RESEARCH ARTICLE

Anaerobic methanotrophic archaea of the ANME-2d clade feature lipid composition that differs from other ANME archaea

Julia M. Kurth1,3,*,†, Nadin T. Smit2,3, Stefanie Berger1, Stefan Schouten2,3, Mike S.M. Jetten1,3,4,‡ and Cornelia U. Welte1,3,4,§

1Department of Microbiology, Institute for Water and Wetland Research, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands, 2NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry and Utrecht University, P.O. Box 59, 1790 AB Den Burg (Texel), The Netherlands, 3Netherlands Earth System Science Center, Utrecht University, Heidelberglaan 2, 3584 CS Utrecht, The Netherlands and 4Soehngen Institute of Anaerobic Microbiology, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

*Corresponding author: Department of Microbiology, Institute for Water and Wetland Research, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands. E-mail: j.kurth@science.ru.nl

One sentence summary: Lipid analysis of microorganisms involved in the anaerobic oxidation of methane helps to understand the prevalence and importance of those organism for the global methane cycle in past and present environments.

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Julia M. Kurth, http://orcid.org/0000-0002-1221-1230
Mike S.M. Jetten, http://orcid.org/0000-0002-4691-7039
Cornelia U. Welte, http://orcid.org/0000-0002-1568-8878

ABSTRACT

The anaerobic oxidation of methane (AOM) is a microbial process present in marine and freshwater environments. AOM is important for reducing the emission of the second most important greenhouse gas methane. In marine environments anaerobic methanotrophic archaea (ANME) are involved in sulfate-reducing AOM. In contrast, Ca. Methanoperedens of the ANME-2d cluster carries out nitrate AOM in freshwater ecosystems. Despite the importance of those organisms for AOM in non-marine environments little is known about their lipid composition or carbon sources. To close this gap, we analysed the lipid composition of ANME-2d archaea and found that they mainly synthesise archaeol and hydroxyarchaeol as well as different (hydroxy-) glycerol dialkyl glycerol tetraethers, albeit in much lower amounts. Abundant lipid headgroups were dihexose, monomethyl-phosphatidyl ethanolamine and phosphatidyl hexose. Moreover, a monopentose was detected as a lipid headgroup that is rare among microorganisms. Batch incubations with 13C labelled bicarbonate and methane showed that methane is the main carbon source of ANME-2d archaea varying from ANME-1 archaea that primarily assimilate dissolved inorganic carbon (DIC). ANME-2d archaea also assimilate DIC, but to a lower extent than methane. The lipid

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characterisation and analysis of the carbon source of Ca. Methanoperedens facilitates distinction between ANME-2d and other ANMEs.

Keywords: lipid analysis; ANME-2d; Methanoperedens; anaerobic oxidation of methane; carbon assimilation

INTRODUCTION

Methane is the second most important greenhouse gas on earth with an atmospheric methane budget of about 600 Tg per year (Conrad 2009; Dean et al. 2018). About 69% of methane emission into the atmosphere is caused by methanogenic archaea (Conrad 2009). On the other hand, aerobic and anaerobic methanotrophic microorganisms can oxidise methane back to carbon dioxide that is a 25-times less potent greenhouse gas than methane. The anaerobic oxidation of methane (AOM) is a microbial process present in marine and freshwater environments. AOM has first been described to be performed by a consortium of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria in microbial mats in the deep sea or in marine sediments (Hoehler et al. 1994; Hinrichs et al. 1999; Boetius et al. 2000; Hinrichs and Boetius 2002; Orphan et al. 2002). ANME archaea are related to methanogens and oxidise methane by using the reverse methanogenesis pathway (Hallam et al. 2004; Arshad et al. 2015; McNulty et al. 2017; Timmers et al. 2017). In addition to sulfate, also oxidised nitrogen compounds (Raghoebarsing et al. 2006; Ettwig et al. 2010; Haroon et al. 2013) as well as iron and manganese (Beal, House and Orphan 2009; Ettwig et al. 2016; Cai et al. 2018) can be used as electron acceptors within the AOM process.

Anaerobic methanotrophic archaea can be assigned to three distinct clusters within the Euryarchaeota, ANME-1, ANME-2 and ANME-3, which are related to the orders Methanococcales and Methanomicrobiales (Knittel and Boetius 2009). The phylogenetic distance between the groups is quite large (16S rRNA gene sequence identity between 75 and 92%) (Knittel and Boetius 2009). Most analysed members of the three ANME clades have been described to perform sulfate-driven AOM in marine environments (Pancost, Hopmans and Sinninghe Damsté 2001; Blumenberg et al. 2004; Niemann and Elvert 2008; Rossel et al. 2008; Wegener et al. 2008; Kellermann et al. 2012). However, members of the ANME-2d cluster have not been found in consortia with sulfate reducers. Instead, ANME-2d archaea are the main players in nitrate-dependent AOM. Microorganisms conducting nitrate AOM have been enriched from anoxic freshwater sediments, digestersludge and rice paddies (Raghoebarsing et al. 2006; Hu et al. 2009; Arshad et al. 2015; Vaksmaa, Guerrero-Cruz, et al. 2017). Denitrifying AOM can either be conducted by a consortium of nitrate-reducing ANMEs, Ca. Methanoperedens sp., and nitrite-reducing NC10 bacteria, Ca. Methylomirabilis sp. (Raghoebarsing et al. 2006; Haroon et al. 2013; Arshad et al. 2015) or by a consortium of those ANME archaea and anammox bacteria (Haroon et al. 2013). In those consortia Ca. Methylomirabilis sp. or anammox bacteria are important to reduce the toxic nitrite produced during nitrate AOM by Ca. Methanoperedens sp.

To understand the prevalence of anaerobic methane oxidation in past and present environments and identify the key players at different environmental sites, it is necessary to identify biomarkers for those organisms. As core lipids are much more stable than DNA over time, lipid biomarkers are a useful tool to trace microorganisms and therefore specific microbial processes back in time. Moreover, intact polar lipids (IPLs) are crucial to examine present microbial communities and to distinguish between different microorganisms (Ruetters et al. 2002; Sturt et al. 2004). Quite some information is available on core and intact polar lipids as well as on carbon assimilation in marine AOM consortia of ANME archaea and sulfate-reducing bacteria (Pancost, Hopmans and Sinninghe Damsté 2001; Blumenberg et al. 2004; Niemann and Elvert 2008; Rossel et al. 2008; Wegener et al. 2008; Kellermann et al. 2012). In contrast, lipids from one of the main players in denitrifying AOM, Ca. Methanoperedens sp., have hardly been studied: a preliminary study on the lipids of a culture containing Ca. Methanoperedens sp. and Ca. Methylomirabilis oxyfera only detected sn2-hydroxyarchaeol as the dominant lipid of the archaeal partner (Raghoebarsing et al. 2006).

Besides the characterisation of lipids in ANME archaea it is also pivotal to understand which carbon source those organisms use for biomass production. The main carbon assimilation pathway in methanogenic Euryarchaeota is the reductive acetyl-CoA pathway (Whitman 1994; Berg et al. 2010). In this pathway a carbonyl group and a methyl group are combined to form acetyl-CoA. In archaea, acetyl-CoA is used for formation of membrane lipids via the isoprenoid compound geranylgeranyolphosphate in the mevalonate pathway, although not all of the enzymes involved in this pathway are known with certainty (Koga and Morii 2007; Matsumi et al. 2011). An ether bond is formed between the glycerol-1-phosphate backbone and the isoprenoid side chains. Subsequently cytidine-diphosphate is attached and finally the unsaturated isoprenoid side chains are reduced to form diphytanylglycerol diether, also known as archaeol (Matsumi et al. 2011).

The isotopic composition of lipids provides information on the carbon source used by the microorganism. The lipids of ANMEs involved in sulfate-driven AOM are usually strongly depleted in $^{13}$C, with $\delta^{13}$C values ranging from $-70$ to $-130\%$ (Elvert, Suess and Whiticar 1999; Pancost et al. 2000; Niemann and Elvert 2008). Such low $\delta^{13}$C values of lipids have been explained by the assimilation of $^{13}$C-depleted methane carbon during methane uptake into biomass (Elvert, Suess and Whiticar 1999; Hinrichs et al. 1999; Pancost et al. 2000; Orphan et al. 2002). Mixed assimilation of CH$_4$ and CO$_2$ has been reported for marine ANME-1, -2a and -2b strains indicating that at least some ANME strains can use methane-derived carbon for biomass production (Wegener et al. 2008). However, for ANME-1 it has been shown that methane oxidation is decoupled from the assimilatory system and that CO$_2$-dependent autotrophy is the predominant mode of carbon fixation (Kellermann et al. 2012). In general, ANME archaea seem to be able to assimilate both, methane and dissolved inorganic carbon (DIC), and the preferred carbon source for assimilation might vary between the different ANME clusters.

In this study, we performed analysis of the lipids from ANME-2d archaea and compared these with previous studies about different ANME lipids. Moreover, we analysed the incorporation of $^{13}$C-labelled methane and bicarbonate in lipids of these archaea to establish the carbon sources used for assimilation.
MATERIALS AND METHODS
ANME-2d bioreactor operation and sampling for lipid analysis

For lipid analysis of Ca. Methanoperedens sp. two different bioreactors were sampled. One bioreactor contained archaea belonging to the ANME-2d clade enriched from the Ooijpolder (NL) (Arshad et al. 2015; Berger et al. 2017) and the other reactor ANME-2d archaea enriched from an Italian paddy field (Vaksmaa, Jetten, et al. 2017; Guerrero-Cruz et al. 2018). The anaerobic enrichment culture dominated by Ca. Methanoperedens sp. strain BLZZ originating from the Ooijpolder (Berger et al. 2017) was maintained in an anaerobic 10 L sequencing batch reactor (30 °C, pH 7.3 ± 0.1, stirred at 180 rpm). The mineral medium consisted of 0.16 g/L MgSO4, 0.24 g/L CaCl2 and 0.5 g/L KH2PO4. Trace elements and vitamins were supplied using stock solutions. 1000 × trace element stock solution: 1.35 g/L FeCl3 × 4 H2O, 0.1 g/L MnCl2 × 4 H2O, 0.024 g/L CoCl2 × 6 H2O, 0.1 g/L CaCl2 × 2 H2O, 0.1 g/L ZnCl2, 0.025 g/L CuCl2 × 2 H2O, 0.01 g/L H3BO3, 0.024 g/L Na2MoO4 × 2 H2O, 0.22 g/L NiCl2 × 6 H2O, 0.017 g/L Na2SeO3, 0.004 g/L Na2WO4 × 2 H2O, 12.8 g/L nitrilotriacetic acid; 1000 × vitamin stock solution: 20 mg/L vitamin B12, 20 mg/L p-aminobenzoic acid, 50 mg/L lipoic acid. The medium supply was continuously sparged with Ar:CO2 (95:5) ratio. Per day 30 mmol nitrate added to the medium were supplied to the bioreactor and were completely consumed. Methane was added by continuously sparging the reactor content with CH4:CO2 in a 95:5 ratio at a rate of 15 mL/min. The reactor was run with a medium turnover of 1.25 L per 12 h. A 5 min settling phase for retention of biomass preceded the reactor was run with a medium turnover of 1.25 L per 12 h. A 5 min settling phase for retention of biomass preceded the A 5 min settling phase for retention of biomass preceded the removal of supernatant. Under these conditions nitrite was not detectable with a colorimetric test with a lower detection limit of 0.1 g/L MnCl2 that cultures with 13C-labelled bicarbonate also contained 12C derived from CO2. Having in mind that 10% of CO2 were added to the culture (pCO2 = 0.1 atm) the CO2 concentration in the solution was calculated to be about 3.36 mM by use of the equation \[ [CO2]_{aq} = pCO2/k_s \] (Henry constant (kH) is 29.76 atm/(mol/L) at 25 °C). Therefore, it has to be assumed that about half of the carbon in the cultures where 13C labelled bicarbonate was added derived from 12C-CO2 dissolved in the medium after gassing with a mixture of 10% CO2/90% Ar gas.

Lipid extraction and analysis
Bligh and Dyer extraction
Bligh and Dyer extraction was used to extract IPLs from the ANME-2d enrichment. Lipids of freeze-dried biomass (between 20 and 70 mg) were extracted by a modified Bligh and Dyer method as described by Bale et al. (2013) using a mixture of methanol, dichloromethane (DCM) and phosphate buffer at pH 7.4 (2:1:0.8 v/v/v). After ultrasonic extraction (10 min) and centrifugation the solvent layer was collected. The residue was re-extracted twice. The combined solvent layers were separated by adding additional DCM and phosphate buffer to achieve a ratio of MeOH, DCM and phosphate buffer (1:1:0.9 v/v/v). The separated organic DCM layer on the bottom was removed and collected while the aqueous layer was washed two more times with DCM. The combined DCM layer was evaporated under a continuous stream of nitrogen.

Acid hydrolysis
Head groups of archaeal lipids were removed using acid hydrolysis. Therefore, this method is used to analyse the core lipids of the microorganisms present in the ANME-2d enrichment. About 20 mg freeze-dried biomass was hydrolysed with 2 mL of a 1.5 N HCl/MeOH solution and samples stirred for 2 h while heated at 130 °C with a reflux system. After cooling, the pH was adjusted to pH 4–5 by adding 2 N KOH/MeOH solution. In total, 2 mL DCM and 2 mL distilled H2O were added. The DCM bottom layer was transferred to a new vial and the MeOH/H2O layer washed twice the closed bottle with argon gas for 10 min. Afterwards, the culture was purged with 90% argon and 10% CO2 for 5 min. In total, 2.5 mM bicarbonate and 18 mL methane (Air Liquide, Eindhoven, The Netherlands) were added. Except for the negative controls, either 13C-labelled methane (99 atom%; Isotec Inc., Matheson Triggs Products Division) or 13C-labelled bicarbonate (Cambridge Isotope Laboratories Inc., Tewksbury, USA) was used in the batch incubations. The bottles were incubated horizontally on a shaker at 50 °C and 250 rpm for 1 or 3 days. All bottles contained sodium nitrate (0.6 mM) at the start of incubation and additional nitrate was added when the concentration in the bottles was close to 0, as estimated by MQquant (Merck, Darmstadt, Germany) test strips. The methane concentration in the headspace was measured twice a day by gas chromatography (GC) with a gas chromatograph (Hewlett Packard 5890a, Agilent Technologies, Santa Clara CA, US) equipped with a Porapac Q 100/120 mesh and a thermal conductivity detector (TCD) using N2 as carrier gas. Each measurement was performed by injection of 50 μL headspace gas with a gas-tight syringe. With this technique a decrease in methane concentration from ~24 to ~20% within 3 days of incubation was observed. After batch incubation, cell material was centrifuged (10000 × g, 20 min, 4 °C) and pellets were kept at −80 °C until subsequent freeze-drying and following lipid and isotope analysis. It has to be considered that cultures with 13C-labelled bicarbonate also contained 12C derived from CO2. Having in mind that 10% of CO2 were added to the culture (pCO2 = 0.1 atm) the CO2 concentration in the solution was calculated to be about 3.36 mM by use of the equation \[ [CO2]_{aq} = pCO2/k_s \] (Henry constant (kH) is 29.76 atm/(mol/L) at 25 °C). Therefore, it has to be assumed that about half of the carbon in the cultures where 13C labelled bicarbonate was added derived from 12C-CO2 dissolved in the medium after gassing with a mixture of 10% CO2/90% Ar gas.

Analysis of the microbial community
For the Ooijpolder enrichment we performed whole genome metagenome sequencing. DNA extraction, library preparation and metagenome sequencing were performed as described before by Berger et al. (2017). Quality-reginning, sequencing adapter removal and contaminant filtering of illumina paired-end sequencing reads were performed using BBTools BBduk 37.76 (BBMap—Bushnell B.—sourceforge.net/projects/bbmap/). All processed paired-end reads were assigned to a taxon using Kaiju 1.6.2 (Menzel, Ng and Krogh 2016) employing the NCBI BLAST non-redundant protein database (NCBI Resource Coordinators 2016).

Batch cultivation of ANME-2d bioreactor cell material for 13C-labelling experiment
In total, 60 mL bioreactor material of a Ca. Methanoperedens sp. BLZZ culture enriched from the Ooijpolder (Arshad et al. 2015; Berger et al. 2017) were transferred with a syringe to a 120-mL serum bottle that had been made anoxic by flushing...
with DCM. Combined DCM layers were dried over a Na₂SO₄ column and the solvent removed by evaporation under a stream of nitrogen.

**BF₃ methylation and silylation**

For GC analysis aliquots of the acid hydrolysed samples were methylated using 0.5 mL of BF₃-methanol and reacted for 10 min at 60 °C in an oven. In total, 0.5 mL H₂O and 0.5 mL DCM were added to the heated mixture to separate the DCM and aqueous layers. Samples were mixed, centrifuged and the DCM layers taken off and collected. The water layer was washed three more times with DCM. The combined DCM layers were evaporated under N₂ stream and water was removed by use of a MgSO₄ column. After dissolving the sample in ethyl acetate, the extract was cleaned over a small silica gel column and lipids were eluted with ethyl acetate. The extract was dried under N₂. For GC analysis, extracts (0.3 to 0.5 mg) were dissolved in 10 μL pyridine and 10 μL BSTFA. Samples were heated for 30 min at 60 °C and afterwards diluted with ethyl acetate to 1 mg/mL.

**GC-MS**

This method was used to analyse bacterial fatty acids as well as archaeal diether lipids. Gas chromatography linked to mass spectrometry (GC-MS) was performed on a 7890B GC system (Agilent) connected to a 7000 GC/MS Triple Quad (Agilent). The gas chromatograph was equipped with a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 CB (0.12 μm film thickness) and a Flame Ionisation Detector (FID). Helium was used as the carrier gas. The samples were injected manually at 70 °C via an on-column injector. The oven temperature was programmed to a temperature increase from 70 to 130 °C with 20 °C/min and a further increase to 320 °C with 4 °C/min to 320 °C was held for 10 min. The mass range of the mass spectrometer was set to scan from m/z 50 to m/z 850.

**GC-IRMS**

To analyse the incorporation of 13C labelled methane or bicarbonate into lipids present in the ANME-2d enrichment GC-IRMS was conducted. Gas chromatography coupled to isotope-ratio mass spectrometry (GC-IRMS) was performed on a TRACE 1310 Gas Chromatograph (Thermo Fisher Scientific) interfaced with a Scientific GC IsoLink II Conversion Unit connected to an IRMS DELTA V Advantage Isotope-ratio mass spectrometer (Thermo Fisher Scientific). The gas chromatograph was equipped with a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 CB (0.12 μm film thickness). Helium was used as the carrier gas. The acid hydrolysed samples containing the core lipids were injected at 70 °C via an on-column injector. The oven temperature was programmed to a temperature increase from 70 to 130 °C with 20 °C/min and a further increase to 320 °C with 4 °C/min to 320 °C was held for 10 min. δ13C values were corrected for methyl group derived from BF₃ methanol in case of carboxylic acid group (bacterial lipids) and methyl groups derived from BSTFA in case of hydroxyl groups (mainly archaeal lipids). Averaged δ13C values are based on experimental triplicates, but not on analytical duplicates.

**UHPLC-APCI-TOF-MS**

UHPLC-APCI-TOF-MS analysis of the acid hydrolysed lipids was conducted in order to obtain information about the tetraether lipids. About 0.4 to 0.8 mg of the acid hydrolysed lipid extract was dissolved in a mixture of hexane/isopropanol 99:1. Extracts were filtered by use of a 0.45 μm, 4 mm diameter PTFE filter. About 2 mg per mL core lipid containing extracts were used for analysis by ultra-high performance liquid chromatography linked to time-of-flight atmospheric pressure chemical ionisation mass spectrometry using a (UHPLC-APCI-TOF-MS). Core lipid analysis was performed on an Agilent 1260 Infinity II UHPLC coupled to an Agilent 6230 TOF-MS. Separation was achieved on two UHPLC silica columns (BEH HILIC columns, 2.1 × 150 mm, 1.7 μm; Waters) in series maintained at 25 °C. The injection volume was 10 μL. Lipids were eluted isocratically for 10 min with 10% B, followed by a linear gradient to 18% B in 15 min, then a linear gradient to 30% B in 25 min, then a linear gradient to 100% B in 30 min and finally 100% B for 20 min, where A is hexane and B is hexane:isopropanol (9:1). Flow rate was 0.2 mL/min and pressure 400 bar. Total run time was 120 min with a 20 min re-equilibration. Settings of the ion source (APCI) are as followed: gas temperature 200 °C, vaporiser 400 °C, drying gas 6 L/min, nebuliser 60 psig. The lipids were identified using a positive ion mode (600–1400 m/z).

**RESULTS AND DISCUSSION**

**Analysis of microbial community**

We performed phylogenetic analysis of the microbial community in the ANME-2d enrichment originating from the Ooijpolder by assigning all processed paired-end reads to a taxon. In total, 23% of the reads were assigned to Ca. Methanopere- dens sp. strain BLZ2, 33% to Ca. Methylophilum sp. acting as nitrite scavenger, 8% to Alphaproteobacteria, 6% to Gammaproteobacteria, 5% to Betaproteobacteria 1% to Deltaproteobacteria, 3% to Terrabacteria, 3% to Sphingobacteria and 1% to Planctobacteria. The only archaeon in the bioreactor was Ca. Methanopere- dens sp. strain BLZ2. 33% to Ca. Methanopere- dens sp. strain BLZ2, 33% to Ca. Methanopere- dens sp. strain BLZ2, 33% to Ca. Methanopere- dens sp. strain BLZ2, 33% to Ca. Methanopere- dens sp. strain BLZ2, 33% to Ca. Methanopere- mens sp. strain BLZ2. Analysis of the microbial community in the Italian paddy field ANME-2d enrichment using a bioreactor approach has been described by Guerrero-Cruz et al. 2018. Metagenome sequencing of the DNA derived from this bioreactor revealed that 83% of 16S rRNA gene reads were assigned to Ca. Methanoperedens nitroreducens strain Verserenteno (Guerrero-Cruz et al. 2018). In this study we mainly show the results derived from lipid analysis of the Ca. Methanoperedens sp. BLZ2 enrichment originating from the Ooijpolder (Arshad et al. 2015; Berger et al. 2017). However, the
results deriving from a Ca. Methanoperedens sp. Versenetto enrichment originating from Italian paddy field soil (Guerrero-Cruz et al. 2018) look very similar, indicating that our results are not dependent on the strain or the environment from which the strain was enriched.

Core lipids of Ca. Methanoperedens sp.

To analyse the lipids of ANME-2d archaea, biomass from a bioreactor containing Ca. Methanoperedens sp. BLZ2 enrichment was sampled and core lipid analysis with GC-MS and UHPLC-APCI-TOF-MS was performed. Shorter archaeal lipids like archaeol (Fig. 1) can be detected via GC-MS, whereas longer archaeal lipids like GDGTs can only be detected via UHPLC-APCI-TOF-MS.

GC analysis of the core lipids released by acid hydrolysis showed the bacterial fatty acids and isoprenoidal archaeal lipids present in the ANME-2d enrichment (Fig. 2). We detected the typical membrane lipids of Ca. Methylmirabilis sp., namely 10-methylhexadecanoic acid (10MeC₁₆:0) and its monounsaturated variant (10MeC₁₆:₁Δ7) (Kool et al. 2012). The archaeal isoprenoids were predominantly composed of archaeol with lower amounts of sn2-hydroxyarchaeol and sn3-hydroxyarchaeol as well as two monounsaturated archaeoals (Fig. 1) that could be identified according to literature references (Nichols and Franzmann 1992). Monounsaturated archaeol has already been described to be present in environmental samples containing lipids of archaea associated with anaerobic methane oxidation in marine environments (Pancost, Hopmans and Sinninghe Damsté 2001; Blumenberg et al. 2005). However, the monounsaturated archaeol might be produced from hydroxyarchaeol during acidic treatment of the lipids and therefore might not be part of native membrane lipid structures (Ekiel and Sprott1992). Alteration of the hydroxyarchaeol structure caused by different reaction conditions during lipid treatment has also been shown by Hinrichs and co-workers (Hinrichs et al. 2000). On the other hand, monounsaturated archaeoals have also been described for Halorubrum lacusprofundi (Franzmann et al. 1988; Gibson et al. 2005), Methanopyrus kandleri (Nishihara et al. 2002), Methanococcoides burtonii (Nichols and Franzmann 1992; Nichols et al. 1993), even if using mild alkaline hydrolysis instead of acidic treatment for lipid extraction (Nishihara et al. 2002). One possibility to distinguish between the different ANME groups is the sn2-hydroxyarchaeol to archaeol proportion (Blumenberg et al. 2004). For ANME-1 this ratio is described to be...
Figure 2. Gas chromatogram of core lipids released by acid hydrolysis from Ca. Methanoperedens sp. (ANME-2d) enrichment. The enriched biomass of ANME-2d originates from the Ooijpolder (NL) (Arshad et al. 2015). Enlarged inserts show the TIC (total ion chromatogram) of the bacterial and archaeal lipids. The most abundant compounds are annotated with their compound name and following abbreviations: Uns-Ar = monounsaturated archaeol, OH-Ar = hydroxyarchaeol.

Table 1. Abundance of archaeol and GDGTs of Ca. Methanoperedens sp.

| Lipid              | Relative abundance (%) | Relative abundance (%) |
|--------------------|------------------------|------------------------|
| Archaeol           | 68 ± 5                 | 68 ± 5                 |
| GDGT-0             | 6 ± 1                  |                        |
| GDGT-1             | 3 ± 1                  |                        |
| GDGT-2             | 2 ± 1                  |                        |
| OH-GDGT-1          | 3 ± 1                  | 32 ± 5                 |
| OH-GDGT-2          | 1 ± 1                  |                        |
| di-OH-GDGT-1       | 3 ± 2                  |                        |
| di-OH-GDGT-2       | 5 ± 2                  |                        |
| Other GDGT-2 derivatives | 9 ± 4              |                        |

Lipid extraction was performed in quadruplicates, error is given as standard deviation. For calculation of the relative abundance of archaeol also peaks derived from archaeol artefacts created during the experimental procedure were used.

Subsequently, UHPLC-APCI-TOF-MS analysis of the lipid extract was conducted in order to obtain information about the tetraether lipids (Fig. 1). This revealed that the relative abundance of archaeol was two times higher than that of glycerol dialkyl glycerol tetraethers (GDGTs) (Table 1). Moreover, several types of GDGTs were present in the enrichment. GDGTs contained either no (GDGT-0), one (GDGT-1) or two (GDGT-2) cyclopentane rings and about 64% of the GDGTs were hydroxylated (OH-GDGTs). The most abundant GDGTs were GDGT-0 with 6% and di-OH-GDGT-2 with 5% of total lipids. In conclusion, ANME-2d archaea synthesise various core-GDGTs, however archaeol and its homologues are the main isoprenoidal core-lipids in this enrichment.

Environmental samples from Mediterranean cold seeps with marine AOM associated archaea mainly contained GDGTs with 0 to 2 cyclopentane rings (Pancost, Hopmans and Sinninghe Damsté 2001). In a study on distinct compartments of AOM-driven carbonate reefs growing in the northwestern Black Sea, GDGTs could only be found in samples when ANME-1 archaea were present, but not when only ANME-2 archaea were found.
which led to the conclusion that ANME-2 archaea are not capable of synthesising internally cyclised GDGT (Blumenberg et al. 2004). Later on in a study on methanotrophic consortia at cold methane seeps, samples associated with ANME-2c were shown to contain relatively high amounts of GDGTs (Elvert et al. 2005). In general, GDGTs are dominant in ANME-1 communities, while in marine ANME-2 and ANME-3 communities archaeol derivatives are most abundant (Niemann and Elvert 2008; Rossel et al. 2008). GDGTs are not only present in marine archaea but are also produced by soil microbiota. For example, several members of the phylum Thaumarcheota from soil environments have been shown to produce GDGTs with cre-narchaeol as the major core GDGT similar to the aquatic thau-marcheota (Sinninghe Damsté et al. 2012). Next to cre-narchaeol, GDGTs with 0 to 4 cyclopentane moieties and GDGTs containing an additional hydroxyl group were detected in Sinninghe Damsté and co-workers. In comparison to ANME-2d archaea, members of the related methanogen order Methanomicrobiales produce relatively high amounts of GDGT-0 (Koga et al. 1998; Schouten, Hopmans and Sinninghe Damsté 2012), whereas other members of the Methanosarcinales produce no or only minor amounts of GDGTs, mainly GDGT-0 (De Rosa and Gambacorta 1988; Nichols and Franzmann 1992; Schouten, Hopmans and Sinninghe Damsté 2012). Hydroxylated GDGTs seem to be relatively rare. In marine sediment samples the hydroxy-GDGT to total core GDGT ratio has been shown to vary between 1 and 8% and the dihydroxy-GDGT to total core GDGT ratio is below 2% (Liu et al. 2012). Hydroxylated GDGTs have so far only been identified in the methanogenic Euryarchaeon Methanothermococcus thermolithotrophicus (Liu et al. 2012) and in several Thaumarcheota (Schouten, Hopmans and Sinninghe Damsté 2012; Sinninghe Damsté et al. 2012). Until now only hydroxy-lated GDGTs with 0 to 2 cyclopentane rings have been found (Liu et al. 2012; Schouten, Hopmans and Sinninghe Damsté 2012; Sinninghe Damsté et al. 2012). In conclusion, the substantial abundance of GDGTs, especially hydroxylated GDGTs, is a distinct feature of the non-marine ANME archaeon Ca. Methanoperedens sp., as GDGTs have so far mainly been described for marine archaea like those of the ANME-1 clade as well as Thaumarcheota present in marine and non-marine environments.

Comparing the results obtained in this study and lipid characterisations of marine ANMEs, it is apparent that the ratio of archaeol and GDGTs are distinctive in the different ANME groups: ANME-1 and partially ANME-2c contain substantial amounts of GDGTs and especially in ANME-1, GDGTs are the predominant membrane lipids (Niemann and Elvert 2008). In contrast to ANME-1, but similar to other ANME-2 and ANME-3, we found that the dominating lipids in the membrane of clade ANME-2d archaea were archaeol variants and not GDGTs. However, about 30% of the membrane lipids in ANME-2d archaea were GDGTs. Most strikingly, the majority of those GDGTs were hydroxylated, which is quite rare and has not been observed for other ANMEs so far.

**Intact polar lipids of Ca. Methanoperedens sp.**

Although IPLs degrade more quickly than core lipids, IPLs are of higher taxonomic specificity and therefore useful to study especially present environments (Ruetters et al. 2002; Sturt et al. 2004). To identify IPLs of Ca. Methanoperedens archaea, UHPLC-ESI-MS was performed.

The three most abundant archaeal IPLs detected were archaeol with a dihexose headgroup and hydroxyarchaeol with either a monomethyl phosphatidyl ethanolamine (MMPE) or a phosphatidyl hexose (PH) headgroup. Further headgroups attached to archaeol were monohexose, MMPE, dimethyl phosphatidyl ethanolamine (DMPE), phosphatidyl ethanolamine (PE) and PH. Next to MMPE and PH, hydroxyarchaeol based IPLs also contained dihexose, monopentose, DMPE, PE, pentose-MMPE, hexose-MMPE and pentose-PE (Fig. 1). Headgroups of GDGTs were found to be diposphatidyl glycerol and dihexose phosphatidyl glycerol. The identification of a pentose as a headgroup of hydroxyarchaeol (mass loss of m/z 132) was unexpected. To our knowledge, this is the first description of a pentose as headgroup for microbial IPLs.

ANME-1 archaea mainly produce diglycosidic GDGTs, whereas lipids of marine ANME-2 and ANME-3 are dominated by phosphate-based polar derivatives of archaeol and hydroxyarchaeol (ANME-2: phosphatidyl glycerol, PE, phosphatidyl inositol, phosphatidyl serine, dihexose; ANME-3: phosphatidyl glycerol, phosphatidyl inositol, phosphatidyl serine) (Rossel et al. 2008). Furthermore, marine ANME-2 archaea produce only minor amounts of GDGT-based IPLs and ANME-3 archaea produce no GDGT-based IPLs at all (Rossel et al. 2008). Intact GDGTs are assumed to be synthesised by head-to-head condensation of two intact archaeol molecules and substitution of the headgroups (De Rosa, Gambacorta and Nicolaus 1980; Nishihara, Morii and Koga 1989; Kellermann et al. 2016). In a study of Wegener and co-workers IPLs of marine ANME-2 and ANME-1 enrichments were analysed (Wegener et al. 2016). In the marine ANME-2 archaea mainly archaeol with a diglycosyl, monoglycosyl or phosphatidyl glycerol headgroup and hydroxyarchaeol with a monoglycosyl or phosphatidyl glycerol headgroup were detected. The ANME-1 enrichment contained mainly GDGTs with a diglycosyl headgroup (Wegener et al. 2016). IPLs of ANME-2d archaea can be distinguished from those of ANME-1 archaea by the prevalence of phosphate containing headgroups as well as archaeol and hydroxyarchaeol based IPLs. Furthermore, ANME-2d can be distinguished from other ANME-2 and ANME-3 archaea by the high abundance of dihexose as headgroup, the rare MMPE and DMPE headgroups and putatively also the pentose headgroup, which so far has not been described in the literature. In contrast to ANME-3 archaea, ANME-2d and marine ANME-2 archaea produce GDGT-based IPLs, albeit only in minor amounts.

In marine environments, a variety of archaeal lipids including those identified in ANME archaea can be found, e.g. those of the abundant Thaumarcheota (GDGTs with hexose or phosphohexose headgroups, Sinninghe Damsté et al. 2012) and uncharacterised archaea (mainly GDGTs with glycosidic headgroups and in subsurface sediments also archaeol with glyco-sidic headgroups, Sturt et al. 2004; Lipp et al. 2008). In freshwater environments, IPLs of methanotrophic archaea have hardly been studied. Two studies on peat samples identified GDGTs with a glucose or glucuronosyl headgroup (Liu et al. 2010) and with a hexose-glycuronic acid, phosphohexose or hexose-phosphoglycerol head group (Peterse et al. 2011). GDGTs with a hexose-phosphoglycerol head group were also identified in our study for ANME-2d archaea. Therefore, ANME-2d together with other archaea might be part of the peat microbial community based on the IPL profile. Using DNA biomarkers, most notably the 16S rRNA gene, Ca. Methanoperedens sp. has been detected in various peat ecosystems (Cadillo-Quiroz et al. 2008; Zhang et al. 2008; Wang et al. 2019).

Other members of the order Methanosarcinales mainly produce archaeol and hydroxyarchaeol with the headgroups glucose, phosphatidyl glycerol (only Methanosarcinaeae), phosphatidyl inositol, PE, galactose (only Methanosetaeae) (Koga et al. 2019).
Nevertheless, the respective lipids were still quite depleted in $\delta^{13}$C in comparison to the incubations with labelled methane ($-2$ to $12\%$, 3 days incubation). Therefore, we concluded that mainly methane and not DIC is incorporated in the lipids of Ca. Methanoperedens sp. Supporting this result, cultures containing marine ANME-1 and ANME-2 were shown to incorporate carbon derived from labelled methane into archaeol, monounsaturated archaeol and biphytanes (Blumenberg et al. 2005). In another study it was found that ANME-1 archaea assimilated primarily inorganic carbon (Kellermann et al. 2012). Incubations with sediments containing ANME-1, 2a & 2b archaea showed that both, labelled methane and inorganic carbon, were incorporated into the archaeal lipids (Wegener et al. 2008). Incubations with fresh-water sediments including ANME-2d archaea followed by RNA stable isotope probing demonstrated that those microbes mainly incorporated methane into their lipids but may have the capability of mixed assimilation of CH$_4$ and DIC (Weber, Habicht and Thamdrup 2017). Our data confirmed that ANME-2d archaea are capable of mixed assimilation of CH$_4$ and DIC, but that methane is the preferred carbon source. In contrast to anaerobic methanotrophs, aerobic methanotrophs require oxygen for methane oxidation and the first step of methane oxidation to methanol is catalysed with the enzyme methane monoxygenase (Dalton 1980). Also the nitrite-dependent intra-aerobic methanotroph Ca. Methyloviribis sp., which is assumed to use nitric oxide to generate internal oxygen to oxidise methane encodes enzymes for the conventional aerobic methane oxidation pathway, including the methane monoxygenase (Ettwig et al. 2010).

Aerobic methanotrophs have different pathways of carbon fixation: proteobacterial methanotrophs assimilate C$_1$ compounds deriving from methane oxidation via the ribulose monophosphate (RuMP) and/or the serine pathway (Dalton 1980) while verrucomicrobial methanotrophs and NC10 bacteria like Ca. Methyloviribis sp. use the Calvin-Benson-Bassham cycle, mainly assimilating DIC (Khadem et al. 2011; Rasigraf et al. 2014). As mentioned before, the reductive acetyl-CoA pathway is the main carbon assimilation pathway in methanogenic Eurysarcarchaeota (Whitman 1994; Berg et al. 2010) and most likely also in ANME-1 and ANME-2d archaea (Hallam et al. 2004; Haroon et al. 2013). In this pathway a carbonyl group and a methyl group are combined to form acetyl-CoA. We were able to show in this study that ANME-2d archaea are capable of mixed assimilation of CH$_4$ and DIC, but preferably incorporate methane in their biomass. For this reason, it can be concluded that the C$_1$ compounds required for the reductive acetyl-CoA pathway derive from oxidation of methane as well as from DIC, whereby methane is the primary carbon source. Although ANME-1 archaea are assumed to use the same carbon fixation pathway (Hallam et al. 2004), they have been shown to primarily assimilate inorganic carbon (Kellermann et al. 2012). Therefore, the type of carbon fixation pathway does not directly allow conclusions on the preferred carbon source used for carbon assimilation of a microorganism.

Conclusion

In this study, we analysed the lipids from the main player in nitrate AOM, Ca. Methanoperedens sp. We found several lipid characteristics that enable distinction between ANME-2d and other ANME groups (Table 2).

ANME-2d archaea therefore can be distinguished from ANME-1 by the higher ratio of archaeol and hydroxyarchaeol instead of GDGTs as well as phosphate containing headgroups. Furthermore, ANME-2d can be distinguished from other ANME-2 and ANME-3 archaea by the high abundance of dihexose as...
Table 2. Lipids of different ANME groups.

| Environment | ANME-1 | ANME-2a/b | ANME-2c | ANME-2d | ANME-3 |
|-------------|--------|-----------|---------|---------|--------|
| Core lipids | Marine | Marine    | Marine  | Freshwater | Marine |
| GDGT        | (OH-) archaeol | (OH-) archaeol, GDGTs | (OH-) archaeol, (OH-) GDGTs | 0.1–0.3 | (OH-) archaeol |
| Sn-2-OH-archaeol/archaeol ratio | 0–0.8 | 1.1–5.5 | 1.1–5.5 | 1.1–5.5 |
| IPLs        | GDGT + dihexose | (OH-) archaeol + PG, PE, PH, PS, dihexose | (OH-) archaeol + PG, PE, PH, PS, Dihexose | PH, PS, Dihexose | PH, PS, Dihexose |
| Main carbon source | DIC | | | | |

For ANME-2d lipid analysis we used Ca. Methanoperedens sp. enriched bioreactor material. For the other ANME groups information was based on publication about the specific lipid characteristic (Blumenberg et al. 2004; Niemann and Elvert 2008) or 13C labelling experiments (Blumenberg et al. 2005; Wegener et al. 2008; Kellermann et al. 2012). GDGT: glycerol dialkyl glycerol tetraether, PE: phosphatidyl ethanolamine, MMPE: monomethyl phosphatidyl ethanolamine, DMPE: dimethyl phosphatidyl ethanolamine, PG: phosphatidyl glycerol, MH: monohexose, DH: dihexose, PH: phosphatidyl hexose, PC: phosphatidyl choline. Features of ANME-2d archaea are written in bold.

headgroup, the rare MMPE and DMPE headgroups and putatively also the pentose headgroup, which so far has not been described in the literature. The appearance of a monopentose as headgroup of ANME-2d lipids is an interesting observation and might be further analysed in the future. In contrast to other ANME groups ANME-2d archaea have been shown to produce relatively rare hydroxylated GDGTs.

ANME groups do not only differ in their membrane lipids itself, but also in the way they incorporate carbon into their biomass. For ANME-1 it has been shown that primarily carbon derived from DIC is incorporated into the lipids (Kellermann et al. 2012). In case of ANME-2d archaea, we were able to demonstrate that both, carbon derived from DIC and from methane, are incorporated into their lipids, with methane as the preferred carbon source.

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