



Depth-related cycling of suspended nitrogen-containing lipids in the northeast Atlantic



Blaženka Gašparović^{a,*}, Abra Penezić^a, Richard S. Lampitt^b, Nilusha Sudasinghe^{c,d}, Tanner Schaub^c

^a Division for Marine and Environmental Research, Ruđer Bošković Institute, POB 180, HR-10002 Zagreb, Croatia

^b National Oceanography Centre, Southampton, United Kingdom

^c Chemical Analysis and Instrumentation Laboratory, College of Agricultural, Consumer and Environmental Sciences, New Mexico State University, Las Cruces, NM 88003, United States

^d Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, United States

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ABSTRACT

We utilized high resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to describe the depth-dependent distribution and molecular nature of nitrogen containing lipids (N-lipids) from suspended particles in an oceanic environment. Samples were collected at the Porcupine Abyssal Plain (PAP) sustained observatory in the northeast Atlantic (49.0°N, 16.5°W). Approximately 12.5% of FT-ICR MS observed lipids contain N. Only 19% of the lipids we recorded have elemental compositions that match those in the Nature Lipidomics Gateway database. Our results illustrate: (i) the proportional and selective accumulation of N-lipids with increased depth; (ii) that N-lipids which contain phosphorus are more stable than those without P; (iii) the majority of the deep Atlantic unsaturated N-lipids are highly unsaturated and (iv) there is depth-related increase in the saturated N-lipids which indicates that saturation is an important process for the export of lipid N and C to the deep ocean. These observations provide a description of N-lipid characteristics, transformation and preservation potential through the water column in the mesotrophic area of the North Atlantic Ocean.

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1. Introduction

Marine organic matter has a key role in the CO₂ sequestration capacity of the world's oceans. The major biochemical components of marine particulate matter are carbohydrates, proteins and lipids. Lipids are an important component of the productivity of seas. They differ to a substantial degree in their chemical structure and functional groups; their common characteristic is solubility in organic solvents. Lipids degrade less readily in comparison to carbohydrates and proteins (Harvey et al., 1995). Hwang and Druffel (2003) suggested that selective lipid accumulation occurs in the water column as particulate organic carbon (POC) sinks – a topic that is widely debated. Conversely, a significant decrease of lipids in POC with depth has been reported by Wakeham et al. (1997) and Loh et al. (2008). This discrepancy may be related to differences in lipid measurements, e.g., determination of total lipid or particular lipid classes. Although molecular structure influences lipid reactivity, the transformation of OM also depends on environmental conditions (Wakeham and Canuel, 2006).

In addition to being rich in carbon, lipids incorporate important heteroatoms including phosphorus, sulfur, oxygen and nitrogen. The identity of organic nitrogen determines the molecular distribution in the water column, recycling and/or loss from the euphotic zone. The chemical forms of N are inorganic N, including nitrous gasses, nitrates, nitrites and ammonia, and organic nitrogen, both in a particulate and dissolved fraction. Particulate organic nitrogen is composed of key molecules such as proteins, DNA, some vitamins and lipids indicating that N is of primary importance for organism growth. Although particulate organic nitrogen represents only a small portion of total N in the ocean N inventory (Reynolds et al., 2007), N cycling is closely coupled to the cycling of carbon. These processes are also of prime relevance for understanding the oceanic carbon cycle, and hence atmospheric CO₂ levels. Because nitrogen is a major nutrient that is required by all life, its availability can control biological productivity and ecosystem structure in both surface and deep-ocean communities (Casciotti, 2016).

Organic matter produced in the surface ocean sinks and is transformed by various processes including biodegradation, photodegradation and autoxidation (Rontani et al., 2011). The biological pump, which is the complex suite of processes that results in the transfer of particulate and dissolved organic matter from the surface to the deep ocean, is poorly understood and analytical techniques for molecular-level OM characterization are sought.

* Corresponding author.

E-mail address: gaspar@irb.hr (B. Gašparović).

Further, while a large fraction of oceanic OM is not characterized, the proportion of the uncharacterized fraction increases with depth and constitutes 70–80% of deep-ocean OM (Lee et al., 2004). Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) provides both high mass resolving power and sub-part-per-million mass measurement accuracy that provide detailed information on the elemental composition and characteristics of the organic matter directly from the measured mass-to-charge ratios. Moreover, this method provides information for the observed compounds about the extent of alkylation and the degree of unsaturation (i.e. number of molecular rings plus double bonds to carbon in each molecule (double-bond equivalents, DBE)). This approach allows determination of the molecular composition of the extremely complex organic matter mixtures including oceanic dissolved organic matter (e.g., Koch et al., 2005) and marine lipids (Gašparović et al., 2016).

While the description of nitrogen-containing organic matter has largely focused on particulate and dissolved organic nitrogen (PON and DON) (e.g., Bronk and Ward, 2005), proteins (e.g., Bridoux et al., 2015) and amino acids (Dittmar et al., 2001), less is known about nitrogen-containing lipids, apart from the studies on betaine lipids, and phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, where the focus is primarily on phosphorous production and cycling (Van Mooy and Fredricks, 2010; Popendorf et al., 2011; White et al., 2015). Recently a gas chromatographic method has been developed for measurement of natural stable nitrogen isotope ratios in lipids (Svensson et al., 2015) and that may improve the description of N-lipid production and cycling. Here we provide the first detailed observation of nitrogen in suspended particulate lipids, their distribution and molecular changes in the northeast Atlantic enabled through sampling throughout a 4800 m water column. We employ FT-ICR MS and thin-layer chromatography-flame ionization detection to provide qualitative and quantitative lipid characterization.

2. Methods

2.1. Study site and sampling

The study site at the Porcupine Abyssal Plain (PAP) in the northeast Atlantic has been a focus of many studies since 1992. The PAP station is isolated from the complexities of the continental slope and the Mid-Atlantic Ridge. A persistent feature of the North Atlantic is undersaturation of CO₂ in surface waters throughout the year, which gives rise to a perennial CO₂ sink and makes this a region of great importance in the global carbon cycle (Hartman et al., 2012). In terms of biogeographical provinces that have dynamical boundaries, it is well within the North Atlantic Drift (NADR) province (Longhurst, 2007). Currents are generally weak (Lampitt et al., 2001) and lateral advection speeds are low but significant (Williams et al., 2006; Hartman et al., 2010).

Sampling was conducted from the RRS James Cook on June 14, 2013. Water samples were collected by predawn deployment of a 24 × 20 l SeaBird CTD-Niskin rosette sampler. Samples were collected at 21 depths from the surface (2 m) to 4800 m (50 m above bottom) to encompass the epipelagic (0–200 m), mesopelagic (200–1000 m), bathypelagic (1000–4000 m) and abyssopelagic (4000–4800 m) zones. Six of the surface productive layer sampled depths (2–50 m depth) corresponded to 97, 55, 20, 7, 5 and 1% of surface irradiance intensity.

2.2. Temperature, salinity, nutrients and chlorophyll *a* analysis

Temperature, salinity, and chlorophyll *a* (Chl *a*) measurements were made using Seabird SBE 37-IM recorders (Sea-Bird Electron-

ics Inc., Bellevue, Washington, USA). Samples for nutrient (total inorganic nitrogen (TIN = nitrate + nitrite + ammonium), orthophosphate (PO₄³⁻ hereinafter termed PO₄) and orthosilicate (SiO₄⁴⁻ hereinafter termed SiO₄) analysis were drawn into 25 ml plastic coulter counter vials from Niskin bottles. The vials were stored in the dark at 4 °C until analysis, which commenced within 24 h of sampling. Nutrients were determined in unfiltered water samples with a Skalar Sanplus segmented flow autoanalyzer and standard colorimetric techniques described by Kirkwood (1996) and Sanders et al. (2007).

2.3. Particulate organic carbon analysis

Seawater samples (1 l) collected from the CTD rosette were prepared by filtering onto pre-combusted 25 mm GF/F filters and stored on board in –20 °C for subsequent particulate organic carbon (POC) analysis. Inorganic carbonates were removed from the filters by acidification with fuming concentrated hydrochloric acid. The filters were dried in a 50 °C oven for 24 h, packaged in pre-combusted tin capsules and analyzed with an Automated Nitrogen Carbon Analysis for Gas, Solids and Liquids (ANCA-GSL) preparation system coupled to a PDZ Europa 20–20 Stable Isotope Analyzer (PDZ Europa Scientific Instruments, Northwich, UK). The mass spectrometer can be tuned using source settings for sensitivity and/or linearity of a standard range. A typical standard range used for linearity is 25–1028 µg carbon, the limit of detection being 3 times the standard deviation of the blank of an analysis. The blank consisting of a tin capsule is analyzed in triplicate.

2.4. Lipid analysis

Particulate lipids were collected on 0.7 mm Whatman GF/F filters combusted at 450 °C/5 h by filtering 5–10 l of oceanic water at 12 kPa vacuum pressures immediately after sampling. Lipids were stored at –80 °C until extraction. Lipids were extracted by a modified one-phase solvent mixture of dichloromethane-methanol-water procedure (1:2:0.8, v:v:v) (Bligh and Dyer, 1959). One µg of the internal standard reserpine was added to each sample before extraction for FT-ICR MS. Ten µg of hexadecanone was added for Iatroscan analysis providing Iatroscan lipid recovery estimation. Extracts were concentrated till dryness under a nitrogen atmosphere and stored at –20 °C until analysis.

The particulate-derived lipid material was analyzed by direct-infusion electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) in both positive and negative ionization modes to provide elemental composition determination for lipids that can serve as diagnostic markers for their origin, transformation and preservation potential through ocean water column. ESI FT-ICR mass spectrometry was performed with a hybrid linear ion trap 7 T FT-ICR mass spectrometer (LTQ FT, Thermo Fisher, San Jose, CA) equipped with an Advion Triversa Nanomate (Advion Biosystems, Inc.) as previously described (Holguin and Schaub, 2013). FT-ICR mass spectra were acquired at a mass resolving power of $m/\Delta m_{50\%} = 400,000$ at m/z 400 (i.e. a time-domain acquisition period of ~3 s). A total of 500 time-domain transients were co-added for each sample in both positive and negative ionization modes prior to fast Fourier transformation and frequency to mass-to-charge ratio conversion. FT-ICR mass spectra were internally calibrated to achieve sub part-per-million mass measurement accuracy which facilitates direct assignment of elemental composition from measured m/z ratio and peak lists were generated from each mass spectrum at $S/N > 10$. Internal calibration of the mass spectra was performed using homologous alkylation series of known compounds where elemental compositions differ by integer multiples of CH₂. High-resolution FT-ICR mass spectra confirm that all observed ions are single charged as

evidenced by the 1 Da spacing between $^{12}\text{C}_c$ and $^{13}\text{C}_1$ $^{12}\text{C}_{c-1}$ peaks for the species with the same molecular formula. IUPAC measured masses ($\text{CH}_2 = 14.01565$ Da) were converted to the Kendrick mass scale ($\text{CH}_2 = 14.0000$ Kendrick mass units) as previously described (Kendrick, 1963) and sorted by the Kendrick mass defect to facilitate identification of homologous series with the same heteroatom composition and the same double-bond equivalents (DBE) but differing in the degree of alkylation. DBE was calculated as follows: $\text{DBE} = \text{C} + 1 - \text{H}/2 + \text{N}/2$ (halogens omitted because they were not observed in our analysis). Mass spectral peak magnitude for each compound was normalized (in both modes) to the internal standard (i.e. reserpine) peak magnitude, so that the mass spectral signals for each compound could be normalized to a fixed volume of seawater.

High mass measurement accuracy and mass resolving power combined with Kendrick mass sorting and isotopic fine structure analysis enables robust determination of elemental compositions for individual lipid compounds present in these extracts. Derived elemental compositions are matched to an in-house assembled lipid library derived from Lipid Maps (<http://www.lipidmaps.org/>). For the purposes of this paper, elemental compositions for which multiple database isomeric matches are possible, further identification was not attempted. In cases where we discuss specific lipid molecular classes, those compounds represent elemental compositions for which only one database match is made. Relative abundance for certain N-lipid class is calculated by normalization of that N-lipid peak magnitude at each depth to the lowest measured that N-lipid peak magnitude across the depth profile.

Additionally, total lipid and lipid class quantitation was performed by Iatroscan thin layer chromatography–flame ionization detection (TLC–FID) (Iatroscan MK-VI, Iatron, Japan). Lipids were separated on Chromarods-SIII thin layer rods and quantified by an external calibration with a standard lipid mixture. Hydrogen flow was 160 ml/min and air flow was 2000 ml/min. Each lipid extract was analyzed in triplicate: for the analysis, 2 μl aliquots of 20 μl of the solution in dichloromethane were spotted by semi-automatic sample spotter. The standard deviation accounted for 0–9% of the signal magnitude of lipid classes. Detailed procedures are described in Gašparović et al. (2015). As this method does not have a protocol to detect N-lipids (apart from phosphatidylcholine and phosphatidylethanolamine) this method served to quantify total lipids that were obtained by summing up all detected lipid classes.

3. Results

3.1. Basic environmental conditions

The environmental conditions were reflected in temperature that varied between 13.7 °C at the surface and 2.6 °C at 4800 m (Fig. 1a), while salinity varied in the range of 35.6 at the surface to 34.9 in the deep Atlantic (Fig. 1b). We observed a depth-related concentration increase of nutrients (Fig. 1c) as the result of surface productive layer consumption and water column remineralization of organic compounds. TIN, PO_4 and SiO_4 concentrations ranged from 5.10 to 22.99 $\mu\text{mol/l}$, from 0.37 to 1.89 $\mu\text{mol/l}$ and from 0.82 to 44.35 $\mu\text{mol/l}$, respectively. N/P ratio decreased from the epipelagic (average 13.8) to the bottom of the water column (12.2) (Fig. 1d).

Chlorophyll *a*, measured by chlorophyll fluorescence (Fig. 1e), was high in the upper 50 m with the highest value (regarding depths of lipid samples) at 30 m. The water column POC profile shows epipelagic POC enrichment (up to 202.3 $\mu\text{g C/l}$) and a decrease in concentration with depth (down to 24.3 $\mu\text{g C/l}$) (Fig. 1f). We do not have POC data for the whole water column due to irreparable damage to the samples during preparation.

3.2. Thin layer chromatography–flame ionization detection

Total lipids (measured by TLC–FID) varied between 3 and 24.3 $\mu\text{g/l}$ with a peak at 30 m depth, while the lowest value was measured at 3500 m depth (Fig. 1g). Total lipid contribution (assuming 70% carbon content in lipids) to POC increased from the epipelagic zone (7.7%) to the bathypelagic (9.2%) zone. The depth variations of total lipids followed that of Chl *a* in the surface productive layer ($R = 0.79$, $p = 0.0227$, $n = 7$).

3.3. Fourier transform ion cyclotron resonance mass spectrometry

ESI FT-ICR mass spectrometry was performed in both positive and negative ionization mode for the lipid extracts collected from the ocean surface to 4800 m depth. Broadband ESI FT-ICR mass spectra acquired for particulate lipids collected at 50 m depth are shown in Fig. 2. With FT-ICR MS, we detected N-lipids in both positive and negative ionization mode. Basic nitrogen-containing compounds are selectively ionized by positive-ion ESI (Hughes et al., 2001; Qian et al., 2001a), whereas acidic nitrogen-containing compounds are selectively ionized by negative-ion ESI (Qian et al., 2001b; Hughes et al., 2002). Therefore, N-lipids detected in the positive and negative ionization modes correspond to lipids containing basic and acidic nitrogen, respectively.

In the positive ionization mode, 377 monoisotopic N-lipids were detected among which 110 (29.2%) had elemental compositions that matched lipid elemental compositions reported in the Nature Lipidomics Gateway database (database-matched). In the negative ionization mode, 1023 monoisotopic N-lipid compounds were detected of which 157 (15.3%) matched known lipid elemental compositions. Altogether only 19% of N-lipid observed elemental compositions match previously reported lipids. We include discussion of differences between those N-lipids for which elemental composition matches previously reported lipids (database-matched) and those that have not been previously reported (novel), below.

In the positive ionization mode, the cumulative signal for N-lipids does not show a significant change with ocean depth (Fig. 3a). The N-lipid molecular diversity changed with ocean depth, with an average of 82 N-lipid elemental compositions observed in the epipelagic layer and an average of 68 in the abyssopelagic zone (Fig. 3b). The contribution of N-lipid molecular formulas to total lipid molecular formulas increased from 6.4% at the surface productive layer to 8.4% in the abyssopelagic zone (Fig. 3c). Database-matched and novel lipids show similar summed signal magnitude at the surface productive layer, but a ~5.5-fold higher signal magnitude is observed for novel N-lipids relative to database-matched lipids below the euphotic zone. For novel N-lipids, the cumulative signal changed only slightly with depth, while database-matched N-lipid cumulative signal decreased 2.4-fold across the water column (Fig. 3d). With increasing depth, the percentage of N-lipid elemental compositions that match database-reported lipids decreased from 55% to 16% (Fig. 3e).

Total lipid and N-lipid cumulative signal obtained in the negative ionization mode decreased about 90% between the epipelagic and abyssopelagic zones (Fig. 3f), similar to results of lipid analysis obtained using the Iatroscan technique. A higher molecular diversity of N-lipids was observed in the negative ion mode than in the positive mode. In general, N-lipid diversity decreased with depth, from 215 monoisotopic N-lipid elemental compositions identified at the surface and 130 identified at 4800 m depth (Fig. 3g). The contribution of N-lipid molecular formulas to total lipid molecular formulas in negative ion mode increased from epipelagic (17.3%) to abyssopelagic (22.1%) depths (Fig. 3h). Both database-matched and novel N-lipid signal decreased across the water column: 94% and

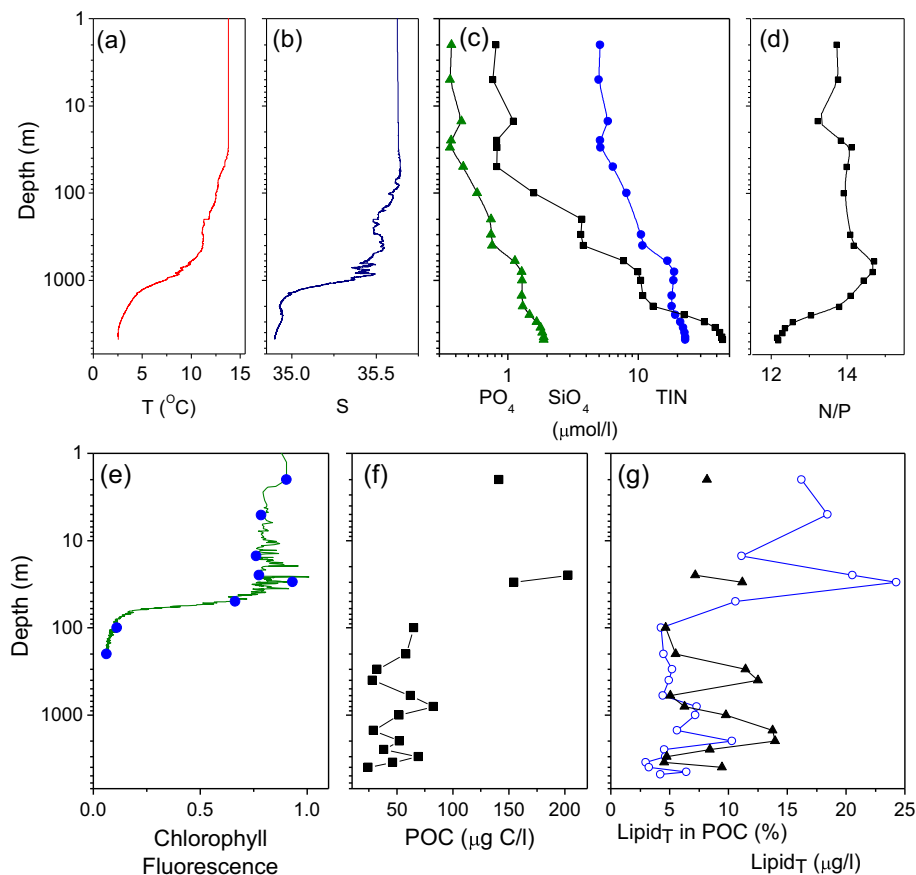


Fig. 1. Depth distribution of: (a) temperature (T), (b) salinity (S), (c) orthophosphate (PO_4) (triangles), total inorganic nitrogen (TIN) (circles) and orthosilicate (SiO_4) (squares), and (d) N/P ratio, (e) chlorophyll fluorescence (as a measure of Chl *a* concentration) with marked depths of lipid samples (circles), (e) particulate organic carbon (POC), and (f) latroscan determined total lipid concentrations.

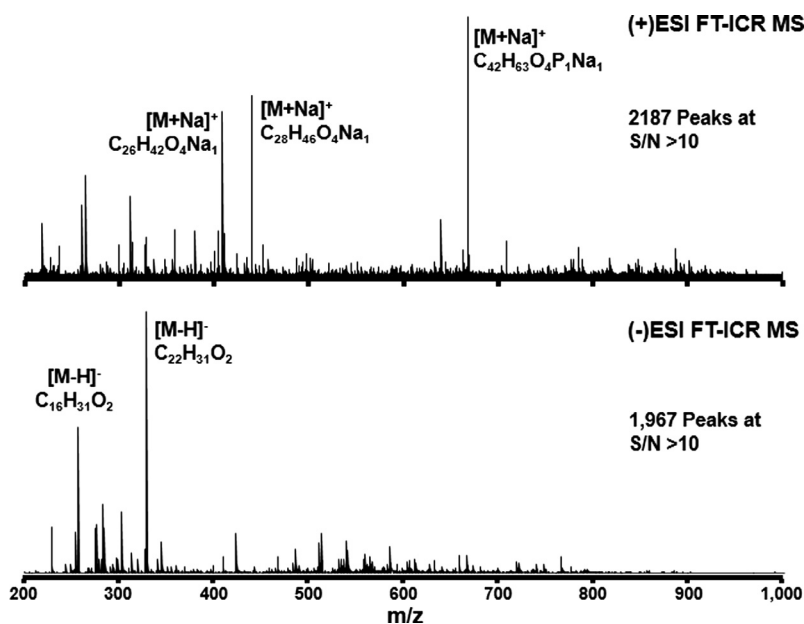


Fig. 2. Broadband positive (top) and negative (bottom) ion ESI FT-ICR mass spectra for particulate lipids collected at 50 m depth.

89%, respectively. The summed signal magnitude of novel lipids obtained in negative ionization was on average 8.7 times higher than that of database-matched lipids (Fig. 3i). The percentage of

N-lipid elemental compositions that match to the Lipid Maps lipid database decreased between surface and deep Atlantic from 22% to 5% (Fig. 3j).

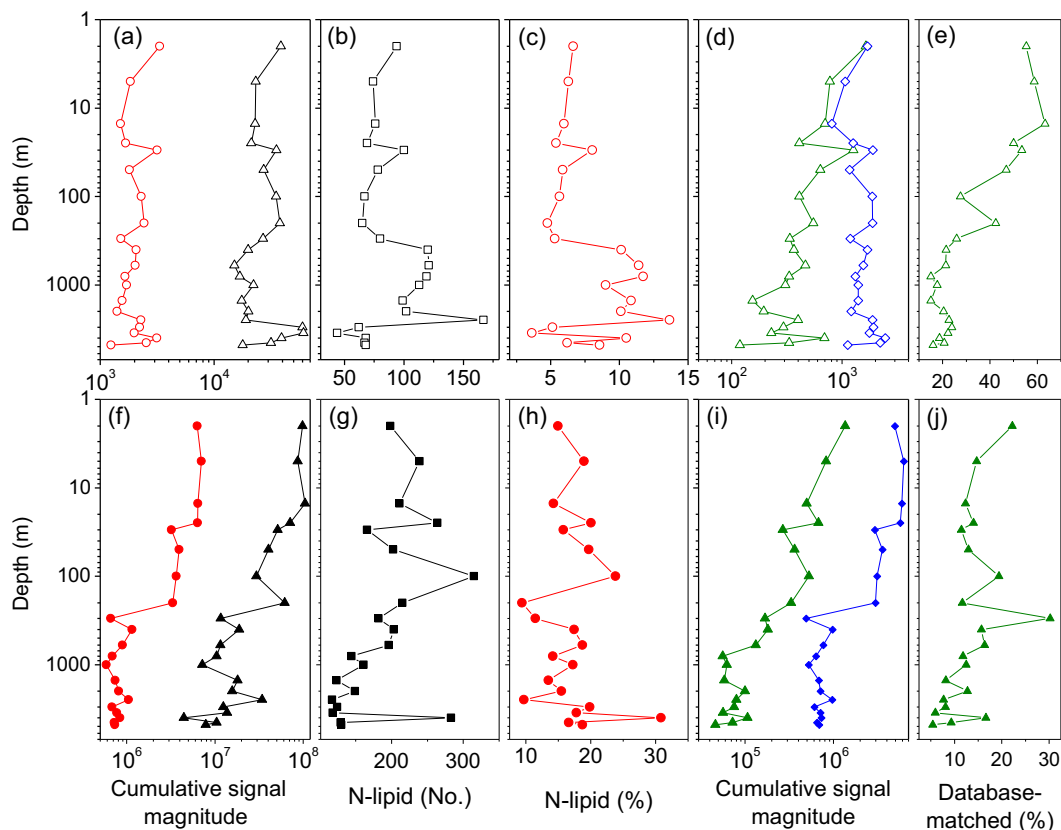


Fig. 3. FT-ICR MS lipid observations for positive (a–e, open symbols) and negative (f–j, full symbols) ionization; (a and f) total particulate lipid (triangles) and N-lipid (circles) FT-ICR MS signal magnitudes, (b and g) the number of monoisotopic N-lipid peaks, (c and h) the contribution of N-lipid number to total number of detected lipids, (d and i) database-matched (triangles) and novel (diamonds) FT-ICR MS signal magnitude and (e and j) the percentage of N-lipid elemental compositions that match to the Lipid Maps lipid database (database-matched).

3.3.1. Positive ions

Among database-matched N-lipids, the majority of nitrogen is found in betaine lipids (Fig. 3). We also detected N-containing phospholipids, sphingolipids, ceramide phosphates, fatty acyl carnitines, amino fatty acids, fatty acyl homoserine lactones, N-acyl ethanolamines, monoacyl betaines, whose summed contribution to the total lipid signal was mainly less than 1%, and are not discussed further here.

The relative abundance of betaine lipids shows a 235-fold decrease from epipelagic to bathypelagic depths (100–4000 m) (Fig. 4a). Changes in betaine lipids include a decreased molecular diversity (34 different betaine lipid molecules at the surface productive layer and 1–3 different molecules in the bathypelagic zone) (Fig. 4b), whose contribution to all lipid molecules was the highest at the euphotic zone (up to 3.3%) (Fig. 4c), low variability of average molecular mass (Fig. 4d) and lower unsaturation in the surface productive layer (on average 6.5) than in the bathypelagic layer (on average 7.0) (Fig. 4e). The most dominant betaine lipids found in the surface productive layer were $C_{38}H_{73}N_1O_7$ (acyl DBE = 3, 28 C atoms in the two acyl chains), $C_{44}H_{83}N_1O_7$ (acyl DBE = 4, 34 C atoms in two acyl chains), $C_{46}H_{81}N_1O_7$ (acyl DBE = 7, 36 C atoms in two acyl chains), and $C_{46}H_{83}N_1O_7$ (acyl DBE = 6, 36 C atoms in two acyl chains).

To characterize the novel positive ion N-lipids, we have tracked those which have N, C, H and O in the molecular formula (termed CHON N-lipids), and those which also have P in the molecular formulas (termed CHONP N-lipids), to evaluate any differences in depth-related cycling between these groups. Additionally, those two N-lipid groupings were tracked as saturated (DBE = 0) and those which have at least one unsaturation (DBE > 0).

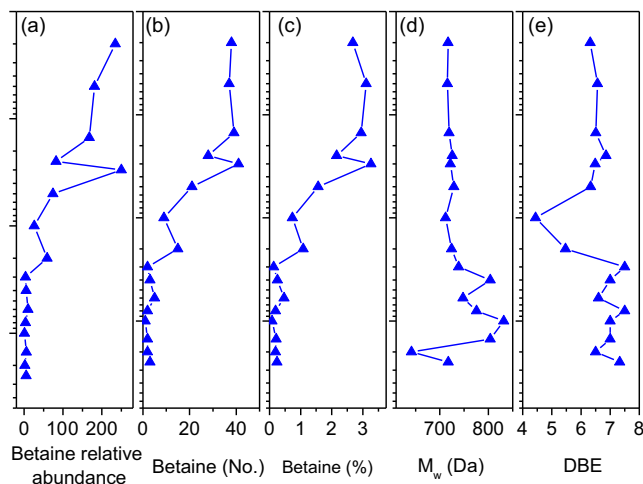


Fig. 4. Betaine lipids: (a) relative abundance, (b) number of compounds, (c) the contribution of betaine lipid number to total number of detected lipids, (d) average molecular mass and (e) double bond equivalents, obtained in positive ionization mode.

The relative abundance of unsaturated CHON N-lipids (Fig. 5a), number of detected compounds (Fig. 5b) and the contribution of number of compounds to total number of detected lipids (Fig. 5c) increased slightly with ocean depth. The observed average molecular masses varied around a value of 563 Da (Fig. 5d) while unsaturation (DBE) varied around a value of 6.5 (Fig. 5e) across the whole water column.

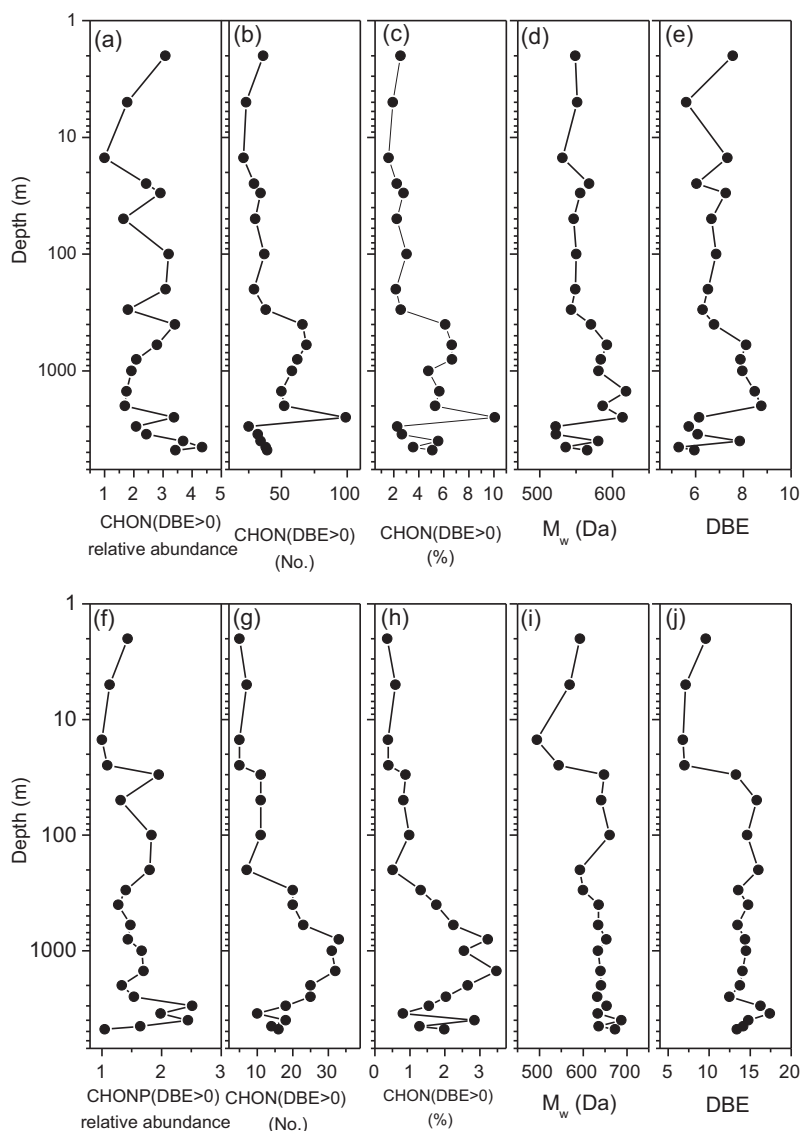


Fig. 5. Novel unsaturated N-lipids that contained C, H, O and N (a–e) and C, H, O, N and P (f–j) in the molecular formula: (a and f) relative abundance, (b and g) the number of monoisotopic peaks, (c and h) the contribution of number of compounds to total number of detected lipids, (d and i) average molecular mass and (e and j) double bond equivalents, obtained in positive-ion ESI FT-ICR MS.

There was a slight decrease in unsaturated CHONP N-lipid relative abundance (Fig. 5f), an increase in the number of compounds, i.e. molecular diversity (average 7 and 16 molecules in epipelagic and abyssopelagic, respectively) (Fig. 5g), and an increase in the contribution of number of compounds to total number of detected lipids (Fig. 5h), an increase in the average molecular mass (average 582 and 665 Da, respectively) (Fig. 5i) and an increase in unsaturation (average DBE = 10 and DBE = 14, respectively) (Fig. 5j) with increasing depth.

The contribution of saturated novel N-lipids, (i.e. both CHON and CHONP) to the total lipid signal was negligible, with a contribution of less than 0.1% and with only 1–3 different molecules present per depth, so these data are not discussed further.

3.3.2. Negative ions

Among database-matched N-lipids, we identified N-containing phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and monoacyl phospholipids) that represented about 90% of the total mass spectral signal, together with ceramides, prostaglandins, amino fatty acids, N-acyl ethanolamines

and sphingolipids where the abundance of those lipids was very low and we have chosen to present those data as summed. Their relative abundance decreased 29-fold (Fig. 6a). The number of database-matched formulas decreased, being on average 31 molecular formulas in the epipelagic zone and 10 in the abyssopelagic zone (Fig. 6b). The contribution of the number of database-matched N-lipids to total number of detected lipids slightly decreased with depth (Fig. 6c). Average molecular mass of database-matched N-lipids varied with an average value of 679 Da (Fig. 6d). Their unsaturation decreased from the epipelagic (average DBE = 4.5) to the abyssopelagic (average DBE = 2) zone (Fig. 6e). Three of the dominant database-matched N-lipids in the whole water column are presented in [Supplementary Table 1](#).

Novel N-lipids obtained in negative ionization mode were also followed as those which have N, C, H and O in the molecular formula and those which also have P in the molecular formulas. Both classes were tracked as saturated and those which have at least one unsaturation. The unsaturated CHON N-lipids average relative abundance decreased ~20 times (Fig. 7a). The number of different molecules decreased ~2-fold between epipelagic and abyssopela-

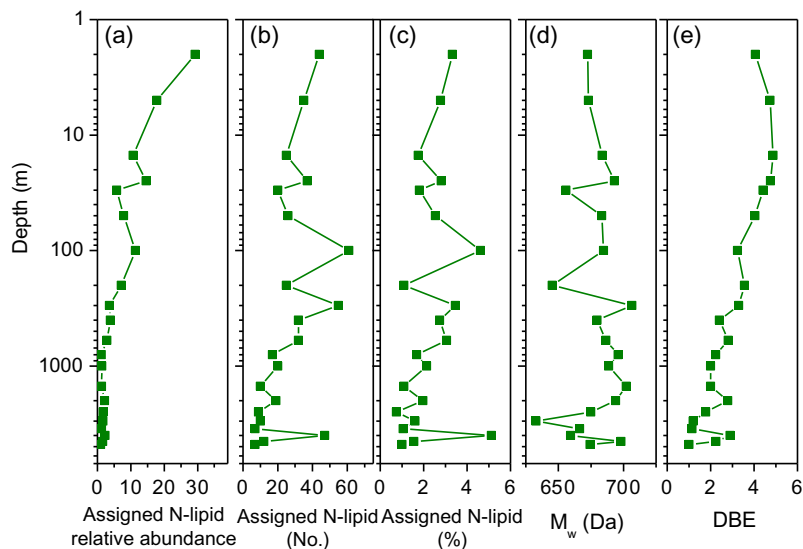


Fig. 6. Database-matched N-lipids: (a) relative abundance, (b) the number of compounds, (c) the contribution number of compounds to total number of detected lipids, (d) average molecular mass and (e) double bond equivalents obtained in negative-ion ESI FT-ICR MS.

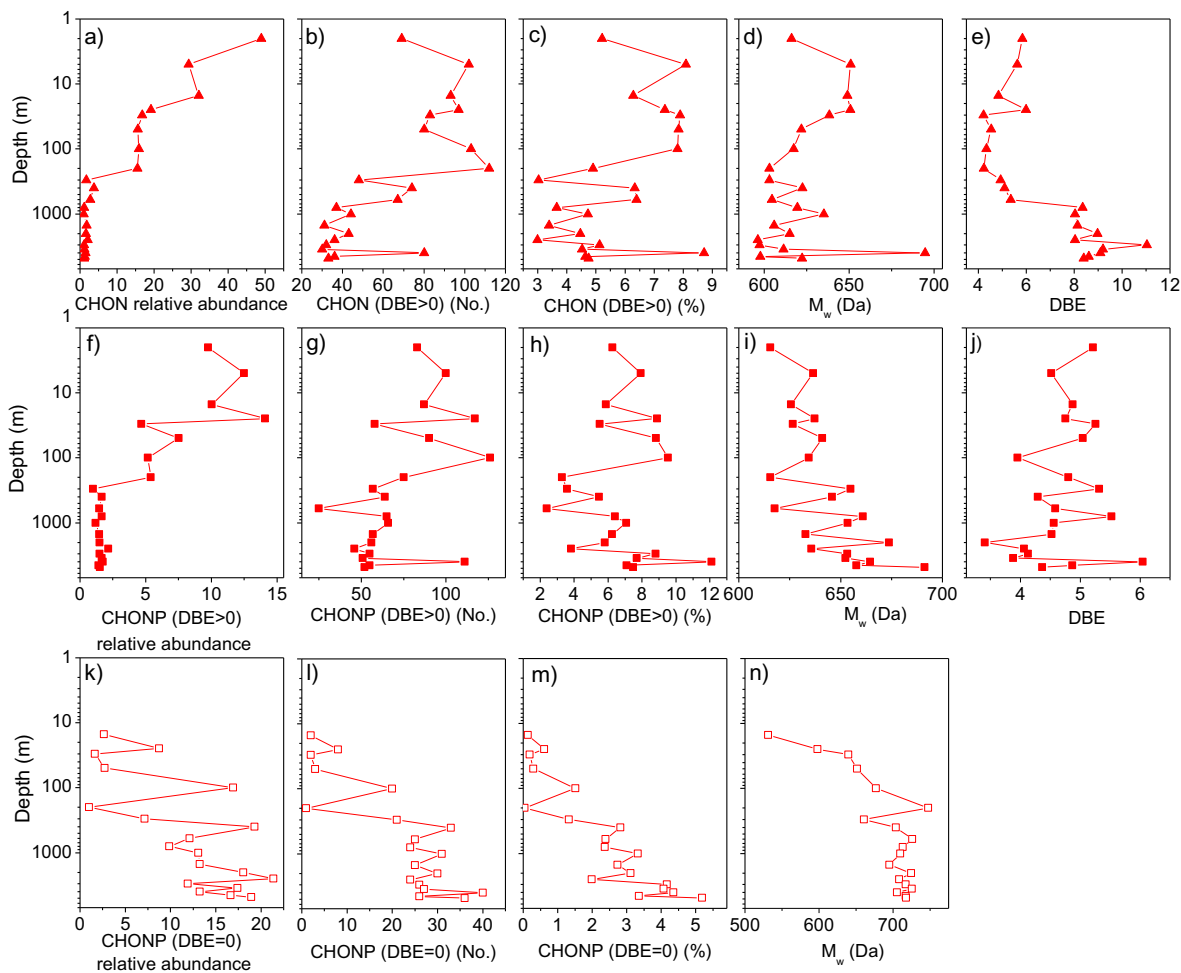


Fig. 7. Novel (a–e) unsaturated N-lipids that contained C, H, O and N, and novel (f–j) unsaturated and (k–n) saturated N-lipids that contained C, H, O, N and P in the molecular formula: (a, f and k) relative abundance, (b, g and l) the number of compounds, (c, h and m) the contribution number of compounds to total number of detected lipids, (d, i and n) average molecular mass and (e and j) double bond equivalents, obtained in negative-ion ESI FT-ICR MS.

gic zones (Fig. 7b), while the contribution of their molecular compounds to total number of lipid compounds slightly decreased between epipelagic (average contribution 7.1%) and abyssopelagic zones (average contribution 6.0%) (Fig. 7c). CHON N-lipid molecular features show a slight decrease in the average molecular mass (Fig. 7d) and increased unsaturation in the deep Atlantic with average DBE = 5.2 in the surface productive layer and average DBE = 8.7 at abyssopelagic depths (Fig. 7e).

The contribution of saturated, novel CHON N-lipids to the total lipid signal in negative ionization was negligible, with a contribution of less than 0.1% and with only 1–3 different molecules present per depth, and so the data are not further discussed.

The signal magnitude of unsaturated, novel CHONP N-lipids decreased on average 6.3 times (Fig. 7f). The number of different molecular formulae decreased with depth being on average 89 elemental compositions in the epipelagic zone and 73 in abyssopelagic zones (Fig. 7g). The contribution of unsaturated, novel CHONP N-lipids to the total number of detected lipids in negative ionization increased between the epipelagic (average 7.2%) and abyssopelagic zones (average 8.9%) (Fig. 7h). Average molecular mass increased somewhat from the surface productive layer (on average 630.4 Da) to the deepest Atlantic (on average 671.2 Da) (Fig. 7i) and the level of unsaturation varied substantially with depth (Fig. 7j).

The contribution of saturated, novel CHONP N-lipids in negative ionization mode increased with depth where the highest contribution of 2.2% was observed at 4800 m depth (Fig. 7k). At the same time, their relative abundance increased ~20-fold (Fig. 7l), as well as their molecular diversity, from an average of 4 molecular formulas in the epipelagic layer to 34 at abyssopelagic depths (Fig. 7m) and also average molecular mass, from an average of 605.2 Da at epipelagic to 713.4 Da at abyssopelagic depths (Fig. 7n).

Fig. 8 shows depth-related average oxygen to carbon (O/C) and hydrogen to carbon (H/C) ratio variations of all elaborated N-lipid classes obtained in the positive and negative ionization mode. There were no significant trends of average O/C ratios with depth. The highest average O/C ratios were recorded for unsaturated CHONP-lipids obtained using negative ionization, which had lowest average H/C ratios. Saturated CHONP-lipids obtained by negative ionization had lowest average O/C ratio and the highest average H/C ratios.

4. Discussion

As biomolecules, lipids are produced by living organisms in the oceans. Primary lipid production occurs in the sunlit ocean (euphotic) zone, where autotrophic plankton are the main lipid producers (Gašparović et al., 2014). During POC sinking out of the surface productive layer, the complex interplay between biological and chemical processes leads to the alteration and remineralization of organic matter. Lipids can indicate OM origin, production, and cycling because of their molecular variety and stable nature compared to carbohydrates and proteins (Harvey et al., 1995). Lipids in marine cyanobacteria, *Prochlorococcus* have been characterized to understand the organism's biochemical adaptations in phosphorus-limiting gyre environments (e.g., Van Mooy et al., 2006). In another study, lipids were characterized to study the viral interaction between *Emiliana huxleyi* virus (EhV86) and *Emiliana huxleyi* algae that leads to the termination of the algal bloom (e.g., Fulton et al., 2014). Lipid biomarkers were used to investigate sources, transport and degradation of terrestrial organic matter in the East Siberian Arctic Shelf (Karlsson et al., 2011). With the development of new techniques new lipids are continuously being discovered (e.g., Fulton et al., 2014).

Our results show that the concentrations of total lipid distribution follow the Chl *a* distribution. This observation indicates that autotrophic plankton were the main lipid producers, as already

observed for the east Atlantic by Gašparović et al. (2014), who showed that bacterial carbon, and therefore also lipid carbon, is much lower than autotrophic plankton carbon. The concentrations of total lipids and POC obtained in this study are within the ranges published for the eastern Atlantic (Neogi et al., 2011; Gašparović et al., 2014). The concentrations of PON and POC found in surface waters of the NADR province, where the PAP station is located, are between 1.1 and 1.4 $\mu\text{mol/l}$ (15.4 and 19.6 $\mu\text{g/l}$) N and 8.5 and 8.7 $\mu\text{mol/l}$ (102 and 104 $\mu\text{g/l}$) C, indicating an important contribution of nitrogen in POC in the eastern Atlantic (Neogi et al., 2011). Only 4–11% of the PON escapes recycling in the ocean surface layer (Pondaven et al., 1999). The increase in the contribution of Iatroscan-measured total lipids to POC from the epipelagic to the bathypelagic indicates their selective accumulation with depth. Regarding various lipid classes, it has been shown that lipid diagenetic reactivity is highly variable (Wakeham et al., 1997; Loh et al., 2008).

Positive and negative ion ESI FT-ICR MS (no overlap of lipids was observed in the two ionization modes) allowed identification of more than one thousand N-lipids at the level of elemental compositions and some lipids are reported with molecular identification based on unique database matching. Those matched lipids include betaine lipids, N-containing phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and monoacyl phospholipids), ceramides, prostaglandins, amino fatty acids, N-acyl ethanolamines and sphingolipids, fatty acyl carnitines, and fatty acyl homoserine lactones. Identified lipids are listed in declining order of their contribution to the total lipid signal. Apart from the identified fraction of N-lipids, FT-ICR MS allowed identification of unknown lipids, whose elemental compositions do not match to the lipids reported in the Lipid Maps database, and are termed “novel”. The results obtained serve as diagnostic markers for N-lipid molecular characteristics, transformation and preservation potential through water column of a mesotrophic system, i.e. at the PAP station in the North Atlantic Ocean.

Here we show that approximately 12.5% of lipids contain N in their molecular formula. The majority of those lipids are novel with a depth-related increasing contribution of N-lipid compounds to total lipid compounds. According to the increased contribution of N-lipid compounds with respect to all lipid compounds, detected at both ionizations (Fig. 3c and h) indicate depth-related selective accumulation of N-lipid among other lipids.

We have observed that CHON-lipids (including betaine lipids) (Figs. 4a and c, and 7a and c) are more prone to degradation than CHONP-lipids (Figs. 5a and c, 6a and c, and 7f and h) based on their depth-related greater decrease in relative abundance, and from their lower contribution of molecular formulas to total lipid molecular formulas with depth. Exceptions were novel CHON-lipids observed in positive ionization (Fig. 5a). There is a statistically significant depth-related decrease of CHON-lipid compounds while for CHONP-lipids such significance is not confirmed (Supplementary Fig. 1). The data suggest that CHONP-lipids carry N efficiently to the deep ocean and that lipids containing N were more efficiently remineralized than those with both N and P. The observed depth-related decreasing inorganic N/P ratio indicates that in the deep NE Atlantic more N-containing organic matter was remineralized than P-containing organic substances. This is consistent with the observed increase in the CHONP-lipid molecular contribution with depth in comparison to the CHON-lipids. Monteiro and Follows (2012) reported on the preferential remineralization of phosphorus over nitrogen in the North Atlantic Ocean.

The availability of nitrogen limits biological production in much of the world ocean (Codispoti, 1989), but this was not the case at the PAP station as the observed epipelagic concentrations of N-nutrients (TIN) (Fig. 1c) were well above threshold values for the phytoplankton uptake, 1 $\mu\text{mol/l}$ (Justić et al., 1995). Measured high

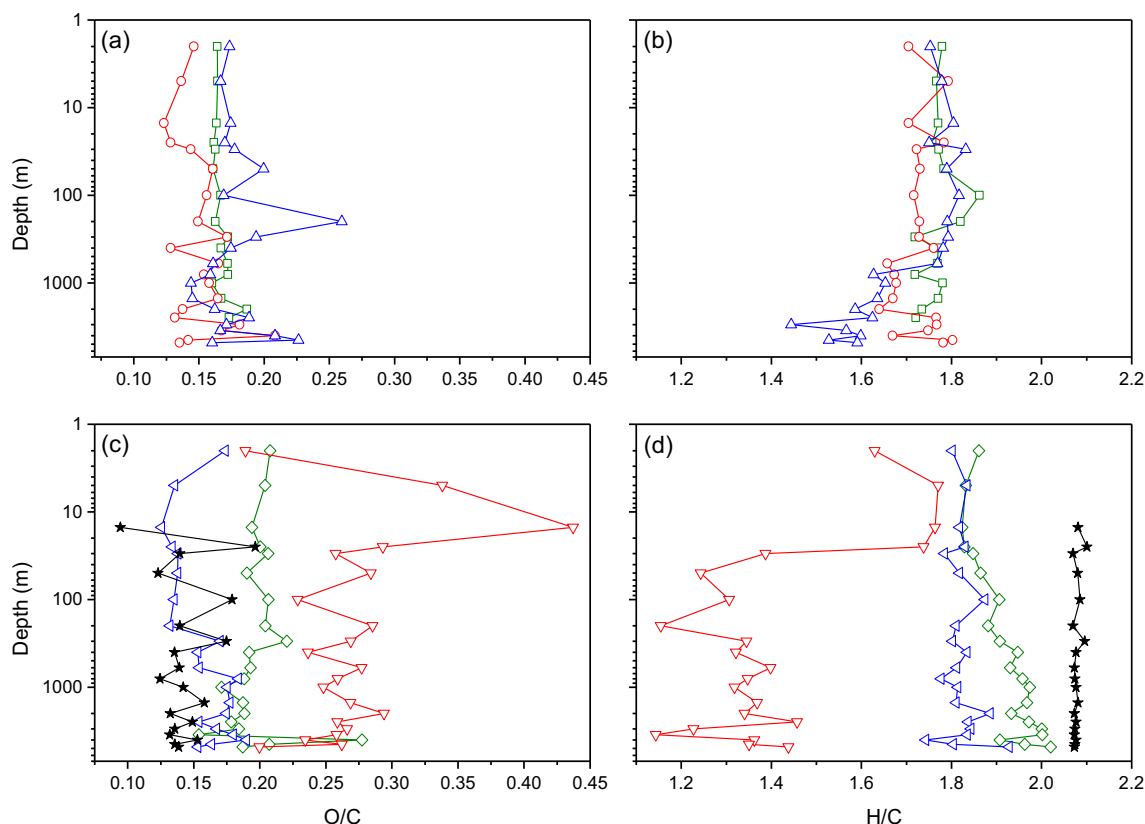


Fig. 8. Depth related (a and c) average oxygen to carbon (O/C) and (b and d) hydrogen to carbon (H/C) ratios of (a and b) unsaturated CHON lipids: betaine lipids obtained in the positive ionization, (squares), novel lipids obtained in the positive (circles) and negative (triangles) ionizations; and (c and d) unsaturated CHONP lipids: obtained in the negative ionization (diamonds), novel lipids obtained in positive (down triangles) and negative (left triangles) ionizations and saturated CHONP lipids obtained in the negative ionization mode.

epipelagic TIN concentrations would imply that surface productive layer preferential remineralization of N-containing organic molecules would not be of prime importance. The surface PO_4 concentrations (Fig. 1c) were also above the reported threshold values for phytoplankton uptake, $0.1 \mu\text{mol/l}$ (Justić et al., 1995).

To explain the difference in preferential removal of CHON-lipids over CHONP-lipids we compared the degree of unsaturation of those lipids assuming that the more unsaturated compounds are more susceptible to degradation in comparison to the saturated ones. Comparisons of DBE values between these two classes indicate that CHON-lipids were not more unsaturated (Figs. 5e, 5j, 7e and 7j). Therefore, a reasonable explanation would be that the difference in the molecular structure and possible functional groups between those two classes is the key factor influencing different reactivities.

Unexpectedly, the level of unsaturation for the majority of observed lipid classes increased with depth, as observed for betaine lipids, positive ion novel CHONP-lipids and negative ion novel CHON-lipids. There was no clear trend in the level of unsaturation for positive ion novel CHON-lipids and negative ion novel CHONP-lipids. The only depth-related unsaturation decrease was observed for database-matched N-lipids that were detected in negative ionization, composed mainly of phospholipids (90%) (Fig. 6e). Extremely high unsaturation of CHONP-lipids obtained in positive ionization from the mesopelagic and bathypelagic (average DBE = 14.5) is not easy to explain with a general observation that unsaturated molecules are more susceptible to degradation than saturated structures (e.g., Canuel and Martens, 1996; Wakeham et al., 1997; Loh et al., 2008).

We do not have a clear explanation for the observation of higher levels of unsaturation for the majority of N-lipids in the depths below the surface productive layer. Those lipids may have

originated from living or fresh organic material that did not experience depth-related alteration. Significant depth-related regeneration of SiO_4 , released from silicate biominerals, may indicate that collected lipids were efficiently exported from the epipelagic zone by mineral ballast, which probably enhances POC fluxes to the deep ocean (Armstrong et al., 2002). As the Niskin bottle collects a mixture of fast, slow and non-sinking particles we may assume a significant contribution of fast sinking particles were present in our samples (which have been shown to be less prone to bacterial degradation than slow-sinking particles; Wakeham et al., 2009). Accordingly, Riley et al. (2012) have shown that fast sinking particles alone were sufficient to explain the abyssal POC flux at the PAP site.

Highly unsaturated lipids are generally associated with living plankton. Given that only ~1% of the sampled lipids in the abyssopelagic may have originated unchanged from living plankton (Gašparović et al., 2016) it is unlikely that those detected in the deep ocean samples derive directly from the living plankton. However, it is known that deep ocean bacteria produce polyunsaturated fatty acids to regulate membrane fluidity and provide protection from oxidative stress (De Carvalho and Caramujo, 2012). If the detected highly unsaturated N-lipids originate from membranes, then fatty acids contained in those lipids should have more than eight double bonds. Our observation of a depth-related molecular weight increase for N-lipids might indicate fatty acid elongation with increased unsaturation in the case that there were fatty acid containing lipids. Finally, long-chain lipid molecules are less reactive than short-chain lipids (Pantoja and Wakeham, 2000).

Lipid molecular alteration in the deep Atlantic may contribute to the formation of highly unsaturated N-lipids that we observed. Cross-linking of unsaturated compounds may lead to the formation

of higher molecular weight lipids with higher degrees of unsaturation (as observed). This process could lead to the formation of humic substances, the most stable products of organic matter decomposition in nature, for which it is shown that lipids are important structural components (Stuermer and Harvey, 1978). Observed elemental composition distributions of marine humic substances have shown that the contribution of N is between 1% and 10% (Rice and MacCarthy, 1991). Increased molecular diversity of novel CHONP- and CHON-lipids obtained in positive ionization mode at mesopelagic and greater depths supports the hypothesis of depth-related N-lipid humic precursor formation. Most of the novel N-lipids observed here could have possibly been formed as a result of molecular transformations of the reported N-lipids.

Depth related molecular transformation of lipids include biotic (heterotrophic) and abiotic (photooxidation and autoxidation) degradations (Rontani et al., 2011, and References therein). Both types include initial formation of more oxygenated species. For example, enzymatic peroxidation of cis-vaccenic acid leads to formation of 10-hydroperoxyoctadec-11(cis)-enoic acid and further on involves reduction to the corresponding hydroxy acid (probably catalyzed by peroxygenases) and cleavage to the corresponding oxoacid, which is then bio-hydrogenated (Rontani and Koblížek, 2008). Photooxidation of sterols in senescent phytoplanktonic cells leads to the photogeneration of Δ^4 -6 α /6 β -hydroperoxides (Rontani et al., 1997). As the average O/C ratios (Fig. 8) did not show a depth-related increasing trend (assuming that lipids from greater ocean depths are more degraded/transformed than those from the upper water column) we suppose that the detected novel lipids do not represent early lipid transformation, but rather higher degrees of transformations.

The relative abundance depth profile (decreasing trend with depth) for database-matched N-lipids (betaine lipids and mainly phospholipids among other database-matched N-lipids analyzed in the negative ionization) suggests that these compounds are biologically produced (Figs. 4a and 6a). Van Mooy and Fredricks (2010) showed that betaine lipids derive primarily from eukaryotic phytoplankton. They also found in the eastern subtropical South Pacific, when phosphate concentrations were relatively high, that like phospholipids, the concentrations of betaine lipids tended to be highest in surface waters and decreased with increasing depth in the water column. Although it is known that phytoplankton substitute betaine lipids for phospholipids when dissolved phosphate is limited (Van Mooy et al., 2009), many types of phytoplankton contain abundant betaine lipids even when phosphate is replete (Dembitsky, 1996). Database-matched N-lipids (Figs. 4 and 6) are efficiently removed from the water column as indicated by a depth-dependent decrease in relative abundance, and decreased molecular diversity, which may result from intrinsic enzymatic activity. Still some betaine lipids were detected at bathypelagic depths, that contribute to about 0.1% to the total lipid signal. Kharbush et al. (2016) also detected betaine lipids in the deep western South Pacific Ocean. These highly unsaturated betaine lipids probably originated from psychrophilic and barophilic bacteria (Wirsen et al., 1987). Although betaine lipids are mainly ascribed to be present in eukaryotes (e.g., Van Mooy and Fredricks, 2010) they are also recognized in bacteria (e.g., Sebastián et al., 2015).

Saturated N-lipids seem to be formed during the N-lipid water column transformation as indicated by a depth-related increase in both the abundance and the number of observed compounds (Fig. 7k and l). Marine bacteria are shown to reduce sterols to the corresponding stanols (e.g. Wakeham, 1989), while oleic and vaccenic acids may be reduced to stearic acid by sedimentary bacteria (Rhead et al., 1971). In a model experiment, marine fungi were shown to perform biohydrogenation of unsaturated ketones (Ferreira et al., 2015). The highest average H/C ratio of NOVEL sat-

urated N-lipids among all N-lipid classes investigated indicate that biohydrogenation is a probable main mechanism for their formation. The higher average molecular mass for those compounds (Fig. 7n) relative to detected unsaturated N-lipids (Fig. 7i), points to complex formation processes and that saturation is an important property for the export of lipid N and C to the deep ocean.

5. Conclusions

Application of FT-ICR MS enabled characterization of particulate N-containing lipids in the northeast Atlantic at the level of elemental composition to provide novel insights into nitrogen cycling in lipids in the ocean. Apart from the database-matched N-lipids that derive from living or fresh organic material, we primarily observed compounds that have not been reported previously. We have shown that N-lipids were selectively preserved with increasing depth. The observed cycling process differed between database-matched and novel N-lipids. Database-matched lipids are effectively removed with water column depth. In contrast, novel lipids were more stable, and saturated N-lipids are apparently produced through cycling. We assume that the majority of observed novel N-lipids are products of N-lipid water column alterations. There are also difference in the cycling of the lipids that contain C, H, O and N in the molecular formula in comparison to those contain C, H, O, N and P. CHON-lipids were more efficiently remineralized as explained by the difference in the molecular structure and functional groups that influence reactivity.

We suggest that the high unsaturation of the majority of novel N-lipids in the deep waters may be due to several alternative processes: (1) efficient export of epipelagic produced unsaturated N-lipids by mineral ballast, (2) synthesis by deep ocean bacteria that are able to produce long polyunsaturated fatty acids, or (3) depth-related formation of those N-lipids as precursors for humic substance formation.

Saturated N-lipids seem to be formed during the N-lipid water column transformation indicating that saturation is an important property for the export of lipid N and C to the deep ocean.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.orggeochem.2017.07.008>.

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