Research Article

Rapid Communications in Mass Spectrometry

Received: 20 January 2015 Revised: 12 August 2015 Accepted: 10 September 2015 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2015**, *29*, 2263–2271
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7393

Compound-specific stable isotope analysis of nitrogen-containing intact polar lipids

Elisabeth Svensson[1*,†], Stefan Schouten[1,2], Axel Stam[1], Jack J. Middelburg[2] and Jaap S. Sinninghe Damsté[1,2]**

[1]Department of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg (Texel), The Netherlands
[2]Department of Earth Sciences, Faculty of Geosciences, Utrecht University, P.O. Box 80021, 3508 TA Utrecht, The Netherlands

RATIONALE: Compound-specific isotope analysis (CSIA) of nitrogen in amino acids has proven a valuable tool in many fields (e.g. ecology). Several intact polar lipids (IPLs) also contain nitrogen, and their nitrogen isotope ratios have the potential to elucidate food-web interactions or metabolic pathways. Here we have developed novel methodology for the determination of $\delta^{15}N$ values of nitrogen-containing headgroups of IPLs using gas chromatography coupled with isotope-ratio mass spectrometry.
METHODS: Intact polar lipids with nitrogen-containing headgroups were hydrolyzed and the resulting compounds were derivatized by (1) acetylation with pivaloyl chloride for compounds with amine and hydroxyl groups or (2) esterification using acidified 2-propanol followed by acetylation with pivaloyl chloride for compounds with both carboxyl and amine groups. The $\delta^{15}N$ values of the derivatives were subsequently determined using gas chromatography/combustion/isotope-ratio mass spectrometry.
RESULTS: Intact polar lipids with ethanolamine and amino acid headgroups, such as phosphatidylethanolamine and phosphatidylserine, were successfully released from the IPLs and derivatized. Using commercially available pure compounds it was established that $\delta^{15}N$ values of ethanolamine and glycine were not statistically different from the offline-determined values. Application of the technique to microbial cultures and a microbial mat showed that the method works well for the release and derivatization of the headgroup of phosphatidylethanolamine, a common IPL in bacteria.
CONCLUSIONS: A method to enable CSIA of nitrogen of selected IPLs has been developed. The method is suitable for measuring natural stable nitrogen isotope ratios in microbial lipids, in particular phosphatidylethanolamine, and will be especially useful for tracing the fate of nitrogen in deliberate tracer experiments. Copyright © 2015 John Wiley & Sons, Ltd.

Intact polar lipids (IPLs) in eukaryotes and bacteria generally comprise fatty acids esterified to glycerol, while in archaea they consist of isoprenoid alkyl chains which are ether bound to glycerol. These lipids often also contain polar headgroups which have a large structural diversity. The most commonly occurring are those with choline or ethanolamine headgroups, such as phosphatidylcholine and phosphatidylethanolamine, while other IPL headgroups can consist of e.g. sugars (glycolipids) or amino acids. Some of these IPLs are source specific and can be used to trace microbes in the natural environment (e.g.[1–3]).

Many methods have been developed to measure the isotopic composition of lipids as a means to obtain the natural stable isotopic signature of specific organisms, particularly of microbes, and for tracing the incorporation of labels in microbes in deliberate tracer experiments (e.g.[4]). Since these compounds generally have to be amenable to gas chromatographic separation, lipid extracts are often analyzed as core lipids (e.g. fatty acids) following base hydrolysis of the ester bonds and derivatization, or, for archaeal lipids, by $HI/LiAlH_4$ cleavage of the ether bonds (e.g.[5,6]). Most of the compound-specific stable isotope analysis (CSIA) of lipids has focused on determining the natural ^{13}C-contents or the incorporation of ^{13}C-labelled substrates into these core lipids, but has also included hydrogen isotope ratios (δ^2H).[7–10] Some studies have also examined the stable carbon isotopic composition ($\delta^{13}C$) of headgroups and the glycerol backbone of IPLs. For example, Takano *et al.*[11] determined the $\delta^{13}C$ values of the glycerol moiety of ether lipids using boron tribromide (BBr_3), and Lin *et al.*[12] developed a method to

* *Correspondence to:* E. Svensson, Department of Geology & Planetary Science, University of Pittsburgh, Pittsburgh, PA 15260, USA.
E-mail: esvensson@pitt.edu
** *Correspondence to:* S. Schouten, Department of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg (Texel), The Netherlands.
E-mail: stefan.schouten@nioz.nl
† Present address: Department of Geology & Planetary Science, University of Pittsburgh, Pittsburgh, PA 15260, USA

measure the ^{13}C-contents of sugar headgroups after methanolysis of the IPLs and aldonitrile derivatization. However, to the best of our knowledge, no studies have been performed to determine the stable nitrogen isotopic composition of IPLs. Nitrogen-containing headgroups are widespread in membrane lipids among both bacteria (e.g. phosphatidylethanolamine; **I** in Fig. 1) and eukaryotes (e.g. phosphatidylcholine, **IV** in Fig. 1) and in intermediaries of lipid biosynthesis (e.g. phosphatidylserine and cytidine diphosphate diacylglycerol; **V** and **VII** in Fig. 1, respectively). Recent studies of IPLs in cultures and the natural environment have shown that there is a large diversity in IPL structures and that a number of them are selective for certain groups of microorganisms. For example, ladderane lipids with a phosphatidylcholine headgroup are indicators for anammox bacteria,[13] while certain glycerol dibiphytanyl glycerol tetraethers with a phosphohexose headgroup are good indicators for the archaeal groups, such as the Thaumarchaeota.[14] Stable nitrogen isotope analysis of headgroups of specific IPLs would thus provide a way to trace nitrogen isotopes in specific groups of bacteria and eukaryotes, which until now was only possible by measuring the $\delta^{15}N$ value of amino acids (e.g.[15,16]). In contrast to IPLs, amino acids are rarely specific for groups of bacteria or eukaryotes. The exception is D-amino acids, such as D-alanine, which occurs predominantly in bacteria and thus its isotopic composition can be used to trace the incorporation of e.g. ^{15}N-labelled substrates in bacterial communities in the natural environment.[15,17]

To analyze the nitrogen-containing headgroups of IPLs, we developed novel methodology for the direct determination of the $\delta^{15}N$ values of the nitrogen-containing lipid headgroups using gas chromatography/combustion/isotope-ratio mass spectrometry (GC/C/IRMS). The method is based on the compound-specific determination of the $\delta^{15}N$ values of amino acids developed and refined by Metges *et al.*[18] and Chikaraishi *et al.*[19,20] The procedure was adapted for IPLs and tested on a number of IPL standards and several bacterial cultures as well as a microbial mat.

EXPERIMENTAL

Standards and culture material

Intact polar lipid standards comprising a number of different N-containing headgroups (Fig. 1; Table 1) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

The photoautotrophic purple sulfur bacterium *Thiocapsa roseopersicina* (strain DSM-217[21]) was grown on a modified Pfenning's medium[22] containing 0.34 g NH_4Cl, 0.34 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.34 g KCl, 0.25 g $CaCl_2.2H_2O$, 1.5 g $NaHCO_3$, 0.4 g $Na_2S.9H_2O$, 0.02 g vitamin B12, and 1 mL trace element solution SL-12 (Pfennig, 1965) per liter of distilled water at a pH of 7–7.5. The culture was incubated in airtight bottles at 25°C and a light intensity of approximately 1300 lux (16 h light, 8 h dark).

The chemolithoautotrophic sulfide oxidizer *Thiobacillus denitrificans* (strain DSM-12475[21]) was grown on a medium containing 2 g KH_2PO_4, 2 g KNO_3, 1 g NH_4Cl, 0.8 g $MgSO_4.7H_2O$, and 2 mL trace element solution SL-4, 5 g $Na_2S_2O_3.7H_2O$, 1 g $NaHCO_3$, 2 mg $FeSO_4.7H_2O$, and 1 mL 0.1 N H_2SO_4 per liter of distilled water at a pH of 7.0. The trace element solution SL-4 contained 0.5 g EDTA, 0.2 g $FeSO_4.7H_2O$, 0.01 g $ZnSO_4.7H_2O$, 3 mg $MnCl_2.4H_2O$, 0.03 g H_3BO_3, 0.02 g $CoCl_2.6H_2O$, 1 mg $CuCl_2.2H_2O$, 2 mg $NiCl_2.6H_2O$, and 3 mg $Na_2MoO_4.2H_2O$ per liter of distilled

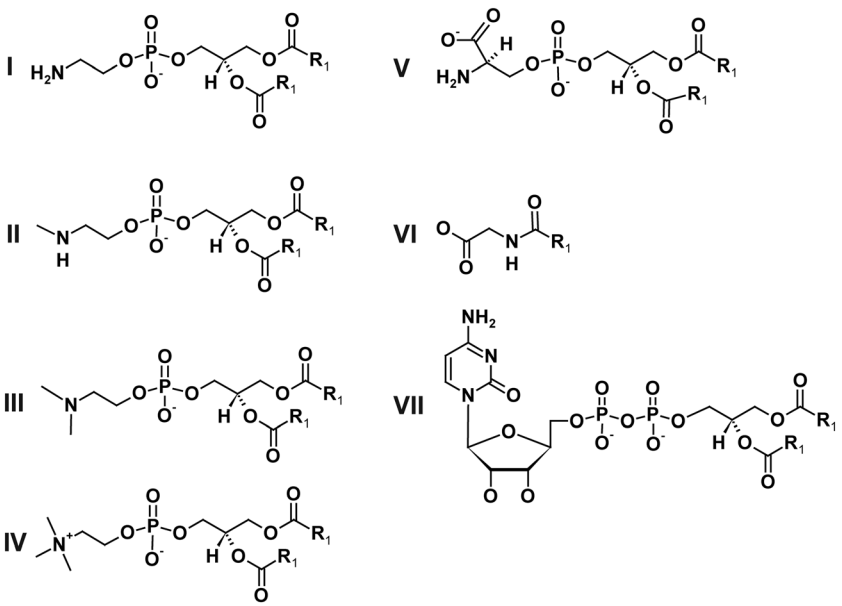

Figure 1. Structures of intact polar lipids tested in this study. **I** = phosphatidylethanolamine (PE), **II** = monomethyl phosphatidylethanolamine (MMPE), **III** = dimethyl phosphatidylethanolamine (DMPE), **IV** = phosphatidylcholine (PC); **V** = phosphatidylserine (PS); **VI** = N-acylglycine, **VII** = cytidine diphosphate (CDP).

Rapid Communications in Mass Spectrometry

Table 1. Stable nitrogen isotopic composition ($\delta^{15}N$ values) of headgroups released from intact polar lipid standards

Polar lipid	Acid Hydrolyzed	Hydrolysis product	$\delta^{15}N_{(CSIA)}$ Avg	$\delta^{15}N_{(CSIA)}$ SD	$\delta^{15}N_{(BULK)}$ Avg	$\delta^{15}N_{(BULK)}$ SD	$\Delta\delta^{15}N$ (CSIA-BULK) Avg	$\Delta\delta^{15}N$ (CSIA-BULK) SD
PE-DAG	yes	Ethanolamine	0.7	0.6	-0.7	0.6	1.4	0.9
monomethyl-PE-DAG	yes	CH_3-ethanolamine	13.5	0.3	–	–	–	–
dimethyl-PE-DAG	yes	No product	–	–	–	–	–	–
PC-DAG	yes	No product	–	–	–	–	–	–
CDP-DAG	yes	Cytidine*	–2.2	0.7	5.5	0.5	–7.6**	0.9
Amino acid								
PS-DAG	yes	Serine	–2.3	0.2	3.0	0.9	–5.3**	1.0
N-palmitoylglycine	no	Glycine	–0.8	–	0.9	0.1	–1.6	–

PE = phosphatidylethanolamine; PC = phosphatidylcholine; PS = phosphatidylserine; CDP = cytidine diphosphate; DAG = diacylglycerol.
See Fig. 1 for chemical structures.
*contains multiple nitrogen
**statistically significantly different (Student's t-test, $p < 0.05$)

water. The *T. denitrificans* cultures were incubated at 25°C. Samples were collected in the stationary phase by centrifugation of the culture samples in Falcon tubes.

Pseudomonas sp., a gram-negative, aerobic gammaproteobacterium, was grown on an ammonium-glucose medium as described in Heinzelmann *et al*.[23] The ammonium-glucose medium contained 5 g glucose, 0.2 g $MgSO_4.7H_2O$, 5 g NaCl, 1.3 g $(NH_4)_2HPO_4$, 1 g KH_2PO_4, 2 mL trace element solution SL-4 per liter of distilled water (pH 7.1). The culture was incubated at 25°C.

Escherichia coli (strain TOP10) culture was grown on Minimal M9 media, amended with glucose and casamino acids, containing 17 g $Na_2HPO_4.12H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl per liter of distilled water, 4.0 mM $MgSO_4$, 0.2 mM $CaCl_2$ and 0.4% of glucose and casamino acid solutions.

Pseudoalteromonas haloplanktis (strain DSM 6060[21]) was grown at 28°C for 48 h in a modified 3M+ medium[24] with 0.5% sodium acetate. The 3M+ media contained 17.6 g NaCl, 1.47 g Na_2SO_4, 0.08 g $NaHCO_3$, 0.25 g KCl, 0.04 g KBr, 1.87 g $MgCl_2.6H_2O$, 0.41 g $CaCl_2.2H_2O$, 0.008 g $SrCl_2.6H_2O$, 0.008 g H_3BO_3, 0.005 g $Na_2SiO_3.5H_2O$, 4.89 mg $FeCl_3.H_2O$, 0.51 g NaCl, 0.23 g K_2HPO_4, and 8.3 g 3-(N-morpholino)propanesulfonic acid buffer (MOPS).

The microbial mat sample was collected from a cyanobacteria-dominated mat on the Dutch island of Schiermonnikoog in November 2009.[25,26]

Extraction of intact polar lipids from cultures and microbial mat

Glassware was heated at 500°C for 4 h before use to remove organic material. All other labware was cleaned thoroughly, rinsed with bidistilled water, and dried at 100–130°C before use.

Extractions were carried out using a modified Bligh and Dyer procedure.[27,28] A known volume of a single-phase solvent mixture of methanol (MeOH)/dichloromethane (DCM)/phosphate buffer (PBS; 50 mM, pH 7–8) (2:1:0.8, v/v/v) was added to 2–3 mg of freeze-dried culture in a glass centrifuge tube and placed in an ultrasonic bath for 10 min. The extract and residue were separated by centrifugation at 2000 *g* for 3 min and the solvent mixture collected in a separate flask. This process was repeated three times. DCM and PBS were then added to the extract to give a new ratio of MeOH/DCM/PBS of 1:1:0.9 (v/v/v), and to induce phase separation. The extract was centrifuged at 2000 *g* for 3 min after which the DCM phase was collected in a round-bottom flask. The residual MeOH/PBS phase was washed two additional times with DCM, and the DCM phase added to the round-bottom flask. The combined DCM phases were reduced under rotary vacuum and transferred to a vial using MeOH/DCM (1:1 v/v) after which the solvents were removed by evaporation under a stream of N_2. The lipid extracts were stored at –20°C until further processing. The IPLs in the lipid extract were identified using high-performance liquid chromatography/electrospray ionization multi-stage mass spectrometry (HPLC/ESI-MS^n) following the method of Sturt *et al*.[29] with some minor modifications.[2,30]

Hydrolysis

The IPL standards (0.2 mg) and Bligh and Dyer lipid extracts from cultures and microbial mat (approximately 2 mg) were dissolved in MeOH/DCM (1:1, v/v) and transferred to 1-mL micro reaction vessels (Supelco, Zwijndrecht, The Netherlands). To release the headgroups from the IPLs, approximately 200 µL of hydrochloric acid (HCl, 6 M) was added to the reaction vials and the mixture was hydrolyzed at 110°C for 18–20 h using a dry bath (VWR, Amsterdam, The Netherlands) (step 1 in Fig. 2). Air was purged from the reaction vial by leaving the valve of a Mininert® cap (Supelco) open until HCl fumes were detected using pH indicator paper. The hydrolysate was filtered using a centrifugal filtration device (10 s, 9000 *g*; GHP Nanosep, Pall Co., Amsterdam, The Netherlands) and the filtrate transferred to a clean reaction vial. Approximately 200 µL *n*-hexane/DCM (3:2, v/v) was added to the filtrate, mixed thoroughly, and the upper organic layer pipetted off after phase separation (3×). The remaining acidic solution (containing the headgroups) was dried by the addition of MeOH and evaporation under N_2 (3×).

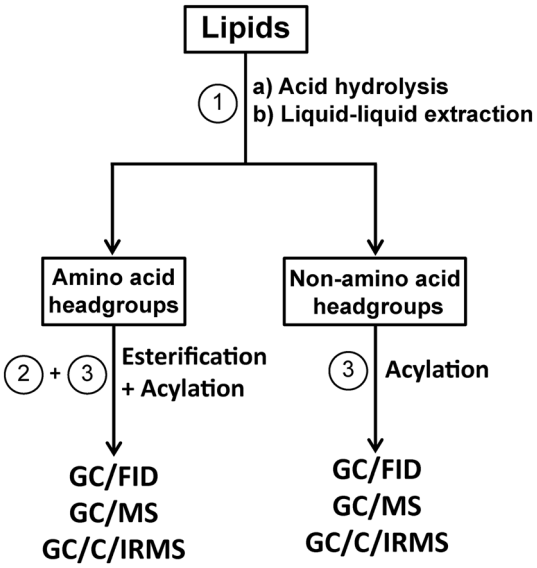

Figure 2. Scheme for release and derivatization of nitrogen-containing headgroups from intact polar lipids.

Derivatization

Nitrogen-containing headgroups were derivatized based on the method described by Metges *et al.*[18] and Chikaraishi *et al.*[19,20] for the compound-specific stable isotope analysis of amino acids. The amino acid headgroups released during acid hydrolysis (e.g. phosphatidylserine, **V** in Fig. 1) were first esterified (step 2 in Fig. 2) followed by acetylation (step 3 in Fig. 2). Esterification was performed in the 1-mL reaction vials using 2-propanol acidified with acetyl chloride (4:1, v/v), at 110°C for 2 h. Air was purged from the reaction vial by leaving the valve of the Mininert cap open for approximately 20 s after placing the vials in the hot dry bath. The samples were cooled down to room temperature and solvents were removed under a gentle stream of N_2. Excess reagents were removed by repeated washes (2×) with approximately 200 µL DCM, and evaporation under a gentle stream of N_2.

Non-amino acid headgroups were acetylated directly (step 3 in Fig. 2) without prior esterification. Acetylation was performed in the reaction vial at 110°C for 2 h, using a mixture of pivaloyl chloride (99%, Sigma-Aldrich, Zwijndrecht, The Netherlands) and DCM (1:4: v/v). Air was purged from the reaction vial by leaving the valve of the Mininert cap open for 10–20 s after placing the vials in the dry bath. Samples were cooled down and solvents were removed under a gentle stream of N_2. To remove excess reagents, approximately 200 µL DCM was added, after which the solvents were removed under a gentle stream of N_2 (2×). Liquid-liquid extraction was performed by the addition of approximately 200 µL bidistilled water and 500 µL of *n*-hexane/DCM (3:2 v/v) to the extracts. After shaking the sample for 10 s, the aqueous and organic phases were allowed to separate completely, after which the top/organic layer, containing the derivatives, was pipetted off and onto a magnesium sulfate column (1–1.5 cm of $MgSO_4$ in a Pasteur pipette). This was repeated three times. Solvents were removed under a gentle stream of N_2 until a few microliters remained. The filtrate was then diluted in dried (1% $MgSO_4$), sonicated, DCM and stored at –20°C until analysis.

Gas chromatography

The derivatized compounds were analyzed using a HP5890 gas chromatograph with a flame ionization detector (FID) (Agilent Technologies, Amstelveen, The Netherlands). Separation of derivatives was achieved on a DB-5ms column (J&W Agilent Technologies, Amstelveen, The Netherlands), either 50 m × 0.25mm i.d., 0.25 µm film thickness or 60 m × 0.32 mm i.d., 0.50 µm film thickness, using the temperature programs described in Supplemental Table 1 (see Supporting Information). The carrier gas was helium at a continuous flow rate of 2 mL min^{-1}. Samples were injected using an on-column injector, which tracked the oven temperature.

Identification of derivatives was done by GC/MS using an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C or 5977A single quadrupole mass-selective detector (MSD). The columns and temperature programs are described in Supplemental Table 1 (see Supporting Information). The carrier gas was helium at a continuous flow rate of 2 mL min^{-1}.

GC/C/IRMS

The $\delta^{15}N$ values of the *N,O*-pivaloyl derivatives were determined by GC/C/IRMS using a Delta V Advantage (Thermo Scientific, Breda, The Netherlands) connected to an Agilent 6890 gas chromatograph. The gas chromatograph and isotope-ratio mass spectrometer were interfaced via a combustion furnace (980°C), reduction furnace (650°C), and a liquid N_2 cold trap. The oxidation oven contained one nickel, one platinum and one copper wire, which were intertwined. The reduction oven contained four intertwined copper wires. Derivatives of the headgroups were analyzed using a 60 m DB-5ms column as described above for the gas chromatography and using an identical temperature program (Supplemental Table 1, see Supporting Information). The carrier gas was helium at a continuous flow rate of 2 mL min^{-1} (29 cm s^{-1}). The injection volumes ranged from 0.4 to 2 µL and were injected through cold column injection, which tracked the oven temperature.

The stable isotope ratios are expressed using the δ notation according to Eqn. (1):

$$\delta = \left(\left(R_{sample}/R_{standard}\right) - 1\right) \quad (1)$$

where $R = {}^{15}N/{}^{14}N$ and expressed in per mil versus N_2 in air. An in-house standard mixture consisting of five derivatized amino acids (glycine, norleucine, glutamic acid, phenylalanine, and tyrosine) with known $\delta^{15}N$ values (determined offline) was used to evaluate daily system performance. The long-term reproducibility (>1 year), based on the standard deviation of multiple injections (n = 52) of this standard, was ±0.7 (glycine), ±0.9 (norleucine), and ±1.2‰ (phenylalanine, glutamic acid, and tyrosine).

Elemental analyzer/isotope-ratio mass spectrometry (EA/IRMS)

The bulk stable nitrogen isotopic ratios of lipid standards and bacterial biomass were determined using a Delta V Advantage isotope-ratio mass spectrometer coupled to a Flash 2000 elemental analyzer (Thermo Scientific). Samples

with a low ratio of nitrogen to carbon atoms were analyzed on a Delta XL isotope-ratio mass spectrometer coupled to a Carlo Erba Instruments Flash 1112 Series elemental analyzer (Thermo Scientific) equipped with a solid CO_2 trap, which removes CO_2 from the sample stream and allows for the nitrogen isotope analysis of a larger amount of sample. An acetanilide standard with a $\delta^{15}N$ value of 1.18 (standard obtained from Arndt Schimmelmann, Indiana University, Bloomington, IN, USA[31]), and known %TOC and %TN content, was used for calibration. The acetanilide was calibrated against IAEA N-standards. The average repeatability of $\delta^{15}N$ determination was 0.2‰, based on repeated analyses of the acetanilide standard over time.

RESULTS AND DISCUSSION

Release and analysis of IPL headgroups

Our method for the determination of the $\delta^{15}N$ values of IPLs headgroups is based on that described by Metges *et al.*[18] and Chikaraishi *et al.*[19,20] for the compound-specific stable isotope analysis of amino acids. The IPL standards and lipid extracts were first acid hydrolyzed (step 1 in Fig. 2), to release the headgroups from the IPLs, followed by derivatization to make the polar compounds GC-amenable. Amino acid containing IPLs require both esterification of the carboxyl group and acetylation of the amine group (steps 2 and 3 in Fig. 2). For other headgroups, acetylation with pivaloyl chloride is sufficient (step 3 in Fig. 2). To test the method, seven IPL standards (Table 1; Fig. 1) were treated following this procedure and the products were analyzed and identified by GC/FID and GC/MS. Of the seven standards, five yielded GC-amenable products identifiable by GC/MS (Table 1). As an example, the products released from phosphatidylethanolamine diacylglycerol (PE; **I** in Fig. 1) are shown in Fig. 3(A) and reveal the presence of two main products. Based on mass spectral interpretation (Fig. 3(C)), the early eluting compound was identified as the pivaloyl derivative of ethanolamine (*N*,*O*-pivaloyl ethanolamine), while the later eluting compound was identified as the pivaloyl derivative of glycerol (*O*-pivaloyl glycerol). Other IPLs for which the derivatized headgroups could be identified were *N*-monomethyl ethanolamine (from N-monomethyl phosphoethanolamine diacylglycerol, MMPE; **II** in Fig. 1), serine (from phosphatidylserine diacylglycerol, PS; **V** in Fig. 1), glycine (from *N*-palmitoylglycine; **VI** in Fig. 1), and cytidine (from cytidine diphosphodiacylglycerol, CDP-DAG; **VII** in Fig. 1) (see Supplemental Figs. 1(A)–1(D) (Supporting Information) for mass spectra). For some IPLs, such as *N*-dimethyl phosphatidylethanolamine diacylglycerol (DMPE; **III** in Fig. 1) and phosphatidylcholine diacylglycerol (PC; **IV** in Fig. 1), we could not identify any products that could be linked to the headgroup of those IPLs. This is probably because pivaloyl chloride readily reacts with primary and secondary amines but not with the tertiary and quaternary amines present in DMPE and PC, resulting in (partially) underivatized products which were too volatile or too polar to be detected by GC analysis. This suggests that our method is not applicable to these types of IPLs and that alternative approaches, such as isolation by preparative HPLC followed by EA/IRMS analysis, are required in order to determine their $\delta^{15}N$ values.

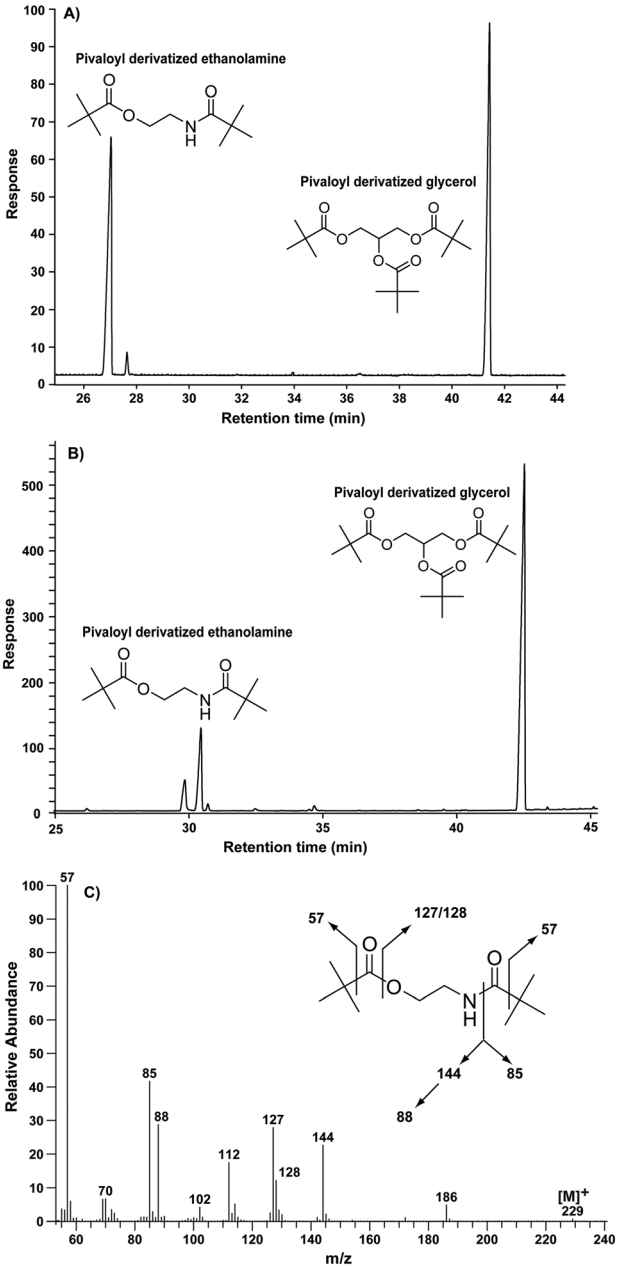

Figure 3. Gas chromatogram of derivatized products released from acid hydrolysis of: (A) phosphatidylethanolamine (PE; see Fig. 1 for structure) analyzed on a 50 m DB-5 column (Supplemental Table 1, see Supporting Information) and (B) a microbial mat from Schiermonnikoog analyzed on a 60 m DB-5 column (Supplemental Table 1). (C) Mass spectrum of the derivatized ethanolamine headgroup.

Isotopic analysis of headgroups

For the IPLs that yielded derivatized headgroups we determined the $\delta^{15}N$ values using GC/C/IRMS and compared them with offline values determined by EA/IRMS. The headgroups were in all but one case depleted in ^{15}N compared with the offline values (1.6 to 7.6‰), suggesting isotopic fractionation during hydrolysis and derivatization (Table 1). The differences in $\delta^{15}N$ values between the online (CSIA) and offline (BULK) values were statistically significant ($p < 0.05$) for PS and CDP but not for

PE and glycine. Isotopic fractionation of nitrogen in amino acids has previously been observed after hydrolysis.[32–34] Bada *et al.*[32] found that the free amino acids released by hydrolysis from bone collagen were enriched in ^{15}N compared with intact collagen although the difference was relatively small (1–2‰). Silfer *et al.*[33] looked at the isotopic fractionation of the glycine-glycine peptide bond and found that isotopic fractionation increased with both higher hydrolysis temperature and increasing yield of hydrolysis, but again the differences in $\delta^{15}N$ values between the free amino acid and bulk material were relatively small (<3‰). Our results for ethanolamine and glycine agree well with these studies, with $\Delta\delta^{15}N_{(CSIA\text{-}BULK)}$ values of IPLs generally <2‰, but large fractionations are observed for CDP and serine. Hofmann *et al.*[34] also found variable differences in isotopic fractionation induced by hydrolysis and derivatization for a variety of amino acids.

Together our results suggest that it possible to determine the $\delta^{15}N$ values of IPLs, but a correction may be needed for the isotopic fractionation induced by hydrolysis as well as derivatization. However, this issue is of lesser importance when the method is applied to trace ^{15}N-label incorporation into IPL headgroups.

Application to cultures and a microbial mat

As the $\delta^{15}N$ value of the derivatized ethanolamine headgroup from PE was not significantly different from that of the offline determined value, it would appear to be feasible to analyze the natural stable isotopic composition of this particular headgroup. Since PE is a common nitrogen-containing headgroup in bacterial lipids, and is rare and in low abundance in eukaryotes and archaea, it can potentially be applied to study the $\delta^{15}N$ values of bacterial biomass in the natural environment.

We first tested our method on IPLs extracted from five bacterial cultures, one photoautotrophic bacterium (*Thiocapsa roseopersicina*), one obligate chemoautotrophic bacterium (*Thiobacillus denitrificans*) and three heterotrophic bacteria (*Pseudomonas* sp., *Pseudoalteromonas haloplanktis*, and *Escherichia coli*; Table 2), which have been reported to contain PE in their cell membranes.[35–39] The presence of PE in all these cultures was confirmed by HPLC/MS analysis of the IPLs, and the derivatized headgroup of PE, *N,O*-pivaloylethanolamine, was detected by GC/MS in all cultures after application of our method. The $\delta^{15}N$ values of *N,O*-pivaloyl ethanolamine varied from –17.0 to –0.3 ‰ and the compound was depleted in ^{15}N compared with bulk biomass by 0.2 to 8.1 ‰ (Table 2; Fig. 4). These results compare well with our recent study,[40] in which the total lipid extracts of several aquatic animals were found to be highly depleted in ^{15}N compared with the bulk biomass. The ^{15}N-depleted amino acid serine was thought to be the cause for this depletion as serine is one of the main sources of nitrogen to PC, which is one of the most abundant IPLs in these animals.[41] As the nitrogen in PE is also derived from the amino acid serine,[42] we determined the $\delta^{15}N$ value of serine released from proteins in the residual, lipid-free, biomass of the bacterial cultures.[18,19] We found that serine in these bacteria was also depleted in ^{15}N compared with the bulk biomass, ranging from –15.6 to –0.3 ‰ (Fig. 4, Table 2). Importantly, the $\delta^{15}N$ value of ethanolamine correlated well with that of serine released from proteins (Fig. 5), suggesting that the $\delta^{15}N$ values of bacterial PE lipids reflects that of the amino acid serine. Interestingly,

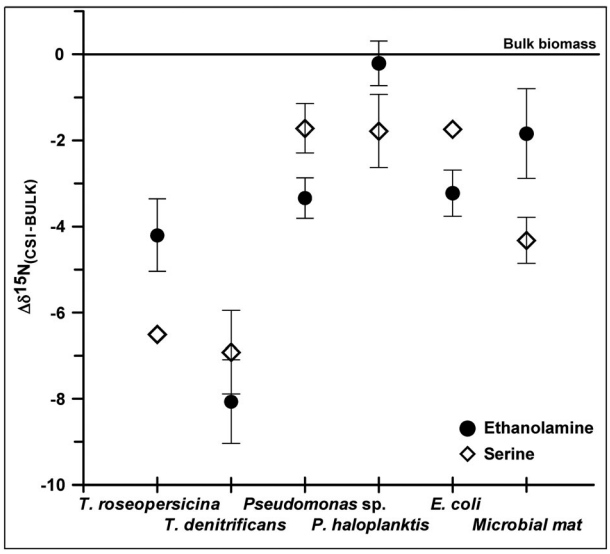

Figure 4. $\delta^{15}N$ values normalized to bulk $\delta^{15}N$ values for phosphatidylethanolamine headgroups (ethanolamine) and serine from five bacterial cultures.

Table 2. Microbial cultures and microbial mat $\delta^{15}N$ values of bulk biomass, phosphatidylethanolamine (PE) and serine (SER)

	Bulk		PE		Serine		$\Delta\delta^{15}N$ (PE-BULK)		$\Delta\delta^{15}N_{(PE\text{-}SER)}$	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Thiocapsa roseopersicina	–5.3	0.7	–9.5	0.6	–11.8		–4.2	0.8	2.3	
Thiobacillus denitrificans	–8.7	0.1	–17.0	1.0	–15.6	1.0	–8.1	1.0	–1.1	1.4
Escheria coli	1.5	0.1	–1.7	0.5	–0.3		–3.2	0.5	–1.5	
Pseudomonas sp.	–11.7	0.1	–15.0	0.4	–13.4	0.6	–3.3	0.5	–1.6**	0.7
Pseudoalteromonas haloplanktis	–3.0	0.1	–3.2	0.5	–4.8	0.8	–0.2	0.5	1.6**	1.0
Microbial mat	1.6	0.5	–0.3	0.9	–2.8	0.2	–1.8	1.0	2.5*	0.9

*statistically significantly different (Student's t-test, $p < 0.1$).
**statistically significantly different (Student's t-test, $p < 0.05$).

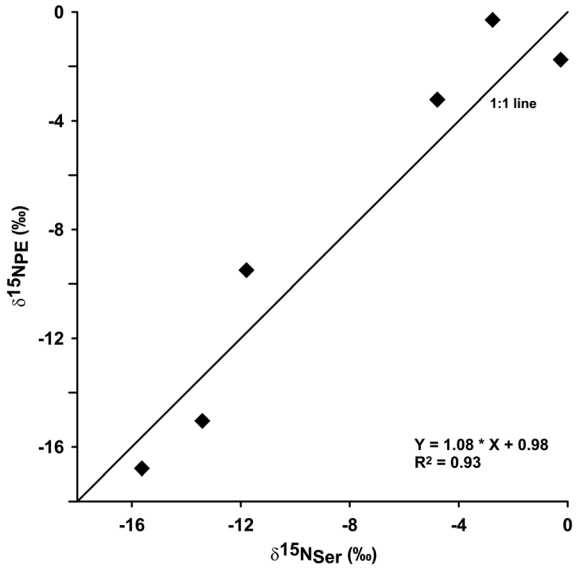

Figure 5. $\delta^{15}N$ values normalized to bulk $\delta^{15}N$ values for phosphatidylethanolamine headgroups (ethanolamine) and serine from five bacterial cultures.

both serine and PE of the autotrophic bacteria (*T. roseopersicina* and *T. denitrificans*) had similar quite negative $\Delta\delta^{15}N_{(CSIA\text{-}BULK)}$ values relative to that of the heterotrophic bacteria (*E. coli*, *Pseudomonas* sp., and *P. haloplanktis*; Fig. 4). This difference in $\Delta\delta^{15}N$ between autotrophs and heterotrophs is probably because the serine was synthesized by the autotrophic bacteria themselves while the heterotrophic bacteria obtained serine from the growth media.

The method was also applied to a cyanobacteria-dominated microbial mat from Schiermonnikoog island in the Dutch Wadden Sea,[26] known to contain PE.[25] The pivaloyl derivative of ethanolamine was detected in the lipid extract from the mat after application of our procedure (Fig. 3(B)). Isotopic analysis of this product (Supplemental Fig. 2, see Supporting Information), as well as of serine derived from proteins, showed that, similar to our heterotrophic bacterial cultures, both ethanolamine and serine were depleted in ^{15}N by 2 and 4 ‰, respectively, compared with the bulk biomass. Interestingly, the depletion in ^{15}N of PE compared with bulk biomass was between that of the autotrophic and heterotrophic bacteria, suggesting that the PE in the microbial mat was sourced from both autotrophic and heterotrophic sources. These results suggest that it is feasible to measure the $\delta^{15}N$ values of PE headgroups in natural samples using the method developed here. Since PE lipids in the environment are predominantly derived from bacteria, and not from eukaryotes or archaea, and the nitrogen isotopic composition of PE reflects that of serine, this is a selective method capable of e.g. tracing the incorporation of ^{15}N-labelled substrates into bacterial biomass under *in situ* conditions. Other methods used to analyze the nitrogen isotope dynamics in microbial communities are rarely selective for certain microbial groups, as most amino acids occur in all domains of life. The exception is D-alanine which is an amino acid predominantly occurring in bacteria and the ^{15}N incorporation of D-alanine has been used to trace the uptake of nitrogen substrates in bacteria.[15] However, D-alanine also occurs in some animals[43] and measurement of the $\delta^{15}N$ values of PE headgroups thus provides an independent method for tracing nitrogen through the microbial domain in the natural environment.

CONCLUSIONS

We have developed a procedure for the measurement of the $\delta^{15}N$ values of nitrogen-containing headgroups from intact polar lipids (IPLs). Our results show that the method works well for a select number of IPLs. Isotopic fractionations resulting from sample preparation were sometimes observed, suggesting that some correction may be needed for some IPLs. No statistically significant fractionation was observed for the $\delta^{15}N$ value of phosphatidylethanolamine, an important bacterial headgroup. Application of this method on five bacterial cultures and a microbial mat showed a similar depletion in ^{15}N of both PE and the amino acid serine compared with the bulk biomass, indicating that the $\delta^{15}N$ signal of PE is reflecting the $\delta^{15}N$ value of its biosynthetic nitrogen source, serine. Autotrophic bacteria PE and serine were in general more depleted in ^{15}N than heterotrophic bacteria, probably because autotrophic bacteria synthesize serine while heterotrophs obtain serine from their food source. Regardless of this, since PE lipids in the natural environment are predominantly sourced by bacteria, the determination of the $\delta^{15}N$ values of the phosphatidylethanolamine headgroups provides the opportunity to investigate the nitrogen isotope dynamics in bacterial biomass under *in situ* conditions.

Acknowledgements

We thank three anonymous reviewers for their valuable comments which improved the manuscript. This work is part of the Waddensleutels project (http://www.waddensleutels.nl/) and supported by the Waddenfonds. The authors would like to thank Laura Villanueva and Sandra Heinzelmann for supplying bacterial cultures, and Lukas J. Stal, Haoxin Fan and Nicole Bale for providing the microbial mat sample, collected as part of the NICYCLE project supported by the Netherlands Organization for Scientific Research (NWO) through the grants 839.08.331 and 839.08.332. Ellen Hopmans, Anchelique Mets, and Monique Verweij are acknowledged for their invaluable assistance with analyses.

REFERENCES

[1] A. Jaeschke, E. C. Hopmans, S. G. Wakeham, S. Schouten, J. S. Sinninghe Damsté. The presence of ladderane lipids in the oxygen minimum zone of the Arabian Sea indicates nitrogen loss through anammox. *Limnol. Oceanogr.* **2007**, *52*, 780.

[2] A. Pitcher, N. Rychlik, E. C. Hopmans, E. Spieck, W. I. C. Rijpstra, J. Ossebaar, S. Schouten, M. Wagner, J. S. Sinninghe Damsté. Crenarchaeol dominates the membrane lipids of *Candidatus* Nitrososphaera gargensis, a thermophilic Group I.1b Archaeon. *ISME J.* **2010**, *4*, 542.

[3] R. A. Gibson, M. T. J. van der Meer, E. C. Hopmans, A.-L. Reysenbach, S. Schouten, J. S. Sinninghe Damsté.

Comparison of intact polar lipid with microbial community composition of vent deposits of the Rainbow and Lucky Strike hydrothermal fields. *Geobiology* **2013**, *11*, 72.
[4] H. T. S. Boschker, J. J. Middelburg. Stable isotopes and biomarkers in microbial ecology. *FEMS Microbiol. Ecol.* **2002**, *40*, 85.
[5] J. Hoefs. *Stable Isotope Geochemistry*. Springer, Berlin Heidelberg, **1997**.
[6] S. Schouten, W. C. M. Klein Breteler, P. Blokker, N. Schogt, W. I. C. Rijpstra, K. Grice, M. Baas, J. S. Sinninghe Damsté. Biosynthetic effects on the stable carbon isotopic compositions of algal lipids: implications for deciphering the carbon isotopic biomarker record. *Geochim. Cosmochim. Acta* **1998**, *62*, 1397.
[7] R. H. Smittenberg, C. Saenger, M. N. Dawson, J. P. Sachs. Compound-specific D/H ratios of the marine lakes of Palau as proxies for West Pacific Warm Pool hydrologic variability. *Quat. Sci. Rev.* **2011**, *30*, 921.
[8] X. Zhang, A. L. Gillespie, A. L. Sessions. Large D/H variations in bacterial lipids reflect central metabolic pathways. *Proc. Natl. Acad. Sci.* **2009**, *106*, 12580.
[9] A. A. Jones, A. L. Sessions, B. J. Campbell, C. Li, D. L. Valentine. D/H ratios of fatty acids from marine particulate organic matter in the California Borderland Basins. *Org. Geochem.* **2008**, *39*, 485.
[10] A. L. Sessions, L. L. Jahnke, A. Schimmelmann, J. M. Hayes. Hydrogen isotope fractionation in lipids of the methane-oxidizing bacterium *Methylococcus capsulatus*. *Geochim. Cosmochim. Acta* **2002**, *66*, 3955.
[11] Y. Takano, Y. Chikaraishi, N. O. Ogawa, H. Nomaki, Y. Morono, F. Inagaki, H. Kitazato, K.-U. Hinrichs, N. Ohkouchi. Sedimentary membrane lipids recycled by deep-sea benthic archaea. *Nat. Geosci.* **2010**, *3*, 858.
[12] Y.-S. Lin, J. S. Lipp, M. Y. Yoshinaga, S.-H. Lin, M. Elvert, K.-U. Hinrichs. Intramolecular stable carbon isotopic analysis of archaeal glycosyl tetraether lipids. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2817.
[13] A. Jaeschke, C. Rooks, M. Trimmer, J. C. Nicholls, E. C. Hopmans, S. Schouten, J. S. Sinninghe Damsté. Comparison of ladderane phospholipid and core lipids as indicators for anaerobic ammonium oxidation (anammox) in marine sediments. *Geochim. Cosmochim. Acta* **2009**, *73*, 2077.
[14] A. Pitcher, L. Villanueva, E. C. Hopmans, S. Schouten, G.-J. Reichart, J. S. Sinninghe Damsté. Niche segregation of ammonia-oxidizing archaea and anammox bacteria in the Arabian Sea oxygen minimum zone. *ISME J.* **2011**, *5*, 1896.
[15] B. Veuger, J. J. Middelburg, H. T. S. Boschker, M. Houtekamer. Analysis of ^{15}N incorporation into D-alanine: a new method for tracing nitrogen uptake by bacteria. *Limnol. Oceanogr. Methods* **2005**, *3*, 230.
[16] M. L. Calleja, F. Batista, M. Peacock, R. Kudela, M. D. McCarthy. Changes in compound specific $\delta^{15}N$ amino acid signatures and D/L ratios in marine dissolved organic matter induced by heterotrophic bacterial reworking. *Mar. Chem.* **2013**, *149*, 32.
[17] B. Veuger, J. J. Middelburg, H. T. S. Boschker, M. Houtekamer. Update of "Analysis of ^{15}N incorporation into D-alanine: A new method for tracing nitrogen uptake by bacteria" (Veuger et al. 2005, Limnol. Oceanogr. Methods 3:230-240). *Limnol. Oceanogr. Methods* **2007**, *5*, 192.
[18] C. C. Metges, K. Petzke, U. Hennig. Gas chromatography/combustion/isotope ratio mass spectrometric comparison of N-acetyl- and N-pivaloyl amino acid esters to measure ^{15}N isotopic abundances in physiological samples: a pilot study on amino acid synthesis in the upper gastro-intestinal tract of minipigs. *J. Mass Spectrom.* **1996**, *31*, 367.
[19] Y. Chikaraishi, Y. Kashiyama, N. O. Ogawa, H. Kitazato, N. Ohkouchi. Metabolic control of nitrogen isotope composition of amino acids in macroalgae and gastropods: implications for aquatic food web studies. *Mar. Ecol. Prog. Ser.* **2007**, *342*, 85.
[20] Y. Chikaraishi, N. O. Ogawa, Y. Kashiyama, Y. Takano, H. Suga, A. Tomitani, H. Miyashita, H. Kitazato, N. Ohkouchi. Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids. *Limnol. Oceanogr. Methods* **2009**, *7*, 740.
[21] DSMZ. Catalogue of Microorganisms. Available: https://www.dsmz.de/catalogues/catalogue-microorganisms.html, n.d.
[22] DSMZ. List of Media for Microorganisms. Available: https://www.dsmz.de/catalogues/catalogue-microorganisms/culture-technology/list-of-media-for-microorganisms.html.
[23] S. M. Heinzelmann, L. Villanueva, D. Sinke-Schoen, J. S. Sinninghe Damsté, S. Schouten, M. T. J. van der Meer. Impact of metabolism and growth phase on the hydrogen isotopic composition of microbial fatty acids. *Aquat. Microbiol.* **2015**, *6*, 408.
[24] J. Östling, A. Goodman, S. Kjelleberg. Behaviour of IncP-1 plasmids and a miniMu transposon in a marine Vibrio sp.: isolation of starvation inducible lac operon fusions. *FEMS Microbiol. Ecol.* **1991**, *9*, 83.
[25] T. Bauersachs, J. Compaoré, I. Severin, E. C. Hopmans, S. Schouten, L. J. Stal, J. S. Sinninghe Damsté. Diazotrophic microbial community of coastal microbial mats of the southern North Sea: Coastal microbial mats of the North Sea. *Geobiology* **2011**, *9*, 349.
[26] H. Bolhuis, L. J. Stal. Analysis of bacterial and archaeal diversity in coastal microbial mats using massive parallel 16S rRNA gene tag sequencing. *ISME J.* **2011**, *5*, 1701.
[27] E. G. Bligh, W. J. Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911.
[28] A. Pitcher, C. Wuchter, K. Siedenberg, S. Schouten, J. S. Sinninghe Damsté. Crenarchaeol tracks winter blooms of ammonia-oxidizing Thaumarchaeota in the coastal North Sea. *Limnol. Oceanogr.* **2011**, *56*, 2308.
[29] H. F. Sturt, R. E. Summons, K. Smith, M. Elvert, K.-U. Hinrichs. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry – new biomarkers for biogeochemistry and microbial ecology. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 617.
[30] S. Schouten, E. C. Hopmans, M. Baas, H. Boumann, S. Standfest, M. Konneke, D. A. Stahl, J. S. Sinninghe Damsté. Intact membrane lipids of "*Candidatus Nitrosopumilus maritimus*", a cultivated representative of the cosmopolitan mesophilic Group I Crenarchaeota. *Appl. Environ. Microbiol.* **2008**, *74*, 2433.
[31] A. Schimmelmann, A. Albertino, P. E. Sauer, H. Qi, R. Molinie, F. Mesnard. Nicotine, acetanilide and urea multi-level ^{2}H-, ^{13}C- and ^{15}N-abundance reference materials for continuous-flow isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3513.
[32] J. L. Bada, M. J. Schoeninger, A. Schimmelmann. Isotopic fractionation during peptide bond hydrolysis. *Geochim. Cosmochim. Acta* **1989**, *53*, 3337.
[33] J. A. Silfer, M. H. Engel, S. A. Macko. Kinetic fractionation of stable carbon and nitrogen isotopes during peptide bond hydrolysis: Experimental evidence and geochemical implications. *Chem. Geol. Isot. Geosci. Sect.* **1992**, *101*, 211.

[34] D. Hofmann, M. Gehre, K. Jung. Sample preparation techniques for the determination of natural 15N/14N variations in amino acids by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). *Isotopes Environ. Health Stud.* **2003**, *39*, 233.
[35] G. F. Ames. Lipids of *Salmonella typhimurium* and *Escherichia coli*: Structure and Metabolism. *J. Bacteriol.* **1968**, *95*, 833.
[36] J. K. Barridge, J. M. Shively. Phospholipids of the thiobacilli. *J. Bacteriol.* **1968**, *95*, 2182.
[37] S. G. Wilkinson, L. Galbraith, G. A. Lightfoot. Cell walls, lipids, and lpopolysaccharides of Pseudomonas species. *Eur. J. Biochem.* **1973**, *33*, 158.
[38] J. F. Imhoff, D. J. Kushner, S. C. Kushwaha, M. Kates. Polar lipids in phototrophic bacteria of the Rhodospirillaceae and Chromatiaceae families. *J. Bacteriol.* **1982**, *150*, 1192.
[39] G. M. Frolova, V. V. Kurilenko, E. P. Ivanova, N. M. Gorshkova, V. V. Mikhailov. Phospholipids of marine proteobacteria of the genus Pseudoalteromonas. *Microbiology* **2000**, *69*, 417.
[40] E. Svensson, S. Schouten, E. C. Hopmans, J. J. Middelburg, J. S. S. Sinninghe Damsté. Stable nitrogen isotope composition ($\delta^{15}N$) of lipids in marine animals reflect the stable nitrogen isotope composition of the base of the food web. **in revision for PLOS ONE**.
[41] L. K. Cole, J. E. Vance, D. E. Vance. Phosphatidylcholine biosynthesis and lipoprotein metabolism. *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids* **2012**, *1821*, 754.
[42] C. Ratledge. *Microbial Lipids*. Academic Press, London, **1989**.
[43] H. Abe, N. Yoshikawa, M. G. Sarower, S. Okada. Physiological function and metabolism of free D-alanine in aquatic animals. *Biol. Pharm. Bull.* **2005**, *28*, 1571.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

