{\max} is expected. The equations for these lines were then used to predict the F_{\max} values for the quinone-like and amino acid-like components identified by the Cory and McKnight (2005) PARAFAC model for both PLFA and SRFA at pH 4 and 7 covering an A_{254} range of 0.05–1.0. The assumption of a linear increase in F_{\max} when $A_{254} \leq 0.3$ was verified by investigation of R^2 values. Threshold A_{254} values, above which the inner-filter correction is no longer able to correct for inner-filter effects, were calculated for each of the quinone-like and amino acid-like components identified by the Cory and McKnight (2005) PARAFAC model for both fulvic acids at pH 4 and 7. Threshold A_{254} values were determined as the A_{254} values above which the difference between the measured and predicted F_{\max} was consistently greater than 5% for a given component.

Results and discussion

Comparison of corrected two-dimensional fluorescence spectra (Ex. = 370 nm) of PLFA at pH 4 at various A_{254} values indicates that the inner-filter correction did not adequately remove inner-filter effects from non-PARAFAC fluorescence spectra at high A_{254} values (Fig. 1). While the two spectra collected at A_{254} values of 0.1 and 0.3 had nearly identical shapes, the shape of the spectra at $A_{254} = 1.0$ was broader. This flattening of the peak at high absorbance would be expected in response to inner-filter effects. These results indicate that differences between measured and predicted PARAFAC component loadings are due to inner-filter effects, and not error associated with

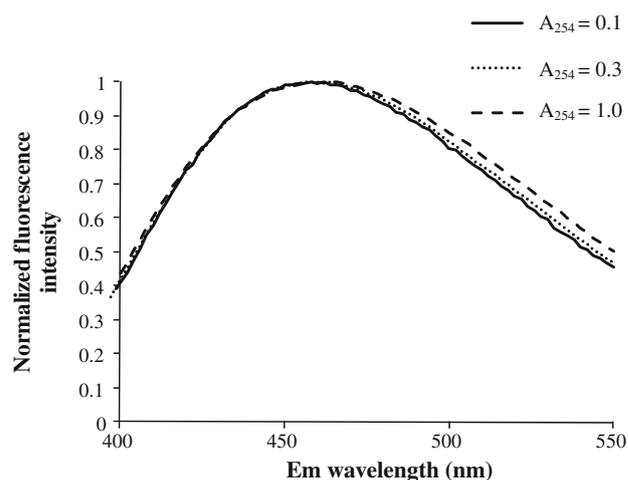


Fig. 1 Emission spectra (Ex. = 370 nm) of corrected PLFA at pH 4 at absorbance values at 254 nm (A_{254}) of 0.1, 0.3, and 1.0. Peak intensities of each spectra were normalized to a value of 1 to allow for easier comparison between spectra

the assignment of fluorescence to each of the predefined PARAFAC components.

For all three-dimensional fluorescence spectra, intensities in the residual EEMs were <10% of the intensities in the measured EEMs. This indicates that all fluorescence scans were fit well by the Cory and McKnight (2005) PARAFAC model (Mladenov et al. 2008). The R^2 values comparing the measured and predicted F_{\max} values at $A_{254} \leq 0.3$ were greater than 0.99 for all quinone-like components for both PLFA and SRFA at pH 4 and 7, with the exception of the SQ3 component identified in SRFA at pH 4. This indicates that, with the exception of the SRFA-pH 4 SQ3 component, the inner-filter effect had been adequately accounted for at $A_{254} \leq 0.3$ by applying the correction. For the amino acid-like components, R^2 values were greater than 0.99 for PLFA-pH 7 as well as the tryptophan-like component for PLFA-pH 4 and SRFA-pH 7. For all other amino acid-like components identified in other samples, there was not a strong linear relationship between measured F_{\max} values and A_{254} in the A_{254} range of 0.05–0.3. Therefore, A_{254} threshold values were not calculated for these components (Table 1). The components for which there was a non-linear relationship between F_{\max} and A_{254} (at $A_{254} \leq 0.3$) contributed $\leq 2\%$ of the total fluorescence to the EEMs. This suggests that there may be a threshold of percent contribution of a given component to the EEM, below which there is a non-linear relationship between F_{\max} and A_{254} .

Applying the criteria outlined above for determining A_{254} threshold values, above which the inner-filter correction is no longer able to account for inner-filter effects, resulted in threshold values for quinone-like components ranging from 0.3 to 0.8 (Table 1). Figure 2 provides an example visual representation of the A_{254} threshold values listed in Table 1 for PLFA at pH 4. Measured F_{\max} values of different components deviated from the predicted values in different directions at A_{254} values greater than the threshold values (Fig. 2). For example, for PLFA at pH 4, the measured F_{\max} values were less than the predicted values for the SQ2 and SQ3 components, whereas the measured values were greater than the predicted values for HQ and the oxidized quinone-like components (Q1, Q2, and Q3). The directions of deviation above A_{254} threshold values were not the same for different fulvic acids at different pH values. In addition to being dependent upon the PARAFAC component in question, the A_{254} threshold values were also dependent on the fulvic acid being modeled and the pH of the sample (Table 1). The generally higher A_{254} thresholds for quinone-like components associated with PLFA as compared to SRFA, and pH 7 as compared to pH 4, is likely a function of differences in the chemical composition of the NOM of these samples.

Our results suggest that the ability of the inner-filter correction to remove the inner-filter effect at a given A_{254}

Table 1 Absorbance values at a wavelength of 254 nm (A_{254}) above which a commonly used inner-filter correction is no longer able to correct for inner-filter effects

Component	PLFA		SRFA	
	pH 4	pH 7	pH 4	pH 7
Reduced Quinone-like components				
SQ1	× ^a	× ^a	0.5	0.8
SQ2	0.4	0.4	0.3	0.3
SQ3	0.6	0.7	NL ^b	0.3
HQ	0.6	0.6	0.5	0.6
Oxidized Quinone-like components				
Q1	0.6	0.6	0.3	0.8
Q2	0.6	0.6	0.3	0.6
Q3	0.6	0.4	0.3	0.7
Amino acid-like components				
Tryptophan	0.6	0.35	NL ^b	0.2
Tyrosine	NL ^b	0.3	NL ^b	NL ^b

Threshold A_{254} values are shown for the quinone-like and amino acid-like components identified by the Cory and McKnight (2005) PARAFAC model for Pony Lake fulvic acid (PLFA) and Suwannee River fulvic acid (SRFA) at pH 4 and 7. Threshold A_{254} values were determined as the A_{254} values above which the difference between measured F_{\max} and predicted F_{\max} (assuming a linear increase in F_{\max} with increasing A_{254}) is consistently greater than 5%

^a '×' indicates that the measured F_{\max} values did not deviate more than 5% from the predicted values for the entire range of A_{254} values investigated

^b 'NL' indicates that there was not a linear increase in measured F_{\max} values over any range of A_{254} values. These components contributed $\leq 2\%$ of the total fluorescence to the EEMs

is dependent upon the PARAFAC component in question as well as the source and chemical quality of the NOM in the samples. Based on the range of measured A_{254} threshold values for quinone-like components (0.3–0.8), we recommend that, for the purpose of accurately identifying and quantifying quinone-like fluorophores using the Cory and McKnight (2005) PARAFAC model, samples should be diluted to A_{254} values less than 0.3 prior to collection of fluorescence spectra. If samples are not diluted to A_{254} values less than 0.3, then fluorescence based measures that are dependent of the ability of the PARAFAC model to quantify quinone-like moieties (e.g. fraction of total fluorescence from quinone-like moieties, RI) are likely to be influenced by inner-filter effects. Given that the tyrosine-like components, identified at low intensities in the fulvic acid solutions, did not follow a strong linear-trend in the A_{254} range of 0.05–0.3 (Table 1), our results are not sufficient to comprehensively evaluate the effects of increasing A_{254} on PARAFAC model fit of the amino acid-like fluorophores. However, for non-fulvic acid samples that have higher fluorescence intensities in the region of the EEM where

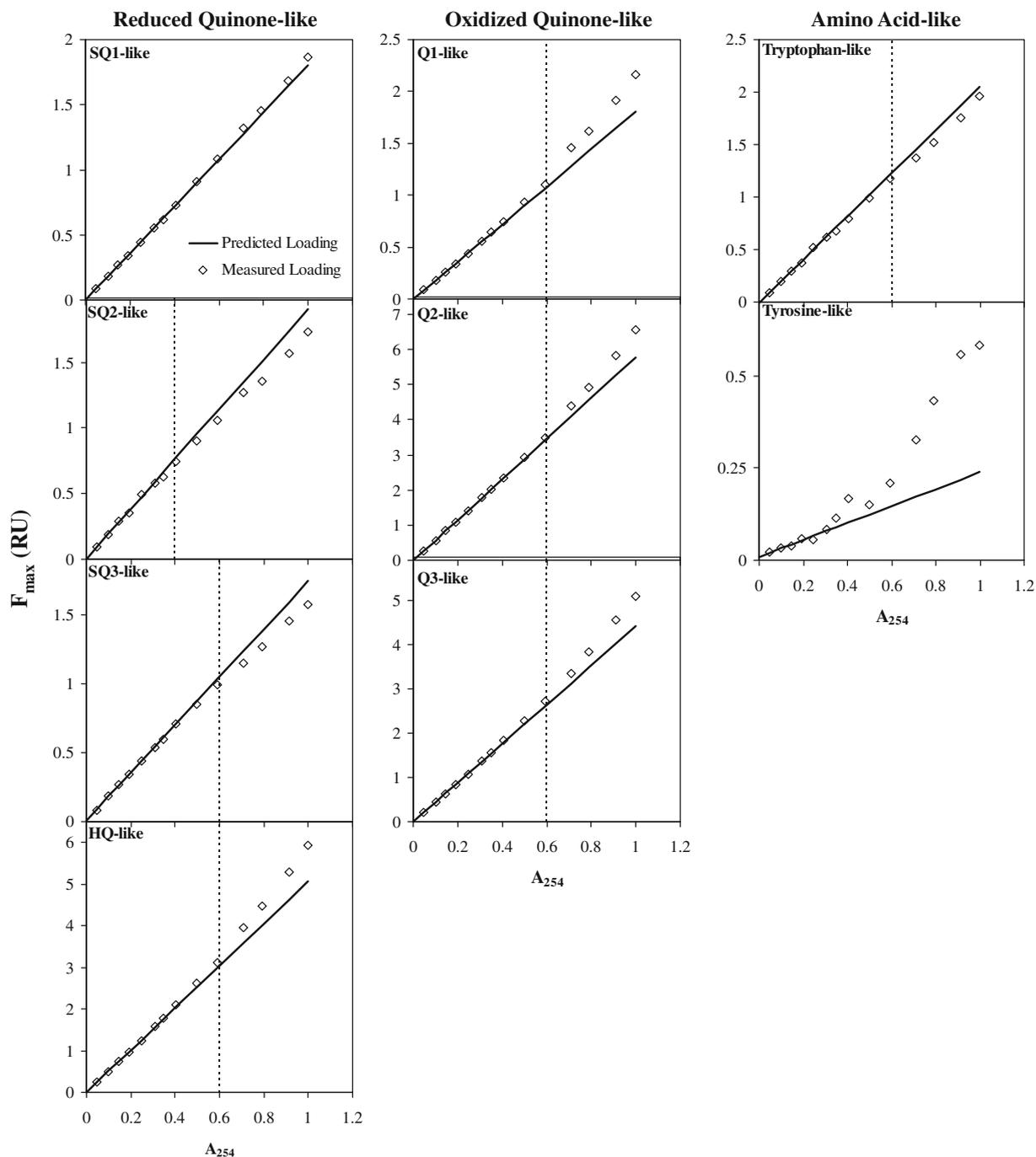


Fig. 2 Changes in F_{\max} values (Raman units) of corrected Pony Lake fulvic acid (PLFA) at pH 4, determined by PARAFAC modeling, for quinone-like and amino acid-like components as a function of increasing absorbance values at 254 nm (A_{254}). The points represent measured F_{\max} values and the solid lines represent the predicted F_{\max} values in the absence of inner-filter effects. Predicted values were calculated based on a linear line-of-best-fit derived from the measured F_{\max} values covering a range of A_{254} values from 0.05 to 0.3, where

amino acids fluoresce, it would be possible to evaluate PARAFAC model fit of the amino acid-like fluorophores in the context of increasing A_{254} .

the inner-filter effect is least pronounced. All reduced quinone-like (SQ1, SQ2, SQ3, and HQ), oxidized quinone-like (Q1, Q2, and Q3) and amino acid-like (tyrosine and tryptophan) components were identified by the Cory and McKnight (2005) PARAFAC model. Vertical dashed lines indicate the A_{254} threshold values. A_{254} threshold values were not identified for SQ1 or the tyrosine-like component. Note the different scales on the y-axes

In their paper, Macalady and Walton-Day (2009) state that, "All dilutions were made with MilliQ water and for untreated samples these were made to bring maximum

UV-visible absorbance values below 1.0.” The authors did not report the wavelength at which absorbance values were diluted to be less than 1.0. Given the “exponential decay-like” form of NOM absorbance spectra, it is likely that, for a sample with a maximum absorbance of 1.0, the A_{254} for the sample would not be less than the value of 0.3 identified above as being the cutoff at which the inner-filter correction is no longer effective for quantifying quinone-like moieties using the Cory and McKnight (2005) PARAFAC model. Macalady and Walton-Day (2009) observed that there was not a shift in PARAFAC component distributions that indicated a consistent change in NOM redox state after experimentally altering the redox state of quinones in NOM. This observation and the subsequent conclusion that “the assignment of components in the PARAFAC model to quinone-like moieties is questionable” would have been strongly influenced by the inability of the inner-filter correction to remove inner-filter effects from their highly absorbing samples. Though we cannot be sure of the A_{254} values of the samples analyzed as part of the Macalady and Walton-Day (2009) study, we encourage all authors to include sufficient analytical details in order to meaningfully compare results.

In the discussion section of the manuscript, Macalady and Walton-Day (2009) cite the five component PARAFAC model presented in Stedmon et al. (2003) and eight component model presented in Stedmon and Markager (2005) as making no claim of fluorophore components from quinone-like moieties. While this is true, it does not indicate that PARAFAC modeling of a dataset of samples from diverse sources, and with diverse chemical composition, is not able to identify components representative of quinone-like moieties. We would like to address the point that a large number of samples are not a guarantee for building a PARAFAC model with a large number of components. Rather, the number of components that are identified by a PARAFAC model is limited by the diversity of the source and chemical composition of the NOM in the dataset. For example, the thirteen component model built by Cory and McKnight (2005), which clearly identified the presence of redox active quinone-like components, included not only diverse source samples including samples from Antarctica (a terrestrial plant-free continent) but also a full range of redox conditions (e.g. anoxic lake bottom waters and oxidized stream water).

In conclusion, we agree that more work is required to effectively evaluate the ability of PARAFAC modeling to quantitatively characterize the redox state of quinone-like moieties associated with NOM. However, the central conclusions of Macalady and Walton-Day (2009), that the Cory and McKnight (2005) PARAFAC model “did not indicate consistent oxidation or reduction of quinones

resulting from experiments designed to alter the redox status of quinones in NOM”, and that “the assignment of components in the PARAFAC model to quinone-like moieties is questionable”, should be assessed in the context of their spectral characterization techniques. Specifically, they do not provide enough evidence to document that their samples, which were diluted “to bring maximum UV-visible absorbance values below 1.0”, were not influenced by the inner-filter effect. Our results suggest that, for the purposes of quantifying quinone-like moieties in NOM with the Cory and McKnight (2005) PARAFAC model, samples should be diluted such that the absorbance at 254 nm is less than 0.3 prior to collection of fluorescence spectra. We welcome future studies on the use of PARAFAC to identify the redox state of quinone-like moieties, and have highlighted some of the important aspects of NOM fluorescence that should be considered in future investigations of this topic.

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