Novel insights from NMR spectroscopy into seasonal changes in the composition of dissolved organic matter exported to the Bering Sea by the Yukon River

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Abstract

Seasonal (spring freshet, summer-autumn, and winter) variability in the chemical composition of dissolved organic matter (DOM) from the Yukon River was determined using advanced one- and two-dimensional solid-state NMR spectroscopy, coupled with isotopic measurements and UV-visible spectroscopy. Analyses were performed on two major DOM fractions, the hydrophobic organic acid (HPOA) and transphilic organic acid (TPIA) fractions obtained using XAD resins. Together these two fractions comprised 64-74% of the total DOM. Carboxyl-rich alicyclic molecules (CRAM) accounted for the majority of carbon atoms in the HPOA (63-77%) and TPIA (54-78%) samples, and more so in winter and summer than in spring samples. 2D and selective NMR data revealed association of abundant nonprotonated O-alkyl and quaternary alkyl C (OC_{np} , $OC_{np}O$ and C_q , 13-17% of HPOA and 15-20% of TPIA) and isolated O-CH structures with CRAM, which were not recognized in previous studies. Spectral editing and 2D NMR allowed for the discrimination of carbohydrate-like O-alkyl C from non-carbohydrate O-alkyl C. Whereas two spring freshet TPIA samples contained carbohydrate clusters such as carboxylated carbohydrates (16% and 26%), TPIA samples from other seasons or HPOA samples mostly had small amounts (< 8%) of sugar rings dispersed in a nonpolar alkyl environment. Though nonprotonated aromatic C represented the largest fraction of aromatic C in all HPOA/TPIA isolates, only a small fraction (~5% in HPOA and 3% in TPIA) was possibly associated with dissolved black carbon. Our results imply a relatively stable portion of DOM exported by the Yukon River across different seasons, due to the predominance of CRAM and their associated nonprotonated C-O and O-C-O structures, and elevated reactivity (bio- and photo-lability) of spring DOM due to the presence of terrestrial inputs enriched in carbohydrates and aromatic structures.

1. INTRODUCTION

Northern permafrost soils contain approximately ~1,300 Petagrams (Pg) of soil organic carbon (OC), most of which is thought to be locked in perennially frozen soils (Hugelius et al., 2014). This pool of organic matter represents an estimated 50% of OC stored globally in soils and is more than twice the size of the current atmospheric carbon (C) pool (Dittmar and Kattner, 2003; Schuur et al., 2008; Tarnocai et al., 2009). There are growing concerns that permafrost thaw due to climate change may cause mobilization of this huge terrestrial OC pool to the atmosphere as carbon dioxide (CO₂) and methane (CH₄) and to aquatic systems as dissolved and particulate C (Frey and McClelland, 2009; Grosse et al., 2011; McGuire et al., 2009). The transfer of OC from land to aquatic ecosystems as dissolved organic matter (DOM) is a fundamental link between terrestrial and aquatic systems (Roulet and Moore, 2006; Spencer et al., 2012). Arctic rivers export large quantities of DOM, and approximately 10% of the global riverine flux of DOM is transferred to the Arctic Ocean, which represents only 1% of the global ocean volume. Because of the large size of this OC pool and the potential sensitivity of soil microbial processes to climate conditions, DOM fluxes from Arctic rivers have received increased attention over the past decade (Dittmar and Kattner, 2003; Guo et al., 2013; Holmes et al., 2012; Holmes et al., 2013; Opsahl et al., 1999; Spencer et al., 2015; Wickland et al., 2012).

Arctic rivers exhibit striking seasonal patterns in water discharge, dissolved organic carbon (DOC) concentrations (Holmes et al., 2012; Striegl et al., 2005; Striegl et al., 2007), DOM chemical composition, and age (Aiken et al., 2014; Guo et al., 2013; Neff et al., 2006; Raymond et al., 2007; Spencer et al., 2008; Spencer et al., 2009). DOM exported during the spring freshet (May 1–June 30) has relatively high lignin phenol content and is typically ¹⁴C-enriched. Under

winter base flow conditions (November 1–April 30), DOM has a greater aged component (Aiken et al., 2014; Neff et al., 2006; Raymond et al., 2007), and shows relatively low lignin phenol content and aromaticity, inferred from optical measurements (Spencer et al., 2008; Spencer et al., 2009). During the summer-autumn period (July 1–October 31), DOM generally exhibits characteristics intermediate between peak flow and winter base flow DOM (Neff et al., 2006; Raymond et al., 2007; Spencer et al., 2008; Spencer et al., 2009).

The full significance of Arctic river DOM for the biogeochemistry of the Arctic Ocean is not currently resolved and largely depends on its susceptibility to microbial and photochemical degradation processes (Cory et al., 2007). Arctic riverine DOM was historically considered refractory, based on the observations of apparent conservative mixing behavior across the continental shelf (Amon and Meon, 2004; Amon, 2004; Cauwet and Sidorov, 1996; Dittmar and Kattner, 2003) and extremely low consumption of Arctic river DOM during extended incubation experiments (Amon, 2004; Lobbes et al., 2000). This view, however, was developed mainly through the analyses of samples collected during mid to late summer after sea ice retreat. Recent incubation studies with better seasonal sampling resolution indicate that spring freshet DOM (Holmes et al., 2008; Mann et al., 2012) has high lability, with 20-40% of spring DOM consumed in incubation studies. Photochemical transformation of Arctic river DOM has been considered of less importance due to the prevailing low solar angle and sea ice cover at high latitudes (Amon and Meon, 2004; Benner et al., 2004; Osburn et al., 2009). However, growing evidence now also points to an enhanced role for photochemistry in DOM cycling within Arctic regions (Cory et al., 2007; Cory et al., 2014).

The chemical composition of DOM, in combination with environmental factors such as nutrient availability (Schmidt et al., 2011; Wickland et al., 2012), determines its susceptibility to

bacterial and photochemical degradation processes, and in turn, influences the fate of Arctic river DOM in fluvial networks and the Arctic Ocean (Del Giorgio and Davis, 2003; Spencer et al., 2015). While many studies have characterized bulk optical/isotopic properties of DOM in Arctic rivers, detailed structural information of DOM in these rivers is lacking. In this study, we aim to assess seasonal variability in the chemical composition of DOM by sampling across the three distinct seasons in a large northern high-latitude river system (Yukon River, Alaska, United States). The Yukon River represents one of only a few remaining great rivers that are uncontrolled, thereby facilitating an unconfounded examination of the response of C cycles to environmental changes in the basin. Distinct from most other major Arctic rivers which flow from south to north, the Yukon River drains from east to west and has a uniformly high latitude (59-69 °N), making it particularly sensitive to ongoing climate change within its basin. In the present work we applied one- and two-dimensional solid-state nuclear magnetic resonance (NMR) spectroscopy, coupled with isotopic measurements and UV-visible spectroscopy, to investigate the chemical composition of major fractions of the DOM from samples collected from distinct phases of the Yukon River hydrograph (spring freshet, summer-autumn, and winter).

2. MATERIALS AND METHODS

2.1. Study area and water sampling

The Yukon River (YR) with an annual water discharge of $\sim 208 \text{ km}^3$ is the largest river in Alaska and the fifth largest river draining to the Arctic Ocean, contributing $\sim 8\%$ of the total freshwater input to the Arctic Ocean via the Bering Strait (Aagaard and Carmack, 1989). The YR basin encompasses $854,700 \text{ km}^2$ of northwestern Canada and Alaska, and covers diverse terrain

including high mountainous regions, forested uplands, and extensive areas of low-lands rich in wetlands and lakes (Wickland et al., 2012). The YR basin is predominantly subarctic or boreal, and much of it is underlain by continuous and discontinuous permafrost, which is presently warming and thawing (Romanovsky et al., 2010).

Sampling was conducted at various times of the year in 2008 and 2009 at Pilot Station, Alaska, to obtain a representative dataset reflecting the changing seasonal hydrograph, including sampling under ice cover during winter (Table 1). Pilot Station is located near the river mouth (latitude 61.93361N, longitude -162.8831W; Figure 1), just above the influence of seawater from the Bering Sea, and therefore best represents the DOM characteristics which are products of the entire basin and its biogeochemical processes. Discharge data were monitored continuously by the U. S. Geological Survey (USGS; gaging station 15565447; <http://nwis.waterdata.usgs.gov.usa/nwis/sw>).

Large volume (40–95 L) water samples were filtered in the field with Gelman AquaPrep 600 capsule filters (0.45 μ m; supor membrane) that were prerinsed with a minimum of 2 L of sample water before sample collection. Samples were filtered into 10-L, high-density polyethylene jugs that were previously cleaned with 0.1 M HCl and distilled water, and rinsed with filtered sample before use. Samples were shipped on ice in the dark to the USGS laboratory in Boulder, Colorado. Early spring samples (before the spring flush) were collected through the ice before breakup while samples during the spring flush were collected when most ice had passed. Water samples were processed by XAD isolation and concentration as described by Aiken et al. (1992). Briefly, samples were acidified to pH 2 with HCl and passed first through a column of XAD-8 resin followed by a column of XAD-4 resin. Each column was then eluted with 0.1 M sodium hydroxide (NaOH) to obtain the XAD-8 fraction (referred to as the hydrophobic organic acids,

HPOA) and XAD-4 fraction (referred to as the transphilic organic acids, TPIA). The eluates were desalted and hydrogen saturated using cation exchange resin, lyophilized, and stored at room temperature. The standard deviation for the mass percentages of the fractionation was $\pm$ 2%.

2.2. Dissolved organic carbon, UV-visible absorbance, elemental analyses, and carbon isotope measurements

Dissolved organic carbon (DOC) measurements were performed using an OI Analytical Model 700 TOC analyzer. Specific UV absorbance ($SUVA_{254}$) was determined by dividing the UV-visible absorbance at $\lambda = 254$ nm, measured on a Hewlett-Packard photo-diode array spectrophotometer (model 8453), by the DOC concentration. Elemental analyses (C, H, O, N, S, and ash) of DOM isolates were performed by Huffman Laboratories (Golden, Colorado) by the method described in Huffman and Stuber (1985). DOM isolates were combusted and converted to graphite targets at the USGS Radiocarbon Laboratory in Reston, Virginia. Radiocarbon abundance determinations were made at the Center for Accelerator Mass Spectrometry at the Lawrence Livermore National Laboratory in Livermore, California. Samples were combusted at 900 °C for 6 hours with cupric oxide (CuO) and silver (Ag) in sealed quartz test tubes to form CO₂ gas. The CO₂ was then reduced to graphite through heating at 575 °C in the presence of hydrogen (H₂) gas and an iron (Fe) catalyst (Vogel et al., 1987). Graphite targets were then analyzed for carbon isotopes ($\delta^{13}C$ in ‰ and ^{14}C as fraction modern carbon) (Davis et al., 1990). $\Delta^{14}C$ data (in ‰) were corrected for isotopic fractionation using measured $\delta^{13}C$ values, and radiocarbon ages were determined from percent modern carbon using the year of sample analysis according to Stuiver and Polach (1977). Ages are presented as modern when the fraction modern

exceeded 1. The Pearson's product momentum correlation coefficient (r^2) is used to estimate linear correlations.

2.3. NMR analysis

All ^{13}C NMR experiments were performed at 100 MHz using a Bruker Avance 400 spectrometer equipped with a 4-mm double-resonance probehead. The ^{13}C chemical shifts were referenced to tetramethylsilane, using the COO^- resonance of glycine in the α -modification at 176.46 ppm as a secondary reference. A summary of NMR methods and associated acronyms used in the present study are listed in Table S1.

The multiple cross polarization (multiCP) technique recently developed by Johnson and Schmidt-Rohr (2014) provides an efficient approach to obtain quantitative solid-state ^{13}C NMR spectra of organic materials with good signal-to-noise ratios, and was applied to all samples. The spectra were measured at a spinning speed of 14 kHz, where spinning sidebands are fairly small (< 3%) and overlap with centerbands is minimal. Corresponding multiCP spectra of nonprotonated C and mobile groups like rotating CH_3 were obtained by combining the multiCP sequence with 68- μs recoupled dipolar dephasing (multiCP/DD) (Johnson and Schmidt-Rohr, 2014). Between 1536 and 4608 scans were collected per spectrum. The total time for a pair of multiCP and multiCP/DD experiments was 2-6 h per sample.

To resolve the overlapping resonances of anomeric (O-C-O) and aromatic carbons between 120 and 90 ppm, the aromatic carbon signals were selectively suppressed using a three-pulse ^{13}C chemical-shift-anisotropy (CSA) filter with a filter time of 68 μs (Mao and Schmidt-Rohr, 2004). Four-pulse total suppression of sidebands (TOSS) (Dixon, 1982) was employed before detection, with two-pulse phase-modulated decoupling (Bennett et al., 1995) applied for optimum

resolution, at a spinning speed of 7 kHz, a cross polarization (CP) time of 1 ms, a ^1H 90° pulse-length of 4.1 μs , and a recycle delay of 0.5 s. The number of scans averaged was 2560-3584 for each spectrum. The CSA filter was also combined with 48- μs dipolar dephasing to isolate signals from nonprotonated anomeric carbons near 100 ppm. The number of scans averaged was 2560-3584 for each CSA-filtered spectrum, and doubled for the corresponding spectrum of CSA filter coupled with dipolar dephasing for each sample.

^1H - ^{13}C two-dimensional heteronuclear correlation (2D HETCOR) NMR experiments (Mao and Schmidt-Rohr, 2006) were performed at a spinning speed of 7.5 kHz. Standard Hartmann-Hahn CP (HH-CP) with 0.5-ms CP time allows for some ^1H spin diffusion and correlates the carbons with protons within a ~ 0.5 nm radius. In addition, a 40- μs dipolar dephasing delay was inserted into the HH-CP HETCOR sequence to reveal multi-bond ^1H - ^{13}C proximities for nonprotonated carbons and mobile groups like CH_3 . The recycle delay was 0.5 s. More long-range proximities were probed by ^1H spin diffusion during a mixing time of 1 ms and 10 ms, correlating the carbons with protons within a ~ 1.6 nm and a 5 nm radius, respectively. Each 2D HETCOR spectrum required 5-27 h of measuring time.

The uncertainty for NMR integrals was reported based on the propagation of analytical uncertainty, estimated for each of the NMR integrals based on the signal-to-noise ratio. Uncertainty (reported as one standard deviation, and shown in Table S2) is $\leq 1\%$ except 1-2% for CH_2/CH and aromatic C-H regions.

3. RESULTS

3.1. Seasonal patterns of DOC concentration, SUVA_{254} , and major chemical isolates

DOC concentrations, ranging from 2.7 to 17.3 mg C L⁻¹, varied with sampling date (Table 1). DOC concentrations peaked during the spring flush (05/22/2009 and 05/29/2009), were lowest during winter base flow (01/21/2009 and 03/26/2009), and intermediate in values before the spring flush event (05/12/2009) and during summer base flow (07/01/2008, 08/12/2008, and 09/24/2008). Similar relationships between river discharge and DOC (concentrations and SUVA₂₅₄) have been reported in earlier Yukon River basin studies (O'Donnell et al., 2012; Spencer et al., 2008; Wickland et al., 2012). SUVA₂₅₄, an indicator of aromaticity, of whole water was higher during spring and summer than in winter months (Table 1).

HPOA were the dominant DOM fraction (ca. 47–57% of total DOC) in all whole water samples, while TPIA accounted for 15-18% (Table 1). The remaining DOM in all samples was largely found in the hydrophilic acid fraction (15-20%), a fraction that was not analyzed further in this work. All DOM fractionation data are given in Figure S1. SUVA₂₅₄ values associated with HPOA and TPIA fractions followed the same trend as whole water SUVA₂₅₄ values ($r^2 = 0.97$ for HPOA, $r^2 = 0.82$ for TPIA). Elemental compositions for HPOA across seasons showed that all HPOA isolates were enriched in C (51.8-55.2%) and O (38.3-42.1%). The H contents ranged from 4.5 to 5.1%, yielding atomic H/C values of 1.0-1.2 for all HPOA samples. The N contents were low (1% or less, except 1.3% for 01/21/2009), resulting in high atomic C/N values (49.3-70.9) associated with the HPOA samples. Sulfur was less abundant than N (0.6% or less). Relative to HPOA isolates, the corresponding TPIA isolates contained less C (47.4-49.1%) but more O (44.0-45.1%). The TPIA isolates were also richer in N (1.5-2.0%) and S (0.7-1.5%), and had lower C/N values (27.6-38.0) than the corresponding HPOA isolates. The $\delta^{13}\text{C}$ values of all HPOA fell into a rather narrow range from -28.32‰ to -27.1‰ indicative of terrestrial sources and within the range previously reported for the Yukon River, its tributaries, and other northern

river basins (Guo and Macdonald, 2006; O'Donnell et al., 2014). The winter HPOA isolates had the most depleted $\Delta^{14}\text{C}$ values (-71.1‰ and -111.5‰), corresponding to the oldest ages (530 ybp and 890 ybp, respectively). Spring HPOA isolates (except 05/12/2009) had greater $\Delta^{14}\text{C}$ values than summer HPOA samples, although both spring and summer samples contained modern DOC.

3.2. Seasonal changes in functional group composition of DOM

The solid-state multiCP ^{13}C NMR spectra for HPOA and TPIA samples from the Yukon River at Pilot Station across different seasons are presented in Figure 2. The unselective ^{13}C spectra of both HPOA and TPIA samples (bold black line) were composed of five relatively well-resolved peaks assigned to signals from nonpolar alkyl C (0-64 ppm), O-alkyl C (64-100 ppm), aromatic or olefinic C (100-160 ppm, also containing anomeric C), COO/NC=O (160-190 ppm), and ketone/aldehyde C (190-220 ppm). In addition, the resonances near 107 ppm due to anomeric C (OCO) were better resolved in the spectra of TPIA isolates (Figure 2(g, h)) than in the spectra of HPOA isolates. MultiCP/DD spectra (thin black line) further identified mobile and nonprotonated C components such as CH_3 , quaternary C (C_q), nonprotonated C-O (O-C_{np}), nonprotonated anomeric C (OC_{np}O), nonprotonated aromatic C-C, and aromatic C-O in phenols and aromatic ethers (143-160 ppm). The intensities of ketone/aldehyde signals remained the same after dipolar dephasing, indicating that these carbonyls were present in the nonprotonated form, i.e., as ketone not aldehyde C, in all HPOA and TPIA isolates. The CP/TOSS spectra after the CSA filter (gray line), only shown in Figure 2(d, h) to avoid overlapping spectra, unambiguously identified anomeric C (OCO, shaded area near 100 ppm). The combination of the CSA filter technique with dipolar dephasing (gray dotted line) further selected nonprotonated anomeric C (OC_{np}O). Isolates within the same seasons shared similar spectral features and were

compositionally very similar (within $\pm 2\%$, Figures S2 and S3). Differences do exist between the two spring TPIA samples: 05/12/2009 was more enriched in O-alkyl and OCO whereas depleted in aromatic C and COO/NC=O compared to 05/22/2009.

Several observations are apparent in the visual comparison of spectra of HPOA/TPIA samples across different seasons (Figure 2). First, signals from OCH₃ and aromatic C (including aromatic C-O) generally characteristic of lignin structural components were more discernible in summer than in winter HPOA, and became even more prominent in spectra of spring HPOA (05/12/2009 and 05/22/2009, Figure 2(c,d)). Second, the O-C peak maximum shifted from 77 ppm in summer and winter HPOA to 74 ppm in spring HPOA (see dashed vertical lines), suggestive of different chemical environments of these O-alkyls. Third, spectra of spring TPIA (05/12/2009 and 05/22/2009) were markedly different from those of summer and winter TPIA samples, with the former showing a sharp O-C peak at 72 ppm and a relatively well-resolved OCO peak at 102 ppm (Figure 2(g,h)).

3.2.1. Seasonal variations of HPOA structures

The HPOA isolates were all considerably more aliphatic than aromatic in composition (Table 2). Alkyl C (0-64 ppm) accounted for 33-42% of the C in HPOA isolates, and its relative abundance increased in the order: 05/12/2009, 05/22/2009, 08/12/2008, and 03/26/2009. The aromatic C fraction (including protonated aromatic C-H, nonprotonated aromatic C-C, and oxygen substituted aromatic C-O) ranged from 19-26% and increased as alkyl C decreased. The relative abundances of O-alkyl C (including O-C and OCO), were generally constant across seasons, accounting for 20-21% of total C. Anomeric C (OCO) was slightly more enriched in spring HPOA (~5%) than in summer and winter HPOA samples (~3%). The COO/NC=O region

constituted 16-17% of the C in all HPOA, with NC=O contributing at most 1-2% of all C based on the high atomic C/N values (49.3-70.9) associated with HPOA isolates. The relative fraction of ketone C was small and constant (~3%) in HPOA isolates across all seasons.

The spring and summer HPOA samples contained more aromatic C-O (and aromatic C-C and aromatic C-H) than the winter HPOA (6-7% vs. 4%). Though the OCH₃ peak at 55 ppm was evident in spectra of spring HPOA, its contribution was <0.5% of all C for all HPOA isolates. Whereas the ratio of the relative fraction of protonated to nonprotonated C-O (OCH_n/OC_{np}) was approximately 1 in summer and winter HPOAs, this ratio doubled in the two spring HPOA samples. This explained the shift of OC peak maximum from 77 to 74 ppm in spring HPOA because OCH_n appeared to resonate at lower chemical shift than OC_{np} (Figure 2). The spring HPOA isolates were also relatively more enriched in protonated anomeric C (OCHO) than summer and winter HPOA. In addition, the summer and winter HPOA isolates contained relatively more nonprotonated aliphatic C (C_q + O-C_{np} + OC_{np}O) than spring HPOA (16% vs. 13%).

3.2.2. Seasonal variations of TPIA structures

Alkyl C (0-64 ppm) was the dominant component in the winter and summer TPIA isolate spectra. Its relative abundance decreased in the order: winter (36%) > summer (32%) > spring (~28%). The dominant component in spring TPIA samples was O-alkyl C (O-C + OCO), which accounted for 38% and 32% of total C in 05/12/2009 and 05/22/2009, respectively. Hence 05/12/2009 TPIA contained more O-alkyl C than 05/22/2009 TPIA, which was sampled only 10 days later. The contribution of O-alkyl C decreased in summer and winter TPIA isolates (~29%). Consistent with the intense COO/NC=O peak, it contributed 20-21% (± 0.5%, Table S2) of C in

all TPIA except for a value of about 18% ($\pm 0.5\%$, Table S2) in 05/12/2009 TPIA. Based on elemental data (Table 1), the relative abundances of NC=O can be at most 3-4% of all C. Aromatic C (including aromatic C-H, aromatic C-C, and aromatic C-O) accounted for 12-17% ($\pm 1\%$) of the C and increased in the order: 03/26/2009 < 05/12/2009 < 08/12/2008 and 05/22/2009. Ketone C constituted only a small portion (2-3%) in all TPIA isolates.

The ratio of the relative fraction of protonated to nonprotonated C-O ($\text{OCH}_n/\text{O-C}_{\text{np}}$) was about 1.0 in summer and winter TPIA, but increased to 3.0 in 05/12/2009 and 2.4 in 05/22/2009, in agreement with the shift of OC peak maximum in spectra of spring TPIA isolates (Figure 2). The spring TPIA contained more protonated anomeric C (OCHO, 05/12/2009 ~5%, 05/22/2009 ~3%) than summer and winter TPIA (< 1%). Similarly, nonprotonated aliphatic C ($\text{C}_q + \text{O-C}_{\text{np}} + \text{OC}_{\text{np}}\text{O}$) was relatively more abundant in summer and winter than in spring TPIA isolates (19-20% vs. 15%).

3.3. Proximities of functional groups in DOM across different seasons

Although quantitative ^{13}C NMR spectra showed definitive differences in functional group distributions among HPOA/TPIA isolates across different seasons, the assignment of structural components associated with these functional groups is ambiguous. For instance, the ^{13}C NMR signals in the 60-110 ppm region are most often assigned to OCH in carbohydrates, but the spectra after dipolar dephasing also showed nonprotonated carbons resonating in this range. For reasons detailed below, this region was assigned to carboxyl-rich alicyclic (CRAM) molecules. Indeed, no significant carbohydrate components are expected in our samples, because unlike ultrafiltration, XAD-8 resin is known not to retain large carbohydrates. A more accurate assignment of the O-C and other signals to different structural components can be aided by ^1H -

¹³C heteronuclear correlation (HETCOR) NMR, which provides through-space ¹H-¹³C correlations and information on binding neighborhood of functional groups.

3.3.1. HPOA structures

Figure 3 displays the 2D HETCOR spectra of 05/22/2009 HPOA (Figure 3(a)) and 03/26/2009 HPOA (Figure 3(b)), which show signals of ¹H within a ~ 0.5 nm radius from the carbon observed in the spectrum, i.e., within the size of a sugar ring or aromatic ring (~ 0.6 nm diameter). The spectrum of 05/22/2009 showed a cross peak of OCH₃, which was absent in the spectrum of 03/26/2009, consistent with their ¹³C multiCP spectra. The cross peaks of O-C and OCO had different patterns in the spectra of 05/22/2009 and 03/26/2009, which were more clearly demonstrated in their corresponding proton slices (Figure 3(c, d)). For 05/22/2009 HPOA, the ¹H spectra associated with O-C (72 ppm) and OCO (102 ppm) showed primarily contributions from O-alkyl protons, but signals from alkyl protons were also seen (Figure 3(c)). In the case of 03/26/2009 HPOA, the ¹H spectrum associated with O-C (77 ppm) contained equal contributions from alkyl and O-alkyl protons. The major band in ¹H spectra associated with OCO (108 ppm) appeared at 2 ppm (Figure 3(d), marked by an arrow), indicating that OCO sites were in close proximity to alkyl protons. This suggests that in the 03/26/2009 HPOA, the O-C and OCO carbons were not primarily associated with carbohydrates; otherwise these C sites would correlate predominantly with O-alkyl protons. For both 05/22/2009 and 03/26/2009 HPOA (Figure 3(c, d)), the ¹H slices extracted at the chemical shifts of aromatic C and aromatic C-O showed contributions from aromatic protons, as well as correlations with alkyl and O-alkyl protons, indicating close association of aromatic and alkyl components. COO and ketone C=O exhibited correlations primarily with alkyl protons, with additional contributions from O-alkyl

protons, indicating that they were attached mostly to alkyl and O-alkyl carbons and that aromatic carboxylic acids are rare in HPOA samples. Linkages between COO and O-alkyl groups can be made either via an ester oxygen, $\text{C}(=\text{O})-\text{O}-\text{CH}_n$, or by a direct carbon-carbon bond, $\text{O}-\text{CH}_n-\text{C}(=\text{O})-\text{O}-$. The latter linkage involved a shorter C-H distance (two-bond C-H) than the former ester linkage (three-bond C-H), and were therefore in better agreement with the rapid H-to-C cross polarization observed in the experiments. In addition, the 2D HETCOR spectra of both 05/22/2009 and 03/26/2009 (Figure 3(a, b)) clearly showed the cross peaks of COO carbons with the acidic COOH protons resonating near 12 ppm (Figure 3(c, d)), which proves the presence of carboxylic acids.

With a mixing time of 1 ms for ^1H spin diffusion, the through-space ^1H - ^{13}C correlations can be extended to a length scale of ~ 1.1 nm radius, the size of 2-3 sugar/aromatic rings. The spectra of 2D HETCOR with 1-ms mixing time of 05/22/2009 HPOA and 03/26/2009 HPOA (Figure S4(a, b)) became more similar, with the major ^1H band associated with all types of C centered near 2 ppm (Figure S4(c, d)) (indicated by arrows), indicating that all C sites were in close proximity to alkyl protons. For 05/22/2009, the ^1H slices associated with O-C and OCO also showed nearly equal contributions from O-alkyl protons. There is strong evidence for sugar rings in 05/22/2009 spring HPOA, but they do not form large carbohydrate domains, as evidenced by the fast spin diffusion equilibration with nonpolar alkyls.

The quantitative NMR data showed the presence of abundant nonprotonated aliphatic C ($\text{C}_q + \text{O}-\text{C}_{\text{np}} + \text{OC}_{\text{np}}\text{O}$, 13-16% of all C, Table 2). The correlations between these nonprotonated carbons and their nearby protons, which are separated by at least two bonds, can be observed selectively in a 2D HETCOR spectrum with 0.5-ms HH-CP and 40- μs dipolar dephasing (Figure 4(a)) and corresponding proton slices (Figure 4(b)) for 03/26/2009 HPOA. The ^1H slice extracted

at 47 ppm (C_q) showed primarily correlations with alkyl protons (~ 2.5 ppm). The 1H spectra associated with $O-C_{np}$ (85 ppm) and $OC_{np}O$ (108 ppm) contained major contributions from alkyl protons resonating near 2 ppm (indicated by arrows), confirming that $O-C_{np}$ and $OC_{np}O$ were found predominantly near alkyl protons, rather than, near O-alkyl protons. These results indicate that these nonprotonated C-O and OCO fractions are not in carbohydrate environments. The 1H spectrum associated with the nonprotonated aromatic C (arom. C-C) showed nearly equal contributions from aromatic protons and alkyl protons (~ 2.5 ppm).

3.3.2. TPIA structures

The 2D HETCOR spectra of 05/22/2009 TPIA and 03/26/2009 TPIA are presented in Figure 5(a, b), with their corresponding proton slices extracted at different ^{13}C chemical shifts shown in Figure 5(c, d). The alkyl carbons (CH_3 , CH_2 , and CH) were mainly associated with their directly attached protons, but also showed correlations with O-alkyl protons, suggesting that alkyl and O-alkyl components were in close proximity. The cross peaks of O-C and OCO carbons with their directly attached protons were more pronounced in the spectrum of 05/22/2009 than in that of 03/26/2009. Alkyl protons also contributed to the proton spectra associated with the O-C and OCO carbons in 05/22/2009, and in 03/26/2009 to a greater extent (Figure 5(c, d), indicated by arrows). The cross peaks of aromatic C were generally weak in both spectra, and their associated proton spectra showed major contributions from aromatic in addition to minor contributions from non-aromatic protons. The 1H spectrum associated with COO (173 ppm) in 05/22/2009 TPIA had a major band centered at 4 ppm, suggesting that COO groups were mostly attached to O-C carbons. The alkyl and O-alkyl protons contributed almost equally to the 1H spectrum associated

with COO in 03/26/2009 TPIA, suggesting that COO groups were connected to both O-C and alkyl carbons. The ketone C=O (190-220 ppm) showed extremely weak cross peaks.

After introducing a mixing time of 1 ms which allowed proton magnetization transfer from more distant sites, the 2D HETCOR spectra of 05/22/2009 TPIA and 03/26/2009 TPIA (Figure S5(a, b)) showed even greater differences. Overall, the major band in ^1H spectra associated with all types of C was centered near 4 ppm for 05/22/2009 but near 2 ppm for 03/26/2009 (Figure S5(c, d)). Specifically, compared to ^1H spectra obtained without mixing time (Figure 5(c, d)), increasing contributions from O-alkyl protons to the ^1H spectra associated with alkyl C were observed for 03/26/2009, and 05/22/2009 to a much greater extent (Figure S5(c, d), arrows indicated larger contribution of alkyl protons for 3/26/2009). The ^1H spectra associated with O-C and OCO carbons instead showed increasing contributions from alkyl protons for both 05/22/2009 and 03/26/2009. This indicated the proximity of alkyl and O-alkyl components. This fast spin diffusion from alkyl protons indicates that these sugar-ring structures did not form large carbohydrate domains in either 05/22/2009 or 03/26/2009 TPIA. Non-aromatic resonances dominated the proton spectra associated with aromatic C for both 05/22/2009 and 03/26/2009, suggesting close proximity of aromatic and aliphatic components and the absence of larger clusters of aromatic compounds. The proton slices extracted at the COO carbon resonances also showed increased contributions from alkyl protons (Figure S5(c, d)) compared to ^1H spectra obtained without mixing time (Figure 5(c, d)). The 2D HETCOR data acquired with 40- μs dipolar dephasing of 03/26/2009 TPIA (Figure S6) indicated that the proton spectra associated with nonprotonated carbons such as quaternary C (C_q , 53 ppm), OC_np (85 ppm), and $\text{OC}_\text{np}\text{O}$ (108 ppm) contained nearly equal contributions from alkyl and O-alkyl protons.

Quantitative NMR data (Table 2) indicated that 05/12/2009 spring TPIA contained substantially more O-C and OCO than those of all other TPIA isolates, and its CP/TOSS spectrum (Figure S7(a)) closely resembled those of coastal and marine DOM isolated by ultrafiltration where carbohydrates are a prominent feature (McCarthy et al., 1993). To examine whether these O-C and OCO carbons were present in large carbohydrate domains, additional 2D HETCOR spectra with longer mixing times ($t_m = 0.01$ ms, 1 ms, and 10 ms) were collected and are shown in Figure S7(b-d). With increasing mixing time (t_m), the OC and OCO carbons predominantly remained correlated with O-alkyl protons, indicating a carbohydrate domain with a radius > 5 nm that did not equilibrate with other ^1H within the 10-ms mixing time. Though the COO carbons showed a cross peak predominantly with O-alkyl protons at short $t_m = 0.01$ ms, they became correlated with alkyl protons with increasing mixing time.

4. DISCUSSION

4.1. Major chemical components in DOM across different seasons

Based on the quantitative ^{13}C NMR, HETCOR NMR, and elemental data, carbons present in the DOM isolates (HPOA and TPIA) throughout different seasons were distributed among the following structural components: carbohydrates, carboxyl-rich alicyclic molecules (CRAM), aromatics, and nitrogen-containing compounds. Figure 6 shows the relative percentages of different functional groups for HPOA and TPIA and their assignments to four structural components. Though the classification scheme is operational and likely an oversimplification, it has been deemed useful for defining compound classes for comparison among samples. In addition, our classification is based on both the quantitative 1D spectra obtained by the multiCP method and the 2D NMR data which provide information on functional group connectivity, an

improved approach in making assignments compared to earlier studies (Abdulla et al., 2010; Zigah et al., 2014). The relative carbon percentages of these four compound class groups or components in HPOA/TPIA isolates are shown in Figure 7.

4.1.1. Carbohydrates

Carbohydrates are an important class of compounds influencing primarily the biolability and solubility of some DOM molecules. These materials, however, can be found in a number of arrangements, such as free carbohydrates or oligosaccharides, or linked to other aliphatic and aromatic compounds, such as terpenoids and flavonoids, through glycoside linkages. In NMR, the carbohydrate compound group is associated with signals of protonated OCO and O-C (i.e., OCHO and O-CH_n, Figure 6) because carbohydrates from biogenic precursors contain primarily OCHO and O-CH_n. While some nonprotonated (or quaternary) anomeric OCO (OC_{np}O) carbons might be found in furanose sugars (Poulin and Lowary, 2010) and keto-deoxyoctulosonate of bacterial lipopolysaccharides (Strain and Armitage, 1985), these structures would not explain the prominent cross peak to nonpolar alkyl protons in HETCOR spectra of Figure 4. Assuming that OCHO, which typically accounts for 1/6 to 1/5 of all carbohydrate C, only occurred as carbohydrates, six times the abundance of OCHO (O-CH_n = 5 × OCHO) provides an upper limit to the concentrations of carbohydrates in these isolates.

Carbohydrate moieties accounted for 1-2% of winter and summer HPOA, and 8% (at most 1-2 sugar rings per 100 C atoms) in spring HPOA isolates. This is generally consistent with results from the 2D HETCOR NMR analyses, which suggested the absence of carbohydrate domains, and that sugars were dispersed in nonpolar alkyl environments, such as in aliphatic glycosides where a carbohydrate is bonded to an aliphatic residue in the same molecule. While carbohydrate

components comprised only 2% of organic carbon in winter and summer TPIA, the fraction increased to 16% and 26% in the two spring TPIA isolates (Figure 7). The higher sugar concentration in 05/12/2009 is consistent with the presence of carbohydrate domains deduced from ^1H - ^{13}C HETCOR spectra. Specifically in the spring TPIA samples, COO was predominantly correlated with O-alkyl protons (Figure 5(a, c) and Figure S7(b)), suggesting the presence of COO-bonded O-alkyl, commonly found in carboxylated or acetylated carbohydrates such as uronic acids and muramic acids. In addition, these carbohydrates may also occur as by-products of cellulose and hemicellulose combustion, such as levoglucosan and mannosan, which have been detected in major Siberian Arctic rivers at all flow regimes (Myers-Pigg et al., 2015).

4.1.2. CRAM

CRAM represents a major class of DOM compounds in various aquatic environments (Hertkorn et al., 2006; Lam et al., 2007; Leenheer, 1994; Leenheer et al., 2003; McCaul et al., 2011; Zigah et al., 2014) that are poorly defined and whose molecular structures are unknown. Here, the CRAM component was assumed to consist of the following functional groups (Figure 6): $\text{CH}_3/\text{CH}_2/\text{CH}/\text{C}_q$, $\text{O}-\text{C}_{\text{np}}$, $\text{OC}_{\text{np}}\text{O}$, COO, ketone C, as well as the remainder of the OCH pool not associated with carbohydrates. Note that contributions from NCH in the 0-64 ppm and $\text{NC}=\text{O}$ in the 160-190 ppm region were estimated based on elemental C and N contents (Table 1) and excluded from the estimation of the CRAM component.

The nonprotonated O-alkyl C (OC_{np} and $\text{OC}_{\text{np}}\text{O}$) accounted for 9-11% of HPOA and 11-15% of TPIA. The ^1H - ^{13}C HETCOR spectra indicated that these O-C moieties are not part of carbohydrate structures. They revealed equal or larger contributions from nonpolar alkyl protons than from OCH (Figure 4(b) and Figure S6(b)), while the immediate proton environments in

carbohydrates would be totally dominated by O-alkyl protons. The cross peak between these nonprotonated O-alkyl carbons and nonpolar alkyl protons is evidence of their association with CRAM structures. Overall, CRAM moieties represented the predominant constituents in both the HPOA (63-77%) and TPIA (54-78%) isolates. The intensity of the C-O resonance in the 64-100 ppm range (Figure 2) indicates that CRAM in TPIA is more oxygen-rich than CRAM in HPOA, consistent with the fact that TPIA isolates are more hydrophilic than corresponding HPOA isolates. Seasonally, CRAM was more enriched in the winter HPOA/TPIA and summer HPOA isolates, than in spring HPOA/TPIA isolates (Figure 7). For spring TPIA isolates the contribution from CRAM-like structures may be even lower than estimated as some COO groups very likely occur in carboxylated or acetylated carbohydrates.

4.1.3. Aromatics

Aromatic compounds in DOM are important for light absorption, metal binding, and photochemical reactivity. In addition, they are responsible for DOM optical properties (absorbance and fluorescence) and, as such, they are important for both remote and in situ sensing of DOM. The aromatic compound class group included contributions from aromatic C-H/C-C/C-O (Figure 6). The contribution of lignin-related compounds in the HPOA isolates was roughly estimated as seven times the fraction of OCH₃ assuming that each aromatic ring in lignin-related compounds is attached to an -OCH₃ substituent, and was < 3.5% in all HPOA isolates. The aromatic components decreased in abundance from spring to summer and winter HPOA/TPIA (Figure 7). The carbon percentages of aromatics from our NMR analyses were linearly correlated to SUVA₂₅₄ ($r^2 = 0.97$; Figure S8), consistent with Weishaar et al. (2003). Some of the aromatic moieties in the HPOA isolates could be derived from lignin, as

characteristic peaks associated with lignin were evident in the spring HPOA and visible in the summer HPOA samples. However, lignin related compounds accounted for only ~3.5% of organic carbon in HPOA isolates based on NMR estimates. Aromatic C abundances were lower in TPIA isolates than in HPOA isolates, but were still considerable (12-17%) and followed the same seasonal trend as those in HPOA isolates. No OCH₃ peak was observed in the TPIA isolates, indicating the insignificance of chemically recognizable lignin in this fraction. This is consistent with a previous study that showed the majority (90–91%) of lignin phenols in the Yukon River was recovered in the HPOA fraction, but only 7% in the TPIA fraction (Spencer et al., 2008). This indicates the possibility of other aromatic carbon sources. For instance, tannins, flavonoids, and other aromatic compound classes associated with plant leaves and vegetative litter might also contribute to phenol-rich aromatics in HPOA/TPIA isolates. Leenheer and Rostad (2004) identified a Suwannee River fulvic acid sub-fraction derived from condensed tannins, through the use of combined fractionation and non-degradative characterization methods such as ¹³C NMR. In addition to the phenol peak at around ~150 ppm, the most diagnostic peak for condensed tannins is from aromatic C at ~107 ppm in dipolar-dephased ¹³C NMR spectra, indicative of C-substituted aromatic carbons between meta-substituted C-O linkages in the A-ring of condensed tannins (Preston et al., 1997; Wilson and Hatcher, 1988). The dipolar-dephased ¹³C NMR spectra of all HPOA/TPIA isolates in the present study (Figure 2(d,h), thin black line and gray dotted line) showed signals in this region, which partially arose from nonprotonated aromatic C, therefore suggesting tannins as potential aromatic precursors.

4.1.4. Dissolved black carbon

Recently, dissolved black carbon (DBC), characterized by prevalent hydrogen deficient molecules with a large aromaticity index, has been identified by Fourier transform ion cyclotron resonance mass spectrometry throughout the oceans (Dittmar and Koch, 2006; Dittmar and Stubbins, 2014). DBC, quantified by the benzenepolycarboxylic acids method, was reported to be between 0.1 and 17.5% of DOC (average $\pm$ SD of $7.1 \pm 3.6\%$) in global rivers including major Arctic rivers such as the Yukon (Jaffé et al., 2013). Our NMR data indicated that nonprotonated aromatic carbons represented the largest fraction of aromatic C, nearly the sum of protonated and O-substituted aromatic C, in all HPOA/TPIA isolates (Table 2). Nevertheless, nonprotonated aromatic carbons also occur in heavily substituted individual rings. Chemical shift can help to distinguish different types of nonprotonated aromatic carbons. The interior carbons in fused aromatic rings of black carbon resonate between 125-132 ppm (Mao et al., 2012a), while most nonprotonated C at the edge of aromatic rings have resonances outside this range. Substitution with O shifts the peak to >140 ppm, and to <125 ppm for second carbon neighbors, while a single bond to carbon in a substituent results in an aromatic-carbon chemical shifts between 132 and 142 ppm. These aromatic components are partially resolved in ^1H - ^{13}C HETCOR after dipolar dephasing (Figure 4(a)). This spectrum of winter HPOA showed the main cross peak between aromatic C and alkyl protons at a carbon frequency near 140 ppm, while the nonprotonated aromatic C near aromatic H resonate closer to 129 ppm, as expected for black carbon. The quantitative dipolar-dephased spectra of HPOA and TPIA isolates appeared to have a black-carbon component resonating near 130 ppm, accounting for less than half of the nonprotonated C not near O, or $\sim 5\%$ and 3% of all C, respectively. DBC therefore comprised $< 4\%$ of total DOC, at the low end of the concentrations reported previous from the benzenepolycarboxylic acids method (Jaffé et al., 2013). Some of the nonprotonated aromatic

structures might arise from low-temperature (100-400 °C) combustion by-products during tundra and forest fires, which have structural characteristics closer to slightly thermally altered organic matter (“brown carbon”) than condensed polycyclic aromatic structures (Anderson et al., 2014; Myers-Pigg et al., 2015). For instance, Myers-Pigg et al. (2015) reported the occurrence of dehydroxyabietic acid, a by-product of thermally altered conifer resin, in major Siberian Arctic rivers, and found such pyrogenic carbon to be particularly labile.

4.1.5. N-containing compounds

Nitrogen-containing compounds (i.e., carbons bonded to nitrogen) were estimated from C/N elemental contents assuming that each N atom was attached to two C atoms. N-containing compounds could account for up to 3% of organic carbon in HPOA isolates and 5-7% in TPIA isolates, as estimated from elemental C/N ratios. Previous ¹⁵N NMR studies of aquatic DOM indicated that secondary amides are the dominant nitrogen moieties, potentially present in the form of N-acetyl amino polysaccharides, protein amino acids, and unhydrolyzable amides (Aluwihare and Meador, 2008; Mao et al., 2012b). N-acetyl amino polysaccharides could be present in the spring TPIA isolates. Other nitrogen-containing moieties, such as protonated pyrrole or indole N, anilide N, identified in coastal and marine DOM (Mao et al., 2012b), can also contribute.

4.2. New insights into DOM composition in Arctic rivers

Our non-target, essentially quantitative NMR analyses of two major DOM isolates (HPOA and TPIA, together accounting for 64-74% of DOC) from the Yukon River across three distinct seasons provide a more comprehensive picture of DOM composition than possible in earlier

studies. Previous studies of Arctic River DOM have focused on analyses of specific components, such as amino acids (Meon and Amon, 2004), carbohydrates (Gueguen et al., 2006), or lignin phenols (Amon et al., 2012; Spencer et al., 2008). Our analyses shed more light on some of these compound classes while providing additional information about the presence of CRAM, which proved to be the predominant DOM component across different seasons (Figure 7). CRAM have been identified in various aquatic environments including oceans (Hertkorn et al., 2006) and temperate rivers and lakes (Lam et al., 2007; Leenheer, 1994; McCaul et al., 2011; Zigah et al., 2014). To the best of our knowledge, the present study is the first to report the occurrence and to provide reliable quantification of CRAM in Arctic rivers. These CRAM components accounted for the majority of DOM in HPOA (63-77%) and TPIA (54-78%) samples (Figure 7), and comprised at least 41%, and in summer and winter more than 49%, of total DOC in the Yukon River. Furthermore, as a novel finding from our selective and 2D NMR data we have identified nonprotonated O-alkyl and quaternary alkyl C (OC_{np} , $OC_{np}O$ and C_q , 13-17% of HPOA and 15-20% of TPIA) as well as some O-CH structures associated with CRAM, which have not been recognized in some prevailing CRAM models (including all isomers in Fig. 13 of Hertkorn et al. (2006)). Notably, nonprotonated O-alkyl (OC_{np} and $OC_{np}O$) structures are only rarely reported in the DOM literature (Helms, 2012; Leenheer et al., 2003; Mao et al., 2012b).

In addition, spectral editing and 2D NMR allowed for the discrimination of carbohydrate O-alkyl C from non-carbohydrate O-alkyl C, which was previously not achievable using 1D NMR alone, and resulted in inaccurate estimates of carbohydrate fractions from NMR. Our results show that only spring freshet TPIA samples were composed of carbohydrate clusters, suggesting larger carbohydrates, such as carboxylated or acetylated carbohydrates (16% and 26%), whereas

TPIA samples from other seasons or HPOA samples contained O-CH dispersed in a nonpolar alkyl environment suggesting the presence of aliphatic glycosides, released as secondary metabolites by plants (Freeman and Beattie, 2008).

4.3. Implications for the fate of DOM exported to the Bering Sea by the Yukon River

The chemical composition of DOM represents an important control on DOM processing, determining its susceptibility to different removal mechanisms such as microbial and photochemical degradation, and thus strongly influences the fate of terrestrial DOM exported by the Yukon River. In the following we explore, from the point of view of the major structural components identified in DOM, the fate of DOM exported to the Bering Sea by the Yukon River in different seasons.

The present study has revealed that CRAM are a major fraction of the DOM in all seasons (> 54% of HPOA/TPIA), particularly in summer and winter periods (Figure 7). The alicyclic structures of CRAM are thought to be resistant to biological oxidation, while the absence of chromophores in CRAM confers resistance to degradation by UV radiation (Leenheer, 1994). The nonprotonated C-O and O-C-O structures associated with CRAM may add to CRAM stability. Such nonprotonated C-O and O-C-O carbons have been proposed as a potential index of humification (Mao et al., 2012b). Specifically, nonprotonated O-C-O carbons have been shown to be present and preserved in biologically refractory carbohydrates (Helms, 2012). Therefore, the predominance of CRAM, with their associated nonprotonated O-alkyl structures, suggests a substantial fraction of relatively stable DOM exported by the Yukon River across different seasons. As they are less susceptible to removal mechanisms such as bio- and photo-

degradation compared to other molecules such as carbohydrates, amino acids, and lignin phenols, CRAM may survive on time scales long enough to reach, and persist, in the Arctic Ocean.

Amon et al. (2012) analyzed the lignin phenol and *p*-hydroxylbenzene composition of Arctic riverine DOM to identify major sources of organic matter to fluvial networks. They pointed to boreal-vegetation-derived leachates as primary DOM sources during spring flood, and peat/moss-derived DOM during low flow periods as indicated by the elevated levels of *p*-hydroxylbenzenes (most abundant in mosses and peat) (Amon et al., 2012). The reported yields of lignin phenols in DOM from the Yukon River were actually < 1% (Amon et al., 2012; Spencer et al., 2008), which agrees with our conclusion that lignin is a minor component (~ 3.5% of HPOA, or ~ 2% of DOC). The predominance of CRAM in DOM from the Yukon River, as well as their detection at high concentration in various aquatic systems in temperate and tropical regions (Hedges et al., 1992; Lam et al., 2007; Leenheer, 1994; Leenheer et al., 2003; McCaul et al., 2011; Zhang et al., 2014; Zigah et al., 2014), suggests that boreal vegetation and peat/moss are not the dominant or immediate sources of a large fraction of the DOM from the Yukon River. CRAM do not resemble any abundant plant biomolecules and have never been detected in plants. Even pine terpenes such as abietic acid and their oxidation products have NMR spectra distinct from CRAM (Leenheer et al., 2003). Rather, CRAM appear to be of microbial origin (Leenheer et al., 2003). A previous study using optical properties identified a seasonal shift from a fresh terrestrial DOM source during peak flow to a more degraded terrestrial DOM pool with a greater microbial imprint during base flow conditions (Walker et al., 2013). Also it has been reported that bacterial DOM produced in bioassay experiments was composed of 30–36% CRAM (Lechtenfeld et al., 2015).

Compared to summer and winter DOM, spring DOM was more enriched in lignin residues (within the HPOA fraction) and carbohydrates, particularly within the TPIA fractions (Figures 2 and 7), which represent contributions from fresh organic materials. This is consistent with observations from previous studies, which showed maximum concentrations of lignin phenols during the spring flush period (Amon et al., 2012; Spencer et al., 2008), and decreased dissolved carbohydrate concentrations from spring to summer/fall (Gueguen et al., 2006). The HPOA isolates in our study had the following order of increasing apparent age: spring DOM, summer DOM, and winter DOM, which agrees with previously published data for DOC collected from the Yukon River at the Pilot Station site during 2004-2005, and with HPOA isolates collected from other locations in the Yukon River basin (Aiken et al., 2014; O'Donnell et al., 2014). These data support the assertion that fresher, less degraded organic matter, likely from the litter and O-horizons of soils in the Yukon River Basin, are major sources of DOM exported in the spring. DOM exported by the Yukon River in spring would therefore be most bio- and photo-reactive, as reported for the Russian Arctic (Siberia's Kolyma River) during the spring freshet (Mann et al., 2012) and the North American Arctic (Kuparuk, Sagavanirktok, and Colville rivers) (Holmes et al., 2008), due to their increased carbohydrates and soluble aromatics.

Furthermore, prior studies have observed that the percent biodegradable DOC increased with greater contribution from so called protein-like fluorophores, the relative abundances of which were typically greatest in winter or immediately before ice breakup and lowest during spring and summer (Mann et al., 2012; Wickland et al., 2012). The N-containing compounds estimated from C/N ratios appear to be most abundant in winter TPIA isolates, but they are insignificant and show no seasonal variability for HPOA isolates (Figure 7). It should be pointed out that while past incubation studies investigated unfractionated DOC, the present study focused on DOM

isolates from adsorption on XAD resins (representing 64-74% of DOC), which may not efficiently retain proteins.

Taken together, our results imply that DOM exported to the Bering Sea by the Yukon River in spring is most susceptible to both microbial and photochemical processes, due to their increased carbohydrates and aromatics. The export of this more reactive organic matter in the spring period is substantial given the increased concentrations during this period, combined with greatly increased river discharge (Striegl et al., 2007). For instance, combining discharge and DOC concentration data for our samples, the instantaneous fluxes of DOM for the spring samples were almost 50 times those for the winter samples and 4 times those for the summer. However, the prevalence of CRAM across all seasons, and their highly substituted alicyclic structure with characteristic nonprotonated alkyl and O-alkyl carbons, underline the relatively stable nature of DOM exported by the Yukon River to the Bering Sea.

5. CONCLUSIONS

Advanced 1D and 2D NMR spectroscopy has provided a more comprehensive picture of, and gained new insights into, DOM composition across different seasons in the Yukon River. CRAM accounted for the majority of DOM in HPOA (63-77%) and TPIA (54-78%) samples, and was more abundant in winter and summer than in spring samples. To our knowledge, this is the first report that identifies and reliably quantifies CRAM in an Arctic river. 2D and selective NMR data revealed association of abundant nonprotonated O-alkyl and quaternary alkyl C (OC_{np} , $OC_{np}O$ and C_q , 13-17% of HPOA and 15-20% of TPIA) and isolated O-CH structures with CRAM, which were not recognized in some previous studies of CRAM. Spectral editing and 2D NMR also enabled the discrimination of carbohydrate O-alkyl C from non-carbohydrate O-alkyl

C, which was previously not achievable using 1D NMR alone. While spring freshet TPIA samples contained carbohydrate clusters such as carboxylated carbohydrates (16% and 26%), the sugar rings in TPIA samples from other seasons or HPOA samples were mostly present in small amounts (< 8%) and likely associated with aliphatic glycosides. The aromatic fraction and lignin signatures (< 17% in HPOA isolates) decreased in abundance from spring to summer and to winter samples. Dissolved black carbon comprised at most 5% and 3% of organic carbon in HPOA and TPIA isolates, respectively. Our results imply the relatively stable nature of DOM exported by the Yukon River across different seasons, due to the predominance of CRAM and their associated nonprotonated C-O and O-C-O structures, even though spring DOM will exhibit elevated biolability and photoreactivity as a result of the presence of fresh terrestrial inputs and aromatic structures.

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Table 1. Seasonal Variations in DOC Concentrations, SUVA₂₅₄, and Major Chemical Fractions of DOM, Ash-Free Elemental Contents, and Isotopic Data of HPOA Fractions, in the Yukon River at Pilot Station (2008-2009).

Sampling Date	Season	DOC (mg C L ⁻¹)	SUVA ₂₅₄ (L mg C ⁻¹ m ⁻¹)	Fraction	% of DOC	SUVA ₂₅₄ (L mg C ⁻¹ m ⁻¹)	Ash-free elemental contents (%) and C isotopic data (‰)							
							C	H	O	N	S	δ ¹³ C	Δ ¹⁴ C	Age (ybp)
07/01/2008	Summer	5.9	3.3	HPOA	54	3.8	55.2	4.5	38.8	1.0	0.5	-27.81	19.6	>Modern
				TPIA	16	2.9	ND	ND	ND	ND	ND	ND	ND	ND
08/12/2008	Summer	7.0	3.2	HPOA	56	3.7	52.8	4.8	41.1	0.9	0.4	-27.67	26.7	>Modern
				TPIA	18	2.8	48.2	4.2	44.5	1.6	1.5	ND	ND	ND
09/24/2008	Summer	5.4	3.0	HPOA	49	3.4	54.0	4.6	40.0	0.9	0.6	-27.57	6.2	>Modern
				TPIA	18	2.4	ND	ND	ND	ND	ND	ND	ND	ND
01/21/2009	Winter	3.3	2.4	HPOA	51	2.8	54.9	4.9	38.3	1.3	0.6	-27.46	-71.1	530
				TPIA	18	2.1	ND	ND	ND	ND	ND	ND	ND	ND
03/26/2009	Winter	2.7	2.2	HPOA	47	2.8	52.7	5.1	40.9	0.9	0.4	-27.1	-111.5	890
				TPIA	17	2.1	48.2	4.8	44.0	2.0	1.0	ND	ND	ND
05/12/2009	Spring	5.7	3.5	HPOA	51	3.9	51.8	4.9	42.1	0.9	0.4	-28.32	6.1	>Modern
				TPIA	17	2.6	47.4	4.8	44.8	1.6	1.5	ND	ND	ND
05/22/2009	Spring	17.3	3.3	HPOA	57	3.9	52.1	5.0	41.7	0.9	0.3	-28.1	92.2	>Modern
				TPIA	16	2.8	48.2	4.6	45.1	1.5	0.7	ND	ND	ND
05/29/2009	Spring	14.9	3.6	HPOA	52	4.1	52.7	4.6	41.4	0.9	0.3	-28.12	82.1	>Modern
				TPIA	15	2.9	49.1	4.3	44.3	1.5	0.7	ND	ND	ND

DOC is dissolved organic carbon, and SUVA₂₅₄ is specific UV absorbance determined at 254 nm. HPOA is hydrophobic acid, and TPIA is transphilic acid. ND, not determined. ybp, years before present.

Table 2. Integrated Areas (in %) from ^{13}C MultiCP NMR Spectra (Total Area = 100%) for HPOA and TPIA Samples Across Different Seasons, and the Structural Moieties Associated with the Spectral Regions. Values >10% are highlighted in bold. Uncertainty (based on signal to noise ratio, reported as one standard deviation, and shown in Table S2) is $\leq 1\%$ except 1-2% for Arom. C-H and CH_2/CH regions.

Samples		ppm										
		220-190	190-160	160-143	143-100		123-100 (OCO)		100-64 (O-C)		64-0 (Alkyl C)	
		Ketone C	COO/ NC=O	Arom. C-O	Arom. C-C ^a	Arom. C-H	OC _{np} O ^b	OCHO ^b	O-C _{np} ^c	OCH _n	C _q ^c	CH ₂ /CH
HPOA	Summer 08/12/2008	3.1	17.3	6.0	11.3	6.0	3.0	0.3	8.0	9.3	5.1	20.3
	Winter 03/26/2009	2.7	17.1	4.4	9.3	5.4	2.5	0.1	8.4	8.6	5.9	23.8
	Spring ^a 05/12/2009	2.9	16.5	6.9	11.9	7.7	4.0	1.3	5.3	10.9	4.7	18.5
	Spring ^a 05/22/2009	2.8	16.1	6.8	12.2	7.2	3.5	1.2	5.3	11.2	4.7	19.6
TPIA	Summer 08/12/2008	3.1	20.7	4.1	7.6	3.8	3.9	0.3	11.4	13.2	4.2	20.0
	Winter 03/26/2009	2.8	20.2	2.8	5.8	3.5	3.5	0.4	11.9	13.3	4.7	21.3
	Spring ^a 05/12/2009	2.1	17.6	3.7	7.1	3.1	4.3	4.7	7.0	21.8	3.7	16.5
	Spring ^a 05/22/2009	2.7	20.0	4.6	8.2	3.9	5.3	2.6	7.1	17.1	3.2	17.6

^a For 05/12/2009 and 05/22/2009 HPOA/TPIA samples, a modified spectral range of 143-93 ppm is assigned to aromatic C-C and aromatic C-H carbons, and ranges of 123-93 ppm and 93-64 ppm are assigned to OCO and O-C carbons, respectively.

^b Based on CSA-filtered CP/TOSS spectrum and CSA-filtered CP/TOSS spectrum after dipolar dephasing.

^c Based on multiCP spectrum after 68- μs dipolar dephasing.

Figure Captions

Figure 1. Map of the Yukon River basin showing the sampling site location.

Figure 2. Solid-state ^{13}C multiCP NMR spectra (bold black line) and multiCP after dipolar dephasing (thin black line) obtained for HPOA and TPIA samples across different seasons. Dipolar dephasing shows primarily nonprotonated and highly mobile carbons. Two CP/TOSS ^{13}C NMR spectra after CSA-filter (gray line, see (d, h)) are shown as examples, for demonstrating the separation of anomeric C (gray shaded area) from aromatic C. Two CP/TOSS ^{13}C NMR spectra after CSA-filter and dipolar-dephasing (gray dotted line, see (d, h)) are shown to demonstrate the separation of nonprotonated anomeric C from nonprotonated aromatic C. All multiCP spectra are scaled to match the intensity of the highest band.

Figure 3. 2D ^1H - ^{13}C HETCOR spectra with 0.5-ms HH-CP of (a) 05/22/2009 HPOA and (b) 03/26/2009 HPOA. (c, d) ^1H slices extracted from the 2D spectra; (c) refers to ^1H slices from spectrum (a), and (d) from spectrum (b). Arrows indicate contributions by alkyl protons.

Figure 4. 2D ^1H - ^{13}C HETCOR spectrum with 0.5-ms HH-CP and 40- μs dipolar dephasing (a) and extracted ^1H slices (b) of 03/26/2009 HPOA. Arrows indicate major contributions from alkyl protons.

Figure 5. 2D ^1H - ^{13}C HETCOR spectra with 0.5-ms HH-CP of (a) 05/22/2009 TPIA and (b) 03/26/2009 TPIA. (c, d) ^1H slices extracted from the 2D spectra; (c) refers to ^1H slices from spectrum (a), and (d) from spectrum (b). Arrows indicate contributions from alkyl protons.

Figure 6. Relative percentages of different specific functional groups for HPOA and TPIA samples across different seasons, and assignments of these functional groups to different compound class groups (carbohydrate, CRAM-like, aromatics, and N-containing). The

998 percentages of carbons bonded to nitrogen (C-N) were estimated from elemental C/N content
999 based on the assumption that each N is bonded to two carbons.

1000 **Figure 7.** The relative carbon percentages of the four compound class groups (carbohydrate,
1001 CRAM-like, aromatics, and N-containing) within HPOA (a), and TPIA (b) isolates.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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