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Research Article

New light on a dark subject: On the use of fluorescence data to deduce redox states of natural organic matter (NOM)

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Abstract. This paper reports the use of excitation-emission matrix fluorescence spectroscopy (EEMS), parallel factor statistical analysis (PARAFAC), and oxidation-reduction experiments to examine the effect of redox conditions on PARAFAC model results for aqueous samples rich in natural organic matter. Fifty-four aqueous samples from 11 different geographic locations and two plant extracts were analyzed untreated and after chemical treatments or irradiation were used in attempts to change the redox status of the natural organic matter. The EEMS spectra were generated and modeled using a PARAFAC package developed by Cory and McKnight (2005). The PARAFAC model output was examined for consistency with previously reported relations and with changes expected to occur upon experimental oxidation and reduction of aqueous samples. Results indicate the implied fraction of total sample fluorescence attributed to quinone-like moieties was consistent (0.64 to 0.78) and greater than that observed by Cory and McKnight (2005). The fraction of the quinone-like moieties that was reduced (the reducing index, RI) showed relatively little variation (0.46 to 0.71) despite attempts to alter the redox status of the natural organic matter. The RI changed little after reducing samples using zinc metal, oxidizing at high pH with air, or irradiating with a Xenon lamp. Our results, however, are consistent with the correlations between the fluorescence indices (FI) of samples and the ratio of PARAFAC fitting parameters suggested by Cory and McKnight (2005), though we used samples with a much narrower range of FI values.

Key words. Fluorescence; redox status; natural organic matter; PARAFAC; EEMS.

Introduction

The ability of aqueous natural organic matter (NOM) to mediate and facilitate a wide variety of redox reactions has been recognized and under investigation for several decades (Skogerboe and Wilson, 1981; Dunnivant, et al., 1992; Macalady and Ranville, 1998; Struyk and Sposito, 2001; Redman, et al., 2002; Kappler and Haderlein, 2003; Peretyazhko and Sposito, 2006). Aqueous phase NOM includes, in this regard, operational isolates (e.g. humic and fulvic acids), natural waters rich in NOM, and NOM precursor solutions made as aquatic extracts of senescent plant materials.

An emerging consensus indicates that quinone or quinone-like moieties in NOM are largely responsible for the observed reversible redox behavior in NOM samples (Tratnyek and Macalady, 1989; Schwarzen-

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bach, et al., 1990; Nurmi and Tratnyek, 2002; Cory and McKnight, 2005). Remaining uncertainties, however, include the extents of the roles of quinones in NOM redox reactions and an incomplete understanding of the nature of NOM redox activity (Ratasuk and Nanny, 2007). For example, results of several studies have indicated that minor fractions of the reducing capacity in NOM samples persist during long periods of exposure to light and molecular oxygen that should oxidize the NOM (Macalady and Ranville, 1998; Klapper, et al., 2002; Bauer, et al., 2007). Therefore, the ability to accurately and routinely assess the redox status of NOM samples is of considerable interest in the aquatic sciences.

The emergence of excitation-emission matrix fluorescence spectroscopy (EEMS) has provided a potentially useful tool for investigations of the redox capacities and status of aqueous NOM samples. In particular, parallel factor statistical analyses (PARAFAC) of EEMS spectral data have been developed that show promise for enabling the quantification of the relative redox capacities and redox states of NOM samples (Stedmon et al., 2003; Chen et al., 2003a, b; Cory and McKnight, 2005; Stedmon and Markager, 2005; Miller et al., 2006). To the extent that they accurately represent the characteristics of the sample, some of these PARAFAC models identify and quantify the total fraction of the fluorescence of a NOM sample that originates from quinone-like moieties and the portion of this quinone-like activity that results from reduced (hydroquinones and semi-quinones) and oxidized (quinones) forms of the quinone-like moieties.

The PARAFAC model developed in the laboratories of Diane McKnight at the University of Colorado includes calculation of a "reducing index" for NOM samples that is defined as the ratio of reduced to oxidized quinone-like fractions identified from EEMS spectra by the PARAFAC analyses (Cory and McKnight, 2005; Miller et al., 2006). This index has been applied to rationalize seasonal changes in redox parameters involving the production and decay of dissolved organic matter (DOM) in natural waters (Hood et al., 2005; Miller et al., 2006; Fimmen et al., 2007).

Here we attempt to establish whether or not this statistical approach can be applied to reliably predict the redox capacity and oxidation/reduction status of NOM in waters dominated by allochthonous NOM from organic litter from forested catchments, or other NOM sources such as grasses and mosses. Accordingly, we obtained EEMS spectra for a wide variety of NOM samples that meet the above criteria. EEMS spectra were also collected from some of these samples after subjection to processes designed to change the redox state of the quinone-like moieties, or at least some of them. The resulting EEMS spectra were analyzed using this PARAFAC model and the utility of the statistical results evaluated.

Experimental considerations

Natural waters or aqueous extracts of senescent, single species plant materials were stored at 4 °C from the time of sampling or isolation until analyses. Natural water samples were all collected by the authors and used without dilution except as noted. Samples of natural waters from New Zealand, Brazil, Canada, and the United States are included. Sampling sites are indicated in Table 1 and sampling protocols are available from the authors upon request. Organic carbon contents of natural waters at sampling time ranged from 1 to 5 mmole CL^{-1} (Seivers 800 Total Organic Carbon Analyzer). No significant changes in organic carbon contents were observed for any of the samples during storage at 4 °C for periods of up to 20 years (data not shown). Concerns about the long storage times for some of the samples can be raised but they are somewhat obviated by the results of samples from 2008 that are consistent with samples stored for longer periods. Also, samples from the same sites involving different storage times showed no apparent differences (q.v.). Certainly changes occur during storage, but there is no evidence that the NOM content or other pertinent characteristics of the NOM samples (e.g. pH, SUVA) changed substantially during long storage at 4 °C. In any case, minor changes that occurred between sample collection and the time of these analyses are not relevant. We are comparing the samples as they existed at the time of analyses with the same samples subjected to processes designed to oxidize or reduce them.

Plant extracts included here were walnut (turnings from American black walnut heart wood, widely known to contain abundant quinones, extract prepared and isolated at Colorado School of Mines (CSM) in 2005) and sphagnum moss (air dried moss obtained from a bog near Eagle, Alaska, prepared and isolated at CSM in 2006). The extracts were prepared by soaking plant materials in distilled water for periods in excess of 90 days, after which they were screened to remove gross solids and centrifuged prior to analyses. Organic carbon contents of the plant extracts were sufficiently high that significant dilution with MilliQ water was required for the present study. Several natural water sites were sampled at multiple times (Table 1). Seasonal variations in NOM properties with time occur at the natural water sites but are not considered in the present study. Values of pH

Table 1. Calculated Results from the 13-factor PARAFAC analyses of the EEMS taken for the 54 NOM samples used in this study. [No., sample number; EEMS, excitation emission matrix spectra; 1/15, 1/5, 1/9, 1/4, 1/3 refer to volumetric dilution of samples; a and b = two different aliquots of sample having the same volumetric dilution; CO, Colorado; NZ, New Zealand; MI, Michigan, AK, Alaska; VA, Virginia; FL, Florida; OR, Oregon; BC, British Columbia]

No.	Sample Designation or Location, Dilution, and Collection Year (where relevant)	Treatment	Date of EEMS Analysis	Total Fraction Quinones	Reduced Quinones	Reducing Index, RI	Fluorescence Index, FI	Fraction Protein
1	Walnut extract, 1/15 a	Untreated	14-May-08	0.74	0.53	0.71	1.23	0.04
2	Walnut extract, 1/15 b	Untreated	14-May-08	0.74	0.51	0.69	1.28	0.05
3	Walnut extract, 1/15 b (repeat)	Untreated	14-May-08	0.74	0.51	0.69	1.28	0.05
4	Walnut extract, 1/15 b	Irradiated	14-May-08	0.71	0.47	0.66	1.23	0.04
5	Walnut extract, 1/15 b	Reduced	14-May-08	0.78	0.48	0.62	1.31	0.07
6	Walnut extract, 1/15 b (repeat)	Reduced	14-May-08	0.77	0.49	0.63	1.28	0.07
7	Walnut extract, 1/15 b	Zn(II) added	19-May-08	0.73	0.51	0.70	1.23	0.05
8	Walnut extract, 1/9	Untreated	11-Aug-07	0.69	0.47	0.68	1.30	0.04
9	Walnut extract, 1/9 (repeat)	Untreated	11-Aug-07	0.70	0.48	0.69	1.26	0.04
10	Well MW-6, CO, 1/5, 2003	Untreated	14-May-08	0.69	0.35	0.51	1.48	0.04
11	Well MW-6, CO, 1/5, 1989	Untreated	2-May-08	0.61	0.29	0.47	1.53	0.08
12	Well MW-6, CO, 1/5, 2003	Oxidized	2-May-08	0.60	0.28	0.46	1.54	0.05
13	Well MW-6, CO, 1/5, 2003	Irradiated	14-May-08	0.69	0.34	0.49	1.51	0.04
14	Well MW-6, CO, 1/5, 2003	Untreated	14-Jun-07	0.70	0.34	0.49	1.50	0.03
15	Inangahua River, NZ, 2008	Untreated	14-May-08	0.74	0.44	0.59	1.31	0.03
16	Inangahua River, NZ, 2008	Irradiated	14-May-08	0.71	0.39	0.56	1.28	0.03
17	Inangahua River, NZ, 2008	Reduced	14-May-08	0.76	0.44	0.58	1.34	0.05
18	Inangahua River, NZ, 2008	Zn(II) added	19-May-08	0.70	0.42	0.60	1.36	0.09
19	Inangahua River, NZ, 1995	Untreated	6-Jun-07	0.75	0.45	0.60	1.30	0.02
20	Black Creek, NZ, 2008	Untreated	14-May-08	0.73	0.46	0.63	1.20	0.03
21	Black Creek, NZ, 2008	Irradiated	14-May-08	0.71	0.42	0.59	1.22	0.03
22	Black Creek, NZ, 2008	Reduced	14-May-08	0.76	0.45	0.60	1.29	0.04
23	Black Creek, NZ, 2008	Zn(II) added	19-May-08	0.68	0.39	0.58	1.20	0.07
24	Black River, MI, 2001	Untreated	14-May-08	0.74	0.40	0.55	1.34	0.03
25	Black River, MI, 2001	Untreated	2-May-08	0.64	0.35	0.55	1.27	0.03
26	Black River, MI, 2001	Oxidized	2-May-08	0.69	0.38	0.55	1.35	0.02
27	Black River, MI, 2001	Irradiated	14-May-08	0.69	0.36	0.52	1.29	0.03
28	Black River, MI, 2001	Reduced	14-May-08	0.74	0.39	0.53	1.37	0.04
29	Black River, MI, 2001	Zn(II) added	19-May-08	0.71	0.38	0.54	1.28	0.04
30	Black River, MI, 2001	pH = 2	19-May-08	0.67	0.33	0.50	1.28	0.05
31	Black River, MI, 2001	pH = 9	19-May-08	0.74	0.39	0.53	1.38	0.08
32	Black River, MI, 2001	Untreated	14-Jun-07	0.70	0.35	0.51	1.29	0.03
33	Black River, MI, 2005	Untreated	14-Jun-07	0.70	0.36	0.51	1.24	0.03
34	Black River, MI, 2001 (repeat)	Untreated	14-Jun-07	0.70	0.36	0.52	1.27	0.03
35	40Mile River, AK, 2003	Untreated	2-May-08	0.70	0.33	0.47	1.24	0.04
36	40Mile River, AK 2003 (repeat)	Untreated	2-May-08	0.70	0.33	0.48	1.24	0.04
37	40Mile River, AK, 2003	Oxidized	2-May-08	0.69	0.31	0.46	1.23	0.04
38	40Mile River, AK, 2003	Untreated	6-Jun-07	0.74	0.37	0.50	1.38	0.03
39	Chincoteague Island, VA, 2002	Untreated	2-May-08	0.68	0.37	0.54	1.26	0.02
40	Chincoteague Island, VA, 2002	Oxidized	2-May-08	0.69	0.38	0.55	1.28	0.02
41	Chincoteague Island, VA, 2002	Untreated	14-Jun-07	0.73	0.39	0.53	1.25	0.03
42	Everglades, FL, 2002	Untreated	2-May-08	0.65	0.33	0.50	1.38	0.03
43	Everglades, FL, 2002 (repeat)	Untreated	2-May-08	0.66	0.33	0.50	1.37	0.03
44	Everglades, FL, 2002	Oxidized	2-May-08	0.68	0.35	0.51	1.40	0.03
45	Everglades, FL, 1/3, 2002	Untreated	6-Jun-07	0.73	0.34	0.46	1.32	0.04
46	Sphagnum Moss Extract, 1/5	Untreated	2-May-08	0.57	0.30	0.53	1.30	0.05
47	Sphagnum Moss Extract, 1/5	Oxidized	2-May-08	0.56	0.28	0.50	1.28	0.04
48	Sphagnum Moss Extract, 1/4	Untreated	11-Aug-07	0.61	0.32	0.53	1.31	0.03
49	Suwannee River, FL, 1/4, 2002	Untreated	6-Jun-07	0.73	0.40	0.55	1.23	0.01
50	Suwannee River, FL, 1/3, 1989	Untreated	6-Jun-07	0.72	0.38	0.53	1.28	0.02
51	Sunset Bay, OR, 2001	Untreated	14-Jun-07	0.77	0.47	0.61	1.21	0.01
52	Mackenzie Creek, Vancouver Island,(Tofino) BC, 2005	Untreated	11-Aug-07	0.67	0.37	0.56	1.28	0.03
53	Rio Negro Brazi;, 2000	Untreated	19-May-08	0.70	0.40	0.57	1.25	0.04
54	Rio Negro, Brazil, 2006	Untreated	19-May-08	0.72	0.42	0.58	1.32	0.03

ranged from about 4 for Suwannee River water to just over 8 for Everglades water. Values of pH were not intentionally adjusted unless otherwise indicated. Some calcium and/or iron-rich samples (e.g. MW-6 groundwater and water from the Everglades) used in this study (Table 1) can show irreversible changes upon pH adjustment or dilution. The most common of such changes is the formation of a precipitate in the

sample, which may or may not, depending on poorly understood variables, re-dissolve when the pH is returned to its original value. As will be discussed below, pH adjustments were made in some of the samples as part of this study.

Aquatic NOM was defined for this study as the organic carbon remaining in solution after centrifugation at 12,000x gravity for 1 hour at 5 °C (Sorvall Evolution RC Centrifuge). However, for some of the earlier samples analyzed in this study (noted below), filtration (0.45 μm cellulose acetate, syringe filter) was used.

All samples were analyzed at the Institute for Arctic and Alpine research (INSTAAR) in the laboratories of Diane McKnight at the University of Colorado. Procedures for obtaining fluorescence and uv-visible spectra have been described in publications originating from this laboratory (Matthew P. Miller, personal communication plus unpublished documents). EEMS spectra and PARAFAC analyses were similarly obtained using procedures developed at INSTAAR (Cory and McKnight, 2005).

All dilutions were made with MilliQ water and for untreated samples these were made to bring maximum uv-visible absorbance values below 1.0. Several samples were analyzed at varying dilutions to investigate the effect of dilution on the EEMS spectra and PARAFAC analyses results. In addition, some samples were analyzed at different dates between June 2007 and May 2008 to check for consistency in the analytical results over an extended time period.

To investigate the effects of oxidation, photochemical processes, and reduction on the EEMS spectra and PARAFAC results, selected samples were treated as described below.

For oxidation, samples were adjusted to pH $\approx$ 11.5 (with 20 % NaOH), where reduced quinones are less stable (have a lower redox potential) and molecular oxygen is a better oxidizing agent (Tratynek and Macalady, 1989, and references therein). Samples were shaken to assure air saturation and allowed to sit at room temperature (ca. 22 °C), with occasional shaking, for 20–24 hours. The pH was then readjusted to near the original pH of the sample with 6 molar (M) HCl immediately prior to analysis. These samples were designated "oxidized." No visible precipitates were observed in the samples during this process, and the samples for which the pH had been altered and then returned to their normal pH had uv-visible spectra (taken as part of the EEMS analyses) that were essentially identical to those of the untreated samples.

For photochemical processes, samples were placed in a 5 cm x 5 cm x 5 cm sealed quartz bottle and placed directly beneath a Xenon arc lamp for 1.5 hours at room temperature (maintained at approximately 22 °C). If dilutions were to be used for the spectrofluorometric analyses, they were made prior to irradiation. Samples treated in this manner are designated "irradiated."

Samples were reduced with zinc (Zn) metal (Tratnek and Macalady, 1989; Blodau et al., 2009). Approximately 100 g of granular Zn was washed with 100 mL of 1.0 M trace metal grade nitric acid (HNO_3) to remove any oxide coatings. The metal was rinsed in a nitrogen (N_2) gas atmosphere four times with 100 mL aliquots of N_2-sparged MilliQ water. Samples to be treated with Zn were sparged for 30 min with ultra pure N_2 before 10–15 g of Zn metal was added to a 40 mL sample, sealed in a 50 ml Nalgene centrifuge tube, and allowed to react with occasional shaking for 12–15 hours. Samples for analyses were decanted from the Zn metal at the time of analysis under a stream of ultra pure N_2 and placed in a sealed quartz spectrophotometer cell. Zn (II) analyses (by ICP-AES) of the filtered samples used for analysis indicated amounts of Zn that had been oxidized ranging from 1.4 to 2.0 mg ZnL^{-1}. Zinc was less than detection ($<$1.0 μg ZnL^{-1}) in all untreated samples. Samples reduced with Zn metal were designated "reduced."

In order to assure that the introduction of Zn (II) to the samples did not itself cause changes in the EEMS spectra and PARAFAC analyses, quantities of 50 mgL^{-1} Zn (II) solution were added to the samples to achieve Zn (II) concentrations equivalent to the reduced samples. Samples so treated are designated as "Zn(II)."

To check for the effect of pH changes on the EEMS spectra and PARAFAC analyses, samples of Black River water (2001) were adjusted to pH 2.0 with 6 M HCl and to pH 9 with 20 % NaOH solution.

Data analyses

The study described herein uses the PARAFAC statistical analyses and the model parameters described in Cory and McKnight (2005) and Miller et al. (2006). Their model identifies and quantifies 13 components in the EEMS spectrum for each sample that account for the variations in the fluorescence data. Of the statistically defined components, three (C2, C11 and C12) have been assigned as resulting from fully oxidized quinone-type moieties in the sample and are designated as components Q2, Q3 and Q1, respectively (Cory and McKnight, 2005). Three others (C5, C7, and C9, designated SQ1, SQ2 and SQ3) have been assigned to semi-quinone moieties. Hydroquinones, or fully reduced quinone-like moieties are assigned to C4, designated HQ. In

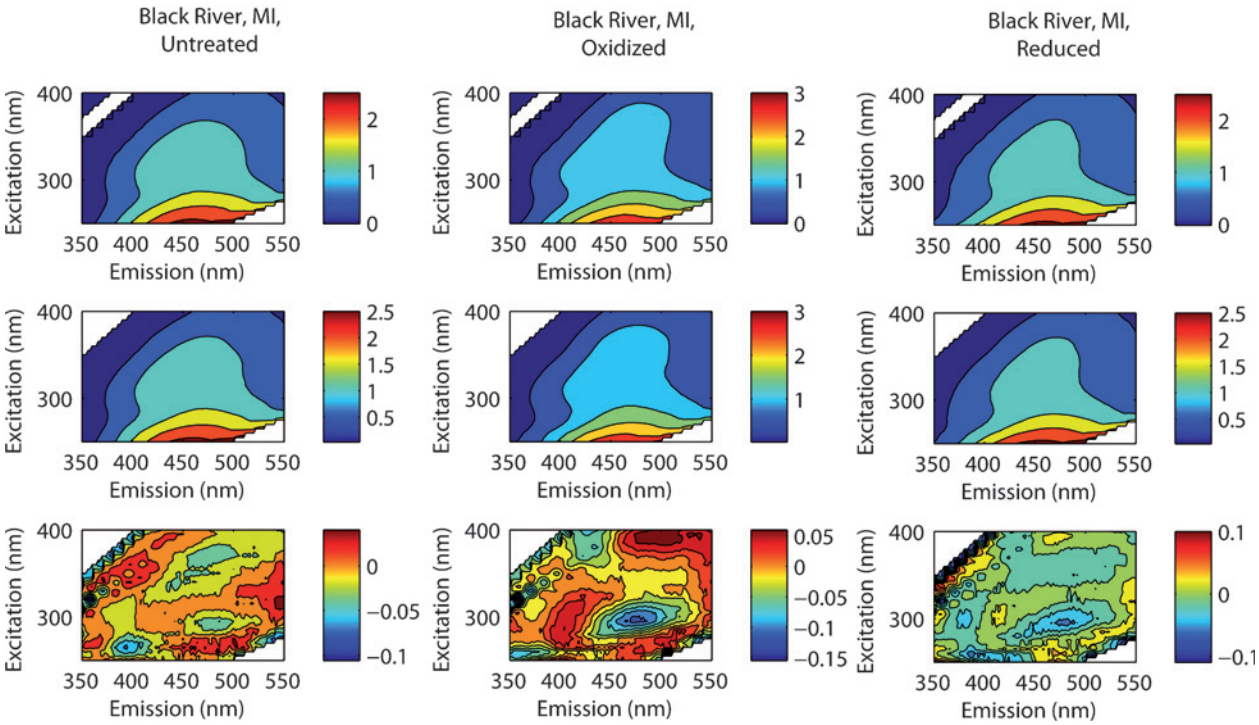

Fig. 1. Comparison of the EEMS spectra for Black River, Michigan (MI) samples. Left, untreated (sample number 25); middle, oxidized (sample number 26); and right, reduced (sample number 28). For each sample the top panel is the measured spectrum, the middle is the PARAFAC model spectrum, and the bottom is the residual (difference between measured and modeled spectra).

addition, two components are assigned to specific protein-like moieties (C8 and C13). The remaining four components (C1, C3, C6 and C10) are not assigned to any specific functional groups or structural components.

The reducing index, RI, (Miller et al., 2006) is the fraction of the quinone-like moieties that are reduced (hydroquinones) or partially reduced (semi-quinones). In the model developed by Cory and McKnight (2005), it is defined in terms of the fractions (or percentages) of the observed fluorescence intensities (designated above) as:

$$RI = (HQ + SQ1 + SQ2 + SQ3) / (HQ + SQ1 + SQ2 + SQ3 + Q1 + Q2 + Q3) \quad (1)$$

The fraction of the total fluorescence intensity that arises from all quinone-like moieties for a given sample is calculated as:

$$(HQ + SQ1 + SQ2 + SQ3 + Q1 + Q2 + Q3) / \Sigma_I CI, \quad (2)$$

where the sum is over all 13 components (CI). Finally, the fraction of the total intensity that arises from protein-like components can be defined as:

$$(C8 + C13) / \Sigma_I CI \quad (3)$$

An additional parameter that has been used to interpret fluorescence data in terms of the relative allochthonous (originating outside of the water body) and autochthonous (originating within the water body) contributions to the NOM in the sample is the fluorescence index (FI) (McKnight et al., 2001; Cory and McKnight, 2005; Fimmen et al., 2007). The FI is defined as the fluorescence emission intensity at 470 nm wavelength divided by the emission intensity at 520 nm when the excitation energy is 370 nm. Values of FI greater than 1.5 generally are interpreted (McKnight et al., 2001) to signify the predominance of microbially derived organic matter (autochthonous), whereas lower values are interpreted to signify varying contributions of terrestrial and soil organic matter sources (allochthonous). Because the PARAFAC analyses used in our study also give FI values, these results are included here to provide further evidence concerning the general applicability of the PARAFAC procedures used.

Results

The results of all analyses are summarized in Table 1 and typical EEMS spectra are shown in figure 1 for three of the Black River, Michigan, samples; untreated, oxidized and reduced. For each sample, the

EEMS spectra shown include the recorded spectrum, the spectrum calculated from the PARAFAC analyses, and the residual difference spectrum which is the difference between the measured and modeled spectra. The fact that the intensities of the residual spectra are very low and that they show no patterns indicate the goodness of the fit of the modeled to the observed spectra (Cory and McKnight, 2005).

Table 1 provides sample numbers, generalized locations, dilutions, experimental alterations, and summary PARAFAC results. Several samples (walnut extract, well MW-6, Inangahua River, New Zealand, and Black River, Michigan) were run repeatedly over a period of 11 months. It should be noted that samples analyzed in 2007 were filtered rather than centrifuged, which may account for some of the observed differences in the results for the same samples analyzed at different times (e.g. 40 Mile River, Alaska (samples 35, 36 and 38 in Table 1) and Chincoteague Island, Virginia (samples 39 and 41)). The data in this table are shown with two digits beyond the decimal point, which is the precision reported in McKnight et al. (2001). Cory and McKnight (2005) indicate a precision of 1% for EEMS data. This level of precision is reasonable, as indicated by results of analyses conducted for the same samples on the same date (cf. samples 2 and 3, 8 and 9, 35 and 36, and 42 and 43 in Table 1).

The first observation we emphasize about our data set is that the fraction of the total fluorescence that is ascribed by the model to quinone-like moieties is remarkably consistent for the entire data set (Fig. 2). With the exception of two of the Well MW-6 samples and the sphagnum extract samples, the quinone-like fraction calculated from the PARAFAC analysis lie between 64 and 78%. This is higher than the similarly narrow range of 50 to 60% for 379 samples reported by Cory and McKnight (2005).

The fraction of the quinones that is calculated to be reduced (reducing index, RI) also shows relatively little variation (Fig. 3), regardless of sample treatment (Figs. 3 and 4). The uncertainties (1 standard deviation) for samples for which multiple analyses were conducted are also shown on figure 4. As this bar graph clearly indicates, the reducing index is not increased upon reduction with Zn metal, nor is it decreased upon oxidation at high pH with O_2. Irradiation of the samples also produces little change in the RI, even though several of the samples (Black River, Michigan, and, especially, well MW-6) contain substantial concentrations of Fe (III), which should promote photo-induced oxidation.

The reduction of quinones and NOM by Zn metal has been clearly established to be rapid and complete (Tratnyek and Macalady, 1989; Blodau et al., 2009).

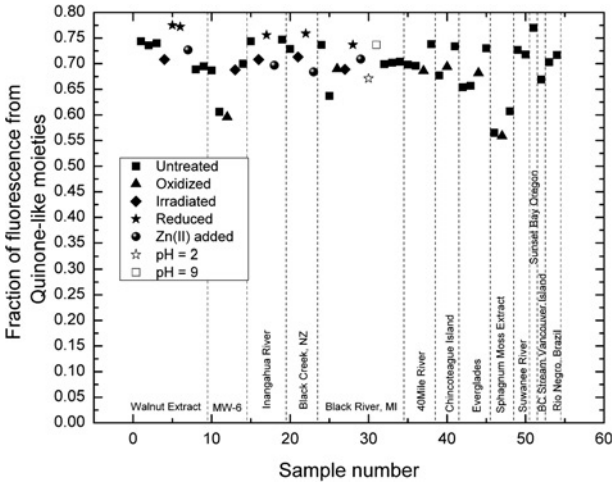

Fig. 2. The fraction of total fluorescence from EEMS spectra due to quinone-like structures as calculated by 13 factor PARAFAC analyses of the 54 samples used in this study. Sample numbers refer to data in Table 1.

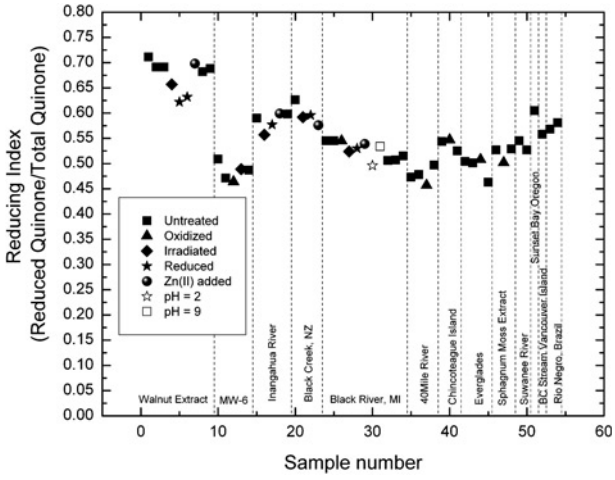

Fig. 3. The fraction of PARAFAC-calculated quinone-like fluorescence in the EEMS spectra due to reduced or partially reduced quinone-like moieties (the reducing index, RI) for the 54 samples used in this study. Sample numbers refer to Table 1.

Thus, though we lack independent confirmation of the reduced status of the Zn-metal-treated samples in this study, the presence of Zn(II) and substantial experience supports the expectation of quinone reduction. A similar argument pertains to the oxidation of the samples with O_2 at elevated pH. Although the literature concerning complete oxidation of hydroquinones to quinones by molecular oxygen is less than totally convincing, there is little doubt that molecular oxygen takes hydroquinones at least to the semiquinone form at higher pH values (Pokhodenko and Khizhnyi, 1966; Roginsky et al., 1999; Roginsky and Barsukova, 2000). Examination of the purported hydroquinone PARAFAC component (C4 or HQ)

Table 2. Percent Hydroquinones Calculated for Selected NOM Samples. [No., sample number; EEMS, excitation emission matrix spectra; 1/15, 1/5, 1/9, 1/4, 1/3 refer to volumetric dilution of samples; CO, Colorado; MI, Michigan, AK, Alaska; VA, Virginia; FL, Florida]

No.	Sample Designation or Location, Dilution, and Collection Year (where relevant)	Treatment	Date of EEMS Analysis	Percent Hydroquinones
11	Well MW-6, CO, 1/5, 1989	Untreated	2-May-08	17
12	Well MW-6, CO, 1/5, 2003	Oxidized	2-May-08	16
25	Black River, MI, 2001	Untreated	2-May-08	22
26	Black River, MI, 2001	Oxidized	2-May-08	22
30	Black River, MI, 2001	pH = 2	19-May-08	21
31	Black River, MI, 2001	pH = 9	19-May-08	23
35	40Mile River, AK, 2003	Untreated	2-May-08	23
36	40Mile River, AK 2003 (repeat)	Untreated	2-May-08	24
37	40Mile River, AK, 2003	Oxidized	2-May-08	23
39	Chincoteague Island, VA, 2002	Untreated	2-May-08	23
40	Chincoteague Island, VA, 2002	Oxidized	2-May-08	24
42	Everglades, FL, 2002	Untreated	2-May-08	19
43	Everglades, FL, 2002 (repeat)	Untreated	2-May-08	20
44	Everglades, FL, 2002	Oxidized	2-May-08	19
46	Sphagnum Moss Extract, 1/5	Untreated	2-May-08	19
47	Sphagnum Moss Extract, 1/5	Oxidized	2-May-08	19

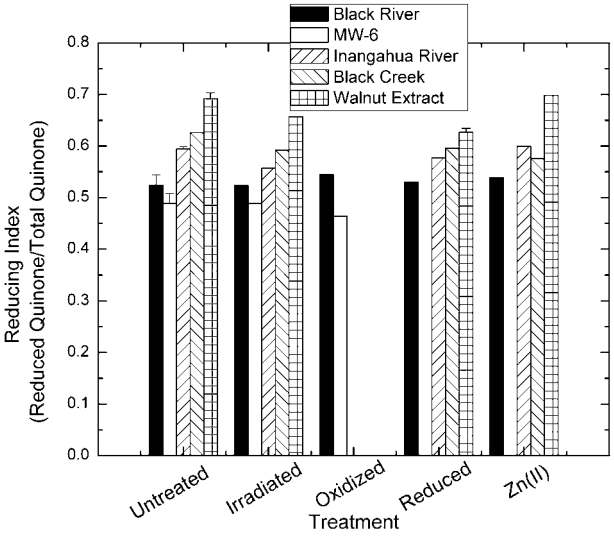

Fig. 4. The reducing index, RI, for samples from five of the sites used in this study as a function of sample treatments defined in the text and listed in Table 1. The uncertainties (1 standard deviation) for samples for which multiple analyses were conducted are shown on top of the bars in the figure.

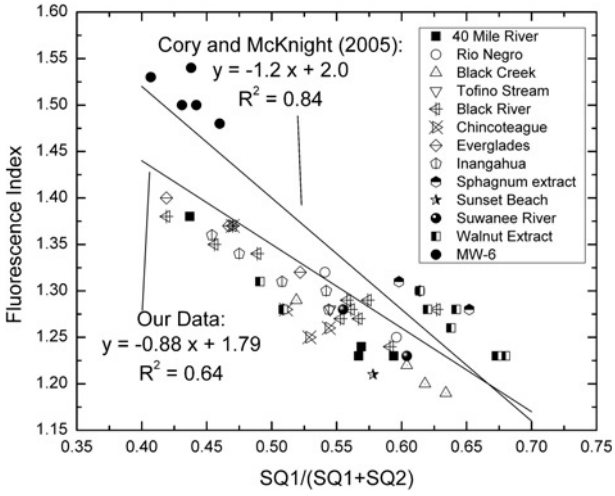

Fig. 5. The fluorescence index, FI as a function of the PARAFAC analyses factor ratio, SQ1/(SQ1 + SQ2) for the 54 samples analyzed in this study. This ratio was reported to be an indicator of relative microbiological vs. higher plant contributions to NOM by Cory and McKnight (2005), and their line is shown, without data points, for comparison.

for raw and oxidized samples analyzed on the same day shows no change in this factor (Table 2). This supports the lack of a correspondence between this factor and reduced quinone moieties in the NOM samples. Interestingly, for several of the samples, reduction, addition of Zn (II), or an increase in pH caused a substantial increase in the apparent fraction of the fluorescence due to protein-like moieties (Table 1).

The relation between FI values and the ratio SQ1/(SQ1+SQ2) was also analyzed in the paper by Cory and McKnight (2005). Here, SQ1 and SQ2 refer to two of the three "semi-quinone" factors identified in the PARAFAC analyses. Using 379 water and fulvic acid samples, they used PARAFAC analyses to establish a linear relation between this ratio and the fluorescence index with a negative slope of 1.2, intercept of 2.0, and an R^2 value of 0.84. Cory and McKnight (2005) provide evidence that SQ2 fluorescence is indicative of a microbial NOM pool, so that the decrease of FI with this ratio implies a decreasing microbial influence in the NOM. Their line is shown without data points in figure 5. The figure also shows that our data follow a quasi-linear pattern with a slope of negative 0.9 (+/- 0.3), intercept of 1.8 (+/- 0.2), and an R^2 of 0.64 (con-

fidence intervals are >97 %). This suggests that our much smaller dataset and that reported in Cory and McKnight (2005) are internally consistent and that no serious method inconsistencies are present.

Our data also cover a much smaller range in FI (about half that of the Cory and McKnight (2005) dataset) and have no examples of waters dominated by microbial influences, which is consistent with the allochthonous origin of the NOM in our samples. In other words, our data are not inconsistent with the association of this PARAFAC factor ratio to fluorescence index and, therefore, the degree of microbial influence in the NOM pool. This correlation does not alone suggest an association of the PARAFAC factors SQ1 or SQ2 with semi-quinone moieties in the NOM pool. It does, however, suggest that our data set does not contain samples that, due to long storage times, have a substantial microbial component.

Discussion

The results outlined above suggest that the 13-component PARAFAC analysis of EEMS data developed by Cory and McKnight (2005) does not produce results that are consistently indicative of the redox status of NOM in natural water or plant extracts. In spite of relatively good agreement between observed and PARAFAC modeled EEMS spectra, PARAFAC models did not indicate consistent oxidation or reduction of quinones resulting from experiments designed to alter the redox status of quinones in NOM. This inconsistency suggests that the assignment of components in the PARAFAC model to quinone-like moieties is questionable. The reasonableness of this conclusion is supported by Boyle, et al. (2009), who analyzed NOM spectral properties, including fluorescence, and concluded that the complex spectral dependence of the steady state and time-resolved emissions cannot be reconciled with a simple superposition of a small number of independently absorbing and emitting chromophores. In addition, Ma, et al. (2008), working with model quinone compounds, reported similar conclusions. Furthermore, work on NOM fluorescence in the laboratories of C.A. Stedmon (Stedmon et al., 2003; Stedmon and Markager, 2005) resulted in a five and eight factor PARAFAC analyses. In the latter study, over 1200 samples of NOM from an estuary and its catchment in Denmark were analyzed. The PARAFAC results yielded four biogenic terrestrial, two anthropogenic, and two protein-like fractions. These factors were used to resolve structural changes in NOM between an estuary and its catchment due to production, transformation, and removal processes, and no claim of fluorophore components from quinone-like moieties was made.

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