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Characterization of IHSS Pony Lake fulvic acid dissolved organic matter by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry and fluorescence spectroscopy





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ABSTRACT

We present the extensive characterization of Antarctic Pony Lake (PL) dissolved organic matter (DOM), an International Humic Substance Society (IHSS) fulvic acid (FA) reference standard, using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) and excitation– emission matrix fluorescence spectroscopy (EEMS). PLFA is the first reference standard available through IHSS derived solely from a microbial source. A number of factors differentiate PLFA from other IHSS standards, including source material, geographic location, sunlight exposure, freeze–thaw conditions, and other in situ environmental influences. ESI FT-ICR MS and EEMS were used to compare the PLFA microbial DOM compositional signature with the IHSS Suvannee River (SR) FA, a standard frequently studied for environmental DOM analysis. Although $C_cH_hO_oN_nS_s$ (n = 0, 1, or 2 and s = 0 or 1) constituents were present in both IHSS samples, PLFA contained more N and S molecular species, whereas SRFA was dominated by $C_cH_hO_o$ compounds. Proteinaceous character was detected with both methods, in greater abundance for PLFA, which we attributed to its microbial source material and labile, potentially more reactive nature than SRFA. Characterization from both analytical techniques resulted in complementary data that reinforce the importance of PLFA as an IHSS reference standard that should be utilized for other microbiological environmental DOM comparisons.

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1. Introduction

Dissolved organic matter (DOM) is a significant component of marine and terrigenous aquatic ecosystems. It is formed from the decay of OM composed of intact or remnant and transformed biomolecules released from living and decaying biota (Mopper et al., 2007). It contains many identifiable classes of compounds such as sugars, amino acids, organic and fatty acids, and humic substances (Hansell and Carlson, 2001; Koch et al., 2005). Because it affects many biogeochemical processes in the environment (i.e. photochemical reactions, metal complexation, microbial growth, and nutrient and contaminant transport), determining its composition is essential for understanding the global carbon cycle (Berner, 1989). DOM derived from different sources (e.g. terrestrial, marine, glacial) has different properties, but its biogeochemical reactivity as a function of chemical nature has yet to be resolved. Moreover, it is challenging with respect to aquatic ecosystems to differentiate DOM derived from primarily autochthonous (microbial) sources

and allochthonous (soil and plant) material; investigating DOM from either source is advantageous for identifying the chemical characteristics unique to various environments and ecosystems.

The International Humic Substance Society (IHSS) was organized in 1981 to promote education about instrumental analysis of humic substances from specifically selected source materials (both solid and liquid phases; http://www.humicsubstances.org/). Humic substances are the product of biogeochemical degradation of detrital biomass and are considered to be the most refractory component of DOM with respect to its resistance to further biodegradation. Aqueous humic substances can be subdivided into two fractions: (i) humic acid (HA) – the major extractable component of humic substances which are dark brown in color and not soluble in water below pH 2, and (ii) fulvic acid (FA) - soluble in water under all pH conditions and lighter in color, ranging from yellow to brown. IHSS standards are available for researchers to critically examine humic substance experimental results from various analytical instruments and represent a DOM reference for comparing and contrasting specific data sets with different DOM samples from other unique environments. Many researchers have focused on extensive DOM characterization by comparison with IHSS

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standards, specifically Suwannee River (SR, a terrestrial HA and FA analog). With the recent addition of Pony Lake (PL) FA, a microbially derived DOM reference sample potentially analogous to other microbially dominated environments is available.

The global reservoir of DOM is substantial: its amount is greater than the quantity of carbon stored as CO_2 in the atmosphere (Gorham, 1991; Hedges, 2002). Marine and terrestrial aquatic systems are thought to be the largest contributors; however, a third reservoir exists that functions as both a source and sink for natural OM, i.e. ice. Until recently, it was believed that glacial environments were devoid of life, but many discoveries of microbial communities and OM in glacial systems have generated attention toward a better understanding of life in these extreme environments and, in turn, studying its contribution to the global carbon cycle (Sharp et al., 1999; Priscu and Christner, 2004; Priscu et al., 2008; Lanoil et al., 2009; Foreman et al., 2011; D'Andrilli et al., unpublished results).

The four main locations for IHSS HA and FA standards are SR (river water in Okefenokee Swamp, Georgia), Elliot Soil (fertile prairie soils, Illinois), Pahokee Peat (agricultural peat soil, Florida Everglades) and Leonardite (a low grade coal, North Dakota), all of which are found in the continental USA and are heavily exposed to higher plant/terrestrial input (site information available at http://www.humicsubstances.org/). PL (77°33'S, 166°9'E) is a coastal pond on Cape Royds, Ross Island, Antarctica. Completely isolated from terrigenous input and with no existing higher plants in the watershed, PL represents an excellent system for studying autochthonous, microbially derived DOM; hence its appeal as an IHSS standard FA sample. The lake is shallow (1-2 m) and perennially ice covered, but areas may melt during the austral summer. It contains a relatively high concentration of DOM (1.3-2.4 mM), compared with other glacial environments, and sustains living organisms such as bacteria, virus-like particles, algae and ciliate protozoans throughout the year (Brown et al., 2004; Foreman et al., 2011; Dieser et al., 2013). It provides a DOM FA reference standard that adds to the repertoire available for future DOM comparisons with different environments. PLFA has been characterized by use of ion chromatography, organic carbon analysis, absorptivity, molecular weight (MW) analysis, high performance size exclu-¹³C nuclear magnetic resonance chromatography, sion spectroscopy (Brown et al., 2004) and excitation-emission matrix fluorescence spectroscopy (EEMS; Cory et al., 2010). However, no detailed molecular qualitative information has been provided for this IHSS standard.

The task of identifying DOM molecular components presents a significant challenge because they are polyfunctional and heterogeneous (containing C, H, N, O and S), and vary greatly in MW and concentration (Mopper et al., 2007). Analyzing DOM has until recently been limited to characterizing its bulk properties or experimenting with very limited/defined fractions not representative of the entire sample (Mopper et al., 2007). Bulk property measurements, while useful, cannot be used as true molecular descriptors because no "average" molecule within DOM defines its entire character. No single analytical technique produces both bulk and detailed molecular information regarding DOM, so it is common for multiple techniques to be applied.

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) at high magnetic field (>7 T) (Marshall et al., 1998; Marshall and Rodgers, 2008) is unrivaled for achieving the resolution and accuracy required to determine DOM molecular formulae over a wide range (200 < m/z < 1000). It is possible to characterize DOM samples from different sources by determining the molecular constituents, as applied for extensive characterization and comparison of other environmental DOM samples with SRFA (Freitas et al., 2001; Kujawinski et al., 2002; Stenson et al., 2003; Sleighter and Hatcher, 2007; Bhatia et al., 2010). We report here complementary

data (from FT-ICR MS and EEMS) for advanced and bulk DOM characterization from this unique Antarctic system in order to highlight PLFA as a useful IHSS reference standard by comparing it with SRFA.

2. Material and methods

2.1. Sampling

PLFA and SRFA reference standards were obtained from the IHSS. Chemical information and source material description can be found at http://www.humicsubstances.org/. The samples were selected for two reasons: (i) PLFA has not been characterized at the molecular level with FT-ICR MS and represents an excellent reference for microbially derived DOM from other glacial, marine and terrigenous ecosystems and (ii) SRFA represents an excellent comparative DOM sample as it has been extensively characterized by FT-ICR MS, as well as being utilized as a terrigenous DOM source reference for many other environments. As noted above, the two IHSS samples represent "end members" regarding environmental source and have high OM concentration. Solutions were created by dissolving the freeze-dried powder of each FA concentrate with 100% MeOH (HPLC grade) in clean combusted amber glass vials (500 µg C/ml). Samples were vigorously shaken and stored in the dark at 4 °C prior to use.

2.2. Electrospray ionization (ESI)

Negatively charged gaseous analyte ions $[M-H]^-$ for PLFA and SRFA were produced by ESI prior to MS analysis. The custom-built micro-electrospray source (Emmett et al., 1998) is equipped with a 50 µm i.d. fused silica tube. A flow rate of 0.5 µl/min was maintained by syringe pump and ESI experimental conditions were: needle voltage -2.1 kV, tube lens, -325 V and heated metal capillary at 11.5 W. The parameters were based on previous experimentation and updated for optimal DOM MS characterization for both samples (Stenson et al., 2003). It is important to note that although another ionization method (atmospheric pressure photoionization) was considered, ESI was selected due to its extensive prior use for SRFA characterization with FT-ICR MS.

2.3. FT-ICR MS

Spectra for PLFA and SRFA were obtained with a custom built 9.4 T superconducting magnet FT-ICR mass spectrometer at the National High Magnetic Field Laboratory (NHMFL) in Tallahassee, Florida, USA (Blakney et al., 2011; Kaiser et al., 2011). The instrument consistently produces ultrahigh resolving power ($m/\Delta m_{50\%}$ > 750,000 at m/z 500) and accuracy (rms mass measurement error < 1 ppm). Instrumental parameters were selected for optimal natural OM MS acquisition and characterization. Excitation ranged from m/z 200–1500 at a frequency sweep rate of 50 Hz/µs and octopole frequency was maintained at 2.0 MHz. Multiple (100–200) time domain acquisitions were co-added, Hanning apodized and zero-filled once before fast Fourier transformation and magnitude calculation (Marshall and Verdun, 1990).

2.4. Mass spectra and molecular formula assignment

Spectra were internally calibrated from two homologous series (differing by number of $-CH_2$ groups) of $[M-H]^-$ ions previously identified and commonly found in natural OM in the range 200 < m/z < 600. Calibration with these homologous series produced an rms mass measurement error < 1 ppm for singly charged molecular ions. A peak list was generated for MS peaks with a

magnitude at least 6x the baseline rms noise, which is a conservative level that potentially yields > 10,000 assignable peaks from one complex DOM spectrum. The signal/noise (S/N) threshold provides a baseline that is sample specific, making it possible to characterize and compare natural OM more accurately. All molecular ions were determined to be singly charged by confirming the m/z1.0034 spacing pattern between ions differing in elemental composition by ${}^{12}C_n$ and ${}^{12}C_{n-1}{}^{-13}C_1$ (Limbach et al., 1991; Brown and Rice, 2000; Kujawinski et al., 2002).

DOM characterization and molecular formula assignment from 9.4 T ESI FT-ICR MS analysis has been described in detail by Stenson et al. (2003). Briefly, large FT-ICR MS data sets (>10,000 peaks) are efficiently and reliably analyzed by conversion to Kendrick mass (Kendrick, 1963; Hughey et al., 2001; Stenson et al., 2003; D'Andrilli et al., 2010a). All possible elemental compositions within ± 1 ppm mass measurement error were considered, subject to the following constraints appropriate for both PLFA and SRFA: ¹²C (0-100), ¹H (0-200), ¹⁴N (0-2), ¹⁶O (0-30), 32 S (0–2), and 13 C (0–1). These compositional constraints were modeled after those reported by Stenson et al., (2003) for natural OM characterization and preliminary data from Brown et al., (2004), that described the elemental constituents C, H, N, O, and S that comprise PLFA samples and the structural functional group identification data regarding the N-containing species reported by Mao et al., (2007) and Fang et al., (2011).

Specific restrictions were applied to eliminate potentially incorrect formula assignments. Elemental composition was assigned to ion peaks for homologous series above the S/N threshold having specific mass error < 1 ppm, MS ¹³C peak confirmation and specified mass spacing patterns. Negative ion peak masses were converted to neutral masses by addition of ¹H⁺ = 1.007276 Da. The data were subsequently sorted by elemental composition ($C_cH_hN_n-O_oS_s$), O content, degree of saturation and unsaturation, aromatic nature, or chemical heteroatom classification. Heteroatom content is defined as C, H, and O species (DOM molecular backbone) with varying numbers of N and/or S atoms.

2.5. Excitation-emission matrix fluorescence spectroscopy (EEMS)

This approach has become relatively more common for probing the composition, concentration, and dynamics of fluorescent OM from various source materials (Coble, 1996; Hudson et al., 2007; Cory et al., 2010). EEMS scans report fluorescence intensity measured over a range of excitation and emission wavelengths (Coble, 1996; Mopper et al., 1996; Hudson et al., 2007).

Absorbance spectra (between 190 and 1100 nm) were collected for both samples by use of a Genesys 10 Series (Thermo-Scientific) Spectrophotometer (1 cm path length) to reduce inner filter effects during post processing (McKnight et al., 2001). Absorbance values were also monitored at 254 nm, a wavelength chosen to evaluate aromatic character of OM in natural waters (absorbance < 0.3 prior to EEMS; McKnight et al., 2001). PLFA values averaged A₂₅₄ 0.231 (*n* = 3), which is below the acceptable threshold, so no dilution was required. SRFA A₂₅₄ values exceeded the threshold and thus were diluted accordingly to produce acceptable values that averaged A₂₅₄ 0.175 (*n* = 3). Dilution factors were recorded and included for post processing calculations.

PLFA and SRFA EEMS spectra were obtained with a Horiba Jobin Yvon Fluoromax-4 Spectrofluorometer equipped with a Xe lamp light source and 1 cm path length quartz cuvette at 25 °C with the following specifications: excitation wavelength 240–450 nm scanned over 10 nm intervals, emission wavelength 300–600 nm recorded in 2 nm increments, data integration period 0.25 s, 5 nm band pass for both excitation and emission monochromators; all data were generated in signal/reference mode to normalize the emission signal relative to the excitation light intensity. Post-collection data manipulation was performed in MATLAB to correct for inner filter effects, Raman scattering and a background blank Milli-Q Water subtraction. Position and intensity (Ex and Em maximum values) for individual fluorophores were determined to gain more information on the composition and character of PLFA and SRFA source material.

3. Results and discussion

3.1. ESI FT-ICR MS of PLFA and SRFA

ESI 9.4 T FT-ICR spectra of PLFA and SRFA are shown in Fig. 1a and b. Although we acknowledge the selectivity effects in the methodology for isolation of both PLFA and SRFA IHSS samples and ESI FT-ICR MS, instrumental parameters were chosen to maximize the production and detection of singly charged ions, reduce ion suppression effects, minimize ion collision and eliminate irreproducible ions between spectral scans (100–200 co-added MS data). Consequently, the data are more extensive in molecular composition coverage than any other single analytical technique. Moreover, our objective at the outset was to characterize the molecular composition of PLFA and subsequently to compare it with that of SRFA to provide further information on its constituents and advantage as an IHSS reference standard.

Although the mass distributions for PLFA and SRFA spanned the same range (200 < m/z < 600), the PLFA distribution was skewed somewhat below m/z 300 relative to the roughly Gaussian distribution for SRFA. More negative ions were observed between m/z200–300 for PLFA (Fig. 1a) than for SRFA (Fig. 1b). The recurring mass spacing patterns common for terrestrial DOM were observed for PLFA and SRFA: 14.01565 Da for homologous series $[(CH_2)_n vs]$. (CH₂)_{*n*+1}], 1.0034 Da (¹³C vs. ¹²C), and 0.0364 Da (CH₄ vs. ¹⁶O), each shown in top and middle insets in Fig. 1a. Insets spanning an m/zrange of ca. 0.25 at nominal mass 311 are depicted in Fig. 1 for both PLFA and SRFA. Above the S/N threshold, negative ESI FT-ICR MS vielded 29 assignable MS peaks for PLFA. (formulae given in Table 1), providing an example of complex DOM having a relatively large number of diverse molecular species within a small mass spectral window (m/z range < 1). Within the same MS window, only 6 mass spectral peaks were identified and assigned formulae above the S/N threshold for SRFA (Fig. 1b inset). Five SRFA MS peaks were common to PLFA at nominal mass 311 (four $C_c H_h O_o$ and one $C_cH_hO_0S_1$ species) and one MS peak for SRFA was assigned a formula not common with PLFA (highlighted in Fig. 1b inset).

The S series $(C_c H_h O_o S_1)$ was present in both PLFA and SRFA; however the heteroatom contribution to the total SRFA composition was much lower than for PLFA (Table 2). PLFA exhibited very different composition proportions from SRFA, although all six classes were present in both samples. Table 2 provides a list of the composition (%) for each molecular species' contribution to the total character of each sample, the carbon number range for formulae assigned, and the composition (%) of each molecular species with exact matching and non-matching molecular formulae for PLFA and SRFA. Composition in Table 2 was calculated by dividing the number of formulae for each class by the total number of formulae in each sample and multiplying by 100. A guarter of the characterized PLFA sample was comprised of only $C_c H_h O_o$, the largest overall contribution to PLFA even though the proportion was considerably lower than the 84.5% value for SRFA. Both overall contributions of N and S containing species for PLFA molecular ions (58.8% and 38.1%) were strikingly different from SRFA (12.1% and 5.8%) and stressed the importance of a heteroatom contribution to the PLFA microbial ecosystem.

PLFA and SRFA data were also evaluated for exact formula matches and non-matches over the entire MS range; 1248 exact



Fig. 1. Negative ESI FT-ICR mass spectra for (a) PLFA and (b) SRFA. Figure (a) shows examples of the mass spacing patterns throughout the distribution: *m*/*z* 305–330 inset (top) and *m*/*z* 0.25 interval inset at nominal *m*/*z* 311 (middle) with formula assignments listed in Table 1. The inset in (b) includes formula assignments for each SRFA peak at nominal *m*/*z* 311 with the unique formula not observed for PLFA highlighted.

matching formulae were identified between each data set with molecular mass within ± 0.00091 Da, whereas 6181 and 301 compositions were determined to be unique to PLFA and SRFA (proportions in Table 2). The largest class of formula matches between PLFA and SRFA was $C_cH_hO_o$ and, while matches existed in all heteroatom groups, they only contributed 16.3% to the total matches between PLFA and SRFA. Again, the major differences were within the N and/or S containing species of unique formulae, accounting for 85.7% of PLFA but only 12.6% of SRFA. We suggest that the different chemical nature resulting from the microbial source of PLFA vs. SRFA is responsible for the higher proportion of negatively charged heteroatom containing ions ionized via ESI and detected with FT-ICR MS (e.g. the 5 series within the heteroatom classes C_c - $H_hO_oS_1$, $C_cH_hO_oN_2$, $C_cH_hO_oN_2$ S₁ assigned from Fig. 1a and listed in Table 1 for PLFA, but not observed for SRFA).

3.2. van Krevelen diagram analysis: Heteroatoms, source material and aromaticity

To extend the observations, entire datasets for PLFA and SRFA were analyzed with van Krevelen diagrams, namely, a plot of H/C vs. O/C atomic ratio (Kim et al., 2003). The diagram is an excellent method for visualizing large DOM datasets and their respective chemical nature with respect to readily ionizable compounds.

Because major chemical classes typically found in DOM have characteristic H/C and O/C ratios, they tend to cluster within specific regions of the diagram. Fig. 2 shows generic van Krevelen patterns, reflecting chemical characterization, reference source material, level of aromaticity and possible reaction pathways.

PLFA and SRFA van Krevelen diagrams, including formula assignments having feasible combinations of $C_c H_h O_o N_n S_s$, are displayed in Fig. 3a and b. The diagrams depict one data point for each formula assigned from one resolved peak in the spectrum. Common and unique constituents of PLFA and SRFA are shown in the van Krevelen diagrams in the Supplementary Information. PLFA data cluster in different regions of the diagram from SRFA. The highest abundance of ionizable biomolecules from ESI FT-ICR MS corresponded to O/C 0.42 and H/C 1.37 for PLFA and O/C 0.53 and H/C 1.20 for SRFA. Both compounds are CHO-containing species: PLFA $C_{19}H_{26}O_8$ and SRFA $C_{15}H_{18}O_8$, which describe compounds very similar in constituents, but differing by a lower degree of oxygenation and a higher degree of hydrogen saturation for PLFA.

Each heteroatom class in Table 2 is also represented in Fig. 3a and b. All classes for PLFA cluster in the middle of the lignin-like species region displaying the common molecular patterns associated with natural OM and reaction pathways depicted in Fig. 2; however, each class also extends from that center (H/C 1.0 and

Table 1

Molecular formulae assigned for the seven 0.0364 Da spaced series in the m/z range ca. 0.25 window at nominal mass 311 displayed in Fig. 1a for PLFA.

	$C_c H_h O_o$	$C_c H_h O_o S_1$	C _c H _h O _o N ₂	$C_c H_h O_o N_2 S_1$
	C ₁₆ H ₇ O ₇	C ₁₃ H ₁₁ O ₇ S ₁	$C_{15}H_7O_6N_2$	$4 \begin{bmatrix} C_{12}H_{11}O_6N_2S_1 \end{bmatrix}$
1	C ₁₇ H ₁₁ O ₆	$2 C_{14}H_{15}O_6S_1$	$\int C_{16}H_{11}O_5N_2$	$C_{13}H_{15}O_5N_2S_1$
	C ₁₈ H ₁₅ O ₅ C ₁₉ H ₁₉ O ₄	$C_{15}H_{19}O_{5}S_{1}$ $C_{16}H_{23}O_{4}S_{1}$	$C_{17}H_{15}O_4N_2$ $C_{12}H_{11}O_8N_2$	
	$C_{20}H_{23}O_{3}$	^L C ₁₇ H ₂₇ O ₃ S ₁	7 C ₁₃ H ₁₅ O ₇ N ₂	
	C ₂₁ H ₂₇ O ₂	$C \begin{bmatrix} C_{10}H_{15}O_9S_1 \end{bmatrix}$	C ₁₄ H ₁₉ O ₆ N ₂	
	C ₁₃ H ₁₁ O ₉	$C_{11}H_{19}O_8S_1$	$- C_{15}H_{23}O_5N_2$	
5	C ₁₄ H ₁₅ O ₈ C ₁₅ H ₁₉ O ₇	C ₁₂ H ₂₃ O ₇ S ₁		
	$C_{16}H_{23}O_{6}$			
	C ₁₇ H ₂₇ O ₅			
	C ₁₈ H ₃₁ O ₄			

Table 2

Percent composition contributions to the total PLFA and SRFA samples calculated from assigned formulae in each category, carbon number ranges, and percent composition for exact matching and non-matching molecular formulae contributions between PLFA and SRFA.

Composition species	PLFA composition (%)	SRFA composition (%)	PLFA carbon # range	SRFA carbon # range	PLFA and SRFA composition matches (%)	Unique PLFA composition (%)	Unique SRFA composition (%)
$C_c H_h O_o$	25.9	84.5	7–42	7-40	83.7	14.3	87.4
$C_c H_h O_0 Si$	15.3	3.41	5-33	9–20	4.26	17.5	0
$C_c H_h O_o N_1$	19.1	9.40	7-32	8-30	11.3	20.7	0
$C_c H_h O_o N_1 s_1$	12.8	0.129	6-29	14-15	0.160	15.3	0
$C_c H_h O_o N_2$	16.9	0.322	7-26	16-24	0.400	20.3	2.00
$C_c H_h O_o N_2 S_1$	9.98	2.25	6-31	13-21	0.240	11.9	10.6



Fig. 2. van Krevelen diagram depicting reference DOM characterization, reaction pathways that extend to higher or lower H/C or O/C ratios, and degree of aromatic nature. The conservative aromaticity threshold (Al > 0.5) is based on Koch and Dittmar (2006).

O/C 0.50) uniquely to different H/C and O/C ratios. Cellulose-like species include formulae containing various N and S classes (CHOS₁, CHON₁S₁ and CHON₂S₁) for PLFA. Compounds in the cellulose-like region in Fig. 3b contain only CHON₂S₁ for SRFA. CHON₂ molecular ions in PLFA extend to proteinaceous-like character, condensed aromatic character and an undefined region having lower O/C ratio (ca. 0.20) and H/C ranging between 0.50 and 1.5. The same characterization existed for CHON₁, CHON₁S₁ and CHO classes, with the latter having the lowest O/C ratio (<0.20) and

H/C value ranging from 0.50-2.0. This region describes molecular ions significantly less oxygenated over varying levels of hydrogen saturation, and has been reported for other glacial and ice core DOM studies (Grannas et al., 2006; Bhatia et al., 2010; Singer et al., 2012; Stubbins et al., 2012). Major differences between PLFA and SRFA exist at this location and also at higher H/C ratio (1.5-2.0)over varying degrees of oxygenation, including molecular ions in each heteroatom class in the lipid-, protein-, amino sugar-, and cellulose-like regions. Variation in H/C and O/C data in the diagrams corresponds to different sources of OM between the two samples. PLFA is comprised of many chemical classes highlighted in Fig. 2, with noticeably more protein-, amino sugar-, cellulose-like, and more condensed aromatic species than SRFA. Protein-like DOM character has been shown to reflect more labile microbially influenced OM and Fig. 3a shows a significant contribution of proteinlike species between H/C 1.5 and 2.0 and O/C 0.20 and 0.50, values not observed for SRFA. The occurrence of N-bearing species in the protein-like region was expected for microbially derived PLFA. We suggest that these compounds with lower MW (200 < m/z < 600) are the biologically produced intermediates or end products of longer chain degraded proteins (500 < m/z < 2500; Chowdhury et al., 1991). Schmidt et al. (2009) reported that shifts to lower O/C and higher H/C ratios of DOM in marine sediments are due to more aliphatic compounds originating from algal detritus and/ or microbial biomass. Kim et al. (2006) suggested that changes in O/C and H/C ratios result from biodegradation, specifically pathways depleting oxygen DOM compounds, leading to a higher degree of hydrogen saturation. Our findings are consistent with the reported studies; we attribute the observed lower O/C and higher H/C ratios to the dynamic microbial character of PLFA relative to SRFA.

Clear similarities in PLFA and SRFA are also seen in Fig. 3a and b, with overlapping data within the lignin-like region, the most predominant feature common to different types of DOM from various



Fig. 3. van Krevelen diagrams for (a) PLFA and (b) SRFA, including compounds containing $C_cH_hO_oN_nS_s$. Various heteroatom classes are represented in different colors and symbols.

environments. Lignin species are complex compounds derived from higher plants, differing in degree of saturation, and are one of the most slowly decomposing components of dead vegetation, hence the refractory nature. It is interesting to observe the lignin-like character for PLFA, an environment completely devoid of higher plants.

Other DOM originating from non-terrigenous environments (Antarctic sea ice, ocean water) displays similar MS characteristics (Koch et al., 2008; D'Andrilli et al., 2010b); we therefore note that the lignin-like classification of van Krevelen diagrams is not, and should not be, exclusively linked to higher plant source material. Other studies have outlined a similar section of the van Krevelen diagram to include carboxylic-rich alicyclic molecules or poly phenolic compounds, which cluster within the lignin region of the diagram (Hertkorn et al., 2006; Stubbins et al., 2010). These types of compounds exhibit strong resistance to further biodegradation as they are the end products of decomposition, therefore possessing refractory character similar to that of lignin and humics. To clarify this point, we stress that the lignin-like section of the van Krevelen diagram merely describes the refractory nature of chemical species that cluster within the outlined H/C and O/C ratios. We suggest that microbial transformation of the biomolecules in PLFA results in lignin-like or more refractory end products, e.g. demethylation of lipids or protein-like residues and dehydrogenation of proteins, as seen in Fig. 2.

Aromatic character was evaluated by use of two calculations: aromaticity index (AI) and modified AI (AI_{mod}), both of which calculate C=C density; the former produces a more conservative

threshold for aromatic character (AI > 0.5) and the latter includes the possibility of C–O bonds as well as C=O bonds, yielding a lower threshold for suggesting condensed aromatic structures (Koch and Dittmar, 2006). Note that this calculation is vital for visualizing unequivocal aromatic character from large DOM datasets based on low H/C nature, but involves some uncertainty regarding highly substituted aromatic species at AI values < 0.5 (examples discussed by Podgorski et al., 2012). Fig. 2 shows a solid black line that corresponds to the conservative threshold of AI > 0.5 indicative of aromatic structures and four downward pointing arrows that lead to more condensed aromatic species at lower H/C ratio (AI \ge 0.67; Koch and Dittmar, 2006). Overall, PLFA contains more highly condensed aromatic compounds with varying amount of heteroatoms (CHO, CHON₁, CHON₂, CHON₁S₁ and CHON₂S₁) than SRFA (Fig. 3a). Fig. 3b shows SRFA condensed aromatic character for compounds containing only CHO and CHON₁.

Photochemical reactions may also explain some of the differences between PLFA and SRFA. Gonsior et al. (2009) compared the nature of North Carolina River and estuary DOM before and after photo-irradation, displaying shifts to molecular ions with higher H/C ratio over a broad range of O/C ratio. The authors related such shifts to the transformation of unsaturated/condensed aromatic compounds (based on AI values) to more saturated, less condensed aromatic molecules. We expected a higher abundance of condensed aromatic molecular data for SRFA than PLFA, for which less aromatic character has been reported (McKnight et al., 2001). However, condensed aromatics could be more readily photochemically altered for SRFA due to its exposure to sunlight and lower probability of freezing and thus not detected in greater abundance vs. PLFA. We propose that the higher condensed aromatic content of PLFA is partly due to its minimal duration of sunlight exposure (seasonally unfrozen for a few weeks during the austral summer) and is therefore less available (but nonetheless susceptible) for photochemical transformation to more saturated material than SRFA.

3.3. Double bond equivalence and carbon number

Additional information was gained by analyzing the double bond equivalent (DBE) values and carbon number distribution. DBEs (number of rings plus double bonds to carbon) measures the hydrogen unsaturation of a molecular ion based on

$$DBE = C - H/2 + N/2 + 1$$
(1)

where C, H, and N represent the numbers of atoms in the formula (McLafferty and Turecek, 1993). A DBE value of 0 describes a fully saturated compound (Purcell et al., 2006). Fig. 4a and b shows isoabundance-contoured plots of DBE vs. carbon number. PLFA and SRFA display the common trend of increasing DBEs with increasing carbon number. However, PLFA extends to higher DBEs (22) than SRFA (17) over a wider carbon number range, due to more unsaturated/condensed aromatic compounds in PLFA, consistent with the van Krevelen data in Fig. 3a. The dark red regions in Fig. 4a and b, correspond to the greatest abundance of biomolecules with the same carbon number and DBE value. Interestingly, they cluster in similar regions between PLFA and SRFA (carbon number \approx 15, DBEs \approx 7–10), with considerably different breadth of distribution and much higher abundances for PLFA than SRFA (49 vs. 19). Compounds in this region were found to contain every heteroatom class, including CHO species for PLFA (49 compositions) spanning H/C 1.20-1.33 and O/C 0.20-0.80, whereas SRFA (19 compositions) contained only CHO, CHOS₁, CHON₁S₁, and CHON₁ classes over H/C 1.20-1.27 and O/C 0.20-0.80 ranges (e.g., carbon number 15 and DBEs 7 in Fig. 4a and b).



Fig. 4. Isoabundance contoured plots of DBE vs. carbon number for (a) PLFA and (b) SRFA.

3.4. EEMS: Source material, photooxidation and reactivity

Complementary data sets from fluorescence spectroscopy are presented in Fig. 5a and b. Similar fluorescence patterns between PLFA and SRFA were observed in the humic-like fluorophore regions labeled A, M and C. We employed the labeled fluorophores A. C. B. T and M as proposed by Coble (1996). Table 3 includes a list of common natural water DOM fluorophores, descriptions and observed fluorescence regions for PLFA and SRFA. The unique fluorescent nature of PLFA in Fig. 5a is evident at low excitation-emission wavelengths where proteinaceous fluorophores are present. Fluorescence in both B and T regions is characteristic of amino acid-like constituents in DOM derived from microbes, and are typically more labile in nature than humic substances. Both the proteinaceous-like and more refractory humic-like fluorescence for PLFA agree with FT-ICR MS data as visualized from the van Krevelen diagram (Fig. 3a). Fig. 5a shows a shift to shorter wavelength (blue shift) for PLFA, which may be characteristic of less refractory humic-like signatures compared with SRFA EEMS in Fig. 5b.

According to the McKnight et al. (2001) fluorescence index (FI) calculation for uncorrected EEMS data, DOM having higher FI values (1.8–1.9) corresponds to microbially derived OM, whereas terrigenous derived OM corresponds to lower FI values (1.3–1.4). For corrected EEMS data, the FI range was considerably broader for microbially derived than terrestrially derived OM (McKnight et al., 2001; Fulton et al., 2004; Schwede-Thomas et al., 2005). FI values calculated by the McKnight et al. (2001) method for PLFA and SRFA corrected EEMS were 1.45 ± 0.002 and 1.29 ± 0.011, within the range reported for these IHSS reference samples with different instruments (Cory et al., 2010). Note, the standard deviation was calculated for n = 3.



Fig. 5. 3D EEMS and identified DOM fluorophores for IHSS samples (a) PLFA and (b) SRFA. Fluorescence intensity was normalized to the integral of the Raman peak from excitation at 350 nm and is given in Raman units (RU).

Biers et al. (2007) describe the role of N in developing chromophoric and fluorescent species in seawater DOM, but also summarize specific works that have confirmed in situ biological activity as a source of marine chromophoric DOM. Other reports confirm that N species are readily incorporated into humic substances in soil (Biers et al., 2007). Because dissolved organic N is susceptible to photochemical reactions (Bushaw et al., 1996; Reitner et al., 2002; Vähätalo and Zepp, 2005), we believe that N containing species are crucial for the differences between PLFA and SRFA composition. Biers et al. (2007) found that microbial processing and photooxidation of dissolved organic and inorganic N compounds produced chromophoric DOM compounds. Therefore, we speculate that similar microbial processing of DOM is occurring for PLFA, producing some of the fluorescent characteristics observed with EEMS (Fig. 5a).

N and S containing functional groups in FA DOM are especially important in determining the reactive nature of the DOM (Chin et al., 1994; McKnight et al., 2002). The amino acid-like fluorophores in the EEMS for PLFA suggest more reactive and labile OM than for SRFA, for which the contributions of the B and T peaks were far less evident. Therefore, we propose that the B and T regions correspond to some N and S containing species that also cluster in the more labile proteinaceous and amino sugar regions in the van Krevelen diagram for PLFA. N and S biogeochemistry occurring for PL and its link to phylogenetic data over the winter season in ice column samples was reported by Foreman et al., (2011). Sulfur-reducing bacteria and sulfur-oxidizing chemolithoautotrophs were identified at PL. both of which involve oxidizing OM either by anaerobic respiration to produce H₂S or by denitrification (reduction of oxidized N species by use of OM as electron donor) (Foreman et al., 2011). The authors reported a pungent odor of H₂S at PL, supporting the occurrence of sulfate reduction by

Table 3

Previously identified common natural water OM fluorophores (Coble, 1996; Marhaba et al., 2000; Marhaba and Lippincott, 2000) and fluorescing regions assigned for PLFA and SRFA.

Fluorophore	Description	Ex/Em Max (nm)	PLFA Ex/Em Maxima (nm)	SRFA Ex/Em Maxima (nm)
А	Humic-like	237-260/400-500	240/440	240/450
С	Humic-like	300-370/400-500	320/425	320/450
M	Marine humic-like	312/380-420	310/400	310/425
В	Protein-like (tyrosine)	225-237/309-321	240/300	240/300
		275/310	270/310	-
Т	Protein-like (tryptophan)	225-237/340-381	240/340	240/340
		275/340	270/340	-

microbes and a potential shift from aerobic to anaerobic conditions with extensive OM uptake in the ice (Foreman et al., 2011). With identified MS compounds containing N and S and with existing data on microbial communities at PL (Foreman et al., 2011; Dieser et al., 2013), it is probable that the most labile material of PLFA originates from the degradation of longer chain proteins and from N and S chemical processes by microbial activity. We also speculate that further transformation and reactions of microbially derived DOM biomolecules occur, producing refractory chemical species due to the data in and around the lignin-like region in the van Krevelen diagrams and humic-like fluorescence regions in the EEMS. However, the data regarding the exact transformation mechanisms of microbially derived DOM and their respective shifts to different DOM character (more humic-like) are considerably limited and further research is needed to understand the N and S microbiological influences on structural diversity, fluorescence and chemical nature of DOM.

4. Conclusions

High magnetic field ESI FT-ICR MS and EEMS are important tools for complementary advanced and bulk characterization of natural OM. IHSS standard and reference samples have been used in the past to compare and contrast different types of DOM constituents with other natural samples in order to better understand its chemical characterization. Most commonly used is SRFA; however, with the addition of PLFA, more information regarding DOM produced solely from microbial sources should soon become available.

PLFA and SRFA samples contain components at varying levels of production and consumption, so the data here represent a mere snapshot of accumulated OM precursors, intermediates and products. Comparing mass spectra and EEMS data gave the most information regarding the labile and recalcitrant nature of both DOM samples. Heteroatom content (N and/or S species) is a relatively new addition for analyzing the constituents of DOM with FT-ICR MS and has gained attention regarding degradation processes, photochemical affinity, source material and reactive character of the DOM. $C_c H_h O_o N_n S_s$ constituents are present in both PLFA and SRFA, but the proportion of each contributing class is different. PLFA is comprised of more labile, microbially derived material, reflected in the higher proportion of N and S containing species over a broad range of O/C and H/C ratios. This conclusion is supported by the presence of protein- and amino sugar-like components identified in the van Krevelen diagram and in the B and T fluorescent regions of the EEMS for PLFA, which were not observed for SRFA. We also report that both labile and refractory DOM constituents are present at PL, based on these data. At present, we can only speculate that the most labile constituents are proteinaceous compounds with varying heteroatom contributions derived from microbial metabolism intermediates or as end products. The more refractory material in PLFA (lignin-, cellulose- and humic-like signature regions) is indicative of degradation of microbially produced OM via

demethylation and potentially dehydrogenation, as this environment receives zero input from terrigenous sources.

Both ESI FT-ICR MS and EEMS produced data that confirm the microbial source of PLFA. Further research is necessary, however, to determine the exact processes involved in microbial production and transformation of DOM, specifically regarding the pathways that may lead to lignin-like, more refractory DOM signatures in PL. However, the results stress the importance of incorporating PLFA as an IHSS standard that can be utilized for other glacial or microbially produced DOM comparisons with many environments. We currently use IHSS PLFA and SRFA as reference standards in other glacial DOM investigations to better understand microbe/DOM interactions and terrestrial vs. microbially produced DOM signatures that can aid in identifying components in ice sheet and glacial ecosystems.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.orggeochem. 2013.09.013.

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