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1 # Carbon isotope fractionation by the marine ammonia-oxidizing
2 archaeon *Nitrosopumilus maritimus*

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10 carbon isotope fractionation, biphytanes, sugars, bulk carbon

11 ## Abstract

12 Ammonia-oxidizing archaea (AOA) are abundant and widely distributed
13 microorganisms in aquatic and terrestrial habitats. By catalyzing the first and rate-
14 limiting step in nitrification, these chemolithoautotrophs play a significant role in the
15 global nitrogen cycle and contribute to primary production. Here, the carbon isotopic
16 fractionation relative to the inorganic carbon source was determined for bulk biomass,
17 biphytanes and polar lipid bound sugars of a marine AOA pure culture. Bulk biomass
18 and biphytanes extracted from *Nitrosopumilus maritimus* showed identical carbon
19 isotope fractionation ($\varepsilon_{DIC/bulk}$ and $\varepsilon_{DIC/byphytanes}$) of ca. −20‰. The glycoside head groups
20 were mainly glucose, mannose and inositol, and exhibited different carbon isotopic
21 compositions. In general, these monosaccharides were enriched in ^{13}C (ε −6.1‰ to
22 −13.8‰) relative to bulk biomass and biphytanes. The fact that the carbon isotope
23 composition of the biphytanes reflected that of the bulk biomass of *N. maritimus*
24 suggests that the depletion of ^{13}C found in both, biomass and biphytanes, resulted mainly

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from the carbon isotope discrimination of the bicarbonate-fixing enzyme in the autotrophic hydroxypropionate/hydroxybutyrate cycle. Our results further revealed that lipid compounds represent suitable biomarkers for determining $\delta^{13}C$ values of archaeal ammonia oxidizers without biosynthetic correction.

1. Introduction

Ammonia-oxidizing archaea (AOA) affiliated with the so-called marine group I crenarchaeota are one of the most widely distributed and abundant groups of microorganisms on Earth and play a significant role in the global N cycle (Francis et al., 2007). The first cultured member of this phylogenetic group, *Nitrosopumilus maritimus*, grows chemolithoautotrophically by gaining energy via the oxidation of NH_3 to NO_2^- and by assimilating HCO_3^- as C source via a hydroxypropionate/hydroxybutyrate cycle (Könneke et al., 2005, Walker et al., 2010). This autotrophic pathway was initially discovered in the thermophilic crenarchaeon *Metallosphaera sedula* (Berg et al., 2007). The glycerol dibiphytanyl glycerol tetraether (GDGT) crenarchaeol was found to be a main component of the core lipids of the marine group I crenarchaeota as well as in cultivated relatives and represents a suitable biomarker for AOA (Sinninghe Damsté et al., 2002; Schouten et al., 2008; de la Torre et al., 2008; Pitcher et al., 2011). The application of carbon isotope analysis to recent or fossil biomarkers represents a strong tool for obtaining information about both metabolic processes and the identity of organisms involved in conversion of carbon (Hayes, 2001). Tracing GDGTs as biomarker for archaeal biomass is of special interest because of their application as paleoproxies and because of the important biogeochemical and ecological role of archaea in marine environments.

In order to further validate and verify carbon isotopic fractionation suggested by environmental biomarker studies of natural mixed archaeal populations, we studied the

1 carbon isotopic fractionation in a pure culture of *N. maritimus* by analysis of bulk
2 biomass and lipid and glycoside components of intact polar lipids relative to the
3 inorganic carbon source.

4 ## 2. Material and methods

5 *N. maritimus* strain SCM1 was grown at 28°C in 15l batch cultures using a defined,
6 HEPES-buffered medium (pH = 7.5) as described previously (Könneke et al., 2005;
7 Martens-Habbena et al., 2009). Cells were harvested in the late exponential phase from
8 three cultures with different bicarbonate concentrations (2, 4, and 8 mM, respectively)
9 with a cross -flow filtration system (Sartocon slice Microsart, Sartorius, Göttingen,
10 Germany). Cells were stored frozen at -20°C. The stable carbon isotopic composition,
11 $\delta^{13}C$, of bulk biomass was determined with an elemental analyzer coupled to an isotope
12 ratio mass spectrometer (Delta V Plus, Thermo Scientific, Bremen, Germany); $\delta^{13}C$ of
13 dissolved inorganic carbon (DIC) used in the batch culture was measured using a gas
14 bench coupled to a ThermoFinnigan MAT 252 mass spectrometer.

15 About half of the biomass (corresponding to ca. $2x10^{11}$ cells) from each batch was
16 extracted after addition of ca. 3 g combusted sea sand using an ultrasonic probe
17 (HD2200, Bandelin electronic GmbH & Co. KG, Germany) and a modified Bligh and
18 Dyer protocol with CCl_3CO_2H buffer (cf. Sturt et al., 2004). The total lipid extract
19 (TLE) was evaporated to dryness under a N_2 stream and stored at -20°C until analysis.
20 Preparation of sugar derivates and biphytanes from intact GDGTs for carbon isotopic
21 analysis was performed on 90% of the TLE according to Lin et al. (2010). Briefly, sugar
22 head groups were cleaved by adding 500 uL of 50% trifluoroacetic acid (TFA) and
23 heating to 70°C for 36 h. After addition of 1 mL deionized water, the GDGT core lipids
24 were extracted with dichloromethane and ether-cleaved with BBr_3 in DCM followed by
25 reaction with superhydride (1M lithium triethylborohydride in tetrahydrofuran). The

1 reaction was stopped by addition of deionized water and the biphytanes were extracted
2 with hexane and purified over a silica gel column. Cholestane (200 ng) was added
3 before injection for quantification. The aqueous phase after TFA hydrolysis was freeze-
4 dried, 3-O-methyl-glucose was added as internal recovery standard and the
5 carbohydrates were derivatized to aldononitriles. The sugar derivates were purified over
6 a silica gel column before analysis.
7 Concentrations of biphytanes and sugar head groups were determined via gas
8 chromatography flame ionization detection (GC-FID) with a ThermoFinnigan Trace GC
9 instrument using published conditions (Lin et al., 2010). Corresponding $\delta^{13}C$ values
10 were analyzed with a TraceGC Ultra instrument coupled via GC-Isolink and ConFlow
11 IV interface to a Delta V Plus isotope ratio mass spectrometer (Thermo Scientific
12 GmbH, Bremen, Germany). The GC temperature programme was as for GC-FID. All
13 $\delta^{13}C$ values are expressed in ‰ relative to Vienna PeeDee Belemnite and are corrected
14 for additional carbon introduced during derivatization with F factors of 1.4‰ for
15 mannose, 2.3‰ for glucose and -3.7‰ for inositol (cf. Lin et al., 2010). Replicate
16 analysis of each batch showed an analytical error of <1‰.

17 ## 3. Results

18 Results for DIC, bulk biomass, biphytanes and glycoside head groups from three
19 cultures of *N. maritimus* are presented in Table 1. The average $\delta^{13}C$ value of HCO_3^- in
20 the medium was -6.5‰ (±0.4‰), and the mean isotope composition of the bulk cell
21 material was -25.8‰ (±0.3‰), corresponding to a ^{13}C depletion of -19.8‰ relative to C-
22 source, $\varepsilon_{DIC/bulk}$, during carbon assimilation and biosynthesis. Significant differences in
23 $\delta^{13}C$ values between the cultures amended with different amounts of DIC were not
24 observed.

1 The membrane lipids comprised GDGT core lipids bound to glycoside or
2 phosphatidyl/glycoside polar head groups. The core lipids comprised acyclic (BP0),
3 monocyclic (BP1), bicyclic (BP2) and the crenarchaeol-derived tricyclic (BPcren)
4 biphytane with two cyclopentyl and one cyclohexyl moieties (Fig. 1A). All biphytanes
5 exhibited a $\delta^{13}C$ value of -25.7‰ (± 0.4), i.e. nearly identical to the bulk cell material.

6 In contrast to the biphytanes, the glycoside head groups showed heterogeneous $\delta^{13}C$
7 values. The major ones were glucose, mannose and inositol (Fig. 1B), which were
8 enriched in ^{13}C relative to bulk cell material and core lipids. The $\delta^{13}C$ value of glucose
9 was most depleted in ^{13}C ($\delta^{13}C$ = -20.0‰), followed by inositol (-16.7‰) and mannose
10 (-12.5‰). The corresponding ε values relative to DIC ranged from -13.8 to -6.1‰.

11 ## 4. Discussion

12 This study provides for the first time information about the carbon isotopic fractionation
13 from a cultured representative of the highly abundant and widely distributed AOA. The
14 isotopic relationship of *N. maritimus* and HCO_3^- is consistent with environmental studies
15 analyzing crenarchaeol ($\Delta\delta^{13}C$ -17 to -23‰; Hoefs et al., 1997; Pearson et al. 2001,
16 Kuypers et al., 2001; Mußmann et al., 2011). The carbon isotopic fractionation is similar
17 to the ammonia-oxidizing bacterium *Nitrosomonas europaea* ($\Delta\delta^{13}C$ =-20‰; Sakata et
18 al., 2008) that uses the Calvin-Benson cycle for inorganic carbon assimilation. But it
19 differed from *M. sedula,* which also uses the hydroxypropionate/hydroxybutyrate
20 pathway and showed only minor carbon isotope fractionation ($\Delta\delta^{13}C$ bulk biomass -
21 1.9‰, biphytanes 0.7‰; van der Meer et al., 2001). The carboxylation in this
22 autotrophic pathway is catalyzed by a single biotin-dependent carboxylase, the final
23 product and universal precursor for further biosynthesis steps being acetyl-CoA (Berg et
24 al., 2007). The differences in isotopic fractionation between both organisms may be
25 attributed mainly to the growth conditions. While *N. maritimus* was cultured under

mesophilic conditions (28°C) and at pH 7.5, *M. sedula* was grown at 65°C and pH 2. First, higher temperatures generally result in smaller isotopic fractionation (Hayes, 2001). Secondly, the low pH pushes the equilibrium between CO_2 and HCO_3^- towards CO_2 resulting in low HCO_3^- concentration; in combination with efficient operation of the acetyl-CoA/propionyl-CoA carboxylase this likely results in a smaller isotopic fractionation vs. *N. maritimus.*

The variability in $\delta^{13}C$ values in monosaccharides is a common characteristic in autotrophic organisms (van Dongen et al., 2002; Teece et al., 2007). Our finding of glycoside head groups enriched in ^{13}C vs. bulk cell material is in line with prior reports of monosaccharides from aquatic algae and terrestrial plants (van Dongen et al., 2002, Copley et al., 2003). The identical isotopic fractionation of *N. maritimus* for biphytanes and bulk biomass likely results from the operation of the mevalonic acid pathway for isoprenoid synthesis, in which all carbon atoms derive from acetyl-CoA (Hayes, 2001). However, biosynthetic pathways of monosaccharide formation in *N. maritimus* have not been unravelled.

In sum, the biphytanes and bulk biomass of *N. maritimus* exhibited the same carbon isotope compositions after growth at autotrophic conditions indicating that the carbon isotope fractionation occurs mainly within the autotrophic hydroxypropionate/ hydroxybutyrate cycle. Our study on a pure culture confirms the interpretation of $\delta^{13}C$ values previously reported for natural samples and supports the application of lipid compounds, like crenarchaeol, as suitable biomarkers for determining the carbon isotopic composition of fossil and active AOA without a biosynthesis-dependent correction.

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4 Tables and Figures

5 Table 1

6 Stable carbon isotope composition of bulk biomass and lipid-derived compounds from
7 *N. maritimus* vs. $\delta^{13}C$ values (ε and $\Delta\delta^{13}C$).

8 Fig. 1. GC-FID chromatograms showing components and carbon isotopic composition
9 of the ether-cleaved lipid fraction from *N. maritimus*. The chromatograms show A,
10 biphytanes and B, a sugar standard reference mixture and the glycoside head groups
11 derived from the polar head groups. Internal standard (ISTD) for biphytane analysis was
12 cholestane; for sugar analysis 3-O-methyl-glucose was added before derivatization.
13 Abbreviations: mannose (Man), glucose (Glc), gulose (Gul), galactose (Gal), inositol
14 (Ino), biphytane (BP) with 0 to 2 rings (BP0, BP1, and BP2) and tricyclic structure
15 (BPcren); see also text for detailed description.

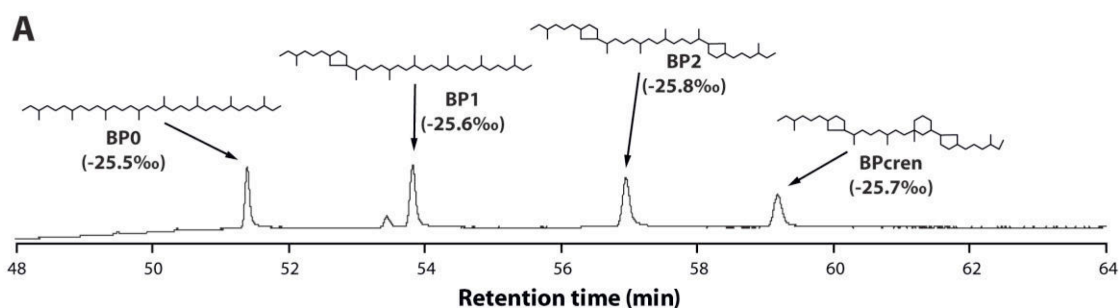
1

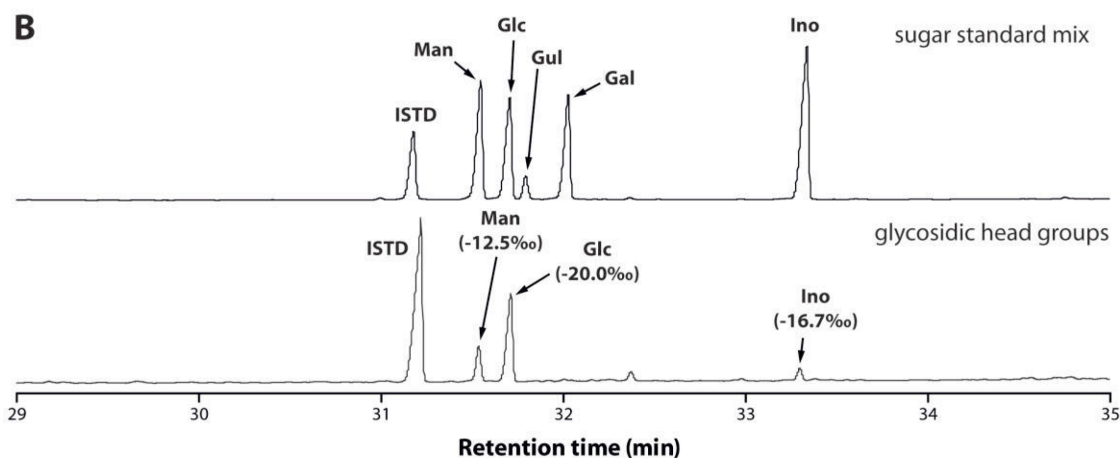
	$\delta^{13}C$ 2 mM DIC	$\delta^{13}C$ 4 mM DIC	$\delta^{13}C$ 8 mM DIC	Mean $\delta^{13}C$ [‰]	Mean $\Delta\delta^{13}C$ [‰]	Mean $\varepsilon_{DIC/x}$ [‰][1]
DIC	- 7.0 ±	- 6.3 ±	- 6.2 ±	- 6.5 ± 0.4		
Bulk biomass	-26.3	-25.7	-25.5	-25.8 ± 0.3	-19.3	-19.8
Biphytanes[2]						
BP0	-26.4	-25.0	-25.2	-25.5 ± 0.6	-19.0	-19.5
BP1	-26.0	-25.3	-25.4	-25.6 ± 0.3	-19.1	-19.6
BP2	-26.4	-25.6	-25.4	-25.8 ± 0.4	-19.3	-19.8
BPcren	-26.0	-25.8	-25.8	-25.7 ± 0.2	-19.2	-19.7
mean BP	-26.2	-25.4	-25.5	-25.7 ± 0.4	-19.2	-19.7
Glycosidic head groups						
Mannose	-12.7	-12.5	-12.3	-12.5 ± 0.2	-6.0	-6.1
Glucose	-20.1	-20.5	-19.3	-20.0 ± 0.5	-13.5	-13.8
Inositol	-17.4	-16.4	-16.2	-16.7 ± 0.5	-10.2	-10.4
mean	-16.7	-16.5	-15.9	-16.4 ± 0.3	-9.9	-10.1

2 [1] = 1000((1000+δDIC)/(1000+δX)-1);

3 [2] BP0, BP1, BP2 and BPcren refer to structures in Fig. 1.

4





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