A History of Molecular Level Analysis of Natural Organic Matter by FTICR Mass Spectrometry and the Paradigm Shift in Organic Geochemistry *

William T. Cooper[1,#], Jeffrey C. Chanton[2], Juliana D'Andrilli[3], Suzanne B. Hodgkins[4], David C. Podgorski[5], Alexandra C. Stenson[6], Malak M. Tfaily[7], Rachel M. Wilson[2]

[1]Department of Chemistry & Biochemistry, Florida State University, Tallahassee, FL 32306

[2]Department of Earth, Ocean and Atmospheric Science, Florida State University, Tallahassee, FL 32306

[3] Louisiana Universities Marine Consortium, Chauvin, Louisiana 70344

[4]Department of Microbiology, Ohio State University, Columbus, Ohio 43210

[5]Department of Chemistry, University of New Orleans, New Orleans, LA 70148

[6]Department of Chemistry, University of South Alabama, Mobile, Al 36688-0002

[7] Department of Environmental Science, University of Arizona, Tucson, Arizona 85721

*This paper is dedicated to Alan Marshall in recognition of his contributions to the field of organic geochemistry through the development of ultrahigh resolution mass spectrometry

Corresponding Author

wcooper@fsu.edu: 1-850-894-9743
Department of Chemistry & Biochemistry
Florida State University
Tallahassee, FL USA 32306-4390

Short Title: FTICR Mass Spectrometry of Natural Organic Matter

Key Words: High field FTICR mass spectrometry, natural organic matter, molecular-level analysis of complex mixtures, environmental metabolomics

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I. Forward
II. Introduction to Natural Organic Matter (NOM) and Organic Geochemistry
III. The Early Years
    A. Combining Electrospray Ionization with Ultrahigh Resolution Mass Spectrometry
    B. Obtaining Molecular Formulas
    C. Visual Representations of Complex UHR Mass Spectra
    D. With a Little Help From Its Friends
IV. Alternative Methods of Ionization
    A. Atmospheric Pressure Photoionization (APPI)
    B. APPI and Selective Ionization of Organic Nitrogen
    C. Desorption Atmospheric Pressure Photoionization (DAPPI) of Black Carbon
V. Some important applications
    A. Marine DOM
    B. Tracing Terrestrial DOM into the Ocean
    C. Peatland Porewaters; Fens and Bogs
    D. Permafrost Peatlands and Climate Change
VI. Particularly Exciting Current Research
    A. General Guidelines on NOM Lability; the van Krevelen Diagram as a Roadmap
    B. Identification of Abiotic Photo-chemical Reactions by FTICR MS
    C. New Insights into the Formation of Humic Substances
VII. An Emerging Application; How Connections of Molecules in the H/C - O/C Space Reveal Specific Microbial Transformations
VIII. Summary

Abstract

Natural organic matter (NOM) is a complex mixture of biogenic molecules resulting from the deposition and transformation of plant and animal matter. It has long been recognized that NOM plays an important role in many geological, geochemical and environmental processes. Of particular concern is the fate of NOM in response to a warming climate in environments that have historically sequestered carbon (e.g. peatlands, swamps) but may transition to net carbon emitters. In this review we will highlight developments in the application of high-field Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR MS) in identifying the individual components of complex NOM mixtures, focusing primarily on the fraction that is dissolved in natural waters (Dissolved Organic Matter, or DOM). We will first provide some historical perspective on developments in FTICR technology that made molecular-level characterizations of DOM possible. A variety of applications of the technique will then be described, followed by our view of the future of high-field FTICR MS in carbon cycling research, including a particularly exciting metabolomic approach.

I. Forward

In this review we will describe how high field FTICR mass spectrometry has fundamentally changed the science of organic geochemistry. It is appropriate that it appears in an issue of *Mass Spectrometry Reviews* devoted to the legacy of Professor Alan Marshall because no one is more responsible for this evolution in molecular-based geochemistry than Alan. And while he and his staff have enthusiastically welcomed other organic geochemists to work with them at the National High Magnetic Field Laboratory, our group has been especially lucky due to proximity and our affiliations with the NHMFL to work quite closely with "the father of FTICR mass spectrometry" for more than 20 years. It all began in the mid-1990s when we approached him with the idea of using the new 9.4 Tesla instrument to study Natural Organic Matter (NOM). It did not hurt that Alan was already familiar with crude oil, a similarly complex mixture. After giving it some thought and accepting that the issues we were interested in were both scientifically valid and suitable for high field FTICR, he agreed with the words: "OK - we'll let you put some of your crud in the instrument." That began a collaboration between our groups that has resulted in pioneering scientific achievements and many fond memories, not to mention fifteen co-authored publications. We became known as the “dirt-sprayers”, as Alan's group liked to call us. On behalf of the entire dirt-spraying community, we thank Alan for his expertise, guidance and willingness to collaborate.

II. Introduction

Organic geochemistry is that field within the spectrum of geosciences that focuses on the role of organic compounds of biological origin in earth processes (Kvenvolden, 2006). The initial recognition that biogenic organic molecules (often referred to as "natural organic matter" or NOM) played a key role in geology dates to the pioneering work of Treibs (Treibs, 1936) who identified porphyrin pigments in crude oil and coal and traced their origins to biological precursors. The specialized field of petroleum geochemistry quickly blossomed when the biological origin of fossil fuels was fully established and the practical advantages of knowing the molecular composition of crude oils and coal were recognized. While early studies in organic geochemistry brought together a wide variety of disciplines such as geology, paleontology and organic chemistry, analytical chemistry played a uniquely important role. Advances in gas chromatography (GC) and later GC combined with mass spectrometry allowed the lipid fractions of crude oil in source rocks and coal formations to be characterized (see for example (Brooks et al., 1969)), greatly advancing the technologies available to locate and identify economically useful fossil fuel reservoirs (Tissot and Welte, 1984).

As analytical chemistry advanced rapidly in the late 20th century, specialized techniques found their way into contemporary organic geochemistry and were applied over a wide range of topics beyond fossil fuel exploration. Particularly, beginning in the 1980s, applications of ^{13}C NMR spectroscopy in organic geochemistry began to flourish (e.g. (Wilson et al., 1981) with the advent of modern solid-state techniques that included superconducting magnets with high fields, Fourier transform (FT) techniques for data acquisition and manipulation, high power proton decoupling, and Cross-Polarization Magic-Angle Spinning (CP-MAS) methods (Opella et al., 1979). The quantitative reliability of sensitivity enhanced cross-polarization solid state ^{13}C NMR was improved with advances in CP-MAS pulse sequences, including ramped amplitude (Ramp-CP) (Metz et al., 1994) and multiple cross polarization at 1-ms periods (Johnson and Schmidt-Rohr, 2014). However, solid state ^{13}C NMR does not provide much in the way of discrete molecular insights for complex biogeochemical mixtures.

These applications of modern analytical chemistry have led to an appreciation of the extent to which natural organic matter in near surface environments such as soils, surface waters and sediments play important roles in a variety of geological processes such as water quality, pollutant bioavailability, metal complexation and nutrient cycling. The importance of microbial communities in controlling the fate of NOM in these environments was also becoming apparent and the specialized field of *Biogeochemistry* began to attract many new practitioners. For example, it is now accepted that carbon stored in some unique environments (e.g. swamps, peatlands, permafrost) and thought to be resistant to degradation is at risk for release to the atmosphere as CO_2 and CH_4 byproducts with rising global temperatures and the resulting increase in microbial activity (Gorham, 1991; Hodgkins et al., 2018; Lin et al., 2012; Tfaily et al., 2014). These issues have driven the search for additional analytical methods suitable for providing more "molecularly-based" information.

Natural organic matter is an extremely complex mixture and much of it is insoluble. Solubilization of targeted fractions is thus required before most molecular-level analyses can proceed. Strong base extraction of NOM with NaOH is a common approach, producing what are generally referred to as "humic substances" which can then be further fractionated into humic acids, fulvic acids and humin based on solubility in strong acid solvents. Indeed, the terms natural organic matter and humic substances are sometimes used interchangeably. However, an especially important fraction of NOM that does not require such harsh isolation procedures is that dissolved in natural waters such as lakes, rivers, oceans and soil porewaters. This dissolved organic matter, or DOM, has received most of the attention for molecular level characterization due to its easy extraction from water and suitability for gas phase analysis such as mass spectrometry.

As noted previously, mass spectrometry played a key role in the early development of organic geochemistry. However, the ionization techniques available then, primarily electron impact, limited molecular level analyses to just a few amenable types of compounds, primarily lipids. It was the advent of softer ionization techniques, and in particular electrospray ionization (ESI)

(Fenn et al., 1989), that made possible the application of mass spectrometry to a wide range of compounds that comprise NOM, and particularly the DOM fraction. The 1990s and early 2000s were thus a period of rapid growth in the use of mass spectrometry for geochemical studies. While these studies began to identify some compositional information about NOM, more importantly they clearly revealed that these natural materials were far too complex to be resolved by low-resolution mass spectrometers. Even more advanced instruments such as Q-TOF (Plancque et al., 2001) and MS/MS (Leenheer et al., 2001) spectrometers could not resolve individual NOM ions.

Fortunately, by the mid-1990s the potential of high-field Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) to resolve very complex mixtures was becoming apparent with the development of the 9.4 T spectrometer at the National High Magnetic Field Laboratory (NHMFL, or Magnet Lab) (Senko et al. 1996; Senko et al. 1997). Researchers in related fields of organic geochemistry quickly recognized the impact this technology would have. Indeed, the first spectrum of an NOM isolate from a contemporary environment produced on this instrument appeared in 1997 (Fievre et al., 1997), but only a few compounds could be resolved. A better understanding of the advantages and limitations of electrospray ionization (ESI) and the marriage of electrospray ionization with the 9.4 T mass spectrometer was required before complex NOM mixtures could be fully resolved and individual formulas identified.

We will begin this review by highlighting the "early years" of ultrahigh resolution mass spectrometry in organic geochemistry, noting the importance of ESI to the success of the technique. We will then discuss the startling realization observed in the first fully resolved ultrahigh resolution (UHR) spectra that DOM was not a random mixture but a highly ordered cohort of molecules related in defined atomic variations. We will also highlight the importance of data reduction techniques such as Kendrick mass defects, van Krevelen diagrams and double bond equivalences (DBE) played in understanding the underlying molecular themes observed in NOM. The unique information alternative ionization modes such as atmospheric pressure photoionization (APPI) and desorption APPI (DAPPI) can provide will then be described. A discussion of applications of UHR FTICR MS to a number of issues in organic geochemistry that our group has been involved in will follow. These issues include the photochemistry of DOM in estuaries and near-shore waters, identifying the role of terrigenous DOM in the marine carbon cycle, variations in DOM composition in peatland porewaters, and the fate of carbon in thawing permafrost. We will conclude by describing the role of UHR MS in identifying microbial pathways of DOM degradation and relevant enzymes involved, an approach that falls within context of "environmental metabolomics" (Bundy et al. 2009), and we will discuss how such a metabolic approach can contribute to climate change science.

This review is not meant to be comprehensive but rather focuses on our group's work with the ICR group at the Magnet Lab. We will, however, make note of some of the important milestones in UHR mass spectrometry of NOM originating from our collaborators and colleagues from around the world. Some researchers deserve special mention and will be cited liberally throughout this paper. These include Pat Hatcher’s group at Ohio State, and later Old Dominion University, as well as many of the students and post-docs who trained with him and built highly visible programs in their own right. Thorsten Dittmar, originally at Florida State

University and now at the University of Oldenburg, made major contributions in characterizing marine DOM and tracing terrestrial DOM into the ocean, and that work included in many cases his frequent collaborator Boris Koch at the Alfred Wegner Institute. Norbert Hertkorn and Phillipe Schmitt-Kopplin at the Helmholtz Zentrum München were very successful in combining UHR mass spectrometry with high resolution ^{1}H and ^{13}C NMR. Regardless of which group is credited with a particular advance, one common feature that binds us all together is that virtually all the original experimental work was carried out at the NHMFL with generous help from and support of the excellent and very cooperative staff of the ICR facility. It is a true testament to the importance of high field FTICR mass spectrometry to organic geochemistry that in the intervening years ultrahigh resolution instruments and associated labs too numerous to list here have been developed in the US and other countries with a specific focus on organic geochemistry.

III. The Early Years

A. Combining Electrospray Ionization with Ultrahigh Resolution Mass Spectrometry

Before the combination of ESI and FTICR MS, high-resolution mass spectrometry of mostly intact molecules came at the expense of sensitivity and involved extremely long single scan times (Marshall and Rodgers, 2004). Both of these limitations reduced the usefulness of mass spectrometry for the analysis of complex mixtures in which each individual component exists in trace amounts (such as NOM and crude oil). ESI (for which John Fenn received the Nobel Prize in 2002) allows for sensitive soft-ionization of molecules within complex mixtures. FTICR MS allows single scans of entire spectra to be taken in less than a second.

The potential usefulness of ESI FTICR MS in the analysis of biogenic organic molecules was evident to our group as soon as the 9.4 T mass spectrometer came on line. However, in the beginning, the greatest challenges were obtaining a good signal, identifying method limitations, and deciding which peaks are the most relevant. Early on, we wrestled with the effect of transient length, spray-solvent composition, and instrument parameters. We struggled to achieve high resolution for the bulk NOM at our disposal, and spent considerable effort investigating selectivity, specifically an apparent low-molecular weight (low-MW) bias, in our data.

Fievre (Fievre et al., 1997) in our group were the first to combine ESI with FTICR MS for humic substances. Full mass resolution of ions comprised of C, H and O that represent the major components of NOM was not achieved in this first, landmark study. However, her work illustrated the changes that ICR mass spectra of NOM undergo as the ions are monitored for longer transient times. Longer transients mean better sampling of peaks (more data points per peak) and better ability to detect high masses, as well as increased resolution between similar m/z ratios. In short, long transients are required for high mass resolution spectra of complex NOM mixtures. However, Fievre's work also demonstrated that more ions with higher *m/z* ratio and higher charge states disappear from the spectrum, the longer ions in complex NOM mixtures spend cycling together in close proximity. These findings, combined with the selective nature of electrospray ionization and the potential of fragmentation even when using such a soft-ionization method are still critical to keep in mind when interpreting FTICR mass spectra of NOM.

Related to these observations, all early work on ESI mass spectrometry of NOM struggled with an apparent low-MW bias. In addition to Fievre (Fievre et al., 1997), Llewlyn (Llewelyn et al., 2002), Brown and Rice (Brown and Rice, 2000), and Kujawinski (Kujawinski et al., 2002a) all noted this bias. Fortunately, by 2002, the challenge of obtaining fully-resolved peaks for bulk humic material was met. Kujawinski et al. (Kujawinski et al., 2002b) were the first to publish fully resolved mass spectra of the bulk of humic substances. In 2002, our group (Stenson et al., 2002) used fully resolved bulk humic spectra to examine the most likely origins of the ubiquitously observed low-MW bias. We concluded that insufficient accounting for charge could be ruled out and that complete dissociation of covalently-bound, high-molecular weight humic substances is unlikely but not as impossible as commonly assumed. The general consensus that emerged from these and other studies was that there really was no actual low-MW bias, but rather that size exclusion chromatography originally used to estimate molecular weights of NOM were providing information on "aggregates" of smaller covalently bonded molecules that were observed by UHR-MS.

By 2002, the major groundwork had been established for allowing ESI FTICR MS to become a powerful tool in the analysis of NOM and its individual components. By keeping NOM concentrations low, reducing bubbles in the ESI capillary (through sonication), choosing long transient-observation times, and signal averaging a large number of transients , the technique produced fully resolved spectra. Major limitations of the technique were also understood. It was then time to obtain as much compositional information as possible from a variety of natural environments and develop visualization techniques that displayed data in the most meaningful fashion.

B. Obtaining Molecular Formulas

One interesting observation from the early data was that NOM spectra exhibited a high degree of internal order represented by repeating mass spacing patterns. In our 2002 work (Stenson et al., 2002), we used the high mass accuracy of FTICR MS to assign elemental differences to these observed mass spacing patterns. Two of the patterns that had not previously been ascribed to formulas in the literature ($\Delta m = 0.0364$ Da (CH_4 vs. O) and $\Delta m = 0.9953$ Da (CH vs. N)) proved especially important in assigning molecular formulas to peaks in bulk NOM spectra.

Building on Hughey's work with crude oil (e.g., (Hughey et al., 2001), we used the combination of sorting humic peaks into homologous series (formulas separated only by an integer number of CH_2 groups) and the CH_4 vs. O mass spacing pattern to assign molecular formulas to most of the peaks in a Suwannee River fulvic acid (SRFA) spectrum (Stenson et al., 2003). In all, we assigned >4600 individual, even neutral-mass formulas belonging to 266 distinct homologous series and spanning a mass range of 316 to 1098 Da. Furthermore, we determined that some odd neutral-mass formulas were not merely isotopomers of the even neutral-mass formulas (i.e., did not simply represent the substitution of one ^{12}C with one ^{13}C atom). Some odd mass formulas were due to the presence of N which could be identified by the 0.9953 Da spacing (i.e. CH vs. N).

Faced with the dilemma of how to excite a journal to publish thousands of formulas in its pages, we thankfully managed to express all CHO-only formulas with one equation and all N-containing formulas with a second. Moreover, we noted that the vast majority of fulvic acid ions amenable to ESI mass spectral analysis fall into two families: one with a family score

(Equation 1) of -1 and another with a family score of +6, as reiterated a little more concisely in Stenson (Stenson, 2008).

$$\text{Family Score} = 0.5|z^*| + (\text{DBE} - \text{O}) \qquad (1)$$

In equation (1), z^* indicates the nominal mass series (i.e., it slices the mass spectrum into 14 mass slices, each containing only every 14th mass, to assist with homologous series grouping), DBE represents double bond equivalence (i.e., it is the combined number of rings and double bonds) and O the number of oxygen atoms in the formula.

Verifying that formulas assigned were accurate represented the next challenge. Originally, we addressed the issue three different ways (Stenson et al., 2003). First, we included the internal standard peaks (polyethylene glycol, PEG) as unknowns in the data set and verified that accurate formulas were assigned to each PEG peak. We then fragmented the same fulvic acid ions in both positive and negative ion mode and verified that the observed neutral losses were consistent with the number and kind of elements allowed for in each corresponding formula. Finally, we examined the formulas (and the patterns between them) against the context of the presumed major components of Suwannee River fulvic acid -- degraded lignin. Kujawinski and Behn (Kujawinski and Behn, 2006) later expanded on this work (and were the first to automate formula assignment) by testing their compound identification algorithm against synthetic data sets. The synthetic data sets were created by randomly picking a formula from a short list of projected humic components then making one of several elemental substitutions commonly observed in humic spectra (such as CH_4 vs. O mentioned above). Between one and five substitutions were randomly picked; the resultant formula was added back to the list to be potentially picked again for different augmentations. Each data set was generated to contain

1000 members. The compound identification algorithm, employing much the same strategies as we did in our 2003 work, was then tested on these data sets. Because modern formula calculators still work on essentially the same principle of assigning low-MW formulas based on direct formula calculation and deriving higher-MW formulas by employing common substitution patterns, the following important conclusions remain relevant: (1) formula error allowed to identify a compound should not exceed instrument error (if the instrument typically provides a mass error < 1 ppm, formulas should not be assigned that have masses > 1 ppm different from the measured mass); (2) Instrument error must be low (≤ 1 ppm) unless significant additional constraints on formula assignments are known; (3) addition of elements (e.g., P, S and Na) more often than not results in inaccurate formula assignments at typical FTICR MS instrument errors and should be treated with caution, except in special situations such as the (+) ESI mode in which [Na^+-M] adducts are frequently observed. .

C. Visual Representations of Complex UHR Mass Spectra

Early on, the NOM community adopted Kendrick plots to visually represent ESI FTICR MS data (e.g., (Kujawinski et al., 2002b; Stenson et al., 2002; Stenson et al., 2003). As illustrated in Figure 1, Kendrick plots allow an entire spectrum (or a substantial fraction of the spectrum as in Figure 1) with several thousand peaks to be condensed into a compact visual space. Kendrick plots are also useful in tracking differences in represented masses (x-axis) or mass defect (y-axis) between samples. Probably the greatest advantage of Kendrick plots is that they include nominal mass and do not require prior formula assignments.

Later, Kim et al. (Kim et al., 2003) proposed the addition of the van Krevelen plot (originally introduced by van Krevelen (van Krevelen, 1950) for reaction processes associated with coal). With it, they introduced the attractive, but somewhat controversial, technique of elemental mapping to NOM analysis. Van Krevelen plots (Figure 2) require previous assignments of molecular formulas; molecular weight information is lost unless it is introduced as a third dimension but that is not commonly done.

Significant prior work had gone into identifying where relevant compound groups fall within a van Krevelen diagram (ovals in Figure 2). This “elemental mapping” remains controversial because each formula could already encompass hundreds or thousands of potential structural- and stereo-isomers; mere ratio relationships of elements therefore cannot, by themselves, identify compound classes. Figure 2 illustrates an example in which the van Krevelen plot itself is useful, but the elemental mapping is not. By showing that more oxygenated (high O/C) humic components elute off a reversed phase chromatography column faster than less oxygenated ones and that oxygenation correlates with desaturation (H/C ratio), it can be assumed that most of the O-containing compounds in this sample consisted of polar, carbonyl-containing groups such as esters and carboxylic acids. However, the plot does not reveal notable differences in compound classes between fractions because, as Kim et al. (Kim et al., 2003) also pointed out, van Krevelen plots for riverine DOM tend to converge towards the middle (lignin and tannins territory) both because of the main starting materials and because of the typical degradative transformations biomolecules tend to undergo.

That a tool is not useful for all applications does not mean it cannot be essential in some. Used with the proper understanding of its limitations, elemental mapping in van Krevelen plots provides valuable insight. For example, Tremblay et al. (Tremblay et al., 2007) compared mangrove porewater DOM with water in the adjacent estuary using elemental mapping. Kendrick plots and raw mass spectra revealed that primarily the heaviest components of the porewater DOM disappeared; elemental mapping provided the complementary information that disappearing components also fell in the condensed hydrocarbon region of the van Krevelen plot. Together, the two pieces of information were consistent with photodegradation of heavy, highly unsaturated (or aromatic) DOM components as the mangrove NOM moved into the estuary, a conclusion supported by additional spectrophotometric analyses (see Section D. that follows.).

D. With a little help from its friends

At the end of its first decade in characterizing NOM, ESI FTICR MS had established itself as a powerful tool for identifying a representative portion of NOM components. Equally important, the major limitations, some listed above (e.g., low-MW bias), some inherent to ESI mass spectral analysis of complex mixtures (overlap of isotopes, limited quantitative information) had also been delineated. Because every analytical technique has both strengths and weaknesses, the most meaningful impact comes from the combination of complementary techniques. Indeed, even in the very first ESI FTICR MS study of NOM, Fievre et al. (Fievre et al., 1997) combined chromatography with ESI FTICR MS and enhanced the informational content derived from the mass spectral data through tandem mass spectrometry. NMR has also commonly been paired with FTICR MS to obtain complementary structural information almost from the beginning (e.g., (Kujawinski et al., 2002a).

Llewelyn et al. (Llewelyn et al., 2002) and Cooper et al. (Cooper et al., 2005) combined chromatography and elemental analysis by Inductively Coupled Plasma (ICP) MS with ESI FTICR MS of selectively extracted phosphorus-containing DOM, commonly referred to as DOP. The elemental analysis revealed that a Florida Treatment Wetland designed to remove inorganic P from water through biological uptake did indeed convert inorganic ortho-phosphates (only peak at the wetland entrance) to a variety of organic phosphates (newly detected peaks at the wetland exit). The compositional ESI FTICR MS analysis of P-containing DOM components in a high-MW retentate (> 1 kDa membrane cutoff) was consistent with previous analyses that indicated a positive correlation between high-MW and refractive nature of organic phosphorus compounds. The overall implication of the combined data was that the wetland does what it is supposed to do and furthermore that high-molecular weight DOP is the most resistant to transformation.

As noted above, Tremblay et al. (Tremblay et al., 2007) combined ESI FTICR MS with UV-Vis (specific absorbance) and Excitation Emission Matrix Fluorescence analyses. The spectrophotometric techniques provided the independent, complimentary confirmation that the components removed from mangrove-derived DOM as porewater ages and moves to become part of the estuary were, indeed, most likely aromatic.

IV. Alternative methods of ionization

At the beginning of the 21st century ESI in both (-) and (+) modes had emerged as the ionization method of choice for broad molecular characterization of NOM. Nevertheless interest in improved ionization approaches identified two methods, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) that provided highly complementary information to ESI. Hertkorn et al. (Hertkorn et al. 2008), in a very significant manuscript, compared (+) and (-) mode APCI, APPI and ESI and demonstrated the complimentary and unique molecular information these ionization methods provided and illustrated how the use of such multiple ionization methods extended the "Event Horizon" of natural organic matter mass spectrometry. Our group participated in this effort to identify useful ionization methods, focusing on APPI. What follows is a brief summary of those efforts.

A. Atmospheric Pressure Photoionization (APPI)

APPI was a relatively new method of soft ionization in mass spectrometry ca. 2006 and rapidly evolved as an important complementary technique to ESI. Ionization selectivity is routinely defined in terms of two fundamental groups of compounds: polar and non-polar. Unlike ESI, APPI is known for its ability to efficiently ionize non-polar molecules directly, through electron detachment or charge transfer between a photo-ionized dopant (e.g., toluene) and a DOM analyte of interest (Mopper et al., 2007; Purcell et al., 2007a). APPI has a greater dynamic range of ionizable molecules (polar and non-polar) and higher signal-to-noise ratio, is more resistant to chemical noise from solvents and salts and exhibits less ion suppression from matrix effects when compared to ESI (Bos et al., 2006; Hanold et al., 2004). A detailed explanation and excellent review of all possible APPI mechanisms in positive and negative modes can be found in Bos et al. (Bos et al., 2006) and Raffaelli and Saba (Raffaelli and Saba, 2003).

APPI performs best for non-polar compounds and therefore the combination of APPI and ESI offers the best approach for expanding the fraction of “characterizable” DOM by FTICR MS. The first reports of APPI coupled to FTICR MS for molecular-level DOM characterization in freshwater, brackish water, and marine ecosystems were published between 2008-2010 (Hertkorn et al. 2008; Hockaday et al. 2009; D'Andrilli et al. 2010). When compared to ESI mass spectra, APPI revealed new spectral features not previously observed and allowed molecular formula assignments over different degrees of hydrogen saturation and oxygenation. Since 2010, the inclusion of APPI in molecular level FTICR MS research of diverse natural organic matter mixtures (e.g., crude oils, freshwater, coastal zones, sea ice, and ocean water), either as the sole ionization source or as an ESI complement, has gained rapid acceptance (see (Cho et al., 2015; Sleighter and Hatcher, 2011) and references within).

B. APPI and Selective Ionization of Dissolved Organic Nitrogen

The dissolved organic nitrogen (DON) fraction of DOM accounts for a considerable portion of the total N pool in most environments. DON represents a potential bioavailable source of N for phytoplankton and bacteria in the microbial loop. The importance of DON in the global N cycle has been identified in fields such as urban watersheds, water purification, soil chemistry, wastewater treatment, and atmospheric chemistry. Therefore, complete molecular-level characterization of DON as a mixture of labile and refractory compounds with significantly different impacts would represent an important advance in DON research.

Because most DOM is composed of molecular species with predominantly highly polar, oxygen-containing compounds with carboxylic functionalities, they are most efficiently ionized by negative ESI and thus suppress ionization of less acidic species, e.g., N-containing compounds.

ESI thus represents only an indirect approach to characterization of DON and typically results in identification of tens to hundreds of N-containing compounds in a complex mixture that potentially contains thousands of unique N-containing molecular formulas. Conversely, APPI offers a direct and highly selective method for ionizing DON in complex DOM mixtures (Podgorski et al., 2012b).

Previous work by Alan Marshall's group (Purcell et al., 2007b) showed speciation of basic and neutral N-containing, heterocyclic compounds in crude oil by positive APPI. We hypothesized that a modified version of this method could be applied to selectively ionize the continuum of DON compounds ranging from those containing basic N (amine) functionalities to neutral and non-polar N-containing degradation products. Moreover, APPI is tolerant to salts eliminating complications in data acquisition and processing resulting from sodium adducts. Figure 3 shows an example of the enhancement in ionization efficiency of N-containing compounds by positive APPI in a deep-sea marine DOM sample. Here, ions with the same neutral molecular formula generated by either positive APPI or negative ESI exhibit a 13-fold (top) and nearly 6-fold (bottom) higher S/N by positive APPI. The trend in increased selectivity of DON by positive APPI covers a wide range of both marine a terrestrially derived DOM (Figure 4). As such, the method has been applied for characterization of DON in rivers, streams, stormwater, and soil leachates. Moreover, the application of positive APPI for selective ionization of DON has promise for tandem mass spectrometry (MS/MS) to provide structural information for DON and characterization of highly aromatic organic nitrogen, or "black nitrogen".

As an example of the utility of APPI FTICR MS in studying DON, we note our study of the bioavailability of DON in surface waters. Nitrogen plays a key role in the biological productivity of surface and ground waters and understanding which fractions of DON are reactive and which are refractory is a critical key in developing best management practices for nutrient input loading. Unlike the inorganic fraction (NO_2^-, NO_3^-, NH_3), the organic component of dissolved N consists of thousands of individual species and therefore little is known about the relationship between DON molecular composition and relative reactive/refractory characteristics. To address this problem, we carried out APPI FTICR MS analyses of riverine DOM obtained from the Caloosahatchee River in southwest Florida, USA, before and after bioassays using a downstream inoculum (Osborne et al., 2013). Initial experiments determined that positive-mode APPI yielded approximately twice the number of identifiable N-containing ions than negative mode. This study can be summarized with a van Krevelen assessment of DON species (Figure 5) that were removed during the five-day bioassay (reactive) and those that were formed. This analysis suggests that most of the reactive DON are "lipid-like" molecules characterized by relatively high H/C and low O/C ratios. Conversely, most of the refractory products are more oxygenated and less saturated. Parallel measurements of Total Dissolved Nitrogen (TDN) and DON established that most of the TDN was DON (96%) but only ~4% of TDN and DON was removed during the five-day bioassay. These results taken together suggest that the microbes in the inoculum are changing the carbon skeleton of the refractive DON fraction but without consumption of N. Thus, from an environmental best management practice perspective, it might be more useful to induce conversion of DON to refractory species rather than try to remove all DON present.

C. Desorption Atmospheric Pressure Photoionization (DAPPI) of Black Carbon

Up to this point our review has focused on a subset of Natural Organic Matter - the dissolved fraction (DOM). However, as we noted in the Introduction, much of NOM of interest to organic geochemists is insoluble in water and requires harsh extraction techniques before it is suitable for any type of molecular analyses. One such insoluble fraction that is receiving much attention of late is Black Carbon (BC), the incomplete combustion product of thermally degraded vegetation. As with most of the characterizations of insoluble NOM, studies of BC have relied heavily on solid state ^{13}C and ^{1}H NMR. However, a variant of traditional APPI, Desorption Atmospheric Pressure Photoionization (DAPPI), offers a unique approach for molecular-level characterization when coupled to high field FTICR MS. In 2011 we presented the first coupling of desorption atmospheric pressure photoionization (DAPPI) to FTICR MS (Podgorski et al., 2012a). DAPPI provides all the advantages of solution APPI, enabling the analysis of compounds spanning a wide range of polarity, while at the same time eliminating any harsh extraction steps that might affect the molecular integrity of original sample. DAPPI was first introduced by Haapala et al. (Haapala et al., 2007). In DAPPI, a heated nebulizer produces a plume of hot gas that includes a solvent that desorbs neutral analyte molecules from a solid surface into the gas phase. The desorbed analytes are then ionized by the same mechanisms as with APPI.

DAPPI is particularly suited for the molecular characterization of BC because it efficiently ionizes non-polar compounds such as aromatic and other highly unsaturated species. The power of DAPPI combined with high field FTICR MS can be summarized in Figure 6, which includes ^{13}C NMR spectra (left panel) and van Krevelen diagram summaries of DAPPI FTICR spectra (right panel) of an oak sample (*Quercus laurifolia*) before and after it was combusted at 250 °C and pyrolyzed at 400 °C. After combustion at 250 °C most formulas in the region associated with resin-like compounds are lost, and a significant number of formulas associated with aromatic and condensed aromatic structures appear. These trends are confirmed by the ^{13}C NMR spectra which show a decrease in O-alkyl carbon and increase in aromatic C. Oak pyrolyzed at 400 °C shows a marked increase in the number of formulas consistent with aromatic and condensed aromatic compounds. Molecular formulas exhibit lower O/C and H/C relative to the parent oak and oak combusted at 250 °C. Furthermore, a complete loss of elemental compositions associated with cellulose-like compounds is observed in the oak pyrolyzed at 400 ^{o}C. Again, the ^{13}C NMR spectra support these conclusions; the predominant NMR peak is in the aromatic region and the O-alkyl carbon is barely observable. The NMR data further support the conclusion that the DAPPI mass spectra are representative of the entire bulk biochar, not just the surface composition.

V. Some Important Applications

By the end of the 2000s FTICR MS had become an established analytical technique for assessing the molecular composition of DOM, albeit a time- and instrument- intensive technique. Organic geochemists were becoming regular visitors to the NHMFL, bringing samples from a wide variety of ecosystems. In the following section we will highlight a few of the pioneering applications that ensued. A comprehensive review is unfortunately beyond the scope of this report.

A. Marine DOM

Determining the composition of DOM and its fate in the many different aquatic environments is essential to understanding the global carbon cycle (Hedges et al., 2002). One particularly important (and large!) environment is the ocean. Indeed, marine DOM represents the largest reservoir of reactive organic carbon on Earth. Marine DOM is known to affect many biogeochemical processes such as metal redox cycling, contaminant transport, microbial growth, gas exchange in surface waters, and the biological carbon pump (Benner et al., 1992; Hansell and Carlson, 2015; Hedges et al., 2000). Initial applications of FTICR MS in marine science spanned several sampling sites, focusing on the heterogeneous nature of marine DOM within a site, as well as similarities and differences between sites. Ocean sites studied included the Weddell Sea (D'Andrilli et al. 2010) and Antarctic bottom waters (Koch et al. 2008), the Pacific Ocean (Hertkorn et al. 2006), and the Angola Basin deep waters (Reemtsma et al. 2008). DOM flux from estuaries into the ocean were also the subject of several studies as well, including from Brazillian Amazon mangroves of the Caeté Estuary (Tremblay et al. 2007) and the Chesapeake Bay (Sleighter et al. 2008). These reports focused on using molecular-level composition assessments to reveal DOM source, formation processes and environmental influences, and potential reactive nature that could not be measured or inferred from other analytical techniques.

These early studies that utilized FTICR MS data inspired such research questions as: “What is marine DOM comprised of?”, “What happens to terrigenous DOM in the ocean?”, “Can we identify marine DOM sources?”, and “What types of marine DOM are labile and refractory?”. From there, advances in FTICR MS resolution, speed and sample throughput, as well as improved strategies for data management of complex spectral datasets, resulted in much better understanding of marine DOM composition. Another major theme that emerged was the use of complementary analytical techniques to better understand abiotic and biotic processes, other environmental drivers of DOM change, and predicted impacts of marine biogeochemical processes in the future (e.g., (Arrieta et al., 2015; Brogi et al., 2018; Flerus et al., 2012; Gonsior et al., 2009; Kujawinski et al., 2009; Osterholz et al., 2015; Schmitt-Kopplin et al., 2012). When viewing these studies, it is important to remember that identical formulas are not necessarily representative of identical molecules. In a very powerful study of marine DOM that utilized ^{1}H and ^{13}C NMR in concert with high resolution FTICR MS, Hertkorn et al. (Hertkorn et al., 2013) identified a class of molecules referred to as carboxyl-rich alicyclic molecules (CRAM) that increased in abundance with depth in the open South Atlantic ocean. These CRAM molecules appear to be a distinguishing feature of marine DOM.

B. Tracing Terrestrial DOM into the Ocean

Understanding the extent to which terrestrially derived organic matter contributes to the marine DOM pool has long been at the forefront of marine science. An underlying assumption in these studies is that differences in the composition of marine DOM will vary from terrestrial DOM from sources such as soils, freshwaters, and coastal mixing zones. Koch et al. (Koch et al., 2005) compared the composition of a marine DOM with almost no terrestrial inputs (Weddell Sea, Antarctica) with terrestrial DOM from the coast of Northern Brazil. These two sample sites were specifically selected to represent vastly different end member environments and were also compared with the terrestrial freshwater reference standard (SRFA). These authors concluded certain processes such as microbial mineralization and photochemical reactions led to abundant similarities between marine DOM and DOM from terrestrial sources.

Photo-chemical transformations of DOM in particular have been the subject of a number of studies that utilized compositional information obtained by FTICR MS coupled with bulk optical spectroscopy measurements. We mentioned earlier the study by Tremblay et al. (Tremblay et al., 2007) of DOM flux from an estuary in Northern Brazil. Similarly, Gonsior et al. (Gonsior et al., 2009) coupled changes in absorbance and fluorescence spectra of riverine and estuarine DOM with FTICR MS compositional data to identify specific photo-degraded compounds and shifts in DOM optical properties and photo-oxygenation after 21 h of controlled irradiation. This study reinforced assumptions about the chromophoric (light absorbing) component of DOM (or CDOM), in particular that the photo-reactivity of CDOM is highly dependent on the molecular composition. This study laid the groundwork for carbon cycling research in inland, coastal zones, and in the open ocean using optical spectroscopy in concert with FTICR MS compositional assays. Other FTICR MS studies have shown similarities in DOM molecular composition in terrestrial to coastal and coastal to open ocean transects (Medeiros et al., 2015; Osterholz et al., 2016; Riedel et al., 2016; Sleighter and Hatcher, 2008; Zark and Dittmar, 2018)

C. Peatland Porewaters: Fens vs. Bogs

Peatlands are a major, climate-sensitive reservoir of carbon. Indeed, there is more carbon stored in peatlands than in the atmosphere. Chanton et al. (Chanton et al., 2008) showed through radiocarbon analyses that CO_2 and CH_4 production was fueled primanrily by DOM in peatland porewaters and not respiration of the solid phase peat. Peatland DOM is composed primarily of cellulose and lignin-derived phenols (Benner et al., 1984; Clymo, 1987) but microorganisms mediate the decomposition of DOM and thereby control greenhouse gas production. A warming climate will result in enhanced microbial productivity making peatland DOM susceptible to increasing degradation. The fear is that peatlands will change from net reservoirs of carbon to net emitters of greenhouse gases, providing a positive feedback to climate change. But fully characterizing their feedbacks with the atmosphere is complicated in part by the complexity of porewater DOM and potential for very different pathways of microbial decomposition as a function of geochemical conditions. Major differences in decomposition between acidic bogs and more alkaline fens have been noted, specifically in their pathways of methane production. That led to a fundamental question: is DOM composition substantially different between bogs and fens, similar to differences observed previously based on location and depth (Harrison et al. 2009), and if so, do these compositional differences drive reactivity differences?

FTICR MS analysis of peat DOM from the Glacial Lake Agassiz Peatlands (GLAP) in northern Minnesota, USA (D'Andrilli et al., 2010a; Tfaily et al., 2013) revealed that the composition of DOM at the surface in sedge-dominated fens is significantly different than that at the surface in Sphagnum-dominated bogs (Figure 7, top), and this difference in composition grows as DOM migrates vertically in the peat column (Figure 7 bottom). In contrast to fens, the majority of formulas in the bogs (often as much as 80%) persist with depth down a vertical profile. Cumulatively these data suggest that hydrogenotrophic methanogenesis is linked with less reactive organic matter in bogs, while higher rates of acetoclasty are favored in systems with more labile unsaturated and aromatic compounds in fens. These results support the "enzyme-latch" hypothesis (Freeman et al., 2001) that the more acidic conditions in the bog inhibit the activity of phenol oxidase, an enzyme responsible for the breakdown of aromatic compounds, contributing to the accumulation of microbially-inhibitory aromatic compounds in bog peat and further contributing to the slow decomposition rates observed there. Furthermore, these differences in DOM respiration rates were linked to differences in microbial communities present (Lin et al., 2014). A detailed analysis of heteroatom content in UHR mass spectra revealed significant depth stratification of DOM decomposition (Tfaily et al., 2014; Tfaily et al., 2018), including a high abundance of highly labile amino sugars and protein-like compounds in the surface DOM which are likely leachates from surface vegetation that are rapidly decomposed with depth.

Further characterization of DOM differences between bogs and fens revealed that CO_2 shedding and DOM reduction are important reactions during peat decomposition that may contribute to elevated CO_2:CH_4 production ratios in peatlands, a characteristic which has significant implications for climate feedbacks given the higher global warming potential of CH_4 relative to CO_2 (Tfaily et al., 2013). That study also included data from several complimentary analytical measurements, including optical spectroscopy, and concluded that optical spectroscopy is a highly complementary bulk method to the detailed molecular information provided by UHR mass spectrometry. This theme of supplementing molecular composition information from UHR mass spectra with optical spectroscopy data (Tremblay et al. 2007; D'Andrilli et al. 2013; Gonsior et al. 2017; Stubbins et al. 2017) was further developed by Tfaily in 2015 (Tfaily et al., 2015) who contrasted the fluorescent properties of DOM in fens and bogs in the GLAP peatlands using excitation emission matrix fluorescence spectroscopy with parallel factor analysis (EEM PARAFAC). The EEM PARAFAC experiments were supported with UHR mass spectrometry data. EEM-PARAFAC identified five distinct fluorescing components and the dynamics of each were evaluated based on their distribution with depth as well as across sites within the peatland. The overlapping analyses by EEM PARAFAC and FTICR mass spectrometry confirmed that different environments within the GLAP peatland contain DOM with distinct chemical properties and that each has a unique fluorescent fingerprint as well.

The determination of differences in composition of DOM in bog and fen porewaters just described relied heavily on the evaluation of van Krevelen diagrams. At this point it is appropriate to note the limitations of such an analysis in which thousands of data points are projected onto a two-dimensional "map" (H/C - O/C axes). Each data point may (and probably does) represent a number of distinct molecules with the same H/C - O/C ratios. To address these limitations, a number of improvements in the van Krevelen analysis have been introduced over the past ten years, many involving the use of a third axis such as molecular weights (Gonsior et al., 2009), aromaticity (D'Andrilli et al., 2010), and DBE or DBE-O (D'Andrilli et al., 2010; Gonsior et al. 2010), among other molecular parameters. Particularly noteworthy was the concept of "elemental mapping" suggested by Rivas-Lubach et al. (2018).

D. Permafrost Peatlands and Climate Change

Climate warming results in thaw of permafrost soils, releasing DOM previously sequestered in frozen porewaters. Thaw also causes land subsidence, leading to changes in hydrology and vegetation, which then leads to changes in newly produced DOM. As temperatures increase, enhanced microbial respiration of this released DOM is also expected (Schadel et al., 2016). Greenhouse gas emissions from permafrost peatlands thus represent a potential major positive feedback to climate change. The extent of such emissions depends on a number of factors, but one important component is the link between thaw, hydrology, vegetation, and organic matter composition and reactivity. These links were investigated by Hodgkins et al. (Hodgkins et al., 2014; Hodgkins et al., 2016) at the Stordalen Mire research site in Northern Sweden. Stordalen

Mire is a peatland in northern Sweden located at the edge of discontinuous permafrost that is currently undergoing permafrost thaw (Johansson et al., 2006). This results in a gradient of habitat types characterized by thaw state and hydrology, from dry palsa underlain by permafrost, through wetter bogs that experience seasonal thawing in the surface active layer, to fully thawed and flooded fen sites. Important controls on DOM dynamics in Stordalen appear to be related to vegetation through the supply of more or less labile source material and the inhibition of the microbial decomposition community by Sphagnum moss (Hodgkins et al., 2014; Hodgkins et al., 2016). Fen DOM has higher abundances of aliphatic and protein-like compounds compared to the bog, which has more carbohydrate, tannin-like, aromatic and condensed aromatic compounds. Similar to results from GLAP, the fen DOM in Stordalen appears to be more bioavailable than bog DOM. FTICR MS analysis suggest that the shift in vegetation with thaw is driving changes in the lability of the DOM (Figure 8), which explains the increasing rates of CO_2 and CH_4 production with thaw (Hodgkins et al., 2014). A key driver in this shift appears to be the presence or absence of Sphagnum moss (Hodgkins et al., 2016), which FTICR MS analyses reveals is associated with high molecular weight, aromatic, oxidized compounds that may inhibit microbial mineralization of organic matter. As in non-permafrost peatlands, microbial respiration is largely driven by DOM composition rather than solid peat.

VI. Particularly Exciting Current Research

A. General Guidelines on NOM Lability; the van Krevelen Diagram as a Roadmap

Because NOM is comprised of a diverse array of molecules representing many chemical classes, the van Krevelen diagram has represented an effective tool for visualizing and interpreting FTICR MS data since its introduction (Kim et al., 2003). When projected onto a van Krevelen plot, NOM species cluster into regions of the diagram that correspond to characteristic compound classes and reactivities (D'Andrilli et al., 2015; Kim et al., 2003; Koch and Dittmar, 2006; Minor et al., 2014; Mopper et al., 2007; Podgorski et al., 2012a). Over the last two decades, researchers have utilized the van Krevelen "elemental mapping" approach for a variety of purposes, including NOM sources, extent of microbial processing, interpretation of reaction pathways, and differences in NOM composition resulting from many chemical/biological/photochemical processes. Taken together, NOM characterization by FTICR MS and interpretation of van Krevelen diagrams generated from formula assignments are now commonly used in the carbon cycling research world.

Another question that van Krevelen analysis can suitably address is NOM lability, although such a process is somewhat controversial in both the biological and chemical communities. D’Andrilli et al. (D'Andrilli et al., 2015) proposed a molecular lability index (MLB) from FTICR MS data that extended our current understanding of NOM reactivity and which is useful when other biological information is unavailable (e.g., heterotrophic bacterial respiration). In this formulation, FTICR MS data can be grouped into more- versus less-labile NOM molecular compositions depending on the degree of hydrogen saturation, with formulas with $H/C \geq 1.5$ over all O/C ratios representing the labile fraction. The relative abundances of labile and recalcitrant formulas are then used to identify the dominant reactive nature of NOM from a particular environment (D'Andrilli et al., 2015; Maizel et al., 2017; Medeiros et al., 2017; Osterholz et al., 2016). The MLB can also be coupled with microbial respiration to help predict short- and long-term trends of biological processing in different aquatic ecosystems (D'Andrilli et al., 2019; Smith et al., 2018). In this way, van Krevelen diagram "elemental mapping" is useful for tracking DOM compositional changes unique to microbial metabolic processes.

Molecular formula assignments from FTICR MS data can also be used in more thermodynamically rigorous estimates of NOM lability. For example, calculation of the nominal oxidation state of carbon (NOSC) can be used as an index of DOM energetics (Keiluweit et al., 2016) especially under anoxic conditions. Calculation of NOSC from FTICR MS data is straightforward:

$$\mathrm{NOSC} = -((-Z + 4C + H - 3N - 2O + 5P - 2S)/C) + 4 \qquad (2)$$

where C, H, N, O, P, and S, are the number of each element in the molecular formula and Z is the net charge of the species. The NOSC can be linked directly to the energy yield upon oxidation (Keiluweit et al., 2016; LaRowe and Van Cappellen, 2011). Because it is directly linked to the thermodynamic forcing, this approach has advantages over inferring organic matter lability based on aromatic content or O/C ratios because knowledge of the NOSC allows the subsequent direct calculation of the Gibbs energy of the oxidation (LaRowe and Van Cappellen, 2011):

$$\Delta G^{\circ}\mathrm{Cox}\ (\mathrm{kJ\ mol\text{-}1\ e\text{-}}) = 13.81 - 3.57(\mathrm{NOSC}) \qquad (3)$$

More positive NOSC values equate to more energetically favorable C oxidation, although whether a particular compound can be favorably oxidized or not also depends on redox conditions. Thus, NOSC is important because it provides a thermodynamically relevant metric for evaluating organic matter quality under the conditions of interest (Wilson and Tfaily, 2018) where the energy yield for oxidizing the NOM can then be directly compared to the availability of terminal electron acceptors (TEAs) to determine the thermodynamic feasibility for degrading each fraction of the NOM (Boye et al. 2017). While other limitations such as enzyme availability and mineral sorption may also influence DOM decomposition, thermodynamic limitations ultimately constrain the lability of NOM. Molecular oxygen has the highest energy yield of available electron acceptors thus in aerobic systems it is thermodynamically feasible to oxidize the full range of natural observed NOSC values (Keiluweit et al., 2016), and decomposition in aerobic systems is then kinetically controlled (Boye et al., 2017). However, under anaerobic conditions, a range of lower energy yield TEAs may be available, as iron has a lower energy yield compared to oxygen and sulfate is even lower. Thus the range of compounds that it is possible to decompose becomes increasingly constrained in low redox conditions. For example, it has been shown that C decomposition can become thermodynamically limited under sulfidic conditions (Boye et al., 2017), resulting in depletion of high NOSC carbohydrates and aromatic compounds and an accumulation of low NOSC proteinaceous and aliphatic-like formulas. Such thermodynamic constraint could account for the accumulation of lipid-like compounds frequently observed in anaerobic systems (Hodgkins et al., 2014; Tfaily et al., 2013) which is in contrast with the preferential decomposition of lipid-like compounds in aerobic environments (Drake et al., 2019; Spencer et al., 2014; Spencer et al., 2015).

At this point it is appropriate to note some problematic issues with this approach to using UHR-MS data to calculate NOSC and free energy values. First, errors in calculating an NOSC lie within the confidence intervals of the molecular formula assignments which can include instrumental limitations and biases. Second, each ion almost certainly represents multiple isomers with different reactivities which, under different environmental conditions, can lead to different processing or preservation mechanisms. We therefore recommend caution with using NOSC as a thermodynamic imprint without sufficient consideration of the limitations of UHR-MS and other quantitative information available. As such, NOSC values estimated from mass spectral data may not represent an imprint, but rather more of a boundary condition through which more specific processes can be predicted.

B. Identification of Abiotic Photochemical Reactions by FTICR MS

While a large number of studies focus on the lability of DOM to microbial degradation, in some systems anaerobic photo-oxidation can actually degrade more DOM than biological processes (Cory et al., 2014). Whereas microbial activity preferentially degrades ancient permafrost (Spencer et al., 2015; Ward and Cory, 2015), photo-degradation appears to favor decomposition of modern DOM (Stubbins et al., 2017). In some systems, strong correlations between aromatic content and photo-lability have been observed (Stubbins et al., 2010) and phenolic compounds were highly susceptible to photo-oxidation (Spencer et al., 2009), although other studies have shown no correlation between DOC concentrations or aromatic content and photo-lability (Cory et al., 2013). Application of FT-ICR-MS and subsequent calculation of oxygen subtracted double-bond equivalents (DBE-O) provided evidence that photo-lability is highly dependent on the initial composition of the DOM (Gonsior et al., 2009). Specifically, compounds with high double bonds relative to oxygen content were most photo-labile (Gonsior et al., 2009). Additionally, the observed molecular changes following photo-degradation have varied among studies. Photo-degradation of DOM resulted in an increase in molecular formulas with both high H/C and high O/C ratios (Stubbins et al., 2017). Applying Kendrick mass defect analysis demonstrated eliminative decarboxylation following exposure to UV light. High-resolution FTICR MS data revealed that photo-lability may be controlled by carboxyl content as well as aromaticity, and aldehyde groups may be photo-irradiated to carboxyl groups as well (Ward and Cory, 2016). Cumulatively, these effects suggest that DOM with high carboxyl content may be degraded to lower O/C DOM following photo-irradiation, while DOM with high aldehyde and ketone content may increase in O content following photo-oxidation (Ward and Cory, 2016), consistent with observations of increasing acid:aldehyde content following photo-irradiation in some studies (Riedel et al., 2016).

During riverine transport, DOM content generally decreases, and FTICR MS characterization of changes in the DOM composition indicates that the removal process appears to be selective, removing high molecular weight aromatics in favor of lower molecular weight aliphatic-like compounds, consistent with changes induced by reaction of the DOM with reactive oxygen species (ROS) produced during photo-oxidation of DOM (Waggoner et al., 2017). The various ROS produced during photo-oxidation (including hydroxyl radicals, superoxide radicals, and singlet oxygen) may all react differently with DOM and because isolating the individual species is difficult, comprehensively characterizing the partial photo-chemical transformation of DOM has proven challenging (Waggoner et al., 2017). These authors used FTICR MS analysis of lignin extracts exposed to various ROS species and showed that all ROS induced an increase in aliphatic-like, lower molecular weight formulas. Comparison of these results with changes in DOM during transport reveal the most overlap with transformations induced by superoxide exposure. Evidence from this study suggests that reactivity is strongly determined by oxygen content of the DOM, but also the relative concentration of ROS produced can influence the resulting transformations. The practical implications of the photo-oxidative transformation of DOM are that, because lignin is transformed during transport, lignin-based biomarker proxies may underestimate the contribution of terrestrially-derived DOM in coastal and marine systems.

Climate warming may enhance the rate at which previously buried DOM can become exposed to sunlight through a variety of processes. As glaciers melt they form drainage streams (Spencer et al., 2016), as permafrost thaws it mobilizes previously frozen DOM which drains to nearby surface waters (Ward and Cory, 2015), and rainwater throughput results in washout of DOM via surficial streams. Although microbial mineralization of DOM to CO_2 has been the primary focus of many studies to date, abiotic oxidation of organic matter may be an important under-constrained mechanism for DOM removal (Amon and Benner, 1996; Cory et al., 2014; Vahatalo et al., 2000). Typical estimates for complete DOM mineralization to CO_2 or CO by abiotic sources are around 10% of the total microbial respiration (Belanger et al., 2006; Graneli et al., 1996), but may exceed microbial respiration rates in some systems (Amon and Benner, 1996). Further, in addition to mineralization to CO_2 (Cory et al., 2014), reaction with reactive oxidative species during photo-degradation may result in photo-chemically modified DOM (Waggoner et al., 2017) and can account for up to 95% of the total DOM processing (Cory et al., 2014). This high rate of photo-oxidative degradation could be critical as photo-oxidation has been shown to significantly enhance the susceptibility of DOM to subsequent microbial mineralization (Vahatalo et al., 2003), although the effect appears to be most pronounced for DOM that has not been previously degraded or undergone prolonged photo-oxidation (Cory et al., 2013).

Other studies have conversely found that photo-oxidation results in less bioavailable DOM (e.g. (Sleighter et al., 2014). Further, DOM may be photo-degraded to highly bioavailable low molecular weight compounds such as pyruvate and glyoxylate which may stimulate microbial CO_2 production (Amon and Benner, 1996). On the other hand, high levels of light radiation inhibit overall bacterial production (Graneli et al., 1996). Thus, there is a tight coupling between photo-degradative and biotic DOM processing encompassing complex interactions between complementary and opposing forces.

In thawing permafrost regimes, abiotic mineralization rates are highest in meltwater streams, and during transport partial photo-oxidation of DOM becomes increasingly important and can contribute to stimulating bacterial respiration in rivers and lakes (Cory et al., 2014). It has been suggested that after an initial enhancement of lability, photo-alteration of previously exposed DOM actually inhibits subsequent bacterial processing (Cory et al., 2013). Conversely, ancient DOM in thawing yedoma permafrost streams is resistant to photo-mineralization, but not photo-oxidation (Stubbins et al., 2017). Young DOM from thawing arctic permafrost appears to be photo-labile and bio-unavailable, while ancient DOM from thawing yedoma appears to be photo-stable and highly bioavailable suggesting the molecular composition of the DOM is critical in determining its photo- and bio-lability.

Outside of the Arctic, the importance of photo-oxidation has been demonstrated in the surface waters of the Amazon River system (Amon and Benner, 1996) where up to 50% of terrestrial DOM may be photo-oxidized in rivers during transport to the coastal ocean. Because of their light absorbing capabilities, it had been suggested that photo-oxidation is governed primarily by aromatic moieties present in DOM (Cory et al., 2014). Additionally, it was assumed that UV-B radiation should have the greatest photo-oxidizing effect because of its strong absorption by DOM, but experiments in Scandinavian lake water found better correlation of photo-oxidation with UV-A distribution (Graneli et al., 1996) and quantum yield estimates suggest that UV-A accounted for 68% of photo-degradation while UV-B accounted for less than 10% (Vahatalo et al., 2000). However, Specific Ultraviolet Absorbance (SUVA) and fluorescence analysis failed to find a compelling correlation between aromaticity and photo-oxidation potential in thermokarst DOM (Cory et al., 2013). Together, these results suggest that the presence of other organic moieties such as phenolic carbon, which may quench photo-chemical reactions, or that differences in carboxyl-C content may be playing a critical role in the susceptibility of DOM to photo-oxidation (Ward and Cory, 2016). FTICR MS analysis of photo-oxidized samples was used to gain a deeper insight into the mechanisms of photo-oxidation on DOM and demonstrated the degradation of higher molecular weight, more oxidized compounds concomitant with the production of lower molecular weight, reduced compounds. Further in a comparison of photo-degradation patterns between organic mat and permafrost-derived DOM, it was observed that similar compounds were photo-degraded in both samples, while the compounds that were produced differed suggesting that while absorption of light by aromatic chromophores is a critical first step, further oxidation to produce CO_2 may be governed by the presence of quenching phenolics or abundance of carboxyl moieties. FTICR MS analysis of O/C and H/C content were combined with ^{13}C-NMR analysis to conclude that photo-mineralization of DOM is primarily accomplished through eliminative decarboxylation (Ward and Cory, 2016).

C. New Insights into the Formation of Humic Substances

Historically, a large portion of natural organic matter has been classified operationally based on solubility in alkaline solution as humic or fulvic acids. The schema of Schulten and Schnitzer (Schulten and Schnitzer, 1997) proposes that humic acids result from novel crosslinking to form humic macromolecules. This is based on structural proposals by Schulten et al., (Schulten et al., 1991) that suggest humic acids are comprised of condensed aromatic acids which form complex hydrogen bonds and are capable of trapping smaller molecular weight compounds inside of their three-dimensional structures. In this view, proteins, lipids, carbohydrates and other organic compounds are trapped within the 3-D structure of humic acids rather than integral to the humic structures themselves. As analytical methods have evolved, however, and higher resolution analysis of the extractable lignin has become possible, a newer picture has emerged. NMR-based studies are not consistent with this view of highly crosslinked 3D macromolecules trapping organic compounds, but rather suggest that plant and microbial residues make up the majority of “humic” structures (Kelleher and Simpson, 2006). In that study, protein, lignin, carbohydrate and aliphatic or lipid-like structures dominated the structure of humic substances.

As early as 1913, Maillard proposed microorganisms were relegated to breaking down macromolecules into sugars and amino acids, and that humic acids formed primarily from non-enzymatic chemical reactions between reducing sugars and amine-containing groups. Waksman (Waksman, 1930), noting the high N content of humic acids which suggests a non-lignin origin, proposed that humic acids result when microbially produced N-compounds (e.g. proteins) react non-enzymatically with lignin residues. This suggests a tight coupling between biological and abiotic processes in the formation of natural organic matter. The high N content of humic materials has been confirmed in more recent analyses (Kelleher and Simpson, 2006; Schulten and Schnitzer, 1997) and is consistent with observations from humic and fulvic-rich natural organic matter, in particular from Sphagnum-dominated peatland ecosystems. The N-content of Sphagnum moss is relatively low compared to other plants species (Painter, 1991) suggesting that microbial activity is nitrogen-limited. Despite this, the N content of peat is correlated with increasing loss of functional groups, a useful definition of humification (e.g. (Mattson, 1955; Smith, 1958; Tfaily et al., 2013). Further complementary NMR and FTIR analyses of peat have demonstrated a decrease in the O-alkyl content with increasing humification suggesting C loss and oxygen shedding from the peat structure itself and an increase in N-alkyl content of the peat during decomposition (Tfaily et al., 2014). This phenomenon has been explained by the creation of highly stable nitrogen-containing compounds; acid-catalyzed incorporation of amines into the solid phase structure with concomitant loss of CO_2 (i.e. decarboxylation) provides a unified explanation of these observations. Explorations of N-involved compounds and transformations using FTICR MS may provide the key to understanding these abiotic reactions and their potential contributions to greenhouse gas (CO_2 and N_2O) production from peatlands.

Finally, an FTICR MS analysis of changes in dissolved organic matter (DOM) with depth in a boreal peatland revealed that overall there is an increase in the diversity of molecular compounds within the 100-1000 Da range with ~6,000 unique compounds identified in the shallow 25cm horizon of the peat and 7,500 unique compounds identified at 2m deep. Correlational analysis with depth reveal accumulation of nitrogen-containing lignin-like and condensed compounds with depth Additionally, contrary to expectations from the 3D humic crosslinking model proposed by Schulten and Schnitzer which results in large macromolecules, the overall average molecular weight of compounds declined with depth in this peatland. These data taken together suggest that the Schulten-Schnitzer model may need to be revised.

VII. An Emerging Application; How Connections of Molecules in the H/C - O/C Space Reveal Specific Microbial Transformations

The Kendrick mass transform analysis described in Section III (Stenson et al., 2003) has become increasingly useful as an extension of the KMD approach. In this technique the data is mathematically transformed to alternate mass scales by means of a conversion factor that allows rapid detection of the loss/gain of specific chemical moieties between individual compounds. Because of the high mass resolution made possible by FTICR MS these changes can be used to infer specific microbial degradation pathways by which organic matter is produced or consumed. This is analogous to the approach used by traditional small-molecule metabolomics for identifying cellular processes but can be performed on the bulk sample matrix encompassing extracellular enzyme activity and interactions among community members (Wilson et al., 2017) and we thus argue that such linkages represent a sub-set of the more general concepts of “environmental metabolomics” (Bundy et al. 2009). This approach has been used in peatlands to identify a novel pathway by which organic matter may act as an electron acceptor to sustain high CO_2 production in otherwise TEA depleted environments (Wilson et al., 2017). This result was important because it suggests a mechanism for CO_2 production that is not tied to the production of another greenhouse gas, CH_4 and may have important implications for peatland-climate feedbacks.

A major current challenge in ecosystem carbon cycling modeling is to couple geochemical and microbial datasets to develop predictive models for testing hypotheses related to climate change. The development of a sequential extraction protocol using increasingly non-polar solvents allowed the recovery of the highest number and diversity of organic compounds from natural samples for elucidating natural organic matter composition (Tfaily et al., 2017). This method improved on previous parallel extractions in that it uses less total starting sample, improves the analytical sensitivity and reduces the variability associated with different methodological approaches. This sequential extraction approach was further expanded to incorporate analysis for other complementary analytical techniques for detecting metabolites, such as GC-MS, LC-MS/MS (lipidomics), and NMR as well as proteomics approaches. All of these analyses use a single sample resulting in a fully-paired metabolomic and proteomic dataset which improves statistical confidence in elucidating the pathways of SOM microbial decomposition. The results of these analyses can then be related to the activity of microorganisms leading to a closer coupling of microbial and geochemical data in models (Tfaily et al., 2019).

Building upon the earlier approaches for transformation identification described by Stenson et al., (Stenson et al., 2003), network analyses have evolved for use with FTICR MS data based on the connections identified from Kendrick mass transform analysis (Longnecker and Kujawinski, 2016; Tziotis et al., 2011). Using freely available networking software such as Cytoscape equipped with the MetaNetter plug-in, the list of m/z values resulting from FTICR MS spectra can be imported and mass differences between each m/z value calculated. In the resulting network, each m/z is represented by a node and the edges connecting nodes represent identified transforms which reflect potential biological and chemical transformations. (Figure 9).

Because of the high complexity of NOM and because each compound may be degraded by multiple different pathways (i.e. more than one edge per node), the resulting networks are often quite complex. The potential degradation pathways can be identified in different ways. One approach is to mathematically calculate all possible mass differences between all nodes present and then match the most frequently observed mass differences to chemical moieties (Longnecker and Kujawinski, 2016). An alternative method is to import a database of known common biological and/or abiotic reactions suspected to occur in the sample from which to build the sample network (Breitling et al., 2006). The first is considered an untargeted approach which is completely unbiased but generates highly complex networks and does not account for chemical alteration during sample preparation or analysis (Breitling et al., 2006). The second, semi-targeted approach ensures that links in the network correspond to chemically feasible reactions allowing degradation pathways to be directly interpreted from network connections, making it is possible to infer changes in the most important carbon cycling pathways across natural gradients, such as vegetation changes, pH, hydrological or other ecosystem gradients (Figure 9). The resulting networks are non-random and allow identification of biochemical pathways which may not have been discovered using classical biochemical methods.

In addition, an increasing number of studies have attempted to link microbial omics techniques with FTICR MS analysis of DOM cycling resulting in a deeper understanding of the complex interactions between microbial ecology and DOM biogeochemistry (Graham et al., 2019; Stegen et al., 2018; Stegen et al., 2015). It was recently shown that high oxidation state C compounds can accumulate in groundwater, despite the overall low DOM concentrations that protect groundwater DOM from microbial degradation (Stegen et al., 2018). In contrast, riverwater is characterized by higher concentrations of low oxidation state DOM which is thermodynamically protected from microbial decomposition. Coupling FTICR MS and microbiome analyses suggested that mixing between these two DOM sources across the hyporheic zone strongly influences biogeochemistry in riverine systems, in part through a priming effect that stimulates the degradation of riverwater DOM. Further, this approach allowed the identification of a "tipping point": as the groundwater fraction increased above 10% of total DOM in the mixing zone, and although NOSC remained high, bioavailability declined due to the low total concentration of DOM available. That study also demonstrated how transform analysis coupled with analyses of the microbiome can be used to infer selective pressures acting on the microbial community. Strong distinctions in N-associated transforms in riverwater vs. hyporheic zone water of the Columbia River were identified, suggesting that microbial communities in the hyporheic zone may be better selected for protein degradation.

While FTICR MS can provide molecular composition information on factors such as molecular weight, C,H,N,O,S,P content, oxidation state and aromatic content on each individual formula, "single-stage" FTICR MS does not provide structural information. Since many isomers are possible for each molecular formula (Reemtsma et al., 2008), identifying functional groups remains a challenge. Two-stage FTICR MS (MS/MS) with subsequent fragmentation of isolated ions has provided some information regarding the presence and abundances of certain polar molecular moieties such as H_2O, CO_2, and CO (Stenson et al., 2003; Witt et al., 2009).

Nevertheless, complementary analyses are becoming increasingly applied to address the remaining gaps. For example, because of the semi-quantitative nature of FTICR MS, critical C cycling processes may be missed if they do not result in complete loss of a given compound. To address complications induced by charge competition and improve confidence in inferring changes based on spectral peak intensities, FTICR MS has recently been increasingly coupled with separation techniques such as LC (Fudyma et al. 2019).. Additionally, critical metabolites in C cycling may be too small (<100Da) or poorly ionized (e.g. carbohydrates) to be detected by FTICR MS. In such cases, NMR is useful for quantifying critical metabolites that are difficult to quantify by other methods, e.g. acetate. Unfortunately the detection limits for NMR are high, but GC-MS can be used to semi-quantitatively identify smaller metabolites at low concentration that may nevertheless be critical to understanding soil processes. In addition, GC-MS and NMR can be used to distinguish between small metabolites that have the same molecular formula but differ in structure (isomers e.g. glucose vs. fructose) FTICR MS still remains superior to GC-MS and NMR in the identification of higher molecular weight metabolites and is unparalleled in identifying novel compounds likely to comprise a large fraction of highly heterogeneous NOM mixtures (Wilson and Tfaily, 2018). Thus, coupling all of these techniques provides a more holistic view of the DOM and processes that are occurring. In Figure 10 we illustrate how different techniques can target specific areas of the van Krevelen space which are poorly or incompletely resolved by FTICR MS to gain a more comprehensive picture of NOM present.

We conclude here with a bit of caution. As is the case with NOSC estimates using UHR-MS data, estimating microbial transformations using H/C - O/C mapping comes with some uncertainties that must be considered. First is differences in ionization efficiencies between samples, and even within the same sample when a transform results in a product with significantly different ionization efficiency than the original substrate. Second, ions separated by the mass of a metabolomic transformation may not be metabolically related. They may be independent and coincidentally present and when these mass spacings are used alone they will not provide unequivocal information about specific microbial pathways. However, coupling this technique with microbial techniques (metagenomic, proteomic, enzyme essays etc.) does provide a more comprehensive approach to better identify biological decomposition pathways and microbial processing as well as the direction of the transformations.

VIII. Summary

In this review we attempted to provide a history of the introduction of ultrahigh resolution FTICR mass spectrometry to the field of organic geochemistry and its impact on a number of disciplines in earth sciences. We began by describing the early stages of this effort which by default occurred primarily at the NHMFL where the only true high field FTICR instrument existed. Once it was recognized that electrospray ionization was the most viable ionization method for dissolved natural organic matter, efforts turned to optimizing FTMS conditions, and by 2002 it was apparent that it was possible to produce spectra with fully resolved major components containing C, H, O, and N with mass-to-charge ratios up to about 500 *m/z*. Attention then turned to data analysis, including automated formula assignments, data reduction, and visualization of the complex mixture composition of DOM. While electrospray ionization in both (+) and (-) modes continued to be the method of choice, we did note that several other ionization techniques provided additional and often complimentary molecular information.

By the mid-2000s FTICR MS had become an accepted and highly valuable analytical tool in organic geochemistry. This fact is reflected in the number of laboratories that brought online additional high-field instruments that were utilized in part or largely by members of the organic geochemistry community. The impact of the technique had led to a true “paradigm shift” in the study of natural organic matter. We provided an overview of some of the most exciting directions that began at this time, including understanding the composition of marine DOM and the molecular signatures in terrestrial DOM that could be used to identify it as it was exported the oceans. We also described how molecular information from UHR MS provides insights into the evolution of DOM in carbon-rich peatland soils, including those in permafrost environments. This molecular information on DOM composition in thawing permafrost is now being used to constrain uncertainties in carbon loss from these sites, an important component of climate change.

We concluded by highlighting some new directions that have emerged with the advent of this “paradigm shift”. One that we find particularly interesting is the linking of chemical transformations of NOM to their microbiological sources. Microbiologists are quite adept these days at identifying individual microbial species present in soils, sediments, and natural waters. Using molecular information from UHR mass spectrometry reduced to the “H/C-O/C space” can reveal which species are actually expressing active enzymes, or what enzymatic functionality a species present may possess.

Acknowledgements

The authors would first like to thank Professor Marshall and all the students, post-docs and staff at the National High Field FT-ICR Facility for their encouragement, technical advice, and enthusiasm for what we have tried to do over these past twenty years. Financial resources are of course necessary, and none of what we have described here would have been possible without the support for the Facility by the U.S. National Science Foundation (NSF-DMR-06-54118). Additional funding for our group came from the National Science Foundation (NSF-EAR-0628349, NSF-EAR- 0819811, NSF-OISE-0710744), the Department of Energy (DE-SC0007144, DE-SC0004632, DE-SC0012272), the National Oceanic and Atmospheric Administration (NA05OAR4311162), and the South Florida Water Management District.

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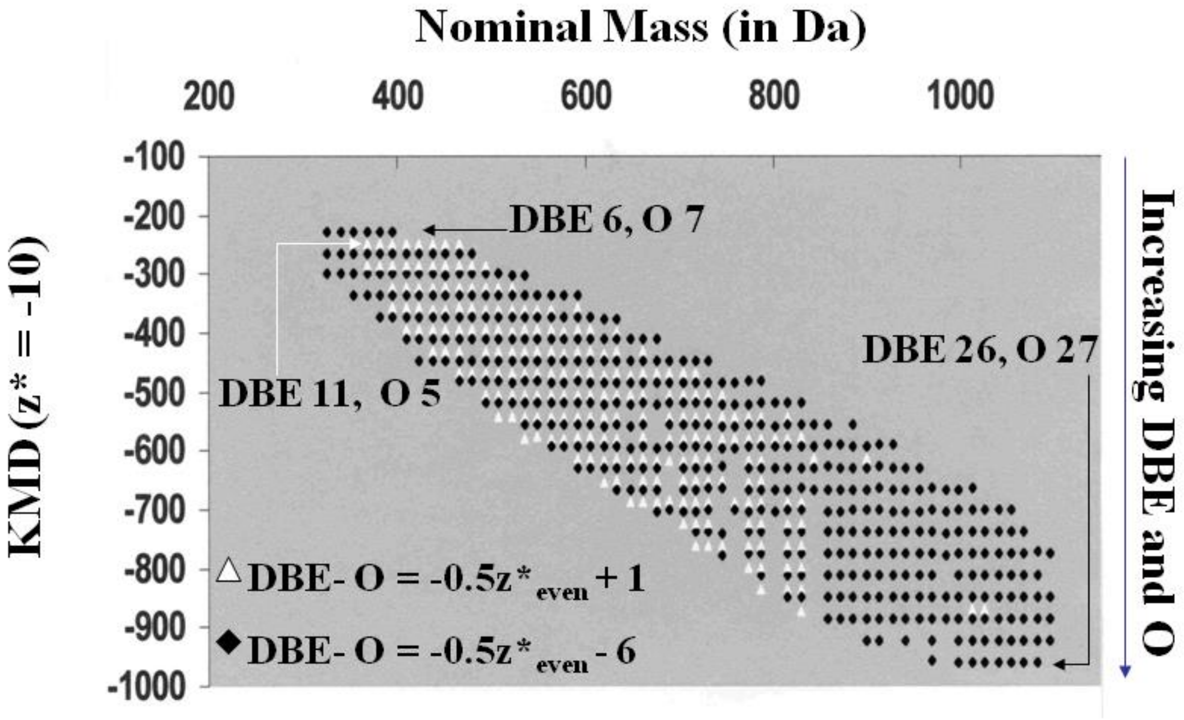

Figure 1. Kendrick Plot of one nominal mass series (z*) of Suwannee River fulvic acid. Kendrick Mass Defect (KMD) for compounds consisting of the elements CHO depends on the number of oxygens and the number of H. The number of H also correlates to degree of saturation. [Figure adapted from Stenson et al., (2003) with permission from The American Chemical Society]

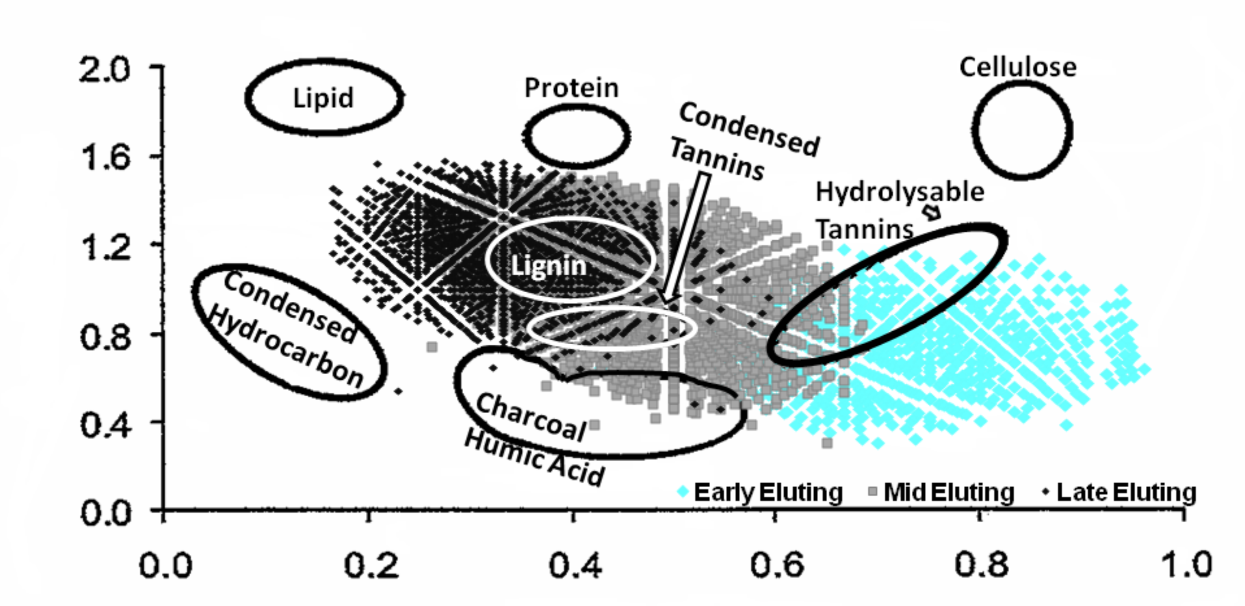

Figure 2. Van Krevelen Plot of three chromatographic fractions of Suwannee River Fulvic Acid. Blue - very early-eluting, most polar fraction. Gray - mid-eluting fraction. Black - late-eluting, far less polar than the blue fraction. Elemental mapping (ovals) adapted from Mopper et al. (Mopper et al., 2007) (see additional references in that citation). [Color figure can be viewed at wileyonlinelibrary.com]

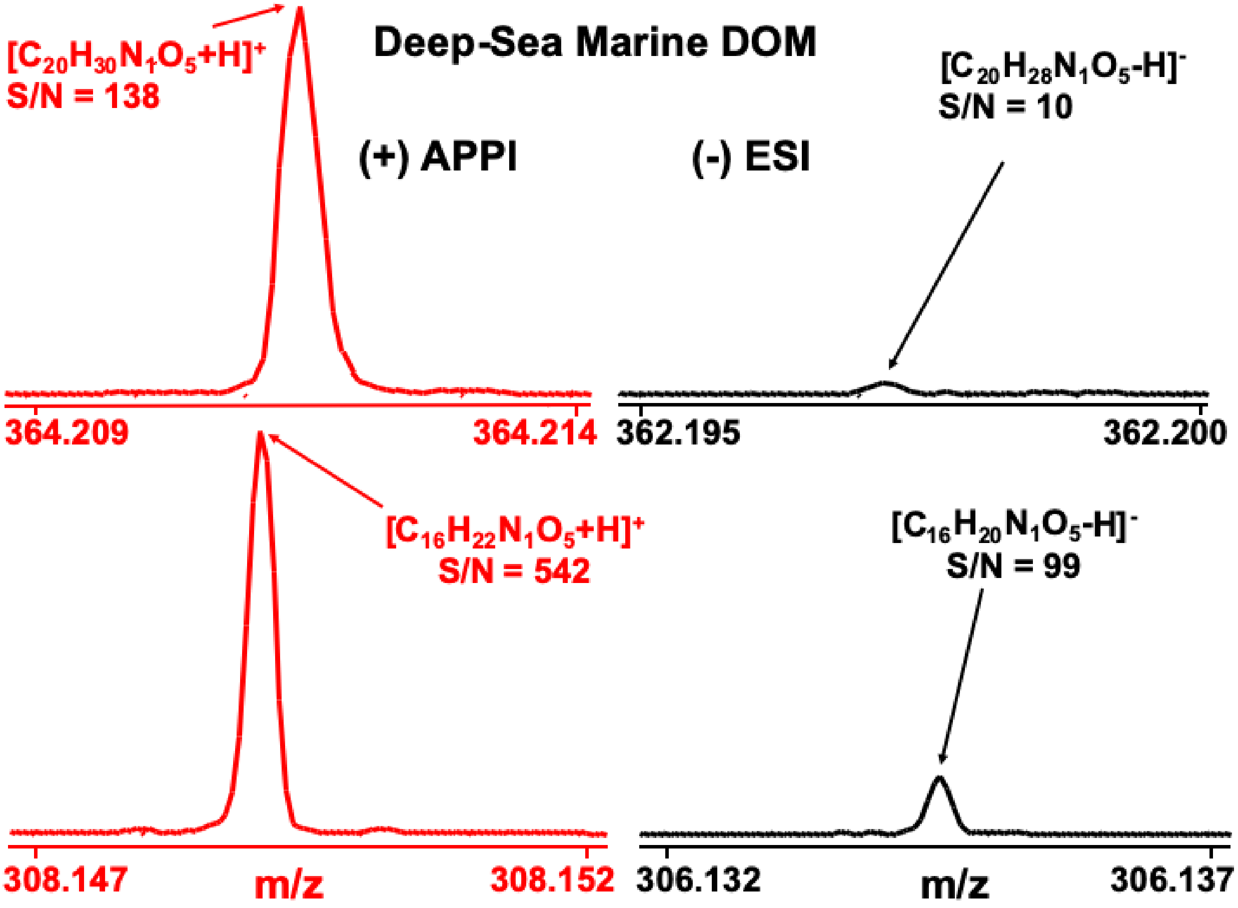

Figure 3. Two m/z ~ 0.01 expanded mass spectral segments for deep- sea marine DOM produced by positive APPI (left) and negative ESI (right). Compounds with a common neutral formula were selected. Note that signal to noise ratio (S/N) is more than 10-fold (top) or 5-fold (bottom) higher for APPI than ESI for the same neutral compound. Reproduced from (Podgorski et al., 2012b), with permission of The American Chemical Society. [Color figure can be viewed at wileyonlinelibrary.com]

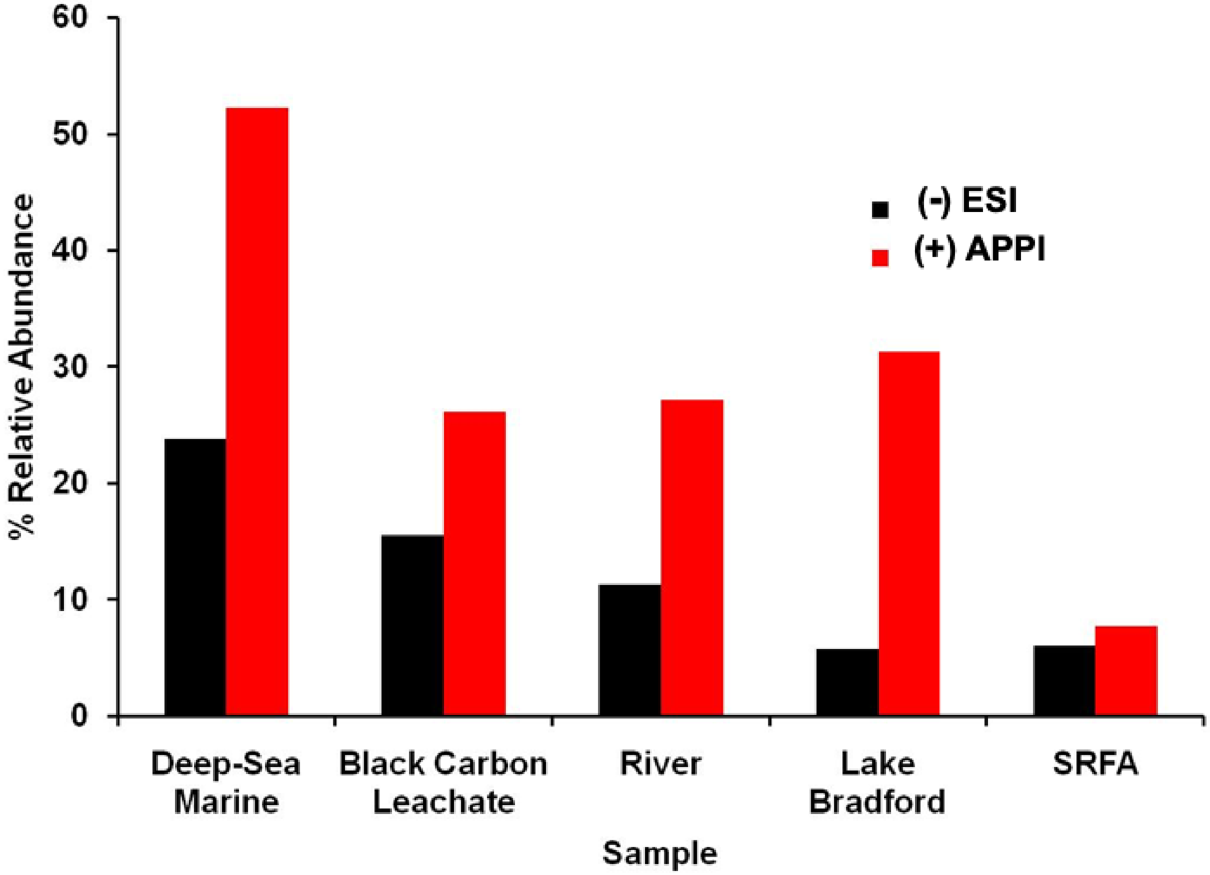

Figure 4. Histogram depicting the percent relative abundances for all nitrogen-containing species representative of five distinct DOM sources for positive ion APPI and negative ion ESI. Reproduced from (Podgorski et al., 2012b), with permission of The American Chemical Society. [Color figure can be viewed at wileyonlinelibrary.com]

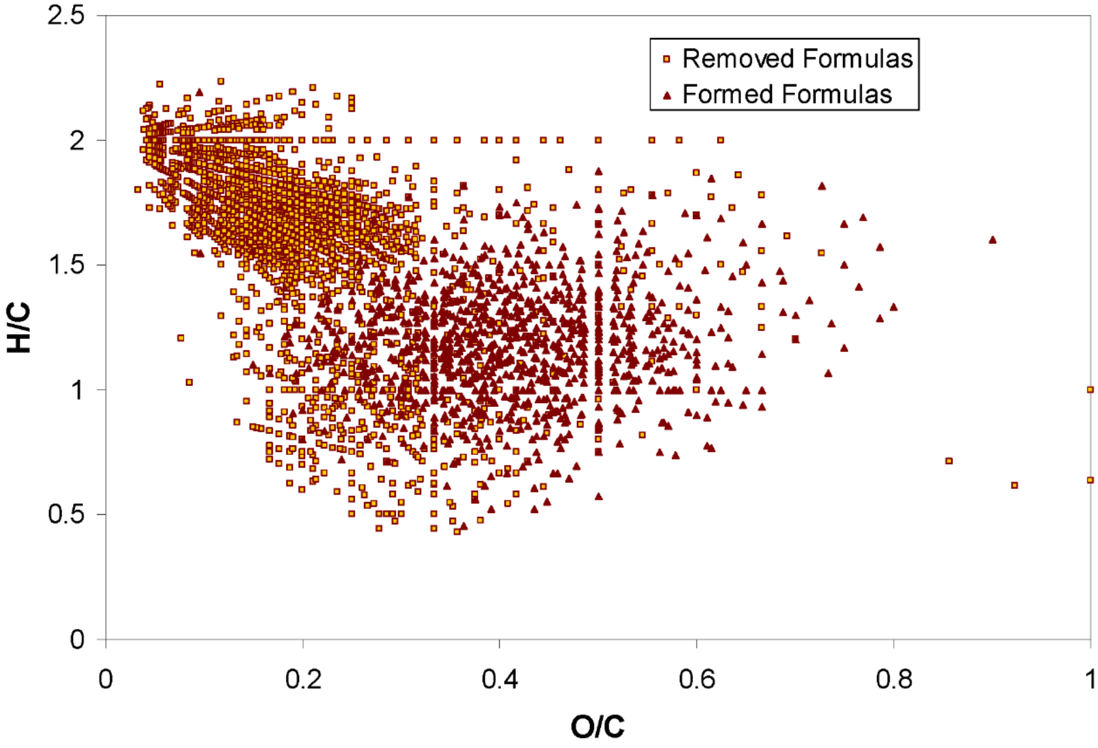

Figure 5. van Krevelen diagram of nitrogen-containing formulas only found in the original sample (T_0, i.e. “removed”, ■) and those only found in five-day assay (T_5, “formed”, ▲). Reproduced from (Osborne et al., 2013). [Color figure can be viewed at wileyonlinelibrary.com]

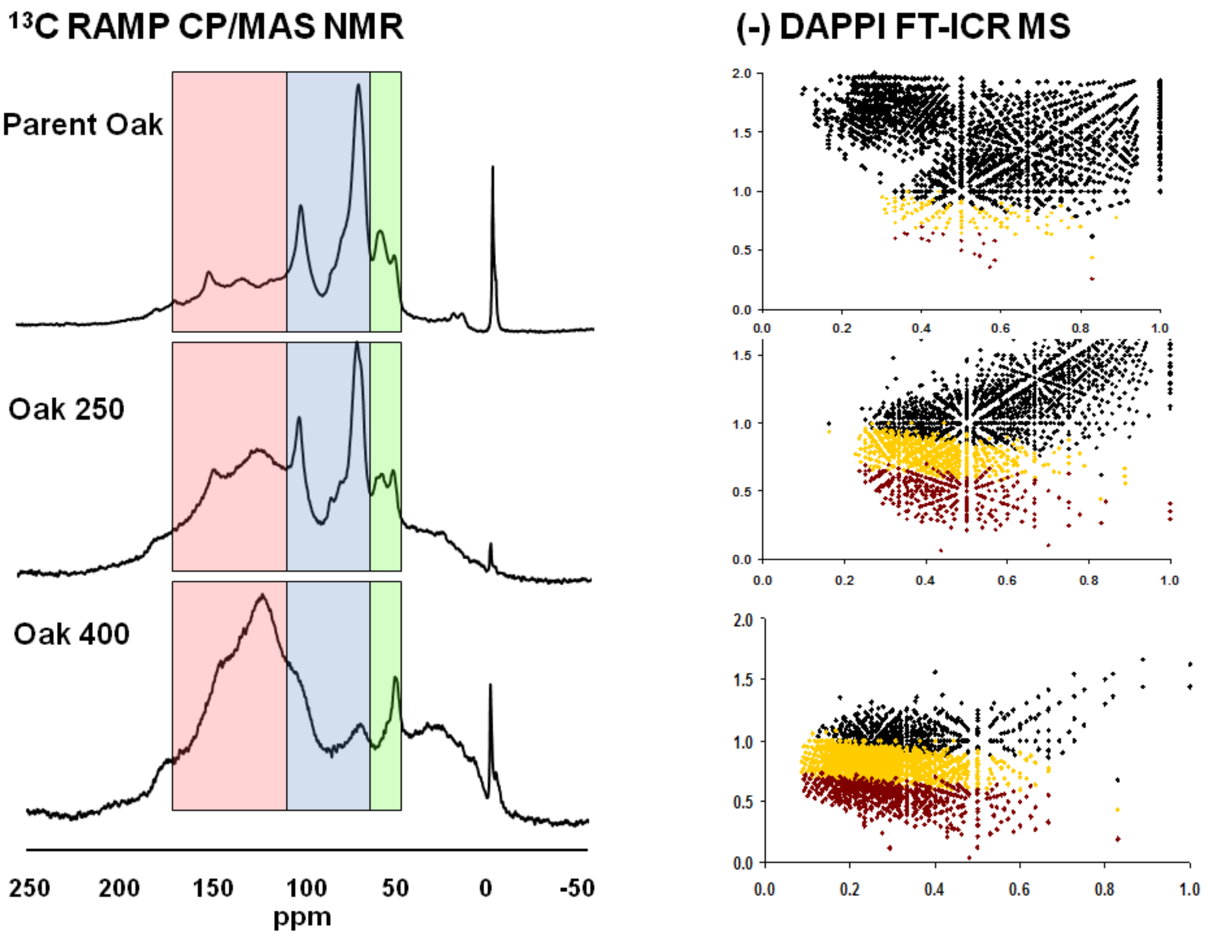

Figure 6. NMR spectrum of parent oak and oak after combustion at 250 °C and pyrolysis at 400 °C (left panel), and corresponding van Krevelen diagrams of the molecular formulas assigned from negative DAPPI FTICR mass spectra (right panel). In left panel N-alkyl and methoxy region highlighted in green, O-alkyl region in blue and aromatic region in pink. Right panel axes represent O/C (x-axis) and H/C (y axis) elemental ratios in formulas identified by FTICR MS. Figure adapted from (Podgorski et al., 2012a). [Color figure can be viewed at wileyonlinelibrary.com]

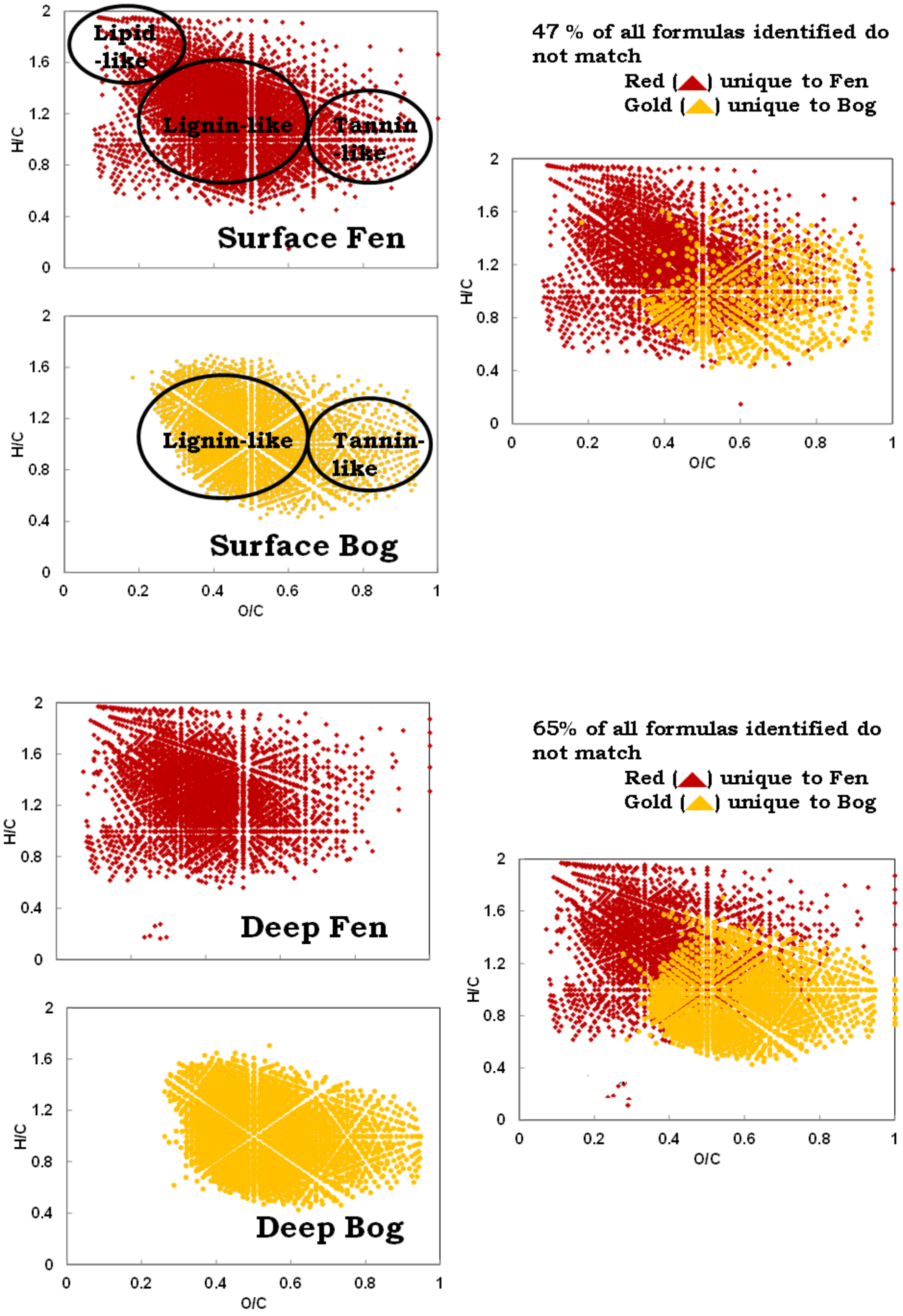

Figure 7. van Krevelen diagrams of porewater DOM from surface (ca. 10 cm, top) and deep (ca. 300 cm) vertical horizons of fen and bog formations in the Glacial Lake Agassiz Peatlands (GLAP) in northern Minnesota. Adapted from (Tfaily et al., 2013). [Color figure can be viewed at wileyonlinelibrary.com]

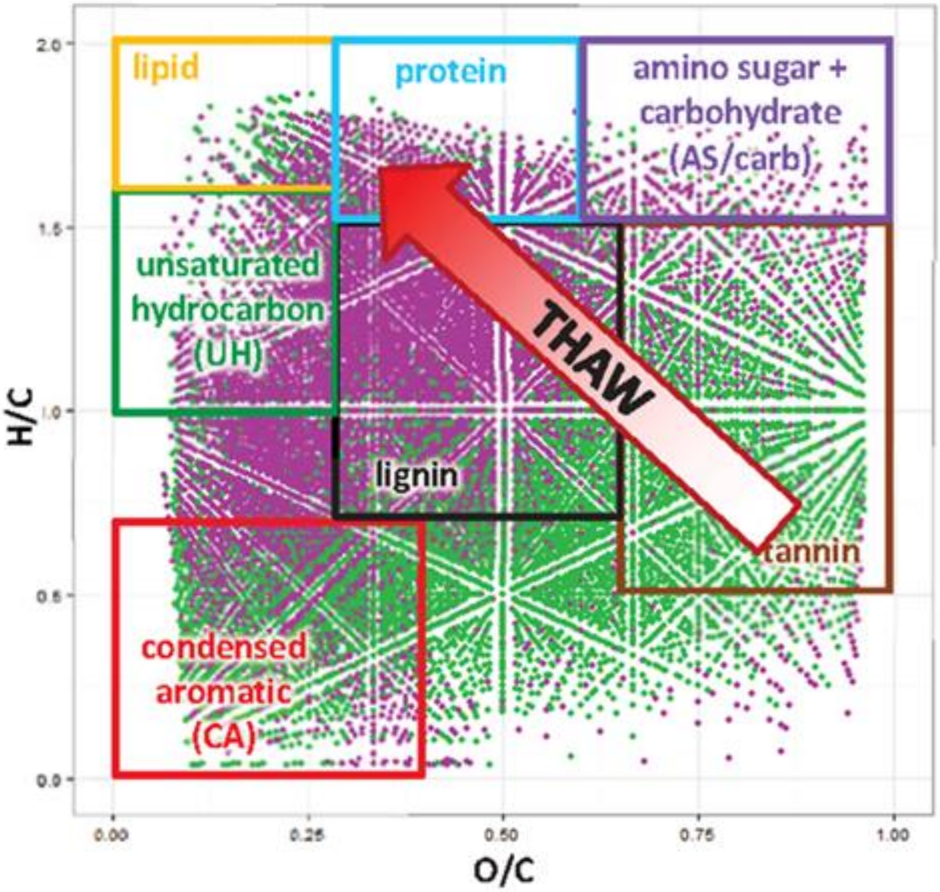

Figure 8. van Krevelen diagram of nonmatching formulas (those not shared between samples) in bog vs. fen DOM at Stordalen Mire. Molecular formulas found only in bog DOM are represented by green circles and formulas found only in fen DOM are represented by purple diamonds. Arrow represents the general trends in elemental ratios of porewater DOM as permafrost thaws. Adapted from (Hodgkins et al., 2014). [Color figure can be viewed at wileyonlinelibrary.com]

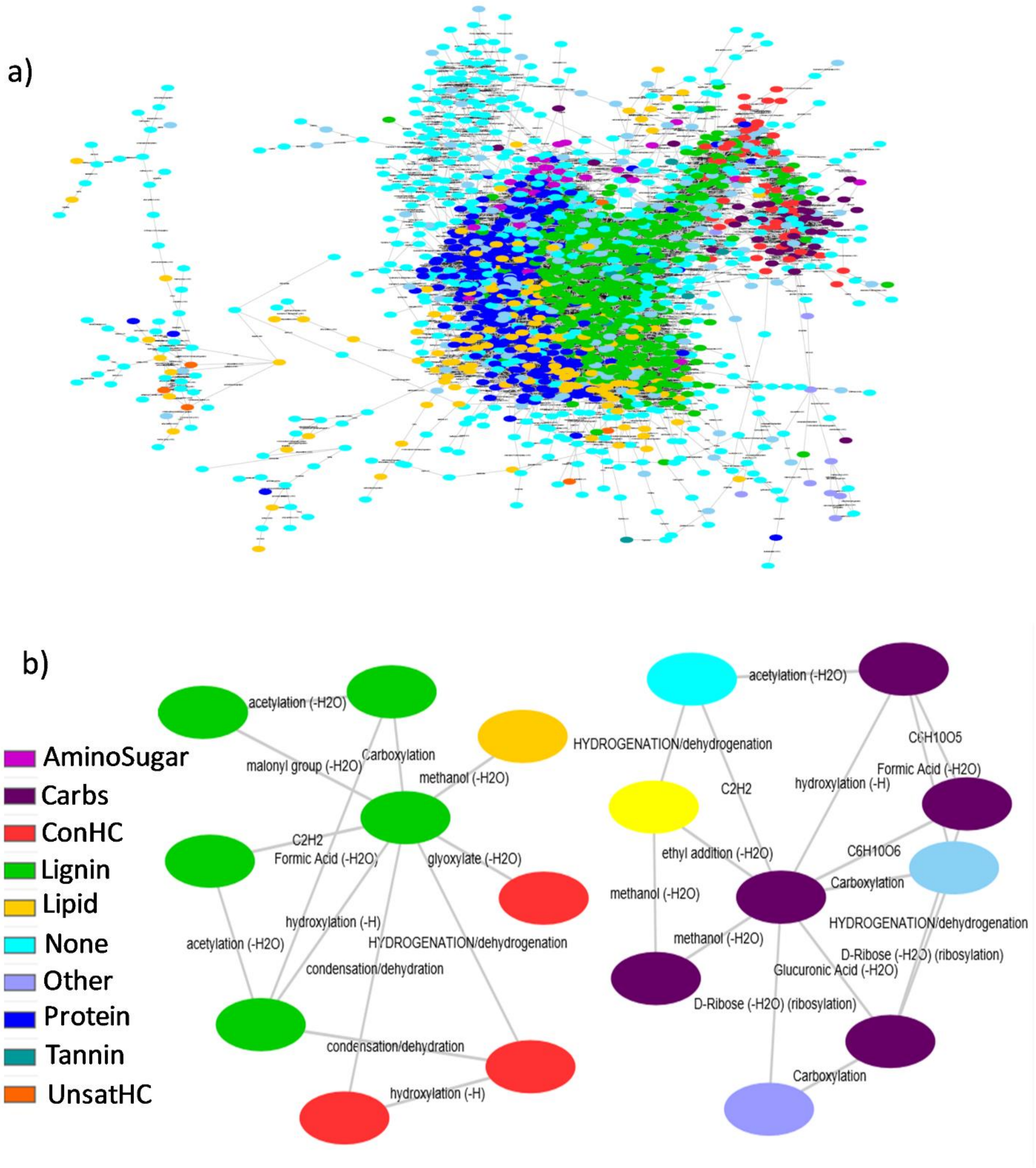

Figure 9. Network analysis of a water extract of soil sample after four weeks of incubation under anaerobic conditions. Different colors represent different classes of compounds; Nodes: represent different peaks observed in the FTICR MS; edges: represent different OM transformation reactions. Panel a) is the network of the whole samples. Panel b) is a subset of the nodes to highlight the connections (transformation) among compounds. [Color figure can be viewed at wileyonlinelibrary.com]

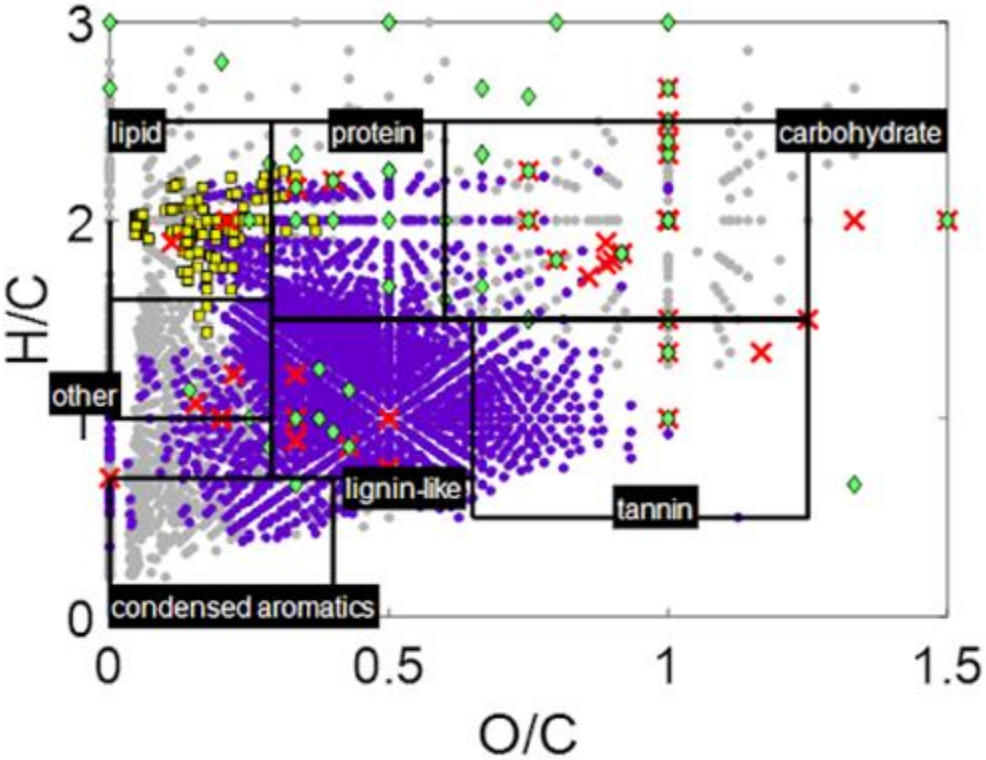

Figure 10. Composite van Krevelen diagram demonstrating how complementary analytical techniques can be used to identify different compound classes to gain a more comprehensive view of NOM. FTICR MS analysis allows identification of a broad range of molecular formula which can be classed with regard to O/C and H/C ratios as well as information on heteroatom content, (e.g. CHO-only compounds in purple vs. nitrogenous compounds in gray). LC analysis can be used to identify lipid compounds (yellow squares) while NMR (green diamonds) and GC-MS (red x's) can provide information on smaller molecular weight and/or poorly ionized compounds such as carbohydrates. (Adapted from (Wilson and Tfaily, 2018). [Color figure can be viewed at wileyonlinelibrary.com]