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Fulvic Acid Oxidation State Detection Using Fluorescence Spectroscopy

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Humic substances are a heterogeneous class of moderate molecular weight, yellow-colored biomolecules present in all soils, sediments, and natural waters. Although humic substances are generally resistant to microbial degradation under anaerobic conditions, some microorganisms in soils and sediments can use quinone moieties in humic substances as electron acceptors. Laboratory experiments have shown that humic substances can act as electron shuttles in the microbial reduction of ferric iron. Field studies of electron shuttling processes have been constrained by the lack of methods to characterize the oxidation state of quinone moieties in humic substances at natural concentrations. All humic substances have fluorescent properties, and fluorescence spectroscopy can indicate differences in precursor organic source of humic substances. Here we show that the quinone moieties responsible for electron transfer reactions contribute significantly to the fluorescence of humic substances. Further we use fluorescence spectroscopy to elucidate the oxidation state of quinone moieties in humic substances at natural concentrations found in sediment interstitial waters.

Introduction

Humic substances are a heterogeneous class of moderate molecular weight, yellow-colored biomolecules present in all soils, sediments, and natural waters (*1*−*4*). From an ecological perspective, humic substances (humics) can be described as ubiquitous, chemically functional, biomolecular detrital material which modulates ecosystem function and elemental cycles in aquatic and terrestrial environments. The importance of the abundant carboxylic acid and phenolic groups of fulvic and humic acids in buffering pH and forming complexes with metals has long been recognized (*5, 6*). Studies in the 1950s demonstrated that the aromatic chromophores of humics could regulate light absorption and the depth of photic zones in lakes (*7*). The presence of organic radicals in humics (*8*) and the fluorescence characteristics of humics have also been studied for several decades (*9*−*19*).

FIGURE 1. Electron shuttling from electron donor (acetate) to electron acceptor (ferric iron) via *Geobacter metallireducens* and humic substances.

Humics are intimately involved in oxidation and reduction reactions of organic compounds, both indirectly as electron shuttles between microorganisms and oxidized metal species and directly as electron acceptors (*20*−*28*). For instance, Lovley et al. revealed that humics can transfer electrons from *Geobacter metallireducens* to ferric iron in the microbial oxidation of acetate (Figure 1) (*25*). Suspecting the involvement of humic quinone functional groups in this electron-transfer process (*20*, *24*), Lovley showed that anthraquinone-2,6-disulfonate (AQDS, a model compound that has been used to study the electron shuttling properties of humics) can also mediate electron transfer between *G. metallireducens* and ferric iron. Without the presence of either humics or AQDS, the oxidation of acetate by *G. metallireducens* is significantly slower. However, oxidation of acetate by *G. metallireducens* does occur in a media containing humics or AQDS yet no iron, further indicating that humics and AQDS can be reduced by *G. metallireducens*. In addition, exposure of the reduced humics to ferric iron results in the formation of ferrous iron.

Direct and indirect methods have been used to investigate the presence of quinones in humics. Electron spin resonance (ESR) spectroscopic data have shown an increase in semiquinone concentration upon the reduction of fulvic acids by either *G. metallireducens* or strong chemical reductants such as $SnCl_2$ or $NaBH_4$ (*8*, *27*, *29*). However, ESR measurements require concentrations that significantly exceed the natural concentrations of humics by several orders of magnitude. Because dihydroquinones and semiquinones have fluorescent properties, we hypothesized that fluorescence spectroscopy could also be used to estimate the oxidation state of humics at natural concentrations, allowing for assessment of the importance of the humic redox couple in field studies. This hypothesis is strengthened by the observation that the three-dimensional excitation−emission matrices (EEMs) of AQDS is significantly different than the EEMs of AHDS (Figure 2).

Fluorescence spectroscopy has been previously investigated as a tool for characterizing the source of humics (*13*, *15*, *17*, *30*, *31*). Humics have been shown to fluoresce, with at least two main excitation−emission maxima detected in their excitation−emission matrices (EEMs). Fluorescence can be readily detected at fulvic acid concentrations (2−5 mg C/L) found in most natural waters and provides information about the source and chemical reactivity of humics (*14*−*16*, *30*, *31*). Dissolved aquatic fulvic acids derived from the degradation of microbial material have fluorescence characteristics different from those of humics derived from plant and soil material (*14*, *31*). We have previously quantified this difference using a simple fluorescence index (the ratio of the emission intensity at 450 nm to that at 500 nm with an excitation of 370 nm (*31*)).

We have focused on fulvic acids (a subset of humics) isolated from three sites in San Diego Bay with differing sources of sedimentary organic carbon (Figure 3). Located

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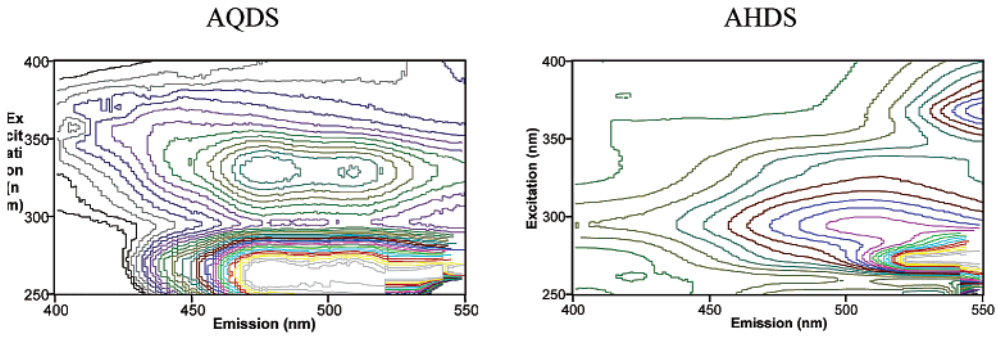

FIGURE 2. Excitation-emission matrices (EEMs) of AQDS and AHDS.

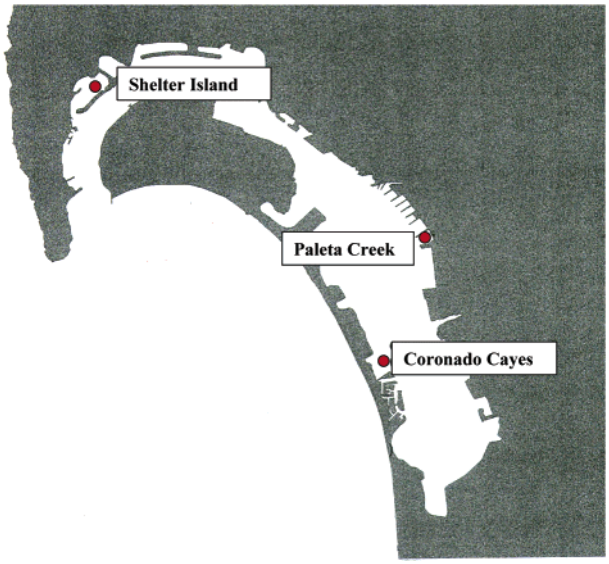

FIGURE 3. Map of the three sites sampled in San Diego Bay: Coronado Cayes, Shelter Island, and Paleta Creek/7th Street Terminal. Map courtesy of the Office of Naval Research.

near Naval Station 7, Paleta Creek is the site of a freshwater inflow carrying terrestrial runoff. Up until recently, this site has received a large amount of petroleum waste, contributing to it being among the most contaminated sites in the Bay. Accordingly, high rates of degradation of polycyclic aromatic hydrocarbons (PAHs) have been observed under sulfate reducing conditions at this site (*32*). Shelter Island is a commercial and recreational boat basin located in northern San Diego Bay. This site receives copper and tributlytin contamination from antifouling hull paints. The final site, Coronado Cayes, is the furthest site from the main land and is relatively uncontaminated.

Materials and Methods

Sample Collection. San Diego Bay sediment samples were obtained on November 1, 1999 from the Naval Ship R/V ECOS. A Van Veen grab sampler was used to collect sediment to a depth of 25 cm. The sediment was transferred into 1 L glass sample jars, capped with seawater, purged with nitrogen, and sealed. The samples were immediately packed on ice for shipping and were subsequently stored in the dark at 4 °C.

Isolation of Fulvic Acids. In March of 2000 and in January of 2001, fulvic acids from San Diego Bay sediment were extracted from 1 L of the sediment obtained on November 1, 1999. To obtain a fulvic acid fraction that may be available as electron shuttles to bacteria and to maintain the integrity of the fulvic acids, a mild extractant ($Na_4P_2O_7$) was used. Standard XAD-8 column chromatography techniques (*33*−*35*) were used to purify the fulvic acids, and the isolates were subsequently freeze-dried.

Microbial Reduction of Fulvic Acids. Cultures of *Geobacter metallireducens* (ATCC 53774) were grown overnight in an anaerobic freshwater medium (*36*) under a N_2−CO_2 headspace. Acetate (20 mM) was used as the electron donor, and ferric citrate (50 mM) was used as the electron acceptor. The cells were harvested under anaerobic conditions and centrifuged for 20 min at 5 K, resuspended in 10 mL of a 30 mM bicarbonate solution, and recentrifuged for 10 min at 10 K. The cells were then resuspended in 3 mL of a 30 mM bicarbonate solution. The microbial reduction of the fulvic acids was conducted in 5 mL solutions of fulvic acid (2 g/L), bicarbonate (30 mM), acetate (10 mM), and 0.15 mL of the cell solution. The solution was then incubated for 2 h at 30 °C. The control experiment was performed in the same manner except that no cells were added to the fulvic acid solution. The samples were then filtered under anaerobic conditions through a 0.2 μm filter, and the filtrate was stored under nitrogen at 4 °C.

Measurement of Electron Accepting Capacity. A 0.9 mL aliquot of the microbially reduced filtrate was transferred under anaerobic conditions into gassed out tubes containing 0.2 mL of a 55 mM Fe(III) citrate solution. After 15 min, the concentration of Fe(II) was determined using the ferrozine assay (*37*).

CPMAS ^{13}C NMR Spectroscopy. Solid-state CPMAS ^{13}C NMR spectroscopy was performed using a Bruker DPX 300 MHz NMR spectrometer. Freeze-dried San Diego Bay fulvic acid samples (25 mg) were placed in a 4 mm rotor and spun at a frequency of 13 kHz at the magic angle. A contact time of 2 ms, recycle delay time of 1 s, a sweep width of 277 kHz, and a line broadening of 100 Hz were used. The ^{13}C NMR data were integrated according to the following regions: 0−60, aliphatic carbon; 60−90, carbohydrate carbons; 90−110, olefinic carbons; 110−160, aromatic carbons; 160−190, carboxyl, ester, and amide carbons; and 190−230, ketonic and aldehydic carbons.

Sample Preparation for Fluorescence Measurements. General Procedure. In a typical experiment to make a solution of "fresh" or "aged" fulvic acid (see text), a solution was made by dissolving 6.4 mg of Paleta Creek freeze-dried fulvic acid into 64 mL of deionized water. In a typical experiment, a solution of "reduced" fulvic acid was made by transferring an aliquot of the reduced Paleta Creek fulvic acid solution (100 μL, 2 g/L) to a sealable cuvette in an inert atmosphere box. This solution was added to 5 mL of degassed and deionized water. The cuvette was then sealed, preventing exposure to air.

Fluorescence Measurements. The fulvic acid solutions were scanned over an excitation range of 250−400 nm at 15-nm increments and an emission range of 400−550 nm at 1-nm increments using a JY-Horiba/Spex Fluoromax-2 spectrofluorometer with DataMax data acquisition software which is built on GRAMS/32 software. Variations in xenon lamp intensity were accounted for by ratioing the fluorescence signal to the reference signal (8% of light from excitation

TABLE 1. Fluorescence Analysis, Electron Accepting Capacity, and Percent Aromaticity for Fulvic Acids Isolated from San Diego Bay Sediment and Humic Substances Isolated from a Variety of Different Aquatic Environments

sample name	sample type[b]	source of organic matter	fluorescence index	electron accepting capacity (μmol Fe/g humics)	% aromaticity
Nymph Lake[a]	sediment FA	terrestrial	0.89	371	22
Paleta Creek	sediment FA	terrestrial	1.04	275	28
Shelter Island	sediment FA	intermediate	1.07	55	29
Coronado Cayes	sediment FA	microbial	1.13	44	24
Green Lakes 4 (Upper Sediment)[a]	sediment FA	microbial	1.13		
IHSS Soil[a]	soil HA	terrestrial	1.15	680	30
Suwannee River[a]	aquatic FA	terrestrial	1.3	66	24
Lake Fryxell[a]	aquatic FA	microbial	1.8	56	13

[a] From ref *34*. [b] FA = fulvic acids; HA = humic acids.

spectrometer). The xenon lamp peak at 467.1 nm was used to reference the excitation spectrometer. The water excitation peak at 350 nm was referenced to an emission wavelength of 397 nm emission in order to calibrate the emission spectrometer. All data manipulation, including inner-filter correction, blank subtraction, and plot generation, was performed using the DataMax software. The intensity of the water Raman scatter peak at Ex/Em = 275/303, obtained daily using distilled water, was used to normalize the intensity of the spectra to account for the decay in lamp intensity over time. In addition, the intensities are reported in units/mg C to account for the varying fulvic acid concentrations used in this study.

Results and Discussion

Characterization data was obtained for the San Diego Bay fulvic acids in order to compare them to fulvic and humic acids from other sources. These data were obtained on San Diego Bay sediment fulvic acids that were isolated after being stored at 4 °C for 4 months (referred to in this paper as "fresh" fulvic acids). Table 1 lists the fluorescence indexes, electron accepting capacity, and percent aromaticity of fulvic acids isolated from the three sites in San Diego Bay. For purposes of comparison the table lists the same data for a few different types of humics, including sediment fulvic acid from two different lakes in Rocky Mountain National Park (Green Lake 4 is an oligotrophic lake with a microbial source of humics and Nymph Lake is a seepage lake with a largely terrestrial source of humics), IHSS soil humic acid, and the two end-members of aquatic fulvic acids, Suwannee River fulvic acid (terrestrial source) and Lake Fryxell fulvic acid (microbial source) (*34*).

The fluorescence indexes of the San Diego Bay sediment fulvic acids showed a trend similar to that of other sediment fulvic acids (Table 1). For instance, the Paleta Creek sediment fulvic acid had a relatively low fluorescence index of 1.04, corresponding to its terrestrial organic carbon source (*31*). The sediment fulvic acid at the Shelter Island site had an intermediate fluorescence index value, reflecting a greater influence of fulvic acid from degradation of phytoplankton and the absence of an adjacent freshwater inflow. The highest fluorescence index value was observed with the Coronado Cayes sample, indicative of the greater influence of microbial organic precursors at this site.

The electron accepting capacity of the isolated San Diego Bay fulvic acids varied greatly with source. Fulvic acid from Paleta Creek sediment had an electron accepting capacity close to the high value of the organic rich Nymph Lake sediment fulvic acid. In contrast, fulvic acid from Coronado Cayes and Shelter Island sediment had a very small electron accepting capacity (Table 1). The electron accepting capacity of these fulvic acids was inversely related to their fluorescence indexes; however, as can be seen by the low electron accepting capacity of the Shelter Island sample and its corresponding intermediate fluorescence index value, this correlation was only a rough approximation.

TABLE 2. Percent Organic Carbon Functional Group Distribution for Fulvic Acids Isolated from the Three San Diego Bay Sediment as Determined Using ^{13}C NMR Spectroscopy

functional group (and range, in ppm)	Paleta Creek	Shelter Island	Coroando Cayes
aliphatic (0−60)	23.6	45.9	36.3
carbohydrates (60−90)	14.4	5.1	15
olefins (90−110)	5.3	2.7	4
aromatic (110−160)	28.1	29.9	23.7
carboxylates, esters and amides (160−190)	15.5	12.9	17.2
aldehydic and ketonic (190−230)	4.2	3.6	3.8

The CPMAS ^{13}C NMR spectroscopic data provided information about the general macromolecular structure of the fulvic acids. The ^{13}C NMR spectroscopy results for San Diego Bay sediment are summerized in Table 2. Shelter Island sediment fulvic acid had a significantly higher aliphatic and a lower carbohydrate carbon content than both the Paleta Creek and Coronado Cayes sediment fulvic acids. Paleta Creek and Shelter Island samples possess higher aromatic carbon content when compared with the Coronado Cayes sample, potentially due to their higher terrestrial inputs. However, all three samples had a relatively high aromatic carbon content when compared to that of purely microbially derived fulvic acids, such as those found in Lake Fryxell in Antarctica (*34*). San Diego Bay samples had a similar aromatic content to that of Nymph Lake and Suwannee River fulvic acids, which contain primarily terrestrial organic carbon precursors.

The first column of Figure 4 shows the EEMs of the isolated fresh fulvic acids. Each contour line represents 3.33% of the maxima intensity. Similar to the EEMs of other fulvic acids, two excitation/emission maxima are observed. The shorter excitation maxima occurs around an excitation wavelength (Ex_{max}) between 250 and 270 nm and an emission wavelength (Em_{max}) ranging between 418 and 504 nm. The longer excitation maxima occurs around Ex_{max}/Em_{max} = 322−358/410−456 nm. These maxima are consistent with a humic signal and are similar to the A (shorter) and C (longer) maxima described by Coble (*15*). Table 3 tabulates the Ex_{max}/Em_{max} and their corresponding intensities (in normalized intensity units/mg carbon) for all of the fulvic acids used in this study.

For all three San Diego Bay sediment fulvic acids, the EEMs of the reduced fulvic acids was considerably different from the EEMs of the fresh fulvic acids and were similar to that of reduced AQDS (AHDS) (Figure 2 and second column of Figure 4). The longer excitation−emission maximum almost completely disappeared in the EEM of the Coronado Cayes reduced fulvic acid and the shorter excitation−

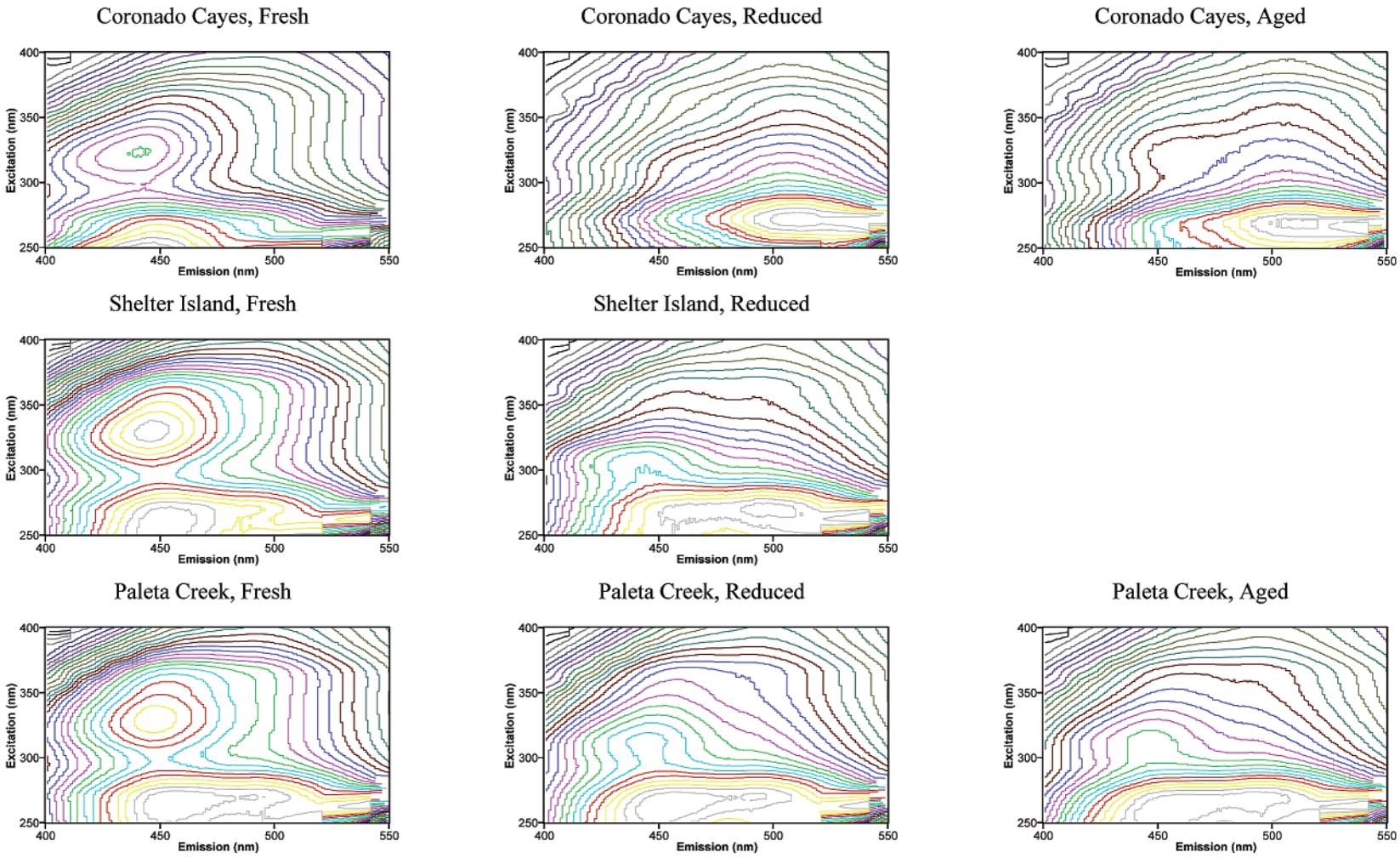

FIGURE 4. EEMs of fresh, reduced, and aged San Diego Bay sediment fulvic acid.

TABLE 3. Ex_{max}/Em_{max} and Their Corresponding Intensities for Fresh, Reduced, and Aged San Diego Bay Sediment Fulvic Acids along with the Ex_{max}/Em_{max} and Their Corresponding Intensities for Suwannee River and Lake Frxyell Fulvic Acids

	fresh				reduced				aged			
	shorter peak		longer peak		shorter pea		longer peak		shorter peak		longer peak	
sample	max.	int.[a]	max.	int.[a]	max.	int.[a]	max.	int.[a]	max.	int.[a]	max.	int.[a]
Paleta Creek	271/504	1.3	329/448	1.4	265/454	0.8	314/443	0.6	270/506	1.0	312/447	1.5
Shelter Island	252/449	1.7	329/445	1.0	270/499	0.3						
Coronado Cayes	250/447	1.8	323/441	1.4	271/507	0.2			270/505	0.7		
Suwannee River	259/445	3.0	331/446	3.7								
Lake Fryxell	241/427	2.3	305/414	3.0								

[a] Intensity units normalized to Ex/Em = 275/303 and mg carbon.

emission maximum significantly red shifted. In the EEMs of the reduced samples for both the Paleta Creek and Shelter Island, the breadth of the longer excitation−emission maximum is significantly diminished and is shifted to a shorter excitation wavelength. The shorter excitation−emission maximum of the reduced Shelter Island sample is significantly broader and red-shifted as compared to the fresh sample; however, no change is observed in the shorter excitation−emission maximum of the Paleta Creek reduced and fresh samples. In all cases, the intensity of the reduced fulvic acids was less than the intensity of the fresh fulvic acids. In addition, the EEMs of the control experiments were similar to that of the fresh fulvic acids.

From these results, we inferred that for the Coronado Cayes samples more of the fluorescence was due to functional groups involved in electron transfer by microbes than in the Paleta Creek and Shelter Island samples. In combination with the similarity of the EEMs of AQDS and AHDS to the EEMs of the fresh and reduced sediment fulvic acids, the dramatically different EEMs of the fresh and reduced fulvic acids suggest that quinone groups are partially responsible for the fluorescence found in humics.

To obtain a larger quantity of material in hopes of studying this process in greater detail, fulvic acids were extracted from San Diego Bay sediment (Paleta Creek and Coronado Cayes sites) that had been stored at 4 °C for 13 months (referred to as "aged" fulvic acids). The EEMs of the aged fulvic acids were dramatically different than the EEMs of the fresh fulvic acids, and more resembled the EEMs of the reduced fulvic acids (third column of Figure 4). Microbial activity would have continued at this temperature. In addition, the supply of available Fe(III) could have become depleted in the sediment, leaving the fulvic acids to act as the terminal electron acceptor. Interestingly, addition of $Fe(NO_3)_3$ to these aged fulvic acids results in a blue-shift of their corresponding emission peaks back to near their original location before reduction (*38*).

These results indicate that EEMs of fulvic acids from oxidizing and reducing zones of marine sediments may be used to assess the oxidation state of humics. In addition, these results indicate that quinones moieties contribute significantly to the fluorescence of humics. Fluorescence index and ^{13}C NMR spectroscopy, both useful for determining the source of humics, are not useful in assessing the electronic state of humics. ESR spectroscopy can determine the amount of free radicals in humics and hence the approximate oxidation state of humics; however, due to the high concentrations necessary to obtain a signal, this method is not useful for field studies. In contrast, fluorescence spectroscopy is a very sensitive technique that can be used on dilute sample solutions and, thus, natural humics concentrations.

The study of humics has traditionally been focused on the linkages between their specific chemical characteristics, the environmental sources of these characteristics, and their ecological roles. The carboxylic acid groups of humics, formed from the microbial oxidation of organic matter, has been demonstrated using pH titration and other analytical methods to be responsible for metal binding and pH buffering in the environment (*1*). As indicated by specific ultraviolet absorbance (SUVA), the chromophoric structures of humics, derived from aromatic precursors, have been tied to photic zone regulation in lakes (*1*). Recent research on microbial electron-transfer involving humics (*25*, *28*) has revealed the ecological significance of electron transfer within humics. Through the use of fluorescence spectroscopy to measure another universal characteristic of humics, we can now investigate the biogeochemistry of electron-transfer reactions in marine and freshwater environments.

Acknowledgments

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