1 **Spectroscopic characterization of oceanic dissolved organic**
2 **matter isolated by reverse osmosis coupled with electrodialysis**

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4 *John R. Helms^{1, 2*}, Jingdong Mao¹, Hongmei Chen^{1, 3}, E. Michael Perdue^{4, 5},*

5 *Nelson W. Green^{4, 5}, Patrick G. Hatcher¹, Kenneth Mopper^{1*} and Aron Stubbins^{6*}*

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¹ Department of Chemistry and Biochemistry, Old Dominion University, Norfolk, VA 23529

² Present address: Biology and Chemistry Department, Morningside College, Sioux City, IA 51106

³ Present address: Oak Ridge National Laboratory, Oak Ridge, TN 37831

⁴ School of Earth and Atmospheric Sciences, Georgia Institute of Technology, Atlanta, GA 30332

⁵ Present address: Department of Chemistry, Ball State university, Muncie, IN 47306

⁶ Skidaway Institute of Oceanography, Marine Sciences Department, University of Georgia, Savannah, GA 31411

* Corresponding Authors: Helms – Phone: 712-274-5412, Fax: 712-274-5101, email: helmsj@morningside.edu;

Mopper – Phone: 757-683-4094, Fax: 757-683-5310, email: kmopper@odu.edu;

Stubbins – Phone: 912-598-2320, Fax: 912-598-2310, email; aron.stubbins@skio.uga.edu.

8 **Abstract**

9 Oceanic dissolved organic matter (DOM) is one of the largest pools of reduced carbon on
10 Earth, yet DOM remains poorly chemically characterized. Studies to determine the
11 chemical nature of oceanic DOM have been impeded by the lack of efficient and non-
12 fractioning methods to recover oceanic DOM. Here, a DOM fraction (~40 to 86%
13 recovery) was isolated using reverse osmosis/electrodialysis (RO/ED) and analyzed by
14 solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Samples were obtained
15 from biogeochemically distinct environments: photobleached surface gyre, productive
16 coastal upwelling zone, oxygen minimum, North Atlantic Deep Water, and North Pacific
17 Deep Water. A ubiquitous ‘background’ refractory carbon pool was apparent throughout
18 the ocean and dominated in the deep Pacific Ocean. Advanced NMR spectral editing
19 revealed that condensed aromatic and quaternary anomeric carbons contribute to this
20 deep refractory DOC pool, the quaternary anomeric carbons being a newly identified and
21 potentially important component of bio-refractory carbohydrate-like carbon.
22 Additionally, our results support the multi-pool (e.g. 3-pool: labile, semi-labile, and
23 refractory) conceptual model of marine DOM biogeochemistry. Surface samples,
24 hypothesized to be enriched in labile and semi-labile DOM, were enriched in
25 carbohydrate-like material consistent with prior studies. High carboxyl signals in the deep
26 Pacific support the hypothesis that a major fraction of the refractory pool consists of
27 carboxyl-rich alicyclic molecules (CRAM).

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29 **Key Words:** Dissolved Organic Matter; Carbon Cycle; Nuclear Magnetic Resonance;
30 Reverse Osmosis-Electrodialysis (RO/ED).

Abbreviations

ALOHA, A Long-term Oligotrophic Habitat Assessment field site (22° 45' N, 158° W); CDOM, chromophoric (or colored) dissolved organic matter; CP, cross polarization; Cq, quaternary carbon(s); CSA, chemical shift anisotropy; DOC, dissolved organic carbon; DOM, dissolved organic matter; DP, direct polarization; ED, electro dialysis; HS, humic substances; ISOW, Iceland/Scotland overflow water; LCPW, lower circumpolar water; MAS, magic angle spinning; NADW, North Atlantic deep water; NMR, nuclear magnetic resonance; POM, particulate organic matter; ppm, NMR chemical shift frequency in parts per million; RO, reverse osmosis; SPE, solid phase extraction; SUVA, specific UV absorbance; TOSS, total suppression of spinning sidebands; UDOM, DOM retained by ultrafiltration; UF, ultrafiltration

1. Introduction

The oceans contain approximately 662 Pg-C of dissolved organic carbon (DOC) (Hansell et al., 2009), a pool on the same order as atmospheric CO₂ (860 Pg-C; Forster et al., 2007). Oxidation of 1% of the oceanic DOC pool would exceed the annual flux (ca. 2000) of anthropogenic CO₂ (Hedges, 2002). Despite its quantitative importance, only a small fraction of marine dissolved organic matter (DOM) has been identified (Repeta, 2015), hindering our understanding of DOM sources and cycling (Dittmar and Stubbins, 2014; Hedges, 2002). One impediment to the development of a comprehensive overview of ocean DOM composition has been the lack of efficient and non-fractionating methods of DOM isolation and salt removal (Mopper et al., 2007).

The two most widely used DOM isolation techniques are solid phase extraction (SPE) (Dittmar et al., 2008; Leenheer, 1981; Leenheer, 2009) and ultrafiltration (UF) (Benner et al., 1992; Swift, 1985). In both methods, a major fraction of ocean DOM is lost during processing resulting in DOC recoveries that range from 17-74% for SPE and 13-38% for UF (Benner et al., 1997; Hertkorn et al., 2013; Mopper et al., 2007; Stubbins et al., 2012b). In addition, as these methods isolate DOM based upon either an apparent size cutoff (UF) or chemical affinity for a resin (SPE), they have failed to produce a representative DOM isolate (Mopper et al., 2007). A system was recently developed that combines concentration of DOM by reverse osmosis and de-salting by electrodialysis (RO/ED) (Gurtler et al., 2008; Koprivnjak et al., 2006; Vetter et al., 2007). Further study has shown that when directly compared with SPE, RO/ED exhibits DOC yields that are typically higher and provides isolates that are more representative of the original organic matter (Green et al., 2014; Helms et al., 2013; Koprivnjak et al., 2009).

69 Mao et al. (2012) and Koprivnjak et al. (2009) applied solid-state CP/MAS ^{13}C
70 NMR and solution ^1H NMR to RO/ED isolates collected in the North Atlantic and
71 observed differences between DOM isolated from a regional transect across the Georgia
72 Bight to the Gulf Stream. The NMR spectra of RO/ED isolates appeared to represent an
73 intermediate of DOM isolated by UF and DOM isolated by XAD, suggesting that RO/ED
74 isolates a more representative fraction of the DOM than these methods. The latter
75 conclusion is also supported by the molar C/N ratios and absorption spectral slopes of
76 RO/ED isolated DOM being more consistent with the original seawater DOM (Green et
77 al., 2014; Helms et al., 2013; Koprivnjak et al., 2009).

78 Studies of SPE and UF DOM isolates have increased knowledge of the reactivity
79 and cycling of organics in the ocean. For example Hedges et al. (1992) used solid-state
80 cross polarization/magic angle spinning (CP/MAS) ^{13}C nuclear magnetic resonance
81 (NMR) spectroscopy to investigate the bulk structural characteristics of humic substances
82 (HS) extracted using XAD from two sites in the Pacific Ocean (DOC recovery ~10%),
83 while Benner et al. (1992) used CP/MAS NMR to characterize UF isolated DOM
84 (UDOM) from three depths in the North Pacific Ocean (DOC recoveries: 22-33%). DOM
85 isolated from the deep ocean contained more aromatic and/or olefinic carbon than surface
86 ocean DOM and deep-ocean UDOM was enriched in carbohydrate-like carbons and
87 depleted in unsubstituted alkyl carbons relative to deep-ocean HS (Benner et al., 1992;
88 Hedges et al., 1992). N. Pacific UDOM was enriched in carbohydrates (oligo- and poly-
89 saccharides) relative to deep and even mesopelagic UDOM (Benner et al., 1992), a trend
90 not seen for HS (Hedges et al., 1992). More recently, Hertkorn et al. (2013) analyzed
91 SPE-DOM from various depths in the South Atlantic Ocean (DOC recovery ~40%) using

NMR and mass spectrometry concluding that, in addition to the prevalence of aliphatic cyclic moieties among carboxylic rich molecules, there is 1) high diversity in aliphatic branching patterns, 2) progressive oxygenation of aromatic C and deoxygenation of aliphatic C in the ocean interior, 3) significant evidence for methylated carbohydrates and both pyranose and furanose sugar derivatives, and 4) evidence of a ubiquitous background DOM including carboxylic rich alicyclic molecules (CRAM) and thermogenic condensed aromatic carbon at all depths.

In addition to CP/MAS, complementary and improved solid-state ^{13}C NMR methods have been developed and applied to natural organic matter. Mao et al. (2011) demonstrated a systematic approach for characterizing humic substances by advanced solid-state NMR techniques. These techniques were used here to characterize the bulk chemical composition of five globally distributed RO/ED-DOM. Previous studies of RO/ED-DOM from specific oceanic regions explored local biogeochemical processing (Koprivnjak et al., 2009; Mao et al., 2012). The current study sampled carefully selected oceanic water masses within the Atlantic and Pacific Oceans that fall along the thermohaline circulation pathway and a global gradient in DOM quality. The RO/ED isolates analyzed here provide in-depth structural characterization of the RO/ED-DOM within productive upwelling zones, an oligotrophic tropical gyre, within an oxygen minimum, and within the deep waters of the North Atlantic and North Pacific Oceans.

2. Material and Methods

2.1. Sample collection and DOM isolation by RO/ED

114 Three Atlantic Ocean and two Pacific Ocean water samples were collected aboard
115 R/V Oceanus and R/V Kilo Moana, respectively. The samples include (i) surface water
116 from the Mauritanian upwelling (5 m; 20.161°N, 17.406°W), (ii) North Atlantic oxygen
117 minimum zone (415 m; 18.995° N, 21.487° W), (iii) North Atlantic deep water (3000 m;
118 18.485° N, 23.397° W), (iv) a surface sample from station ALOHA in the North Pacific
119 (5 m; 22.450° N, 158.00° W), and (v) a deep sample from station ALOHA (3500 m;
120 22.450° N, 158.00° W).

121 The Mauritanian upwelling is an area of high primary production and represents
122 waters impacted by fresh algal DOM enriched in labile and/or semi-labile DOM
123 (Carlson, 2002; Reinthaler et al., 2008; Teira et al., 2001). The 415 m North Atlantic
124 RO/ED-DOM from the oxygen minimum layer was expected to contain DOM that is
125 degraded during and after export to the ocean interior from surface waters or released
126 during the degradation of POM in the nearby shelf sediments (Carlson, 2002; Hansell,
127 2002; Hansell and Carlson, 2001). The 3000 m North Atlantic sample was representative
128 of the Iceland-Scotland overflow water (ISOW), which is a component of North Atlantic
129 Deep Water (NADW) (Hernes and Benner, 2006; LeBel et al., 2008). ISOW forms in the
130 sub-polar North Atlantic before sinking to depths of ~2500 to 3000 m and flowing
131 southward. The surface water sample collected at Station ALOHA (5 m North Pacific
132 sample) represents oligotrophic surface waters that cover the majority of Earth's surface
133 and is largely dominated by semi-labile (Carlson, 2002) and extensively photobleached
134 DOM (Kitidis et al., 2006; Nelson et al., 2010; Siegel et al., 2005). The 3500 m North
135 Pacific sample collected at Station ALOHA represents Lower Circumpolar Water
136 (LCPW) (Johnson and Toole, 1993), which exhibits old apparent ¹⁴C DOC ages (~6000

y) (Williams and Druffel, 1987), suggesting that DOM from this water mass consists largely of highly-degraded and refractory DOM that resists degradation over multiple ocean overturn cycles (Hedges et al., 1997).

Sample volumes ranging from 142 L to 473 L were collected using Niskin bottles (General Oceanics) from which they were sterile-filtered directly through pre-cleaned 0.1 μm capsule filters (Whatman, PolycapTM TC). The RO/ED method is described in detail elsewhere (Green et al., 2014; Vetter et al., 2007). Samples were desalted by ED using anion and cation exchange membranes (Selemion AMV/CMV, AGC, Japan) and concentrated by RO (FilmTec TW30-4021, Dow, USA). Samples were recirculated through both RO and ED systems until a sufficiently low conductivity ($<50 \mu\text{S cm}^{-1}$) and volume ($\sim 5 \text{ L}$) were obtained. Pacific Ocean samples were frozen shipboard and processed on Hawai'i within 9 days. All other sterile-filtered samples were refrigerated before starting RO/ED within 6 hours. Sample handling times ranged from 6.5 to 32.2 hours after which RO/ED isolates were frozen immediately. Each sample was freeze-dried (without further treatment) for analysis by solid-state NMR.

2.2. UV-visible spectroscopy and dissolved organic carbon analysis

Absorption spectra of chromophoric dissolved organic matter (CDOM) were collected for seawater and isolated samples using an Agilent 8453 diode array spectrophotometer with a 1 cm, 5 cm or 10 cm pathlength quartz cuvette (Helms et al., 2008). Data output from the spectrophotometer were in the form of dimensionless absorbance or optical density (OD) and were subsequently converted to the Napierian absorption coefficient, $a \text{ (m}^{-1}\text{)}$; Hu et al., 2002). Spectral slope (S) and slope ratio (S_R)

were calculated (Helms et al., 2013). DOC was measured as non-purgeable organic carbon using high-temperature catalytic combustion by high temperature combustion (720 °C) on a Shimadzu TOC-VCPH (Shimadzu Scientific Instruments; Chen et al., 2014). Specific UV absorbance (SUVA) was calculated by dividing decadic absorbance in units of m^{-1} by the DOC concentration in units of mg C L^{-1} (Weishaar et al., 2003).

2.3. Solid-state ^{13}C NMR

NMR experiments were performed using a 400 MHz (^1H) Bruker Avance II with a dual resonance probe. Samples were packed in 4 mm diameter zircon NMR tubes with Kel-F rotor caps (Bruker-Biospin). A detailed description of NMR methods is given in Supplementary Material. Briefly, semi-quantitative compositions of the RO/ED-DOM were investigated using ^{13}C cross polarization/total sideband suppression (CP/TOSS) NMR experiments (Bennet et al., 1995; Dixon, 1982). Signals from nonprotonated carbons or mobile carbons such as rotating CH_3 groups were isolated using 40 μs dipolar dephasing (Opella and Frey, 1979).

The signals of anomeric carbons (e.g., stereoisomeric carbons from cyclic sugars; O-C-O) were differentiated from those of aromatic and olefinic carbons using a five-pulse ^{13}C chemical-shift anisotropy (CSA) filter (Mao and Schmidt-Rohr, 2004) to selectively suppress the signals of sp^2 hybridized carbons. The CSA-filter technique was combined with dipolar dephasing to isolate signals from unprotonated and highly mobile aliphatic carbons and, with a short CP time (50 μs), to isolate protonated aliphatic carbons.

A CP/TOSS spectrum was recorded using a short-pulse CP of 50 μ s and a CP/TOSS spectrum was recorded using a short CP of 50 μ s and 40 μ s dipolar dephasing. The difference of the two spectra provided the spectrum of protonated carbons including immobile CH₂ and CH carbons, with a small CH₃ contribution (Mao et al., 2007).

High-speed ¹³C direct polarization/magic angle spinning (DP/MAS) NMR and high-speed quantitative ¹³C DP/MAS NMR with recoupled dipolar dephasing provided quantitative structural information (Mao et al., 2000; Mao and Schmidt-Rohr, 2003).

To determine the analytical uncertainty, the NMR signal-to-noise ratio (S/N) was determined for each integration region. Based on the inverse relationship between S/N and relative standard deviation (RSD), the standard deviation of the integral was calculated (Skoog et al., 2006). Additional uncertainty arises from the assignment of chemical shift ranges used for integration and from the unrecovered fraction of DOM. The former was partly alleviated by the use of spectral editing to differentiate overlapping signals and by the consistent application of integration ranges. The uncertainty resulting from incomplete DOM recovery was not considered in our interpretations, based on the assumption that RO/ED isolation provides DOM samples that are intercomparable (Koprivnjak et al., 2009; Vetter et al., 2007) and the finding that DOC recoveries of nine replicate samples of deep oceanic DOM collected at the Natural Energy Laboratory of Hawaii Authority (NELHA; 674m) with the RO/ED system used in this study led to consistent DOC recoveries of $68 \pm 2\%$ (Green et al., 2014; Table 1).

3. Results

3.1 Bulk organic and inorganic properties of RO/ED isolates.

Initial seawater sample DOC concentrations were 39 to 88 $\mu\text{mol L}^{-1}$ and were concentrated by RO/ED to 1076 to 4298 $\mu\text{mol L}^{-1}$ (Table 1; Chen et al., 2014a). DOC recoveries for the RO/ED process ranged from 40 to 68% in the North Atlantic Ocean samples (3 samples average = 51%) and 83 to 86% in the Pacific Ocean samples (2 samples average = 84%; Chen et al., 2014a). The lowest recovery (40%) was obtained on the first sample that was processed by a new RO/ED system (the 3000 m North Atlantic sample) and was likely due to the system not being adequately conditioned prior to use, as opposed to the DOM in this sample having anomalous extraction behavior (Chen et al., 2014a). Because its spectroscopic properties appeared to be consistent with the other samples, it was kept as part of this study. With the exception of this sample, DOC average recoveries (71%) are consistent with the results of Koprivnjak et al. (2009) (69%).

CDOM was concentrated effectively along with the bulk DOM (Table 1; Supplementary Material). Generally, the RO/ED isolates exhibited slightly steeper S and higher S_R and SUVA values than the initial seawater samples; however, the 415 m North Atlantic and 5 m North Pacific isolates had slightly lower SUVA values. RO/ED isolates showed lower absorbance in the 190-230 nm region compared to the original samples (Helms et al., 2013) indicative of the removal of dissolved nitrate, nitrite, and bromide.

Electrodialysis cannot remove uncharged inorganic solutes such as B(OH)_3 and Si(OH)_4 , which were co-concentrated with the DOM. Inductively coupled plasma measurements indicated that the inorganic fraction of the freeze-dried powders contained primarily sodium tetraborate (~75 to 82%) and silica (~0.3 to 17%), the latter increasing with sample depth. Consequently, the freeze-dried isolates from deep ocean samples

contained less organic carbon as a fraction of total solids than the surface ocean samples. The 5 m North Atlantic and 5 m North Pacific freeze-dried powders contained 22% and 17% organic carbon respectively. The 415 m North Atlantic freeze-dried powder contained 15% organic carbon, while the 3000 m North Atlantic freeze-dried powder contained 8% organic carbon and the 3500 m North Pacific freeze-dried powder contained 7% organic carbon.

3.2 Comparison of direct and cross polarization NMR spectra

Direct polarization (DP/MAS) ^{13}C NMR provides quantitative data for carbon content but is time intensive. Cross polarization (CP/TOSS) NMR spectra are quicker to obtain (~5 hours per spectrum) but rely on indirect polarization of carbon atoms.

Comparison of DP/MAS ^{13}C NMR and CP/TOSS NMR spectra obtained for the surface North Atlantic (Mauritanian upwelling zone) sample reveal that CP/TOSS NMR peak area integrations are semi-quantitative, having lower signal for aromatic carbons and carboxyl carbons than DP NMR spectra (Fig. 1). Due to excessively long run times required for DP NMR (≥ 73 hours per spectrum for these samples) and the moderate differences between DP and CP NMR spectra, the compositional differences between samples were explored using CP NMR (Mao et al., 2002).

3.3 NMR signatures of RO/ED isolates

With the exception of the deep Pacific sample, the solid-state CP/TOSS ^{13}C NMR spectra for the RO/ED-DOM isolates are markedly similar (Fig. 2; Table 2). All five samples contained very little aromatic material (90-160 ppm), but rather a mixture of

substituted (50-120 ppm) and un-substituted (0-50 ppm) aliphatic carbons as well as a significant contribution from carboxyl carbons (acids, esters and amides; 160-190 ppm). All samples showed strong carbohydrate-like signals (60-90 ppm C-O and 90-120 ppm O-C-O) though signals in carbohydrate-like regions were weaker for the 3500 m North Pacific Ocean sample. The 3500 m North Pacific and 415 m North Atlantic samples expressed the strongest carboxyl signals.

The 90-115 ppm region of the CP/TOSS reference spectrum represents the combined contributions of anomeric and some aromatic carbons, while the CSA-filtered spectra exclude aromatic signals. Anomeric carbons clearly dominated this region (Fig. 3 “Aliphatic C”). Comparing the CSA-filtered/dipolar-dephased spectrum and the CSA-filtered spectrum with short-pulse CP showed that the majority of anomeric moieties were protonated (Fig. 3). The signal from the 90-115 ppm region of the CSA-filtered/dipolar dephased spectrum, (O-C_q-O; quaternary anomeric carbon; Supplementary Material), was least abundant in the surface Pacific and most abundant in the deep Pacific sample. The 90-165 ppm regions of the spectra labeled “Non-protonated C” and “Protonated carbons only” in Fig. 3, corrected for the contribution from anomeric carbons, estimated relative contributions from protonated and non-protonated aromatic carbons (Table 3). This spectral region includes signals from substituted ring and substituted olefinic carbons, plus carbons in the interior of condensed aromatic structures, such as black carbon. The Mauritanian upwelling sample (5 m North Atlantic) showed the highest percentage of carbohydrate-like carbons (~75 ppm and ~100ppm) and the lowest percentage of non-protonated aromatic carbons (Tables 3 and 4). The surface sample from station ALOHA (5 m North Pacific) was similar to the Mauritanian

upwelling zone sample, but with slightly lower carbohydrate-like carbon and slightly higher aromatic and/or olefinic carbon. The alkyl carbon signals of the two surface samples (0-50 ppm) were also similar (Tables 3 and 4). The ISOW (3000 m North Atlantic) DOM contained the lowest percentage of carboxyl carbon, while the 3500 m North Pacific DOM contained the lowest percentage of carbohydrate-like material (but highest percentage of quaternary anomeric carbons), lowest percentage of protonated aromatic carbon, and highest percentages of non-protonated carbon and carboxyl carbon (Table 3).

4. Discussion

4.1. Characterization of RO/ED-DOM by solid-state ^{13}C NMR

In general, the DOM recoveries were higher for RO/ED than those reported for other isolation techniques such as ultrafiltration or XAD-SPE, which typically recover ~10-30% of oceanic DOC (Benner et al., 1992; Dittmar et al., 2008; Hertkorn et al., 2013; Mopper et al., 2007) and isolated a higher proportion and an apparently more representative fraction of oceanic DOM than is routinely isolated using PPL-SPE (Dittmar et al., 2008; Green et al., 2014; Hertkorn et al., 2013). For example, in the only large scale, direct comparison of the techniques, PPL-SPE recovered 61% of deep ocean DOM, whereas RO/ED recovered 68% from the same water mass, with an additional 14% of DOC recovered in a sodium hydroxide rinse of the RO/ED system (Green et al., 2014).

The most abundant inorganic materials in the isolates were silica and sodium tetraborate. The large difference between residual inorganic solids in the deep and surface

seawater samples was mainly due to silica, which follows typical nutrient distributions in the oceanic water column, with very low surface ocean concentrations and elevated concentrations due to remineralization at depth (Chester, 1990). Beyond their dilution of ^1H and ^{13}C , silica and borate are not expected to cause significant artifacts in the CP/TOSS NMR spectra (Tao et al., 2000).

DP/MAS ^{13}C NMR (Fig. 1) provided spectra that were more quantitative than CP/TOSS (Mao et al., 2011). However, time constraints meant that DP/MAS spectra were not obtained for all samples. CP/TOSS was moderately biased against carboxylic moieties (165-190 ppm) and aromatic carbons (100-165 ppm), which are thus underestimated by ~15% and 11% respectively in Tables 3 and 4.

Compared to surface samples, DOM isolated from the deep North Pacific and North Atlantic oxygen minimum zone showed lower abundance of O-alkyl carbons (60-120 ppm) and higher abundance of carboxylic functionality (160-190 ppm; Fig. 3; Table 2). These two samples exhibited the greatest contribution from quaternary alkyl carbons (50-100 ppm; Fig. 3) and the weakest anomeric carbon signals (90-115 ppm; Fig. 3). The surface samples, which were expected to contain the largest fraction of freshly produced algal DOM, showed the highest content of carbohydrate-like material, more protonated carbons, and a lower content of carboxyl carbons than the deep ocean samples (Figs. 1 and 3; Tables 3 and 4). This trend is consistent with ultrahigh resolution MS data for the same samples, which indicated that surface samples had higher average H/C ratios than deeper samples (Chen et al., 2014a).

4.2. Comparison with previously published results

Aromatic carbon (NMR-based) in surface waters typically vary from ~25% of organic carbon in DOM-rich freshwater (RO isolated Suwannee River NOM; Perdue and Ritchie, 2003) to 10% of organic carbon in coastal seawater (isolated by RO/ED; Koprivnjak et al., 2009; Mao et al., 2012) and 4-5% in open ocean surface water (Table 4). NMR data (Table 4) show that deep Pacific UDOM is similar to the RO/ED-DOM (using the average of the two different deep Pacific UDOM results), while surface Pacific UDOM is more enriched in O-alkyl groups and depleted in unsubstituted alkyl groups (Benner et al., 1992; Sannigrahi et al., 2005) compared to RO/ED-DOM, suggesting that UF preferentially isolates HMW carbohydrate-like material. Isolation of DOM from acidified seawater with XAD-2 resin (Hedges et al., 1992) recovers a fraction of marine DOM that is enriched in alkyl, aromatic, and carboxyl carbons, and depleted in O-alkyl carbons relative to comparable RO/ED isolates (Table 4) and thus appears to preferentially extract the LMW humic-like fraction from the marine DOM pool (Koprivnjak et al., 2009; Mopper et al., 2007; Stubbins et al., 2008).

Hertkorn et al. (2006, 2013) showed evidence of a refractory DOM component referred to as carboxylic rich alicyclic molecules (CRAM) based on characterization of UDOM and SPE-DOM samples using 2D NMR and ultra-high resolution MS. While the one-dimensional NMR results presented here cannot confirm these specific DOM structures, the abundance of carboxylic functional groups as a fraction of DOC is highest in the deep Pacific sample, supporting the findings of Hertkorn et al. (2006). Our identification of quaternary anomeric moieties is consistent with the recent identification of α/β -furanose sugars in open ocean SPE-DOM (Hertkorn et al., 2013).

4.3. Compositional and bulk structural changes to oceanic DOC

An aged background refractory DOM pool is hypothesized to circulate the oceans and is best represented by ~6,000 year old DOM at the end of the deep ocean conveyor belt deep in the North Pacific Ocean (Bauer, 2002; Druffel et al., 1989; Hansell and Carlson, 2001). DOM concentrations are elevated wherever newly produced DOM is added to the ocean and slowly degrade back to background levels with increasing journey time along the deep ocean conveyor belt. Correspondingly, the current suite of samples range from low-concentration, refractory DOM in the deep North Pacific Ocean (the end of the conveyor belt) to elevated DOC concentration due to additions of labile or semi-labile DOM in the highly productive upwelling waters off Mauritania (Table 1). Thus, the DOC in the RO/ED samples is assumed to have the following order of biological reactivity: 5 m North Atlantic > 5 m North Pacific > 415 m North Atlantic > 3000 m North Atlantic > 3500 m North Pacific (Carlson, 2002; Hansell and Carlson, 1998; Repeta, 2015).

In order to estimate the impact of diagenesis (i.e., long-term degradation) on the composition of DOM, several moieties quantified by solid-state ^{13}C NMR are shown in order of their estimated biogeochemical reactivities (Fig. 4). Alkyl carbons did not show a trend as a fraction of DOC (Fig. 4B) but, based upon DOC concentrations (Table 1), decreased in absolute concentration indicating that some alkyl carbons (aliphatic C) are part of the labile and/or semi-labile fraction of DOC. Carbohydrate-like compounds decreased as a fraction of DOC, presumably as DOM undergoes diagenesis within the ocean (Fig. 4A) in agreement with Benner et al. (1992; 1997) and Repeta (2015).

Chen et al. (2014a) report wet chemical hydrolyzed carbohydrate yields for the current sample suite. Estimates of hydrolyzed and NMR carbohydrate-carbon were correlated ($R^2 = 0.98$, $p = 0.0006$; Supplementary Material), although with a significant offset from zero on the NMR carbohydrate scale (22%). Although this discrepancy may have arisen from sugar monomers being degraded during hydrolysis or non-carbohydrate NMR signals that resonate in the 60-100 ppm range (McCarthy et al., 1996; Skoog and Benner, 1997), we suggest the intercept of the two carbohydrate analyses indicates the presence of difficult-to-hydrolyze carbohydrates, as suggested previously (Benner et al., 1992; Mao et al., 2010; Skoog and Benner, 1997). The chemical resistance of these difficult-to-hydrolyze carbohydrates may explain their recalcitrance in the deep ocean.

Quaternary anomeric (O-Cq-O) carbon distributions differed significantly from the overall carbohydrate pool (Table 3). Samples expected to contain the freshest algae-derived DOM had the lowest O-Cq-O percentages, while the oldest, most-degraded DOM samples were enriched in O-Cq-O. The overall O-alkyl signal decreases significantly with apparent age/degree of DOM degradation (Table 3; Fig. 4A) Thus, it appears likely that quaternary anomeric carbon (O-Cq-O) constitutes part of the refractory and/or non-hydrolyzable carbohydrate signature in the NMR data.

Quaternary anomeric carbons are present in some furanose sugars (Poulin and Lowary, 2010) and sugars that are functionalized at the anomeric carbon, e.g. keto-deoxyoctulosonate in bacterial lipopolysaccharides (Herschberger and Binkley, 1968; Strain and Armitage, 1985). Furanose-containing glycoconjugates are common constituents of bacterial cell walls, but furanose-containing sugars (e.g. fructose, sucrose), oligosaccharides and polysaccharides are also produced by plants, fungi, and

archaea (Poulin and Lowary, 2010). Thus, oceanic quaternary anomeric carbons may be produced by bacteria or released during microbial degradation of organic matter (Ogawa et al., 2001), or released/preserved during bacterial mortality (Nagata and Kirchman, 1999).

The above possibilities are consistent with increasing O-Cq-O abundance with apparent DOC diagenetic state; however a bacterial source would also explain the greater abundance of O-Cq-O in highly productive Mauritanian upwelling surface waters compared to the oligotrophic North Pacific surface waters, as a larger source of algal DOM would support a larger and more productive bacterial community (Ducklow and Carlson, 1992). The difference between these two samples is based on the dual filtered (CSA and dipolar dephasing) spectra for which the 5 m N. Pacific sample has a low signal-to-noise ratio (see spectrum in Fig. 3 and Supplementary Information), suggesting that these results should be interpreted with care. Further efforts to identify specific, naturally produced carbohydrates with O-Cq-O structures and investigations of their sources and cycling in the marine environment are clearly needed.

Aromatic carbons were observed in relatively low abundance, consistent with the UV-visible spectrophotometric (Table 1; Supplementary Material) and ultra-high resolution MS data for the same samples (Chen et al. 2014a). Aromatic carbons do not trend with depth or diagenetic state (Fig. 4C). The non-protonated aromatic carbons (Table 3) likely include carbons from condensed aromatic structures (Jaffé et al., 2013; Masiello and Druffel, 1998; Ziolkowski and Druffel, 2010). Condensed aromatic compounds are typically pyrogenic and reach the ocean via riverine export (Hockaday et al., 2006; Jaffé et al., 2013) and atmospheric deposition (Flores-Cervantes et al., 2009;

Hockaday et al., 2006; Watanabe et al., 2010), although hydrothermal vents (Dittmar and Koch, 2006) and photochemical production (Chen et al., 2014b) have also been speculated as sources. Oceanic distributions and radiocarbon data (Dittmar and Paeng, 2009; Ziolkowski and Druffel, 2010) indicate that black carbon is one of the oldest and most recalcitrant components of ocean DOM. Our results concur, with the percentage non-protonated aromatic carbon increasing with diagenesis (Table. 3) and peaking in the deep North Pacific (Table 3), suggesting non-protonated aromatic carbon is preserved in the deep ocean. Photochemical removal in surface waters has been posited as the main sink for oceanic dissolved black carbon (Helms et al., 2014; Stubbins et al., 2012b). Therefore, photo-removal likely explains the low abundance of non-protonated aromatic carbons in sunbathed surface waters (Helms et al., 2014; Stubbins et al., 2012b). There were few condensed aromatic or black carbon-like molecular formulae identified by ultrahigh resolution MS of these samples (Chen et al., 2014a), either due to their low abundance in the samples or low ionization efficiencies.

4.4. Cycling of semi-labile and refractory DOM in the ocean

Of particular importance to understanding the possibility of long-term survival of otherwise labile DOM are recent studies dealing with the extreme dilution of ocean DOM (Arrieta et al., 2015; Dittmar, 2015) and the structural and compositional complexity of the DOM pool (Chen et al., 2014a; Hertkorn et al., 2013; Jiao et al., 2010). However, the primary focus of this study was to gain insight into the relationship between DOM composition and biogeochemical reactivity. Attempts to conceptualize the production, processing, and preservation of DOM within the ocean have led to a multi-pool DOC

model in which DOC is classified according to time scales over which it is removed or preserved (Carlson, 2002; Hansell et al., 2009). In this model, bio-labile carbon is released during photosynthesis, lysis, and grazing and is rapidly removed or recycled by heterotrophic activity (Carlson et al., 2002); semi-labile carbon is identified as DOM that is removed over months to years (Carlson and Ducklow, 1995; Kirchman et al., 1993; Ogura, 1972); and refractory carbon resists degradation, remaining in the ocean for centuries or millennia (Williams and Druffel, 1987).

The marked compositional similarity of DOM isolated from different oceanic regimes (Fig. 2) suggests that a large fraction of all of the samples is comprised of an apparently recalcitrant (or refractory) “background” DOM. This DOM is best represented in our sample set by the “old” deep North Pacific sample, where near-minimal DOC concentrations and maximum DOC apparent ages are observed in the global ocean (Bauer, 2002; Hansell et al., 2009). Above this background, compositional differences between the samples (Figs. 1 and 3) are likely due to localized processes of production and removal (Table 3; Carlson 2002).

In considering the distribution of structural moieties within our set of samples, several clear trends emerge. The dominant constituents of semi-labile DOC were carbohydrate-like compounds followed by protonated alkyl groups and N–C–H groups, presumably from peptides and/or amino sugars (Aluwihare et al., 2005; McCarthy et al., 1998; McCarthy et al., 1997). These components generally occurred at concentrations above the refractory background signal in the surface samples, but diminished with depth and diagenesis (Tables 2 and 3; Figs. 3 and 4). Refractory DOC was enriched in quaternary aliphatic DOM, which is not abundant in algal DOM (Mao et al., 2007), but

which may have arisen from abiotic condensation or cross-linking reactions (Harvey et al., 1983; Keil et al., 1994; Kieber et al., 1989) and/or survived microbial degradation of macromolecular DOM (Amon and Benner, 1996). Proton-poor, condensed or substituted aromatic rings typically increased as a fraction of total aromaticity with degree of biological and/or diagenetic processing (Table 3), and might be indicative of black-carbon moieties (Dittmar and Koch, 2006; Dittmar and Paeng, 2009; Ziolkowski and Druffel, 2010).

DOM that survives in surface waters of the subtropical gyres can be expected to be photo-refractory due to vertical stratification and long-term exposure to light. Perhaps counter-intuitively, protonated aromatic and olefinic DOC (Fig. 3) appeared to accumulate in the North Pacific gyre. Aromatic-rich and extensively conjugated DOM is typically photo-labile (Osburn et al., 2001; Stubbins et al., 2012a; Stubbins et al., 2010). This incongruence may be explained by a fraction of the photochemically altered aromatic (unsaturated) DOM having blue-shifted absorption spectra (i.e. transparent to solar radiation; Helms et al., 2008). Thus, the NMR signal in this spectral region could be due to compounds containing single rings (or rings connected by non-conjugated structures such as peptides or proteins containing tyrosine or phenylalanine) that absorb at shorter wavelengths than condensed aromatic compounds (Zhang et al., 2006), single-ring aromatic compounds that are substituted with electron-withdrawing groups (Stubbins et al., 2008), or olefinic or isoprenoid moieties, which contain some sp^2 hybridized carbons but absorb at wavelengths shorter than the solar irradiance cut-off (~295 nm).

4.5. Conclusions

480 The RO/ED-isolated oceanic DOM provided an apparently more representative
481 fraction of open-ocean DOC compared with previously described UDOM and SPE
482 isolates. Examining a suite of samples ranging from high productivity surface waters,
483 through oligotrophic surface gyres, to the carbon-limited deep ocean enabled the
484 structural character of semi-labile, photo-refractory and deep-ocean refractory DOM
485 pools to be elucidated. This study showed for the first time that photo-refractory,
486 protonated conjugated organic compounds accrued in subtropical gyre surface waters,
487 suggesting that a pool of aromatic compounds is able to escape photo-degradation in
488 these sundrenched waters. Quaternary anomeric carbons were identified for the first time
489 as a component of biologically refractory carbohydrate-like material. Additionally,
490 advanced NMR spectroscopy confirmed that refractory DOM is enriched in non-
491 protonated aromatic compounds, and carboxylic-rich material consistent with CRAM and
492 that semi-labile DOM is enriched in carbohydrate-like and aliphatic compounds.
493

Acknowledgements

The authors gratefully acknowledge the crews of the R/V Oceanus and R/V Kilo Moana for assistance with sample collection and processing. Susan Hatcher and Junyan Zhong at Old Dominion University's College of Science Major Instrument Cluster (COSMIC) maintain the Nuclear Magnetic Resonance instrument used for this study. Richard C. Zimmerman as well as six anonymous reviewers provided helpful comments on earlier versions of this manuscript. This research was supported by National Science Foundation grant numbers OCE-0728634 and OCE-0728050.

Figure Captions

Fig. 1. A freeze-dried RO-ED extract (5 m N. Atlantic) was measured using both (A) direct polarization and (B) cross polarization ^{13}C NMR.

Fig. 2. Solid-state ^{13}C NMR spectra of the freeze-dried RO-ED samples measured by cross polarization with magic angle spinning and total suppression of sidebands (CP-TOSS). Samples collected at (A) a depth of 5 m from the Mauritanian upwelling zone in the N. Atlantic, (B) a depth of 5 m from station ALOHA in the N. Pacific, (C) a depth of 415 m in the eastern N. Atlantic, (D) a depth of 3000 m in the eastern N. Atlantic (Iceland Scotland Overflow Water), and (E) a depth of 3500 m from station ALOHA in the N. Pacific.

Fig. 3. Solid-state ^{13}C CP/TOSS NMR spectra and CP/TOSS NMR spectra with spectral editing were collected for (A) RO/ED isolated DOM from the N. Atlantic and (B) RO/ED isolated DOM from the N. Pacific. CP/TOSS spectra “All Carbon” are shown as reference spectra. CP/TOSS with dipolar dephasing: “Non-protonated C,” CSA-filtered spectra: “Aliphatic C,” short CP and CSA-filter: “Prot. aliphatic C,” CSA-filter and dipolar dephasing: “Non-prot. Aliph. C.” The spectra labeled “Protonated carbons only” show the difference between spectra collected using a short CP time and spectra combining the short CP time and dipolar dephasing.

Fig. 4. Functional group relative abundance displayed on a carbon percentage basis. (A) O-alkyl, (B) alkyl carbons, (C) aromatic carbons, and (D) carboxyl carbons quantified in

528 the RO/ED isolates by CP-TOSS ^{13}C NMR. Black circles represent CP/TOSS results.
529 Gray circles represent spectral editing results. Error bars represent analytical uncertainty
530 calculated using the signal-to-noise ratios of the CP/TOSS, CSA-filtered, dipolar
531 dephased, and/or combined CSA-filtered/ dipolar-dephased spectra. They are shown as
532 two standard deviations (95% confidence interval).

533

534

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536

537 **Table 1**

538 Summary of optical properties measured for initial seawater samples and RO/ED isolates.

539 Values in parentheses are the absorption coefficients predicted based on the measured

540 seawater values, RO/ED concentration factors and DOC recoveries. Values in brackets

541 are total recoveries including DOC recovered in NaOH rinse, but not in a form readily

542 available for NMR characterization at the time of this work. DOC values were previously

543 published by Chen et al. (2014a).

	Seawater samples				
	5 m N. Atl.	5 m N. Pac.	415 m N. Atl.	3000 m N. Atl.	3500 m N. Pac.
a_{254} (m^{-1})	1.54	1.19	0.99	0.90	0.80
a_{300} (m^{-1})	0.62	0.34	0.46	0.32	0.29
DOC ($\mu\text{M C}$)	88	69	54	41*	39*
SUVA254	0.591	0.591	0.664	0.664	0.525
SUVA300	0.255	0.168	0.308	0.308	0.191
$S_{300-700}$ (nm^{-1})	0.0087	0.0084	0.0071	0.0090	0.0067
$S_{275-295}$ (nm^{-1})	0.0225	0.0289	0.0156	0.0203	0.0174
$S_{350-400}$ (nm^{-1})	0.0101	0.0092	0.0064	0.0166	0.0095
S_R	2.22	3.13	2.42	1.22	1.83
	RO/ED isolates				
	5 m N. Atl.	5 m N. Pac.	415 m N. Atl.	3000 m N. Atl.	3500 m N. Pac.
a_{254} (m^{-1})	77.8 (75.1)	14.1 (23.0)	24.9 (28.2)	16.7 (23.7)	19.1 (25.6)
a_{300} (m^{-1})	28.0 (30.2)	3.6 (6.6)	8.7 (13.0)	5.8 (8.4)	7.2 (9.3)
DOC ($\mu\text{M C}$)	4298 (1104)	1325 (435)	1523 (421)	1076 (323)	1254 (343)
SUVA254	0.673	0.376	0.617	0.647	0.530
SUVA300	0.242	0.097	0.216	0.223	0.201
$S_{300-700}$ (nm^{-1})	0.0097	0.0125	0.0086	0.0080	0.0123
$S_{275-295}$ (nm^{-1})	0.0284	0.0351	0.0275	0.0279	0.0235
$S_{350-400}$ (nm^{-1})	0.0100	0.0112	0.0083	0.0097	0.0127
S_R	2.85	3.15	3.33	2.87	1.85
	CDOM/DOC Recovery (%)				
	5 m N. Atl.	5 m N. Pac.	415 m N. Atl.	3000 m N. Atl.	3500 m N. Pac.
a_{254} (%)	70	51	42	28	64
a_{300} (%)	63	46	32	27	67
DOC (%)	68 [79]	83 [102]	46 [56]	40 [50]	86 [106]

544 * DOC values from previous studies (Hansell and Carlson 1998; Carlson et al. 2010).
545 MeasuredDOC were higher, 62 $\mu\text{M C}$ (N. Atlantic 3000 m) and 52 $\mu\text{M C}$ (N. Pacific
546 3500 m), and were likely contaminated during the sub-sample collection.

547 **Table 2**

548 Integration ranges and normalized areas based on solid-state ^{13}C CP/TOSS NMR spectra.

549 Values are given as integrated signal (normalized to 100% total area).

CP/TOSS Integrations	Functional Groups	3000 m N. Atl.	415 m N. Atl.	5 m N. Atl.	3500 m N. Pac.	5 m N. Pac.
0-25	CH ₃ , CH ₂	13.3	13.8	12.4	12.5	11.3
25-35	CH ₂ , CH	10.2	8.4	8.2	8.3	7.5
35-50	CH, CH ₂ , Cq	14.5	14.3	13.1	13.8	12.9
50-60	Cq, OCH ₃ , HC-NH	9.1	9.3	9.5	9.1	9.0
60-90	Carbohydrate-like	30.5	30.2	34.8	25.0	35.0
90-110	Anomeric O-C-O, Aromatic C	7.9	7.8	8.0	7.9	8.8
110-130	Aromatic C-H, Cq	2.3	2.2	1.9	2.4	2.8
130-150	Aromatic C-H, Cq	1.8	1.9	1.4	2.7	1.6
150-165	Aromatic C-O	1.3	1.2	0.8	1.3	0.8
165-190	COO-/COOH, ester, amide	8.6	10.8	9.6	16.3	9.9
190-220	Aldehyde, ketone, quinone	0.7	0.1	0.3	0.8	0.7

550

551 **Table 3**

552 The relative abundances (%) of functional groups in RO/ED isolated DOC estimated
 553 based on NMR integration data (Table 2) and NMR spectral editing results (Fig. 3).

Integrated Region	Functional Groups	3000 m N. Atl.	415 m N. Atl.	5 m N. Atl.	3500 m N. Pac.	5 m N. Pac.
0-25	CH ₃	11.2	10.2	8.9	10.5	7.6
15-45	CH ₂	13.6	13.9	13.3	12.0	12.0
35-50	Aliphatic CH	11.9	9.1	8.7	8.5	11.6
35-60	Aliphatic Cq	5.1	5.1	4.1	6.6	4
50-60	Aliphatic C-OH and NCH	7.1	7.2	7.9	6.1	7.2
60-110	Carbohydrate-like C	31.1	31.8	38.1	25	34
90-110	O-Cq-O	2.3	4.4	2.6	5	1.8
100-165	Protonated aromatics	4.1	2.4	3.0	2	5.5
100-165	Non-protonated aromatics	4.0	3.4	2.6	5	4.5
165-190	Carboxyl C	9.1	11.7	10.3	17.7	10.3
190-220	Carbonyl C	0.7	0.1	0.3	0.9	0.7

554

555 **Table 4**

556 Comparison of CP/TOSS results for RO/ED isolates with CP/MAS results from previous
557 studies.

Chemical Shift Range, ppm	0-60	60-110	110-160	160-190	190-220
Structural Category	Alkyl	Alkoxy	Aromatic	Carboxyl	Carbonyl
<i>Open Ocean DOM Isolated by RO/ED</i>					
5 m N. Atl. (upwelling)	42.8	43.2	3.8	9.9	0.3
5 m N. Pac. (5 m)	40.3	44.0	4.9	10.1	0.7
415 m N. Atl. (O ₂ min.)	45.8	37.8	5.1	11.1	0.2
3000 m N. Atl.	47.0	38.2	4.9	9.2	0.7
3500 m N. Pac.	45.3	32.4	5.2	16.8	0.3
<i>Coastal Marine DOM isolated by RO/ED</i>					
2 m Ogeechee R. (Koprivnjak et al., 2009)	43	32	10	11	4
20 m GA Bight* (Koprivnjak et al., 2009)	40	38	8	12	2
84 m GA Bight (Koprivnjak et al., 2009)	44	36	7	11	2
<i>DOM isolated by UltraFiltration</i>					
10 m N. Pac. (Benner et al., 1992)	26	54	7	13	3
20 m N. Pac. (Sannigrahi et al., 2005)	26	66	0	8	
4000 m N. Pac. (Benner et al., 1992)	27	25	19	15	5
4000 m N. Pac. (Sannigrahi et al., 2005)	46	43	0	11	
<i>HA and FA isolated with XAD-2 resin</i>					
5 m N. Pac. (Hedges et al., 1992)	45	18	18	15	4
4200 m N. Pac. (Hedges et al., 1992)	46	17	19	15	3
<i>Freshwater NOM (RO isolated)</i>					
Suwannee R. (Perdue and Ritchie, 2003)	27	24	25	19	5

558 *Average of two samples.

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Figure 2

Figure 3

Figure 4