



Metabolic Capabilities of Microorganisms Involved in and Associated with the Anaerobic Oxidation of Methane

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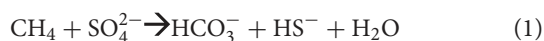
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In marine sediments the anaerobic oxidation of methane with sulfate as electron acceptor (AOM) is responsible for the removal of a major part of the greenhouse gas methane. AOM is performed by consortia of anaerobic methane-oxidizing archaea (ANME) and their specific partner bacteria. The physiology of these organisms is poorly understood, which is due to their slow growth with doubling times in the order of months and the phylogenetic diversity in natural and *in vitro* AOM enrichments. Here we study sediment-free long-term AOM enrichments that were cultivated from seep sediments sampled off the Italian Island Elba (20°C; hereon called E20) and from hot vents of the Guaymas Basin, Gulf of California, cultivated at 37°C (G37) or at 50°C (G50). These enrichments were dominated by consortia of ANME-2 archaea and Seep-SRB2 partner bacteria (E20) or by ANME-1, forming consortia with Seep-SRB2 bacteria (G37) or with bacteria of the HotSeep-1 cluster (G50). We investigate lipid membrane compositions as possible factors for the different temperature affinities of the different ANME clades and show autotrophy as characteristic feature for both ANME clades and their partner bacteria. Although in the absence of additional substrates methane formation was not observed, methanogenesis from methylated substrates (methanol and methylamine) could be quickly stimulated in the E20 and the G37 enrichment. Responsible for methanogenesis are archaea from the genus *Methanohalophilus* and *Methanococoides*, which are minor community members during AOM (1–7% of archaeal 16S rRNA gene amplicons). In the same two cultures also sulfur disproportionation could be quickly stimulated by addition of zero-valent colloidal sulfur. The isolated partner bacteria are likewise minor community members (1–9% of bacterial 16S rRNA gene amplicons), whereas the dominant partner bacteria (Seep-SRB1a, Seep-SRB2, or HotSeep-1) did not grow on elemental sulfur. Our results support a functioning of AOM as syntrophic interaction of obligate methanotrophic archaea that transfer non-molecular reducing equivalents (i.e., via direct interspecies electron transfer) to obligate sulfate-reducing partner bacteria. Additional catabolic processes in these enrichments but also in sulfate methane interfaces are likely performed by minor community members.

Keywords: anaerobic oxidation of methane, archaea, syntrophy, methanogenesis, disproportionation, physiology

INTRODUCTION

In the anoxic marine subsurface large amounts of the potential greenhouse gas methane are formed by microbial and thermal degradation of organic matter. Hence methane is highly abundant in the marine subsurface (Reeburgh, 2007). The efflux of methane from sediments into the water column is however limited, which is mostly due to the effective barrier of methanotrophic microorganisms. The quantitatively most important sink is the coupling of methane oxidation to the reduction of sulfate (AOM) according to the net reaction:



with an energy yield of only -34 kJ per mol substrate turnover at standard conditions (Knittel and Boetius, 2009). AOM is performed in dual species microbial consortia of anaerobic methane-oxidizing archaea (ANME), which are closely related to known methanogens, and partner bacteria affiliated to canonical sulfate reducers of the *Desulfosarcina/Desulfococcus* clade (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001; Knittel et al., 2005) or of the HotSeep-1 group (Krukenberg et al., under review). Currently three major clades of ANME archaea are known. ANME-2 is the most prominent methanotrophic clade at marine cold gas seeps (Orphan et al., 2002; Mills et al., 2003; Wegener et al., 2008b; Knittel and Boetius, 2009). The temperature at those sites is usually between 4 and 14°C (Knittel and Boetius, 2009). ANME-3 often occurs at mud volcanoes (i.e., Håkon Mosby Mud Volcano; Niemann et al., 2006 *in situ* temperature -1.5°C) and the Eastern Mediterranean seepages (14°C; Omoregie et al., 2008). To our knowledge so far ANME-3 does not proliferate *in vitro*. The third phylogenetic group ANME-1 has been originally described at cold seeps (Hinrichs et al., 1999), but it is particularly abundant in diffusive sulfate methane interfaces (Thomsen et al., 2001; Lanoil et al., 2005; Harrison et al., 2009; Aquilina et al., 2010) and in microbial mats and chimney structures at methane seeps in the Black Sea (Michaelis et al., 2002), *in situ* temperature of 10°C. In hydrothermally heated sediments such as in the Guaymas Basin (AOM activity up to 70°C) ANME-1 perform thermophilic methane oxidation (Teske et al., 2002; Holler et al., 2011b; Dowell et al., 2016). All ANME clades form dense consortia with deltaproteobacterial partners, which belong either to Seep-SRB1a from the *Desulfosarcinales/Desulfococcus* subcluster; (Schreiber et al., 2010), Seep-SRB2 from the *Desulfobacterales* subcluster (Kleindienst et al., 2012) or *Desulfobulbus* (mostly ANME-3; Niemann et al., 2006). The partner of thermophilic ANME-1 is HotSeep-1 (Holler et al., 2011b; Wegener et al., 2015). Different naturally enriched AOM communities proliferated *in vitro* (Nauhaus et al., 2002; Krüger et al., 2005; Holler et al., 2009), however cultivation at low temperatures ($\leq 20^\circ\text{C}$) repeatedly selected for ANME-2, although several source sediments were dominated by other clades (ANME-1 from the Black Sea or ANME-3 at Håkon Mosby Mud Volcano; Holler et al., 2009;

own data). The principles underlying this selective growth of ANME-2 *in vitro* have so far not been resolved. Only cultivation at elevated temperatures sustained ANME-1 (Holler et al., 2011b).

The potential of ANME to perform methanogenesis has been repeatedly suggested. This hypothesis based on experiments with natural enrichments (Bertram et al., 2013), on thermodynamic constraints (Alperin and Hoehler, 2009) and on the phylogenetic proximities and genomic similarities of ANME and known methanogens (Lloyd et al., 2011). Furthermore, using radiotracer co-occurrence of AOM and methane formation have been repeatedly measured (Treude et al., 2007; Orcutt et al., 2008) and ANME-1 archaea have been found to be abundant in potentially methanogenic sedimentary horizons (Lloyd et al., 2011). However, tracer transfer from product (DIC) into the reactant pool (methane) might also be explained as inherent process of AOM as suggested by Holler et al. (2011a).

The certainly least understood feature of AOM is how archaea and bacteria interact in the characteristic dual-species consortia. The activation and complete oxidation of methane via a reversal of the well-described methanogenesis pathway can be confidently assigned to the ANME archaea (Hallam et al., 2004; Meyerdierks et al., 2010; Thauer, 2011; Stokke et al., 2012; Wang et al., 2014). The fate of the released electrons including the localization of sulfate reduction is instead so far controversial. Based on their phylogenetic classification as *Deltaproteobacteria* (Knittel et al., 2003; Schreiber et al., 2010; Kleindienst et al., 2012) and the presence of genes and enzymes of sulfate reduction (Milucka et al., 2013; Wegener et al., 2015), all different partner bacteria are likely involved in the sulfur cycle. Different mechanisms for the interaction of ANME and partner bacteria have been suggested. For the sediment-free Isis Mud Volcano AOM enrichment (Mediterranean Sea), incomplete sulfate reduction in ANME-2 and zero-valent sulfur transfer to disproportionating partner bacteria was proposed (Milucka et al., 2012). Instead, for AOM communities in Hydrate Ridge sediments (Coast off Oregon, USA) cytochrome-mediated direct interspecies electron transfer between ANME-2 and their sulfate-reducing partner was proposed (McGlynn et al., 2015). For the interaction of thermophilic ANME-1 and their sulfate-reducing HotSeep-1 partner direct interspecies electron transfer via nanowires and cytochromes was proposed (Wegener et al., 2015).

Here we retrieved three sediment-free AOM enrichments derived from methane-percolated coastal sands off the Mediterranean Island Elba (Italy; enriched at 20°C; E20) as well as a mesophilic enrichment (37°C; G37) and a thermophilic enrichment culture (G50) from the Guaymas Basin. We described community compositions and membrane lipid patterns of these enrichments and performed physiological experiments to test metabolic capabilities attributed to AOM community members including chemoautotrophy, methanogenesis and sulfur disproportionation. Findings were evaluated in further AOM enrichments obtained from different seep sites.

MATERIAL AND METHODS

Production of AOM Enrichments and Maintenance

Source material for the E20 enrichment were clastic sediments sampled by scuba diving in 2010 from the coastal hydrocarbon seeps off the Mediterranean island Elba (*in situ* water temperature 12–27°C and 12 m water depth, further described in Ruff et al., this issue). By shaking and collecting the supernatant we concentrated slowly settling microbial biomass from rapidly sinking mineral particles (sand). The concentrated biomass (<1% of the sediment weight) retained 60% of the microbial methane-dependent sulfate reduction rate of the sediment. The G37 and G50 enrichments derived from the methane-rich hydrothermally heated sediments of the Guaymas Basin, Gulf of California, sampled during RV ATLANTIS cruise AT15-56 with the submersible ALVIN in 2009. After determination of applicable cultivation temperatures in a temperature gradient block (Holler et al., 2011b), sediments from distinct temperature horizons have been enriched at the determined temperature optima of AOM (37, 50, 60°C). All enrichments were incubated with marine sulfate reducer medium (Widdel and Bak, 1992) and supplied with methane and sulfate as sole potential redox couple for at least 3 years. Medium was exchanged when sulfide concentrations exceeded 15 mM and biomass was diluted (1:2 or 1:4) when sulfide production exceeded approximately 0.2 mmol l⁻¹ day⁻¹. Community structures in the 50 and 60°C enrichment were highly similar, thus experiments presented here were performed at 50°C. The additional sample “GF” (here only studied for microbial diversity and sulfur disproportionation), derived from the methane seeps in the vicinity of the Gullfaks oil field in the North Sea and was sampled during RV ALKOR cruise 267 in October 2005 (Wegener et al., 2008b). Sediment-free AOM enrichments from this site were produced by incubation under AOM conditions at room temperature and subsequent dilution as described above. Further methanotrophic enrichment cultures from the Mediterranean (NAUTNIL expedition with RV L Atalante in 2003), from Hydrate Ridge (RV SONNE expedition SO148 in 2000), the Black Sea (RV POSEIDON expedition POS 148 in 2004) and Gulf of Mexico (RV SONNE expedition SO 174), which were only screened for their microbial diversity, were retrieved and cultivated as described (see Supplementary Table 1).

Cultivation of Methanogens from the AOM Enrichments

To determine potential methanogenesis activity in the AOM enrichments E20, G37, and G50, triplicate sulfate-free culture aliquots of 10 ml (1:10 dilution) were incubated in 20 ml Hungate tubes with alternative substrates [hydrogen (0.2 MPa), carbon monoxide (0.05 MPa), formate, acetate, methylamine, and methanol (all 20 mM)] for 30 days at their distinct temperature. The development of methane in the headspace was measured using gas chromatography coupled to flame ionization detection (Focus GC, Thermo equipped with a Poropak column; Analytical columns). In this time interval, methane formation

was only observed with methylamine and methanol (for both, E20 and G37), but substrates were already fully turned over after 10 days. For these substrates experiments were repeated with more frequent sampling intervals (Figure 4A). Furthermore, for the substrates methanol and methylamine triplicate dilution-to-extinction series with factor 10 dilutions were prepared (down to 10⁸). Methane formation was repeatedly measured and the highest active dilutions (1:10⁵) were further diluted (1:1000). From freeze-thawed pellets of aliquots of these cultures we identified the enriched microbes by direct 16S rRNA gene amplification using the primer pair Arch20F (Massana et al., 1997)/Arc1492R (Teske et al., 2002) and sequencing (PCR and sequencing as described below for AOM enrichments).

Cultivation of Sulfate Reducers from the AOM Enrichments

To determine potential methane-independent sulfate reduction in the AOM enrichments, triplicate culture aliquots of 10 ml were incubated with possible alternative substrates [hydrogen (0.1 MPa), carbon monoxide (0.05 MPa), methyl sulfide (0.05 MPa) formate, acetate, methylamine, and methanol (all 20 mM)] for 30 days at their distinct temperature. The development of sulfide was measured by a copper sulfate assay and spectroscopic analysis (Cord-Ruwisch, 1985). Dilution-to-extinction series (down to 10⁸ dilution) were set up from active AOM enrichments (G37, G50) with hydrogen as only used alternative electron source, and incubated at their respective temperature for 2 months. After a subsequent second dilution step (1:100) of the highest sulfide-producing dilutions, enriched microbes were identified by 16S rRNA gene amplification (primer pair GM3/GM4; Muyzer et al., 1995) from freeze-thawed pellets of culture aliquots and direct sequencing (PCR and sequencing as described below for AOM enrichments).

Experiments on Alternative Sulfur Sources in the AOM Enrichments

To identify the spectrum of sulfur sources used by the microbial communities in the E20, G37, and G50 AOM enrichments, sulfate-free culture aliquots were incubated with the alternative sulfur sources sulfite (5 mM), thiosulfate (20 mM), and with sulfate (20 mM) as control. Furthermore, we incubated with colloidal (zero-valent) sulfur (ca. 50 mM) that was prepared according to Steudel et al. (1988), and dissolved in anaerobic deionized water (approximately 0.5 mol S⁰ per liter). Triplicate incubations with and without methane (0.2 MPa CH₄:CO₂; 90:10) were performed for each substrate. We measured sulfide production calorimetrically using the copper sulfate assay (Cord-Ruwisch, 1985). Dilution-to-extinction series (as described above) were performed for alternative substrates which showed substantial sulfide production (only in zero-valent sulfur enrichment). The highest active dilutions were further diluted (1:100) and enriched microbes were identified by 16S rRNA gene amplification (primer pair GM3/GM4; Muyzer et al., 1995) from freeze-thawed pellets of culture aliquots and direct sequencing (PCR and sequencing conditions as described above for AOM enrichments).

To study the underlying principle of zero-valent sulfur disproportionation, we tested the stoichiometry of sulfur disproportionation by simultaneously measuring sulfate [by ion chromatography; 761 Compact ion chromatograph (Metrohm) with a Metrosep A SUPP 5 column] and sulfide production (copper sulfate assay; Cord-Ruwisch, 1985) in the AOM enrichments and in the zero-valent sulfur enrichments.

Extraction and Analysis of Archaeal Intact Polar Lipids from the AOM Enrichments

Cell pellets from 30 ml AOM enrichment cultures were spiked with an internal standard (phosphatidylcholine C_{21:0/21:0}) and 3 g of combusted sand and extracted using a modified Bligh and Dyer protocol (Sturt et al., 2004). The obtained TLEs were stored at -20°C until analyses. IPLs were analyzed by high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS). Separation of IPLs was achieved on a Dionex Ultimate 3000 UHPLC equipped with a Waters Acquity UPLC BEH amide column (150 \times 2.1 mm, 1.8 μm particle size). Chromatographic conditions included constant flow rate of 0.4 ml/min with eluent A [75% acetonitrile; 25% dichloromethane; 0.01% formic acid; 0.01% ammonium hydroxide solution (NH₃ aq.)] and eluent B [50% methanol 50% Milli-Q water; 0.4% formic acid; 0.4% NH₃ aq. as previously published (Wörmer et al., 2013)]. Under a constant flow, the HPLC routine applied: 99% A and 1% B for 2.5 min, increasing to 5% B at 4 min, followed by a linear gradient to 25% B at 22.5 min and then to 40% B at 26.5 min. Thereafter a 1 min washing step with 40% B followed and afterwards reset to the initial conditions for 8 min to achieve column re-equilibration. Compound detection was conducted on a maXis Ultra-High Resolution qToF-MS (Bruker, Bremen, Germany). IPLs were measured in positive ionization mode scanning a mass-to-charge (m/z) range of 150–2000, with automated data-dependent MS/MS fragmentation of base peak ions. Compound identification was achieved by monitoring exact masses of possible parent ions (present mainly as H⁺ and NH₄⁺ adducts) in combination with characteristic fragmentation patterns (Sturt et al., 2004; Yoshinaga et al., 2011). The reported relative distribution of microbial lipids is based on the peak areas of the respective molecular ions without differentiating for potential differences in response factors; results should therefore be considered as semi-quantitative.

Determination of Microbial Carbon Sources and Growth Efficiencies in the AOM Enrichments

To determine the role of methane and bicarbonate as carbon sources in the AOM enrichment and their assimilation rate in relation to AOM, we incubated triplicate culture aliquots of E20, G37, and G50 (4 ml) in 5 ml Hungate tubes with ¹⁴C-bicarbonate [380 kBq, equilibrated with 0.2 MPa CH₄:CO₂ or N₂:CO₂ (90:10)] or with ¹⁴C-methane [14 kBq; equilibrated with 0.2 MPa CH₄:CO₂ (90:10)]. After 5 days of incubation cell material was transferred to membrane filters (GSWP, 0.2 μm pore size). To remove non-fixed inorganic carbon we washed the

filters with saline water (0.5 M NaCl). Potential residual inorganic carbon was removed by exposing the dried filters to a HCl atmosphere for 24 h. Total radioactivity was determined from liquid incubation aliquots (0.1 ml) and incorporated radioactivity was determined from the particulate organic carbon fraction (POC) collected on filters by liquid scintillation counting (scintillation mixture; Filtercount or Permafluor; Perkin Elmer, Waltham, MA, USA; scintillation counter; 2900TR LSA; Packard, Waltham, MA, USA). Counts for ¹⁴C-compound in POC were corrected for background values. To determine carbon fixation efficiencies (CFE) values were normalized to the added amount of radiotracer and the rate of sulfate reduction

$$\text{CFE}(\%) = \frac{[^{14}\text{C} - \text{POC}_i(\text{kBq})/^{14}\text{C} - \text{total}_i(\text{kBq}) \times \text{conc}_{\text{CS}}(\text{mmol}) \times 100]}{\text{SRR}(\text{mmol})} \quad (2)$$

where ¹⁴C-POC defines the concentration of radiotracer in the particulate organic carbon (biomass) and ¹⁴C-total defines the concentrations of added radiotracer (¹⁴CH₄ or ¹⁴C-inorganic carbon) in an experiment “*i*,” and conc_{CS} is the concentration of the carbon source (either methane or inorganic carbon) and the respective sulfate reduction rate (SRR) in replicate vials determined as described below.

Radiotracer Measurement of Inorganic Carbon Fluxes in the AOM Enrichments

To track the carbon fluxes between methane and inorganic carbon in AOM enrichments E20, G37, and G50, replicate culture aliquots (4 ml) were incubated in 5 ml Hungate tubes equilibrated with 0.2 MPa CH₄:CO₂ (90:10). After 5 days of pre-incubation the vials were completely filled with methane-saturated medium and carrier-free ¹⁴C-bicarbonate (approximately 66 kBq per sample) or ³⁵S-sulfate (100 kBq per sample) was injected into 5 replicates, respectively according to protocols described by Holler et al. (2011a). Concurrently controls inactivated by formaldehyde addition were performed to estimate impurities or abiotic reactions. Samples were incubated for 2 days and reactions were stopped by transferring samples into sodium hydroxide solution (0.5 N) or zinc-acetate solution (20 % w/v), respectively for ¹⁴C and ³⁵S labeling. The ¹⁴C-bicarbonate and the ³⁵SO₄ samples were processed as described before (Kallmeyer et al., 2004; Holler et al., 2011a). The turnover rates of bicarbonate were inferred by calculating the portion of radiotracer transferred into the methane pool multiplied by the concentration of DIC and divided by the total tracer content in the experiment. Sulfate reduction rates were calculated as described below.

Sequencing of 16S rRNA Gene Libraries of AOM Enrichments

DNA from AOM enrichments was extracted as described before (Zhou et al., 1996). The protocol encompassed three cycles of freezing and thawing, chemical lysis in a high-salt extraction buffer (1.5 M NaCl) by heating of the suspension in the presence of sodium dodecyl sulfate and hexadecyltrimethylammonium bromide, and treatment with proteinase K, followed

by chloroform:isoamylalcohol extraction (24:1) and isopropanol based nucleic acid precipitation. To analyze the phylogeny of the dominant members of the enrichment and to obtain representative full length 16S rRNA gene sequences, the bacterial and archaeal 16S rRNA genes were amplified from the extracted DNA using the primer pair GM3/GM4 (Muyzer et al., 1995) and 20F (Massana et al., 1997)/Arc1492R (Teske et al., 2002), respectively. PCR reaction mixtures were prepared as previously described (Holler et al., 2011b) and subjected to the following cycle conditions: 95°C for 5 min; 26 cycles, each 95°C for 1 min, 46°C (GM3/GM4) or 58°C (Arch20F/Arc1492R) for 1.5 min, and 72°C for 3 min; and a final step at 72°C for 10 min. The amplicons of three replicate PCR reactions were pooled. Following gel electrophoresis bands were extracted from an agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Purified amplicons were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* (One Shot Top10 cells; Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Taq cycle sequencing was performed using ABI BigDye Terminator chemistry and an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA).

Pyrosequencing of 16S rRNA Genes Retrieved from AOM Enrichments

To obtain an overview about the diversity of rare microbial phyla we performed massive parallel tag sequencing of 10 long-term AOM enrichment cultures. In addition to the four enrichments presented in detail (E20, GB37, GB50, and GF) we also investigated six enrichment cultures that were obtained with samples from Amon Mud Volcano, a Black Sea microbial reef, Black Sea sediments, Caldera Mud Volcano, the Gulf of Mexico, and Hydrate Ridge (see Supplementary Table 1). The latter six enrichment cultures were used for comparison, but are not focus of this study. From DNA that was extracted as described above, we amplified 16S rRNA genes using the primer pairs GM3/907RM (Muyzer et al., 1995, 1998) for bacteria and Arch20F/Arch958RV (Massana et al., 1997; Pires et al., 2012) for archaea. The amplicon libraries were prepared, and the V3–V5 region of these amplicons was sequenced on a 454 Genome Sequencer GS FLX+ (Roche, Basel, Switzerland) at the Max Planck Genome Centre (Cologne, Germany). The raw read data was processed based on a standard operating procedure (Schloss et al., 2011) using Mothur (release 1.33, 02/2014; Schloss et al., 2009). Reads were denoised based on PyroNoise (Quince et al., 2009), trimmed, preclustered (Huse et al., 2010), and chimeras were removed (Edgar et al., 2011). After quality filtering we had a total of 122,363 archaeal and 102,762 bacterial reads forming 13,935 unique archaeal and 17,237 unique bacterial sequences with an average length of 488 and 435 nucleotides, respectively. The alignment and taxonomic classification of the sequences was based on the SILVA small subunit reference database (release 119, 07/2014; Quast et al., 2013). Operational taxonomic units were clustered at 98% 16S rRNA gene V3–V5 sequence identity using average neighbor clustering. The datasets were subsampled to

account for unequal sampling effort prior to community analyses and multivariate statistics.

Comparison of 16S rRNA Tags and 16S rRNA Gene Libraries

To investigate whether the same organisms are present in gene libraries as well as tag datasets we compared the results of the two methods. We made a sequence database of the 16S rRNA gene tags using BLAST (Boratyn et al., 2013) and then searched this database with target 16S rRNA gene sequences from the enrichments. The headers of the resulting output were matched with an OTU_{0.02} list created by mothur (Schloss et al., 2011) to find the sequences that were present in both datasets. We only used sequences that matched the whole length of the sequence and had an *E*-value of basically 0.

Phylogenetic Analysis of Retrieved 16S rRNA Gene Sequences

Retrieved partial 16S rRNA gene sequences (from AOM enrichments and alternative substrate enrichments) were aligned with the SILVA Incremental Aligner (SINA; Pruesse et al., 2007) and classified based on the SILVA small subunit database (release 115; Quast et al., 2013). Phylogenetic analysis was performed with representative, nearly full length (>1200 bp) sequences from AOM enrichments, and from methanogenic, sulfate-reducing and sulfur-disproportionating enrichments (see above) using the ARB software package (Ludwig et al., 2004). Maximum likelihood based trees were calculated by RAxML (Stamatakis, 2006) with GTRCAT as nucleotide substitution model including 235 bacterial and 148 archaeal nearly full length sequences (>1200 bp). A base frequency filter was employed to consider only alignment regions which were at least 50% conserved. 100 bootstrap replicates were used to estimate branch support.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences were archived in the NCBI public nucleotide sequence databases under the accession numbers KT899714, KT899739–KT899743 (bacteria) and KT899737, KT899738 and KM605124 (archaea). Pyrosequencing raw reads were deposited in the sequence read archive under study accession number SRP065102.

Catalyzed Reporter Deposition Fluorescence *In situ* Hybridization (CARD-FISH)

For CARD-FISH culture aliquots were fixed in 2% formaldehyde for 2 h at room temperature, washed with 1 × phosphate buffered saline (PBS; 8.2 g l⁻¹ NaCl, 0.2 g l⁻¹ KCl, 1.8 g Na₂HPO₄ × 2H₂O, 0.24 g KH₂PO₄, adjusted to pH 7.4) and stored in 1 × PBS:ethanol (1:1) at –20°C. Fixed cells were treated with mild sonication (Sonoplus HD70, Bandelin, Berlin Germany) for 30 s at 10 W and filtered onto GTTP filter (0.2 μm pore size). CARD-FISH was performed as described previously (Perntaler et al., 2002). For cell wall permeabilization, filters were sequentially incubated in lysozyme solution (10 mg ml⁻¹

lysozyme, lyophilized powder (SigmaAldrich) in 0.1 M Tris-HCl, 0.05 M EDTA, pH 8) for 30 min at 37°C, proteinase K solution (15–150 $\mu\text{g l}^{-1}$ proteinase K (Merck) in 0.1 M Tris-HCl, 0.05 M EDTA, 0.5 M NaCl, pH 8) for 2 min at room temperature and optionally in sodium dodecyl sulfate solution (0.5%) for 10 min at room temperature. Endogenous peroxidases were inactivated by incubating the filters in 0.15% H_2O_2 in methanol (30 min, room temperature). Oligonucleotide probes were synthesized by Biomers (Ulm, Germany) and applied with formamide concentrations in the hybridization buffer according to literature values. For dual CARD-FISH, peroxidases of the first hybridization were inactivated by 0.3% H_2O_2 in methanol (30 min, room temperature). Catalyzed reporter deposition was combined with the fluorochromes Alexa Fluor 488 and Alexa Fluor 594 (Thermo Fisher Scientific). Filters were stained with DAPI (4,6-diamidino-2-phenylindole). Micrographs were obtained by confocal laser scanning microscopy (LSM 780; Zeiss, Oberkochen, Germany).

RESULTS AND DISCUSSION

Cultivation, Microbial Diversity and Archaeal Intact Polar Lipids in the Studied Enrichment Cultures

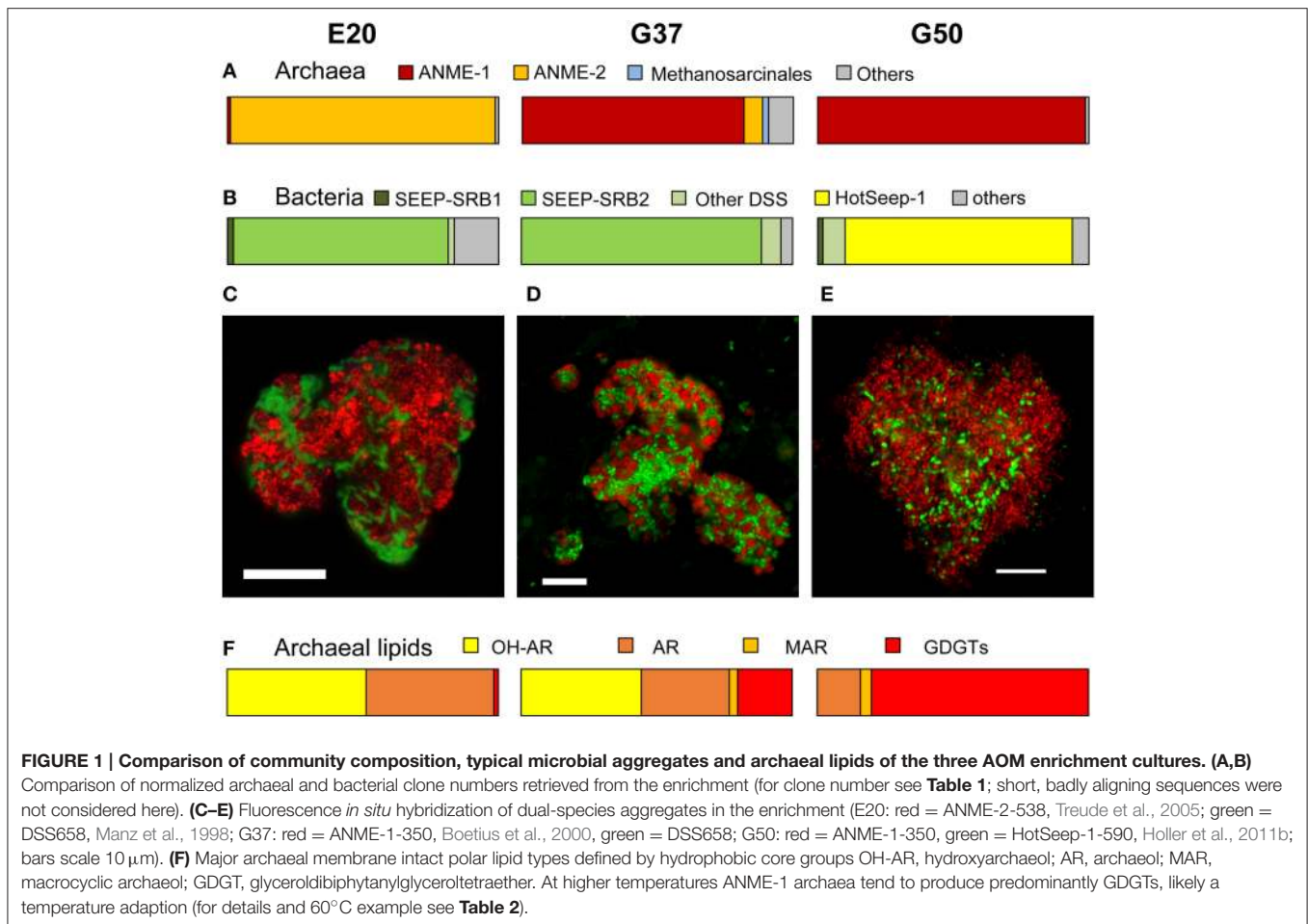
The original sediment samples from Guaymas Basin and the Elba seeps showed already high methane-dependent sulfide production when incubated at AOM conditions (about 0.15 $\mu\text{mol g}_{\text{dw}}^{-1}$; gram dry weight; E20; Ruff et al., this issue to 0.5 and 1.25 $\mu\text{mol g}_{\text{dw}}^{-1}$ in Guaymas Basin; G37 and G50; Holler et al., 2011b). In E20, cells were separated from the sandy matrix (see Materials and Methods). All samples were further enriched for AOM by cultivation in anoxic marine sulfate-reducer medium equilibrated with 0.225 MPa methane and 0.025 MPa carbon dioxide headspace. Cultivation was performed at the respective temperature optima of 20°C (E20) and 37°C and 50°C (G37/G50). From the development of sulfide production rates and dilution frequencies we estimated doubling times of 69 days (G37) and 55 days (G50; Supplementary Figures 1A,B). Due to repeated subsampling for experiments similar required long-term incubations are yet not available for E20, but we expect doubling times in the range of other cold-adapted enrichments (2–7 month; Girguis et al., 2005; Nauhaus et al., 2005). The studied meso- and thermophilic cultures from Guaymas Basin grew faster than the before studied cold-adapted deep sea AOM enrichments (i.e., 7 month in Hydrate Ridge enrichments; Nauhaus et al., 2007). Hence, after repeated dilution and cultivation a sediment-free state (<100 mg background sediment per liter culture) was reached after 1.5–2 years in the Guaymas Basin cultures. Cultures were maintained at sulfide production rates of 100–250 $\mu\text{mol l}_{\text{inoculum}}^{-1} \text{d}^{-1}$. The microbial composition of the three enrichments were analyzed by sequencing archaeal and bacterial 16S rRNA genes (Figures 1A,B, Table 1, Supplementary Figure 2). In E20 the most sequence-abundant archaeal group was ANME-2 (subgroups ANME-2a, ANME-2b, ANME-2c). Using catalyzed

reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) we showed that ANME-2 archaea formed tightly packed consortia with Seep-SRB2 partner bacteria (Figure 1C). G37 consisted of likewise densely packed dual-species consortia of ANME-1 and Seep-SRB2 partner bacteria. (Figure 1D) The dominance of ANME-1 and Seep-SRB2 is typical for moderately heated surface sediments of the Guaymas Basin seeps (Dowell et al., 2016). As shown for 60°C thermophilic AOM enrichments before also the G50 enrichment was dominated by ANME-1 and their partner bacteria HotSeep-1 (Wegener et al., 2015; Figures 1A,B). Compared to the low and medium temperature enrichment cell types were strongly mixed but less densely packed (Figure 1E), which may indicate a less established partnership than in the low-temperature enrichments.

Our results of the E20 enrichment and also prior *in vitro* cultivation at low-temperatures ($\leq 20^\circ\text{C}$; i.e., Hydrate Ridge, Mediterranean seeps such as Amon Mud Volcano, Black Sea; Holler et al., 2009) showed that low-temperature enrichments of mixed communities always led to ANME-2-dominated enrichments (Supplementary Figure 2), whereas ANME-1 is usually not sustained *in vitro*. In contrast, cultivation at elevated temperatures ($\geq 37^\circ\text{C}$) led to ANME-1-dominated enrichments, even from sites that harbored mixed communities (Table 1, c.f.; Holler et al., 2011b; Kellermann et al., 2012). The different temperature optima and growth ranges of ANME-1 and ANME-2 might be due to their cell membrane structure. The ANME-2 in the E20 enrichments assemble their membranes from double layers of diether lipids (intact archaeols) such as hydroxylated (PG)phosphatidylglycerol archaeol (Figure 1F, Table 2). ANME-1 are instead able to condense diethers to tetraether lipids (Kellermann et al., under review). Hence in the G37 enrichment culture an about 1:1 mixture of diether and tetraether lipids (i.e., glyceroldialkylglyceroltetraether GDGTs) was detected, whereas the high-temperature enrichments (G50 and also G60; the latter only shown in Table 2) contained between 80 and 94% tetraether lipids. The formation of GDGT might allow higher temperature optima (Kellermann et al., 2012) or better resistance in starvation periods (Schouten et al., 2003; Rossel et al., 2008). This observation might also explain the predominance of ANME-1 in most deep sulfate-methane interfaces or in inner parts of microbial chimneys where they have to survive under often minimal substrate concentration. The adaption to harsh conditions or limited substrate availability may, on the other hand, also explain their inability to compete with ANME-2 during cultivation at low temperatures and high substrate availability.

Origin of Biomass Carbon in AOM-Performing Microbial Enrichments

To interpret natural biomass stable isotope signals and to perform stable isotope studies the dominant biomass carbon sources of the active organisms need to be identified. For AOM methane and inorganic carbon have been suggested as carbon sources (Hinrichs et al., 1999; Blumenberg et al., 2005; Wegener et al., 2008a; Kellermann et al., 2012). Here we studied inorganic carbon and methane assimilation into AOM communities using a radiotracer assay with respective labeled carbon sources and



tracked the assimilation into the bulk sample. In all three cultures mainly inorganic carbon was assimilated, whereas only 3–15% of the biomass carbon derived from methane (**Figure 2**). In the absence of methane as energy source, assimilation of inorganic carbon dropped to about 1/10 of the values measured under AOM conditions. This shows that the microbial activity and carbon fixation in the studied cultures strongly depended on the presence of methane and the process of AOM, respectively. During the oxidation of 1 mol methane only 10–40 mM of carbon (mostly of inorganic origin) were incorporated. The rates of inorganic carbon assimilation measured here are in the upper range of growth/ carbon fixation reported in earlier studies (Nauhaus et al., 2007; Wegener et al., 2008a). However, in those studies extremely slow-growing AOM enrichments were investigated with doubling times of approximately 7 months, e.g., for enrichments from Hydrate Ridge.

The predominant use of inorganic carbon as carbon source for assimilation is in line with earlier observations stating “chemoorganoautotrophy” for mesophilic ANME-1 (Kellermann et al., 2012). This growth mode seems to be consistent in cold-adapted and thermophilic methane-oxidizing enrichments. The minor amounts of methane carbon incorporation observed here and in earlier studies (Wegener et al., 2008a) should also be interpreted as assimilation of methane-derived inorganic carbon.

The assimilation of methane-derived CO_2 and further isotope fractionation might also explain the extremely low carbon isotope values. Carbon fixation in ANME proceeds most likely via the acetyl-CoA pathway (Koga and Morii, 2005; Meyerdierks et al., 2010), which causes the highest ^{13}C -discrimination (Preuß et al., 1989). It is furthermore consistent with the observation of lowest ^{13}C -lipid values in highly active AOM sites, where pore water inorganic carbon derives mostly from methane, thus is also strongly depleted in ^{13}C . In less active AOM sites rather moderate ^{13}C -signatures of archaeal lipids are observed (Elvert et al., 2005).

Methanogenesis in the AOM Cultures

Using radiotracer isotope assays (i.e., $^{14}\text{CO}_2$) transfer of inorganic carbon into the methane pool has been shown for many different AOM systems. This phenomenon has been repeatedly interpreted as capacity of ANME to thrive as methanogens (Orcutt et al., 2008; Lloyd et al., 2011). However, alternatively this tracer transfer was related to enzymatic back reactions. All three studied cultures showed substantial tracer transfer from DIC into the methane pool amounting to 2–5% of the methane-dependent sulfate reduction rate (**Figure 3**). This tracer transfer is independent of a net formation of methane, as in none of the three cultures methane formation was observed without addition of further methanogenic substrates. Hence, in agreement with

TABLE 1 | Analyzed clones from 16S rRNA gene libraries established from sediment-free methane-oxidizing anaerobic enrichment cultures from Elba and the Guaymas Basin.

Phylogenetic group	Enrichment				
	E20	G37	G50	G60	G50 & 60
ARCHAEA					
Euryarchaeota					
Methanomicrobia					
ANME-1					
ANME-1a		71 (83%)	85 (99%)	70 (88%)	155 (93%)
ANME-1b	1 (1%)				
Methanosarcinales					
ANME-2					
ANME-2a-2b	9 (11%)				
ANME-2b	23 (30%)				
ANME-2c	46 (58%)	6 (7%)			
Others		3		1	1
Thermoplasmata		6	1	6	7
Thermococci				1	1
Thaumarchaeota				1	1
Crenarchaeota				1	1
Total sequences analyzed	79	86	86	80	166
BACTERIA					
Proteobacteria					
Deltaproteobacteria					
HotSeep-1			41 (48%)	48 (74%)	89 (59%)
Seep-SRB1	7 (9%)		1 (1%)		1 (1%)
Seep-SRB2	35 (46%)	60 (88%)			
Others	2	5	4		4
Betaproteobacteria				1	1
Bacteroidetes	6				
Spirochaetes	3				
Chloroflexi	1				
Planctomycetes	5				
Firmicutes	3				
Candidate division OP-3			37	3	40
Candidate division OP-8	1			6	6
Candidate division JS1		2			
Others	13	1	3	7	10
Total sequences analyzed	76	68	86	65	151

Bold numbers represent sequences targeted by probes used for CARD-FISH (Figures 1C–E).

earlier hypothesis (Holler et al., 2011a) the observed tracer flux should be seen as intrinsic back reaction during the oxidation of methane in ANME, which proceeds on the same pathways as methanogenesis (Hallam et al., 2004), but is not an energy conserving net reaction.

We furthermore aimed to induce methanogenesis in the AOM enrichments with typical substrates for methanogens. Therefore, we incubated 1:10 diluted AOM enrichments in sulfate-free medium with different methanogenic substrates and screened those enrichments for methane formation. Hydrogen, acetate and carbon monoxide addition did not cause methane formation in any of the 3 studied enrichments (Table 3), also after extended

incubation times of several months (data not shown). However, in the E20 and the G37 cultures methylated substrates (methanol, methylamine) were largely converted to methane within 18 days of incubation (Figures 4A–D). In contrast, the G50 AOM enrichment culture did not show methanogenic activity even after prolonged incubation of 60 days with these two substrates.

Using the *dilution-to-extinction* approach with methylamine or methanol we yielded cultures of methanogenic archaea from the E20 and G37 enrichments. Sequencing of the 16S rRNA gene amplified from the enrichments identified all methylamine cultures as relatives of *Methanococoides* spp., whereas organisms in methanol cultures were identified as relatives

TABLE 2 | Relative composition of archaeal lipids in the three studied enrichments, and for comparison, composition of lipids in G60.

	ANME-2		ANME-1	
	20°C	37°C	50°C	60°C
DIETHER LIPIDS				
Archaeol-based lipids				
1Gly-AR	9			
2Gly-AR	18	7	9	3
GN-1G-AR	3			
PG-AR	12	25	6	2
Pent-PG-AR	2			
PE-AR	2			
Macrocylic archaeol based lipids				
PE-MAR		3	4	2
Hydroxy-archaeol based lipids				
1Gly-OH-AR	31			
PG-OH-AR	19	1		
PE-OH-AR	1	44		
PI-OH-AR	1			
	98	80	19	7
TETRAETHER LIPIDS				
PG-GDGT-PG				
PG-GDGT		5		
1Gly-GDGT	1		1	5
2Gly-GDGT	1	14	79	88
SUMMARY				
AR	46	32	15	5
MAR		3	4	2
OH-AR	52	45		
Tetraether	2	20	80	94

Values given in percent (%). Headgroups: Gly, glycosyl; GN-1G, (N-acetyl)-glucosamine-monoglycosyl; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PE, phosphatidylethanolamine. Core lipid: AR, archaeol; MAR, macrocylic archaeol; OH-AR, hydroxyarchaeol; GDGT, glyceroldibiphytanylglyceroltetraether.

of *Methanohalophilus* spp. (Figure 4E). As methylotrophs, both methanogenic cultures grow on methanol and methylamine. Generally, methylotrophic methanogens grow rapidly, and are hence relatively easy to cultivate (Sowers and Ferry, 1983; Kendall and Boone, 2006). We also retrieved those groups in archaeal 16S rRNA gene tag datasets of the enrichments (Table 4). Both groups contributed between 1 and 3% of all archaeal sequences retrieved from the E20 and G37 enrichments. Furthermore, we screened the additional low temperature (4–20°C) methanotrophic enrichment cultures (Supplementary Table 2) for methanogens. All those enrichments contain few but also up to 10% sequences that align with *Methanococcoides* or *Methanohalophilus*. In contrast, in the G50 only a single read aligned to *Methanococcoides*. ANME archaea, however, were not enriched in any of the methanogenic enrichments which clearly indicates that ANME cannot thrive as methanogens.

Minor populations of methanogens also regularly appear in sulfate methane interfaces (Wegener et al., 2008b; Ruff et al., 2015), where they likely also thrive on methylated

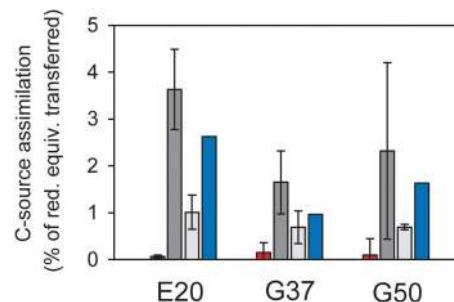


FIGURE 2 | Assimilation of carbon sources in relation to reducing equivalent transfer assuming an average oxidation state of organic carbon of 0. Red = methane carbon assimilation; light gray = DIC assimilation in the absence of methane; dark gray = DIC assimilation in the presence of methane; error bars = standard deviation, $n = 3$ per treatment; blue bars = methane-dependent DIC assimilation as difference between incubations with and without methane, therefore no error bars. In all cultures assimilation of inorganic carbon strongly exceeds methane carbon assimilation, suggesting that the latter is likely methane-derived DIC assimilation.

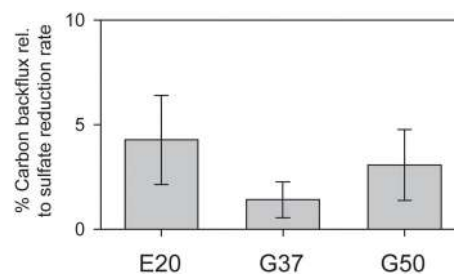


FIGURE 3 | Production of ^{14}C -methane from ^{14}C -bicarbonate relative to AOM rates (here determined by production of ^{35}S -sulfide from ^{35}S -sulfate) in the three studied AOM enrichments incubated under AOM conditions at their respective temperature optima (error bars = standard deviation $n = 5$ per treatment).

substrates. These substrates (i.e., methanol, methylamines, and methyl sulfides) are not competitively used by other anaerobic microorganisms with potential higher energy yields including sulfate reducers (King, 1984; Kiene et al., 1986; Lovley and Klug, 1986). Yet, the source of methylated substrates in those environments and in the studied laboratory enrichments is unclear. Three possible sources are outlined here: (I) Active ANME may leak methylated compounds, as was shown for aerobic methanotrophs (Xin et al., 2004). This might be in particular true for ANME-1 which lack the methylenetetrahydromethanopterin reductase (Mer) enzyme from a strict reversal of methanogenesis. Meyerdierks and coworkers proposed a bypass via the formation of methanol or methylamine as intermediates which would be oxidized via alcohol dehydrogenases (Meyerdierks et al., 2010). Leakage of these intermediates would be a source of methylated compounds. (II) The strong reversibility of the enzymes involved in AOM, particular of the methyl-CoM reductase (Thauer and Shima, 2008; Holler et al., 2011a) may lead to the formation of trace amounts of methylated substrates. These trace production of

TABLE 3 | Stimulation of methanogenesis and sulfate reduction in enrichments from Elba (E20) and Guaymas Basin (G37 and G50) using different substrates (methanogenesis w/o sulfate).

Substrate	E20	G37	G50
Methanogenesis			
AOM control	+	+	+
No-substrate control	0	0	0
Hydrogen	0	0	0
Formate	0	0	0
Acetate	0	0	0
Methanol	+++	+++	0
Methylamine	+++	+++	0
Sulfate reduction			
AOM control	+	+	+
No-substrate control	0	0	0
Hydrogen	0	++	++++
Carbon monoxide	0	0	0
Methyl sulfide	0	0	0
Methanol	0	0	0
Acetate	0	0	0
Formate	0	0	0
Propionate	0	0	0

"+", expected rate measured; "++", instant rates low, but rates exceed AOM after longer time; "+++", instant rate low, but rapidly higher than AOM; "++++", rate instantly 3 times higher than during AOM; "0", no rate detectable.

methylated substrates should not be confused with the proposal of methyl sulfide as intermediates between ANME and partner bacteria (Moran et al., 2008). Trace production of methylated compounds during AOM or (III) alternatively during decay of microbial biomass might be sufficient to sustain the low numbers of methanogens observed in our enrichments and in sulfate methane interfaces. An experimental detection of these compounds is however challenging as they are efficiently consumed by the methanogenic side communities. In G50 methanogenesis could not be stimulated. The considerably higher maintenance energy at elevated temperatures (Tijhuis et al., 1993) might be the reason for the lack of methanogens and stimulation of methanogenesis here. Our results allow an alternative explanation for the observed stimulation of methane and lipid production in Black Sea mats by methylated compounds as demonstrated by Bertram et al. (2013). This production is unlikely caused by ANME archaea, but should be rather interpreted as growth of specific methanogenic side communities.

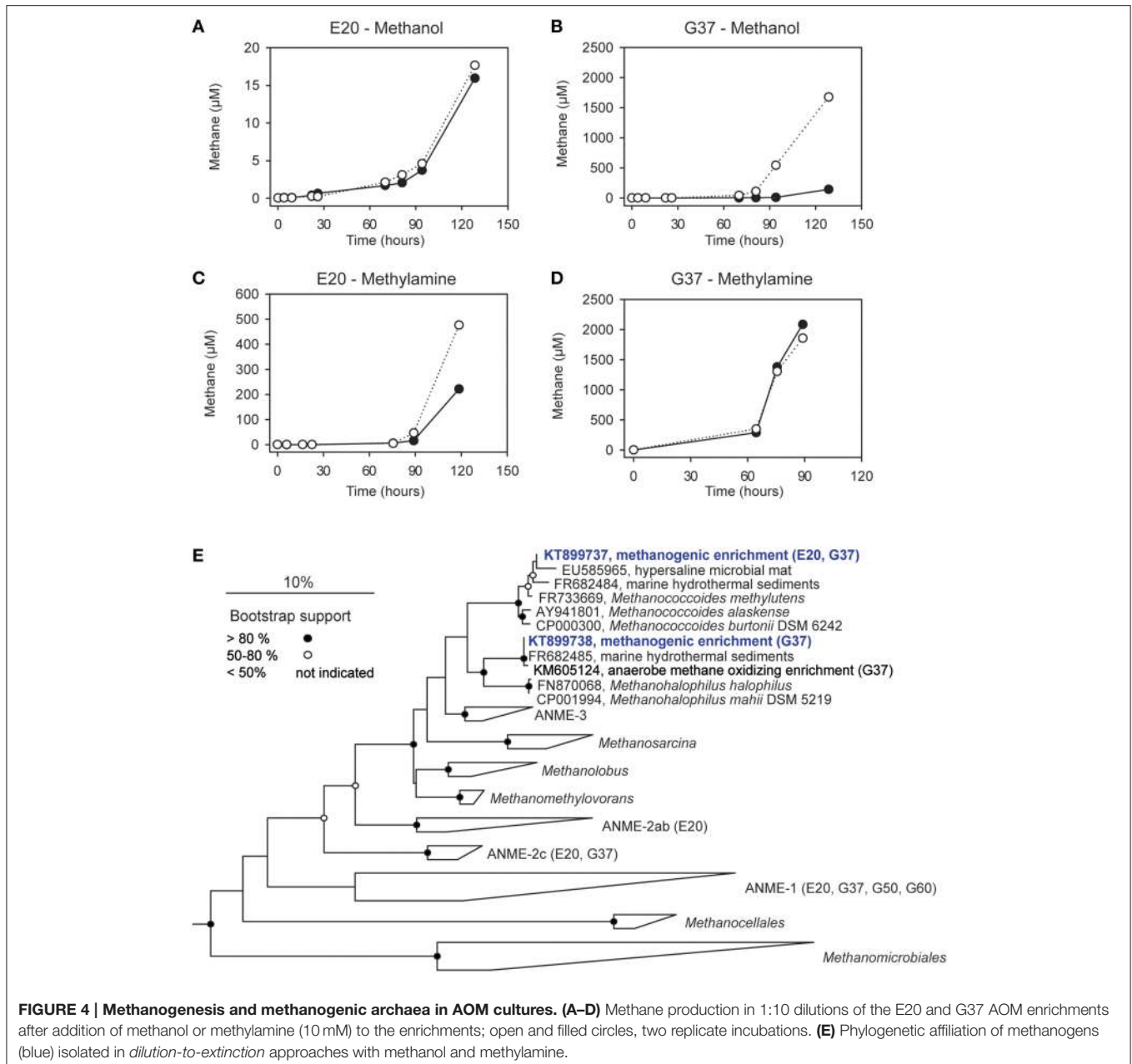
Hydrogenotrophic Sulfate Reduction and Sulfur Disproportionation in the AOM Enrichments

We tested the capabilities of the three enrichments to metabolize sulfate with alternative energy sources. As shown before HotSeep-1, the sulfate-reducing bacterium in thermophilic

AOM, instantly reacts on hydrogen with elevated sulfide production and growth uncoupled from ANME-1 (Wegener et al., 2015). However, besides G50, also the G37 culture showed sulfide production on hydrogen as substrate. Rates quickly exceeded those of parallel incubations on methane. Following this observation we cultivated the sulfate reducers from the sediment-free AOM enrichment using the *dilution-to-extinction* approach. The retrieved cultures were characterized by direct 16S rRNA gene sequencing. The 16S rRNA gene sequence obtained from one of the cultures affiliated to the larger cluster of Seep-SRB2 bacteria, but was clearly not identical (only 93% sequence similarity which is below the proposed threshold of 94.5% for a genus; Yarza et al., 2014) with the Seep-SRB2 partner bacterium found in this mesophilic and in the cold-adapted AOM culture (Figure 5F). In another hydrogenotrophic culture a bacterium related to *Desulfatitalea tepidiphila* was obtained. The mesophilic *D. tepidiphila* was described to grow as autotroph by hydrogen-dependent sulfate reduction or alternatively by using thiosulfate as electron acceptor and various organic carbon sources as electron donor (Higashioka et al., 2013). Hydrogenotrophic sulfate reduction could not be stimulated in the E20 culture, which likewise is dominated by Seep-SRB2 partner bacteria. Hence it is unlikely that the meso- or psychrophilic AOM partner bacteria can thrive on hydrogen, therewith confirming earlier results which excluded hydrogen as intermediate in low-temperature AOM or as (alternative) substrate of their partner bacteria (Nauhaus et al., 2002).

To investigate the response of the AOM cultures to additions of zero-valent sulfur and therewith to test the observation made by Milucka et al. (2012) in an ANME-2 dominated AOM enrichment derived from Mediterranean mud volcano, we supplied aliquots of the three cultures with freshly prepared colloidal sulfur solution and tracked the development of the chemical endmembers of disproportionation, sulfide and sulfate. As described before (Wegener et al., 2015) sulfur disproportionation was absent in the thermophilic AOM culture (Figure 5C). In contrast, E20 and G37 responded to elemental sulfur addition with rapid sulfide and sulfate production tightly following the 3:1 stoichiometry characteristic for the disproportionation of elemental sulfur (Figures 5D,E). Disproportionation stopped when sulfide concentrations reached approximately 3 mM (E20) or 7 mM (G37). A 7:1 stoichiometry between sulfide and sulfate production, as described for another Mediterranean enrichment (Isis Mud Volcano; cultivated at 20°C and dominated by ANME-2; Milucka et al., 2012) has not been observed in any of our enrichments.

Using a *dilution-to-extinction* approach with colloidal sulfur as only available electron donor we repeatedly isolated specific strains of sulfur-disproportionating bacteria from the two natural AOM enrichments. Interestingly in the dilution series from G37 we repeatedly isolated a single bacterium (hereon called GB-DISP1) that is basically identical to the one isolated on hydrogen (Figure 5F). GB-DISP1 is a rare member in the G37 AOM enrichment, accounting for about 9‰ of the bacterial 16S rRNA gene sequences. The under AOM conditions dominant bacterium Seep-SRB2 however, did not respond to additions of elemental sulfur, hence pointing toward a neutral



role of elemental (zero-valent) sulfur in mesophilic AOM proceeding in the G37 enrichment. Growth experiments with the enriched GB-DISP1 showed that it can grow as sulfate-reducing hydrogenotroph (with activity doubling time of 3 days), it can couple sulfur reduction to hydrogen oxidation (activity doubling time 1 day) or it grows as sulfur-disproportionating bacterium (with activity doubling times of about 1 day). Using hydrogen as electron donor GB-DISP1 thrives at sulfide concentrations of up to 20 mM. Instead via sulfur disproportionation GB-DISP1 grows well to sulfide concentrations of up to 5 mM. Above this value sulfide production slows down and sulfide production levels off at around 7 mM. At these sulfide concentrations the energy yield of sulfur disproportionation at 37°C is reduced to approximately

–10 kJ mol⁻¹ elemental sulfur turnover (Finster, 2008), which is about the minimum free energy yield (ΔG_{\min}) to sustain microbial metabolism (Hoehler, 2004).

The E20 *dilution-to-extinction* series with elemental sulfur yielded several replicates of a single bacterium, hereon called Elba-DISP1, with high identity to the uncultivated deltaproteobacterial bacterium MSBL7 (Pachiadaki et al., 2014) and the isolated disproportionating species *Desulfurivibrio alkaliphilus* (Sorokin et al., 2008). *D. alkaliphilus* was described as halophilic chemoautotrophic sulfate reducer, capable to thrive on sulfur disproportionation even without supplying a sulfide sink (Poser et al., 2013). Unlike described for *D. alkaliphilus*, we did not succeed to grow Elba-DISP1 as hydrogenotrophic

TABLE 4 | Methanogenic and sulfur-disproportionating minor community members.

OTU.02*	E20	GB 37	GB 50	GF	Organism**	Sequence accession number
A-Otu00017	2.8	1.3	0.1	4.3	<i>Methanococcoides</i>	KT899737
A-Otu00024	2.9	1.8	–	7.0	<i>Methanohalophilus</i>	KT899738
B-Otu00016	–	–	–	1.0	<i>Desulfocapsa</i>	KT899741
B-Otu00114	8.6	–	–	–	Elba-DISP1	KT899742
B-Otu00373	–	2.2	–	–	GB-DISP1	KT899739; KT899740

*Based on 454 pyrosequencing of the 16S rRNA V3-V5 region; **presented organisms had a taxonomy quality score of 100; numbers report detected sequences as parts of 1000 (‰).

sulfate or elemental sulfur reducer, thus Elba-DISP1 may exclusively thrive as sulfur-disproportionating bacterium. Furthermore, we searched for sulfur disproportionation in the cold seep AOM enrichment culture “GF” retrieved from the Gullfaks oil field (Norwegian North Sea). Indeed also this culture responded to elemental sulfur addition with its disproportionation. The microorganism enriched from the GF culture was *Desulfocapsa sulfoexigens* (>99% 16S rRNA gene identity), one of the first described sulfur-disproportionating microorganism (Finster et al., 1998). In contrast to many other sulfur-disproportionating enrichments we were able to proliferate the enriched sulfur-disproportionating cultures without the addition of iron as sulfide sink as so far only shown for halophiles by Poser et al. (2013). However, due to the limited sulfide tolerance and expected low growth yields of all studied cultures their cell densities remained rather low. We searched for sequences related to disproportionating bacteria in the 16S rRNA gene tag libraries of the other AOM enrichments that proliferated for up to 15 years in the laboratory. The genus *Desulfocapsa* was found in 5 of 10 enrichments, whereas Elba-DISP1 appeared only in the Black Sea enrichment with more than 0.5‰ of the sequences (Supplementary Table 2). Bacteria related to GB-DISP1 were not found in other enrichments than in the GB37 enrichment. Hence we conclude that disproportionating bacteria are a general impurity of AOM enrichment cultures. The abundant partner bacteria, namely Seep-SRB1a, Seep-SRB2 and HotSeep-1, neither respond to elemental sulfur additions nor to any other potential added molecular intermediate (except hydrogen in G50 as discussed above, see Table 3). The absence of stimulation by potential intermediates supports an obligate syntrophic role of Seep-SRB1a, and Seep-SRB2, and an electron transfer in AOM by direct interspecies electron transfer as suggested for thermophilic and psychrophilic AOM (McGlynn et al., 2015; Wegener et al., 2015). Due to the lacking cultivability of these dominant partner bacteria, zero-valent sulfur as primary intermediate exchanged in AOM is less likely.

Sulfur-disproportionating bacteria such as *Desulfocapsa* sp. have also been repeatedly identified in reduced ecosystems and in particular at cold seeps (Lloyd et al., 2006; Sylvan et al., 2012; Ruff et al., 2015). A direct connection of these groups to AOM is meanwhile unlikely in those environments, as they appear in rather low numbers compared to the known partner bacteria (Table 4; Supplementary Table 2). The sulfur source for the disproportionating bacteria in the strictly anaerobic laboratory AOM enrichments is so far unknown. It might be elemental

sulfur produced by ANME archaea, which show characteristic sulfur inclusions (Milucka et al., 2012). Furthermore, also the cultivation medium will provide at least trace amounts of elemental sulfur that is produced when sodium sulfide is used as reducing agent. Furthermore, also any leak of oxygen during cultivation will lead to formation of zero-valent sulfur. Activity directly after medium exchange (medium is prepared with about 0.5 mM sulfide) is likely sufficient for the responsible sulfur-disproportionating bacteria to survive later inactivity at increased sulfide concentrations (regular medium change at approximately 12–15 mM sulfide). These short periods of activity are likely sufficient to thrive in the infrequently diluted AOM enrichments. At higher temperatures increased demands of maintenance energy may not have allowed survival of disproportionating bacteria in the G50 culture. In the environment they might thrive on elemental sulfur produced by sulfide-oxidizing bacteria or chemical oxidation of sulfide or being involved in the cryptic sulfur cycle rather below the sulfate methane interfaces (Holmkvist et al., 2011).

CONCLUSIONS

Here we described physiological characteristics of AOM communities at different temperatures from the Elba cold seeps and the Guaymas Basin hydrothermal vent area. We identified inorganic carbon as the dominant carbon source of AOM communities in all three tested AOM cultures, and hence provide additional evidence that all studied ANME and their partner bacteria are autotrophs. Further stable isotope probing experiments should consider this finding with respect to the selection of labeled carbon sources. We found no indications for a capability of ANME to reverse their metabolism towards net methanogenesis. In contrast, we showed the presence of specific known methanogens (*Methanococcoides* spp., *Methanohalophilus* spp.) in all studied low and medium temperature AOM enrichments. Those methanogens can be enriched and isolated using methylated compounds. In the enrichments but also in methane-rich sediments their substrate might be formed as byproduct of AOM or as decay product of AOM biomass. Furthermore, we were able to enrich sulfur-disproportionating bacteria from different non-thermophilic AOM enrichments, which however, are not identical with the known and abundant AOM partner bacteria (Seep-SRB1, Seep-SRB2, or HotSeep-1), but represent known or novel disproportionating bacteria. In the thermophilic (G50) enrichment, neither sulfur

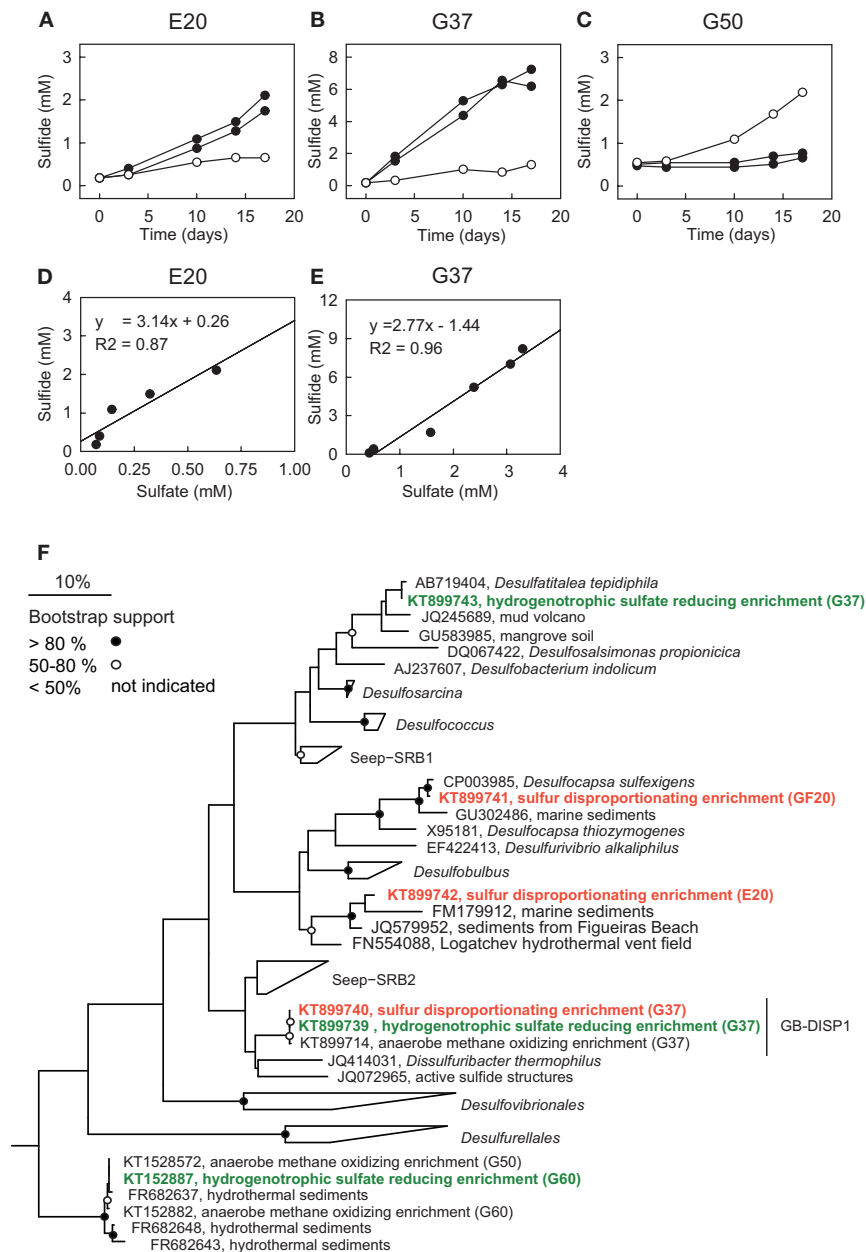


FIGURE 5 | Sulfur disproportionation and sulfate reduction in AOM enrichment cultures. (A–C) Comparison of developments of sulfide concentrations in the three AOM enrichments under AOM conditions [methane (0.2 MPa) plus sulfate (20 mM; open circles)] and during addition of colloidal sulfur (20 mM; filled circles; two replicates) within 18 days. **(D,E)** Comparison of sulfide and sulfate production in zero-valent sulfur amendments of E20 and G37; disproportionation has not been observed in G50. The observed approximate 3:1 stoichiometry between sulfide and sulfate production is characteristic for disproportionation of elemental (zero-valent) sulfur. **(F)** Phylogenetic affiliation of sulfur-disproportionating (red) and sulfate-reducing (green) bacteria within the Deltaproteobacteria based on nearly full-length 16S rRNA sequences retrieved from high dilutions of AOM-active cultures supplied with elemental sulfur.

disproportionation nor disproportionating taxa were observed. Also the prominent AOM partner bacteria in the studied low and intermediate temperature enrichment did not respond to elemental sulfur addition, which makes transfer of zero-valent sulfur in these enrichments highly unlikely. In summary, we narrowed down metabolic capabilities of the AOM core community, the ANME and their syntrophic partner bacteria.

ANME thrive as obligate methane-oxidizing, but autotrophic organisms, which, however, depend on specific partner bacteria that are obligate autotrophic sulfate-reducers. Other metabolic processes observed in AOM cultures and natural enrichments, such as methanogenesis and sulfur disproportionation, are meanwhile likely performed by specialized minor community members.

AUTHOR CONTRIBUTIONS

GW and VK conceived this study, GW and VK performed microbiological experiments, MK did lipid analysis. GW, VK, SR, and KK performed molecular work and analyzed sequence data. GW and VK wrote the manuscript with input of all authors. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00046>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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