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Marine Chemistry 92 (2004) 23–37

MARINE CHEMISTRY

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Probing molecular-level transformations of dissolved organic matter: insights on photochemical degradation and protozoan modification of DOM from electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry

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Received 11 November 2003; received in revised form 17 May 2004; accepted 30 June 2004
Available online 1 October 2004

Abstract

Molecular-level characterization of natural organic matter (NOM) has been elusive due to the inherent complexity of natural organic mixtures and to the fact that individual components are often polar and macromolecular. Electrospray ionization (ESI) is a "soft" ionization technique that ionizes polar compounds from aqueous solution prior to injection into a mass spectrometer. The highest resolution and mass accuracy of compounds within NOM have been achieved when ESI is combined with an ultrahigh-resolution mass spectrometer such as the Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS). With this technique, individual molecules within a variety of natural organic mixtures can be detected and their elemental composition can be determined. At low mass-to-charge (m/z) ratio, the resolution is high enough to assign exact molecular formulas allowing specific components of these mixtures to be identified. In addition to molecular identification, we can now use ESI FT-ICR MS to examine molecular-level changes in different organic mixtures as a function of relevant geo-processes, such as microbial alterations and photochemistry. Here we present the results from the application of ESI FT-ICR MS to two geochemical questions: (1) the effect of photoirradiation on the molecular composition of fulvic acids and (2) the role of protozoan grazers in the modification of DOM in aquatic systems.

Keywords: ESI FT-ICR MS; FT-MS; DOM; Fulvic acids; Photochemistry; Protozoan grazing

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doi:10.1016/j.marchem.2004.06.038

1. Introduction

The structure and composition of natural organic matter (NOM) plays a critical role in a number of geochemical processes such as metal redox cycling, contaminant transport and microbial growth and community structure (e.g., Hassett and Anderson, 1981; Santschi et al., 1997; Redman et al., 2002; Sobczak et al., 2002; Crump et al., 2003). Organic matter within aquatic systems is affected by a number of biotic and abiotic geochemical processes. For example, reduced carbon produced by photosynthesis is remineralized and modified within the microbial loop comprised of bacteria and protozoan grazers (e.g., Azam et al., 1983; Caron et al., 1988; Hagstrom et al., 1988; Sherr and Sherr, 2002), whereas humic material is degraded photochemically in the surface waters (e.g., Schmitt-Kolpin et al., 1998; Del Vecchio and Blough, 2002; Brinkmann et al., 2003). The structure and composition of the organic matter within each of these pools affects their respective rates and extent of degradation. Predicting the fate of organic matter in these systems is possible only when the structure and composition of OM is known.

Organic matter in aquatic systems is a complex mixture of compounds with diverse functional groups and thus widely varying physicochemical properties. To date, OM structural characterization studies have relied on gas chromatography (GC) or nuclear magnetic resonance (NMR). NMR has been used extensively to estimate the relative contributions of functional groups such as aliphatic and aromatic moieties (e.g., Hatcher et al., 1980; Aluwihare et al., 1997; Haiber et al., 2001; Dria et al., 2002). However, the spectra cannot give molecular-level detail but instead assess the general character of organic matter. To discern molecular-level information, small volatile (and semivolatile) molecules are examined by GC techniques coupled with flame ionization detection (FID) and/or mass spectrometry (MS). Large molecules are degraded or dissociated either chemically or thermally to generate small volatile pieces that can be analyzed by GC/MS (e.g., TMAH methods- delRio and Hatcher, 1998; delRio et al., 1998). Once degraded, the structure of the original macromolecule is lost and cannot be reconstituted. Furthermore, the presence of polar functional groups, such as alcohols and carboxylic acids, limits compound volatility. Thus, molecular-level structural characterization has been limited to small nonpolar compounds, a minor fraction of the total organic matter in aquatic systems.

A polar ionization technique, electrospray ionization mass spectrometry (ESI MS), has shown promise in the study of molecular-level transformations of polar components of natural organic matter. In the following paper, we summarize and review prior applications of this technique to geochemical questions. In addition, we use this technique to discern molecular-level transformations due to photochemical degradation of fulvic acids and to protozoan modification of biological dissolved organic matter (DOM).

2. ESI FT-ICR MS

The characterization of polar compounds is now increasingly possible with a new ionization technique, electrospray ionization (ESI). This new technique has been widely applied by the biochemical community for analysis of large polar compounds such as proteins and their complexes with metals and small peptides. Electrospray ionization preferentially ionizes polar compounds with functional groups such as amines ($-NH_2$) and carboxylic acids ($-COOH$; for an excellent review, see Gaskell, 1997). During ESI, a voltage is applied across the gap between the sample needle and the mass spectrometer. The sign of the voltage determines the ion charge sign. For example, a negative voltage difference attracts only positively charged particles into the mass spectrometer. Depending on the chemical composition of the molecule, multiple charges are possible. Thus, large molecules can be observed within the mass range of the mass spectrometer when the mass-to-charge ratio (m/z) is lowered by the presence of multiple charges. ESI MS is a "soft" ionization technique, and generally, the molecular ion remains intact.

ESI has a number of advantages for use in the geosciences. First, ESI has a large mass range, ionizing compounds $10<m/z<3000$ as quasi-molecular ions, $(M+nH)^{n+}$ or $(M-nH)^{n-}$, eliminating the need for compound degradation inherent in frag-

mentation-based GC/MS. Second, compounds are introduced into ESI through water-based solvent mixtures (usually an alcohol/water mix), thus making it ideal for the analysis of water-soluble compounds. Third, ESI can be coupled with high-resolution mass spectrometry, separating numerous compounds with the same nominal (i.e., integer) mass value. ESI has been coupled to a number of mass spectrometers for the analysis of natural organic matter, including ion-traps (Leenheer et al., 2001), triple–quadrupole analyzers (McIntyre et al., 2002), quadrupole time-of-flight analyzers (Moulin et al., 2001; Plancque et al., 2001; Kujawinski et al., 2002b) and Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS; Fievre et al., 1997; Brown and Rice, 2000; Kujawinski et al., 2002b; Stenson et al., 2002). The main advantage of ESI MS over other ionization techniques such as matrix-assisted laser desorption ionization (MALDI) or fast-atom bombardment (FAB) is the reduced frequency of fragmentation of parent molecular ions.

The highest mass accuracy and resolution have been achieved with the Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (an excellent review is presented in Marshall et al., 1998). Briefly, the ions generated by ESI or another ionization source are mass-selected, stored and transmitted by electric multiple ion optics through a series of differentially pumped stages. The ion cyclotron resonance cell resides in the horizontal bore of a 9.4 T magnet. As the ion beam enters the cell, ions are trapped by the magnetic field and a quadrupolar electrostatic potential, and undergo cyclotron rotation at very small (sub-mm) orbital radii. The radius of the orbit is expanded by the application of a rf-pulse across two opposed excitation electrodes. As the ions pass by one of two detection electrodes, they induce a current differential between the two electrodes. This current differential is amplified and monitored as a function of time. The frequency of orbit of each ion is determined by Fourier transform of the current versus time signal. Because the frequency of cyclotron rotation varies approximately inversely with ion m/z, the frequency spectrum may be converted to a mass spectrum if at least two experimental m/z are known (Ledford et al., 1984; Shi et al., 2000). The maximum resolution and mass accuracy of an ICR spectrum increase directly with the strength of the magnet (Marshall and Guan, 1996).

Electrospray ionization mass spectrometry has been applied by a number of investigators to the structural characterization of natural organic matter mixtures such as humic acids, organic acids in drinking water and riverine organic matter (McIntyre et al., 1997; Brown and Rice, 2000; Kujawinski et al., 2002b,c; McIntyre et al., 2002; Stenson et al., 2002; Kim et al., 2003b; Stenson et al., 2003). The resolution and mass accuracy of peaks in the mass spectra is substantially enhanced by combination with FT-ICR MS. With the ultrahigh resolution and mass accuracy of this technique, thousands of compounds can be detected and identified in each of these samples. For example, the negative ion mode spectrum of Suwannee River fulvic acid shows approximately 4000 peaks within $300<m/z<1000$, with approximately 20 peaks per odd nominal mass (Fig. 1) at baseline resolution. In the low mass range ($m/z<500$), elemental formulae can often be determined due to the mass accuracy of the spectrum and the limited number of chemical formulae with the appropriate mass. Each mass spectrum is externally calibrated first by a standard test mixture and then is internally calibrated by a series of known peaks (usually a homologous series of compounds separated by $–CH_2$ groups) or added standards (such as polyethylene glycol polymers). After combined external and internal calibration, the mass accuracy is less than 1 ppm, i.e., <0.001 Da for singly-charged ions of $300<m/z<500$. Although the mass accuracy decreases with increasing m/z, elemental formulae can often be determined for high m/z compounds if they are related to low m/z compounds by the addition of simple functional groups such as methylene ($–CH_2$; Hughey et al., 2001a).

Compounds in humic acid and DOM samples have been shown to be primarily singly charged in ESI mass spectra, based on a separation of ~1.003 Da between $^{12}C_n$ and $^{12}C_{n-1}{}^{-13}C_1$ forms of the same molecule (Brown and Rice, 2000; Kujawinski et al., 2002c). Using polyacrylic acid as a proxy for humic acids, Leenheer et al. (2001) suggested that ESI MS generates both fragments and multiply-charged species. However, an elegant study by

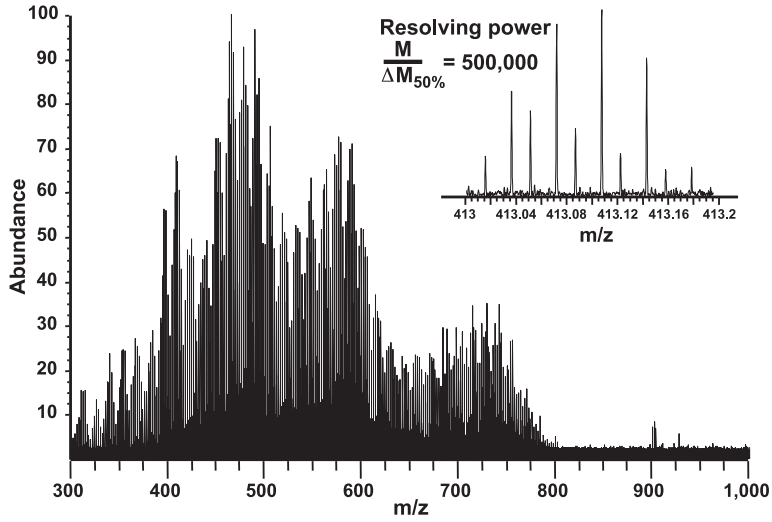

Fig. 1. Negative ion mode ESI FT-ICR mass spectrum of Suwannee River fulvic acids (SRFA). The mass spectrum was collected by use of 0.5 mg/mL SRFA in 1:1 MeOH:H_2O. The spectrum represents 200 co-added scans on a 9.4 T ESI FT-ICR MS. The inset shows the expanded region near m/z 413, highlighting the ultrahigh resolution of this technique.

Stenson et al. (2002) showed that humic acids are not fragmented in ESI MS in the same manner as polymers such as polyethylene glycol. Furthermore, the same study showed that humic acids do not accommodate multiple charges easily and so occur primarily as singly-charged species.

This technique generates large data sets (>1 MWord) for each mass spectrum or set of spectra and efficient data analysis is required. Studies focusing on descriptive structural analysis employ techniques that separate compounds into categories based on elemental composition. Kendrick mass analysis was first developed in the petroleum research community as a way to visually separate compounds with identical chemical backbones but differing numbers of $–CH_2$ groups (Kendrick, 1963). Kendrick mass analysis was adapted by researchers at the National High Magnetic Field Laboratory (Tallahassee, FL) to visually separate polar petroleum compounds (Hughey et al., 2001a). This technique normalizes the measured mass to a "Kendrick" mass, in which the mass of $–CH_2$ is defined as 14.000 Da (instead of 14.01565 Da).

$$\text{Kendrick mass } (KM) = \textit{IUPAC mass}\ (14.000\ \text{Da}/14.01565\ \text{Da}) \quad (1)$$

The difference between the Kendrick mass and the Kendrick nominal mass is the Kendrick mass defect (KMD).

$$\text{Kendrick mass defect } (KMD) = \textit{Nominal Kendrick mass} - \textit{Kendrick mass} \quad (2)$$

The KMD is constant for all compounds with the same chemical backbone but different numbers of $–CH_2$ groups. This technique has proved to be invaluable for determining elemental formulae for the majority of compounds within petroleum-based samples (Hughey et al., 2001a,b; Qian et al., 2001). The elemental formula is assigned for the compound with the lowest mass in each homologous series, thus determining the masses for all compounds in each series.

Kendrick mass analysis proved less appropriate for nonpetroleum materials (Kujawinski et al., 2002c) because the Kendrick series were much shorter for non-CH_2-based compounds such as humic and fulvic acids. To circumvent this limitation, Stenson et al. (2003) modified the Kendrick mass approach and separated compounds based on the integer remainder after the IUPAC nominal mass was divided by 14 Da (Z^* in Stenson et al., 2003).

$$Z^* = \textit{Nominal IUPAC mass}/14\ \text{Da} \quad (3)$$

By use of this approach, they noted a number of interesting patterns in elemental composition in their humic acid sample. They had noted earlier that peaks at each nominal mass were separated by 0.036 Da, the difference between CH_4 and O. After separating the compounds in their mixtures by the Z^* value, they noted that there were two sets of homologous series in each Z^* category, one with high double-bond equivalent (DBE) and low oxygen number (O) and the other with relatively lower DBE and higher O values. The striking degree of compositional similarity among all the compounds in humic and fulvic acids had been hypothesized in previous work but not confirmed by molecular-level studies. In fact, the correlation between DBE and number of oxygens for elemental composition, $C_cH_hN_nO_o$, is a direct consequence of the relation (McLafferty and Turecek, 1993),

$$Ring + Double\ bonds = DBE = c - h/2 + n/2 + 1 \quad (4)$$

Thus replacement of CH_4 by O must be accompanied by the addition of one ring or double bond. More recently, data visualization has taken another step forward with the work by Kim et al. (2003a). These investigators use the Van Krevelen diagram to separate compounds based on their elemental ratios (H:C and O:C for hydrocarbons) and thus compare compound types within different samples (see Kim et al., this volume).

To date, most studies have used ESI FT-ICR MS as a descriptive tool due to its inability to generate high-quality quantitative data. ESI MS, in general, is not a quantitative technique because ionization efficiencies are very sensitive to changes in sample matrix and composition. In addition, variations in ionization efficiencies among compounds within a mixture can vary over two orders of magnitude, making quantitative assessments of compound changes difficult. Thus, the relative abundances of ions in an ESI mass spectrum do not necessarily reflect the relative abundances of the corresponding neutrals in the original sample. However, the ultrahigh resolution and mass accuracy of ESI FT-ICR MS make it an attractive option for examining structural changes in organic matter as a function of biogeochemical processes. As long as changes in peak height are not used as indicators of structural change, but instead the presence of particular peaks are used, ESI FT-ICR MS will be a very sensitive technique for elucidating molecular-level transformations in organic matter.

The comparative power of the ESI FT-ICR MS is only just beginning to be exploited. Small changes in the composition of complex mixtures can be identified with the ultrahigh resolution and mass accuracy of this technique, making it extremely sensitive to experimental processes that affect molecular-level composition. For example, changes in mixture composition have been assessed with ESI FT-ICR MS after fuel processing (Hughey et al., 2001b) and weathering (Rodgers et al., 1999, 2000b). In addition, it has been shown that isotopic incorporation into cellular OM can be easily identified (Rodgers et al., 2000a). It seems likely, then, that this technique will be applicable to questions of geochemical importance, such as the molecular-level transformations of organic matter mediated by both abiotic and biotic processes. With molecular-level resolution of compounds within NOM, we can assess changes to specific chemical families within these mixtures as a function of numerous factors such as microbial activity, photochemistry and geopolymerization. In the following sections, we describe the application of this technique to two geochemical processes—photochemical degradation and protozoan grazing.

3. Application 1: photochemical degradation of DOM

3.1. Introduction

The molecular-level effects of photochemical degradation are not well understood for heterogeneous mixtures such as aquatic dissolved organic matter. The absorption spectra of chromophoric DOM from numerous sources are known to decrease exponentially with increasing wavelength, with no discernible structure at wavelengths greater than 300 nm. Little is known at the molecular level about the constituents or interactions that produce this long-wavelength absorption, although we recently proposed that this absorption may arise from intramolecular charge transfer interactions between hydroxy-aromatic donors and quinoid acceptors formed by the partial oxidation of

lignin precursors (Del Vecchio and Blough, 2004; see also Del Vecchio and Blough, 2002; Goldstone et al., 2004). The goal of our preliminary study was to examine molecular-level transformations in DOM after exposure to polychromatic light over differing wavelength ranges.

3.2. Methods

Suwannee River fulvic acids (SRFA), obtained from the International Humic Substances Society (IHSS), were used as a model system for chromophoric DOM due to their relatively lower degree of heterogeneity. SRFA was dissolved in Milli-Q water at a concentration of 1 mg/mL. Aliquots (300 μL) were placed in short pathlength (1 mm) quartz irradiation cells and exposed to polychromatic light under aerobic conditions as previously reported (Del Vecchio and Blough, 2002). The cells were capped to minimize evaporation. The polychromatic irradiations employed an ILC Technology Illuminator Power Supply and a 300-W Xenon lamp with the samples placed 50 cm from the lamp housing. A 25-cm cell containing Milli-Q water was placed between the lamp and the sample to eliminate sample heating by absorption of infrared radiation. Different long pass cut-off filters (>305 and >360 nm) were employed to select different irradiation ranges (λ_{irr}). Irradiation lasted for one week or more depending on the cut-off filter employed (170 and 270 hrs with 305 and 360 nm cut-off filters, respectively). The light intensity was measured daily employing an Ocean Optic USB2000. Absorption spectra were regularly recorded during time exposure with a Hewlett Packard 8452A spectrophotometer. Milli-Q water was the blank. Absorption spectra recorded over the wavelength range from 200 to 800 nm were baseline-corrected by subtracting the averaged absorption values at wavelengths greater than 650 nm. After irradiation, the sample was placed in an Eppendorf tube and frozen until ESI FT-ICR MS analysis.

ESI FT-ICR MS analyses were conducted at the NSF National High Field FT-ICR MS Facility in Tallahassee, FL on the home-built 9.4 T ESI FT-ICR MS (Senko et al., 1996b). Samples were diluted 1:1 with MeOH prior to analysis and infused into the ESI system at 400 nL/min (approximately 36 μL were infused for each analysis). The samples were run in negative ion mode. Negative ion mode was chosen because the sample constituents easily form negative ions in neutral-pH aqueous solutions. In addition, complexes of Na^+ and H^+ with individual fulvic acids are minimized in negative ion mode. The capillary needle voltage was set at −2600 V. Ions were accumulated in the external octopole (frequency set at 1.6 MHz) for 15 sec before transfer to the ion cyclotron cell. Data was collected (4 MWord) by the MIDAS data station (Senko et al., 1996a; Blakney et al., 2002). Numerous scans (150—approximately 90 min for each analysis) were co-added prior to Hamming apodization, zero-fill and Fourier transformation.

3.3. Results and discussion

SRFA absorption spectra before and after exposure to polychromatic light are reported (Fig. 2). The ESI FT-ICR mass spectra of SRFA were also compared before and after irradiation. While substantial differences were observed between the mass spectra of irradiated and nonirradiated samples (see below), no observable molecular-level differences were found between samples of SRFA irradiated at wavelengths >305 and >360 nm. This result indicates that a common set of structures was destroyed over these two spectral ranges. Although this observation is consistent with the recently proposed charge transfer model (Del Vecchio and Blough, 2004), other explanations are also possible and additional work will be needed to test this model definitively. For illustrative purposes, the SRFA mass spectrum following irradiation at λ>305 nm was chosen for detailed analysis.

The differences in the mass spectra prior to and after irradiation are visually striking when one nominal mass region is expanded and examined (Fig. 3). For example, at m/z 413, every other peak is missing from the irradiated sample. Furthermore, the peaks that have been removed differ from one another by multiples of 0.036 Da, the difference between CH_4 and O, likely from replacement of a methyl group by an aldehyde or ketone: RCH_2CH_3 versus RCHO (Stenson et al., 2003). This phenomenon can be seen more generally when different nominal mass regions are examined at the same time. With the modified Kendrick mass analysis of Stenson

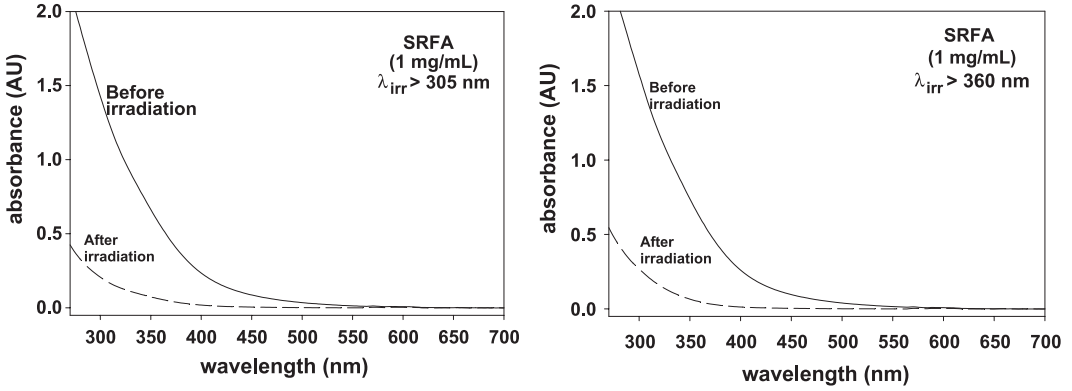

Fig. 2. Absorption spectra for SRFA samples irradiated above 305 and 360 nm. SRFA samples (1 mg/mL) were irradiated with polychromatic light above 305 (A) and above 360 nm (B). Absorption spectra were compared for SRFA samples before (—) and after (- - -) irradiation.

et al. (2003), the peaks in both the irradiated and nonirradiated SRFA mass spectra were divided into categories based on the remainder after division by 14 Da (the mass of a $–CH_2$ group). Once sub-divided, the peaks were plotted as a function of their nominal mass and Kendrick mass defect.

The category containing the *m*/*z* 413 nominal mass region was chosen for further analysis (Fig. 4). In this group, the chemical formulae were determined for each chemical family. As noted by Stenson et al. (2003), alternating horizontal lines incorporate the 0.036 Da shift by increasing the double bond equivalent (DBE) and the oxygen number (*O*) by one increment. DBE values represent the number of multiple bonds (double or triple) and rings that must be present within a compound so that all carbon atoms have four bonds and obey the "octet rule". In our sample and other fulvic acids, highly oxygenated compounds with low DBE alternate with chemical families characterized by low oxygen values and high DBE. By comparing irradiated to nonirradiated SRFA by use of this

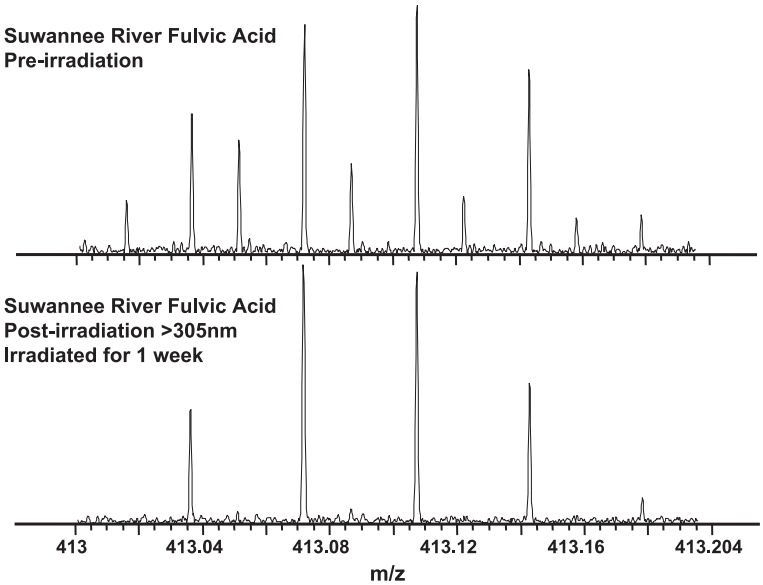

Fig. 3. Effect of irradiation on SRFA. Negative ion mode ESI FT-ICR mass spectra were acquired for SRFA samples before and after photobleaching. The expanded region near *m*/*z* 413 is shown for both the nonirradiated (top) and irradiation (bottom) samples.

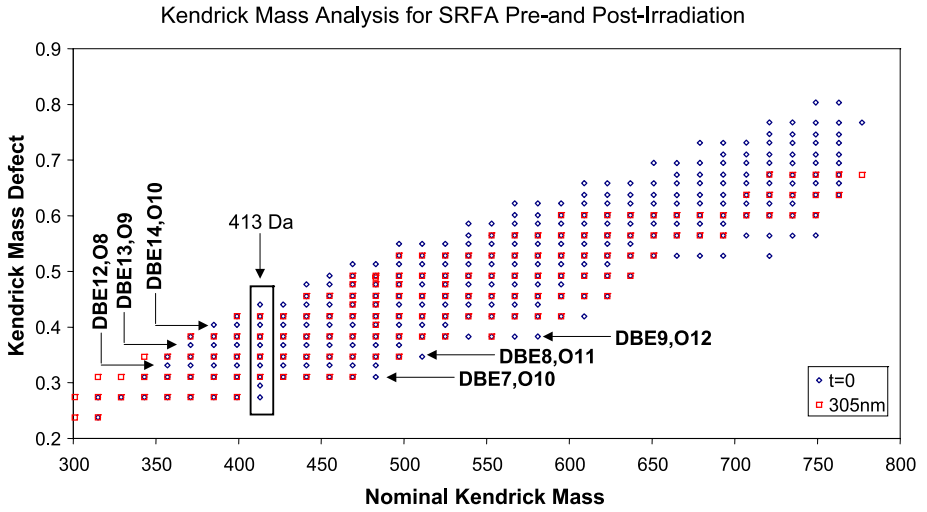

Fig. 4. Kendrick mass analysis for a subset of the SRFA sample. Compounds present before irradiation (◇) and after irradiation above 305 nm (□) have a Z^* value of −6 (for definition, see above and Stenson et al., 2003). The expanded region near m/z 413 shown in Fig. 3 is highlighted with a box in this figure. The double-bond equivalent (DBE) and O values are given for six homologous series.

approach, we observe that the chemical families characterized by high DBE and low oxygen number are preferentially missing from the irradiated SRFA mass spectrum. Because high DBE values indicate an increased prevalence of rings and double bonds, we hypothesize that the moieties that are destroyed are conjugated to some degree, although it is not yet clear whether these species represent single- or multiple-ring aromatic groups or extended polyenes. Our work indicates that these compounds are preferentially destroyed by exposure to longer-wavelength polychromatic light. Compounds with low DBE but high oxygen content remain after photobleaching, suggesting that these compounds are less photoactive (Schmitt-Kolpin et al., 1998). This hypothesis could be examined further with elemental composition analysis, which will be conducted in our future studies.

Interestingly, we did not observe the production of new compounds in the ESI mass spectra after photobleaching. Previous studies had shown that the average molecular weight of DOM decreases after photobleaching and NMR studies showed that oxygen content increased after photobleaching (Schmitt-Kolpin et al., 1998). Our data does not contradict earlier work, but adds the interesting observation that new ionizable chemical families are not produced by photobleaching. We cannot rule out the possibility that all compounds produced by photobleaching are too small to be seen by our mass spectrometer (m/z<300). However, it seems unlikely that HMW compounds produce only compounds below m/z 300 after photobleaching. Instead, chemical families with high DBE and low O values may be converted to the other chemical families (low DBE, high O) as a result of photobleaching. Another possibility is that compounds were converted to neutral, non-ionizable molecules. For example, because the compounds detected in SRFA were primarily singly charged, the loss of a carboxylic acid group would render these compounds incapable of carrying a negative charge and thus they would not be detected by ESI MS. Although dissolved organic carbon (DOC) concentrations were not measured directly on these samples, previous work indicates that photobleaching of this magnitude can remove up to 15% of the DOC in fulvic acids (Miller and Zepp, 1995), not the 50% loss of peaks observed in our spectra, suggesting the conversion of ionizable compounds into nonionizable compounds.

3.4. Conclusions

This preliminary study shows that striking molecular-level differences can be detected in ESI mass spectra as a result of photoirradiation at long wave-

lengths. The next step in this work will be to link specific molecular characteristics to susceptibility to photodegradation. Elemental formulae cannot be converted to chemical structures without additional structural information such as fragmentation patterns. The peaks in this particular sample are too close together to isolate a single compound for MS/MS analysis in the 9.4 T ESI FT-ICR MS. However, based on comparison of fragmentation patterns at a nominal mass before and after irradiation, fragments of the peaks that are impervious to photodegradation can be separated from the fragments of the compounds that are destroyed by photobleaching and thus some structural features should be inferred.

4. Application 2: protozoan modification of DOM

4.1. Introduction

The role of the microbial web in the cycling of organic matter and nutrients has been well-established in both freshwater and marine environments (Pomeroy, 1974; Azam et al., 1983; Goldman and Caron, 1985; Caron et al., 1988; Sherr and Sherr, 1994). The microorganisms within this web recycle over 90% of fixed carbon, with the highest recycling efficiencies occurring in nutrient-poor, oligotrophic regimes. Protozoan grazers feed on bacteria, small phytoplankton and small detrital particles (Sherr and Sherr, 1994, 2002). Protozoa have been shown to have a large impact on carbon and nutrient cycles both in laboratory culture (Goldman and Caron, 1985; Nagata and Kirchman, 1990, 1992; Tranvik, 1994; Barbeau et al., 1996, 2001) as well as in field studies (Lochte, 1991; Nagata and Kirchman, 1997; Barbeau and Moffett, 2000). During grazing, protozoa feed primarily on particles within the 0.1–1.0 μm size class, affecting both the chemical composition of organic matter and the particle size spectrum. Extensive chemical alterations are possible due to the extreme chemical conditions present in the protozoan digestive vacuole. Initial stages of digestion are characterized by low pH levels (approximately 2) that lyse prey cells. Later stages include high concentrations of digestive enzymes and less acidic pH levels (approximately 5; Fok et al., 1982). To date, the study of the role of the microbial web in altering organic matter has focused on the changes in quantity of organic matter and the distribution of particle sizes. Studies are now needed that address such changes on a molecular level, and more importantly, that identify key structural modifications that are diagnostic of microbial processes such as protozoan grazing. A preliminary study was designed to examine the molecular-level differences in protozoan-modified DOM, relative to a bacterial control. Our goal was to test the applicability of ESI FT-ICR MS to this type of study and identify compounds that would be characteristic of protozoan grazing and the subsequent DOM modifications.

4.2. Methods

Bacterial prey, *Enterobacter aerogenes*, (American Type Culture Collection (ATCC) #13048) were grown on 0.04% yeast extract and rinsed three times prior to inoculation with protozoan predator, *Bodo caudatus* (ATCC #50361). A control culture with no protozoan predator was also established. Once stationary growth had been achieved in the grazing culture, both cultures were filtered through 0.2-μm polycarbonate filters. Polycarbonate filters have been shown to contribute little contamination to DOC filtrates (Barbeau et al., 2001; Kujawinski et al., 2002a). The filtrate was then acidified with HCl to pH 3 to protonate all carboxylic acid functional groups. The acidified DOM was then concentrated on a C18 extraction disk and eluted with 5 mL MeOH (Kim et al., 2003b). The DOM extract from each culture was then analyzed without further sample preparation in positive ion mode on the 9.4 T ESI FT-ICR MS as described above. Because DOM had been isolated through acidification and subsequent extraction onto C18 disks, most compounds will be protonated and thus carry a positive charge. The most efficient mode for ESI MS analysis will therefore be the positive ion mode. DOC concentrations were not measured for either culture.

Little chemical similarity was expected for these compounds, so an additional data analysis approach was employed. Lists of all peak m/z values with magnitudes greater than 3× the noise level were generated for each mass spectrum by use of the MIDAS data analysis software (Senko et al., 1996a).

Peak lists from selected mass spectra were then integrated into a master peak list by use of a matrix-based data analysis program written for this purpose (an example is shown in Table 1). The program is based within Matlab™. In brief, this program collated peaks in selected mass spectra into a master peak list, or matrix. Non-detected peaks are denoted by a zero magnitude. Elemental or functional group mass changes within and across relevant mass spectra can be identified by examining mass differences between peaks. For example, if we were interested in identifying compounds that differed by the addition of $–CO_2$ groups, we looked for the difference of the exact mass difference (or its integral multiples) between peaks in a master list generated from the spectra of both cultures. Tentative identification of a proposed formula alteration was based on the observation of the accurate mass difference within a 1-ppm error window. The elemental formulae of low-molecular weight compounds were determined from molecular mass alone (using the elements C, H, N and O). The relationships determined by this program were used to predict molecular formulae of high-molecular weight (HMW) compounds. Functional groups examined in this analysis included the addition of –O, $–CO_2$, $–CH_2$, –CH, $–CH_2CH_2O$, $–H_2$, –CHCHO and –NH. Predicted formulae were inserted into the original Kendrick mass analysis of the same spectra and additional formulae were determined by use of relationships inherent in the Kendrick mass analysis (Stenson et al., 2003). These two data analysis approaches were used iteratively until the predicted and actual molecular weights of all identified compounds differed by less than 5 ppm (Table 1). This analysis was used to determine elemental formulae for 301 of 393 peaks in the protozoan culture (77%) and 241 of 313 peaks in the bacterial control (77%). Additional peaks might be identified if other elements were considered, namely P and Na, but only with additional elemental information or MS/MS analysis of specific peaks.

4.3. Results and discussion

The spectra for the biologically derived DOM in this study were significantly less complex than those of fulvic acids (above). Although fulvic acids can have a biological origin (degradation of lignin), for the purposes of this manuscript, we use "biologically derived" DOM to represent that material present in our culture experiments and derived from the activities of the bacteria and protozoa therein. Within an expanded mass region, one can visually identify those compounds that are common to both the grazing and

Table 1
Peaks observed in the mass range $653<m/z<658$ (shown in Fig. 5) in both the bacterial and protozoan grazing cultures

Observed mass	Magnitude in bacterial DOM	Magnitude in protozoan DOM	Elemental formula	Predicted molecular weight	Error (ppm)
652.2848	0	1	$C_{20}H_{44}O_{16}N_8$	652.2864	−2.50
652.4199	1	1	$C_{37}H_{56}O_6N_4$	642.4186	2.02
653.4224	1	1	$C_{30}H_{55}O_7N_9$	653.4211	2.10
654.2639	0	1	$C_{19}H_{42}O_{17}N_8$	654.2657	−2.77
654.3781	0	1	$C_{39}H_{50}O_5N_4$	654.3769	1.95
654.3803	0	1	$C_{28}H_{50}O_8N_{10}$	654.3801	0.39
655.3818	0	1	$C_{33}H_{45}O_2N_{13}$	655.3808	1.54
656.0285	0	1	ND	n/a	n/a
656.1961	1	1	$C_{30}H_{32}O_{13}N_4$	656.1958	0.45
656.2427	0	1	$C_{18}H_{40}O_{18}N_8$	656.2451	−3.65
656.3624	1	1	$C_{31}H_{52}O_{11}N_4$	656.3620	0.63
656.5280	1	0	ND	n/a	n/a
656.5300	1	1	ND	n/a	n/a

The charge on each peak is assumed to be +1, thus the peak location (m/z value) was assumed to be equivalent to the molecular mass plus a proton ($M-H^+$). For each peak, the elemental formula, the predicted molecular weight, the observed mass (corrected for the proton, H^+) and the mass accuracy error are given. Because ESI MS is not quantitative, the magnitude of each peak is set at "1" if the peak is present and at "0" if the peak is absent. "ND" indicates an elemental formula could not be determined by use of either exact mass calculation or our combined Kendrick–matrix approach.

control cultures and those that are unique to one culture (Fig. 5). As expected, Kendrick mass analysis indicates that compounds within our culture experiments do not have the same structural similarity as fulvic acids. That finding is not surprising because compounds within biologically derived DOM have diverse function and, thus, structure (e.g., carbohydrates, proteins and lipids). This contrast was especially evident when the elemental compositions of fulvic acids and biological DOM were compared by use of the van Krevelen diagram (Fig. 6). This figure shows the stark difference in chemical composition between fulvic acids and biologically produced DOM. Compounds present in the two biological cultures have higher H:C and lower O:C ratios than compounds present in SRFA, suggesting that biological DOM is significantly more aliphatic than fulvic acids. Although this finding is not surprising, it shows that ESI MS is analyzing a subfraction of the sample that is representative of bulk DOM. Because linear relationships on the van Krevelen diagram indicate particular chemical processes such as hydrogenation or methylation (Kim et al., 2003a), this graphical analysis confirms our conclusion from the Kendrick mass analysis that the compounds within SRFA are structurally more similar to each other than the compounds in either of the biological DOM samples.

This work is the first study showing molecular-level transformations in DOM as a result of protozoan grazing. We can now identify specific compounds that are produced during protozoan grazing and those excreted by bacteria in the control culture. By use of our matrix-based data analysis approach, we determined the elemental formulae of the majority of these compounds. As expected, the compounds were more aliphatic and less oxygenated than the compounds detected in SRFA. Many of the identified compounds in biological DOM contained nitrogen (Table 1). It is not clear whether increased dissolved organic nitrogen (DON) is released in these cultures, or the positive ion mode preferentially detected nitrogen-containing compounds. Nitrogen atoms can

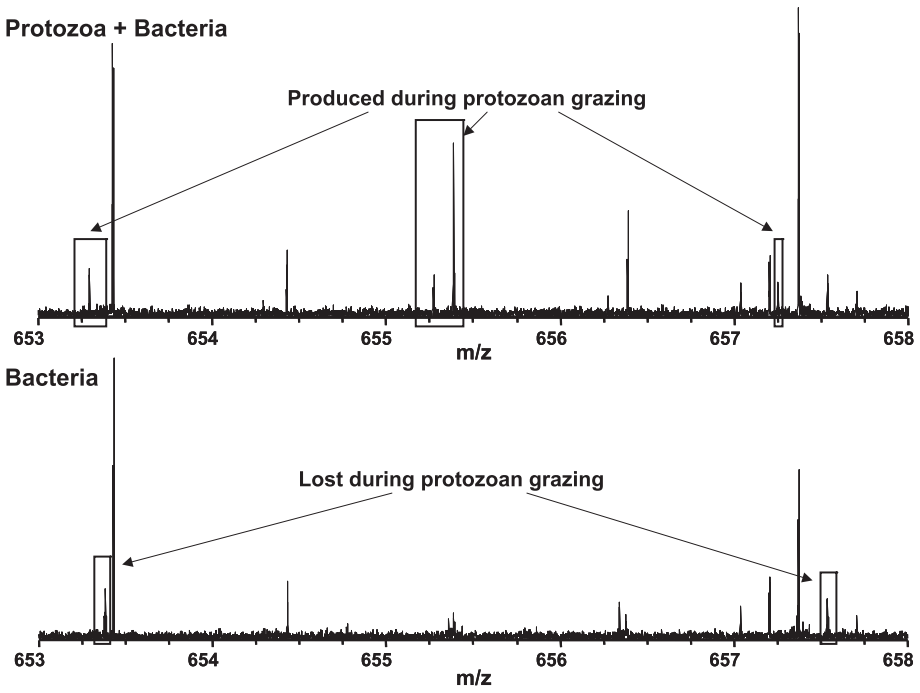

Fig. 5. Mass spectra for DOM from protozoan and bacterial cultures. Positive ion mode ESI FT-ICR mass spectra were acquired for C18-extracted protozoan and bacterial DOM (Kim et al., 2003b). The expanded $653<m/z<658$ segment is shown for both protozoan (top) and bacterial DOM (bottom). Compounds unique to each spectrum are highlighted for both spectra.

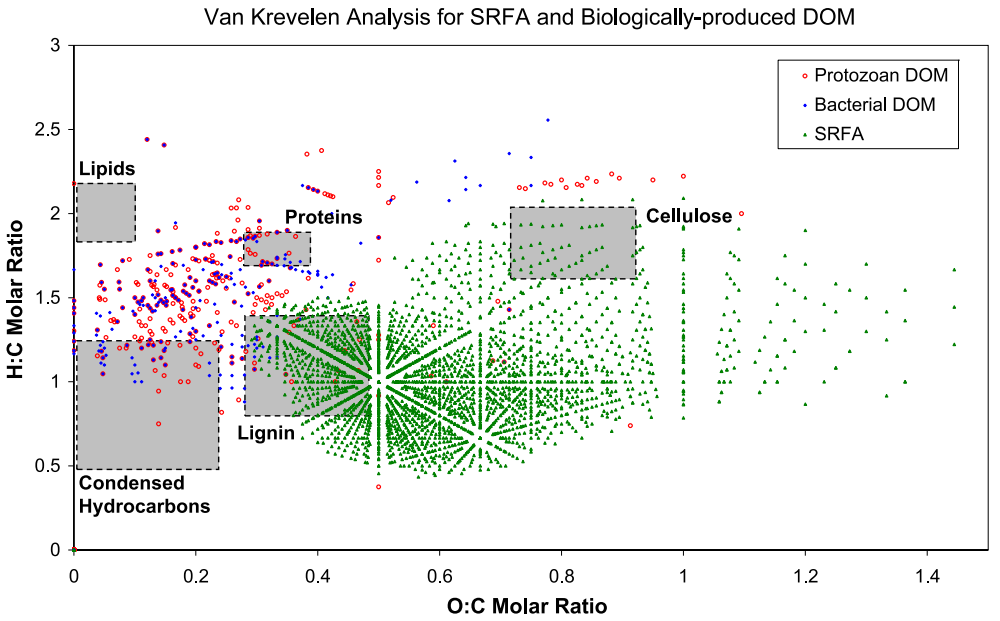

Fig. 6. Van Krevelen diagram for DOM from protozoan and bacterial cultures and SRFA. Elemental compositions for all peaks were determined by use of exact mass calculators, Kendrick mass analysis and the matrix-based program described in the text. Peaks found in the protozoan culture DOM (○); peaks found in the bacterial culture DOM (◆); and peaks found in SRFA (▲). Regions corresponding to general compound classes are also shown (values taken from Kim et al., 2003a).

easily accommodate a proton and so nitrogen-containing compounds can be ionized almost an order of magnitude more easily than oxygen-containing compounds in positive ion mode. The matrix-based data analysis identified important functional group differences in compounds within biological DOM. For example, the most commonly observed mass difference was the addition of integral multiples of $–CH_2CH_2O$, instead of $–CH_2$ used in Kendrick mass analysis. The underlying chemistry of this observation is not clear at this time.

Elemental formulae do not give structural information about compounds and thus further work is needed to identify the structure of the compounds unique to the protozoan-modified DOM. MS/MS analysis will be ideal in this situation because there are fewer compounds per nominal mass and so one compound can be isolated and fragmented. Combining ESI FT-ICR MS with more traditional geochemical techniques such as GC/MS will verify detection of different chemical fractions such as lipids. Preconcentration of different chemical fractions by liquid chromatography or solvent extraction can help enhance detection of specific chemical groups (e.g., Llewellyn et al., 2002). Isolation of DOM during different growth stages of protozoa will lead to a comparison of molecular-level changes during stationary and exponential growth phases.

5. Conclusions and future directions

ESI FT-ICR MS is an effective technique for elucidating molecular-level differences in DOM and other natural organic matter mixtures as a function of source and experimental conditions. Abiotic and biotic geochemical processes such as photodegradation and protozoan grazing produce discernible differences in ESI FT-ICR mass spectra. In the photodegradation study, compounds with high DBE and low *O* content are preferentially destroyed relative to compounds with low DBE and high *O* content. In addition, we showed that the novel products of photobleaching were either all too small ($m/z<300$) to be detected in our mass spectrometer, or they were converted to neutral nonionizable molecules, or they had the identical elemental composition to the compounds remaining after photobleaching. We did not detect any new compounds after photobleaching,

an interesting finding given the previous work in this field. In the protozoan grazing study, we detected numerous compounds that were unique to the protozoan grazing culture relative to a bacterial control. We determined elemental compositions of >75% of the detected peaks in both bacterial and protozoan grazing cultures. Many compounds contained nitrogen, suggesting preferential ionization of DON relative to bulk DOC. Future MS/MS analysis will elucidate key structural features for these compounds and extend the utility of this technique to biological experiments.

The application of ESI FT-ICR MS to geochemical questions represents a new ability to examine environmental processes on a molecular level. With the appropriate experimental design, this approach can elucidate key chemical reactions within geochemical processes. For example, one could use isotopic labeling to monitor the incorporation of a nutrient (e.g., ^{13}C-glucose or ^{13}C-acetate) into DOM. Over time, the isotopic label would move through different DOM fractions and the rates of different degradation pathways for different types of compounds could be determined. In another example, DOM from different depths in a water column could be compared and contrasted. Compounds found at all depths could be structurally characterized with MS/MS to determine the common structural features of refractory materials. Moreover, ancillary information on the depth profiles such as nutrient and oxygen concentrations could be used to explain the occurrence of unique compounds at each depth. By use of this type of data, an organic geochemist could design experiments to elucidate specific chemical reactions within natural systems and eventually formulate predictive models for DOM degradation and cycling.

Acknowledgments

Funding for this project was provided by Barnard College (EBK), the donors of the ACS Petroleum Research Fund (EBK), the Hughes Science Pipeline Project at Barnard College (EBK), the Office of Naval Research (N00014-95-10201, NVB), the National Science Foundation (CHE-9909502, NHMFL), Florida State University and the National High Magnetic Field Laboratory. Karen Chang (Barnard College '04) and Ruth Kang (BC '04) provided technical assistance. The comments of two anonymous reviewers substantially improved the manuscript.

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