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

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## 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 isotopes have the potential to elucidate food-web interactions or metabolic pathways. Here we have developed novel methodology for the determination of  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  values of 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  $\delta^{15}\text{N}$  values of ethanolamine and glycine were not statistically different compared to 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

We developed a method to enable CSIA of nitrogen of selected IPLs. The method is suitable for measuring natural stable nitrogen isotopes in microbial lipids, in particular phosphatidylethanolamine, and will be especially useful to trace the fate of nitrogen in deliberate tracer experiments.

## Introduction

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.<sup>[e.g. 1,2,3]</sup>

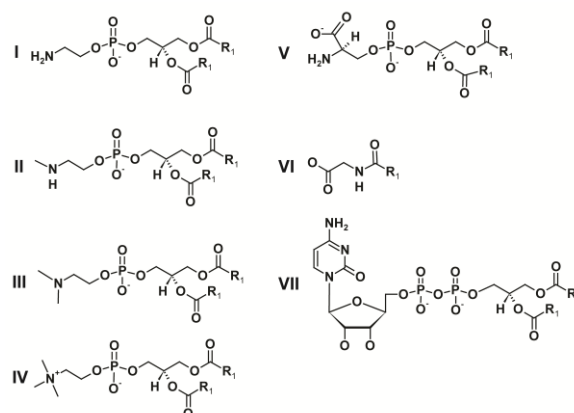
Many methods have been developed to measure the isotopic composition of lipids as a means to obtain the natural stable isotopic signature of specific organisms, particularly of microbes, and for tracing the incorporation of labels in microbes in deliberate tracer experiments.<sup>[e.g. 4]</sup> Since 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<sub>4</sub> cleavage of the ether bonds.<sup>[e.g. 5,6]</sup> Most of the compound specific stable isotope analysis (CSIA) of lipids has focused on determining the natural <sup>13</sup>C-contents or the incorporation of <sup>13</sup>C-labelled substrates into these core lipids, but has also been extended to include hydrogen isotopes,  $\delta^2\text{H}$ .<sup>[7–10]</sup> Some studies have also examined the stable carbon isotopic composition ( $\delta^{13}\text{C}$ ) of headgroups and the glycerol backbone of IPLs. For example, Takano et al.<sup>[11]</sup> analyzed the  $\delta^{13}\text{C}$  of the glycerol moiety of ether lipids using boron tribromide (BBr<sub>3</sub>), and Lin et al.<sup>[12]</sup> developed a method to analyze the <sup>13</sup>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 Figure 1) and eukaryotes (e.g. phosphatidylcholine, **IV** in Figure 1) and in intermediaries of lipid biosynthesis (e.g. phosphatidylserine and

cytidine diphosphate diacylglycerol; **V** and **VII** in Figure 1, respectively). Recent studies of IPLs in cultures and the natural environment has 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<sup>[13]</sup>, while certain glycerol dibiphytanyl glycerol tetraether with a phosphohexose headgroup are good indicators for the archaeal groups, such as the Thaumarchaeota<sup>[14]</sup>. 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 analyzing the  $\delta^{15}\text{N}$  of amino acids.<sup>[e.g. 15,16]</sup> 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.  $^{15}\text{N}$ -labelled substrates in bacterial communities in the natural environment.<sup>[15,17]</sup>

To analyze the nitrogen-containing headgroups of IPLs, we developed novel methodology for the direct determination of the  $\delta^{15}\text{N}$  values of the nitrogen-containing lipid headgroups using GC/C/IRMS. The method is based on the compound-specific determination of  $\delta^{15}\text{N}$  values of amino acids developed and refined by Metges et al.<sup>[18]</sup> and Chikaraishi et al.<sup>[19,20]</sup> The procedure was adapted for IPLs and tested on a number of

IPL standards and several bacterial cultures as well as a microbial mat.



**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-acyl-glycine; VII = cytidine diphosphate (CDP).

## Experimental

### Standards and culture material

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

The photoautotrophic purple sulfur bacterium *Thiocapsa roseopersicina* (strain DSM-217<sup>[21]</sup>) was grown on a modified Pfenning's medium<sup>[22]</sup> containing 0.34 g  $\text{NH}_4\text{Cl}$ , 0.34 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.34 g  $\text{KCl}$ , 0.25 g  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ , 1.5 g  $\text{NaHCO}_3$ , 0.4 g  $\text{Na}_2\text{S} \cdot 9 \text{H}_2\text{O}$ , 0.02 g vitamin B12, and 1 mL trace element solution SL-12 (Pfenning, 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).

**Table 1. Stable nitrogen isotopic composition of headgroups released from intact polar lipid standards. PE=phosphatidylethanolamine; PC=phosphatidylcholine; PS=phosphatidylserine; CDP=cytidine diphosphate; DAG=diacylglycerol. See Figure 1 for chemical structures.**

Polar lipid	Acid Hydrolyzed	Hydrolysis product	$\delta^{15}\text{N}_{(\text{CSIA})}$		$\delta^{15}\text{N}_{(\text{BULK})}$		$\Delta\delta^{15}\text{N}_{(\text{CSIA-BULK})}$	
			Avg	SD	Avg	SD	Avg	SD
PE-DAG	yes	Ethanolamine	0.7	0.6	-0.7	0.6	1.4	0.9
monomethyl-PE-DAG	yes	CH <sub>3</sub> -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
<b><u>Amino acid</u></b>								
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	-

\*= contains multiple nitrogen

\*\*= statistically significant different (Student's t-test,  $p < 0.05$ )

The chemolithoautotrophic sulfide oxidizer *Thiobacillus denitrificans* (strain DSM-12475<sup>[21]</sup>) was grown on a medium containing 2 g KH<sub>2</sub>PO<sub>4</sub>, 2 g KNO<sub>3</sub>, 1 g NH<sub>4</sub>Cl, 0.8 g MgSO<sub>4</sub>·7 H<sub>2</sub>O, and 2 mL trace element solution SL-4, 5 g Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·7 H<sub>2</sub>O, 1 g NaHCO<sub>3</sub>, 2 mg FeSO<sub>4</sub>·7 H<sub>2</sub>O, and 1 mL 0.1 N H<sub>2</sub>SO<sub>4</sub> 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<sub>4</sub>·7 H<sub>2</sub>O, 0.01 g ZnSO<sub>4</sub>·7 H<sub>2</sub>O, 3 mg MnCl<sub>2</sub>·4 H<sub>2</sub>O, 0.03 g H<sub>3</sub>BO<sub>3</sub>, 0.02 g CoCl<sub>2</sub>·6 H<sub>2</sub>O, 1 mg CuCl<sub>2</sub>·2 H<sub>2</sub>O, 2 mg NiCl<sub>2</sub>·6 H<sub>2</sub>O, and 3 mg Na<sub>2</sub>MoO<sub>4</sub>·2 H<sub>2</sub>O per liter of distilled water. *T. denitrificans* cultures were incubated at 25°C. Samples were collected in the stationary phase by centrifugation of culture samples in Falcon tubes. *Pseudomonas* sp., a gram-negative, aerobic gammaproteobacterium, was grown on an ammonium-glucose medium as described in Heinzemann et al.<sup>[23]</sup> The ammonium-glucose medium contained 5 g glucose, 0.2 g MgSO<sub>4</sub>·7 H<sub>2</sub>O, 5 g NaCl, 1.3 g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 1 g KH<sub>2</sub>PO<sub>4</sub>, 2 mL trace element solution SL-4 per liter of distilled water (pH 7.1). The culture was incubated at 25 °C.

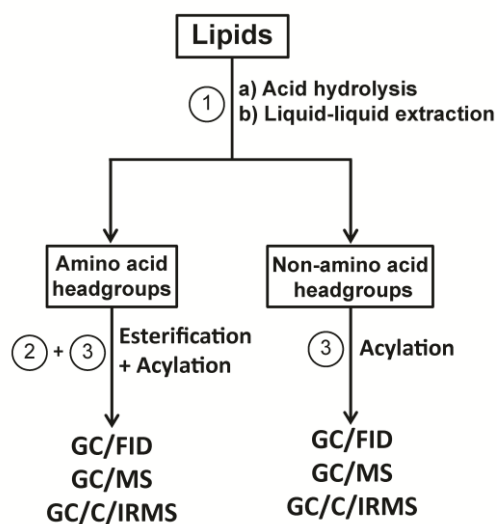
*Escherichia coli* (strain TOP10) culture was grown on Minimal M9 media, amended with glucose and casamino acids containing 17 g Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 3.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g NaCl, 1.0 g NH<sub>4</sub>Cl per liter of distilled water, 4.0 mM MgSO<sub>4</sub>, 0.2 mM CaCl<sub>2</sub> and 0.4% of glucose and casamino acid solutions.

*Pseudoalteromonas haloplanktis* (strain DSM 6060<sup>[21]</sup>) was grown at 28°C for 48 h in a modified 3M+ medium<sup>[24]</sup> with 0.5% sodium acetate. The 3M+ media contained 17.6 g NaCl, 1.47 g Na<sub>2</sub>SO<sub>4</sub>, 0.08 g NaHCO<sub>3</sub>, 0.25 g KCl, 0.04 g KBr, 1.87 g MgCl<sub>2</sub>·6 H<sub>2</sub>O, 0.41 g CaCl<sub>2</sub>·2 H<sub>2</sub>O, 0.008 g SrCl<sub>2</sub>·6 H<sub>2</sub>O, 0.008 g H<sub>3</sub>BO<sub>3</sub>, 0.005 g Na<sub>2</sub>SiO<sub>3</sub>·5 H<sub>2</sub>O, 4.89 mg FeCl<sub>3</sub>·H<sub>2</sub>O, 0.51 g NaCl, 0.23 g K<sub>2</sub>HPO<sub>4</sub>, 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.<sup>[25,26]</sup>

### 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.



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

Extractions were carried out using a modified Bligh and Dyer procedure.<sup>[27,28]</sup> A known volume of 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, transferred to a vial using MeOH/DCM (1:1 v/v) after which the solvents removed by evaporation under a stream of N<sub>2</sub>. Lipid extracts were stored at -20°C until further processing. IPLs in the lipid extract were identified using HPLC/ESI-MS<sup>n</sup> following the method of Sturt et al.<sup>[29]</sup> with some minor modifications.<sup>[2,30]</sup>

### Hydrolysis

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, 6M) 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 Figure 2). Air was purged from the reaction vial by leaving the valve of a Mininert<sup>®</sup> cap (Supelco, Sigma-Aldrich, Zwijndrecht, The Netherlands) 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 (3x). The remaining acidic solution (containing the headgroups) was dried by

addition of MeOH and evaporation under N<sub>2</sub> (3x).

### **Derivatization**

Nitrogen-containing headgroups were derivatized based on the method described by Metges et al.<sup>[18]</sup> and Chikaraishi et al.<sup>[19,20]</sup> for the compound-specific stable isotope analysis of amino acids. Amino acid headgroups released during acid hydrolysis (e.g. phosphatidylserine, **V** Figure 1) were first esterified (step 2 in Figure 1) followed by acetylation (step 3 in Figure 1). 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. Samples were cooled down to room temperature and solvents were removed under a gentle stream of N<sub>2</sub>. Excess reagents were removed by repeated washes (2x) with approximately 200 µl DCM, and evaporation under a gentle stream of N<sub>2</sub>.

Non-amino acid headgroups were acetylated directly (step 3 in Figure 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) 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<sub>2</sub>. To remove excess reagents, approximately 200 µl DCM was added, after which the solvents were removed under a gentle stream of N<sub>2</sub> (2x). Liquid-liquid extraction was done by

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<sub>4</sub> in a Pasteur pipette). This was repeated three times. Solvents were removed under a gentle stream of N<sub>2</sub> until a few microliters remained. The filtrate was then diluted in dried (1% MgSO<sub>4</sub>), sonicated, DCM and stored at -20°C until analysis.

### **Gas chromatography**

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

Identification of derivatives was done with GC/mass spectrometry (GC/MS) using an Agilent 7890A gas chromatograph interfaced with a 5975C or 5977A mass selective detector (MSD). Columns and temperature programs are described in Supplemental Table 1. Carrier gas was helium at a continuous flow of 2 mL min<sup>-1</sup>.

**Continuous flow gas chromatography-isotope ratio mass spectrometry (GC/C/IRMS)**

The  $\delta^{15}\text{N}$  values of *N,O*-pivaloyl derivatives was 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  $\text{N}_2$  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 headgroups were analyzed using a 60 m DB-5ms column as described for the GC and using an identical temperature program (Supplemental Table 1). Carrier gas was helium at a continuous flow of  $2 \text{ mL min}^{-1}$  ( $29 \text{ cm sec}^{-1}$ ). Injection volumes ranged from 0.4 – 2  $\mu\text{l}$  and were injected through cold column injection which tracked the oven temperature.

Stable isotope ratios are expressed using the  $\delta$  notation in units per mil according to:

$$\delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right), \quad \text{[Equation 1]}$$

where  $R = {}^{15}\text{N}/{}^{14}\text{N}$

and expressed in per mil versus air. The isotopes values are referenced against external  $\text{N}_2$  gas. An in-house standard mixture consisting of five derivatized amino acids (glycine, norleucine, glutamic acid, phenylalanine, and tyrosine) with known  $\delta^{15}\text{N}$  values (determined offline) was used to evaluate daily system performance. Long-term reproducibility (>1 year), based on the standard deviation of multiple injections ( $n=52$ ) of this standard was  $\pm 0.7$  (glycine),

$\pm 0.9$  (norleucine), and  $\pm 1.2\text{‰}$  (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, Breda, The Netherlands). 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 (Thermo Scientific, Breda, The Netherlands) elemental analyzer and equipped with a solid  $\text{CO}_2$  trap, which removes  $\text{CO}_2$  from the sample stream and allows for the nitrogen isotope analysis of a larger amount of sample. An acetanilide standard with a  $\delta^{15}\text{N}$  value of 1.18 (standard obtained from Arndt Schimmelmann, Indiana University, Bloomington, IN, USA<sup>[31]</sup>), and known %TOC and %TN content, was used for calibration. The acetanilide was calibrated against IAEA N-standards. The average repeatability of  $\delta^{15}\text{N}$  determination was 0.2‰ based on repeated analysis of the acetanilide standard over time.

**Results & Discussion****Release and analysis of IPL headgroups**

Our method for the determination of  $\delta^{15}\text{N}$  values of IPLs headgroups is based on the method described by Metges et al.<sup>[18]</sup> and Chikaraishi et al.<sup>[19,20]</sup> for the compound-specific stable isotope analysis of amino acids. The IPL standards and lipid extracts were first acid hydrolyzed (step 1 in Figure 2), to release the headgroups from the IPLs,

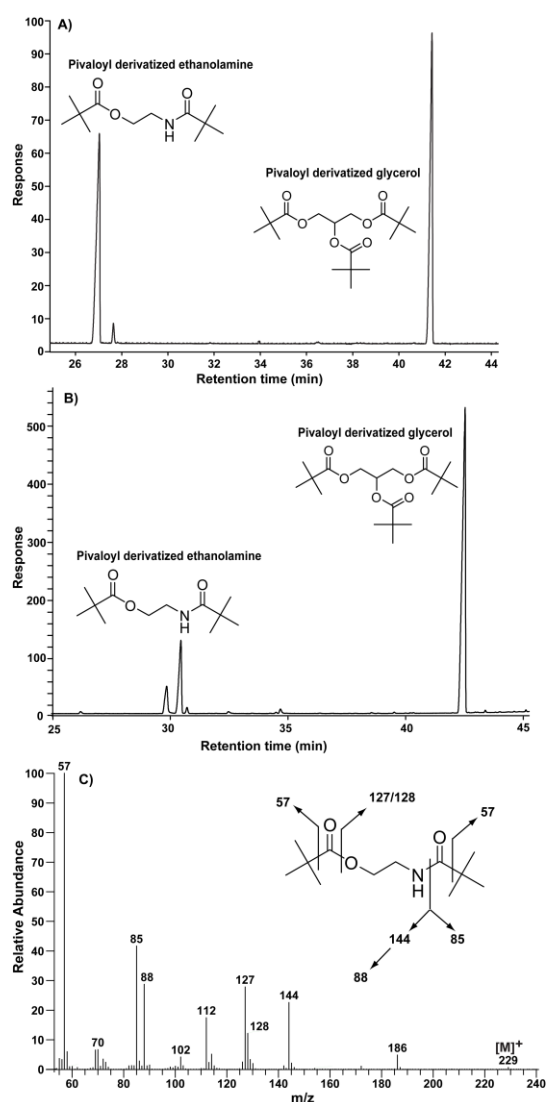


followed by derivatization to make polar compounds GC-amenable. Amino acid containing IPLs require both esterification of the carboxyl group and acetylation of the amine group (step 2 and 3; Figure 2). For other headgroups, acetylation with pivaloyl chloride is sufficient (step 3, Figure 2). To test the method, seven IPL standards (Table 1; Figure 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 Figure 1) are shown in Figure 3A and reveal the presence of two main products. Based on mass spectral interpretation (Figure 3C), 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 Figure 1), serine (from phosphatidylserine diacylglycerol, PS; **V** in Figure 1), glycine (from *N*-palmitoylglycine; **VI** in Figure 1), and cytidine (from cytidine diphospho- diacylglycerol, CDP-DAG; **VII** in Figure 1) (see Supplemental Figure 1A-D for mass spectra). For some IPLs, such as *N*-dimethyl-phosphatidylethanolamine diacylglycerol (DMPE; **III** in Figure 1) and phosphatidylcholine diacylglycerol (PC; **IV** in Figure 1), we could not identify any products which could be linked to the headgroup of

those IPLs. This is likely due to the fact that 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 analyze their  $\delta^{15}\text{N}$  values.

#### **Isotopic analysis of headgroups**

For the IPLs which yielded derivatized headgroups we determined the  $\delta^{15}\text{N}$  values using GC/C/IRMS and compared them with offline values determined by EA/IRMS. Headgroups were in all but one case depleted in  $^{15}\text{N}$  compared with the offline values (1.6 to 7.6‰), suggesting isotopic fractionation during hydrolysis and derivatization (Table 1). The difference in  $\delta^{15}\text{N}$  values between online (CSIA) and offline (BULK) values was 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.<sup>[32–34]</sup> Bada et al.<sup>[32]</sup> found that the free amino acids released by hydrolysis from bone collagen were enriched in  $^{15}\text{N}$  compared to intact collagen although the difference was relatively small (1–2‰). Silfer et al.<sup>[33]</sup> looked at the isotopic fractionation of the glycine-glycine peptide bond and found that isotopic fractionation increased with both higher hydrolysis



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

temperature and increasing yield of hydrolysis, but again, differences in  $\delta^{15}\text{N}$  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  $\Delta\delta^{15}\text{N}_{(\text{CSIA-BULK})}$  values of IPLs generally <2‰ but large fractionations are observed for CDP and serine. Hofmann et al.<sup>[34]</sup> also found variable differences in isotopic fractionation induced by hydrolysis and derivatization for a variety of amino acids.

Together our results suggests that it possible to determine the  $\delta^{15}\text{N}$  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  $^{15}\text{N}$  label incorporation into IPL headgroups.

#### **Application to cultures and a microbial mat**

As the  $\delta^{15}\text{N}$  value of the derivatized ethanolamine headgroup from PE was not significantly different from that of the offline determined value, it appears that it would 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  $\delta^{15}\text{N}$  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.,

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

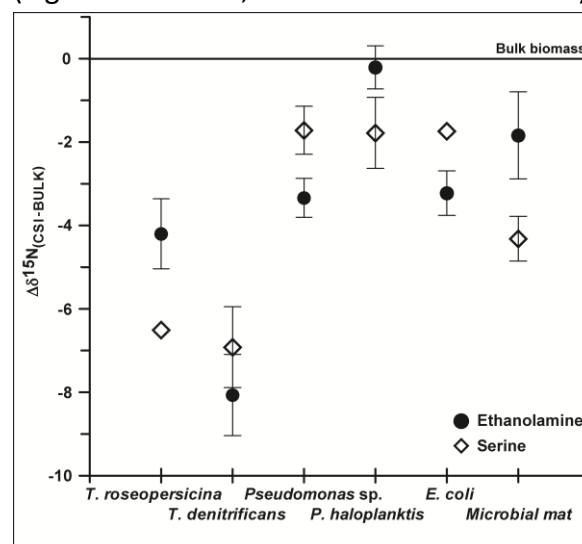
	BULK		PE		Serine		$\Delta\delta^{15}\text{N}_{(\text{PE-BULK})}$		$\Delta\delta^{15}\text{N}_{(\text{PE-SER})}$	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
<i>Thiocapsa roseopersicina</i>	-5.3	0.7	-9.5	0.6	-	11.8	-4.2	0.8	2.3	
<i>Thiobacillus denitrificans</i>	-8.7	0.1	-	1.0	-	15.6	-8.1	1.0	-1.1	1.4
<i>Escheria coli</i>	1.5	0.1	-1.7	0.5	-0.3		-3.2	0.5	-1.5	
<i>Pseudomonas sp.</i>	-	0.1	-	0.4	-	0.6	-3.3	0.5	-	0.7
<i>Pseudoalteromonas haloplanktis</i>	11.7		15.0		13.4				1.6**	
<i>Pseudoalteromonas haloplanktis</i>	-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 significant different (Student's t-test,  $p < 0.1$ )

\*\* = statistically significant different (Student's t-test,  $p < 0.05$ )

*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 of these cultures was confirmed by HPLC/MS analysis of IPLs, and the derivatized headgroup of PE, *N,O*-pivaloyl ethanolamine, was detected by GC/MS in all cultures after application of our method. The  $\delta^{15}\text{N}$  values of *N,O*-pivaloyl ethanolamine varied from -17.0 to -0.3 ‰ and the compound was depleted in  $^{15}\text{N}$  compared to bulk biomass by 0.2 to 8.1 ‰ (Table 2; Figure 4). These results compare well with a recent study of ours [40] in which the nitrogen isotopic composition of total lipid extracts of several aquatic animals were found to be highly depleted in  $^{15}\text{N}$  compared to bulk biomass. The  $^{15}\text{N}$ -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  $\delta^{15}\text{N}$  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  $^{15}\text{N}$  compared to bulk biomass, ranging from -15.6 to -0.3 ‰ (Figure 4, Table 2).



**Figure 4.  $\delta^{15}\text{N}$  values normalized to bulk  $\delta^{15}\text{N}$  values for phosphatidylethanolamine headgroups (ethanolamine) and serine from five bacterial cultures. CSIA = compound specific isotope analysis.**

Importantly, the  $\delta^{15}\text{N}$  value of ethanolamine correlated well with that of serine released

from proteins (Figure 5), suggesting that the  $\delta^{15}\text{N}$  values of bacterial PE lipids reflects that of the amino acid serine.

Interestingly, both serine and PE of the autotrophic bacteria (*T. roseopersicina* and *T. denitrificans*) had similar quite negative  $\Delta\delta^{15}\text{N}_{(\text{CSIA-BULK})}$  values relative to that of the heterotrophic bacteria (*E. coli*, *Pseudomonas* sp., and *P. haloplanktis*; Figure 4). This difference in  $\Delta\delta^{15}\text{N}$  between autotrophs and heterotrophs is likely caused by the fact that 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,<sup>[26]</sup> known to contain PE.<sup>[25]</sup> The pivaloyl-derivative of ethanolamine was detected in the lipid extract from the mat after application of our procedure (Figure 3B). Isotopic analysis of this product (Supplemental Figure 2), as well as of serine derived from proteins, showed that, similar to our heterotrophic bacterial cultures, both ethanolamine and serine were depleted in  $^{15}\text{N}$  by 2 and 4 ‰, respectively, compared to bulk biomass. Interestingly, the depletion in  $^{15}\text{N}$  of PE compared to bulk biomass was in between that of the autotrophic and heterotrophic bacteria, suggesting that PE in the microbial mat was sourced both from autotrophic and heterotrophic sources. These results suggest that it is feasible to measure the  $\delta^{15}\text{N}$  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, it provides a selective method capable of e.g. tracing the incorporation of  $^{15}\text{N}$  -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, e.g. 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  $^{15}\text{N}$  incorporation of D-alanine has been used to trace the uptake of nitrogen substrates in bacteria<sup>[15]</sup>. However, D-alanine also occurs in some animals<sup>[43]</sup> and measurement of  $\delta^{15}\text{N}$  values of PE headgroups thus provides an independent method to trace nitrogen through the microbial domain in the natural environment

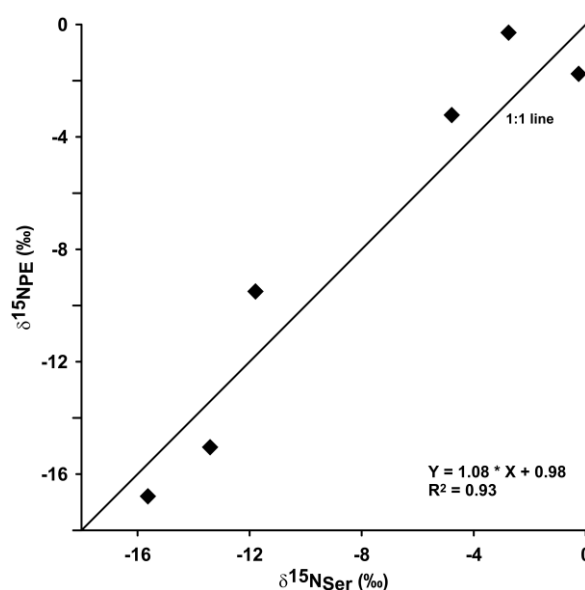


Figure 5.  $\delta^{15}\text{N}$  values of serine (SER) versus  $\delta^{15}\text{N}$  values of the phosphatidylethanolamine headgroup (PE).

## Conclusions

We developed a procedure for the measurement of  $\delta^{15}\text{N}$  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 some correction may be needed for some IPLs. No statistically significant fractionation was observed for the  $\delta^{15}\text{N}$  value of phosphatidylethanolamine, an important bacterial headgroup. Application of this method on five bacterial cultures and a microbial mat showed a similar depletion in  $^{15}\text{N}$  of both PE and the amino acid serine compared to bulk biomass, indicating that the  $\delta^{15}\text{N}$  signal of PE is reflecting the  $\delta^{15}\text{N}$  value of its biosynthetic nitrogen source, serine. Autotrophic bacteria PE and serine were in general more depleted in  $^{15}\text{N}$  compared to the heterotrophic bacteria, probably because autotrophic bacteria synthesize serine while heterotrophs obtain serine from their food source. Regardless, since PE lipids in the natural environment are predominantly sourced by bacteria, the analysis of the  $\delta^{15}\text{N}$  value of phosphatidylethanolamine headgroups provides the opportunity to investigate the nitrogen isotope dynamics in bacterial biomass under in situ conditions.

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Table S1

Species	Tissue	%lipid	Bulk biomass		Lipid-free biomass		Total lipid extract	
			d15N	C:N	d15N	C:N	d15N	C:N
Atlantic herring	Gill	30	11,5	5,6	11,8	3,3	6,3	NA
		32	10,6	8,0	10,7	3,2	5,2	NA
		25	10,6	6,0	10,7	3,2	NA	NA
		19	11,2	4,8	13,0	3,3	NA	NA
		30	11,1	6,0	12,0	3,0	9,2	NA
Atlantic herring	Muscle	21	14,4	6,6	13,7	3,1	0,4	62,9
		8	13,7	3,4	14,2	3,1	2,7	NA
		34	11,7	6,1	12,5	3,1	2,9	NA
		8	11,4	3,3	12,2	3,0	-0,5	23,2
		32	11,8	5,8	12,8	3,1	NA	NA
		40	11,5	5,8	13,2	3,1	2,4	NA
Brown trout	Gill	7	16,2	3,3	16,3	3,0	3,1	14,6
		10	15,0	3,9	15,3	3,2	11,5	NA
		8	13,9	3,8	14,2	3,2	7,2	19,5
		9	16,3	3,8	16,3	3,3	11,0	NA
		6	16,0	4,0	16,6	3,5	NA	NA
		3	15,9	3,7	16,6	3,2	NA	NA
Brown trout	Muscle	7	16,1	3,8	16,7	3,2	8,5	42,2
		25	15,3	5,1	15,5	3,1	5,8	NA
		5	13,7	3,2	14,4	3,0	4,3	NA
		9	16,1	3,3	16,6	3,2	7,7	NA
		6	15,9	3,2	16,8	3,1	NA	NA
		8	16,5	3,3	17,0	3,1	9,7	21,3
Twait shad	Gill	15	16,4	4,0	16,8	3,0	7,0	NA
		9	16,7	4,2	17,0	3,3	NA	NA
Twait shad	Muscle	6	14,7	4,3	14,8	3,6	NA	NA
		6	17,1	3,2	17,9	3,1	10,3	14,1
Green shore crab	Muscle	6	15,8	3,2	16,7	3,1	NA	NA
		4	13,2	3,8	13,4	3,6	8,2	NA
		2	15,8	3,7	16,5	3,3	NA	NA
Common cockle	Muscle	6	13,9	5,3	14,3	4,4	NA	NA
		5	11,3	4,6	12,0	4,1	NA	NA
		5	11,5	4,9	11,9	4,3	7,2	NA
		5	12,1	4,7	11,7	4,1	6,9	NA
		4	12,6	4,1	12,7	3,7	NA	NA
Pacific oyster	Muscle	3	12,0	4,5	11,9	4,1	NA	NA
		6	12,3	3,3	13,3	3,3	7,0	NA
Lugworm	Head	2	12,3	3,0	13,5	3,2	6,0	NA
		5	12,4	4,7	11,3	4,0	8,5	NA
Lugworm	Whole	3	11,7	4,2	10,8	3,7	8,4	NA
		7	6,7	3,1	6,4	3,5	7,4	NA

Table S2. Fatty acid composition, indicated by number of carbons and degree of unsaturation, of most abundant intact polar lipids (IPLs) in different aquatic species in this study. N.d. = not detected. PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; MMPE: monomethyl-PE; PI: Phosphatidylinositol; Plasmalogen: Fatty acid with vinyl linkage to glycerol backbone; Lyso: IPL where one fatty acid has been removed (lysed); Sphingo: Sphingobase. See Figure 3 for structures.

	Atlantic herring		Brown trout		Green shore crab	Common cockle	Pacific oyster	Lugworm
	muscle	gill	muscle	gill	muscle	head	muscle	muscle
<b>PC</b>	38:6	34:1	38:6	34:1	34:1	36:2	38:4-	34:1
	34:1	32:1	36:6	32:1	32:1	34:1	38:6	36:5
		40:2	34:1	36:1		32:0	36:6	32:0
				36:2		32:1	32:1	32:1
						34:2	37:5	30:0
<b>Plasmalogen-PC</b>	n.d.	34:0	38:5	34:0	36:3	n.d.	n.d.	n.d.
				36:0	32:0			
					38:4			
					40:5			
<b>Lyso-PC</b>	15:0	17:0	15:0	15:0	17:1	17:1	21:6	19:1
	19:1		17:1	17:0	15:0	15:0	15:0	15:0
	15:0		21:6	16:3	16:0	15:1	20:5	17:1
						16:1	17:0	13:0
						14:0	15:0	
<b>Sphingo-PC</b>	n.d.	n.d.	42:2	42:2	32:1	n.d.	n.d.	n.d.
				34:1	34:1			
				32:1	36:1			
<b>PE</b>	n.d.	n.d.	n.d.	n.d.	38:5	n.d.	38:3	40:3
					38:6		38:4	
							40:5	
<b>MMPE</b>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	38:3	
<b>Betaine</b>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	32:1	n.d.
<b>PI</b>	n.d.	n.d.	41:4	n.d.	n.d.	36:6	n.d.	n.d.