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Molecular level characterization of methyl sugars in marine high molecular weight dissolved organic matter

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ABSTRACT

Dissolved organic matter (DOM) is the largest active organic carbon reservoir in the ocean (662 GT C), a major fraction (>95%) of which remains chemically uncharacterized. The concentration and isolation of DOM from seawater by ultrafiltration facilitates its chemical characterization by spectroscopic techniques. Using ultrafiltration, silver cation preparative chromatography and gas chromatography coupled with mass spectrometry (GC-MS), we identified 50 novel sugar compounds after hydrolysis of the high molecular weight dissolved organic matter fraction (HMWDOM; the fraction of DOM isolated after ultrafiltration). Sugars were identified by comparison of their mass spectra with those of chemically synthesized standards and with spectra previously described in the literature. Our results showed that mono- and di-methylated hexoses; mono- and di-methylated pentoses; mono- and di-methylated 6-deoxysugars, as well as heptoses, methylated heptoses, 3,6-dideoxysugars and 1,6 anhydrosugars (levoglucosan, mannosan, and galactosan) are components of HMWDOM, which may explain the low apparent yields of sugars recovered by molecular level (HPLC) analyses of HMWDOM after hydrolysis.

From three depths spanning the surface (15 m) to bathypelagic (1800 m) ocean in the North Pacific near Hawaii our results showed that mono- and di-methylated hexoses were most abundant in the surface sample (64% of the total identified methylated sugar compounds), while at 1800 m monomethylated 6-deoxysugars were the dominant sugars (42% of the total identified methylated sugar compounds). The high diversity of mono- and di-methylated hexoses in the surface sample most likely suggests an algal and/or bacterial source, while the high abundance of methylated 6-deoxy hexoses in the deep sample points toward an important bacterial contribution because the latter sugars are mostly found in bacterial lipopolysaccharides as well as highly degraded organic material.

1. Introduction

More than 98% of organic carbon in the ocean is sequestered as dissolved organic carbon (DOC), which is defined as the fraction of carbon that passes through a 0.1–0.7 μm filter and is nonvolatile at $\text{pH} \leq 2$. Although several studies have acknowledged the important role of DOC in a variety of oceanic processes (Carlson, 2002; Bronk, 2002; Nelson and Siegel, 2002; Mopper and Kieber, 2002; Wells, 2002) affecting as such the global carbon cycle (Hansell et al., 2009), much less is known about its origins and chemical composition. Dissolved organic matter (DOM) includes a wide range of constituents that have different compositions, reactivities and ages (Benner, 2002; Repeta and Aluwihare, 2006; Eglinton and Repeta, 2004). Only a small

part of this organic matter is recycled on short time scales, while the largest part (especially the deep-water DOM) is sequestered for long periods of time (Williams and Druffel, 1987; Druffel et al., 1992; Hedges, 1992; Bauer, 2002). Currently, only 4–5 GT C of DOC (~10% of DOC) are characterized at the molecular level as a sum of amino acids, lipids and sugars in surface waters (Benner, 2002). In deep waters the amount of characterized DOC is about 34 GT C (<5% DOC).

Seawater concentrations of dissolved amino acids, simple sugars, and lipids are in the low nanomolar range and are very close to the detection limits of the analytical techniques employed for their characterization. Most DOM is thought to be more complex than the simple biomolecules measured by direct analysis. To access and characterize this fraction of DOM, particularly in the presence of high concentrations of sea salt, a number of isolation techniques including tangential flow ultrafiltration, solid-phase extraction, dialysis, size-exclusion chromatography, and electrodialysis combined with reverse osmosis (Tedetti et al., 2006; Mopper et al., 2007 and references therein; Vetter et al., 2007) have been explored.

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Organic matter isolates have been characterized by very powerful analytical tools such as direct temperature-resolved chemical ionization (DT-MS), Fourier transform ion cyclotron mass spectrometry (FT-ICR-MS), high field nuclear magnetic resonance (NMR) spectroscopy, as well as high performance liquid chromatography (HPLC) coupled to Fourier transform infrared spectroscopy (HPLC-FT-IR) thereby providing important insights into DOM chemical composition (Minor et al., 2002; Panagiotopoulos and Sempéré, 2005 and references therein; Kujawinski and Behn, 2006; Koch et al., 2008; Reemtsma et al., 2008; Hockadey et al., 2009; Bhatia et al., 2010; Landry and Tremblay, 2012; Minor et al., 2012). For example, Boon et al. (1998) used DT-MS, to show the presence of amino sugars, furfurals, and methylated sugars in marine ultrafiltered high molecular weight dissolved organic matter (HMWDOM). Other studies using solid state NMR and FT-ICR-MS showed the presence of carboxylic rich alicyclic molecules (CRAM) as important refractory components of the DOM pool (Hertkorn et al., 2006; Woods et al., 2010).

All of these studies reported general structural characteristics of complex DOM. Detailed information of component molecules can complement this approach, and provide insight into subtle chemical transformations that may help to explain and to understand organic matter characteristics (e.g. complexity, apparent age, interactions with organisms etc.) (Repeta and Aluwihare, 2006; Goldberg et al., 2011; Hansell et al., 2012).

Previous studies have shown that most HMWDOM in the upper ocean is comprised of carbohydrates (acylated carbohydrates or APS), but only a minor fraction of the carbohydrate has been identified at the “molecular level” (as monosaccharides after hydrolysis, i.e. fucose, rhamnose, arabinose, galactose, glucose, mannose, and xylose; Fig. 1) (Benner et al., 1992; Aluwihare et al., 1997; Repeta et al., 2002). The discrepancy between NMR derived estimates of HMWDOM carbohydrate (50–70% C) and molecular level analyses (10–20% C) has not been explained, and no new sugars have been identified in HMWDOM in about 10–15 years (McCarthy et al., 1996a; McCarthy et al., 1998; Aluwihare et al., 1997). The carbohydrate component (APS) of the HMWDOM is water soluble (hydrophilic), exhibits similar $\Delta^{14}\text{C}$ values with the surface dissolved inorganic carbon (DIC), and it is composed from sugar polymers (poly-, oligosaccharides) (Repeta and Aluwihare, 2006; Panagiotopoulos et al., 2007).

In a recent study, Panagiotopoulos et al. (2007) isolated a methyl sugar fraction from the acid hydrolysis products of HMWDOM (APS-F₃; Fig. 1) and identified new methylated sugars by 1-D and 2-D NMR spectroscopy. Because NMR required μmoles of sample at the time, only the most abundant methyl sugars of the samples were identified.

In this study, we further characterized the APS-F₃ component of HMWDOM in terms of methyl sugar composition using gas

chromatography mass spectrometry (GC-MS). To do so we first chemically synthesized methylated sugar standards which were then used to identify most of the APS-F₃ methyl sugar components. Here we report the presence of a diverse suite of sugars and methyl sugars within the HMWDOM collected from 3 depths in the North Pacific Ocean. The distributions and the possible sources of these compounds are also discussed.

2. Materials & methods

2.1. HMWDOM sampling

Epipelagic (15 m), mesopelagic (900 m), and bathypelagic (1800 m) seawater samples were collected from the North Pacific Ocean (15 m; 19° 43' N; 156° 03' W, 900 m and 1800 m; 24° 25' N, 156° 18' W) in December 2003 (15 m sample) and June 2002 (900 m and 1800 m samples). The samples were filtered to remove bacteria and small particles using a cleaned (10% HCl) Suporflow dual stage (0.8 μm and 0.2 μm) Gelman polyether sulfone cartridge filter (Pall Corp.) fitted to an Advanta stainless steel housing. HMWDOM samples were collected using a large volume cross flow ultrafiltration system consisting of a stainless steel centripetal pump and membrane housings and a fluorinated high density polyethylene reservoir. The system was plumbed with Teflon tubing and fitted with PVDF valves. We used two ultrafiltration membranes (90 ft² each; Separation Engineering, Escondido, CA) that nominally retain organic matter of a molecular weight greater than 1000 Da (>98% rejection of vitamin B₁₂). Membranes were cleaned using isopropanol, detergent (0.01% micro), HCl (0.01 N) and NaOH (0.01 N), stored in sodium azide (0.55 mM), and rinsed with water and seawater immediately before use. The two filters were plumbed in parallel. Between 2763 L (900 m sample) and 8640 L (15 m sample) of seawater was concentrated over several days to approximately 40 L, frozen and returned to Woods Hole for further processing. The HMWDOM samples were concentrated to 2 L using a smaller system fitted with a 10 ft² membrane, desalted by repeated (10 \times) diafiltration with high purity, low carbon, deionized water (Milli-Q water, 2 L), and reduced to 2 L. The final concentrate gave no visible precipitate (AgCl) when an aliquot of permeate was mixed with a 5 mg/50 ml solution of AgNO₃. After diafiltration was complete, the sample (2 L) was lyophilized to a fluffy off-white powder that was 24–35% by weight carbon with a C/N ratio of 13–16.

2.2. Sample preparation

HMWDOM samples (200–300 mg) were hydrolyzed in 2 M trifluoroacetic acid (TFA) at 120 °C under a helium atmosphere. Approximately 14.5 g of TFA was added to 43 g of water in a 250 mL round bottom flask. The solution was heated to temperature and the HMWDOM was added as a solution in approximately 2–3 mL of water. After 4 h, the dark brown solution was cooled, frozen at –20 °C, and freeze-dried. The dried sample was dissolved in 2–3 mL of water and applied to a 6–8 mm i.d. column packed with 4 g anion exchange resin (OH[–] form, Bio-Rex® 5 resin, Bio-Rad Laboratories, Hercules, CA). The column was preconditioned by rinsing with 15 mL of NaOH (0.5 M) followed by Milli-Q water until the pH returned to approximately 7. The column was drained of water and the sample was applied. Carbohydrates were eluted with 20–40 mL of water, collected, concentrated to a small volume (few mL) by rotary evaporation and freeze dried. The recovery of HMWDOM (by weight) after hydrolysis and ion exchange was 85–90%.

2.3. Chromatography

Chromatography was performed on a Waters pump (Model 510) and a Shodex RI-71 refractive index detector. Sample injection was performed with a manual Rheodyne injector fitted with a 100 μL sample loop. Carbohydrates were separated by the degree of

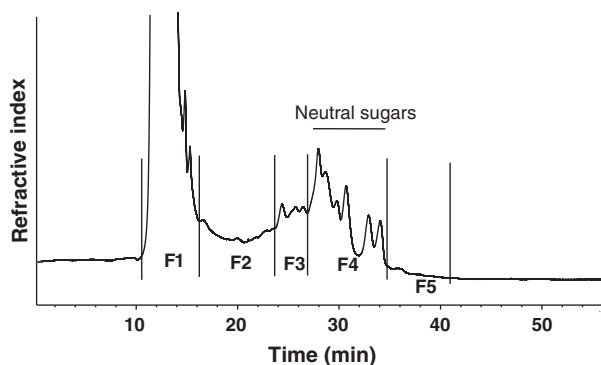


Fig. 1. Silver ion chromatography of the APS component of the HMWDOM from the 1800 m sample. Sugars were separated on two Ag⁺ cation exchange columns connected in series at 80 °C and eluted with high purity water at 0.5 ml min^{–1}. The detection was made by refractive index (RI) and the fractions F₁–F₅ were collected manually for further chemical characterization.

polymerization on two sulfonated styrene–divinylbenzene gel analytical columns (30 cm × 7.8 mm i.d.; particle size 8 μm; cross linking 8%) in the silver form (Supelcogel™ Ag, Supelco, Inc., Bellefonte PA) connected in series. The columns were placed into a column heater (Tiberline TL-50) set at 80 °C. Sugars were eluted with water at a flow rate of 0.5 mL min⁻¹. Prior to use, the mobile phase was degassed with He for 30 min. The total weight of the sample injected onto the column did not exceed 10 mg/injection. Retention times for neutral sugars and oligosaccharides were determined from authentic fucose, rhamnose, arabinose, galactose, glucose, mannose, xylose, and maltooligosaccharides (DP 2–7). Fig. 1 depicts a typical chromatogram of a hydrolyzed APS sample. The suite of the seven neutral sugars (fucose, rhamnose, arabinose, galactose, glucose, mannose, and xylose) were identified within the APS-F₄ fraction, while the APS-F₃ fraction contained the methylated-, and the methylated deoxy-sugars (Panagiotopoulos et al., 2007). The different carbohydrate fractions (F₁–F₅) were collected manually for further chemical characterization. The sugars within the APS-F₃ fraction were reduced and acetylated (see below) to produce alditol acetate derivatives which were further analyzed by GC–MS.

2.4. Preparation of partially methylated alditol acetates (PMAAs)

2.4.1. Acquisition and preparation of partially methylated methyl glycosides

Sugar standards of fucose, rhamnose, arabinose, glucosamine, galactose, mannose, xylose, ribose, quinovose (6-deoxy glucose), 2-deoxy ribose, gluco-heptose, and manno-heptose were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sugars were methylated according to the protocol of Ciucanu and Kerek (1984) with some important modifications (Fig. 2). Briefly 10 mg of a standard sugar was dissolved in 1 mL of 40 mg mL⁻¹ NaOH/DMSO. The mixture was well vortexed for 1 min. Then 0.1 mL of MeI was added and the solution was centrifuged for 4 min. After 11 min at ambient temperature, 1 mL of Milli-Q water and 1 mL of CH₂Cl₂ were added to the solution. The organic layer (lower) was transferred in a second vial and the CH₂Cl₂ was removed under a stream of N₂. In contrast to the protocol of Ciucanu and Kerek (1984) that produces fully permethylated sugars which are further recovered in the CH₂Cl₂ phase, we found that the aqueous phase contained most of the mono-, di-, and tri-methylated sugars. Therefore, the aqueous layer was not discarded but kept and freeze dried to remove the excess of DMSO and the water. The dried residue was extracted with a few mL of ethyl acetate, combined with the CH₂Cl₂

extract, filtered through quartz wool (precombusted at 500 °C for 3 h), and dried under a stream of N₂.

2.4.2. Reduction and acetylation of methylated standard sugars

Partially methylated methyl glycosides obtained in the previous step were dissolved in 1 mL of 2 M TFA and hydrolyzed for 2 h at 100 °C (Wang et al., 2007). This step converts the ketal group of sugars to hemiacetal and thus facilitates the subsequent step of reduction. The methylated standard sugars obtained after treatment with TFA were reduced and acetylated to their corresponding alditol acetate derivatives according to the protocol of Panagiotopoulos and Wurl (2009). This procedure (reduction and acetylation) was applied as well to our APS-F₃ samples prior to their GC–MS analysis (Fig. 2). Briefly, in a 4–5 mL vial sugar samples/standards were dissolved in 0.25 mL of 1 M NH₄OH/NaBH₄ (or NaBD₄). The solution was flushed with N₂, and kept at room temperature for a minimum of 1 h. The reaction was quenched with a few drops of glacial acetic acid until bubbling ceased. Acetic acid was removed by adding 0.5 mL methanol and the solution was evaporated under N₂. This last step was repeated three times. The dry alditols were acetylated with 100 μL of acetic anhydride and 20 μL of 1-methyl imidazole. The sample was mixed thoroughly and let stand for 15 min. Excess acetic anhydride was quenched with 0.5 mL of Milli-Q water. After 10 min at room temperature, 0.5 mL of CH₂Cl₂ was added and the solution was vortex-mixed. The organic layer containing the alditol acetates was transferred to a 4 mL clean vial and dried over anhydrous sodium sulfate. The solution was then filtered through quartz wool (precombusted at 500 °C for 3 h) and the organic layer was transferred to another clean 4 mL vial. The CH₂Cl₂ was removed under a stream of N₂. The alditol acetates were then dissolved in ethyl acetate (1–2 mL) and analyzed by GC–MS. All methylated sugar standards and samples were run in duplicate. Alditol acetate derivatives are stable for 3–4 months when kept at 4 °C in the dark.

2.5. Gas chromatography mass spectroscopy (GC–MS)

Standards of PMAA and APS-F₃ samples were analyzed using an Agilent 5975C mass spectrometer connected to a 6850 gas chromatograph operating in electron impact mode. The following operating conditions were employed: 30 m × 0.25 mm (i.d.) fused silica capillary column coated with DB-5 (5% phenyl-methylpolysiloxane; film thickness 0.20 μm). The following conditions were used: oven temperature programmed from 90 to 150 °C at 10 °C min⁻¹ after a delay of 1 min and then from 150 to 230 °C at 2 °C min⁻¹; helium

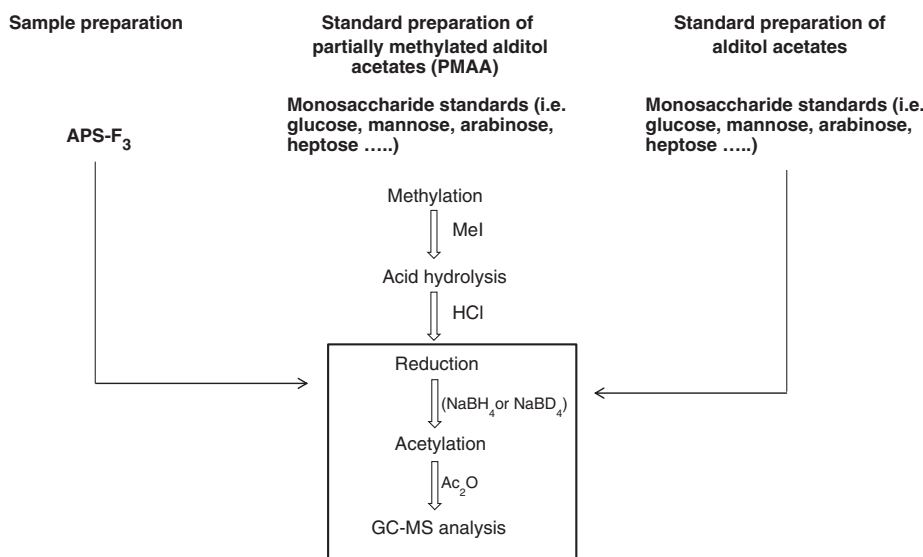


Fig. 2. Summary of the experimental procedures used in this study.

was used as carrier gas at 1 ml min⁻¹; injector (pulsed splitless) temperature, 250 °C; electron energy, 70 eV; source temperature, 230 °C; and cycle time, 0.2 s. Alditol acetates were eluted between 10 and 30 min (Fig. 3). Data acquisition and processing were performed with Agilent Chem-Station software (Agilent Technologies).

3. Results and discussion

3.1. Identification and interpretation of PMAAs mass spectra

Gas chromatography–mass spectrometry is a powerful tool for the partial characterization of simple sugars. However, epimeric hexoses (glucose, mannose, and galactose) and their derivatives (methyl- and deoxy-hexoses), which are abundant in HMWDOM, display nearly identical mass spectra, and can only be distinguished by their chromatographic behavior. For example, GC–MS can easily distinguish hexoses methylated at different positions (2-OMe vs 6-OMe) since they exhibit different fragmentation patterns, but cannot distinguish 2-OMe glucose and 2-OMe-mannose without comparing retention times to authentic compounds. We therefore performed partial chemical synthesis of PMAA to generate a chromatographic and spectral database of the methylated derivatives for each of the major monosaccharides found in APS.

The elution order of PMAAs depends generally on their degree of methylation (Table 1; Fig. 3). Under the chromatographic conditions used in our analysis, permethylated sugars (which are less polar) eluted first followed by tetra-, tri-, di-, and mono-methylated sugars (Sasaki et al., 2005; Wang et al., 2007). Fig. 3 depicts a typical TIC (Total Ion Current) chromatogram of the partially methylated galactose. Previous studies indicated that PMAAs mass spectra are characterized by primary and secondary fragments (Bjørndal et al., 1967). Primary fragments result from the fragmentation of the parent alditol, while secondary fragments arise from primary fragments after the elimination of acetic acid (*m/z* 60), ketene (*m/z* 42), MeOH (*m/z* 32), CH₂O (*m/z* 30) or acetic anhydride (*m/z* 102). It is worth noting that PMAAs mass spectra do not display the molecular ion (Fig. 4). The identification of standard PMAAs was made by the comparison of mass spectra with literature data (Sasaki et al., 2005;

Wang et al., 2007). To confirm PMAAs structure attributions, the reduction step was also performed using sodium borodeuteride (NaBD₄). This reagent induces an asymmetry in the alditol acetates through the introduction of deuterium at the reducing carbon and allows the discrimination of symmetrical derivatives such as 2,3-OMe₂ glucose and 4,5-OMe₂ glucose; 3-OMe glucose and 4-OMe glucose (Sasaki et al., 2005). In general, for each monosaccharide 10–14 methylated derivatives were identified (Fig. 3).

3.2. Identification and distribution of sugars in the APS-F₃ samples

Sugars in our APS-F₃ samples were identified by comparing their respective retention time and mass spectra to the authentic standards of PMAA. Likewise, we identified most of the major peaks within the 12–30 min chromatographic window, while several unknown peaks appeared at >30 min (see below; Fig. 5). In total, we identified 38 methylated monosaccharides (3 methylated heptoses, 19 methylated hexoses, 6 methylated pentoses, and 10 methylated 6-deoxy hexoses), one heptose, two 2-amino 6-deoxy hexoses (fucosamine and quinovosamine), three 1-6 anhydro hexoses (galactosan, levoglucosan, and mannosan), one 2-deoxy pentose (xylose or arabinose), two 3,6-dideoxy hexoses (3-deoxy fucose and 3-deoxy rhamnose) and two tetroses (Table 2; Fig. 6). Due to the imperfect separation between fractions F₃ and F₄, we also found within the APS-F₃ fraction the seven major neutral sugars identified in the APS-F₄ fraction, where they prevailed (Repeta and Aluwihare, 2006). Unidentified compounds eluting between 10 and 40 min represented 32, 45, and 24% of the total chromatogram (integrated area) for the 15, 900, and 1800 m samples, respectively (Fig. 5). Unidentified peaks at >30 min most likely correspond to mono- and di-methylated heptoses as well as monomethylated amino sugars. Based on their characteristic fragments however, their structures could not be fully resolved.

3.2.1. Methylated sugars

The methylated sugar fraction APS-F₃ accounted between 2 and 3% of the total integrated area of the sample chromatograms (Fig. 1). Relative area provides a semi-quantitative estimate of the contribution of methyl sugars to APS as it does not account for likely differences in

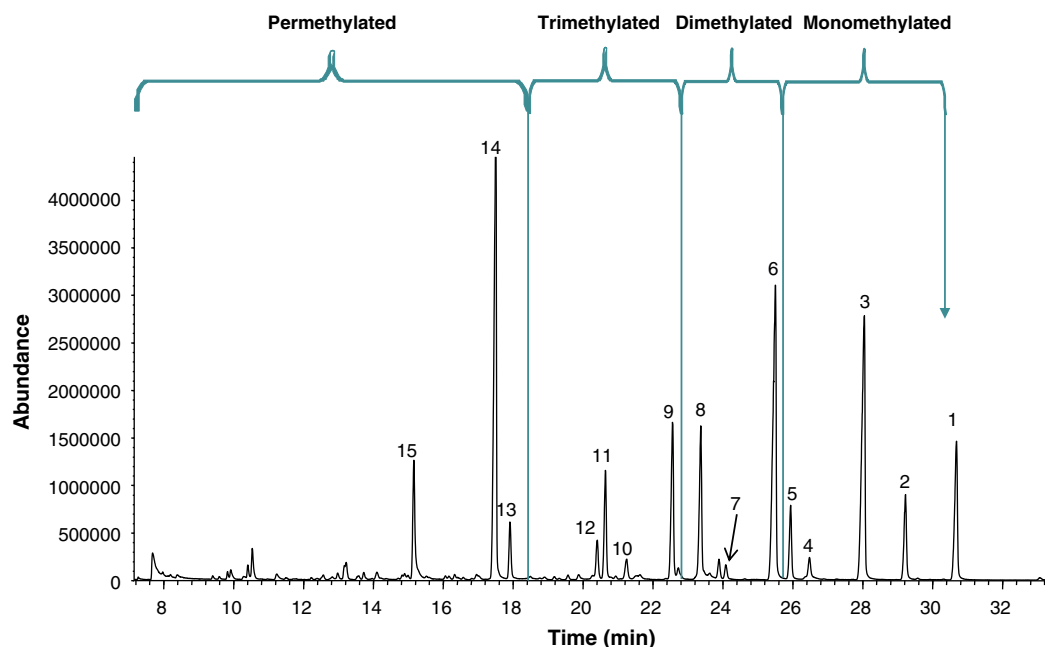


Fig. 3. Chromatogram of a standard of PMAAs of galactose. The number on each peak corresponds to the compound name given in Table 1. Peaks were identified as a function of their mass spectra and comparison with literature data (Sasaki et al., 2005; Wang et al., 2007).

Table 1

Partially methylated alditol acetates (PMAAs) of galactose identified by GC-MS. Abbreviations: Gal.: galactose, RT: retention time.

Peak no	PMAA galactose standard	RT (min)	Characteristic fragments ^a
1	Gal.	30.69	85, 115, 145, 170, 187, 217, 259, 289, 361
2	3-O-Me Gal. ^b	29.24	85, 87, 99, 127, 129, 159, 189, 261, 333
3	2-O-Me Gal.	28.05	87, 97, 117, 129, 139, 159, 231, 259, 333
4	2,4-OMe ₂ Gal.	26.48	87, 101, 117, 129, 139, 159, 189, 233, 305
5	6-O-Me Gal.	25.93	87, 97, 115, 129, 139, 157, 184, 259, 333
6	2,3-OMe ₂ Gal.	25.50	85, 87, 99, 101, 117, 127, 159, 187, 201, 261
7	3,6-OMe ₂ Gal.	24.07	87, 99, 113, 129, 189, 233
8	2,6-OMe ₂ Gal.	23.36	58, 87, 101, 117, 129, 143, 185, 231, 305
9	2,3,5-OMe ₃ Gal.	22.58	85, 101, 117, 127, 143, 159, 201, 233
10	3,4,6-OMe ₃ Gal.	21.20	74, 87, 101, 117, 129, 143, 161, 189, 233
11	2,3,6-OMe ₃ Gal.	20.66	87, 99, 101, 117, 129, 131, 161, 173, 233
12	2,5,6-OMe ₃ Gal.	20.41	59, 87, 89, 97, 117, 129, 143, 185, 231, 305
13	2,3,4,6-OMe ₄ Gal.	17.90	71, 87, 101, 117, 129, 145, 161, 205
14	2,3,5,6-OMe ₄ Gal.	17.51	59, 89, 101, 117, 129, 143, 161, 205
15	2,3,4,5,6-OMe ₅ Gal.	15.17	59, 87, 89, 101, 117, 131, 161, 175

^a Typical fragments (unit m/z) used for structural identification of PMAAs.

^b For simplicity we indicated 3-O-methyl galactose, nevertheless the full name of this compound is 1,2,4,5,6-penta-O-acetyl-3-O-methyl galactitol. The same holds for all the compounds identified the standard including those found in the sample.

the refractive index of major neutral sugars (Section 2.3), methyl sugars and polysaccharides. Interestingly enough, methylated 6-deoxysugars also occur in the unhydrolyzed APS-F₁ polymer (Panagiotopoulos et al., 2007). Quan and Repeta (2007) using periodate oxidation also showed the presence of methylated sugars within the APS pool, however as MeOH (end product of -OMe group after periodate oxidation) was not measured, the total amount of methylated sugars in APS was not estimated. Currently, we do not know the exact contribution of the methylated sugar component in the whole APS pool and the HMWDOM in general.

The most abundant methylated sugars in the surface sample were the 2-O-methyl glucose (10% of the total APS-F₃ sugars), 3-O-methyl rhamnose

(6%), 2,4 di-O-methyl galactose (6%), 4-O-methyl glucose (5%), and 2-O-methyl rhamnose (5%) in agreement with previous results (Panagiotopoulos et al., 2007). At 1800 m depth, the major methylated sugars were the 2-O-methyl fucose (24%), 2-O-methyl galactose (18%), 2-O-methyl rhamnose (5%), 2-O-methyl glucose (4%), and 2,4 di-O-methyl galactose (4%). Trimethylated and permethylated sugars were not detected in our samples indicating that mono-, and di-methylated sugars prevail in APS pool.

Previous investigations have indicated changes in the relative amounts of dissolved, hydrolyzable neutral sugars with depth in different oceanic basins (Benner et al., 1992; McCarthy et al., 1996a; McCarthy et al., 1998; Skoog and Benner, 1997; Sempéré et al., 2008; Kaiser and Benner, 2009; Goldberg et al., 2011) and we find similar trends in unidentified and methyl sugars within the APS-F₃ fraction. A large body of work on carbohydrate concentrations in seawater indicates that combined neutral sugar concentrations, total sugar yields (total sugar-C/DOC%), and mol % glucose, galactose and mannose + xylose are higher in surface vs deep waters pointing to a recent accumulation of DOM (Skoog and Benner, 1997; Sempéré et al., 2008; Kaiser and Benner, 2009; Goldberg et al., 2011). Our results on methylated sugar composition are in agreement with these observations indicating that surface APS-F₃ sugars are a less diagenetically altered material. Finally, our findings on methylated sugar composition may explain in part the discrepancy between the amount of total carbohydrate measured colorimetrically and that of neutral carbohydrates measured by HPLC since methylated sugars were never included in HPLC or GC measurements (Borch and Kirchman, 1997; Benner, 2002).

The number of methylated sugars decreased in our samples from 36 in the surface sample, to 26 at 900 m and 25 at 1800 m. Mono-, and di-methylated hexoses in particular accounted for 41% and 24%, respectively of the total methyl sugars in the surface sample, whereas these percentages dropped to 35% and 12% for the 1800 m sample (Fig. 7). These results indicate that there is a net loss of diversity and relative mass of mono- and di-methylated hexoses with depth.

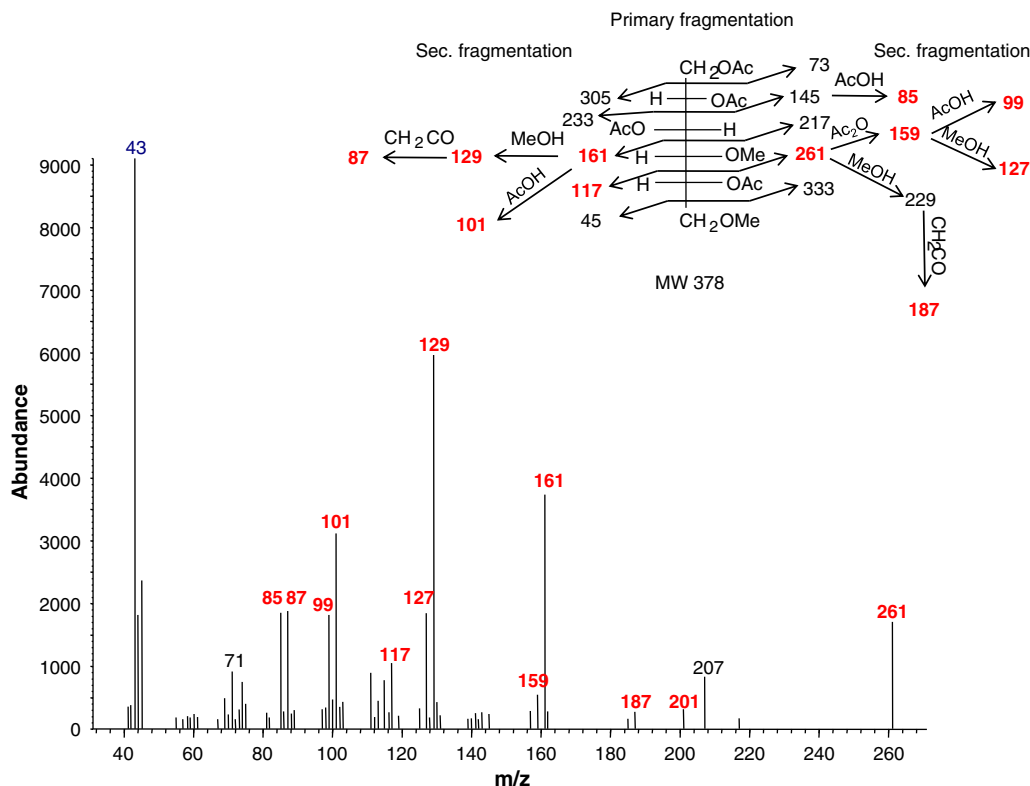


Fig. 4. Mass spectra and fragmentation patterns (primary & secondary fragmentation) of 4,6-OMe₂ mannose.

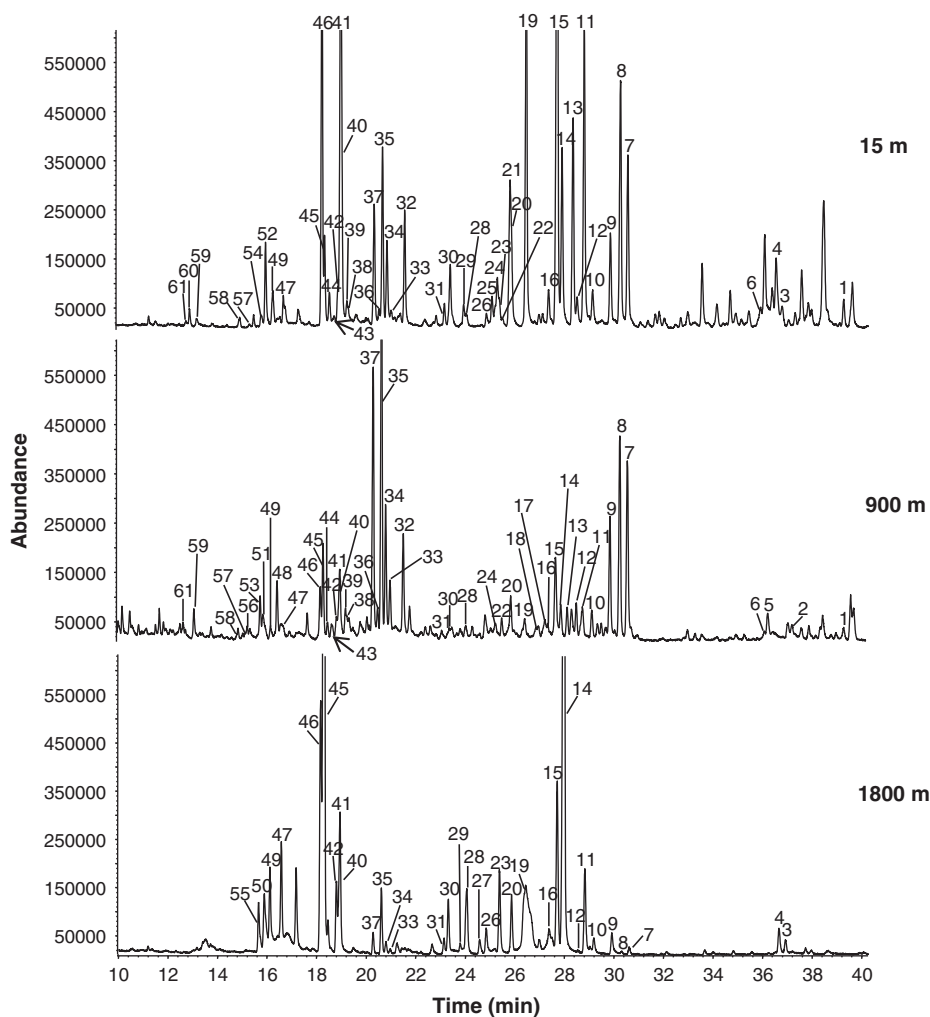


Fig. 5. GC-MS chromatograms for the APS-F₃ sample at 15, 900, and 1800 m depths. The numbers on the peaks correspond to the compounds in Table 2.

Although the mechanisms responsible for these changes with depth are not clear, microbial demethylation may be active (Carpenter et al., 2004; Neufeld et al., 2008; McCarren et al., 2010).

On the other hand, monomethylated 6-deoxy hexoses increased with depth from 23% to 43% of total APS-F₃ sugars in the surface and deep samples, respectively (Fig. 7). This increase in relative abundance may suggest either the selective preservation of monomethyl 6-deoxysugars during APS degradation, or the addition of these sugars by mesopelagic bacteria. Monomethylated 6-deoxysugars and 6-deoxysugars (i.e. fucose, rhamnose, quinovose) have been reported in bacterial lipopolysaccharides (Table 3) and/or highly degraded material in soils as part of humic and fulvic substances (Ogner, 1980).

3.2.2. Non-methylated sugar compounds

Non-methylated sugar compounds included heptoses, tetroses, anhydrosugars, 3,6-dideoxy-hexoses, 2-deoxy-pentoses, 2-amino 6-deoxy hexoses, a branched monosaccharide (yersiniose), and 6-deoxy hexose (quinovose). With the exception of quinovose, our results showed that these compounds were mainly found in the mesopelagic sample (900 m) where they accounted 6% of the total APS-F₃ sugars (Table 2). Galactosan was the most abundant non-methylated sugar compound accounting 1.2% of the total identified sugars followed by levoglucosan, quinovose, and 2-deoxy pentose which contributed about equally (~0.9%).

3.3. Sources and biogeochemical importance of methylated sugars

Methylated sugars have been found in sediment trap samples (Bergamaschi et al., 1999), diatom frustules (Chiovitti et al., 2005), coccoliths (e.g. *Emiliania huxleyi*; Fichtinger-Schepman et al., 1979), and mucilage material (Evans et al., 1974). Klok et al. (1984) reported a plethora of 52 methylated sugars in sediments from the Black Sea and Namibian continental shelf (Table 3). However, the full structure of some of these compounds was not completely resolved (tentative identification). Other studies showed that methylated sugars are common components of plant cell walls (Popper et al., 2004) and bacterial lipopolysaccharides, where they account for 1–4% of the cell dry weight (Weckesser et al., 1970; Choma et al., 1987; Fox, 1999).

Currently very little is known about the origins and the biogeochemical role of methylated sugars within HMWDOM. Previous studies using DT-MS showed the presence of methylated, deoxy and N-acetyl amino sugars within HMWDOM which were attributed to marine prokaryote sources (Boon et al., 1998). Other studies using ¹H and ¹³C NMR showed similar functional group distributions between seawater HMWDOM and HMWDOM produced by *E. huxleyi*, *Phaeocystis* sp., *Thalassiosira weissflogii*, and *Chaetoceros neogracile* (Aluwihare et al., 1997; Aluwihare and Repeta, 1999; Repeta et al., 2002; Gogou and Repeta, 2010). In agreement with these observations, other investigations showed that *E. huxleyi* and *Chlamydomonas eugametos* (biflagellate unicellular green alga) cell walls are composed

Table 2
Identified monosaccharides within the APS-F₃ fraction. In bold all the new compounds. Abbreviations: Man.: Mannose; Gal.: Galactose; Glc.: Glucose; Xyl.: Xylose; Ara.: Arabinose; Fuc.: Fucose; Quin.: Quinovose; Rha.: Rhamnose; Rib.: Ribose; GlcN.: Glucosamine; GalN.: Galactosamine; FucN.: Fucosamine; QuiN.: Quinovosamine; RT.: Retention time.

Peaks	Monosaccharides	RT (min)	Depth (m)	Characteristic fragments (m/z)
1	Mannoheptose	39.41	15–900	85, 115, 145, 157, 187, 259, 289, 361, 433
2	GalN.	37.29	900	84, 102, 139, 144, 156, 193, 259, 318
3	2-OMe heptose^a	36.92	15–1800	73, 99, 117, 145, 159, 169, 201, 261, 303, 405
4	2-OMe heptose^a	36.64	15–1800	73, 99, 117, 145, 159, 169, 201, 261, 303, 405
5	GlcN	36.31	900	84, 102, 139, 144, 156, 193, 259, 318
6	2-OMe mannoheptose	36.18	15–900	73, 99, 117, 145, 159, 169, 201, 261, 303, 405
7	Gal.	30.64	15–900–1800	85, 115, 145, 170, 187, 217, 259, 289, 361
8	Glc.	30.36	15–900–1800	85, 115, 145, 170, 187, 217, 259, 289, 361
9	Man.	29.90	15–900–1800	85, 115, 145, 170, 187, 217, 259, 289, 361
10	3-OMe Gal.	29.20	15–900–1800	87, 99, 129, 145, 189, 217, 261, 333
11	4-OMe Glc.	28.89	15–900–1800	87, 99, 127, 129, 159, 189, 201, 217, 261, 333
12	3-OMe Man.	28.57	15–900–1800	87, 99, 127, 129, 159, 189, 201, 217, 261, 333
13	3-OMe Glc.	28.43	15–900	87, 99, 127, 129, 159, 189, 201, 217, 261, 333
14	2-OMe Gal.	28.01	15–900–1800	87, 97, 117, 129, 139, 159, 231, 259, 289, 333
15	2-OMe Glc.	27.76	15–900–1800	87, 97, 117, 129, 139, 159, 231, 259, 289, 333
16	2-OMe Man.	27.41	15–900–1800	87, 97, 117, 129, 139, 159, 231, 259, 289, 333
17	FucN.^b	27.27	900	60, 84, 102, 117, 144, 201, 231, 242, 260, 302
18	QuiN.^b	26.90	900	60, 84, 102, 117, 144, 201, 231, 242, 260, 302
19	2,4-OMe₂ Gal.	26.41	15–900–1800	87, 101, 117, 127, 129, 139, 159, 189, 233, 305
20	6-OMe Glc./6-OMe Gal.	25.90	15–900–1800	87, 115, 129, 139, 157, 184, 231, 259, 289
21	2,4-OMe₂ Man.	25.82	15	87, 101, 117, 127, 129, 139, 159, 189, 233, 305
22	6-OMe Man.	25.51	15–900	87, 115, 129, 139, 157, 184, 231, 259, 289
23	2,3-OMe₂ Gal.	25.40	15–1800	87, 101, 117, 127, 129, 159, 189, 233, 261
24	2,4-OMe₂ Glc.	25.30	15–900	87, 101, 117, 129, 139, 159, 189, 233, 305
25	2,3-OMe₂ Glc.	25.08	15	87, 101, 117, 127, 129, 159, 189, 233, 261, 305
26	2,3-OMe₂ Man.	24.85	15–1800	87, 101, 117, 127, 129, 159, 189, 233, 261, 305
27	Yersiniose^c	24.60	1800	95, 109, 113, 143, 155, 189, 215
28	3,6-OMe₂ Gal./3,6-OMe₂ Man.	24.03	15–900–1800	45, 87, 99, 113, 129, 159, 189, 233
29	3,6-OMe₂ Glc	23.92	15–1800	45, 87, 99, 113, 129, 159, 189, 233
30	2,6-OMe₂ Glc./2,6-OMe₂ Gal.	23.38	15–900–1800	87, 97, 117, 129, 143, 159, 231, 281, 305
31	2,6-OMe₂ Man.	23.14	15–900–1800	87, 97, 117, 129, 143, 159, 231, 281, 305
32	Xyl.	21.52	15–900	85, 103, 115, 127, 145, 158, 170, 187, 217, 289
33	Qui.	20.96	15–900–1800	99, 103, 115, 128, 145, 157, 170, 217, 289, 303
34	Ara.	20.80	15–900–1800	85, 103, 115, 127, 145, 158, 187, 217, 289
35	Fuc.	20.60	15–900–1800	99, 103, 115, 128, 145, 157, 170, 217, 289, 303
36	Rib.	20.48	15–900	85, 103, 115, 127, 145, 158, 187, 217, 289
37	Rha.	20.28	15–900–1800	99, 103, 115, 128, 145, 157, 170, 217, 289, 303
38	4-OMe Fuc.	19.23	15–900	85, 87, 99, 129, 131, 143, 159, 189, 261
39	3-OMe Fuc.	19.16	15–900	87, 101, 117, 129, 143, 159, 189, 203, 261
40	3-OMe Xyl.	18.97	15–900–1800	87, 99, 117, 129, 159, 189, 203, 261
41	3-OMe Rha.	18.95	15–900–1800	87, 101, 117, 129, 143, 159, 189, 203, 261
42	2-OMe Xyl.	18.80	15–900–1800	85, 87, 99, 117, 127, 159, 201, 261
43	4-OMe Rha.	18.65	15–900	85, 87, 99, 129, 131, 143, 159, 189, 261
44	2-OMe Ara.	18.42	15–900	85, 87, 99, 117, 127, 159, 201, 261
45	2-OMe Fuc.	18.29	15–900–1800	87, 117, 129, 141, 159, 173, 201, 231, 275
46	2-OMe Rha.	18.16	15–900–1800	87, 117, 129, 141, 159, 173, 201, 231, 275
47	2,4-OMe₂ Fuc.	16.59	15–900–1800	89, 101, 117, 131, 159, 173, 187, 201, 233
48	Galactosan^d	16.39	900	81, 98, 115, 157, 186, 229, 243
49	2,3-OMe₂ Fuc.	16.14	15–900–1800	69, 89, 101, 117, 131, 143, 203
50	2,4-OMe₂ Ara.	15.90	1800	87, 99, 101, 117, 129, 159, 189, 233
51	2-deoxy Xyl./2-deoxy Ara.	15.87	900	99, 117, 129, 142, 145, 159, 184, 231
52	2,3-OMe₂ Rha.	15.85	15	69, 89, 101, 117, 131, 143, 203
53	Levoglucozan^d	15.70	900	81, 98, 115, 157, 186, 229, 243
54	2,4-OMe₂ Xyl.	15.68	15	87, 99, 101, 117, 129, 159, 189, 233
55	2,3-OMe₂ Ara.	15.67	1800	71, 87, 99, 101, 117, 129, 189, 233
56	Mannosan^d	15.18	900	81, 98, 115, 157, 186, 229, 243
57	3-deoxy Fuc.^e	15.10	15–900	74, 85, 87, 115, 142, 157, 184, 245
58	3-deoxy Rha.^e	14.78	15–900	74, 85, 87, 115, 142, 157, 184, 245
59	Tetrose^a	13.02	15–900	55, 73, 115, 145, 217
60	2,3,4,5-OMe₄ Rha.	15.74	15	74, 87, 101, 114, 129, 141, 157, 217
61	Tetrose^a	12.55	15–900	55, 73, 115, 145, 217

^a Isomers.

^b 2-Amino 6-deoxy hexoses. Mass spectra data from Fox et al. (1998).

^c 3,6-Dideoxy-4-C-(1-hydroxyethyl)-D-xyllo-hexose. Mass spectra data from Gorshkova et al. (1984).

^d 1,6-Anhydro-hexoses. Mass spectra data from Simkovic et al. (2003).

^e 3,6-Dideoxy-hexoses.

of methylated sugars pointing to a phytoplanktonic source (Fichtinger-Schepman et al., 1979; Schuring et al., 1987).

Our results together with literature data suggest that the surface HMWDOM sample has algal and bacterial components, based on the

polydisperse diversity of the methylated sugar compounds, while the deep HMWDOM sample could have a more pronounced bacterial contribution due to the high abundance of monomethylated 6-deoxysugars which are often found in bacterial lipopolysaccharides

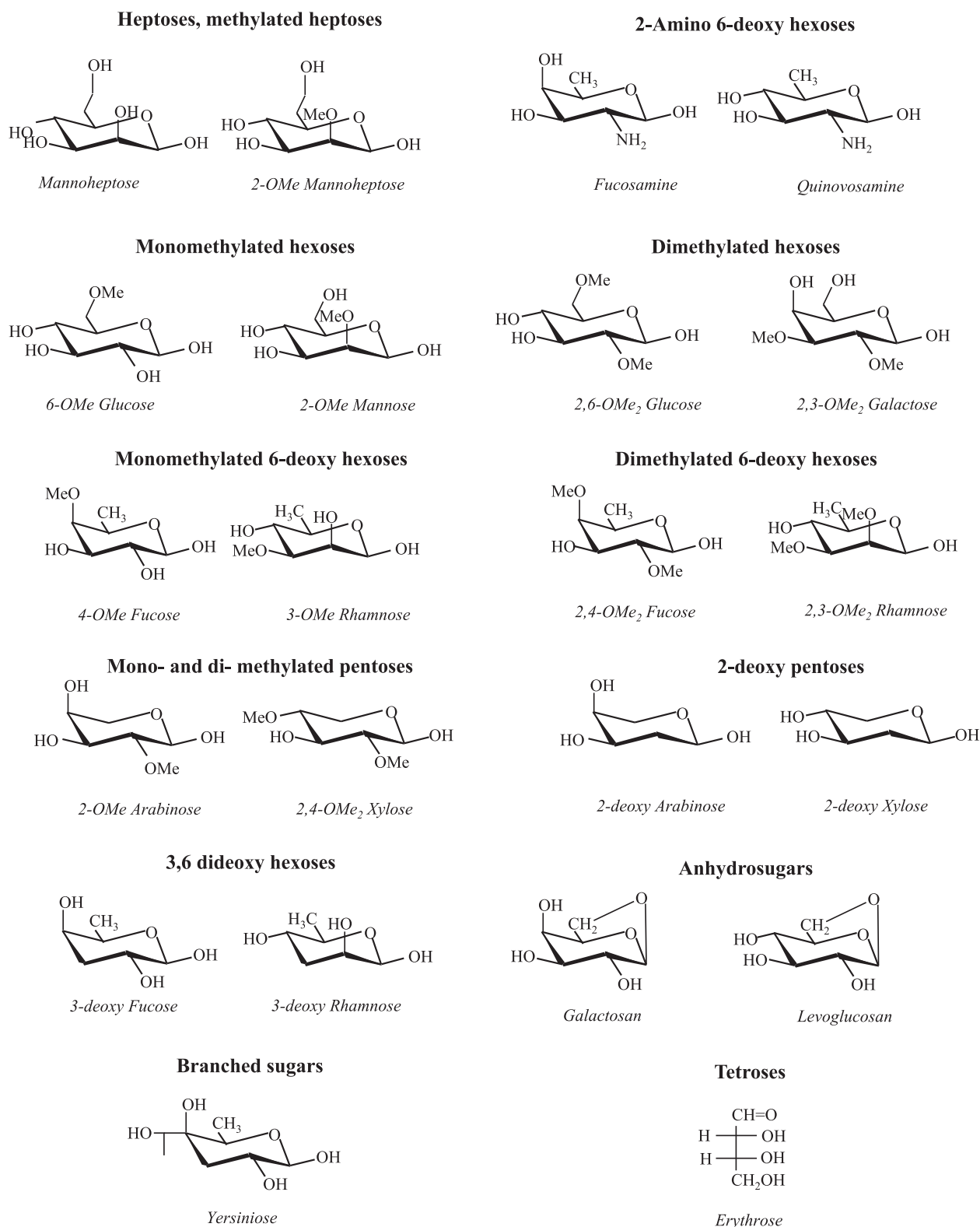


Fig. 6. Chemical structures of some of the identified compounds in the APS-F₃ fraction. The whole list of compounds is given in Table 2 along with their respective retention time and mass spectrum fragments.

(Table 3). Previous investigations based on amino acid measurements in HMWDOM also showed that HMWDOM contains algal and bacterial components and are in agreement with the present study (McCarthy et al., 1998; Dittmar et al., 2001; Jones et al., 2005; Kaiser and Benner, 2008).

3.4. Sources and biogeochemical importance of non-methylated sugars

In contrast to methylated sugars, non-methylated sugars (quinovose, yersiniose, dideoxy hexoses, heptoses, deoxy-pentoses, amino deoxy hexoses) have been reported almost exclusively in O-antigenic

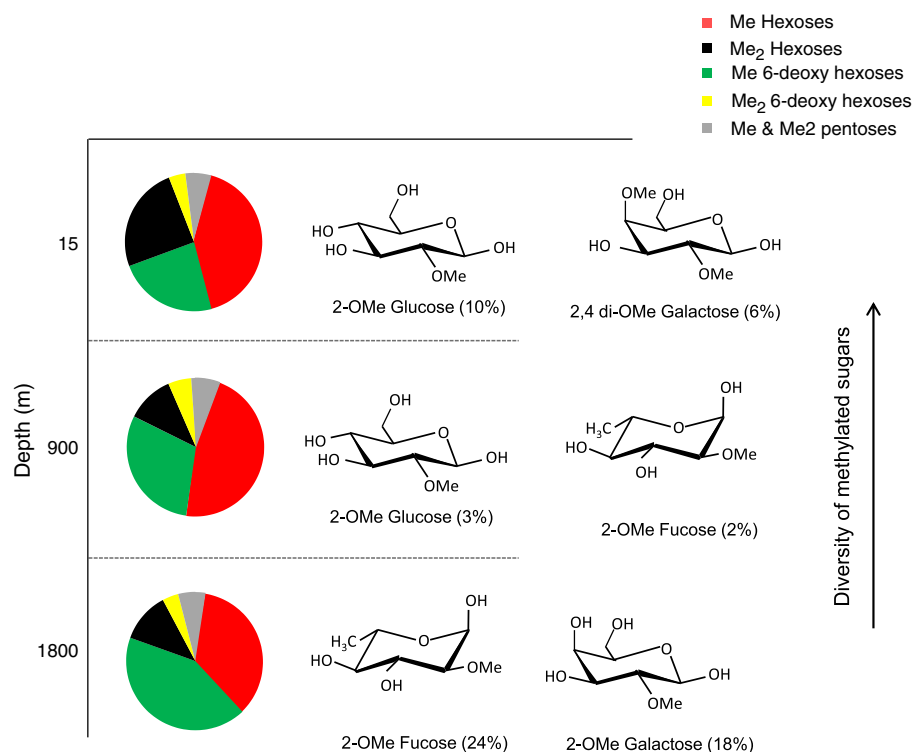


Fig. 7. Relative abundance of methylated sugars identified in this study at 15, 900, and 1800 m depths. Mono- and di-methylated hexose, 6-deoxy hexose, and pentose structures are given in Table 2. Mono- and di-methylated pentose results are pooled. The two most abundant sugars at each depth are also given.

polysaccharides of several species of gram-negative bacteria (Table 3). However, it is worth noting that the presence of these compounds in other organisms, such as planktons, algae, photosynthetic bacteria etc. has not been studied. Yersiniose has been reported in bacterial lipopolysaccharides of diazotrophs isolated from soils, roots, and plant leaves (Mattos et al., 2005), and further investigations are warranted to determine if it is also present in marine diazotrophs.

Anhydrosugars (levoglucosan, galactosan, and mannosan) were detected in the 900 m sample. These compounds are generally found in the airborne particulate matter as the thermal degradation products of cellulose (levoglucosan) and hemicelluloses (mannosan and galactosan) (Simoneit et al., 1999) and have never been detected in seawater. Reported levoglucosan concentrations in atmospheric samples over the ocean vary between 0.0008 and 0–15 ng m⁻³ (Simoneit and Elias, 2000), while > 1000 ng m⁻³ values were recorded in atmospheric samples of urban air sheds. Although we have detected anhydrosugars in the APS-F₃ fraction, our preliminary results suggest that anhydrosugars were concentrated in the APS-F₅ fraction (Fig. 2). Therefore, anhydrosugars were not quantified in this study. Nevertheless, these results indicate that the HMWDOM-900 m sample might include carbon from terrestrial biomass burning. Additional measurements of δ¹³C of pure levoglucosan in the sample and rigorous tests of the analytical protocol are necessary to establish the source of anhydrosugars in HMWDOM.

4. Summary and concluding remarks

This study showed the presence of a much broader suite of sugars (in total 50 compounds; Table 2; Fig. 6) within HMWDOM carbohydrate than previously recognized. Our results suggest that methyl sugars (APS-F₃) account for approximately 2–3% of APS, and that the relative distribution of sugars changes significantly with depth. Mono- and di-methylated hexoses and monomethylated 6-deoxysugars represent ~90% of the methylated sugars identified in our samples. Mono- and di-methylated hexoses dominated the surface sample (64% of the

total methylated compounds), while at 1800 m depth monomethylated 6-deoxysugars were the most abundant sugars (42% of the total methylated compounds) indicating a net loss of diversity and mass of methylated sugars with depth. Other sugar compounds identified in this study comprised the heptoses, deoxy hexoses (quinovose), amino deoxy hexoses, deoxy pentoses, dideoxy hexoses, anhydrosugars, and branched sugars (yersiniose) and accounted 1–6% of the total identified compounds.

Methylated-, deoxy-, amino deoxy-, dideoxy-sugars, and heptoses are broadly distributed within plants and bacteria and currently their presence in HMWDOM cannot provide information to distinguish specific sources of DOM (algal and bacterial contributions; Table 3). Nevertheless, very few studies have identified and quantified these compounds in marine samples (e.g. phytoplanktonic organisms, sediment trap material, prokaryotic bacteria) and therefore, their potential as biomarkers requires a more thorough examination of potential sources.

Much more work is left to be done on the polysaccharide component (APS) of HMWDOM. For example, the F₁ fraction (Fig. 1) is not hydrolyzed by strong acid, and has not yet structurally characterized. Nevertheless, ¹H NMR suggests that it is composed by 6-deoxysugars (Panagiotopoulos et al., 2007) including perhaps quinovose, identified in this study. Anhydrosugars, also appear to be a part of the HMWDOM carbohydrate fraction. Do they have a terrestrial or a marine origin?

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Table 3

Summary of methylated sugar markers, identified in different environmental sources as alditol acetates, on GC-MS analysis. In bold the compounds identified in this study. Same abbreviations as in Table 2. ManN.: Mannosamine; GlcA.: Glucuronic acid.; LPS.: Lipopolysaccharides. §19 out of 52 compounds were identified tentatively.

Compound	Species	Location	Environmental source	References
3-OMe Rha.	<i>Rhodospseudomonas capsulata</i>	LPS of bacterial cell walls (Gram-negative)	Bacterial strain	Weckesser et al., 1970
2-OMe Xyl.; 2-OMe Fuc.; 3-OMe Gal.; 4-OMe Gal.	<i>Sinapis alba</i> , <i>Zostera marina</i> , <i>Posidonia australis</i>	Polysaccharide	Leaves of deciduous trees	Bacon and Cheshire, 1971
2,3-OMe ₂ Xyl.; 2,4-OMe₂ Xyl.; 2,3,6-OMe ₃ Gal.; 2,4,6-OMe ₃ Gal.; 2,4-OMe₂ Gal.; 2,6-OMe₂ Gal.; 3,6 dideoxy Gal. 6-OMe GlcN.	<i>Rhodella maculata</i>	Polysaccharide	Extracellular mucilage of red alga	Evans et al., 1974
3-OMe Man. 3-OMe l-Rha. 3-OMe Rib. 2-OMe Gal. 2,3-OMe₂ Gal.	Photosynthetic bacteria, cyanobacteria	LPS of bacterial cell walls (Gram-negative)	Bacterial strain	Mayer et al., 1974
6-OMe D,L Man. 2,3-OMe₂ l-Rha. 3-OMe Xyl.	<i>Emiliana huxleyi</i>	Acidic polysaccharide	Coccoliths culture	Tharanathan et al., 1978
3,6 dideoxy hexose; 3-OMe Man.; 4-OMe Man.; ManN; 2,3,4- OMe ₃ Ara.	Cyanobacterium <i>Synechococcus</i>	LPS of bacterial cell walls (Gram-negative)	Bacterial strain	Fichtinger-Schepman et al., 1979
Mono-, di-, and tri-methylated 6-deoxysugars; di-, tri-, tetra-methylated hexoses and pentoses		Humic and fulvic acids	Soil	Schmidt et al., 1980
3,4-OMe ₂ Rha. 3-OMe Glc. 2-OMe Rha. 2,3-OMe₂ Fuc.	Mycobacterium	Glycopeptidolipid antigens	Serovar	Ogner, 1980
Mono, di, and tri- methylated 6-deoxysugars; 3,6 -dideoxy hexose; monomethylated pentoses; mono, di-methylated hexoses; heptose, 2-OMe heptose (Total 52 comp.)			Marine sediments	Brennan et al., 1981
2-OMe l-Rha 3-OMe l-Rha	<i>Spirulina platenesis</i>	Polysaccharide content	Culture of blue green algae	Klok et al., 1984 [§]
2-OMe Fuc.; 3-OMe Fuc.; 3-OMe Rha.; 2-OMe Gal.	Nitrogen-fixing bacteria	LPS of bacterial cell walls (Gram-negative)	Roots	Shekharam et al., 1987
4-OMe Xyl.; 2-OMe Ara.; 3-OMe Gal.; 6-OMe Man.; 3-OMe Glc.	<i>Chlamydomonas eugametos</i>	Surface-membrane-associated glycoconjugates	Strains of green algae	Choma et al., 1987
Mono methylated pentoses, hexoses and 6-deoxysugars Heptoses	<i>Helicobacter pylori</i> (bacteria)	LPS of bacterial cell walls (Gram-negative)	Peat core	Schuring et al., 1987
3-OMe Rha. 2-OMe Rha.	<i>Bacillus anthracis</i> , <i>Bacillus cereus</i>	Bacterial cell walls oligosaccharides des Gram-positive	Human stomach	Moers et al., 1990
Methylated sugars non-identified 2-OMe l-Rha. 3-OMe l-Rha.	<i>Phaeocystis</i> sp. <i>Chlorella vulgaris</i>	Acidic polysaccharide	Pathogenic organisms	Conrad et al., 1992
2,3,4-OMe ₃ Rha.; 2,3,4-OMe ₃ Ara.; 2,3,4-OMe ₃ Glc.; 2,3,4,6-OMe ₄ Glc	<i>Passiflora edulis</i> , <i>Passiflora edulis flavicarpa</i> , <i>Passiflora ligularis</i> , <i>Passiflora molissima</i>	Glycosylated conjugates Compounds	Mucus Chlorococcal algae	Fox et al., 1993
2-OMe Rha.; 2-OMe Fuc.; 3-OMe Rha.; 3-OMe Fuc. 3-OMe Xyl.; 2,3-OMe ₂ Xyl.; 6-OMe Glc.; 6-OMe Man.; 6-OMe Gal.; 2-OMe Glc.; 2-OMe Gal.; 2-OMe Man.; 3-OMe Man.			Fruit passion	Janse et al., 1996
Heptoses Quin. 3-OMe Rha.	<i>Salmonella typhimurium</i> <i>Bacillus subtilis</i> <i>Chara coralina</i> , <i>Coleochaete scutata</i> , <i>Klebsormidium flaccidum</i> , <i>Anthoceros caucasicus</i> , <i>Lycopodium pinifolium</i> , <i>Huperzia selago</i> , <i>Diphasiastrum alpinum</i>	LPS Spore polysaccharide Polysaccharides of cell walls	Sediment trap, sediments	Ogawa et al., 1997
Yersiniose	Nitrogen-fixing bacterium	LPS of bacterial cell walls (Gram-negative)	Human stomach	Chassagne et al., 1998
2-OMe Rha.; 3-OMe Rha.; 2,3-OMe₂ Rha.; 3-OMe Xyl.; 4-OMe Xyl.; 2-OMe GlcA	Diatom frustules	Polysaccharides of cell walls	Peat core	Moers et al., 1990
3-OMe Man.; 3-OMe Gal.; 4-OMe Gal.	<i>Arianta arbustorum</i> , <i>Achatina fulica</i> , <i>Arion lusitanicus</i> , <i>Planorbarius corneus</i>	N-glycans and O-glycans	Charophytic green algae, hornwort plant and lycopodiophytes	Conrad et al., 1992
			Soil & rhizosphere	Fox, 1999
			Bacterial strain	Fox, 1999
			Charophytic green algae, hornwort plant and lycopodiophytes	Popper et al., 2004
			Soil & rhizosphere	Mattos et al., 2005
			Algal cell cultures	Chiovitti et al., 2005
			Snails	Stepan et al., 2010

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