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Research Article

RESEARCH ARTICLE

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

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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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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 ^{13}C 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; McAnulty *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 *Methanosarcinales* 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, digester sludge 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 geranylgeranylphosphate 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 BLZ2 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 $MgSO_4$, 0.24 g/L $CaCl_2$ and 0.5 g/L KH_2PO_4. Trace elements and vitamins were supplied using stock solutions. 1000 × trace element stock solution: 1.35 g/L $FeCl_2 \times 4\ H_2O$, 0.1 g/L $MnCl_2 \times 4\ H_2O$, 0.024 g/L $CoCl_2 \times 6\ H_2O$, 0.1 g/L $CaCl_2 \times 2\ H_2O$, 0.1 g/L $ZnCl_2$, 0.025 g/L $CuCl_2 \times 2\ H_2O$, 0.01 g/L H_3BO_3, 0.024 g/L $Na_2MoO_4 \times 2\ H_2O$, 0.22 g/L $NiCl_2 \times 6\ H_2O$, 0.017 g/L Na_2SeO_3, 0.004 g/L $Na_2WO_4 \times 2\ H_2O$, 12.8 g/L nitrilotriacetic acid; 1000 × vitamin stock solution: 20 mg/L biotin, 20 mg/L folic acid, 100 mg/L pyridoxine-HCl, 50 mg/L thiamin-HCl × 2 H_2O, 50 mg/L riboflavin, 50 mg/L nicotinic acid, 50 mg/L D-Ca-pantothenate, 2 mg/L vitamin B12, 50 mg/L p-aminobenzoic acid, 50 mg/L lipoic acid. The medium supply was continuously sparged with Ar:CO_2 in a 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 CH_4:CO_2 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 removal of supernatant. Under these conditions nitrite was not detectable with a colorimetric test with a lower detection limit of 2 mg/L (MQuant test stripes, Merck, Darmstadt, Germany). Growth conditions and operation of the bioreactor containing ANME-2d archaea enriched from an Italian paddy field soil are described by Guerrero-Cruz *et al*. (2018). Sampled material from both reactors 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.

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-trimming, 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 ^{13}C-labelling experiment

In total, 60 mL bioreactor material of a *Ca*. Methanoperedens sp. BLZ2 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 the closed bottle with argon gas for 10 min. Afterwards, the culture was purged with 90% argon and 10% CO_2 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 ^{13}C-labelled methane (99 atom%; Isotec Inc., Matheson Trigas Products Division) or ^{13}C-labelled bicarbonate (Cambridge Isotope Laboratories Inc., Tewksbury, USA) was used in the batch incubations. The bottles were incubated horizontally on a shaker at 30°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 MQuant (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 Porapaq Q 100/120 mesh and a thermal conductivity detector (TCD) using N_2 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 ^{13}C-labelled bicarbonate also contained ^{12}C derived from CO_2. Having in mind that 10% of CO_2 were added to the culture ($pCO_2 = 0.1$ atm) the CO_2 concentration in the solution was calculated to be about 3.36 mM by use of the equation $[CO2]_{aq} = pCO_2/k_h$ (Henry constant (k_h) 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 ^{13}C labelled bicarbonate was added derived from ^{12}C-CO_2 dissolved in the medium after gassing with a mixture of 10% CO_2/90% 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 H_2O were added. The DCM bottom layer was transferred to a new vial and the MeOH/H_2O layer washed twice

with DCM. Combined DCM layers were dried over a Na_2SO_4 column and the solvent removed by evaporation under a stream of nitrogen.

BF_3 methylation and silylation

For GC analysis aliquots of the acid hydrolysed samples were methylated using 0.5 mL of BF_3-methanol and reacted for 10 min at 60°C in an oven. In total, 0.5 mL H_2O 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 a N_2 stream and water was removed by use of a $MgSO_4$ column. After dissolving the sample in ethyl acetate, the extract was cleaned over a small silicagel column and lipids were eluted with ethyl acetate. The extract was dried under N_2. 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 with 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 ^{13}C 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, 320°C was held for 10 min. $\delta^{13}C$ values were corrected for methyl group derived from BF_3 methanol in case of carboxylic acid group (bacterial lipids) and methyl groups derived from BSTFA in case of hydroxyl groups (mainly archaeal lipids). Averaged $\delta^{13}C$ 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-TOFMS). 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).

UHPLC-ESI-MS

This method was used to analyse the IPLs of the ANME-2d enrichment. In total, 0.3 to 0.7 mg of Bligh and Dyer sample was dissolved in an injection solvent composed of hexane/isopropanol/water (72:27:1;v/v/v) and filtered through a 0.45 μm regenerated cellulose filter with 4 mm diameter prior to analysis by ultra-high performance liquid chromatography linked to ion trap mass spectrometry using electrospray ionisation (UHPLC-ESI-MS). UHPLC separation was conducted on an Agilent 1200 series UHPLC equipped with a YMC-Pack Diol-120-NP column (250 × 2.1 mm, 5 μm particle size) and a thermostated autoinjector, coupled to a Thermo LTQ XL linear ion trap with Ion Max source with electrospray ionisation (ESI) probe (Thermo Scientific, Waltham, MA). Solvent A contained 79% hexane, 20% isopropanol, 0.12% formic acid, 0.04% ammonium and solvent B 88% isopropanol, 10% H_2O, 0.12% formic acid, 0.04% ammonium. Lipids were eluted with 0% B for 1 min, a linear gradient from 0 to 34% B in 17 min, 34% B for 12 min, followed by a linear gradient to 65% B in 15 min, 65% B for 15 min and finally a linear gradient to 100% B in 15 min. The IPLs were identified using a positive ion mode (m/z 400–2000) and a collision energy of 35 eV.

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*. Methanoperedens sp. strain BLZ2, 33% to *Ca*. Methylomirabilis 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*. Methanoperedens 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 Verserenetto (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

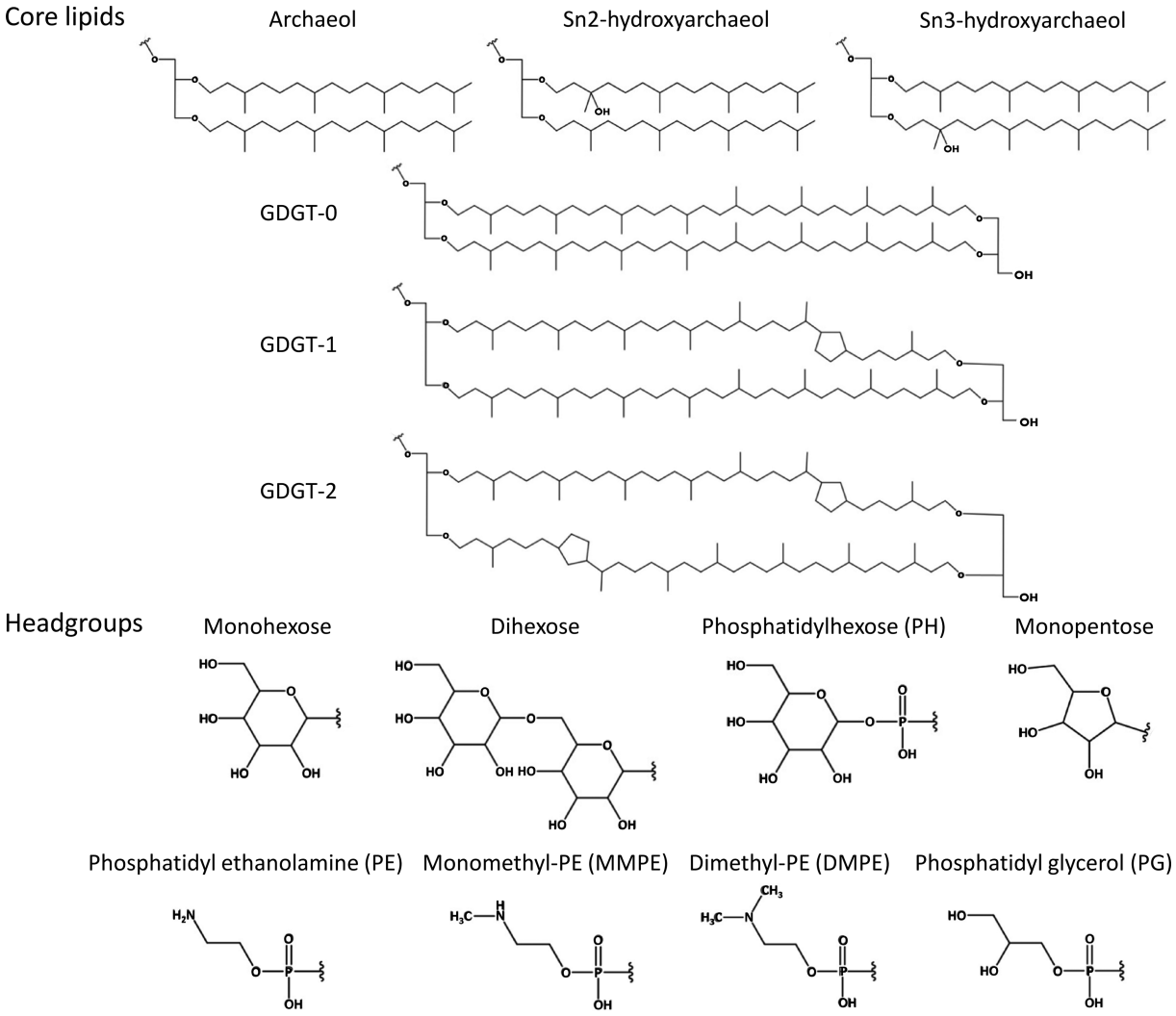

Figure 1. Structures of archaeal core lipids and headgroups. The main core lipids and headgroups that we found to be present in ANME-2d archaea are shown. The exact structure of monounsaturated archaeol as well as the pentose headgroup is not known. Intact polar lipids consist of a core lipid and one or two headgrops (only GDGTs can contain two headgroups). GDGT = glycerol dialkyl glycerol tetraethers.

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*. Methylomirabilis sp., namely 10-methylhexadecanoic acid ($10MeC_{16:0}$) and its monounsaturated variant ($10MeC_{16:1\Delta7}$) (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 archaeols (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 Sprott 1992). 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 archaeols 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

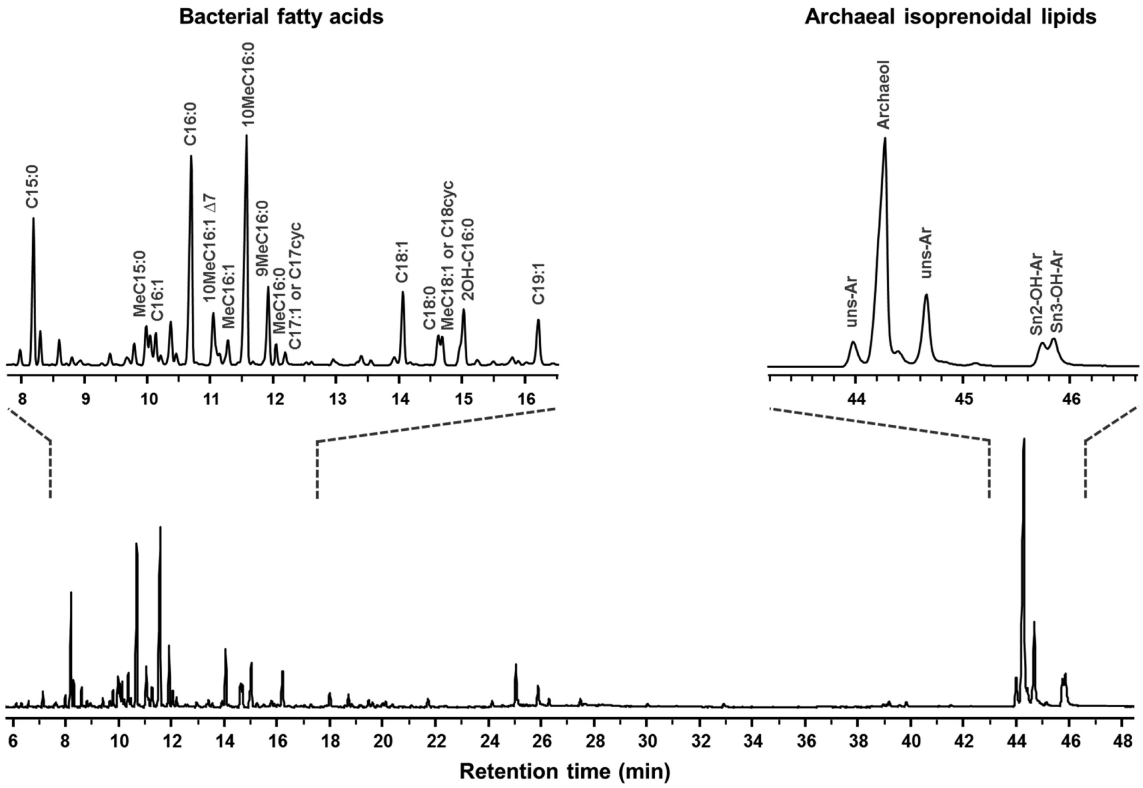

Figure 2. Gas chromatogram of core lipids released by acid hydrolysis from *Ca.* Methanoperedens sp. (ANME-2d) enrichment. The enriched biomass of ANME-2d originates from the Ooijpolder (NL) (Arshad *et al.* 2015). Enlarged inserts show the TIC (total ion chromatogram) of the bacterial and archaeal lipids. The most abundant compounds are annotated with their compound name and following abbreviations: Uns-Ar = monounsaturated archaeol, OH-Ar = hydroxyarchaeol.

0–0.8, for marine ANME-2 1.1 to 5.5 and for ANME-3 within the range of ANME-2 (Blumenberg *et al.* 2004; Niemann *et al.* 2006; Nauhaus *et al.* 2007; Niemann and Elvert 2008). In our study with the non-marine ANME-2d archaea we observed a sn2-hydroxyarchaeol to archaeol ratio of around 0.2. As mentioned before, the monounsaturated archaeol species might be an artefact of hydroxyarchaeol. If the monounsaturated archaeols are added to that of the sn2-hydroxyarchaeol abundance, the ratio would still be only around 0.3. That means that the hydroxyarchaeol to archaeol ratio of ANME-2d is more similar to that of ANME-1 archaea than to that of other ANME-2 or ANME-3 archaea. Members of the related methanogen order *Methanomicrobiales* only contain archaeol and GDGT-0 in their membranes but not hydroxyarchaeol (Koga *et al.* 1998). In the order *Methanosarcinales* the lipid composition varies between the different members. Most strains produce archaeol and hydroxyarchaeol, but the ratio differs and also the type of hydroxyarchaeol isomer varies. *Methanosarcinaceae* mainly produce the sn2-isomer, whereas *Methanosaetaceae* mainly produce the rare sn3-isomer (Koga *et al.* 1993; Koga *et al.* 1998).

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.

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.

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 Thaumarchaeota from soil environments have been shown to produce GDGTs with crenarchaeol as the major core GDGT similar to the aquatic thaumarchaeota (Sinninghe Damsté *et al.* 2012). Next to crenarchaol, GDGTs with 0 to 4 cyclopentane moieties and GDGTs containing an additional hydroxyl group were detected in by 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 Thaumarchaeota (Schouten, Hopmans and Sinninghe Damsté 2012; Sinninghe Damsté *et al.* 2012). Until now only hydroxylated 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 Thaumarchaeota 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 diphosphatidyl 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: phospatidyl glycerol, PE, phosphatidyl inositol, phosphatidyl serine, dihexose; ANME-3: phospatidyl 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 phoshatidyl glycerol headgroup and hydroxyarchaeol with a monoglycosyl or phoshatidyl 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 Thaumarchaeota (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 glycosidic 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 *Methanosarcinaceae*), phosphatidyl inositol, PE, galactose (only *Methanosaetaceae*) (Koga

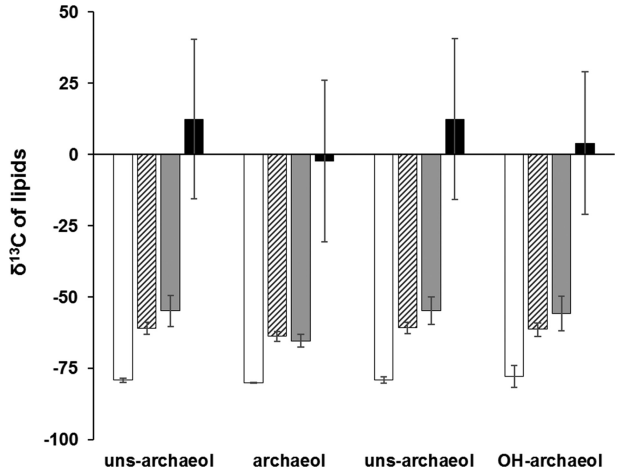

Figure 3. $\delta^{13}C$ values (in ‰) of *Ca*. Methanoperedens sp. lipids after batch cultivation with labelled bicarbonate or methane. ANME-2d reactor material originating from the Ooijpolder was incubated in anaerobic batch cultures with either ^{13}C labelled bicarbonate for 3 days (striped columns) or ^{13}C labelled methane for one (light grey columns) or 3 days (black columns). Controls contained only non-labelled carbon sources (white columns). Incubations were performed in triplicates, error bars = standard deviation. $\delta^{13}C$ values were obtained by analysing acid hydrolysed samples that only contained the core lipids by GC-IRMS. Peak identification was conducted with the help of GC-MS analysis of the same samples, showing that lipid extracts contained archaeol, hydroxyarchaeol and two monounsaturated archaeols (Fig. 1). Uns-archaeol = monounsaturated archaeol.

et al. 1998). On the other hand, members of the related order *Methanomicrobiales* contain GDGT-0 and archaeol with the lipid headgroups glucose, galactose, phosphatidyl aminopentanetetrols, phosphatidyl glycerol (Koga *et al*. 1998). Therefore, IPLs from *Ca*. Methanoperedens sp. differ from methanogen IPLs by the high abundance of dihexose, MMPE and phosphatidyl hexose as lipid headgroup and the absence of the quite common headgroup phosphatidyl serine.

Incorporation of carbon derived from methane and bicarbonate in lipids

We were not only interested in characterising the lipids of *Ca*. Methanoperedens sp., but also in answering the question if the organism incorporates carbon derived from methane or from DIC in its lipids. In a labelling experiment from 2006 with an ANME-2d enrichment culture, incorporation of carbon derived from methane could hardly be detected for archaeal lipids (Raghoebarsing *et al*. 2006). To establish the carbon sources for *Ca*. Methanoperedens sp. we incubated the enrichment culture with ^{13}C labelled bicarbonate and methane and analysed lipid extracts for $\delta^{13}C$ depletion by GC-IRMS (Fig. 3).

Analysis of the isotopic composition of archaeol and its derivatives showed that ANME-2d archaea incorporated carbon derived from both methane and bicarbonate into their lipids. However, the main carbon source for biomass production seemed to be methane and not DIC as the former resulted in a higher degree of labeling in the archaeal lipids. However, it has to be considered that the cultures to which ^{13}C labelled bicarbonate was added did not exclusively contain ^{13}C-DIC. About half of the DIC in the cultures derived from ^{12}C-CO_2 dissolved in the medium after gassing with a mixture of 10% CO_2/90% Argon gas (calculations in the methods part). Considering this, the $\delta^{13}C$ values of the archaeol isomers without ^{12}C-DIC in the incubations would vary most probably between −40 and −60‰. 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 freshwater 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 monooxygenase (Dalton 1980). Also the nitrite-dependent intra-aerobic methanotroph *Ca*. Methylomirabilis 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 monooxygenase (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*. Methylomirabilis 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 Euryarchaeota (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.

	ANME-1	ANME-2a/b	ANME-2c	**ANME-2d**	ANME-3
Environment	Marine	Marine	Marine	**Freshwater**	Marine
Core lipids	GDGT	(OH-) archaeol	(OH-) archaeol, GDGTs	**(OH-) archaeol, (OH-) GDGTs**	(OH-) archaeol
Sn-2-OH-archaeol/archaeol ratio	0–0.8	1.1–5.5	1.1–5.5	**0.1–0.3**	1.1–5.5
IPLs	GDGT + dihexose	(OH-) archaeol + PG, PE, PH, PS, dihexose	(OH-) archaeol + PG, PE, PH, PS, Dihexose	**(OH-) archaeol + dihexose, hexose, pentose, PH, PE, MMPE, DMPE**	(OH-) archaeol + PG, PH, PS
Main carbon source	DIC			**CH_4**	

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 ^{13}C 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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REFERENCES

Arshad A, Speth DR, de Graaf RM *et al.* A metagenomics-based metabolic model of nitrate-dependent anaerobic oxidation of methane by *Methanoperedens*-like archaea. *Front Microbiol* 2015;**6**:1–14.

Bale NJ, Villanueva L, Hopmans EC *et al.* Different seasonality of pelagic and benthic Thaumarchaeota in the North Sea. *Biogeosciences* 2013;**10**:7195–206.

Beal EJ, House CH, Orphan VJ. Manganese- and iron-dependent marine methane oxidation. *Science* 2009;**325**:184–7.

Berger S, Frank J, Dalcin Martins P *et al.* High-quality draft genome sequence of "*Candidatus* Methanoperedens sp." strain BLZ2, a nitrate-reducing anaerobic methane-oxidizing archaeon enriched in an anoxic bioreactor. *Genome Announc* 2017;**5**:e01159–17.

Berg IA, Kockelkorn D, Vera WHR *et al.* Autotrophic carbon fixation in archaea. *Nat Rev Microbiol* 2010;**8**:447–60.

Blumenberg M, Seifert R, Nauhaus K *et al.* In vitro study of lipid biosynthesis in an anaerobically methane-oxidizing microbial mat. *Appl Environ Microbiol* 2005;**71**:4345–51.

Blumenberg M, Seifert R, Reitner J *et al.* Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. *Proc Natl Acad Sci* 2004;**101**:11111–6.

Boetius A, Ravenschlag K, Schubert CJ *et al.* A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* 2000;**407**:623–6.

Cadillo-Quiroz H, Yashiro E, Yavitt JB *et al.* Characterization of the archaeal community in a minerotrophic fen and terminal restriction fragment length polymorphism-directed isolation of a novel hydrogenotrophic methanogen. *Appl Environ Microbiol* 2008;**74**:2059–68.

Cai C, Leu AO, Xie G-J *et al.* A methanotrophic archaeon couples anaerobic oxidation of methane to Fe (III) reduction. *ISME J* 2018;**12**:1929–39.

Conrad R. The global methane cycle: recent advances in understanding the microbial processes involved. *Environ Microbiol Rep* 2009;**1**:285–92.

Dalton H. Oxidation of hydrocarbons by methane monooxygenases from a variety of microbes. *Adv Appl Microbiol* 1980;**26**:71–87.

Dean JF, Middelburg JJ, Roeckmann T *et al.* Methane feedbacks to the global climate system in a warmer world. *Rev Geophys* 2018;**56**:207–50.

De Rosa M, Gambacorta A, Nicolaus B. Regularity of isoprenoid biosynthesis in the ether lipids of archaebacteria. *Phytochemistry* 1980;**19**:791–3.

De Rosa M, Gambacorta A. The lipids of archaebacteria. *Prog Lipid Res* 1988;**27**:153–75.

Ekiel I, Sprott GD. Identification of degradation artifacts formed upon treatment of hydroxydiether lipids from methanogens with methanolic HCl. *Can J Microbiol* 1992;**38**:764–8.

Elvert M, Hopmans E, Treude T *et al.* Spatial variations of methanotrophic consortia at cold methane seeps: implications from a high-resolution molecular and. *Geobiology* 2005;**3**:195–209.

Elvert M, Suess E, Whiticar MJ. Anaerobic methane oxidation associated with marine gas hydrates: superlight C-isotopes from saturated and unsaturated C_{20} and C_{25} irregular isoprenoids. *Naturwissenschaften* 1999;**86**:295–300.

Ettwig KF, Butler MK, Le Paslier D *et al.* Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 2010;**464**:543–8.

Ettwig KF, Zhu B, Speth D *et al.* Archaea catalyze iron-dependent anaerobic oxidation of methane. *Proc Natl Acad Sci USA* 2016;**113**:127922–12796.

Franzmann PD, Stackebrandt E, Sanderson K *et al.* *Halobacterium lacusprofundi* sp. nov., a halophilic bacterium isolated from Deep Lake, Antarctica. *Syst Appl Microbiol* 1988;**11**:20–7.

Gibson JAE, Miller MR, Davies NW *et al.* Unsaturated diether lipids in the psychrotrophic archaeon *Halorubrum lacusprofundi*. *Syst Appl Microbiol* 2005;**28**:19–26.

Guerrero-Cruz S, Cremers G, van Alen TA *et al.* Response of the anaerobic methanotroph "*Candidatus* Methanoperedens nitroreducens" to oxygen stress. *Appl Environ Microbiol* 2018;**84**:1–17.

Hallam SJ, Putnam N, Preston CM *et al.* Reverse methanogenesis: testing the hypothesis with environmental genomics. *Science* 2004;**305**:1457–63.

Haroon MF, Hu S, Shi Y *et al.* Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* 2013;**500**:567–70.

Hinrichs K, Boetius A. The anaerobic oxidation of methane: new insights in microbial ecology and biogeochemistry. In: Wefer G, Billett D, Hebbeln D *et al.*(eds). *Ocean Margin Systems*. Berlin: Springer-Verlag, 2002, pp. 457–77.

Hinrichs K-U, Hayes JM, Sylva SP *et al.* Methane-consuming archaebacteria in marine sediments. *Nature* 1999;**398**:802–5.

Hinrichs K-U, Pancost RD, Summons RE *et al.* Mass spectra of sn-2-hydroxyarchaeol, a polar lipid biomarker for anaerobic methanotrophy. *Geochem Geophys Geosyst* 2000;**1**:2000GC000042.

Hoehler TM, Alperin MJ, Albert DB *et al.* Field and laboratory studies of methane oxidation in an anoxic marine sediment: evidence for a methanogen-sulfate reducer consortium. *Global Biogeochem Cycles* 1994;**8**:451–63.

Hu S, Zeng RJ, Burow LC *et al.* Enrichment of denitrifying anaerobic methane oxidizing microorganisms. *Environ Microbiol Rep* 2009;**1**:377–84.

Kellermann MY, Wegener G, Elvert M *et al.* Autotrophy as a predominant mode of carbon fixation in anaerobic methane-oxidizing microbial communities. *Proc Natl Acad Sci* 2012;**109**:19321–6.

Kellermann MY, Yoshinaga MY, Wegener G *et al.* Tracing the production and fate of individual archaeal intact polar lipids using stable isotope probing. *Org Geochem* 2016;**95**:13–20.

Khadem AF, Pol A, Wieczorek A *et al.* Autotrophic methanotrophy in Verrucomicrobia: *Methylacidiphilum fumariolicum* SolV uses the Calvin-Benson-Bassham Cycle for carbon dioxide fixation. *J Bacteriol* 2011;**193**:4438–46.

Knittel K, Boetius A. Anaerobic oxidation of methane: progress with an unknown process. *Annu Rev Microbiol* 2009;**63**:311–34.

Koga Y, Morii H, Akagawa-Matsushita M *et al.* Correlation of polar lipid composition with 16S rRNA phylogeny in methanogens. Further analysis of lipid component parts. *Biosci Biotechnol Biochem* 1998;**62**:230–6.

Koga Y, Morii H. Biosynthesis of ether-type polar lipids in Archaea and evolutionary considerations. *Microbiol Mol Biol Rev* 2007;**71**:97–120.

Koga Y, Nishihara M, Morii H *et al.* Ether polar lipids of methanogenic bacteria: structures, comparative aspects, and biosyntheses. *Microbiol Rev* 1993;**57**:164–82.

Kool DM, Zhu B, Rijpstra WIC *et al.* Rare branched fatty acids characterize the lipid composition of the intra-aerobic methane oxidizer "*Candidatus* Methylomirabilis oxyfera." *Appl Environ Microbiol* 2012;**78**:8650–6.

Lipp JS, Morono Y, Inagaki F *et al.* Significant contribution of archaea to extant biomass in marine subsurface sediments. *Nat Lett* 2008;**454**:991–4.

Liu X, Leider A, Gillespie A *et al.* Organic geochemistry identification of polar lipid precursors of the ubiquitous branched GDGT orphan lipids in a peat bog in Northern Germany. *Org Geochem* 2010;**41**:653–60.

Liu X-L, Lipp JS, Simpson JH *et al.* Mono- and dihydroxyl glycerol dibiphytanyl glycerol tetraethers in marine sediments: identification of both core and intact polar lipid forms. *Geochim Cosmochim Acta* 2012;**89**:102–15.

Matsumi R, Atomi H, Driessen AJM *et al.* Isoprenoid biosynthesis in archaea – biochemical and evolutionary implications. *Res Microbiol* 2011;**162**:39–52.

McAnulty MJ, Poosarla VG, Kim K-Y *et al.* Electricity from methane by reversing methanogenesis. *Nat Commun* 2017;**8**:15419.

Menzel P, Ng KL, Krogh A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nat Commun* 2016;**7**:1–9.

Nauhaus K, Albrecht M, Elvert M *et al.* In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. *Environ Microbiol* 2007;**9**:187–96.

NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 2016;**44**:7–19.

Nichols PD, Franzmann PD. Unsaturated diether phospholipids in the Antarctic methanogen *Methanococcoides burtonii*. *FEMS Microbiol Lett* 1992;**98**:205–8.

Nichols PD, Shaw PM, Mancuso CA *et al.* Analysis of archaeal phospholipid-derived di- and tetraether lipids by high temperature capillary gas chromatography. *J Microbiol Methods* 1993;**18**:1–9.

Niemann H, Elvert M. Diagnostic lipid biomarker and stable carbon isotope signatures of microbial communities mediating the anaerobic oxidation of methane with sulphate. *Org Geochem* 2008;**39**:1668–77.

Niemann H, Lösekann T, de Beer D *et al.* Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. *Nature* 2006;**443**:854–8.

Nishihara M, Morii H, Koga Y. Heptads of polar ether lipids of an archaebacterium, *Methanobacterium thermoautotrophicum*: structure and biosynthetic relationship. *Biochemistry* 1989;**28**:95–102.

Nishihara M, Morii H, Matsuno K *et al.* Structural analysis by reductive cleavage with LiAlH4 of an allyl ether cholinephospholipid, archaetidylcholine, from the hyperthermophilic methanoarchaeon *Methanopyrus kandleri*. *Archaea* 2002;**1**:123–31.

Orphan VJ, House CH, Hinrichs K-U *et al.* Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proc Natl Acad Sci USA* 2002;**99**:7663–8.

Pancost RD, Hopmans EC, Sinninghe Damsté JS. Archaeal lipids in mediterranean cold seeps: molecular proxies for anaerobic methane oxidation. *Geochim Cosmochim Acta* 2001;**65**:1611–27.

Pancost RD, Sinninghe Damsté JS, de Lint S *et al.* Biomarker evidence for widespread anaerobic methane oxidation in

mediterranean sediments by a consortium of methanogenic archaea and bacteria. *Appl Environ Microbiol* 2000;**66**:1126–32.

Peterse F, Hopmans EC, Schouten S *et al.* Identification and distribution of intact polar branched tetraether lipids in peat and soil. *Org Geochem* 2011;**42**:1007–15.

Raghoebarsing AA, Pol A, van de Pas-Schoonen KT *et al.* A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* 2006;**440**:918–21.

Rasigraf O, Kool DM, Jetten MSM *et al.* Autotrophic carbon dioxide fixation via the Calvin-Benson-Bassham cycle by the denitrifying methanotroph "*Candidatus* Methylomirabilis oxyfera." *Appl Environ Microbiol* 2014;**80**:2451–60.

Rossel PE, Lipp JS, Fredricks HF *et al.* Intact polar lipids of anaerobic methanotrophic archaea and associated bacteria. *Org Geochem* 2008;**39**:992–9.

Ruetters H, Sass H, Cypionka H *et al.* Phospholipid analysis as a tool to study complex microbial communities in marine sediments. *J Microbiol Methods* 2002;**48**:149–60.

Schouten S, Hopmans EC, Sinninghe Damsté JS. The organic geochemistry of glycerol dialkyl glycerol tetraether lipids: a review. *Org Geochem* 2012;**54**:19–61.

Sinninghe Damsté JS, Rijpstra WIC, Hopmans EC *et al.* Intact polar and core glycerol dibiphytanyl glycerol tetraether lipids of group I.1a and I.1b Thaumarchaeota in soil. *Appl Environ Microbiol* 2012;**78**:6866–74.

Sturt HF, Summons RE, Smith K *et al.* 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**:617–28.

Timmers PHA, Welte CU, Koehorst JJ *et al.* Reverse methanogenesis and respiration in methanotrophic archaea. *Archaea* 2017;**2017**:1654237.

Vaksmaa A, Guerrero-Cruz S, van Alen TA *et al.* Enrichment of anaerobic nitrate-dependent methanotrophic "*Candidatus* Methanoperedens nitroreducens" archaea from an Italian paddy field soil. *Appl Microbiol Biotechnol* 2017;**101**:7075–84.

Vaksmaa A, Jetten MSM, Ettwig KF *et al.* *McrA* primers for the detection and quantification of the anaerobic archaeal methanotroph "*Candidatus* Methanoperedens nitroreducens." *Appl Microbiol Biotechnol* 2017;**101**:1631–41.

Wang M, Tian J, Bu Z *et al.* Structural and functional differentiation of the microbial community in the surface and subsurface peat of two minerotrophic fens in China. *Plant Soil* 2019;**437**:21–40. DOI: 10.1007/s11104-019-03962-w

Weber HS, Habicht KS, Thamdrup B. Anaerobic methanotrophic archaea of the ANME-2d cluster are active in a low-sulfate, iron-rich freshwater sediment. *Front Microbiol* 2017;**8**:1–13.

Wegener G, Krukenberg V, Ruff SE *et al.* Metabolic capabilities of microorganisms involved in and associated with the anaerobic oxidation of methane. *Front Microbiol* 2016;**7**:1–16.

Wegener G, Niemann H, Elvert M *et al.* Assimilation of methane and inorganic carbon by microbial communities mediating the anaerobic oxidation of methane. *Environ Microbiol* 2008;**10**:2287–98.

Whitman WB. Autotrophic acetyl coenzyme A biosynthesis in methanogens. In: Drake H L (ed). *Acetogenesis*. New York: Chapman & Hall., 1994,pp. 521–38.

Zhang G, Tian J, Jiang N *et al.* Methanogen community in Zoige wetland of Tibetan plateau and phenotypic characterization of a dominant uncultured methanogen cluster ZC-I. *Environ Microbiol* 2008;**10**:1850–60.