Advanced Instrumental Approaches for Characterization of Marine Dissolved Organic Matter: Extraction Techniques, Mass Spectrometry, and Nuclear Magnetic Resonance Spectroscopy

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1. Introduction

1.1. Importance of Marine Dissolved Organic Matter

Dissolved organic matter (DOM) is operationally defined as the fraction that will pass through a filter of a nominal pore size (usually between 0.1 and 1.0 μm). DOM accounts for around 90% of the organic carbon in the oceans, making it one of the Earth's largest active C pools (∼700 Pg C), approximately equal to the atmosphere's CO_2 load (750 Pg C). Thus, DOM is an important component of the global C cycle, and even minor changes in its size and dynamics can potentially impact many of the Earth's biogeochemical systems. For instance, the net mineralization of just 1% of the marine DOM pool would generate more atmospheric CO_2 than annually produced by fossil fuel combustion.[1] Therefore, an understanding of marine DOM dynamics is essential for predicting the responses of global biogeochemical cycles and local ecosystems in a warming climate.

Increasing research in DOM distribution and dynamics has uncovered many aspects of its multifarious biogeochemical functions. Significantly, the bioavailable fraction of DOM is now recognized as an important component in the marine microbial loop, being rapidly consumed by heterotrophic microbes[2] and released by zooplankton grazing, microbial exudation, and cellular lysis.[3] Due to its rapid turnover, the bioavailable fraction of the marine DOM pool does not accumulate and, thus, is found at low concentrations, typically between <1% and ∼6% of the total DOM pool,[4] with the upper end of the range in the surface waters (0 to ∼200 m). Consequently, it is biologically refractory compounds that dominate the bulk of marine DOM and largely define the characteristics of the DOM pool. As evidence of its refractory nature, the deep ocean DOM pool has an apparent ^{14}C average age of approximately 6000 years BP,[5] ∼7−8 times the oceanic cycling time. Furthermore, it has been suggested that the chemical characteristics that impart such inertness to these survivor molecules[1] may hold the "molecular Rosetta stone" for unraveling the degradation pro-

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Aron Stubbins is a Postdoctoral Research Scholar at Old Dominion University. He studied Marine Biology at the University of Newcastle-upon-Tyne (England) and Marine Biogeochemistry at Newcastle and Plymouth Marine Laboratory, receiving a Ph.D. in 2001. Since then, he has worked as a Research Scientist at the universities of Newcastle-upon-Tyne and Edinburgh, as well as at the Centre for Ecology and Hydrology–Edinburgh (Scotland), Plymouth Marine Laboratory, and Old Dominion University. His main research centers upon the aquatic carbon cycle, particularly dissolved organic matter photochemistry, microbial carbon processing, and air–sea gas exchange of carbon trace gases.

cesses responsible for transformation of the labile DOM fraction.[6]

DOM also plays key roles in metal chelation, influencing metal toxicity and bioavailability.[7,8] Furthermore, the colored fraction of DOM, CDOM, is the principal chromophore in marine waters, initiating photoreactions and influencing the heat budget of surface waters and the penetration of both photosynthetically active radiation and bio-inhibitory UV light.[9−11] Finally, there is a growing recognition that the multifarious constituents of the DOM pool provide useful

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information about both their own and their parent water's source and history. For example, recent studies have demonstrated that the composition and concentration of lignins within the DOM pool vary with source and diagenesis[12,13] and that the radiocarbon clocks associated with different compounds in the DOM pool can be used to infer information about their age and reactivity.[14] Despite the importance of marine DOM in oceanic and global biogeochemical processes, its cycling and chemical composition is poorly constrained at present, with any net shifts in size, function, and composition largely obscured by current analytical limitations.

1.2. Need for Detailed Chemical Characterization of DOM

The major obstacles to an improved understanding of DOM chemistry and composition are (1) difficulties in extracting unbiased (i.e., not altered by the extraction) and

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sufficiently large amounts of DOM from seawater needed for detailed analyses and (2) the low resolution of most previously applied instrumental approaches.

Ultra-high-resolution techniques are necessary because the marine DOM pool contains tens of thousands of molecules of unknown complexity resulting from the degradation/diagenesis of biota that vary widely in community structure in different regions of the oceans, as well as with depth in the water column. Recent advances in the biosciences, particularly in the fields of genetics and proteomics, have led to an appreciation of the intrinsic heterogeneity of biomolecules and helped place the complexity of marine DOM in context. Therefore, even to analyze in detail one class of biomolecules, i.e., proteins, within a single defined biochemical system requires high-resolution techniques that can separate and identify numerous compounds at vastly different concentrations. Non-protein biomolecules add additional complexity, as does their diagenesis both within and outside the organism. Marine DOM is the sum of (1) all intact biomolecules exuded, excreted, leached, and otherwise released from the living and decaying biota present in a water body; (2) the remnant and transformed biomolecules from organisms that previously inhabited the water, plus (3) chemically and biologically altered biomolecules from surrounding waters, atmospheric deposition, sediments, and terrestrial sources. Therefore, we can expect the chemical complexity of the marine DOM pool to be orders-of-magnitude greater than for any single organism. Confronted with this enormous complexity and heterogeneity, just the task of describing DOM component structures will be a monumental challenge because the molecules are polyfunctional, heterogeneous, polyelectrolytic, polydisperse in molecular weight, at low concentrations (typically less than picomolar to low micromolar), and dissolved in a ~0.7 M ionic strength inorganic salt matrix.

Until recently, analytical limitations have restricted researchers to either describing broad, bulk properties or characterizing in detail small fractions of the total DOM pool. Measurements of DOC, C:N ratios, bulk isotopic composition, colored DOM, and fluorescent DOM all come under the first category, whereas measurements of identifiable lignins, amino acids, sugars, proteins, nucleic acids, and other biomolecules belong to the latter. While these approaches have yielded significant advances, as demonstrated by the quantity and scope of the current DOM literature (for a recent review, see ref 15), they are fundamentally limited. The first approach assumes that the complex DOM pool can be approximated by a nonexistent, average DOM molecule and, thus, yields only qualitative information about shifts in the bulk composition of this hypothetical mean molecule. The second approach allows individual compounds to be identified and tracked as they are cycled. However, at present, the identifiable fraction represents <11% of oceanic DOC.[16] Therefore, identifying sources and understanding the processing of DOM components in the ocean has been hindered by methodologies in which only bulk parameters were ascribed or for which a significant pool of the DOM resided outside our analytical window,[17,18] with both approaches providing incomplete, and perhaps incorrect or highly biased, insights.

While the task of determining the detailed composition and structure of the major fraction of marine DOM is daunting, it offers unparalleled rewards, for, if the usefulness of a set of tracers is defined by their informational richness, it is apparent that the molecules within the DOM pool, diverse as they are in source, reactivity, and history, as well as carrying stable isotopic signatures and radiochemical clocks, represent a unique set of biogeochemical tracers capable of providing important insights into the origins of their parent waters and the diagenetic alterations that have occurred within those waters during transport. The prospect of mining this vast information store is a truly exciting challenge, leading John Hedges to comment that "the future of oceanographic research belongs in large part to those who can learn to read these molecular messages".[1] Here, we review a number of advanced analytical techniques with the potential to meet this challenge.

Many of these analytical techniques stem from advances in the biosciences, particularly in the area of proteomics, which are producing sophisticated tools with the power to bring a major fraction of DOM within our analytical window. We present an overview of the most promising of these avant-garde analytical techniques for the characterization of marine DOM, i.e., Fourier transform ion cyclotron mass spectrometry (FT-ICR-MS, section 2), and advanced nuclear magnetic resonance (NMR) spectroscopy involving spin-editing and multidimensional techniques (section 3). We also summarize advances made in sample preparation (i.e., desalting/extraction techniques) that are critical for taking advantage of the high-resolution capabilities of FT-ICR-MS and NMR. In particular, techniques for obtaining the 20−100 mg of representative (i.e., unbiased and uncontaminated)

marine DOM sample required for NMR are currently unavailable, although progress is being made in that direction (section 4). The capabilities of these advanced instrumental techniques are addressed, including how they are currently being used in the study of DOM in marine and terrestrial environments. Where techniques have yet to be applied to marine samples, their potential adaptability is presented. Potential applications are suggested by addressing current limitations and whether these are likely to be overcome, thereby identifying areas for needed development. The principal aim of this review is to provide the marine biogeochemistry community with insights into the capabilities of these emergent high-resolution technologies, so that they can be used advantageously in the future. So, in many ways, this review is actually a preview in that many of the techniques discussed have yet to be extensively applied to marine DOM.

2. High-Resolution Mass Spectrometry

2.1. Mass Spectrometry in DOM Studies

Mass spectrometry (MS) has been used for the past three to four decades by the marine community in the study of DOM. In most of these studies, a separation technique was coupled to MS, usually to improve the resolution and selectivity. These coupled, or "hyphenated", techniques include LC-MS, GC-MS, pyrolysis-GC-MS, and direct temperature-resolved MS (DT-MS). As the main focus of this review is the application (and potential application) of emerging high-resolution instrumental techniques, specifically FT-ICR-MS, to analysis of marine DOM, we will not present a comprehensive review of the extensive non-ICR-MS marine literature but rather present representative examples of the major applications to date. In addition, we will not be reviewing studies dealing with compound-specific analyses by radio-isotope MS, i.e., accelerator MS (AMS), and stable isotope MS, i.e., isotope ratioing MS (IRMS), which have been used to probe the marine biogeochemical cycling of specific compounds, as well as their metabolism by marine organisms.[19−25] The reader is referred to recent reviews that cover these techniques in depth.[14,26] However, it is worth noting that compound-specific radiocarbon analysis is poised for a major breakthrough. At present, these analyses rely on off-line collection of chromatographically separated DOM fractions that are then processed individually for AMS. This multistaged operation is time-consuming and labor-intensive, greatly limiting sample throughput and elevating the costs. These problems are now being addressed by continuous-flow AMS, the coupling of which to liquid chromatography (LC-AMS) will facilitate on-line monitoring of ^{14}C abundance in chromatographically separated compounds for the first time.[25]

With a few exceptions (see below), MS has been mainly used in past studies for identifying and quantifying specific fractions or trace components within the marine DOM pool, as opposed to deciphering the structure and composition of bulk DOM. The main classes or species include specific biomolecules, such as proteins,[27,28] amino acids,[29] sugars and complex carbohydrates,[30] lipids including sterols, alkenes and fatty acids,[31,32] polyphenolics and dissolved lignin[33,34] and pheromones and other chemotaxis compounds;[35,36] volatile organic compounds and trace organic trace gases;[37−39] organic ligands;[40] anthropogenic/pollutant compounds such as polyaromatic hydrocarbons, PCBs, pesticides and brominated compounds;[41,42] oil spill residues;[43,44] detergents and surfactants including alkylbenzenesulfonates and nonylphenol-ethoxylates;[45,46] pharmaceuticals and caffeine;[47] organic iodine species[48] and organic anti-foulants.[49−50]

In the above-cited studies, a variety of common ionization techniques, including electron impact (EI), atmospheric pressure electrospray ionization (AP-ESI), atmospheric pressure chemical ionization (AP-CI), matrix-assisted laser desorption/ionization (MALDI), and tandem MS-MS with and without collisionally induced dissociation (CID), were employed in various combinations with either ion-trap, quadrupole, or time-of-flight MS. The MS ionization techniques were usually coupled (either on-line or off-line) with a variety of extraction and pre-separation techniques to further enhance selectivity and facilitate spectral interpretation. These pretreatment techniques include steam distillation, solvent extraction combined with fractionation by normal-phase column chromatography, saponification of fatty acids, solid-phase extraction (SPE), solid-phase microextraction (SPME), ultrafiltration (UF) or tangential flow filtration, precipitation and gel electrophoresis (e.g., SDS−PAGE for proteins), HPLC (e.g., reverse-phase, size-exclusion, and affinity) with and without analyte derivatization, membrane introduction for volatiles, purge-and-trap for volatiles (with or without cryogenic trapping), GC with and without analyte derivatization, GC-inductively coupled plasma (ICP) mainly for organometallics and metalloids, Curie-point-pyrolysis GC, and direct temperature-resolved chemical ionization MS (DT-MS).

The latter technique, in contrast to most previous MS studies that focused on specific (and usually minor or trace) components within the DOM pool, has been successfully used to characterize the major components within the previously uncharacterized (and dominant) fraction of marine DOM and POM. The chemical information obtainable by DT-MS is intermediate between that obtained by detailed wet chemical analyses and one-dimensional solid-state NMR (see below). The DT-MS approach, in combination with multivariate statistical analyses, revealed the high abundance of polysaccharides (consisting of neutral sugars, methylsugars, N-acetyl aminosugars and acidic sugars), as well as furfural, and alkylphenols in DOM extracted from various mid-latitude coastal and estuarine waters.[51,52] When DT-MS was preceded by size-exclusion chromatography, it was found that the high-molecular-weight (HMW) fractions were enriched in methylsugars, aminosugars, and deoxysugars, while the low-molecular-weight (LMW) fractions were enriched in neutral hexoses.[53] When combined with ultrafiltration and C-18 SPE, it was found (for Chesapeake Bay waters) that the HMW fraction (UF retentate) was enriched in degraded polysaccharides and aminosugars, while the C-18 SPE extract of the LMW UF permeate was enriched in aromatic compounds, presumably from lignin and aromatic amino acids.[54] DT-MS was found also useful for evaluating the impact of DOM produced in a productive coastal bay (Chincoteague Bay, Virginia and Maryland) on the immediate coastal waters.[55]

Stabenau and Zika (2004)[56] combined hydrophilic−lipophilic SPE with AP-ESI-MS to obtain information about the distribution of masses within riverine and marine DOM and the origin of chromophoric DOM in southwestern Florida coastal waters. All riverine DOM samples showed a bimodal

mass distribution centered at about 400 and 1400 Da. In a river-to-coast transect, the latter maximum gradually decreased to about 1230 Da with increasing salinity. Combining MS and optical data, the authors concluded that chromophoric DOM in coastal water is largely altered terrestrially derived material.

While the previous MS techniques have yielded important insights into the composition of specific marine DOM components, as well as some insights into the "uncharacterized" DOM fraction, they were still limited by insufficient MS resolution needed to separate highly complex samples, like DOM, which contains an enormous number of compounds (as discussed in section 1.2). Thus, the purpose of this section is to present an emerging ultra-high-resolution MS technique, specifically Fourier transform ion cyclotron resonance MS (FT-ICR-MS), which holds the promise of greatly improving our knowledge of marine DOM composition and structure.

During the latter half of the 20th century, great strides were made in attainable mass accuracy in MS, which is important as, theoretically, if the mass of an ion can be measured with sufficient accuracy and precision, the exact elemental composition of that ion can be calculated,[26] a major breakthrough for the characterization of unidentified molecules. Today, advanced MS instrumentation allows ultra-precise mass determinations of ions,[57] making it possible to assign masses so accurate (e.g., mass $\div 10^{11}$) that molecular bonds can be weighed. These ultra-precise techniques are, however, only applicable for ions of nearly identical masses (i.e., mass-to-charge ratios, m/z) and, therefore, are not applicable to mass measurements of aquatic DOM. For such heterogeneous mixtures, accurate mass measurement techniques capable of resolving tens of thousands (or even hundreds of thousands) of peaks over a broad range of m/z values ($\sim$200 to $\sim$5000 Da) are required. From a recent review of modern MS techniques and their use in proteomics,[58] it is apparent that the only technique that is currently capable of yielding the mass accuracy and resolution needed to provide definitive elemental formulas for the range of ion masses displayed by DOM is FT-ICR-MS.

2.2. Introduction to FT-ICR-MS

The potential of FT-ICR-MS (occasionally referred to as FTMS) to directly identify the individual compounds that comprise the highly complex DOM pool with little sample preparation is currently unrivaled. FT-ICR-MS was developed about three decades ago.[59] The numerous reviews and articles describing the principles and applications of FT-ICR-MS attest to its popularity and versatility.[60−63] (For a thorough introduction to the technique, see Marshall et al., 1998.[64])

The heart of any ICR mass spectrometer is the ICR cell, typically about a centimeter in radius and located in the horizontal bore of a powerful magnet. Ions are usually produced externally by various ion sources (addressed below, section 2.3) and introduced via various ion guides into the ICR cell, where they are entrained into sub-millimeter orbits (cyclotron rotations) perpendicular to the magnetic field. Ion cyclotron rotation is initially random. Application of a spatially uniform electric field (by means of a radio frequency pulse) perpendicular to the magnetic field increases the radii of ion rotations and also brings all the ions of various masses and charges into phase. The now coherently orbiting ion packet induces a differential current between two opposed detection plates; this signal is modeled as a current source. The radio frequency pulse is then turned off, and the complex pattern of frequencies associated with the mixture of ions (brought about by constructive and destructive interference of all the ions, each undergoing its own cyclotron motion due to the different masses and charges) is then recorded in the time domain while the excited ions' coherent and extended orbitals return to a random state of ion cyclotron motion in the cell during an observation period of $\sim$1 s. Ions decaying from coherent orbitals of about 1 cm in radius will travel approximately 30 km during a 1 s observation period.[64] This long path length is a major reason that FT-ICR-MS can offer much higher mass resolution than conventional MS techniques.

The diminishing time domain signals are digitized and converted to the frequency domain by Fourier transformation. Frequencies can be measured with very high accuracy, which is another major reason for the ultra-high resolution of FT-ICR-MS. Importantly, under a constant magnetic field, the frequency is only dependent on the m/z value, allowing m/z values to be readily calculated by rearrangement of the cyclotron equation:

$$\upsilon = 1.535611 \times 10^7\, T/(m/z) \qquad (1)$$

where υ is the calculated frequency in hertz, m is mass, z is the charge of the ion, and T is the magnet field strength in tesla. This equation demonstrates that, if all the parameters are unchanged, the shift in frequency per m/z unit, and therefore mass resolution and accuracy, scales linearly with magnet field strength.[64] Therefore, projected increases in field strength will yield concurrent improvements in mass resolution. For example, current state-of-the-art instruments are equipped with magnets between 9 and 15 T. A 9.4 T instrument routinely yields an accuracy and precision of approximately 100 μDa at 500 Da.[65] A 14.5 T instrument is reported to have a resolution of 25 μDa,[65] and a 110 mm diameter bore, 21 T superconducting magnet (resolution of 5 μDa at 500 Da) for FT-ICR-MS is presently under development at the National High Magnetic Field Laboratory (Tallahassee, FL).

2.3. Ion Sources for FT-ICR-MS in Relation to DOM Studies

The use of FT-ICR-MS (like all other MS techniques) requires that analytes be ionized prior to analysis by MS. An ideal ionization source for the quantitative characterization of DOM should be both nonselective (i.e., ionizing all compounds equally) and soft (i.e., ionizing compounds without fragmentation). There are currently three popular soft ionization sources in routine use: MALDI,[66] atmospheric pressure electrospray ionization (normally referred to in the literature as ESI), and atmospheric pressure photoionization (APPI). MALDI is not favored for ultra-high-resolution MS analyses, as ions produced by MALDI have slightly varying energies and velocities, leading to broadening of mass spectral peaks and reduced resolution.

ESI was first reported by Fenn et al. in 1989,[67] and was combined with FT-ICR-MS later that year.[68] It has become the most popular ionization mode for FT-ICR-MS. This

ionization technique transfers *pre-existing* ions from solution phase to gas phase with no or negligible fragmentation, thereby allowing molecular weight determination of large macromolecular ions. Although ESI coupled to FT-ICR-MS has mainly been used for protein studies, this approach has also been applied to freshwater and marine DOM analyses.[65,69−77] From these studies, several critical questions regarding the ionization of DOM by ESI have emerged. For instance, Koch et al. (2005)[76] found that the average MW of DOM determined by ESI-MS was always somewhat lower than that obtained using size-exclusion chromatography for the same samples. Since ESI is presumed "soft" enough not to cleave covalent bonds, fragmentation of DOM during ionization by this source has been attributed to disaggregation of weak, non-covalently bonded complexes or associations.[69,72] This explanation is consistent with the hypothesis that HMW DOM, humic substances, and colloidal natural organic matter are noncovalent aggregations of smaller molecules or complexes[78−81] partly held together by polyvalent metal cation bridging.[82] Alternatively, ESI may preferentially ionize a lower molecular weight fraction of the DOM pool. In fact, ESI is known to be selective, ionizing only molecules that contain both polar regions (for holding the charge) and nonpolar regions (for enhancing surface activity).[83] For example, although peptide-like substances are present within DOM and peptides give multiply charged species during ESI, multi-charged species are a rarity in ESI of DOM (see section 2.6). This inconsistency is currently ill understood, and, to date, there have been no systematic studies to examine the variables that control the selective ionization of DOM. As the questions regarding the ionization behavior of DOM during ESI are addressed in future studies, important new insights about the basic structure and chemistry of DOM are likely to emerge.

Among the currently available alternative ionization techniques, atmospheric pressure photoionization appears to hold the greatest potential for DOM analysis. Two recent reviews covering the technique's development provide a thorough introduction to the theory and application of APPI.[84,85] In APPI, ionization occurs mainly by charge transfer between an *added*, easily photoionized dopant (e.g., benzene in the presence of O_2) and the analyte. Thus, in contrast with ESI, in APPI the analyte does not need to be a pre-existing ion in solution; i.e., in APPI, neutral molecules are also ionized and, thus, detectable by MS. Thus, APPI has potentially much to offer regarding the composition of DOM owing to its ability to ionize what was previously an invisible molecular fraction. Other key advantages of APPI over ESI for DOM characterization include greater sensitivity, particularly for nonpolar organics, greater dynamic range and signal-to-noise ratio, less chemical noise from solvents and salts, and less ion suppression from matrix effects.[86,87] The greater sensitivity to analytes and robustness in the presence of salts are particularly advantageous for studies of marine DOM, where most analytes are at vanishingly low concentrations in a matrix of inorganic salts. Examples of analytes that are not readily detected using ESI, but are amenable to APPI, include polycyclic aromatic hydrocarbons (PAHs) extracted from sediments[88] and microbial respiratory ubiquinones and menaquinones in environmental samples and cell cultures.[89] Further studies will likely extend this range through the use of different dopants and tunable excitation wavelengths. However, like ESI, APPI also has some unresolved issues with respect to DOM analyses. Purcell et al. (2004)[90] observed that protonated molecules, deprotonated molecules, and radical molecular ions are formed simultaneously in the APPI source, which complicated their spectra (>12 000 peaks per mass spectrum and up to 63 peaks of the same nominal mass for complex organic matter samples, i.e., crude oil) and precluded the use of the "nitrogen rule" for nominal mass determination of number of nitrogen atoms in a molecule.

Figure 1 shows APPI FT-ICR and ESI FT-ICR mass spectra of an aquatic DOM extract (Lake Drummond, Dismal Swamp, VA) that was directly infused into the ionization sources of a 9.4 T instrument. Visual inspection of the spectra clearly shows that APPI exceeds ESI in terms of molecular-level information. For example, the inset shows the resolution of peaks at nominal mass 639. The mass error between the theoretical formula weights and measured masses is <500 ppb, and the resolving power is >450 000 at 500 Da. Detailed analysis of the entire APPI spectrum[91] reveals the presence of a diverse suite of aromatic biomolecules, as well as novel arene and azarene compounds, e.g., a homologous series of PAHs extending to $C_{60}H_{16}$. Clearly, much more work is required to determine the impact of selective ionization, macromolecular disaggregation, and matrix effects in both ESI and APPI sources with regard to biasing the interpretation of DOM composition and structure.

2.4. Interpretation of FT-ICR Mass Spectra of DOM

The high degree of mass accuracy and precision, coupled with the ability of FT-ICR-MS to detect ions over a wide range of *m*/*z* values, facilitates assignment of exact molecular weights and subsequently molecular formulas to individual components within DOM without the need for prior separation by chromatographic or other methods.[70,77] However, even with high levels of accuracy and precision, the identification of all components within the DOM pool is not straightforward. Fortunately, there are a number of "rules" that can be applied to complex DOM mass spectra to eliminate certain elemental formulas, in particular, the nitrogen rule[92] and the double bond equivalents (DBE) rule.[92] In brief, the nitrogen rule,[92] derived from the valence of chemical bonding, dictates that, for even-electron N-containing ions [$(M + nH)^{n+}$ or $(M - nH)^{n-}$],

even-mass ions have odd numbers of nitrogen atoms

odd-mass ions have even numbers of nitrogen atoms

and that for odd-electron N-containing ions (e.g., radical cations, $M^{+\bullet}$),

even-mass ions have even numbers of nitrogen atoms

odd-mass ions have odd numbers of nitrogen atoms.

The double bond equivalents (DBE) rule states that, for every ring or double bond present, the number of hydrogen atoms is reduced by 2. Therefore, the sum of rings and double bonds per molecule (double bond equivalents, DBE) for an organic compound of composition $C_cH_hN_nO_o$ can be calculated as

$$\text{ring} + \text{double bond equivalents} = \text{DBE} = c - h/2 + n/2 \pm 1 \quad (2)$$

so that the DBE of uncharged molecules must be an integer

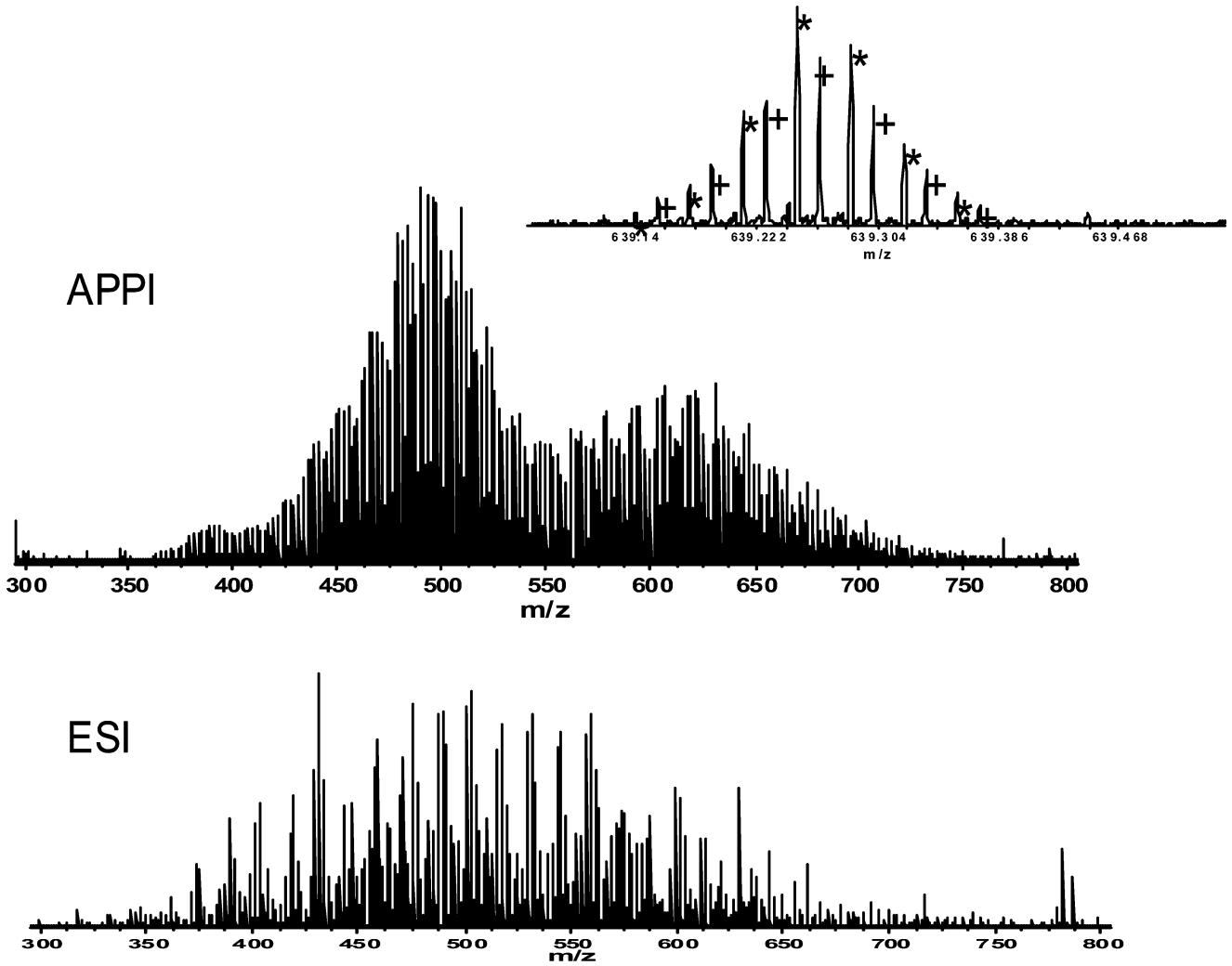

Figure 1. Negative-ion ESI and APPI-FT-ICR mass spectrum of Lake Drummond DOM. (Data were obtained on the NHMFL 9.4 T ESI FT-ICR instrument.) The inset shows a single nominal mass region ($m/z = 639$), with the asterisk indicating peaks from direct ionization of DOM and the plus sign indicating peaks from chemical ionization via a dopant (toluene).The APPI spectrum shows many more peaks than the ESI spectrum due to the ionization of a wider range of molecules.

value. Therefore, molecules of the same nominal mass cannot differ in elemental composition by NH_2 versus O, for example.

Furthermore, the number of different molecules possible within a DOM sample is constrained, as not all elemental formulas are chemically allowed.[65] In addition, some chemically allowed elemental formulas can also be ruled out on the basis that they are highly unlikely to occur in natural samples. For example, the maximum number of hydrogen atoms per organic molecule can be assumed to be less than $2C_n + 2$, and molecules comprised of all oxygens or all sulfurs can be ruled out.[65] Additionally, because the natural abundance of ^{13}C is only 1%, the relative abundance of molecules with more than two ^{13}C atoms is so low that their number falls below detection and they can be ignored.[65] Finally, compounds containing one or two ^{13}C atoms must have analogous all-^{12}C counterparts in the mass spectrum. If a corresponding all-^{12}C compound cannot be detected, then assignment to an elemental formula containing ^{13}C can also be ruled out.[65] Applying the above rules and constraints, Kim et al. (2006)[65] calculated that, at a resolution of ~100 μDa, typical of a 9.4 T instrument, it is possible to assign unique elemental compositions (e.g., C, H, N, O, and S) to all ions within complex samples, such as humic substances and DOM, up to a mass of ~500 Da. The mass limit can now be readily extended to higher masses, given the availability of commercial instruments with higher magnetic field strengths, e.g., 12 and 15 T, which have significantly better mass resolution (as mentioned in section 2.2).

After exact elemental formulas have been calculated, further data analysis is required in order to deal efficiently with the vast data sets that comprise FT-ICR-MS of DOM and to reveal information about DOM component structures. To date, researchers have employed a number of techniques that use elemental composition to categorize compounds. First, Kendrick mass analysis, originally used to identify series of compounds with identical chemical backbones but differing numbers of $-CH_2$ groups,[93] was later adapted to separate polar petroleum compounds[94] and to help categorize DOM.[95] In brief, the measured mass is converted to a "Kendrick mass", where the mass of $-CH_2$ is defined as 14.000 Da, instead of the IUPAC mass, 14.01565 Da. A further calculation yields the Kendrick mass defect (KMD = IUPAC mass − Kendrick mass), which is constant for compounds with identical chemical backbones but different numbers of $-CH_2$ groups. The elemental formula of the lightest compound in the family can then be assigned, thus determining the masses for all compounds in each series.

Subsequent studies have expanded this treatment to non-CH_2 building blocks (e.g., $-CO_2$).[77,96] Stenson et al. (2003)[70] also further developed Kendrick mass analysis, separating compounds within a humic acid sample on the basis of the integer remainder after the IUPAC nominal mass was divided by 14.000 Da, and defined this term *Z**. Using this approach, they noted two sets of homologous series, one with high DBE and low oxygen (O) and the other with lower DBE and higher O. The observed correlation between DBE and O for the elemental composition $C_cH_hN_nO_o$ is an outcome of the DBE relationship (eq 2).[92] Consequently, the substitution of CH_4 by O must be accompanied by the addition of a ring or a double bond.

The concept of carbon-normalized double bond equivalents (DBE/C) was introduced by Hockaday et al. (2006).[97] These authors found that a threshold DBE/C value of 0.7 can be used for identifying species with condensed aromatic ring structures (CARS). On the basis of this criterion, Hockaday et al. identified CARS of the same mass (within 1 ppm) and empirical formulas in soil black carbon, volcanic ash soil humic acid from Japan, and Amazonian Rio Negro DOM. This similarity of water-soluble condensed aromatics present within, as well as exported from, fire-impacted soils of geographically and climatically disparate ecosystems led the investigators to conclude that these CARS are molecular fingerprints of black carbon degradation in soils. The identification of such fingerprints should provide new insight to black carbon degradation and cycling. Kim et al. (2003)[73] developed the use of van Krevelen diagrams[98] (plots of H:C ratios on the *y*-axis versus O:C ratios on the *x*-axis, Figure 2) for elemental formulas identified by FT-ICR-MS in order

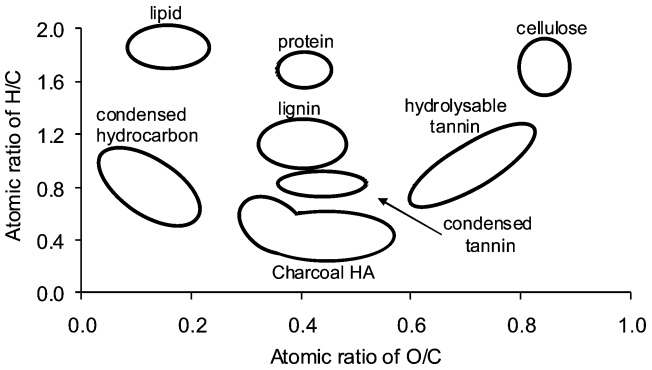

Figure 2. The van Krevelen diagram of major biopolymer components, adapted from Hockaday et al., 2006.[101]

to separate compounds in a DOM sample on the basis of their elemental ratios. Using this approach, complicated mass spectra can be visualized in two ways: (1) as possible reaction pathways and (2) as qualitative analyses of major classes of compounds that comprise the spectra. Figure 2 shows the positions in which major biomolecular components occur on a van Krevelen diagram.[73,99−101] Additionally, the van Krevelen diagram was expanded to a 3D plot by either incorporating peak intensities or relative peak intensities on a *z*-axis or using contour plots (e.g., Figure 3).[73] The 3D van Krevelen diagram allows for an estimation of the relative abundance of structurally related compounds and can also be useful for discerning compositional differences among samples. Later, Kim et al. (2006)[102] applied similar van Krevelen analyses to elemental formulas of DOM components to reveal that hydrogen-deficient molecules with low H:C ratios (assigned to black carbon-derived molecules) are

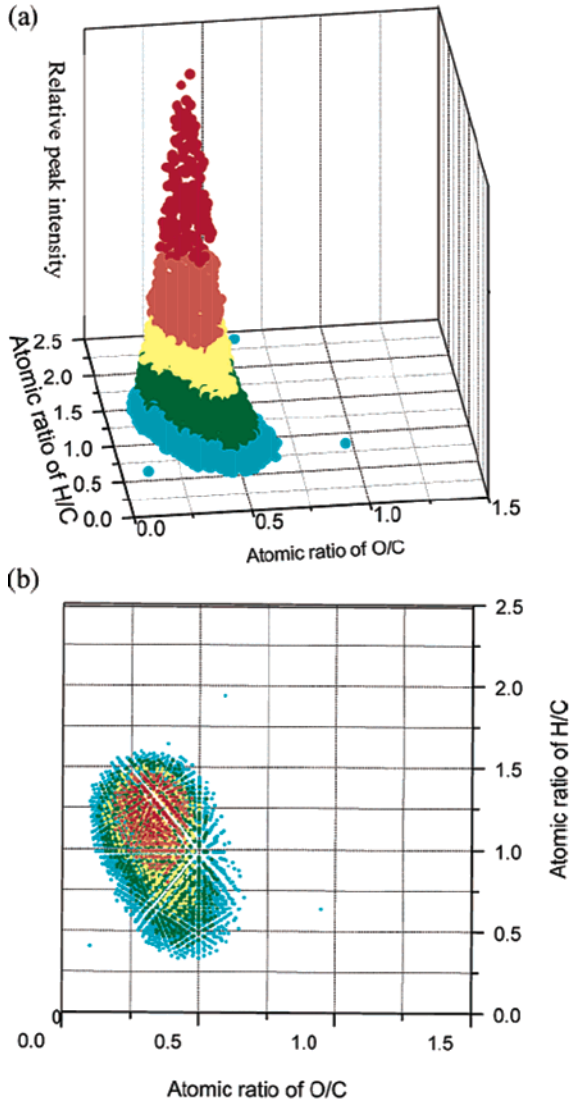

Figure 3. 3D display of the van Krevelen plot (a) of the peak intensities and elemental data obtained from the ultra-high-resolution mass spectrum of McDonalds Branch DOM and plan view (b). Colors of points were varied according to relative peak intensities. The intensities increase in the order blue, green, yellow, orange, and red. (Adapted with permission from Kim et al., 2003[74].)

present and generally not metabolized in temperate and tropical streams and therefore may be transported and persist in the oceans.

Other approaches to data mining in order to extract DOM compositional information can be envisioned. For example, one can combine van Krevelen analysis with Kendrick mass analysis[73,96] to examine formulas that constitute part of a homologous series within a narrow range of the van Krevelen plot. This approach allows one to extract formulas from a region of the diagram assigned to specific groups of compounds (e.g., lignin, lipids, black carbon, etc.) and to query the edited dataset for elemental relationships. Another possible data mining strategy is to employ discriminant or principal component analyses, as applied to pyrolysis/mass spectrometry data for marine particulate organic matter by Minor and Eglinton (1999).[103] This type of approach could potentially be used to identify groups of elemental compositions within FT-ICR-MS sample datasets that belong to specific DOM source end-members.

2.5. Application of FT-ICR-MS to Marine and Freshwater DOM

The only reported FT-ICR-MS characterizations of marine DOM have been conducted using 7 T FT-ICR-MS with an ESI source.[76,77] Koch et al. (2005)[76] analyzed DOM isolated by solid-phase extraction from two contrasting aquatic environments: autochthonous marine samples isolated from the waters of the Weddell Sea, Antarctica, and a terrestrially dominated sample isolated from the pore waters of a tropical mangrove sediment sample in northern Brazil. Hertkorn et al. (2006)[77] analyzed two samples of ultrafiltered DOM (>1 kDa; UDOM) isolated from the surface (2 m) and deep (4000 m) waters of the Pacific Ocean. Both UDOM isolates had been extensively characterized using more conventional methods, providing supportive supplemental data. For example, the C:N ratios of the surface and deep samples were 16.1 and 18.4, respectively, and their stable carbon isotopic signatures were −21.4 and −21.8‰, indicating a predominantly marine source for both samples.[104] These UDOM samples had also been analyzed for amino sugars,[105] neutral sugars,[106,107] and hydrolyzable amino acids.[106] Both publications regarding FT-ICR-MS characterizations of marine DOM[76,77] report a number of findings, which we summarize below and use, together with examples from the non-marine literature, to highlight the current capabilities of FT-ICR-MS, where future advances are likely to come, and how we should proceed in order to utilize this powerful technique to its fullest.

Both refs 76 and 77 report hundreds to thousands of resolvable peaks between 200 and 1000 *m*/*z*, indicating that the average MW of marine DOM is not on the order of thousands of Da, which is a particularly incongruous finding in the case of Hertkorn et al. (2006),[77] given that their UDOM protocol should greatly favor the isolation of compounds exceeding 1 kDa. As for non-marine DOM, all resolved ions appeared to be singularly charged. This conclusion was based upon the occurrence a separation of ~1.003 Da between $^{12}C_n$ and $^{12}C_{n-1}-^{13}C_1$ forms of the same molecule[95,108] and the finding that humic substances do not readily accommodate multiple charges.[69] However, polyacrylic acid, when used as a humic acid proxy, has been shown both to fragment and to obtain multiple charges during ESI.[109] Although further study is required to clarify the charge state of DOM following ESI, the current consensus is that DOM molecules are predominantly singularly charged, and thus all *m*/*z* values represented unique molecular ions, as opposed to species with variable, multi-charges.[69,76,77,97,105] In addition, each nominal mass region showed more than one peak, usually >20 peaks. The latter finding implies that there are thousands of peaks in the spectra and that each peak represents either at least one compound with a unique elemental formula (as afforded by the high mass accuracy) or, more likely, numerous compounds having the same elemental formulas but different structures.

Koch et al. (2005)[76] used C-18 SPE to extract the DOM in their samples prior to analysis by FT-ICR-MS. This concentration procedure probably resulted in artifacts. For example, C-18 SPE is known to be biased against hydrophilic analytes, as SPE selectively extracts the hydrophobic constituents (i.e., those that are hydrophobic at all pH values as well as those that become hydrophobic by ion suppression at low pH; see section 4). Artifact-prone sample isolation and concentration steps may be avoided by advances in instrument sensitivity. For example, a 12 T FT-ICR-MS instrument has sufficient sensitivity to obviate the need for preconcentrating DOM in terrestrial waters.[74] FT-ICR mass spectra have been successfully obtained by direct infusion of a Lake Drummond (Great Dismal Swamp, VA) DOM-rich water sample into the ESI source (P. G. Hatcher, unpublished results). Tests are currently underway to determine the viability of analyzing whole estuarine and coastal seawater samples by direct infusion into the source. Direct analysis of seawater samples is facilitated by the use of APPI, as it is moderately tolerant of salts (section 2.3).

Irrespective of whether their MS data accurately reflect the original in situ macromolecular composition, the studies of Koch et al. (2005)[76] and Hertkorn et al. (2006)[77] provide important new insights into the composition and structure of marine DOM. Specifically, the overwhelming majority of resolved peaks could be assigned unique elemental formulas, allowing the authors to make observations about the likely chemical structures present. For instance, both groups report a mass spacing pattern of 14.0156 Da for all samples, consistent with previous ESI FT-ICR-MS studies of riverine DOM isolates[69,75] and more recent work with DOM isolates from both lakes and rivers.[75] This mass difference corresponds to $-CH_2-$ groups, indicating that a major fraction of the DOM pool is made up of a series of compounds with homologous chemical backbones but varying numbers of $-CH_2$ groups, simplifying somewhat the task of compound identification. In addition to these series, Hertkorn et al. (2006)[77] identified a series of mass spacing patterns separated by 2.0157 Da, related to variations in DBE/H_2, 1.0034 Da, the mass difference between ^{13}C and ^{12}C, and 0.0364, due to exchange of CH_4 versus oxygen.

One of the main advances made by Hertkorn et al. (2006)[77] was their identification of a major component of UDOM (DOM isolated by ultrafiltration using cutoff 1 kDa), i.e., a class of compounds they term refractory carboxylic-rich alicyclic molecules (CRAM). This breakthrough was achieved through the use of both FT-ICR-MS and multidimensional NMR. Based on their NMR results, CRAM is composed mainly of carboxylic acids. Previously, Kim et al. (2003)[73] had pointed out that the application of Kendrick mass defect analyses was not limited to CH_2 units but could be used for any fragment. Thus, Hertkorn et al. (2006)[77] applied Kendrick mass defect analysis to the two prominent building blocks of CRAM, CH_2 and CO_2, and identified 156 series by CH_2-based analysis, but only 4 series by CO_2-based analysis. The apparent lower prevalence of the CO_2-based series was mainly due to mass truncation compared to the CH_2 series, principally because of the greater mass spacing of CO_2 (44 Da) versus CH_2 (14 Da).

Interestingly, Koch et al. (2005)[76] observed a large degree of spectral similarity between the mass spectra of their marine and mangrove samples, which may be related to biases of the solid-phase extraction technique used (see above). Koch et al. (2005)[76] suggested that this similarity was due to either a major fraction of terrestrial DOM that persists in the ocean or the diagenesis of both marine and terrestrial DOM, resulting in the preservation of similar sets of survivor molecules that subsequently come to dominate their respective DOM pools. Insights into whether such recalcitrant molecules exist in the terrestrial DOM pool and survive to have an impact in the oceans will be gained with improved and detailed descriptions of the complex marine and terrestrial DOM pools and their diagenetic alterations. FT-ICR-MS and advanced NMR techniques (section 3) seem the most appropriate of the current analytical techniques to address this task.

3. NMR Applications in DOM Studies

3.1. Introduction to NMR Applications

Nuclear magnetic resonance (NMR) is undoubtedly the most widely used technique for structural characterization of molecules. Although originally used for small, relatively simple organic compounds, it has gained widespread popularity as a method for DOM characterization. NMR utilizes the magnetic properties of nuclei that possess a magnetic moment for detection of the chemical environment in the vicinity of these nuclei. When placed in the presence of a strong magnetic field (B_0), these nuclei orient themselves in the lowest possible energy configurations with respect to B_0 with a quantized energy difference between the two orientation states (aligned and opposed to the field). The lower energy state is slightly more populated, as determined by the Boltzmann equation. Application of a radio frequency (RF) pulse for a short period (a few microseconds) excites the sample nuclei and inverts the spin population such that a slight excess of spins is in a higher energy state. Relaxation back to the equilibrium state involves a subsequent energy change and emission of electromagnetic radiation that is detected as a signal. As the excited nuclei of a varied population of different structural units relax, however, the excited nuclei on those diverse structural units exhibit slightly different resonances (measured as chemical shifts on the order of parts per million of frequency) due to (1) shielding by the local electron clouds, (2) deshielding by nearby functional groups, and (3) the proximity of other nuclei (coupling). Thus, investigation of the chemical structure of the sample occurs via nuclei "manipulation" without destroying it or changing its chemical nature.

When one-dimensional ^{1}H and ^{13}C NMR were first applied to DOM, NMR spectroscopists observed complicated spectra with broad and unresolved peaks due to the vast diversity of major functional groups present in DOM.[110] Additionally, high quality NMR spectra were difficult to obtain due to very low signal-to-noise ratios, especially for ^{13}C NMR because of the very low natural abundance of ^{13}C atoms in organic samples.

The correlation between NMR chemical shift ranges and structural composition can easily be made by comparison of unknown spectral data to those of published ^{1}H, ^{13}C, and ^{15}N NMR databases, many of which are available on the Internet. For DOM samples, there are several published works detailing chemical shift ranges for structural entities commonly found.[111−113] In these studies, the areas under the peaks corresponding to the various chemical shift ranges are integrated to obtain estimates of the relative contributions made by the corresponding functional group to the entire spectrum. Table 1 shows typical assignments for ^{13}C NMR spectra, and Figure 4 shows a typical marine DOM spectrum obtained as a solid by the technique of cross-polarization with magic angle spinning (CPMAS). Below, we discuss the various aspects of both liquids and solids NMR as applied to marine DOM and summarize what has been and continues to be accomplished with these approaches. We also include discussion of advanced NMR techniques, which offer a wealth of structural information to studies of marine DOM that has yet to be exploited.

Before launching into the wealth of new information that is obtainable with new NMR techniques, it is appropriate to summarize what has been learned about marine DOM from the traditional 1D techniques. The following are some of the important observations made for marine DOM:

1. It is now well recognized from ^{1}H NMR spectra of marine DOM that certain aliphatic functional groups are important constituents. Repeta and his group[114−116] have argued for some time that acetate functional groups associated with many amino sugars, with chemical shifts in the range of approximately1−2 ppm, are clearly major components of marine DOM.

2. Both ^{13}C and ^{1}H NMR spectra reveal that marine DOM is mostly aliphatic, attributed to its predominantly autochthonous source, and that aromatic functional groups are not significant contributors to structural entities.[117,118]

3. In the upper mixed layer of oceanic waters, complex polysaccharides are the most important components of HMW DOM (>1000 Da), as demonstrated by solid-state ^{13}C NMR spectra[118] and solution ^{1}H NMR spectra.[116] In deeper layers of the ocean, carbohydrates are minor, being replaced by predominantly aliphatic structures.

To date, only one-dimensional NMR techniques have been used in studies of marine DOM. As the latter is a complex mixture of thousands of compounds consisting of freshly released to highly degraded/altered biomolecules, difficult peak quantification and spectral interpretational problems (i.e., peak assignment errors) have arisen in past studies due to overlapping peaks from different functionalities and peak broadening. Consequently, one-dimensional NMR spectra based on ^{13}C CP/MAS techniques cannot be considered quantitative, as assumed in many past studies of marine DOM. These problems are confounded by small sample size

Table 1. Chemical Shift Regions Used for Integration of Peak Areas in ^{13}C NMR Spectra of NOM and Their Respective Assignments (after Dria et al., 2002[112])

integration regions (ppm)	identity
0−45	paraffinic carbons from lipids and biopolymers
45−60	methoxyl, mainly from lignin, and amino groups
60−90	carbohydrate carbons
90−120	carbohydrate anomeric and proton-substituted aromatic carbons
120−140	carbon-substituted aromatic carbons, mainly from lignin and non-hydrolyzable tannins
140−160	oxygen-substituted aromatic carbons, mainly from lignin and hydrolyzable tannins
160−190	carboxyl and aliphatic amide carbons from degraded lignin and fatty acids
190−220	aldehyde and ketone carbons

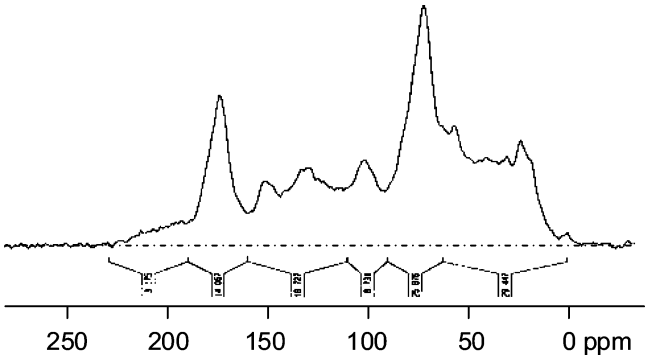

Figure 4. CPMAS ^{13}C NMR spectrum of marine DOM showing the integration values (in % normalized to the total of 100% C) for the various spectral regions. The spectrum is of ultrafiltered DOM (> ~1 kDa) from the Gulf of Mexico. (Courtesy of Tom Bianchi, Texas A&M University.)

and the presence of paramagnetic species. To address the latter problem, prior to NMR analysis of marine DOM samples, paramagnetic metals are usually extracted by ion exchange followed by neutralization and evaporation to dryness. However, these demetalation steps may significantly alter the original structures and, thereby, contribute to the difficulties with peak quantification and assignments. NMR specificity can be greatly improved by use of spectral-editing and multidimensional NMR techniques, which have been available for years but not commonly used by marine chemists.

3.2. Solid-State NMR

3.2.1. Background to Solid-State NMR

In the mid-1970s, significant strides were made in NMR spectroscopy for characterizing materials that are insoluble in typical NMR solvents. Notably, the cross-polarization (CP) technique, which was developed by Pines et al. (1972, 1973)[119,120] to facilitate rapid detection of dilute spins (e.g., ^{13}C, ^{15}N, ^{29}Si, and others), was combined with magic angle spinning (MAS). The latter technique minimizes chemical shift anisotropy, while dipolar couplings are removed by high-power decoupling.[121] In MAS NMR, the sample is placed into a rotor that spins at the "magic angle" of 54.7° to the magnetic field direction. CPMAS NMR has allowed geochemists to routinely analyze natural organic matter (NOM), including DOM, to obtain important information about the relative proportion of various constituent functional groups and structural entities. The reader is referred to numerous review articles[113,122] and textbooks[123,124] for background and details of the CPMAS technique and its applicability to NOM. Recent reviews by Cook (2004)[125] and Cardoza et al. (2004)[126] give comprehensive evaluations of the various solid-state NMR spectroscopic approaches used to date, including CPMAS as well as newer, advanced approaches for obtaining solid-state NMR spectra of NOM, some of which provide much more information than CPMAS alone. In this current review, we discuss many of the modern approaches, even though some have yet to be applied to marine DOM but potentially could be applied.

Marine DOM must be isolated from its salt matrix (including bound paramagnetic trace metals) and prepared as a solid (usually by freeze-drying) prior to analysis by solid-state NMR. Thus, given that one often can choose between solids and liquids NMR techniques, one may intuitively believe that liquids NMR, discussed below, would be a better choice mainly because DOM is soluble and lends itself to modern 2D and 3D techniques, as well as to ^{1}H NMR spectroscopy. However, there are some important advantages of solids NMR over liquids NMR, and these have traditionally provided the incentive for utilizing solids NMR preferentially. First, there is a 4-fold gain in sensitivity in solids CPMAS NMR, which is derived from a distortion of the spin populations when CP is employed. Second, the sample is placed inside the receiver coils of the NMR probe at its highest concentration, i.e., as a solid. NMR spectroscopy is inherently an insensitive technique; therefore, it is usually necessary to maximize the amount of sample placed in the NMR probe. In liquids NMR, the intensity of the detected signal is dependent on the amount of DOM that can be dissolved into approximately 1 mL of solvent in a 5 mm i.d. NMR tube. However, dissolving too much DOM usually results in aggregation, which can lower the signal-to-noise ratio and spectral resolution because aggregates change the spin dynamics of soluble molecules and affect spin−spin interactions.[74] Third, there is less sample handling in solids NMR, and one need not worry about solvent effects that may alter chemical shifts, introduce new peaks (i.e., solvent peaks), or eliminate peaks (e.g., loss of peaks from exchangeable ^{1}H).

However, there are also disadvantages to solid-state NMR. One is that the spin−lattice relaxation process, T_1, which determines the delay time between spectral acquisitions, is on the order of seconds to minutes, compared with tenths of seconds in liquids. Typical spectral runs of DOM often require greater than 10 000 or more scans for signal-averaging needed to achieve adequate signal-to-noise ratios; therefore, the longer delay time for solids NMR can result in acquisition times of days to weeks for one spectrum! These long run times are particularly problematic for the more quantitative direct polarization technique (DPMAS), where long inter-scan delays (usually minutes) and MAS are used, but a standard 90° RF pulse is employed rather than CP.[125] The CPMAS technique largely overcomes this problem, but at the cost of quantitative reliability.[125,127−129] Another disadvantage is the fact that the instrumentation for solids NMR is not standard, in that it requires special NMR probes, high power decouplers, and high signal generation power levels.

In deciding whether to utilize liquids over solids NMR for DOM, a careful evaluation is needed to determine which approach is more appropriate, which requires consideration of many criteria. Of course, if one has both liquids and solids NMR capabilities available, it is advisable to try both to determine which one is more appropriate.

In our experience, institutions having both capabilities often have both on the highest magnetic field instrumentation in the NMR facility (usually >12 T or 500 MHz). However, this high field presents problems for solids NMR of DOM. Whether one uses CPMAS, DPMAS, or other approaches, the sample is housed in a rotor spinning rapidly at the magic angle. High rotor spinning frequencies are needed to remove spinning sidebands that are derived from chemical shift anisotropy of sample powders and can interfere (i.e., overlap) with analyte spectral peaks. For a 500 MHz spectrometer analyzing for ^{13}C, a rotor spinning frequency of about 25−30 kHz is needed, which is currently unavailable. However, at 400 MHz, a spinning speed of approximately 20 kHz is now routine on newer spectrometers. It is important to collapse spinning sidebands into the main peak because they represent part of the signal associated with the isotropic chemical shift line. If the signal is redistributed to other parts of the spectrum, they can cause large errors in measuring the intensities of the isotropic signals of other functionalities that give signals in the same region as the spinning sidebands.

The most common example of this is the spinning sidebands for aromatic carbons (~130 ppm) which often overlap the aliphatic carbon region of the spectra (0−50 ppm). Thus, it is inappropriate to utilize a high-field instrument, e.g., 400 MHz, with slow spinning probes, as was recently reported by Sannigrahi et al. (2005).[130] Under these conditions, one can expect that structural components of the DOM that display a high degree of anisotropy (e.g., aromatic and carboxyl/carbonyl/ketone resonances) will not be quantitatively represented because a significant portion of the signal will be spinning sidebands. Mao et al. (2000)[131] showed that aromatic and carboxyl carbons are systematically underrepresented, in part due to incorrect spinning speeds. Side-

band suppression techniques have been developed for overcoming these errors,[131,132] but these have yet to be applied to marine DOM. From a practical perspective, the optimum spectrometer frequency is about 200−300 MHz,[112,129] but few such spectrometers exist because instrument manufacturers tend to advocate higher field systems and also because of the general belief by researchers that higher field instruments are superior. If possible, an appropriately configured solids NMR spectrometer (e.g., 300 MHz and a high spinning frequency such as 13 kHz or faster) should be dedicated to DOM studies.

A difficulty can arise when attempting to overcome the spinning sideband problem by spinning at higher frequencies. This problem derives from the fact that the Hartmann−Hahn matching during cross-polarization is affected by the rotor frequency.[133] With slow spinning, this problem is not significant, but at spinner frequencies above about 10 kHz, the matching condition is significantly modulated, resulting in different structures behaving differently in their matching characteristics.[125] To overcome this problem, Metz et al. (1994)[134] proposed a ramp CP approach, which we[112] and others[135] have adopted. This ramp CP variation was utilized for studies of riverine and marine DOM.[136−138] Dria et al. (2002)[112] demonstrated that spectra virtually identical to those obtained at slow spinning and lower magnet field strengths could be obtained at higher magnetic fields (300 MHz spectrometer frequency) and high spinning frequencies (13 kHz).

3.2.2. Application of Solid-State CPMAS ^{13}C NMR Technique to Marine DOM

Soon after its introduction,[139] the CPMAS technique for obtaining solids ^{13}C NMR spectra was applied to NOM from terrestrial and marine sources (see Wilson, 1987,[123] where much of the early literature is reviewed). The first application to marine DOM was on DOM samples isolated by XAD resins and compared the spectrum with those of soluble exudates of marine algal cultures processed in a similar manner.[140] The marine DOM spectrum was dominated by aliphatic carbon signals and contained little aromaticity. Malcolm (1990)[141] examined another marine DOM sample processed similarly and observed the same dominance of aliphatic polymethylene signals, which, he pointed out, is a feature characteristic of many riverine samples. Hedges et al. (1992)[117] examined DOM from Amazonian rivers, isolated by XAD resins, and compared these NMR spectra to those of oceanic DOM isolated similarly from waters of the east equatorial and north central Pacific. They concluded that the low aromaticity of marine DOM, together with its stable isotopic signature, was inconsistent with the notion that it could be derived from riverine DOM. This work laid the foundation for examining DOM from waters of the north Pacific (station ALOHA), isolated by tangential flow ultrafiltration (~ >1 kDa), which recovers significantly more of the DOM pool than XAD resins[118] and also, very likely, a different pool of organic matter (see section 4). In this study, solid-state CPMAS ^{13}C NMR showed that DOM underwent substantial molecular transformation on going from surface to deep waters. Polysaccharides were found to be the main bio-reactive DOM components in surface waters but were lost preferentially at greater depth, presumably by microbial utilization. More recent CPMAS ^{13}C NMR studies of DOM and particulate organic matter (POM), also isolated by ultrafiltration (~ >1 kDa) from the same area of the north Pacific, showed similar depth trends in DOM composition.[130] In contrast, POM did not follow this trend of decreasing carbohydrate content with depth, which agrees with the results obtained by Baldock et al. (2004)[142] from the same area using the same NMR approach. Baldock et al. (2004)[142] introduced a modeling approach to deconvolute the CPMAS ^{13}C NMR spectra into component biochemical constituents. Using this technique, they could estimate the amounts of carbohydrates, proteins, lignin, black carbon, and lipids in the sample. It should be pointed out that this model assumes that the defined constituents are the sole contributors to the spectral areas, which is a problem if overlapping, unknown components are present or if spinning sidebands interfere with spectral lines, as was the case in the work by Sannigrahi et al. (2005).[130]

Van Heemst (2000)[136] used CPMAS ^{13}C NMR spectroscopy to examine the changes in the composition of ultrafiltered terrestrial DOM as it transited through the Ems−Dollart Estuary in The Netherlands. Interestingly, little change was observed in the NMR spectra over a wide salinity range, which was interpreted to mean that the input of fresh material to the estuarine DOM pool was small, and that the DOM was mainly old and refractory and, thus, largely unaffected by estuarine processes (e.g., microbial degradation). Engelhaupt and Bianchi (2001)[143] examined the composition of HMW DOC in a tidal stream by CPMAS ^{13}C NMR. The authors concluded that the dominant sources of HMW DOC were terrestrial plant leachate and soil organic matter introduced during flooding events.[143]

Much of the early work that used CPMAS ^{13}C NMR focused on terrestrial DOM and soil extracts,[123] where spin dynamics and quantification of the technique were thoroughly evaluated. These studies showed that the technique is approximately quantitative for humic and fulvic acid soil extracts if samples are carefully demetallated prior to analysis. Recent studies confirmed that thorough removal of inorganic species from soil samples and extracts significantly improves quantification.[129,144]

Cook (2004)[125] reviewed and evaluated all published laboratory-based studies showing conditions required for obtaining quantitative signals in CPMAS NMR spectroscopy. In contrast to past terrestrial DOM studies, little effort has been devoted to performing the necessary and laborious NMR experiments needed to verify quantitative behavior for marine DOM. To establish quantitative behavior, one must conduct spin-counting experiments by adding measured amounts of pure standards, obtain spectra using DPMAS, where large recycled delay times of more than 60 s are used between acquisitions, and conduct variable contact time experiments.[125] Moreover, one is faced with the enormous task of isolating DOM from tens to hundreds of liters of seawater to recover sufficient amounts, about 25−50 mg, needed for obtaining an adequate signal-to-noise ratio for CPMAS ^{13}C NMR spectra within a reasonable run time (e.g., several hours). It should be pointed out that the approach selected to isolate marine DOM, e.g., tangential flow ultrafiltration, C_{18} reverse-phase extraction, XAD, or another method, may generally bias the NMR spectra obtained.[137,145] Problems related to DOM isolation techniques are discussed in section 4.

3.2.3. Solid-State ^{15}N and ^{31}P NMR of Marine DOM

Only a few marine applications of nuclei other than ^{13}C have been reported for solids NMR. Both ^{15}N and ^{31}P nuclei

impose significant analytical constraints when analyzed by the CPMAS technique. These constraints are similar to those discussed above for ^{13}C, but more pronounced for ^{15}N, as it is much less abundant than ^{13}C, comprising 0.36% of N isotopes. In addition, its main resonance frequency is rather low, further adding to its low sensitivity. NOM applications of ^{15}N NMR evolved mainly from studies by Heike Knicker as part of her Ph.D. dissertation,[146] and numerous papers have been published since then, particularly in studies related to the incorporation and cycling of N in soils,[112] marine sediments,[147] marine POM,[148] and coals.[149] McCarthy et al. (1997)[150] used the natural abundance ^{15}N NMR to demonstrate that marine ultrafiltered DOM (> ~1000 Da) from surface and deep samples in the north Pacific Ocean exhibited NMR spectra primarily comprised of one broad peak in the region of the spectrum that is normally assigned to peptidic N. Because hydrolyzable amino acids were found to constitute only a minor fraction of their DOM samples, the authors suggested that most of this nitrogen exists as a nonhydrolyzable peptidic N, perhaps associated with chitin or peptidoglycans from bacteria. The lack of additional published studies in this area is undoubtedly due to the fact that an enormous effort must be expended to yield very noisy spectra containing only one broad peak.

Like ^{15}N, few studies applying ^{31}P solids NMR have been reported for marine DOM. Clark et al. (1998)[148] examined HMW marine DOM from the north Pacific Ocean and found signals corresponding to phosphate esters and phosphonate functionalities. The NMR peaks were broad, and spinning sidebands were prevalent due to the strong chemical shift anisotropy for phosphorus compounds. Dissolved HMW phosphorus in seawater was isolated by tangential flow ultrafiltration and characterized by solid-state ^{31}P NMR spectroscopy.[151,152] A prominent peak for orthophosphate esters was observed in addition to a minor peak for phosphonates.[151,152] For additional information, we refer the reader to an extensive review on the application of ^{31}P NMR in characterizing phosphorus in environmental samples.[153]

3.2.4. Advanced Solid-State NMR Techniques

Recently, a wide variety of powerful spectral-editing techniques for solid-state NMR have been adapted and developed for probing molecular connectivity and functional group identification for humic substances.[154] These techniques are based on application of spin quantum mechanics to selectively excite and detect functional groups such as CH, CH_2, alkyl OCHO, alkyl OCO, CN, C in fused aromatic rings, and nonprotonated C.[155−157] To date, there are a multitude of these techniques in use by the Schmidt-Rohr (Iowa State University) group, an example of which is saturation pulse induced dipolar exchange with recoupling (SPIDER). In this technique, ^{14}N saturation pulses create recoupling and partial dephasing of $^{14}N-^{13}C$ dipolar coupling for selective detection of ^{14}N bonded to ^{13}C at normal MAS spinning speeds (~5 kHz) and relatively low RF frequencies. Thus, with this technique, one can determine the nature of structures to which N is bonded. For example, Schmidt-Rohr et al. (2004)[154] were able to determine that humic acids in rice-cropping agricultural systems contained N bonded directly to aromatic ring carbons.

In solid-state CPMAS ^{13}C NMR, the fact that the through-space $^{1}H-^{13}C$ dipolar couplings (as opposed to through-bond couplings) are essential for a strong signal intensity greatly facilitates the selective detection of nonprotonated carbons, which is achieved using a technique called dipolar dephasing.[125,126] In this technique, one turns off the high-power decoupler for a fixed portion of the pulse cycle, during which only protonated carbons, or those that have strong dipolar couplings between ^{1}H and ^{13}C, lose their signal intensity. One can remove from the spectrum the signals for protonated carbons and, consequently, edit the spectrum to select for only nonprotonated or methyl carbons. Methyl groups, though protonated, are observed because their rapid rotation diminishes their dipolar $^{1}H-^{13}C$ interaction. Examples of functional groups in NOM that Schmidt-Rohr and others have observed by spectral editing using dipolar dephasing, alone or in combination with other structure-selective NMR techniques, include (1) quaternary alkyl carbons bonded to one oxygen atom, (2) nonprotonated aromatic carbons resonating near 100 ppm, (3) ketones and quinones, (4) several types of nonprotonated aromatic carbons bonded to nitrogen, (5) carboxybenzenes, and (6) phenolic OH hydrogen-bonded to carboxybenzene groups.

These powerful, advanced solids NMR techniques have yet to be applied to marine DOM, even though they can now be implemented by appropriately trained operators using modern commercial spectrometers. We illustrate the potential of these techniques for studies of marine DOM by showing results of unpublished work by Klaus Schmidt-Rohr and Jingdong Mao (Iowa State University), who collaborated with Dianne McKnight and Rose Cory (University of Colorado) to obtain a series of "edited" solid-state ^{13}C NMR spectra for DOM from Pony Lake, Antarctica, as shown in Figure 5.

Spectrum a in Figure 5 was obtained by DPMAS with high-speed spinning, in which the acquisition conditions were verified to be quantitative and the signals were determined to represent all the carbon structures in the sample. Spectrum b was obtained by DPMAS and recoupled dipolar dephasing, which shows only the nonprotonated carbons or mobile CH_n groups, mostly methyl groups. Spectrum c was obtained by DPMAS but was further manipulated by filtering, which removes signals from all but the quaternary carbons, methyl groups, and mobile methylene groups. The dipolar dephasing technique was applied previously to marine DOM.[117] Spectrum d shows only quaternary aliphatic structures obtained by a chemical shift filtering technique.[159] In spectrum e, a chemical-shift-filtered, short CP pulse sequence was used to selectively quantify aliphatic CH_n structures. Spectrum f was obtained using a short CP technique combined with subtraction of a dipolar dephasing spectrum,[157] which selectively detects all CH_n groups, while spectrum g, obtained using a DPMAS experiment with a short recycle delay, shows only CH_2 (i.e., methylene) groups. Spectrum h shows only those structures bonded to one H, obtained via a three-spin coherence technique.[158] Finally, spectrum i was obtained by a distortionless enhanced polarization transfer (DEPT) technique, which detects OCH and NCH carbons very clearly.

From this example, it is apparent that much new information about marine DOM is potentially obtainable by implementing spin-editing techniques. Unfortunately, due to the sophistication of these filtering techniques, marine geochemists who wish to apply them to DOM analysis will need to await training on these new approaches by the few NMR spectroscopists who are currently fluent in them. However, this added complication begs the question, "Do I really need

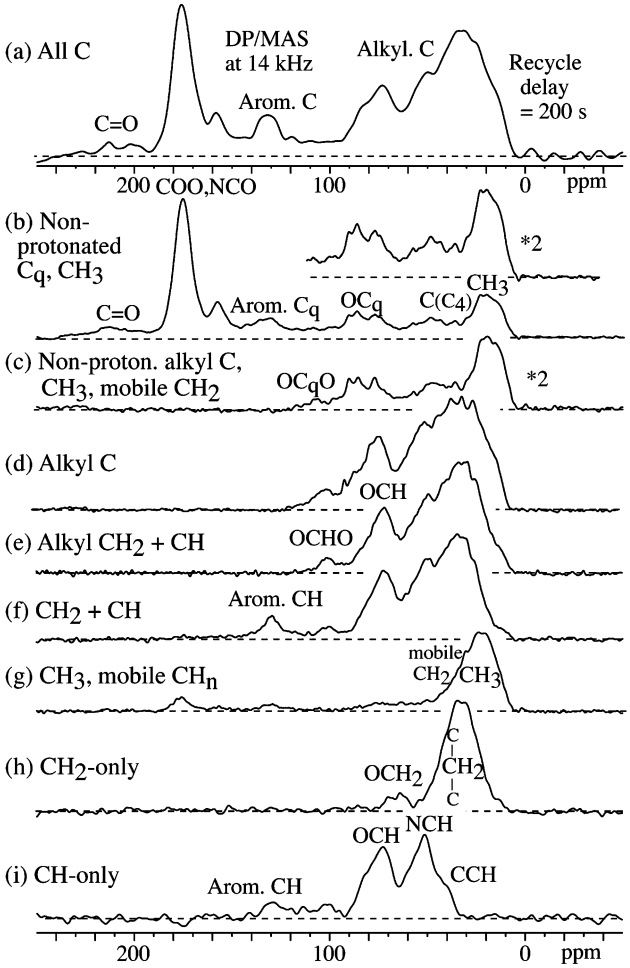

Figure 5. Solid-state NMR spectral editing applied to a fulvic acid from a coastal lake in Antarctica. (a) ^{13}C direct polarization (DP/MAS) at 14 kHz, with a recycle delay of 200 s. (b) DPMAS spectrum with a 70 μs recoupled dipolar dephasing. (c) Spectrum obtained by the same pulse sequence as (b) but including a chemical shift filter. (d) Chemical-shift-filtered CPMAS spectrum. (e) Chemical-shift-filtered spectrum, with short CP. (f) Short CP minus short CP after dipolar dephasing. (g) DPMAS spectrum with a 1.5 s recycle delay. (h) Three-spin-coherence selection at 5.8 kHz MAS. (i) Dipolar DEPT at 4 kHz MAS. The weak aliphatic signals are mostly due to slow motion during the relaxation delay, not from C−N segments. C_q refers to quaternary carbons. (Figure kindly provided by Rose M. Cory and Dianne McKnight, University of Colorado, and Jingdong Mao and Klaus Schmidt-Rohr, Iowa State University.)

these advanced techniques to gain the information I want from DOM?" The answer lies in the level of information desired. If one is simply examining carbon aromaticity, or some average structural parameter and its variations with respect to sources or level of transformation, then simple 1D spectra may be all that are warranted, especially considering the level of sophistication and time required to obtain spin-edited spectra. However, if one is delving into detailed structural changes in DOM brought about by diagenetic alterations or short-term photochemical/biochemical alterations, then spin-editing methods can be well worth the effort to implement. For example, Mao and Schmidt-Rohr (2004)[159] and Kramer et al. (2004)[96] showed that spin-editing techniques provide estimates of condensed aromatic carbon content of humic substances, or black carbon. This information can be used to evaluate the black carbon content of marine DOM, providing a complementary technique to FT-ICR-MS.[75] As another example, Schmidt-Rohr et al. (2004)[154] showed that spin-editing methods can provide estimates of the extent to which N is directly bonded to aromatic rings in sedimentary humic acids. Thus, this approach can be used to study pathways of N sequestration into DOM, perhaps explaining how N-containing molecules such as peptides can be incorporated into DOM and preserved from biodegradation. Finally, spin-editing techniques allow for positive peak identification, thereby avoiding errors in peak assignments due to potentially overlapping signals. For example, in past NMR studies of marine DOM,[115,116] several peaks were erroneously assigned to lipids.[157] From just these few examples, it is clear that, if we are to gain knowledge of the manner in which specific DOM functional groups are bonded or altered within DOM as it undergoes transformation in the water column and sediment, then we will need to expend the effort to obtain spin-edited spectra.

3.3. Liquid-State NMR

3.3.1. One-Dimensional Liquid-State NMR Studies of Marine DOM

While ^{13}C nuclei are usually preferred over ^{1}H nuclei for direct observation due to their greater spectral dispersion, the low natural abundance of ^{13}C (1.1%) usually prevents suitable detection in liquid-state NMR. High sample concentrations and isotopic enrichment are often used to enhance the sensitivity of ^{13}C; these approaches are generally not applicable to natural organic matter (see discussion of CPMAS solid-state NMR in sections 3.2.1 and 3.2.2 for additional details). To date, the ^{1}H nucleus (~100% natural abundance) has been the mostly widely used probe for liquid-state NMR studies of DOM.

Early investigations of the structure and composition of marine and terrestrial fulvic acids were conducted using continuous-wave (CW) ^{1}H NMR.[110] In the CW technique, the radio frequency is varied at a constant magnetic field and resonances are measured sequentially. Unfortunately, the poor sensitivity of the CW technique produces NMR spectra that have low signal-to-noise ratios. Nevertheless, Stuermer and Payne (1976)[110] were able to glean important insights into the chemical nature of marine and terrestrial fulvic acids. The ^{1}H and ^{13}C NMR spectra showed that marine fulvic acids have a relatively high abundance of aliphatic carbons (0−50 ppm), while terrestrial fulvic acids have a relatively high aromatic content. This study highlighted the potential of NMR in the study of marine DOM and the fundamental compositional differences between marine DOM and terrestrially derived DOM (i.e., microbial sources vs higher plant sources).

Substantial improvements in the NMR signal-to-noise ratio and sensitivity were achieved with the introduction of pulsed Fourier transform techniques, in which a broadband RF pulse is used to excite all nuclei simultaneously. The RF emissions from relaxing nuclei are then recorded in the time domain and transformed into the frequency domain to yield a spectrum that closely resembles the CW spectrum, but with significantly improved resolution and detection limit. Hatcher et al. (1980)[160] employed this technique to obtain liquid-state FT NMR spectra of marine humic acid isolated from sediments by alkali extraction followed by precipitation. This study revealed that marine humic acid possessed a higher proportion of aliphatic carbons than previously thought. In

the subsequent studies, marine fulvic acids that were characterized by pulsed ^{1}H NMR,[140,141,161,162] and the spectra supported the conclusions of earlier studies that marine DOM appeared to be much more aliphatic in structure than terrestrial DOM isolated by similar techniques. These studies demonstrated how advances in chemical and spectroscopic techniques could be applied to determine the origin of DOM.

In the aforementioned studies, DOM was isolated from natural waters using XAD-2 and XAD-7 resins, which are inefficient at extracting DOM, especially from marine waters (see section 4). Moreover, artifact peaks in NMR spectra may result from the chemical alteration of sample during XAD extraction and organic contamination from the resin itself. These artifacts are clearly observed as sharp peaks; however, their contributions to the total signal areas are usually minor.[137,145] In recent years, ultrafiltration has become the preferred extraction technique. For example, Aluwihare et al. (2002)[114] used ultrafiltration to isolate HMW DOM from Mid-Atlantic Bight samples, which were subsequently analyzed by liquid-state ^{1}H NMR on a 400 MHz instrument. The observed ^{1}H chemical shifts were consistent with large contributions by carbohydrates, acetate, and lipids. The authors proposed that these compounds were major components of macromolecular structures, referred to as acylated polysaccharides.[114,115] High-field (i.e., 500 MHz), one-dimensional proton and carbon NMR was later employed to characterize the bulk chemical structure of refractory DOM.[77] The higher sensitivity gained by using the more powerful magnet unveiled subtle variations in DOM composition that were previously indistinguishable on low-field instruments. Further discussion of this study is given in the two-dimensional techniques section below (section 3.3.2).

Chromophoric dissolved organic matter (CDOM) has been examined using liquid-state ^{1}H NMR to determine whether CDOM molecular composition can be linked to in situ biological production of chlorinated aromatic acids, which are known to be present in marine organisms.[116] Repeta et al. (2002)[116] first fractionated the CDOM by high-performance liquid chromatography (HPLC) and then analyzed those fractions by proton NMR, only to reveal that the resonance signals in the aromatic region (7−8 ppm) were very complex and essentially unresolved. The high degree of complexity in the aromatic region suggested that the dominant structural components of their CDOM fractions were poly-substituted aromatic compounds. The positions of protons directly bound to the aromatic rings (e.g., ortho, meta, para) were assessed by measuring NMR coupling constants of protons in close proximity to each other (i.e., a lack of coupling between two protons suggests that the protons are para to each other in the aromatic ring, whereas stronger coupling means that protons are at ortho or meta positions). Repeta et al. (2002)[116] were able to use their experimental coupling constants, together with the UV/vis spectra of the CDOM isolates, to conclude that 2,4-dichlorobenzoic acid and isomers of tetrachlorobiphenyl carboxylic acids were in significant concentrations and were believed to be metabolic byproducts of marine microorganisms.

Kovac et al. (2002)[163] used ^{1}H NMR spectroscopy to examine macroaggregation of organics (i.e., colloid and "gel" formation) and their degradation in seawater. Macroaggregate formation was facilitated by temporal increases in organic silica content, which most likely formed cross-linked structures with aliphatic moieties or esterification reactions with esters and amides. The authors suggested that silicon and carbohydrates stabilize aliphatic compounds that may, in turn, contribute to the seasonal persistence of macroaggregates in seawater. In contrast, when in situ macroaggregate formation was low, a decrease in polysaccharide content was observed, which they speculated was caused by microbial and photochemical degradation.[163]

Interpretation of ^{1}H NMR spectra of DOM can be limited, however, by moderately to strongly overlapping chemical shifts. For example, Mao et al.[157] pointed out that DOM peaks assigned to lipids in several past studies of DOM[115,116] were most likely due to methyl groups within polysaccharides. Peak assignments in ^{1}H NMR spectra can be particularly difficult if DOM is present at low concentrations (signal peaks will be indistinguishable from background noise) and if there is inadequate suppression of signal from the solvent (usually accomplished by solvent suppression pulse sequences). In those instances, researchers can only make generalized interpretations of DOM structure, as opposed to specific molecular assignments and quantitative integration of peak areas, even though modern high-field instrumentation (500−800 MHz) is capable of high resolution and sensitivity.[125] Two-dimensional NMR is able to disperse overlapping resonance signals into a second dimension, resulting in more distinct and interpretable chemical shifts that can often be related to specific structural components of DOM.[125,126]

3.3.2. Two-Dimensional Liquid-State NMR Studies of Marine DOM

Heteronuclear NMR is a powerful alternative to one-dimensional ^{1}H NMR. During a heteronuclear single quantum correlation (HSQC) experiment, magnetization is transferred from a highly abundant nucleus,^{1}H, to the inherently less sensitive and less abundant nuclide (e.g., ^{15}N or ^{13}C) that is directly attached to the proton. This transfer of energy results in spectral cross-peaks that intersect the chemical shifts of ^{1}H and the ^{13}C (or ^{15}N) nuclei to which it is bonded. Furthermore, sensitivity for the ^{13}C or ^{15}N is enhanced while producing an additional dimension in which changes in chemical environment can be dispersed and interpreted. Heteronuclear multiple bond correlation (HMBC) experiments are similar to HSQCs, but during HMBC, magnetization from the excited protons is transferred to the less sensitive nuclei that are two, three, or even four bonds away.

2D NMR has particular nuances that must be addressed. Very large molecules often have short transverse (T_2) relaxation times, often shorter than the data acquisition time, which may not allow them to be detected. Conversely, molecules with longer T_2 relaxation times are preferentially detected. When assigning chemical resonances to such spectra, it is commonplace to base the ranges of chemical shifts and signal intensities on those of model organic compounds that could potentially exist within the unknown sample. Simpson et al. (2004)[164] used American Chemistry Development (ACD) software to generate an NMR database of chemical shifts of model lignin compounds, which could then be cross-referenced to spectra of fulvic acids believed to be comprised, in part, of "lignin-like" compounds. When developing such chemical databases, it is important to consider solvent effects; i.e., the solvent employed in the analysis of the unknown should closely match that used for the model compounds, otherwise spectral mismatches (incorrect assignments) will result.

Despite the nuances and potential for misinterpretation of NMR data, inverse detection experiments (like HMBC,

where ^{13}C signals are detected through the ^{1}H's to which they are attached or in close proximity) have been successfully applied to identify the H−C bond connectivities in complex humic extracts of soils and sediments,[82,165−172] as well as riverine DOM.[137]

To date, there have been only a few studies that have applied 2D NMR techniques to marine DOM. Hertkorn et al. (2006)[77] used heteronuclear 2D NMR to characterize refractory marine DOM isolated by ultrafiltration. Based on chemical shifts, the $^{1}H-^{13}C$ HSQC spectra clearly showed molecular substructures within refractory DOM. Seven substructural peaks were defined, as shown in Figure 6, and

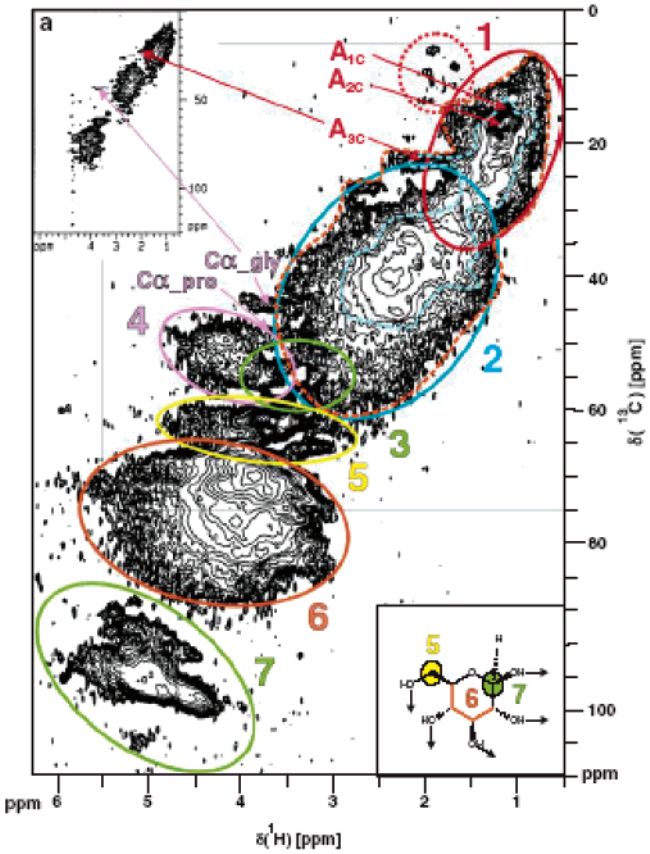

Figure 6. Two-dimensional NMR spectrum of refractory DOM. $^{1}H-^{13}C$ HSQC NMR spectrum of surface marine ultrafiltered DOM (i.e., UDOM). Seven groups of major constituents are shown, as discussed in the text. Top left (inset a): A_{1c-3c} represents branched aliphatic CH pairs. (Reprinted with permission from Hertkorn et al., 2006.[77] Copyright 2006 Elsevier.)

defined by the various numbered regions: (1) methyl bound to carbon and sulfur (dotted circle), (2) methylene and methine cross-peaks, (3) low-intensity methoxy cross-peaks, (4) dicarboxylic acids, (5) carbohydrate methylene cross-peaks, (6) carbohydrate methine cross-peaks, and (7) anomeric carbons in cyclic form. The chemical shift and intensity of cross-peaks in the 2D spectrum also allowed peptides and aliphatic polycarboxylic acid functionalities to be detected. In one-dimensional NMR, such specific assignments would be tentative, at best, due to strong peak overlap. By combining their heteronuclear 2D NMR results with results from another high-resolution technique, FT-ICR-MS (see section 2), for the same samples, Hertkorn et al. (2006)[77] concluded that a major fraction of refractory DOM consists of carboxylated and fused alicyclic rings, with few hydrogen atoms attached to double bonds. They called this material carboxyl-rich alicyclic molecules (CRAM).

3.3.3. Multidimensional (3D) NMR Techniques

Multidimensional NMR correlates the interactions of different nuclei along the *x*, *y*, and *z* dimensions. The *x* and *y* dimensions (or axes) usually consist of proton−proton couplings, e.g., $^{1}H-^{1}H$ total correlation spectroscopy (TOCSY), that are correlated to another nucleus in the *z* plane (e.g., $^{1}H-^{13}C$ HSQC). For clarification, we preface our discussion of multidimensional NMR techniques with a brief introduction to homonuclear NMR. Homonuclear 2D NMR experiments based on ^{1}H nuclei consist of a spectral diagonal that represents a one-dimensional spectrum of protons. Cross-peaks that do not fall along the diagonal indicate protons that are coupled to each other (i.e., coupling of two protons results in a displacement from the diagonal that is related to the extent of the coupling). For each cross-peak, a perpendicular line can be dropped to the *x* and *y* axes to determine the chemical shifts of the coupled protons. $^{1}H-^{1}H$ correlation spectroscopy (COSY) distinguishes protons that are interacting through one bond, while TOCSY identifies protons that are interacting within two to three bonds, depending on the spin system.

Homonuclear $^{1}H-^{1}H$ couplings can be used in combination with HSQC or HMBC correlations to generate a molecular map consisting of proton couplings and the carbon atoms to which they are connected. Although the "generic" H−C backbone of a molecule can be readily determined using this approach, connectivities cannot be mapped through heteroatoms, such as oxygen, because these disrupt the ^{1}H correlation continuity. Despite this drawback, heteronuclear techniques still provide important structural information about complex biochemical and biogeochemical samples. For example, Cook et al. (2003)[172] and Simpson et al. (2003)[169] used three-dimensional NMR, i.e., TOCSY heteronuclear multiple quantum correlation (HMQC) to characterize soil humic fractions. Simpson et al. (2003)[169] clearly showed that expanding NMR spectroscopy into a third dimension simplified a complicated spectrum by selective detection of spin-interacting structural components within chosen one-dimensional slices. Thus, one could examine a specific one-dimensional peak in a ^{1}H NMR spectrum and show a 2D spectrum of how the ^{1}H's and ^{13}C's coupled to that peak are themselves coupled to each other. Such information allows for detailed structural assignments to possible components within NOM.

The application of multidimensional NMR (e.g., TOCSY-HMQC) to DOM is still in its infancy. Widespread application of 3D NMR to studies of DOM is hindered by the time required to run those experiments, typically days of spectrometer time. Fortunately, cryogenic probes have been shown to be much more sensitive than ambient temperature probes. Cryogenic probes allow for multidimensional NMR spectra to be obtained much faster, on the order of hours rather than days.[169] Use of pulsed field gradients have significantly maximized sensitivity and further reduced the total analysis time. Gradient selection in either the *z*-axis (vertical) or in the *x*, *y*, and *z* axial directions significantly reduces t_1 noise associated with intense signals from solvents, as is commonly observed in multidimensional spectra.

Multidimensional NMR has proven to be effective for analyzing complex, heterogeneous samples, even without using supplemental instrumentation or other analytical

techniques.[126,169,172] If possible, assignments of chemical classes within NOM using multidimensional NMR techniques should be verified with other analytical techniques, spanning from simple wet chemical methods to sophisticated high-resolution FT-ICR-MS techniques. Multidimensional NMR methods, especially if used collaboratively with other high-resolution techniques, offer great promise in answering major unsolved questions regarding the composition and structure of marine DOM, as recently demonstrated by Hertkorn et al. (2006).[77]

As in the case for solids NMR (section 3.3), one must critically examine the need for implementing higher order sophistication in liquids NMR studies of DOM. The time and learning effort involved in the use of the 2D and 3D techniques are substantial when compared to 1D NMR. Typically, 1D spectra provide average measurements of functional group compositions for DOM (e.g., aromaticity, polysaccharide contents, CRAM content, etc.). Spectral overlap among the various structural groups often precludes absolute quantitative measurements and can lead to errors in peak assignments,[157] even at higher fields. For example, olefinic groups have resonances that are co-incident with those of aromatic groups.

If one is simply interested in assessing the general degree of unsaturation for DOM to discern source characteristics or general reactivity, then obtaining 1D spectra is usually sufficient. On the other hand, if detailed molecular information or structural subtleties are desired, then one must resort to 2D and 3D NMR techniques to tease out the required information in the form of cross-peaks. These techniques allow for the determination of structural connectivities for the various functional groups, and one can begin to visualize molecular structural constituents. For example, Simpson et al. (2003)[169] showed that various known structural units derived from cuticles, lignin, and lipids in humic substances could be identified by use of 3D liquids NMR. Used in combination with other advanced techniques such as FT-ICR-MS, discussed in section 2, the molecular makeup and sources of DOM can begin to be unraveled. Thus, studies that focus on the determination of the sources of DOM components or that build upon detailed structural changes brought about by biodegradation, photodegradation, or diagenesis of DOM would likely benefit significantly from incorporation of the advanced liquids NMR approaches. Another important justification for implementing 2D and 3D techniques is enhanced sensitivity. At present, 1D liquids ^{13}C or ^{15}N NMR spectroscopy is not practical due to poor sensitivity. However, using indirect detection methods, such as HSQC, in which ^{13}C or ^{15}N signals are detected via their interaction with ^{1}H's, significant gains in sensitivity are readily obtained. Thus, one can obtain spectra for these dilute spins in a few hours rather than days.

4. Isolation and Desalting of DOM Samples from Saline Waters

4.1. Introduction to Isolation and Desalting Techniques

In the previous sections, high-powered MS and NMR techniques, which have the potential to yield significant new insights into the composition and structure of marine DOM, were reviewed (or previewed). However, despite the great promise of these sophisticated techniques, the old adage still holds: "garbage in, garbage out". That is, if DOM cleanup, concentration, and extraction procedures result in a contaminated, altered, or strongly biased samples, then the validity or usefulness of the results will be, at best, questionable. In the case of FT-ICR-MS, due to its high sensitivity and low detection limit, it may be possible that a simple desalting step is all that is required to make a seawater sample amenable to analysis; furthermore, APPI sources may allow direct infusion of seawater without desalting. However, even with this high sensitivity, FT-ICR-MS would benefit from higher concentrations, as, at natural levels, trace molecules will likely fall below current detection limits (~100 ions).[64] For NMR, the level of sensitivity required to analyze marine DOM at in situ concentrations may never be reached, with current NMR techniques requiring about 5−500 mg of C, making extraction and preconcentration a necessity.

Even though isolation and concentration steps are critical for realizing the full potential of advanced instrumental techniques in the analysis of marine DOM, these steps are problematic due to the very high concentrations of inorganic salts (20−35 g L^{-1}) compared to the very low DOM concentrations (1−3 mg L^{-1}). Unlike soil and freshwater organic matter, there is neither a robust protocol for the quantitative isolation of marine DOM nor any commercially available marine reference sample with which to compare extraction efficacy or DOM characteristics of the isolate. An ideal isolation and concentration method should (1) recover all DOM, (2) produce a conserved (i.e., unbiased and uncontaminated) distribution of all solutes and chemical properties that existed in the original sample (i.e., minimize chemical or physical alteration of the sample), (3) be able to process very large volumes of water in minimal time, and (4) minimize the retention of inorganic salts.

Of the available concentration and isolation techniques, ultrafiltration (UF) and solid-phase extraction are currently the most commonly used for marine DOM. These methods typically yield only 10−30% of the total marine DOM, and thus the extracted DOM should not be regarded as representative, unless it can be shown otherwise. These extracts are biased toward that fraction of the DOM "targeted" by the physical and/or chemical interactions governing its extraction. Given the great importance of desalting, isolation, and extraction procedures, we conclude this review with a discussion of the current techniques, their limitations and suggestions for future approaches that appear promising.

4.2. Ultrafiltration

Boundaries within the size continuum of marine DOM are blurred.[173,174] For instance, Culler and McClellan (1976)[175] and Verdugo et al. (2004)[174] describe overlapping size distributions and reactivity of marine viruses, macromolecular organic assemblages or gels, colloidal organic matter, and "truly" dissolved organic matter that exist within the operationally defined dissolved size regime of $<0.1-0.2\ \mu$m. Nonetheless, the traditional and simpler view of DOM size classes persists, and many of the isolation and fractionation schemes for marine DOM are still based on size. The most prominent methods for isolating and fractionating marine DOM by size are tangential-flow and cross-flow UF, both of which yield high- and low-molecular weight fractions.[118] There are recognized problems with UF, including membrane fouling associated with sorption of organics and scaling by organic−divalent metal complexes[176−179] and variable rejection efficiencies of different commercially available mem-

branes.[180−185] Gustaffson et al. (1996)[186] discussed many of the factors that compromise UF membranes and the isolation of marine DOM by UF. Dai et al. (1998)[187] detailed stringent cleaning and operating protocols that address many of those factors.

To date, most seawater DOM studies have focused on the HMW fraction (operationally defined as organic material retained by a 1 kDa membrane). The HMW fraction contains a much smaller fraction of inorganic salts than the LMW fraction, which permeates through the 1 kDa membrane with the inorganic salts. Diafiltration or ion-exchange chromatography is typically used to remove residual inorganic ions from the HMW retentate; however, losses of 10−35% of HMW carbon have been reported after desalting.[118,188−190] Final recoveries of marine HMW DOM can range from 10 to 40% (25 ± 8%) based on DOC mass balances (Table 2).

Table 2. Percent of High-Molecular-Weight Organic Matter Isolated from Seawater (Salinity ≥20) by Ultrafiltration, Retained by 1 kDa Membranes

		% HMW			
sample	*n*	range	mean	S.D.	ref
Mid-Atlantic Bight	13	8−23	13.9	5.0	118
Mid-Atlantic Bight	7	29.0−32.8	31.5	1.5	*b*
Mid-Atlantic Bight	7	18.9−28.3	23.7	3.4	*c*
Gulf of Mexico[a]	17	13−45	28.8	8.0	193
Gulf of Mexico	10	23.7−34.5	28.7	3.4	*b*
Gulf of Mexico	4	21.1−32.3	27.1	5.7	34
Station Aloha	3	22−35	26.7	5.7	130
North Pacific	3	15−16	15.7	0.7	*d*
total seawater	64	8−45	25	8	
freshwater	68	14−99	66	24	191

[a] Final recovery after solid-phase extraction followed by ultrafiltration and diafiltration. [b] Santschi, P. H.; Guo, L. D.; Baskaran, M.; Trumbore, S.; Southon, J.; Bianchi, T. S.; Honeyman, B.; Cifuentes, L. *Geochim. Cosmochim. Acta* **1995**, *59*, 625. [c] Hernes, P. J.; Benner, R. *Mar. Chem.* **2006**, *100*, 66. [d] Midorikawa, T.; Tanoue, E. *Mar. Chem.* **1998**, *62*, 219.

In contrast, approximately 66 ± 24% of freshwater DOM is routinely retained by 1 kDa UF filters.[191] HMW marine DOM contains a high concentration of chromophores that strongly absorb at UV and visible wavelengths.[192] Spectroscopic detection of organic material in retentates and permeates overestimates the quantities of isolated DOC.[190]

Benner and Opsahl (2001)[193] reported marked decreases in the yield of HMW DOM with increasing salinity (from 0 to 36) in the Gulf of Mexico. This phenomenon was attributed to flocculation and salting-out of terrigenous-related, higher MW organic matter crossing through the mixing zone between freshwater and seawater environments.[193−195] Guo et al. (1994)[196] showed the same trend for the Bering Sea but at overall greater salinities (31−33 psu). Wheeler (1976)[192] and Guo and Santschi (1996)[189] suggested that retention of HMW DOM is significantly influenced by the concentration factor of the UF method, i.e., the % HMW DOM retained by 1 kDa membranes decreases with increasing concentration factor. The causes for this decreased retention are not clearly known, but one possible explanation is the loss of DOM due to increased sorption onto membrane surfaces or the UF system plumbing due to increased hydrophobic organic−organic interactions at greater DOM concentrations.

4.3. Nanofiltration and Reverse Osmosis

Nanofiltration and reverse osmosis (RO) have been widely used by environmental engineers for desalinization and potable water generation because these methods separate nearly all organic and inorganic solutes from natural waters.[197−199] Portable RO systems can rapidly concentrate the solutes in hundreds of liters of surface freshwater by 20−25-fold in 4−8 h, retaining 80 to >90% of the DOM.[201−204] Inorganic cations can be effectively removed from RO concentrates by cation exchange chromatography with H^+-saturated resins, leaving moderate concentrations of residual SO_4^{2-} and H_4SiO_4 (10−30% by weight) in the freeze-dried products.[201,205] Currently, RO cannot be used to concentrate marine DOM because of the very high inorganic salt concentrations. However, the development of high-throughput desalting front-end methodologies (e.g., electrodialysis, section 4.5.2) may facilitate the use of this technique for concentrating marine DOM.[206−208]

4.4. Sorption by Functionalized Solid Phases

Activated charcoal,[209,210] hydrophobic bonded-phase silica sorbants (or solid-phase extraction),[211] and Amberlite XAD resins[188] have been used to isolate DOM from acidified seawater, with XAD being the most widely used. In the late 1970s and early 1980s, standard protocols were developed for the isolation of freshwater humic substances using the XAD suite of resins (Rohm and Haas, Philadelphia, PA). Under these protocols, humic substances were operationally split into two fractions: (1) hydrophobic organic acids (HPOA, humic + fulvic acids) that sorb to XAD-2 and XAD-8[212−216] and (2) hydrophilic organic acids (HPIA) isolated using XAD-4 resin.[217,218] Elemental analysis, potentiometric titrations, and ^{13}C NMR spectroscopy show that HPIA have greater oxygen content, greater carboxyl concentrations, greater % alkyl, and lower % aromaticity than HPOA isolated from the same DOM sample.[205,219−221] Typical recoveries of freshwater HPOA and HPIA range from 19 to 90% (54 ± 14%) and from 5 to 51% (26 ± 13%), respectively, representing ~80% of the total DOC.[191] Shuman (1988)[222] and Town and Powell (1993)[223] have discussed in detail the potential biases of XAD methods.

Preparation and purification of functionalized solid phases for DOM extraction are time-consuming, and their use in DOM extraction necessitates that the DOM experiences major shifts in matrix pH, salinity, and polarity. Activated carbon and XAD resins must be thoroughly cleaned by Soxhlet extraction with sequences of organic solvents and rinsed multiple times with base and acid. C-18 (octadecyl bonded-silica) SPE media (i.e., cartridges and filters) are first activated by polar organic solvents (e.g., methanol or acetonitrile) and then rinsed with aqueous salt solutions. Samples of pre-filtered water (0.45 or 0.2 μm) must be acidified to pH ~2−2.5 before they are passed through the extraction columns. At low pH, the majority of carboxyl groups on DOM solutes are protonated, reducing their solubility in aqueous media and enhancing their sorption onto the hydrophobic surfaces. The sorbed hydrophobic DOM is then eluted by a strong base, such as 0.1 M NaOH, 1 M NH_4OH, or an alkaline methanol mixture. All three elution methods have artifacts. There are frequent reports of substantial contamination due to bleeding,[209,224,225] reaction of ammonium ion with DOM functional groups (e.g., carbonyls), and the inability to elute the strongly sorbed DOM from the sorbant surfaces.[210]

Reported recoveries of marine DOM (on a % carbon basis) using activated charcoal, SPE sorbants, and XAD resins are significantly smaller than those for freshwaters (Table 3).

Table 3. Recoveries of Seawater DOM (salinity ≥20) by Sorption to Functionalized Solid Phases

		% isolated			
sample	*n*	range	mean	S.D.	ref
Activated Charcoal					
Sargasso Sea and coastal Rhode Island	4	25−72	43.5	20.2	209
Atlantic Ocean	4	37−61	53.3	11.0	210
Amberlite XAD-2					
Sargasso Sea and coastal Massachussetts	5	3.7−22.5	8.1	8.0	*g*
Gulf of Mexico	7	6.8−87.7	35.0	27.6	161
Equatorial Pacific		5−15			225
Antarctic seawater[a]	1	17	17		229
Antarctic seawater[a,b]	3	37−56	44.3	10.2	229
Halifax Harbor	2	28.4−30.0	29.2	1.1	227
Amberlite XAD-8					
coast of Portugal[c]	2	51.2−60.2	55.9	5.6	228
coast of Portugal[c,d]	2	76.7−81.9	79.2	3.2	199
Adriatic Sea[e]	6	7.2−18.7	10.5	4.7	*h*
C-18 SPE					
Chesapeake Bay	2	38.4−39.1	38.8	0.4	190
Chesapeake Bay[f]	2	67−68	68	0.7	190

[a] Algal-derived humic substances doped with ^{14}C; detection by scintillation. [b] Tandem column extractions with XAD-2, XAD-7, and XAD-4. [c] Recovery based on UV detection at 250 nm. [d] Tandem column extractions with XAD-8 and XAD-4. [e] Surface-active substances and shallow marine. [f] Total recovery of HMW DOM retained on 1 kDa membrane + C-18 extracted LMW DOM. [g] Stuermer, D. H.; Harvey, G. R. *Deep-Sea Res.* **1977**, *24*, 303. [h] Vojvodic, V.; Cosovic, B. *Mar. Chem.* **1996**, *54*, 119.

Activated charcoal was able to recover ~10−15% more DOM than the XAD-2 or XAD-8 alone (Table 3). Fu and Pocklington (1983)[226] reported that XAD-2 and XAD-8 have similar efficiencies for isolating the HPOA fraction from seawater, although XAD-2 is preferred over XAD-8 for seawater because the latter tends to bleed and contaminate samples.[227] However, Little and Jacobus (1985)[224] reported substantial bleeding from XAD-2 resins as well. Removal of inorganic cations by cation exchange with H^+ did not enhance the sorption of marine DOM onto XAD-2 resin over acidification with HCl, i.e., 28% vs 30% sorption, respectively.[227] Esteves et al. (1995)[228] reported that 51−60% of chromophoric marine DOM (UV absorbance detection) could be isolated using XAD-8 alone, but that amount increased to 77−82% using tandem XAD-8 and XAD-4 columns. However, Lara and Thomas (1994)[229] recovered only ~44% of marine DOM from seawater samples using tandem XAD-2, XAD-4, and XAD-7 resins beds (in various orders), compared to 17% isolated by XAD-2 alone. C-18 SPE is somewhat more efficient at isolating marine DOM than XAD-2 resins (on a % carbon basis) and favors the sorption of most of the chromophoric DOM.[190,230] Fluorescence spectrophotometry and ^{13}C NMR characterization of C-18 and XAD-8 hydrophobic acid isolates from the same freshwaters revealed nearly identical chemical compositions.[145]

4.5. Desalting

The latest FT-ICR mass spectrometers (i.e., 9.4 T and higher) appear to be approaching the sensitivity required to analyze marine DOM at in situ concentrations, although this has yet to be empirically demonstrated. A major concern is the rapid "salting-out" of inorganic ions and inorganic−organic complexes when filtered marine DOM is mixed with organic modifiers (i.e., 50% acetonitrile) required for sample dispersion during ESI. If salts can be effectively removed from larger volume samples (e.g., tens to hundreds of liters), then the desalted sample can be concentrated using conventional methods for freshwater samples (e.g., freeze-drying, rotary evaporation, and reverse osmosis), producing minimally biased DOM samples at concentrations amenable to NMR and other, lower sensitivity techniques. Currently, there are two possible techniques which offer the potential to desalt DOM samples with minimal introduction of bias due to fractionation or contamination.

4.5.1. Desalting by Size-Exclusion Chromatography

Preparative size-exclusion chromatography (SEC) and gel permeation chromatography (GPC) have generally been used in research on humic substances as fractionation techniques rather than purification methods. Under ideal separation conditions, i.e., mobile phases with well-buffered pH values,[231] ionic strengths of >0.02,[232] and no hydrophobic solute−stationary phase interactions,[233,234] solutes with the largest sizes migrate through the column at the greatest rate, followed by solutes with progressively decreasing sizes,[235] with no solutes eluting after the permeation limit (i.e., the total permeation volume of the column). Only a few studies have used SEC to analyze marine and estuarine DOM.[53,236−238] Huber and Frimmel (1994, 1996),[236,237] using on-line TOC detection, observed that the retention time of DOM solutes (either whole seawater or HMW isolates) is a function of both DOM molecular size and structural subunits. Marine DOM constituents tend to elute in the order of (1) large organic colloids, (2) HMW carbohydrate-like molecules, (3) humic and fulvic acids, (4) LMW acids and phenols, (5) "salt trough", (6) LMW neutrals, and (7) amphiphilic molecules.[236,237] The exact boundaries between these divisions overlapping to some degree due to coelution.[236,237]

Inorganic salts, being relatively small in size compared to organic solutes, usually elute immediately before the total permeation volume of the column, with the exact timing recognizable by a "salt trough" that is detected as a very narrow spike in conductivity or a depression in UV absorbance.[231,238−240] Furthermore, as the salt trough is a semiconserved feature, it may be possible to use preparative SEC as an alterative method for desalting DOM samples. As only a minor fraction of the DOM coelutes with the salts (principally small, highly charged organic acids), the collection of the eluted mobile phase before and after the salt trough can recover >90% (~80% before the salt trough, ~10% after the salt trough, ~10% within the salt trough) of the initial DOM placed on the column (J. Ritchie, unpublished results).

The most practical use of preparative SEC would be for desalting reconstituted DOM samples (i.e., HMW ultrafiltrates and RO isolates) from freeze-dried powders rather than for whole, unconcentrated seawater. When scaling-up SEC to generate a large volume of desalted DOM solution, the main limitation is the sample injection volume. To avoid overloading the column, the injection volume should be kept below 4% of the total bed volume.[231] The size of the column and volume of SEC media needed to efficiently fractionate

enough marine DOM at its natural concentration would be cost- and time-prohibitive. Commercially available SEC media are expensive, on the order of thousands of dollars per 500−1000 cm^3. Hypothetically, 1 L of seawater at 83 μM DOC (1 mgC L^{-1}) would require ~25 L of stationary phase packed in an absurdly large column to process 2 mg of DOM (assuming that DOM is 50% carbon by weight). Furthermore, hundreds of SEC elutions would be needed to accumulate 100 mg or greater of sample for some instrumental analyses (e.g., solid-state ^{13}C NMR). In contrast, 20 mg of freeze-dried DOM from RO or UF isolation that is reconstituted with deionized water to 10−20 mL (500−1000 mg L^{-1}) could easily be processed on a 0.25−0.5 L preparative column.

4.5.2. Electrodialysis

Although in its infancy for freshwater and seawater samples, electrodialysis is currently being examined as a suitable desalting procedure prior to analysis or to concentration of DOM using RO.[206,208] During electrodialysis, chloride and strong cations (Na^+, Ca^{2+}, Mg^{2+}, Sr^{2+}, and K^+) are easily stripped from solution by accelerated dialysis through semipermeable ion-exchange membranes, driven by an electric field. Following electrodialysis, the low concentrations of residual cations (μmol L^{-1}) can be removed by simple cation-exchange chromatography, while the majority of excess chloride can be eliminated by freeze-drying due to its relatively high vapor pressure under vacuum. Over-dialyzing the sample to near zero concentration of salt, however, significantly increases the risk of loss of organic matter through increased sorption onto ion-exchanging dialysis membranes. Koprivnjak et al. (2006)[208] showed that 4−6 L samples of surface freshwater and salt marsh water require 8−12 h of processing to reduce background ion concentrations to <10% of their initial concentrations, while retaining 90% of the original DOC, with 6−10% of DOC sorbing onto the ion-exchange membranes that may be recovered later by washing the membranes with dilute base.

An important impetus for exploring electrodialysis as a front-end desalting method is for the analytical removal of sulfate.[208] Unlike chloride, sulfate is not volatile under vacuum and could induce destructive, acid-driven reactions with the organic matter during RO concentration and freeze-drying. Due to the relatively large size of the sulfate ion, the kinetics of sulfate exchange with electrodialysis membranes is significantly slower than for chloride ion. Therefore, complete sulfate removal requires prolonged electrodialysis with continual loading of NaCl to maintain a high-enough and constant specific conductance across the electrodialysis system to drive sulfate into the ion-exchange membranes. Once sulfate is removed, additional electrodialysis will remove the remaining NaCl and other salts to near-zero concentrations.

E. M. Perdue and co-workers at Georgia Institute of Technology (Atlanta, GA) have recently scaled-up their portable electrodialysis−RO system for shipboard work in coastal Atlantic waters and the Sargasso Sea (personal communication). Starting with 100−200 L of coastal and Sargasso Sea water, Perdue and co-workers were able to remove >99% of sea salt (starting salinities 30−35), followed by a 20−50-fold concentration by RO, to yield 5−10 L of concentrated marine DOM with 65−85% recovery (by carbon mass balance), with only micromolar concentrations of residual sea salt (E.M. Perdue, unpublished data).

The electrodialysis−RO technique has three main advantages over UF and chemical isolation methods. First, there is no manipulation of solution pH that could facilitate unintentional acid or alkali hydrolysis reactions. Second, the DOM never comes into contact with organic solvents or other eluting agents. Finally, size-fractionation and loss of DOM would be minimal relative to those observed for diafiltration of samples after concentration. Although electrodialysis is still under development, it may provide an effective way to initially desalt unconcentrated marine samples for direct analysis by FT-ICR-MS, and it also has the potential to desalt large volumes of sample, which can then be concentrated for NMR analyses.

5. Future Directions

5.1. Future Directions for Isolation of Marine DOM

Currently, membrane and functionalized solid sorbates can isolate <50% (typically only 10−30%) of the total organic carbon in seawater. Some studies have explored the possibility of combining methods to increase total DOM recoveries. Fu and Pocklington (1983)[226] were able to absorb 53−100% of DOM from estuarine and marine waters onto tandem columns of XAD-2 and activated carbon. However, the quantitative removal and recovery of the sorbed DOM from the XAD and activated carbon required several sequential extractions with weak base, methanol, and base/methanol solutions. Simjouw et al. (2005)[190] first isolated HMW DOM by UF (1 kDa) and then extracted the LMW permeate with C-18 SPE. By coupling these methods, they were able to recover 68% of the DOC from a Chesapeake Bay mouth sample (salinity ~20−30), approximately twice the recovery achieved with UF, XAD-2 or XAD-8, and C-18 SPE alone (Tables 2 and 3). It should be pointed out that the combination of C-18 and UF used by Simjouw et al. (2005)[190] may yield lower recoveries for true oceanic samples because the Chesapeake Bay DOM contained significant amounts of terrestrial carbon; however, this approach has yet to be examined for oceanic samples.

Future research may be directed toward analytical fractionation (and subsequent isolation) of marine DOM (similar to the XAD-8/XAD-4 fractionation scheme of Leenheer and Huffman, 1976[214]) using combinations of commercially available nonpolar (C18, C8, C2, and phenyl), weakly polar (cyano), weakly ionic (tertiary amine), and strongly ionic (quaternary amine, anion and cation exchange) functionalized bonded-silica sorbants. However, the most promising approach appears to be electrodialysis[208] as an initial desalting technique that can be coupled with established DOM concentration techniques, such as freeze-drying, rotary evaporation, and RO. Even though this technique is still in development for marine waters, electrodialysis coupled with RO holds the potential to isolate a minimum of 65−85% of marine DOM when optimized, without the artifacts associated with UF and solid sorbant methods.

5.2. Future Directions for FT-ICR-MS

Current FT-ICR-MS studies of marine DOM have been restricted, and perhaps biased, by two main factors, namely the need to concentrate and desalt the analyte and the selective ionization of the ESI source, thus skewing our analytical window. Future advances in FT-ICR-MS technol-

ogy will likely reduce the need for sample isolation as instruments become more sensitive, with increasing magnet strength[64] and improvements in the mechanical and electrical components comprising FT-ICR mass spectrometer. In addition, other novel MS technologies may prove more suitable to DOM analysis. However, other advances will still be required to negate the selectivity and matrix sensitivity of current ionization sources. The use of APPI instead of ESI appears to be a step in that direction. Future refinements, or the advent of new ionization approaches, will likely result in sources with much lower selectivity. Alternatively, although not ideally, biases may be removed by careful calibration, i.e., examining the ionization efficiency of a large group of model compounds whose structural makeup is similar to that of the types of molecules expected to comprise DOM.

5.3. Future Directions for NMR

While the use of 1D NMR spectroscopy has been commonplace for studies of DOM, the future directions for liquid-state NMR applications are clearly toward the multidimensional realm and, for solid-state MAS NMR applications, the use of advanced spectral-editing techniques, where one can identify specific functional groups on the basis of nuclear spin dynamics. From our perspective, the major advances that need to be made are in the area of spectral interpretation and familiarity with use of multidimensional and spectral-editing techniques. The expansive dataset of cross-peaks provided by the former NMR approach is quite daunting to those who only have experience with 1D spectra. Supplementing multidimensional solution-state NMR experiments with solid-state spectral editing, however, may be an advantageous approach to accurate spectral interpretation. Spin editing, for example, can greatly minimize peak overlap and detect chemical functionalities that may remain obscure in other experiments. Moreover, when deciding to implement a specific NMR technique, one should pay close attention to the research question at hand. If one is interested in obtaining detailed structural information caused by diagenetic alterations, then spin-editing and multidimensional solution-state experiments may be well worth the effort. If, on the other hand, one is concerned with characterizing a given structural parameter of DOM related to various sources, then simple 1D experiments may be all that are warranted.

Proper application of multidimensional liquids NMR requires familiarity with multidimensional databases of molecules that are likely to be similar to those existing in DOM. Moreover, the databases are often not adequate for nuclei other than carbon or hydrogen, and so, one must seek additional approaches to making structural assignments for these other nuclei. Ab initio calculations of chemical shifts for representative compounds likely to be found in DOM are possible, but these have yet to be applied for DOM studies. Another approach involves studies of biomacromolecules likely to contribute to DOM. These often show simpler NMR spectra that can often be interpreted more readily, providing chemical shift information on structural entities that can ultimately be a part of DOM. 3D NMR (i.e., HSQC-TOCSY) is highly useful in dispersing spectral data into a third dimension, a process that can potentially reduce peak overlap that would limit chemical shift assignments in a 2D spectrum.

Perhaps the greatest challenge to researchers not intimately familiar with NMR instrumentation is gaining access to time on an advanced NMR spectrometer and selecting the appropriate pulse sequence for the information desired, especially for solid-state NMR spectral-editing techniques. While 3D studies are highly useful in reducing spectral overlap by dispersing chemical shifts into a third dimension, these experiments require significant time that can be on the order of days. Certain multidimensional NMR experiments require pulse sequences that incorporate solvent suppression to minimize the overwhelming signal from water. In addition, sensitivity-enhanced pulse sequences may be required for samples that have low concentrations of analyte. Regardless of these challenges, multidimensional and spectral-editing NMR techniques offer great promise in the structural elucidation of complex biogeopolymers.

5.4. Future Directions: Conclusion

The main advances in DOM characterization are likely to come after the development of unbiased, non-altering methods for extracting most of the DOM (~75−100%) from seawater in sufficient quantities to permit analyses using multiple, complementary techniques, a current tidemark for marine science being the recent use of multidimensional NMR techniques together with FT-ICR-MS to characterize a major fraction of the HMW DOM pool.[77] The handling and interpretation of the voluminous and complicated datasets generated will require further innovation and borrowing from the bioscience community.[241] However, as these technologies develop, the coming decades will undoubtedly see major advances in the field of DOM characterization, yielding new and exciting insight into the composition and functioning of the marine DOM pool,[242] delivering us into a future of oceanographic research where we can, indeed, learn to read the information-rich molecular messages encoded in the DOM pool.

6. Abbreviations and Acronyms

ACD	American Chemistry Development
APPI	atmosphere pressure photoionization
COSY	correlation spectroscopy
CPMAS	cross-polarization with magic angle spinning
DBE	double bond equivalents
DEPT	distortionless enhanced polarization transfer
DPMAS	direct polarization magic angle spinning
ESI	electrospray ionization
FT-ICR-MS	Fourier transform ion cyclotron mass spectrometry
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
HMW	high-molecular-weight
HR-MS	high-resolution mass spectrometry
HSQC	heteronuclear single quantum correlation
LMW	low-molecular-weight
MALDI	matrix-assisted laser desorption/ionization
MAS NMR	magic angle spinning nuclear magnetic resonance
NMR	nuclear magnetic resonance
RF	radiofrequency
SEC	size exclusion chromatography
SPIDER	saturation pulse induced dipolar exchange with recoupling
TOCSY	total correlation spectroscopy
T_1	spin−lattice relaxation time
T_2	transverse relaxation time

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