ELSEVIER

Marine Geology 217 (2005) 67–96

MARINE GEOLOGY
INTERNATIONAL JOURNAL OF MARINE GEOLOGY, GEOCHEMISTRY AND GEOPHYSICS

www.elsevier.com/locate/margeo

Biomarker and 16S rDNA evidence for anaerobic oxidation of methane and related carbonate precipitation in deep-sea mud volcanoes of the Sorokin Trough, Black Sea

A. Stadnitskaia[a,b,*], G. Muyzer[a,c], B. Abbas[a], M.J.L. Coolen[a], E.C. Hopmans[a], M. Baas[a], T.C.E. van Weering[a], M.K. Ivanov[b], E. Poludetkina[b], J.S. Sinninghe Damsté[a]

[a]*Royal Netherlands Institute for Sea Research (NIOZ), Departments of Marine Biogeochemistry and Toxicology and Marine Chemistry and Geology, P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands*
[b]*UNESCO-MSU Center for Marine Geosciences, Moscow State University, Faculty of Geology, Vorobijevi Gory 1, 119899 Moscow, Russia*
[c]*Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands*

Received 22 June 2004; received in revised form 26 January 2005; accepted 15 February 2005

Abstract

Many mud volcanoes were recently discovered in the euxinic bottom waters of the Sorokin Trough (NE Black Sea). Three of them, i.e., NIOZ, Odessa, and Kazakov, were selected for a detailed biogeochemical investigation. Four methane-related carbonate crusts covered with microbial mats, and sediments ('mud breccia') from these mud volcanoes were collected during the 11th Training-Through-Research cruise (TTR-11) in 2001, the first finding of methanotrophic microbial mats associated with authigenic carbonates in the deep Black Sea. We measured the concentrations and $\delta^{13}C$ values of methane and specific archaeal and bacterial lipids, and determined archaeal and bacterial 16S rRNA gene sequences. The $\delta^{13}C$ of the microbial lipids reflected the carbon isotopic values of the methane, indicating that methane was the main carbon source for microorganisms inducing carbonate formation. Anaerobic oxidation of methane (AOM) in these settings was performed by archaea affiliated with the so-called ANME-1 group. None of the identified archaeal sequences were closely related to known methanogens. The combined 16S rRNA gene sequence and biomarker data revealed a distinct difference in archaeal assemblage between the carbonate crusts and mud breccias. Besides gene sequences of sulfate-reducing bacteria, DNA analysis of bacterial communities revealed a diversity of bacteria with apparent contrasting metabolic properties. The methane utilization via AOM processes was detected in the uppermost sediments where it subsequently induces authigenic carbonate precipitation most probably below seafloor. The results of integrated biomarker and 16S rRNA gene study reveal a crucial role of AOM processes in formation of authigenic carbonates in methane seep environments.

Keywords: Black Sea; mud volcanoes; AOM; carbonates; lipids; 16S rRNA

* Corresponding author. Fax: +31 222 319 674.
E-mail address: alina@nioz.nl (A. Stadnitskaia).

0025-3227/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.margeo.2005.02.023

1. Introduction

Mud volcanoes (MVs) represent locations at which subsurface fluids escape from the subsurface through the seafloor. They develop as a result of a strong lateral or vertical compressions, which allow deep-lying sediments to move upward and over the seafloor. MVs can be expressed as mounds, extending up to 100 m above the seabed with diameters of a few kilometers or as negative collapse structures, caused by catastrophic eruption of fluids, especially hydrocarbon gases (predominantly methane), hydrogen sulfide, carbon dioxide, and petroleum products (Ivanov et al., 1998). Generally, fluids (composed of gases, pore water, and sediment) still migrate upwards after the initial eruption. This results in the development of highly diverse and productive ecosystems based on chemosynthesis below and/or at the seafloor. One of the most important biogeochemical processes fuelling these communities at these locations is the anaerobic oxidation of methane (AOM).

Comprehensive biogeochemical, structural, and stable isotope analysis of lipids (Schouten et al., 1998; Thiel et al., 1999, 2001; Hinrichs et al., 1999, 2000a,b; Pancost et al., 2000, 2001a,b; Zhang et al., 2002, 2003) and molecular ecological studies (Boetius et al., 2000; Orphan et al., 2001a,b; Teske et al., 2002; Michaelis et al., 2002) revealed that AOM is generally performed by syntrophic consortia of methanogenic archaea operating in reverse and the sulfate-reducing bacteria (SRB) (Hoehler et al., 1994; Hoehler and Alperin, 1996; Valentine and Reeburgh, 2000). It is hypothesized that the use of hydrogen as an electron donor by the SRB results in low partial pressure of H_2, creating thermodynamically favorable conditions for methanogenic archaea to act as methane-oxidizers (Reeburgh, 1976; Zender and Brock, 1979; Alperin and Reeburgh, 1985; Hoehler et al., 1994; Hoehler and Alperin, 1996). Phylogenetic analyses of ribosomal RNA (rRNA) gene sequences have revealed two distinct lineages among the *Euryarchaeota* capable of anaerobic methanotrophy: the ANME-1 cluster, which does not contain any cultured relatives (Hinrichs et al., 1999), and the ANME-2 cluster affiliated to the cultured members of the *Methanosarcinales* (Boetius et al., 2000; Orphan et al., 2001a,b). The indication of anaerobic methanotrophy was first demonstrated by compound-specific carbon isotopic study of archaeal lipids: their distinctively depleted $\delta^{13}C$ signals indicate that archaea are able to use methane as a carbon source (Hinrichs et al., 1999, 2000b; Elvert et al., 2000; Pancost et al., 2000, 2001a,b; Thiel et al., 1999, 2001; Aloisi et al., 2002; Teske et al., 2002; Zhang et al., 2002, 2003; Schouten et al., 2003). ^{13}C-depleted lipids of SRB also indicate their involvement in the AOM process. Collectively, these results indicate a close metabolic association between SRB and methanotrophic archaea (Pancost et al., 2000, 2001b). Conclusive evidence for the co-existence of archaea and SRB, i.e. AOM consortia, was obtained by fluorescence in situ hybridization (FISH) (Boetius et al., 2000; Michaelis et al., 2002) and secondary ion mass spectrometry (SIMS) analyses of individual cell aggregates (Orphan et al., 2001a). Various studies have also revealed a large variety in distribution and composition of archaea/SRB microbial biomass in gas venting settings (Boetius et al., 2000; Orphan et al., 2001a; Pancost et al., 2001a; Michaelis et al., 2002). For example, sulfate-reducing bacteria affiliated to members of the genera *Desulfosarcina* and *Desulfococcus* have been found in association with ANME-1 and ANME-2 archaeal cells, representing in both cases putative methanotrophic consortia (Boetius et al., 2000; Orphan et al., 2001a,b; Michaelis et al., 2002). An independent *Methanosarcinales*-related ANME-2 lineage composed of bacteria-free archaeal cells has been also reported (Orphan et al., 2001a).

In spite of the diversity of microbes involved in AOM, the associated or AOM-induced "end-products" are similar in most cases. For example, the co-occurrence of neoformed diagenetic carbonates is a widespread phenomenon in cold seep settings (Ritger et al., 1987; Roberts and Aharon, 1994; Von Rad et al., 1996; Peckmann et al., 1999a,b, 2001; Aloisi et al., 2000, 2002; Michaelis et al., 2002). These authigenic carbonates exhibit a broad range of morphologies, mineralogy, and stable carbon and oxygen isotopic compositions (Hovland et al., 1987; Roberts and Aharon, 1994; Peckmann et al., 1999a,b, 2001, 2002; Stakes et al., 1999; Aloisi et al., 2000, 2002). Chemical analyses showed that these carbonates are depleted in ^{13}C which led to the suggestion that carbon in such carbonates is derived from methane (Peckmann et al., 1999a,b, 2001, 2002; Stakes et al., 1999; Aloisi et al., 2000, 2002). It was proposed that the precipitation of authigenic methane-

derived bicarbonate is the result of increased alkalinity created by AOM (Ritger et al., 1987; Von Rad et al., 1996; Thiel et al., 1999), i.e. AOM serves as a source of inorganic carbon for the formation of carbonate cement, concretions, crusts, and other carbonate build-ups, which are known in the geological record from the Middle Devonian (Peckmann et al., 1999b). However, only the recent finding of living methanotrophic mats associated with carbonate build-ups in the Black Sea (Michaelis et al., 2002) directly proved that anaerobic microbial consortia performing AOM indeed induce carbonate precipitation in methane seepage environments.

Here we report the results of a lipid biomarker study integrated with a survey of 16S rRNA gene sequences to characterize microbial communities in four methane-related carbonate crusts covered by microbial mats and mud volcanic deposits (mud breccia matrix) in an environment where oxygen cannot play a role in the oxidation of methane. Molecular signatures, such as lipids and nucleic acid sequences, have been used in this study as tools to identify specific microorganisms involved in AOM, to reveal microbial ecological relationships and their metabolic potential in the context of the specific environments created by migrated fluids. Samples were collected from three MVs at the anoxic seafloor of the Sorokin Trough (NE Black Sea) during the 11th Training-Through-Research expedition (TTR-11) in 2001 (Kenyon et al., 2002). This is the first finding of methanotrophic microbial mats associated with carbonates in the deep (ca. 2000 m) Black Sea.

2. Materials and methods

2.1. Samples

On the basis of comprehensive geophysical surveys, carried out during the TTR-6 (1996), and the TTR-11 (2001) cruises, a considerable number of MVs were discovered in the Sorokin Trough (Ivanov et al., 1996, 1998; Woodside et al., 1997; Bouriak and Akhmetzhanov, 1998; Kenyon et al., 2002). Three of them, i.e., NIOZ (44°19′ N; 35°04′ E, water depth of ca. 2020 m), Odessa (44°23′ N; 35°09′ E, water depth of ca. 1830 m) and Kazakov (44°18′ N; 35°11′ E, water depth of ca. 1920 m), were chosen for a detailed geochemical investigation (Fig. 1). These MVs are characterized by a set of characteristic features such as mound morphology (Woodside et al., 1997; Ivanov et al., 1998), composition and sources of hydrocarbon gases (Ivanov et al., 1998), and the occurrence of cold seep-associated features, such as gas hydrates, authigenic carbonates of various types and morphology, and the presence of microbial mats (Ivanov et al., 1998; Bouriak and Akhmetzhanov, 1998; Kenyon et al., 2002).

Bottom sampling was performed according to standard TTR-procedures (Ivanov et al., 1992). All samples were taken using a 6 m long, 1500 kg gravity corer with an internal diameter of 14.7 cm. Sediments, carbonate crusts, and microbial mats were described, photographed and stored at −20 °C until further geochemical and molecular biological analyses.

A carbonate crust, CC-1, was collected from sampling site TTR-11 BS-336G located on the crater of the Odessa MV. The crust appeared at the boundary of the sapropel layer (Unit 2) (Degens and Ross, 1972) and the mud breccia interval (12–13 cm). It is a porous well-cemented precipitate with its lower surface covered by a pink microbial mat (Fig. 2a). The same type of biofilm also filled the cavities and pores within the crust.

Three carbonate crusts were collected from the crater of the NIOZ MV. Two flat, well-cemented carbonate crusts, CC-2 and CC-3, were found at sampling site TTR-11 BS-328G located on the northern edge of the crater. No sediment was recovered from the site since the core catcher was blocked by the carbonates. These crusts are parts of prolonged carbonate pavements forming a positive seafloor relief. Both of them contained brown, semi-transparent gel-like microbial mats that filled the pores and interstices on the surfaces and within the crusts (Fig. 2b). Carbonate crust CC-4 from core TTR-11 BS-325G formed a thin, well-cemented layer, which was confined to the mud breccia at a sub-seabed interval depth of 12–14 cm (Fig. 1). Occasional microbial mats similar to these found on the CC-2 and CC-3 crusts were clearly visible within the inner pores of the precipitate. This sampling site was located ca. 500 m to the south from the TTR-11 BS-328G, in the central part of the crater of the NIOZ MV.

All studied carbonate crusts were associated with microbial mats. Therefore, the reader has to be aware

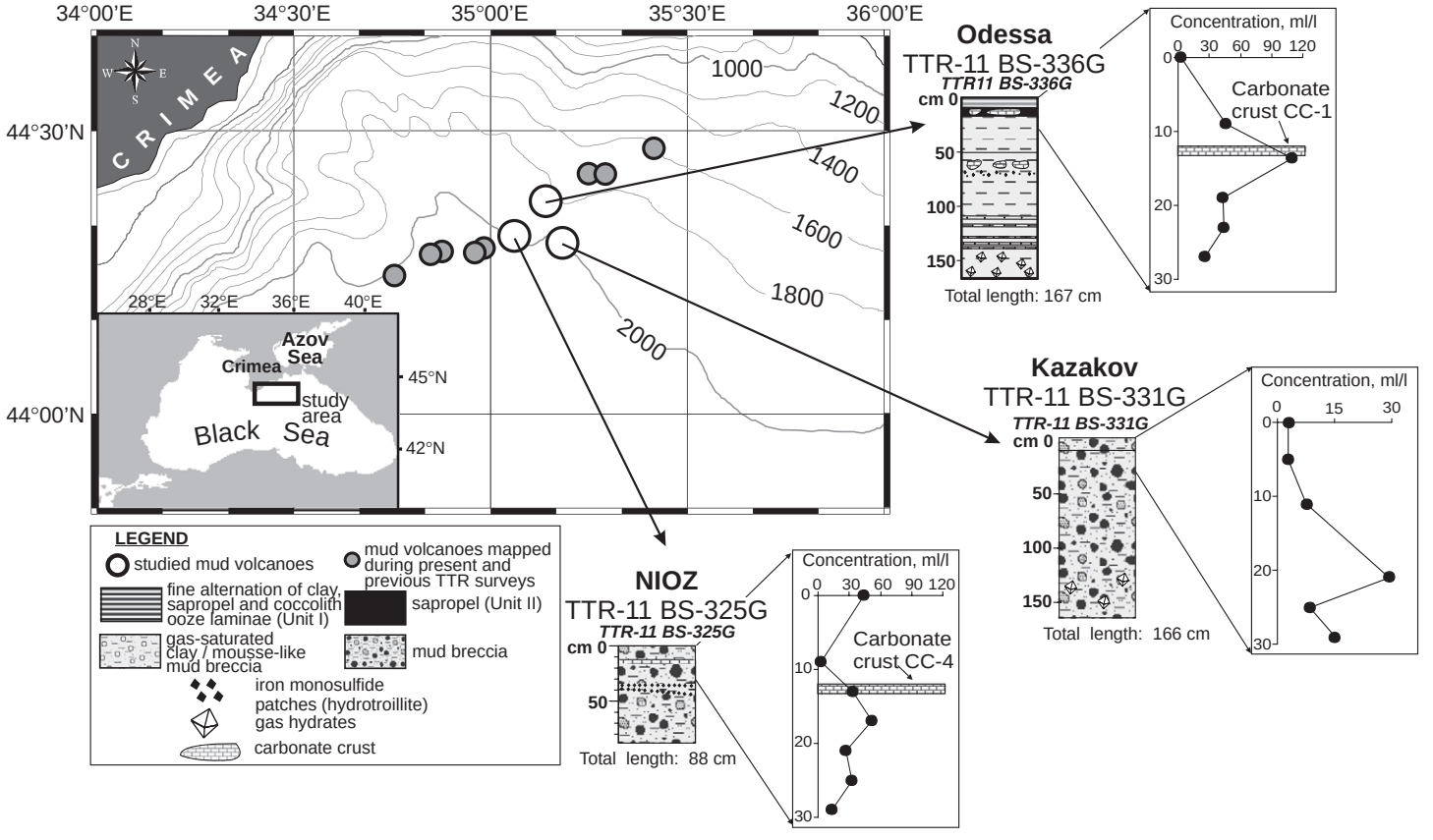


Fig. 1. Location of studied mud volcanoes in the Sorokin Trough of the Black Sea and the sediment cores obtained with schematic lithology. The insets show methane concentration profiles measured in the uppermost 30 cm for the studied cores.

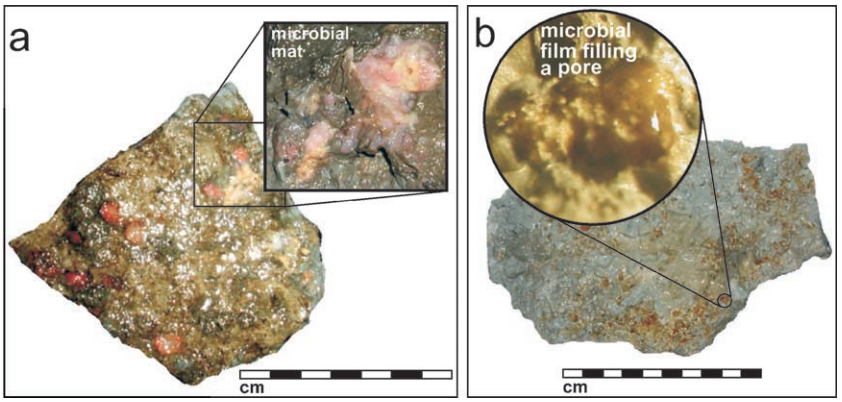


Fig. 2. Two main types of investigated carbonate crusts and microbial mat. a) The Odessa mud volcano, sampling site TTR-11 BS-336G. The inset assigns the location of the microbial mat. b) The NIOZ mud volcano, sampling site TTR-11 BS-328G. The inset is a binocular image with typical appearance of microbial films within pores and interstices.

that their molecular signatures principally indicate living microbial communities within these methane-related carbonate crusts. For reasons of conciseness, we decided to name our carbonate/mat samples as CC-1, CC-2, CC-3, CC-4, using predominantly in the text the word "carbonate" rather then "a microbial mat associated with a carbonate" or "carbonate with microbial mat".

Sediments from the Kazakov MV (TTR-11 BS-331G) were collected from the eastern edge of the crater. They are represented by gas saturated mud breccia containing variety of rock clasts of different lithology and roundness. Drop-sized gas hydrates were found at the base of the core.

2.2. Gas measurements

Hydrocarbon gases were sampled using head-space methods, adapted for shipboard conditions (Bolshakov and Egorov, 1987). The gas phase was transferred into sterile glass jars filled with saturated NaCl solution and stored at −5 °C. A gas chromatograph with a flame-ionization detector was used for quantification of methane.

The results of methane concentration were calculated according to the volume of wet sediment from which gases were extracted. It should be noted that in spite of the absolute notations for the methane, significant part of the hydrocarbon had been lost due to active degassing of the recovered sediments. However, in order to see the trend of methane distribution profiles and to show rough level of methane saturation even with its relatively high loss, it was decided to present methane data in ml/l of wet sediments.

The stable carbon isotopic composition of methane was measured on a Finnigan Delta S mass spectrometer with a HP 5890 GC and a GC-combustion interface. Methane was separated on a molsieve 5 Å plot column using split- or splitless injection, depending on the concentrations. Results are reported using the delta (δ) notation in per mil (‰), with respect to the Vienna Pee Dee Belemnite (PDB) standard.

2.3. Lipid extraction and separation

About 100–120 g of each carbonate crust and ca. 50 g of each mud breccia sample were freeze-dried, crushed to a fine powder, and extracted with an automatic Accelerated Solvent Extractor (ASE 200/DIONEX) using a solvent mixture dichloromethane (DCM) : methanol (MeOH) (9 : 1, v/v) at 1000 psi and 100 °C PT conditions. Elemental sulfur was removed from the total extract by elution over a small pipette filled with activated copper. An aliquot of the total extract was chromatographically separated into apolar and polar fractions using a column with activated (2 h at 150 °C) Al_2O_3 as stationary phase. Apolar compounds were eluted using hexane : DCM (9 : 1, v/v), and polar compounds, including glycerol ether core membrane lipids, were obtained with MeOH : DCM (1 : 1, v/v) as eluent. Alcohols were transformed into trimethylsilyl-

derivatives by addition of 25 μl of pyridine and 25 μl of BSTFA and heating at 60 °C for 20 min.

In order to remove saturated normal hydrocarbons and to enrich branched/cyclic compounds, the apolar fraction was filtered over silicalite using cyclohexane as an eluent.

To check for the presence of bacteriohopanepolyol derivatives, fresh extracts of mud breccia from the NIOZ MV were treated with periodic acid and sodium borohydrite according to Rohmer et al. (1984).

2.4. Analysis and identification of biomarkers

Gas chromatography (GC) was performed using a Hewlett Packard 6890 gas chromatograph equipped with an on-column injector and a flame ionization detector. A fused silica capillary column (CP Sil5 25 m × 0.32 mm, d_f=0.12 μm) with helium as a carrier gas was used. The samples were injected at 70 °C. The GC oven temperature was subsequently raised to 130 °C at a rate of 20 °C/min, and to 320 °C at 4 °C/min. The temperature was then held constant for 15 min.

All fractions were analyzed by gas chromatography–mass spectrometry (GC–MS) for compound identification. The structural designation of lipids was evaluated by the comparison of their mass spectral fragmentation patterns and pseudo Kovats retention indices with reported data. GC–MS was conducted using a Hewlett Packard 5890 gas chromatograph interfaced to a VG Autospec Ultima Q mass spectrometer operated at 70 eV with a mass range of *m/z* 50–800 and a cycle time of 1.8 s (resolution 1000). The gas chromatograph was equipped with a fused silica capillary column (CPsil 5 25 m × 0.32 mm, d_f=0.12 μm) and helium as a carrier gas. The temperature program used for GC–MS was the same as for GC.

2.5. Isotope-ratio-monitoring gas chromatography–mass spectrometry (IRM–GC–MS)

IRM–GC–MS was performed on a Finnigan MAT DELTA ^{plus} XL instrument used for determining compound-specific $\delta^{13}C$ values. The GC used was a Hewlett Packard 6890 A series and the same analytical conditions were used as described for GC and GC–MS.

With the purpose to achieve better separation and more accurate $\delta^{13}C$ values for the cluster of pentamethylicosenes (PMEs), the silicalite non-adducted hydrocarbon fractions isolated from carbonates CC-1 and CC-4 were also analyzed using DB-1MS 60 m × 0.25 mm with d_f=0.25 μm capillary column. Samples were injected at 70 °C followed by increasing the temperature to 140 °C at a rate of 25 °C/min, and to 320 °C at a rate of 3 °C/min. The temperature was then held constant for 20 min.

For carbon isotopic correction of the added trimethylsilyl groups, the carbon isotopic composition of the used BSTFA was determined (−49.30 ± 0.5‰). Obtained values are reported in per mil relative to the VPDB standard, and have been corrected for the addition of $Si(CH_3)_3$ group due to the derivatisation procedure. In order to monitor the accuracy of the measurements, the analyses were carried out with co-injection of two standards, C_{20} and C_{24} *n*-alkanes, which have known carbon isotopic composition.

2.6. High performance liquid chromatography–mass spectrometry (HPLC–MS)

To determine the distribution and composition of intact glycerol dialkyl glycerol tetraethers (GDGTs), carbonate crust and mud breccia samples were analyzed using a HPLC–MS method for their direct analysis (Hopmans et al., 2000).

2.7. DNA extraction

Table 1 summarizes general details on samples analyzed using molecular biological techniques. Samples of carbonate crusts and sediments were directly stored at −20 °C after collection, and at −80 °C after return to home institute. Genomic DNA was extracted from about 1 g of sample using the UltraClean Soil DNA Isolation Kit (MoBio, Carlsbad, USA). The quality and quantity of the extracted DNA was checked by standard agarose gel electrophoresis.

2.8. PCR amplification of 16S rRNA genes

Amplification of archaeal 16S rRNA gene fragments that were suited for denaturing gradient gel electrophoresis (DGGE) was conducted by using a new combination of published primers as recently described by Coolen et al. (2004). Parch519f (*E.coli* positions 519–533; 5′-CAG CCG CCG CGG TAA-3′), which is complementary to reverse sequence of

Table 1
General information of samples studied

Sample ID	Mud volcano	Sampling site	Latitude	Longitude	Water depth (m)	Interval (cm, b.s.f)
Carbonate crusts with microbial mats						
CC-1	Odessa	TTR-11 BS-336 G	44°23′	35°09′	1816	12–13
CC-2	NIOZ	TTR-11 BS-328 G	44°19′	35°04′	2020	Seafloor surface
CC-3						
CC-4		TTR-11 BS-325 G	44°19′	35°04′	2015	12–14
Mud breccia						
MB1A	Kazakov	TTR-11 BS-331 G	44°18′	35°11′	1918	2–4
MB1B						8–10
MB1C						20–22
MB2A	NIOZ	TTR-11 BS-325 G	44°19′	35°04′	2015	8–12 (above CC-4)
MB2B						16–20 (below CC-4)

primer Parch519r published by Øvreås et al. (1997) and ARC915r (*E.coli* positions 915–934; Stahl and Amann, 1991). The stability of the archaeal 16S rRNA gene fragments in the DGGE was obtained by attaching a 40-bp long GC-clamp (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3′ Schäfer and Muyzer, 2001) to the 5′-end of the ARC915r primer (Coolen et al., 2004). PCR conditions included an initial denaturation step of 4 min at 96 °C, followed by 35 cycles including a denaturation step for 30 s at 94 °C, a primer annealing step for 40 s at 57 °C, and a primer extension step for 40 s at 72 °C. A final extension was performed for 10 min at 72 °C. Partial bacterial 16S rRNA genes were amplified using primers 341f [*E. coli* positions 341–357; 5′-CCT ACG GGA GGC AGC AG-3′ (Muyzer et al., 1993) including the 40-bp GC-clamp and 907r [*E.coli* positions 907-926; 5′-CCG TCA ATT CCT TTR AGT TT-3′ (Lane, 1991)]. PCR conditions were comparable to those described for the amplification of archaeal 16S rRNA genes, except that 32 cycles were applied. The fragment lengths of the archaeal and bacterial PCR products including the 40-bp long GC-clamp were 436 and 606 bp, respectively.

All PCR amplifications were performed with a Geneamp PCR System 2400 (Perkin-Elmer) using a mixture of the following components: 5 μl of 10X PCR-buffer (100 mM Tris–HCl [pH 9.0], 15 mM $MgCl_2$, 500 mM KCl [Pharmacia Biotechnology, Upsalla, Sweden]), 10 mM of dNTP's, 0.5 μM of each archaeal (Parch-519f, ARC-915r+GC-clamp) and bacterial (341f+GC-clamp, 907r) primers, 20 μg of bovine serum albumine, and 1 unit of Taq DNA polymerase (Pharmacia). Between 2 and 5 ng of template DNA from each carbonate crust, and up to 20 ng from each sediment sample was subjected to PCR. The final volume of the mixture was adjusted to 50 μl with molecular-grade water (Sigma, Saint Louis, MO, USA).

2.9. DGGE analysis of 16S rRNA genes

All PCR-products were analyzed by DGGE (Schäfer and Muyzer, 2001), carried out in a Bio-Rad D Gene system (Biorad, München, Germany). PCR samples were applied directly onto 6% (wt/vol) polyacrylamide gels (acrylamide/*N*,*N*′-methylene bisacrylamide ratio, 37:1 [w/w]) in 1 × TAE buffer (pH 8.3), which had been prepared from sterile solutions and casted between sterilised glass plates. The gels contained a linear gradient of denaturant from 20–70% (100% denaturant is 7 M urea plus 40% [v/v] formamide). Electrophoresis proceeded for 5 h at 200 V and 60 °C (archaeal 16S rDNA) and 15 h at 100 V and 60 °C (bacterial 16S rDNA). Afterwards, gels were stained for 30 min in sterile double-distilled water containing ethidium bromide, destained for 60 min in sterile double-distilled water, and photographed. DGGE fragments were excised from the gel with a sterile scalpel and rinsed with molecular-grade water (Sigma, Saint Louis, MO, USA). The DNA of each fragment was eluted in sterile 10 mM Tris–HCl (pH 8.0) by incubation for 24 h at 2 °C and served as template DNA for re-amplification. PCR conditions for re-amplification of PCR bands included an initial denaturation step of 4 min at 96 °C, followed

by 30 cycles for 40 s at 57 °C, and a primer extension step for 40 s at 72 °C. A final extension was performed for 10 min at 72 °C. Primers without GC-clams were used.

2.10. Sequencing of DGGE bands

Prior to the sequencing reactions, primers were enzymatically removed from the reamplified DGGE bands using the ExoSAP-IT™ kit (Amersham Bioscience, Roosendaal, the Netherlands) following the descriptions of the manufacturer. Cycle sequencing reactions were performed with the ABI Prism Big Dye Terminator V3.0 kit (Applied Biosystems, CA, USA) using the forward or reverse primer (without GC clamp) at a final concentration of 0.2 μM, and 10 ng of template DNA. The reaction volume was adjusted to a volume of 20 μl with molecular grade water (Sigma). The following reaction conditions were employed: 1 s initial denaturing at 96 °C, followed by 25 cycles of 10 s at 96 °C, 5 s at 45 °C, and 4 min at 60 °C. Nucleotide positions were determined using an automated ABI-310 capillary sequencer (Applied Biosystems). Complementary sequences were aligned and manually edited using the AutoAssembler software package (Version 2.1.1; Applied Biosystems).

2.11. Comparative analysis of 16S rRNA gene sequences

The partial sequences were analyzed using BLAST in the NCBI database (http://ncbi.nlm.nih.gov/BLAST) and added together with the most important BLAST hits, to an alignment of about 1400 homologous bacterial 16S rRNA gene sequences by using the aligning tool of the ARB software package. Trees were generated by neighbor-joining, with the correction method of Felsenstein as implemented in ARB. Bootstrap analysis (1000 replicates) was performed in PAUP version 4. Names of the sequences consisted of the prefix DGGE, indicating that the sequences were obtained from excised DGGE bands with ARC for Archaea, BAC for Bacteria, and the number of the excised band. The numbers used in the DGGE are the same as those used in the trees. The accession numbers for the archaeal and bacterial sequences are AY847616–AY847624 and AY847598–AY847615, respectively.

3. Results and discussion

3.1. Methane

Colds seeps, especially actively degassing MVs, are focal sources of fluids containing methane. The occurrence of gas and its migration through the sediments have been recognized in seismic recordings of the Sorokin Trough in many ways (Ivanov et al., 1998; Bouriak and Akhmetzhanov, 1998). Gas measurements revealed that the main gas components in the area are hydrocarbons, nitrogen, and carbon dioxide, with methane being the most abundant (Ivanov et al., 1998). Hydrocarbon gases from the NIOZ and Odessa MVs are mainly composed of methane (99.1–99.9% of total hydrocarbon gases). The average carbon isotopic composition of methane is ca. −63‰ at the NIOZ MV and −68‰ at the Odessa MV, indicating the biogenic origin of methane (Rice and Claypool, 1981) at these sites. The abundance of methane in the sediments of the Kazakov MV is ca. 95% of the total hydrocarbon gases and the average $\delta^{13}C_{CH4}$ value is −56‰. These results indicate a thermogenic contribution to the methane at the Kazakov MV.

The methane distribution in the Kazakov MV displayed the characteristic concave-up-curve at a depth interval of ca. 10–13 cm below seafloor (bsf) (Fig. 1), which is consistent with anaerobic methane consumption (Martens and Berner, 1974; Barnes and Goldberg, 1976; Reeburgh, 1976; Alperin and Reeburgh, 1984; Valentine and Reeburgh, 2000). In contrast, methane profiles in the NIOZ and Odessa MVs showed an irregular pattern. Maximum methane concentrations occurred in both settings just below the methane-related authigenic carbonate crust layers (Fig. 1). These carbonate crusts were characterized by depleted $\delta^{13}C$ values (~−40‰), indicating their origin, at least in part, from carbon dioxide produced by AOM (Kovalenko and Belenkaia, 2002; Mazzini et al., 2002).

3.2. Archaeal and bacterial lipid variability

Lipid analysis of the carbonate crusts associated with microbial mats (C1, C2, C3, and C4) and the mud breccias matrix revealed a quite diverse set of biomarkers diagnostic for different archaea and bac-

teria. A remarkable difference in the molecular composition and distribution of biomarkers was observed, especially for the carbonate crusts CC-2, CC-3 and CC-4, which all originate from the same venting structure (i.e. the NIOZ MV). Lipid extracts of the mud breccia from the Kazakov (TTR-11 BS-331G) and from the NIOZ (TTR-11 BS-325G) MVs represented a complex mixture of components indigenous to the erupted mud breccia and archaeal and SRB lipids formed in situ. In general, the concentrations of archaeal and bacterial biomarkers were substantially lower in the mud breccia than observed in the carbonate crusts.

3.2.1. Irregular acyclic isoprenoids

The acyclic isoprenoids in all crusts are dominated by the irregular, tail-to-tail linked C_{25} isoprenoid, 2, 6, 10, 15, 19-pentamethylicosane (PMI; I, see Appendix for structures) and its unsaturated counterparts (Figs. 3 and 4). PMI is considered to be a specific biomarker for methanogenic Archaea and has been detected in cultures of methanogens, such as *Methanobacterium*

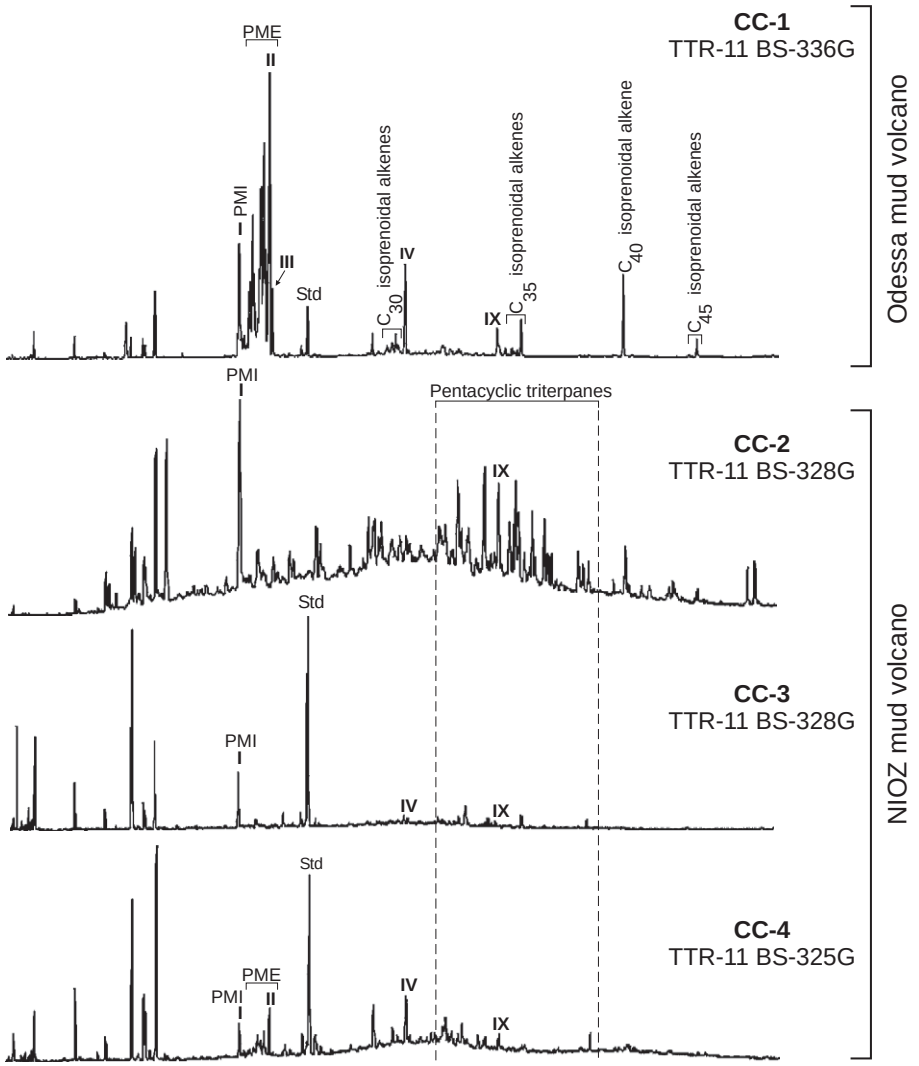


Fig. 3. Total ion current traces of the silicalite non-adduct (branched and cyclic hydrocarbons of the apolar fraction) of the four carbonate crusts. Roman numbers refer to structures shown in the Appendix.

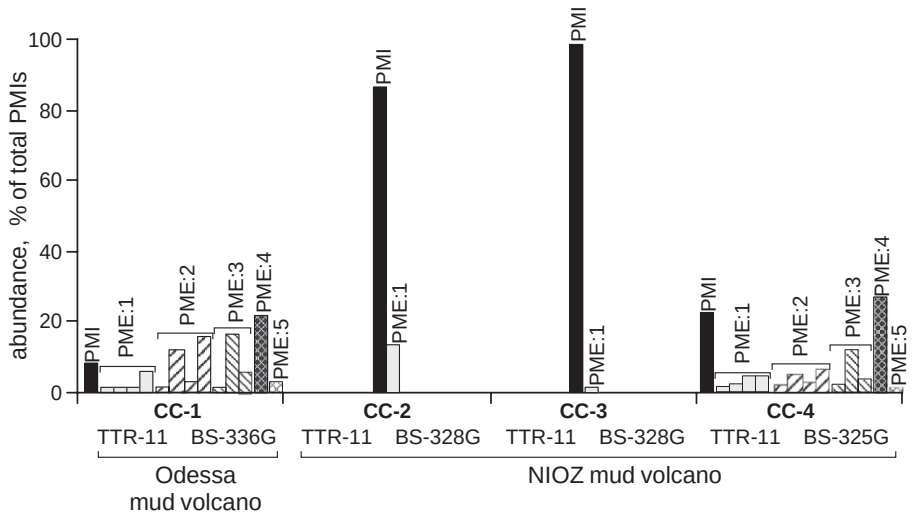


Fig. 4. The distribution of PMEs in the four carbonate crusts.

thermoautotrophicum and *Methanosarcina barkeri* (Holzer et al., 1979), *Methanosarcina mazei*, and *Methanolobus bombayensis* (Schouten et al., 1997). PMI has also been frequently encountered in marine settings with a high rate of methanogenesis or AOM (Wakeham, 1990; Kohnen et al., 1992; Pancost et al., 2000; Elvert et al., 2000; Thiel et al., 2001). Mono-, di- and polyunsaturated pentamethylicosenes (PMEs) possessing up to five double bonds are also abundant biomarkers in some crusts (Figs. 3 and 4). PMEs have been identified in the methanogenic archaea *M. mazei*, *M. barkeri* and *M. bombayensis* (Holzer et al., 1979; Tornabene et al., 1979; Schouten et al., 1997; Sinninghe Damsté et al., 1997). Carbon-isotope depleted, PMEs have also been found in cold-vent sediments, and their origin has been ascribed to archaea involved in AOM (Elvert et al., 1999; Pancost et al., 2000, 2001a,b; Thiel et al., 2001). The distribution of PMEs in carbonates CC-1 and CC-4 is similar to that reported for the Eastern Mediterranean cold seep sediments (Pancost et al., 2001a,b). They are particularly abundant in crust CC-1, comprising ca. 26% from the total identified apolar compounds. Crust CC-4 is characterized by less abundant PMEs, but their composition is similar to that observed in the CC-1 (Fig. 4). In both carbonates the most abundant PME is 2, 6, 10, 15, 19-pentamethylicosa-2, 6, 14, 18-tetraene (Pseudo Kovats index (CP Sil 5), 2337, II), occurring in the methanogenic archaeon *M. mazei* (Schouten et al., 1997; Sinninghe Damsté et al., 1997). The PME with 5 double bonds, pentamethylicosa-2, 6, 10, 14, 18-pentaene (Pseudo Kovats index (CP Sil 5), 2346, III), which was previously found in cultures of *M. mazei* and *M. bombayensis* (Schouten et al., 1997; Sinninghe Damsté et al., 1997), was also identified in crusts CC-1 and CC-4. In carbonates CC-2 and CC-3 PMI is abundant but PMEs are almost absent (Fig. 4). The carbon isotopic compositions of PMI range from −101‰ in the crust CC-1 to −89‰ in the crust CC-2 (Table 2). The $\delta^{13}C$ values of PMEs in all crusts are quite similar to that of PMI (Table 2). Their depleted δ values indicate that these compounds are derived from archaea involved in AOM.

In the mud breccias, PMI and PMEs were only found in the Kazakov MV. In this MV, a mixture of PMI and PMEs appeared in the uppermost 10 cm of the sediments. Down-core at depth of 20 cm, only PMI and PME (III) were identified. The concentrations of these hydrocarbons decrease with depth and this drop is accompanied with its ^{13}C enrichment. In the uppermost sediments $\delta^{13}C$ of PMI is −81‰, while at 20 cm depth $\delta^{13}C$ values of ca. −71‰ (±3‰) were measured. The distribution of PMEs in Kazakov mud breccias resembles that of the carbonate crusts CC-1/CC-4.

Another microbial lipid present in the carbonate crusts is the irregular C_{30} isoprenoid squalene (2, 6, 10, 15, 19, 23-hexamethyltetracosa-2, 6, 10, 14, 18,

Table 2
Carbon isotopic composition ($\delta^{13}C$) of archaeal and bacterial lipids in carbonate crusts with microbial mats and mud breccia (‰ by PDB standard)

Biomarker	Odessa mud volcano	NIOZ mud volcano			Kazakov mud volcano	
	Carbonate crusts with microbial mats				Mud breccia	
	TTR-11 BS-336 G (CC-1)	TTR-11 BS-328 G (CC-2)	TTR-11 BS-328 G (CC-3)	TTR-11 BS-325 G (CC-4)	0–10 cm b.s.f.	10–20 cm b.s.f.
PMI (I)[a]	−101[b]	−89[b]	−96[b]	−99	−81 ± 3‰[c]	−71 ± 3‰
PMI:1	n.d.*	−83	−97	n.d.	n.d.	n.d.
PMI:1 (Σ)[d]	−99	–	–	–	n.d.	–
PMI:2 (Σ)	−97	–	–	–	n.d.	–
PMI:3 (Σ)	−98	–	–	−92	n.d.	–
PMI:4+PMI:5 (II, III)	−96	–	–	−103	n.d.	n.d.
Squalene (IV)	−71	−57	n.d.	−40	n.d.	n.d.
Tricos-1-ene ($C_{23:1}$) (V)	−95	−100	−94	n.d.	n.d.	n.d.
Tetracos-1-ene ($C_{24:1}$) (VI)	−91	n.d.	n.d.	n.d.	n.d.	n.d.
Diploptene (VII)	−84	−61	−82	n.d.	n.d.	n.d.
Isoprenoidal DGDs						
Archaeol (X)	−106	−116	−102	−107	−85 ± 3‰	−70 ± 3‰
sn-2-hydroxyarchaeol (XI)	n.d.	n.d.	–	n.d.	−79 ± 3‰	n.d.
sn-3-hydroxyarchaeol (XII)	n.d.	n.d.	−112	n.d.		
Macrocyclic DGDs						
XIII	−106	n.d.	−104	−111	–	–
XIV	−115	n.d.	−111	−116	–	–
Non-isoprenoidal DGDs						
XV	−86	−96	−95	–	−70 ± 3‰	–
XVI	−70	−79	−92	–	−72 ± 3‰	–
XVI isomer	−90	n.d.	n.d.	–	–	–
XVII	−87	–	–	−87	–	–
XVIII	−106	–	n.d.	–	–	–
XVIII isomer	−96	–	–	–	–	–

For polar compounds obtained values have been corrected for carbon atoms added by derivatisation.
n.d.*-not determined.
[a] Roman numbers refer to the structures in the Appendix.
[b] −89—co-elution with PMI:1 isomer.
[c] Mud breccias show considerably lower concentrations of archaeal and SRB lipids compare to the carbonate crusts. Therefore, an error of δ^{13} C measurements of reported compounds for mud breccias increases up to ± 3‰.
[d] (Σ)—δ^{13} C values for the sum of C_{25} isomers with the same amount of double bonds.

22-hexaene; IV) (Fig. 3). Carbon isotopic analyses showed that squalene in carbonate CC-1 is isotopically enriched (−71‰) relative to the cluster of PMI and PMEs. Furthermore, this $\delta^{13}C$ value is the most depleted squalene of all carbonate crusts (Table 2). A distinct feature of CC-1 is the occurrence of relatively high amount of squalene and the presence its C_{35}, C_{40} and C_{45} pseudo-homologues (Fig. 3). None of these "squalene-like" lipids were detected in other carbonate crusts or in the mud breccia. The carbon isotopic compositions of ΣC_{30}, ΣC_{35}, C_{40} and C_{45} squalene pseudo-homologues were, −55‰, −74‰, −80‰ and −82‰, respectively. The isotopic compositions of these components suggest the potential incorporation of methane or methane-derived substrates by the larger bacterial cold seep community.

3.2.2. Isoprenoidal dialkyl glycerol diethers (DGDs)

Analysis of polar fractions of carbonate crusts and mud breccias revealed a suite of isoprenoidal DGDs

diagnostic for various archaea (De Rosa and Gambacorta, 1988; Sprott et al., 1990, 1993; Nishihara and Koga, 1991; De Rosa et al., 1991; Koga et al., 1993) (Fig. 5). Archaeol (bis-*O*-phytanyl glycerol diether, X), *sn*-2-(XI) and *sn*-3-(XII) hydroxyarchaeols (Hinrichs et al., 1999, 2000a,b; Pancost et al., 2000) and two macrocyclic diethers possessing one and two cyclopentane rings within the biphytane chain (XIII and XIV, respectively; Stadnitskaia et al., 2003) were identified (Fig. 5). Archaeol is a common membrane lipid of such ecologically contrasting archaeal groups as thermophiles, halophiles and methanogens (De Rosa and Gambacorta, 1988; De Rosa et al., 1991; Koga et al., 1993). It was detected in all carbonate crusts as the most abundant DGD and in the mud breccias from both MVs. In carbonate crusts CC-2 and CC-3 (from the NIOZ MV), archaeol is the most abundant compound of the polar fractions (Fig. 5) with the highest concentration of 5.2 μg/g of dry sediment in CC-2. Unlike archaeol, both the *sn*-2 and *sn*-3 isomers of hydroxyarchaeol (hydroxydiether lipids) have only been reported to occur in methanogenic archaea, i.e. in all cultured members of the *Methanosarcinales* (Ferrante et al., 1988; Sprott et al., 1990, 1993; Nishihara and Koga, 1991; Koga et al., 1993), suggesting that these lipids are a chemotaxonomic characteristic of this genus. It was also reported that hydroxylation at the C-3 position of the *sn*-2 chains is exclusively present in all of the *Methanosarcina* spp., whereas the *sn*-3 isomer has been shown to be produced by a *Methanosaeta* sp. (Sprott et al., 1993). The hydroxyarchaeol isomers occur in all carbonate crusts with abundances substantially lower than that of archaeol and varying between the crusts (Fig. 5). In crust CC-3, the *sn*-2-isomer was not detected.

All carbonate crusts are characterized by extremely depleted δ^{13}C values of archaeol and the hydroxyarchaeols. The δ^{13}C of archaeol in crust CC-1 and CC-4 is nearly identical (−106‰ and −107‰, respectively). Crust CC-2 is characterized by a more depleted ^{13}C value of archaeol (−116‰), whereas archaeol in crust CC-3 is slightly enriched in δ^{13}C value (−102‰) (Table 2). The isotopic composition of *sn*-3-hydroxyarchaeol could only be obtained in crust CC-3 (Table 2) and is 10‰ depleted relative to archaeol.

In the mud breccia from the Kazakov MV archaeol and the *sn*-2 and *sn*-3 isomers of hydroxyarchaeol were only detected in the uppermost 20 cm of the core. Archaeol represents the dominant DGD as observed for the crusts. Its concentration at 10 cm was 1.4 μg/g and decreased to 0.3 μg/g at 20 cm depth. As noticed for PMI and PMEs, deeper sediments show decreasing levels of archaeol and the hydroxyarchaeols. This change was also reflected in their carbon isotopic composition (Table 2). For example, the δ^{13}C of archaeol was −85‰ in the uppermost interval and ca. −70‰ at 20 cm depth. The δ^{13}C value of the composite *sn*-2-and *sn*-3 hydroxyarchaeols was −79‰ in the topmost sediment.

In addition to these well-known archaeal DGDs, two macrocyclic diphytanyl glycerol diethers possessing one (XIII) and two (XIV) cyclopentane rings were identified in all carbonate crusts. Although their basic molecular structure is similar to acyclic macrocyclic DGD previously identified in the thermophilic methanogen *Methanococcus jannaschii* (Comita et al., 1984), their occurrence in cold seep settings suggests that these macrocyclic DGDs are not restricted to thermophilic methanogens (Stadnitskaia et al., 2003). The δ^{13}C of the macrocyclic DGD XIV was 5–9‰ depleted relative to macrocyclic DGD XIII, which have similar δ^{13}C values as that of archaeol in all carbonate crusts (Table 2). The macrocyclic DGDs were only found in the carbonate crusts.

3.2.3. Glycerol dibiphytanyl glycerol tetraethers (GDGTs)

Isoprenoidal GDGTs are biomarkers for a wide group of archaea. They can represent the main constituents of archaeal membranes and can contain 0–8 cyclopentane rings (De Rosa et al., 1983; Langworthy, 1985; De Rosa and Gambacorta, 1988). In the carbonate crusts and mud breccias GDGTs prevail over all other identified archaeal lipids. The total GDGTs in the carbonates ranged from 36 to 136 μg/g. These values are substantially higher than those for total archaeal DGDs (sum of archaeol, OH-archaeols and macrocyclic diethers; 2.1–5.6 μg/g). All GDGT distributions are dominated by GDGTs with 0–2 cyclopentane rings (XIX, XX and XXI) except those of the mud breccias below crust CC-1 and above crust CC-4 (Fig. 6), and, thus, reveal AOM "fingerprints". Such patterns are similar to those previously observed in the Eastern Mediterranean cold seeps, MVs and carbonate crusts (Pancost et al., 2001b; Aloisi et al., 2002), in the Gulf of Mexico methane

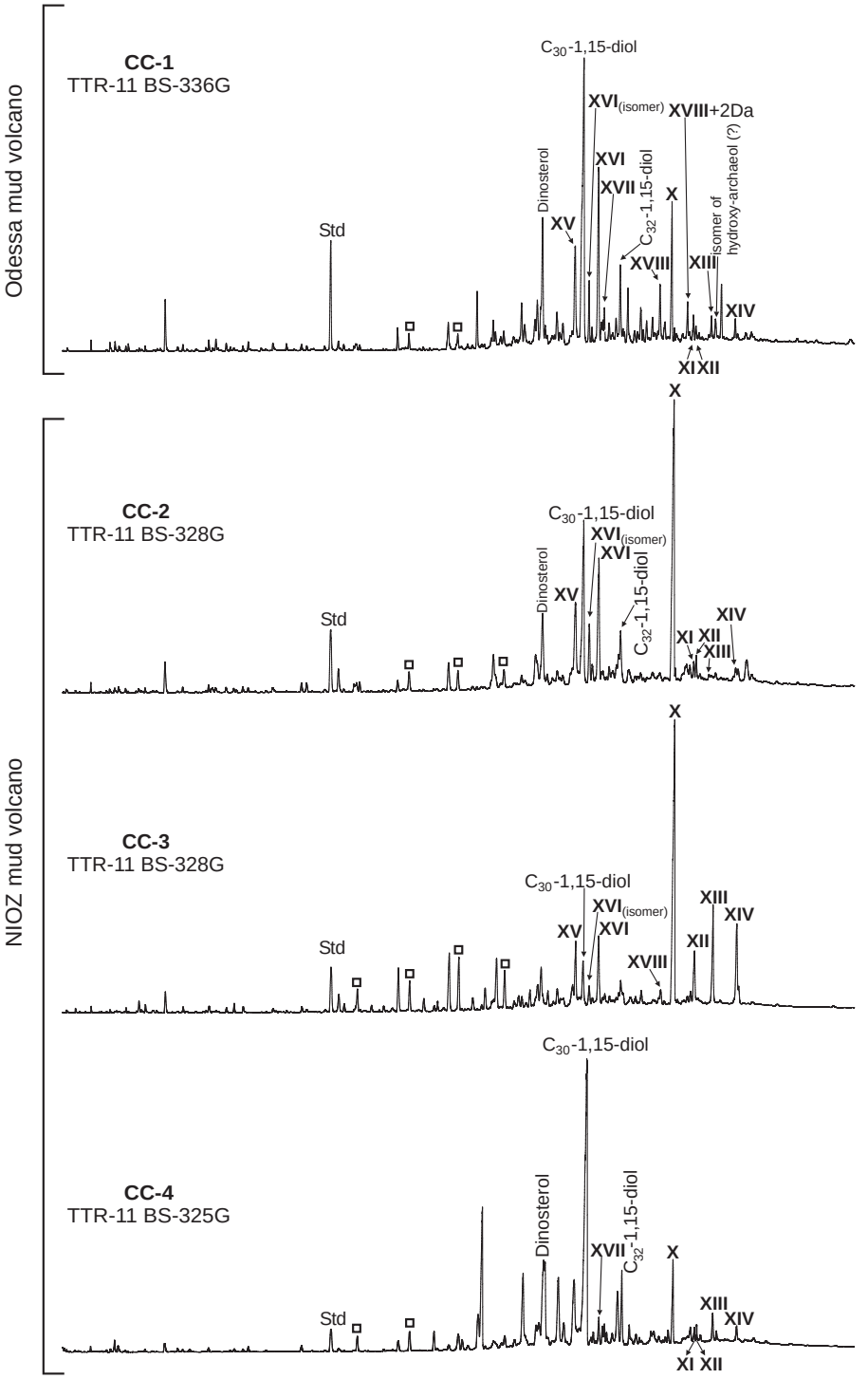


Fig. 5. Gas chromatograms of the polar fractions of the four carbonate crusts. Roman numbers refer to structures in the Appendix. Open squares indicate straight-chain alcohols. All alcohols were analyzed as their trimethylsilyl derivatives.

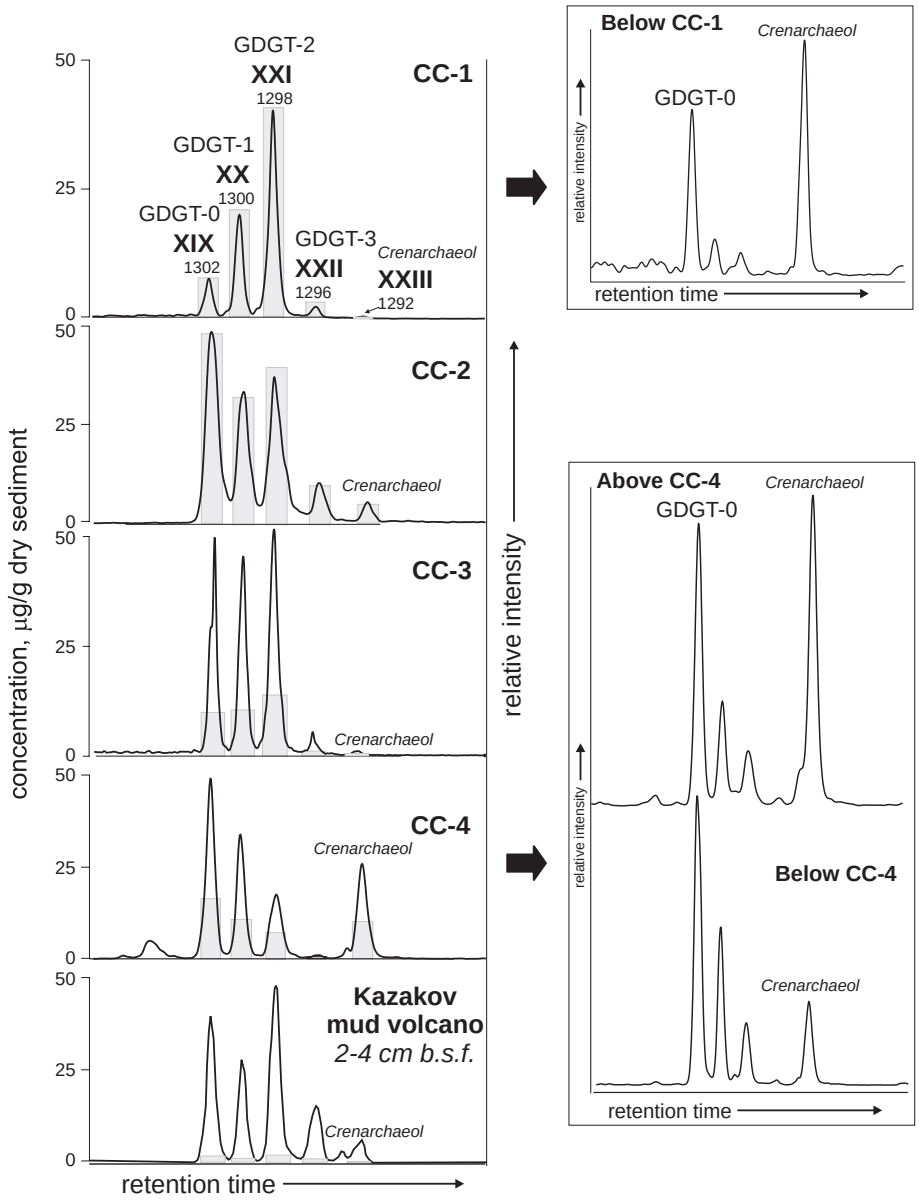


Fig. 6. Base peak HPLC chromatograms of the polar fractions obtained from the carbonate crusts and mud breccias. The number after ≪GDGT≫ indicates the number of cyclopentane rings within the biphytane chains. Roman numbers refer to structures in the Appendix. Arabic numbers below the Roman ones indicate the dominant [M+H]+ions. Grey bars designate the abundances of GDGTs and refer to the scales at the left side of the plot.

seepages (Zhang et al., 2003), and in the deep (>700 m) Black Sea water column (Wakeham et al., 2003).

Fig. 7 shows the GDGT profiles in mud breccias from the Kazakov and the NIOZ MVs. Although the GDGT concentrations in the Kazakov mud breccias are more than one order of magnitude lower than in the carbonate crusts and substantially lower than in mud breccias from the NIOZ MV, the GDGT distribution profile in the Kazakov mud breccia indicates active anaerobic methanotrophy (Fig. 7a). It reveals the predominance of GDGT-0 (XIX) and GDGT-2 (XXI), with the latter as the most prevalent

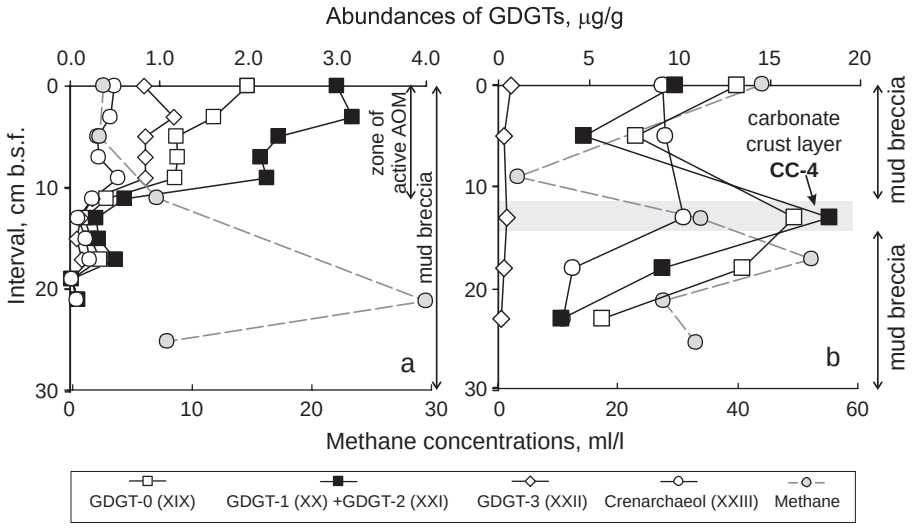


Fig. 7. Abundances (mg/g of dry sediment) versus depth of GDGTs and methane (ml/l) for (a) the Kazakov mud volcano, TTR-11 BS-331G; and (b) the NIOZ mud volcano, TTR-11 BS-325G. Roman numbers refer to structures in the Appendix.

one (Fig. 7a). In contrast, the predominance of GDGT-0 (XIX) and crenarchaeol (XXIII) in mud breccias from the NIOZ MV (Fig. 7b) and in sediments below crust CC-1 and above crust CC-4 (Fig. 6) does not indicate the presence of archaea involved in AOM. Such distributions have previously been observed in seawater particulate organic matter and in marine sediments from different geographical settings (Schouten et al., 2000; Pancost et al., 2001a,b; Wakeham et al., 2003; Wuchter et al., 2003). Crenarchaeol (XXIII) is a marker of ubiquitous planktonic crenarchaeota (Hoefs et al., 1997; Schouten et al., 1998, 2000, 2001; Sinninghe Damsté et al., 2002a,b; Wakeham et al., 2003), which also produce other GDGTs with 0 and, to a lesser degree, 1–3 cyclopentane rings. The crenarchaeotal GDGTs are most probably indigenous to the mud breccias and, thus, represent fossil material and do not reflect living crenarchaeotal biomass. The presence of crenarchaeol in the carbonate crusts in relatively small amounts is attributed to the inclusion of mud breccia particles during carbonate formation.

3.2.4. Non-isoprenoidal DGDs

Non-isoprenoidal DGDs, inferred before as a marker of SRB in cold seep carbonate crusts and MV deposits in the Eastern Mediterranean (Pancost et al., 2001a,b; Werne et al., 2002), were identified in all carbonates and in mud breccia from the Kazakov MV. Two series of these DGDs were found. The first series includes DGDs possessing anteiso pentadecyl moiety attached at the *sn*-1 position with either an anteiso C_{15} alkyl chain (C_{33}, XV) or a cyclopropyl-containing C_{16} alkyl chain at the *sn*-2 position ($C_{34:1}$, XVI) (Pancost et al., 2001a,b). The second series was represented by DGDs possessing a cyclopropyl-containing C_{17} alkyl chain at the *sn*-2 position with either an *n*-C_{14} alkyl ($C_{34:1}$, XVII; RI=3633) or a C_{17} ω-cyclohexyl alkyl moiety ($C_{37:2}$, XVIII; RI=3927) at the *sn*-1 position (Pancost et al., 2001a,b). With the exception of CC-4, the most abundant diether in the carbonate crusts was XVI and its tentatively identified isomer (Fig. 5). The non-isoprenoidal DGD XVII was detected only in crusts CC-1 and CC-4. A novel non-isoprenoidal DGD was tentatively identified in crust CC-1 (Fig. 5). Its mass spectrum resembled that of XVIII (Pancost et al., 2001a,b), but the molecular ion is 28 Da higher. The $\delta^{13}C$ values of the non-isoprenoidal DGDs in the crusts were generally 10–20‰ heavier than those of the isoprenoidal and macrocyclic DGDs (Table 2). An exception is XVIII, which was characterized by a $\delta^{13}C$ similar to that of archaeol.

The DGDs XV and XVI were only found in the uppermost interval of the mud breccia from the Kazakov MV. Their carbon isotopic composition was ca. −71‰. In the mud breccia the non-isoprenoidal DGDs co-occurred with archaeal DGDs, PMI and PMEs. It does not indicate a relation of sulfate-reducing

bacteria with archaea, but their ^{13}C-depleted signatures do indicate that the carbon source most probably was methane.

3.2.5. Cyclic triterpenes

The hopanoid diploptene (hop-22(29)-ene; VII), widely occurring in the bacterial domain, was detected only in the carbonate crusts (Fig. 3). The low $\delta^{13}C$ values of diploptene (up to −84‰; Table 2) indicates an incorporation of methane-derived carbon into the biomass of diploptene-biosynthesizing bacteria. The presence of ^{13}C-depleted diploptene exclusively in the carbonates may suggest specific bacterial populations involved in the process of carbonate precipitation via AOM.

Two other lipids, tetrahymanol (VIII) and bishomohopane-32-ol (IX), were detected in the uppermost 25 cm of the mud breccia of the NIOZ MV. Tetrahymanol and bishomohopane-32-ol were relatively depleted in ^{13}C, i.e. ca. −49‰ at the interval 16–22 cm bsf. Since bishomohopane-32-ol is probably derived from C_{35} bacteriohopanepolyol derivatives, fresh extracts were also subjected to chemical degradation in order to detect all bacteriohopanepolyol derivatives (Rohmer et al., 1984, 1992). However, no indications for the presence of intact C_{35} bacteriohopanepolyol derivatives were obtained.

Hopanoids are derived from precursors in the membranes of bacteria. They occur mainly in aerobic bacteria, such as methylo- and methanotrophs, heterotrophs, cyanobacteria, and facultative anaerobic photosynthetic purple non-sulfur bacteria (Rohmer et al., 1984, 1992; Kenneth and Moldovan, 1993; Schoell et al., 1994; Summons et al., 1999). Recent biomarker studies revealed the occurrence of hopanoids in anoxic environments (Pancost et al., 2000; Elvert et al., 2000; Thiel et al., 2003), suggesting that the occurrence of hopanoids is not restricted to aerobic bacteria. Recently, it has indeed been shown that strictly anaerobic bacteria capable of anaerobic ammonium oxidation are able to biosynthesize hopanoids, including diploptene and bacteriohopanepolyols (Sinninghe Damsté et al., 2004).

It is still unclear which microorganisms are capable to biosynthesize tetrahymanol in strict anoxic environments. Tetrahymanol was first isolated from the ciliate protozoan *Tetrahymena pyriformis* (Mallory et al., 1963). Besides eukaryotes such as in ferns, fungi, and other ciliates, tetrahymanol was found in addition to hopanoids in the phototrophic purple non-sulfur bacterium *Rhodopseudomonas palustris* (Kleemann et al., 1990) and in the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (Bravo et al., 2001).

3.2.6. Straight-chain alkenes

All carbonate crusts contain *n*-tricosene ($C_{23:1}$; V) and *n*-tetracosene ($C_{24:1}$; VI). Based on the relative retention time, the $C_{23:1}$ alkene was tentatively identified as *n*-tricos-10(*Z*)-ene, previously reported in a Black Sea microbial mat (Thiel et al., 2001). The $C_{24:1}$ alkene has not been previously reported in AOM settings. The $\delta^{13}C$ signatures of these alkenes ($\delta^{13}C$ = ca. −95‰; Table 2) indicated that *n*-$C_{23:1}$ and *n*-$C_{24:1}$ were biosynthesized by microorganisms involved in AOM (cf. Thiel et al., 2001). It is presently unknown which microorganisms involved in anaerobic methanotrophy are capable to biosynthesize these straight-chain hydrocarbons. However, their presence only in methane-related carbonates and not in mud breccias indicates peculiar trophic association of microorganisms, which may be involved in carbonate precipitation via AOM.

3.3. 16S rRNA gene sequence analysis

3.3.1. Archaeal sequences

DGGE of PCR products obtained with primers specific for the 16S rRNA encoding gene of Archaea (Coolen et al., 2004) resulted in one intensely stained band at the same position in the gel for carbonate crusts CC-1 and CC-4 (Fig. 9, lanes 2 and 5) as well as for CC-2 and CC-3 (Fig. 9, lanes 3 and 4). The DGGE profiles of the PCR products obtained with DNA from the mud breccias showed more than one band. The mud breccias from the Kazakov MV showed two bands at 2–4 and at 8–10 cm, and three bands at 20–22 cm (Fig. 9, lanes 7–9, respectively). In contrast, the mud breccias from the NIOZ MV showed no bands at 8–12 cm, just above carbonate crust CC-4, and ca. seven relatively weak bands at 16–20 cm depth, just below the crust (Fig. 8, lanes 10–11).

Most of the bands were excised from the gel, reamplified, and sequenced to infer the phylogenetic affiliation of the community members. None of the identified archaeal sequences was closely related to known methanogens. Bands at the same position in the

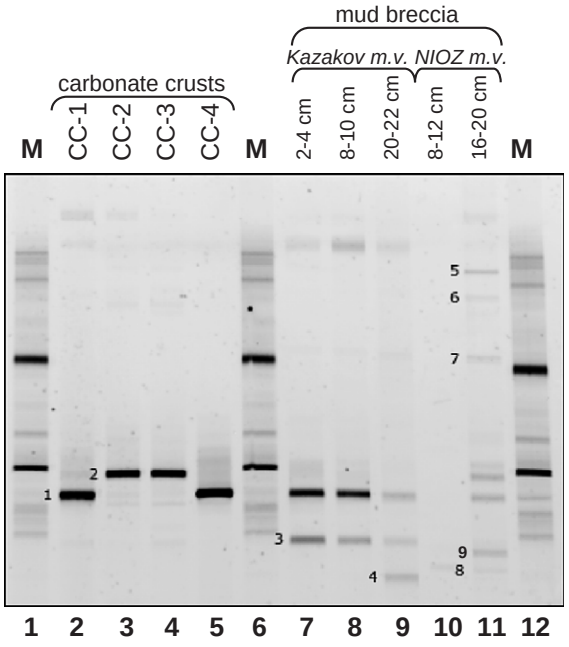


Fig. 8. DGGE analysis of PCR products obtained with primers specific for the 16S rRNA encoding gene of Archaea and genomic DNA extracted from the carbonate crusts and mud breccias (for detail on the samples, see Table 2). Lanes 1, 6, and 12 are marker fragments. Bands that were excised and sequenced are numbered; band no. 1 is named DGGE-ARC1, band no. 2 is DGGE-ARC2, etc. (see Fig. 9 for their phylogenetic position).

gel had identical sequences. Comparative analysis of the sequences obtained from bands no. 1, 2 and 3, and sequences stored in nucleotide databases indicated a close relationship of these populations with Archaea grouped in the ANME-1 cluster (Fig. 9). A bootstrap value of 100% confirmed this strong affiliation. The sequence obtained from band no. 7 clustered with sequences of Archaea of the Marine Benthic Group D within the *Euryarchaeota* (Vetriani et al., 1999; Teske et al., 2002). The sequences of the other excised bands grouped with sequences of *Crenarchaeota*. Bands no. 5 and 6 grouped within the Marine Benthic Group B (Vetriani et al., 1999), and bands no. 4, 8, and 9 within the cluster of the Miscellaneous Crenarchaeotal Group (Inagaki et al., 2003). No sequences of mesophilic archaea affiliated with the group of pelagic crenarchaeota were identified (Fig. 9).

3.3.2. Bacterial sequences

A diversity of bacterial 16S rRNA gene fragments was detected in DNA isolated from the microbial mats associated with carbonate crusts and from mud breccias. DGGE analysis of 16S rRNA gene fragments obtained with bacterial primers (Fig. 10) showed a more complex pattern than those for Archaea (Fig. 8). To determine the identity of the bacteria, DGGE bands were excised, reamplified, and sequenced. Comparative sequence analysis indicated the presence of bacteria affiliated to different phylogenetic groups (Fig. 11). Three sequences, DGGE-BAC2, -BAC4 and -BAC7, grouped with bacteria for which no cultured representatives have been isolated so far, i.e. the OP8 candidate division (Hugenholtz et al., 1998). Two other sequences, i.e. DGGE-BAC1 and -BAC13, grouped with sequences belonging to bacteria from hydrocarbon seeps or benzene-mineralizing consortia within the Haloanaerobiales. Three sequences, DGGE-BAC5, -BAC8 and -BAC12, clustered with SRB within the δ-subdivision of the Proteobacteria. These sequences belong to the Desulfobacteraceae, including all the sequences between *Desulfobacterium niacini* and *Desulfostipes sapovorans* with a bootstrap value of greater than 90%. However, they do not belong to the *Desulfosarcina* cluster. They form a separate group together with the sequence of the uncultured bacterium 63-2 found in the Benguela Upwelling System (Schaefer et al., unpublished data). One sequence, DGGE-BAC17, grouped with members belonging to the genus Sphingomonas. Four sequences, DGGE-BAC14, -BAC15, -BAC16, and -BAC18, were affiliated with the α-subdivision of the Proteobacteria (Fig. 11). Interesting is the affiliation of DGGE-BAC18 with known *Methylobacterium* species, and DGGE-BAC15 with methanotrophic bacteria (Fig. 11).

3.4. Microbial diversity in carbonate crusts and mud breccia: insight from 16S rDNA sequences and biomarker data

3.4.1. Archaea

Despite the crucial role of archaea in AOM, no species are available in pure culture. Therefore, their precise biomarker composition is still unknown. The combination of the lipid biomarker and 16S rDNA data is, however, a powerful means to identify the main microbial community members involved in AOM. Our 16S rRNA gene sequence survey only

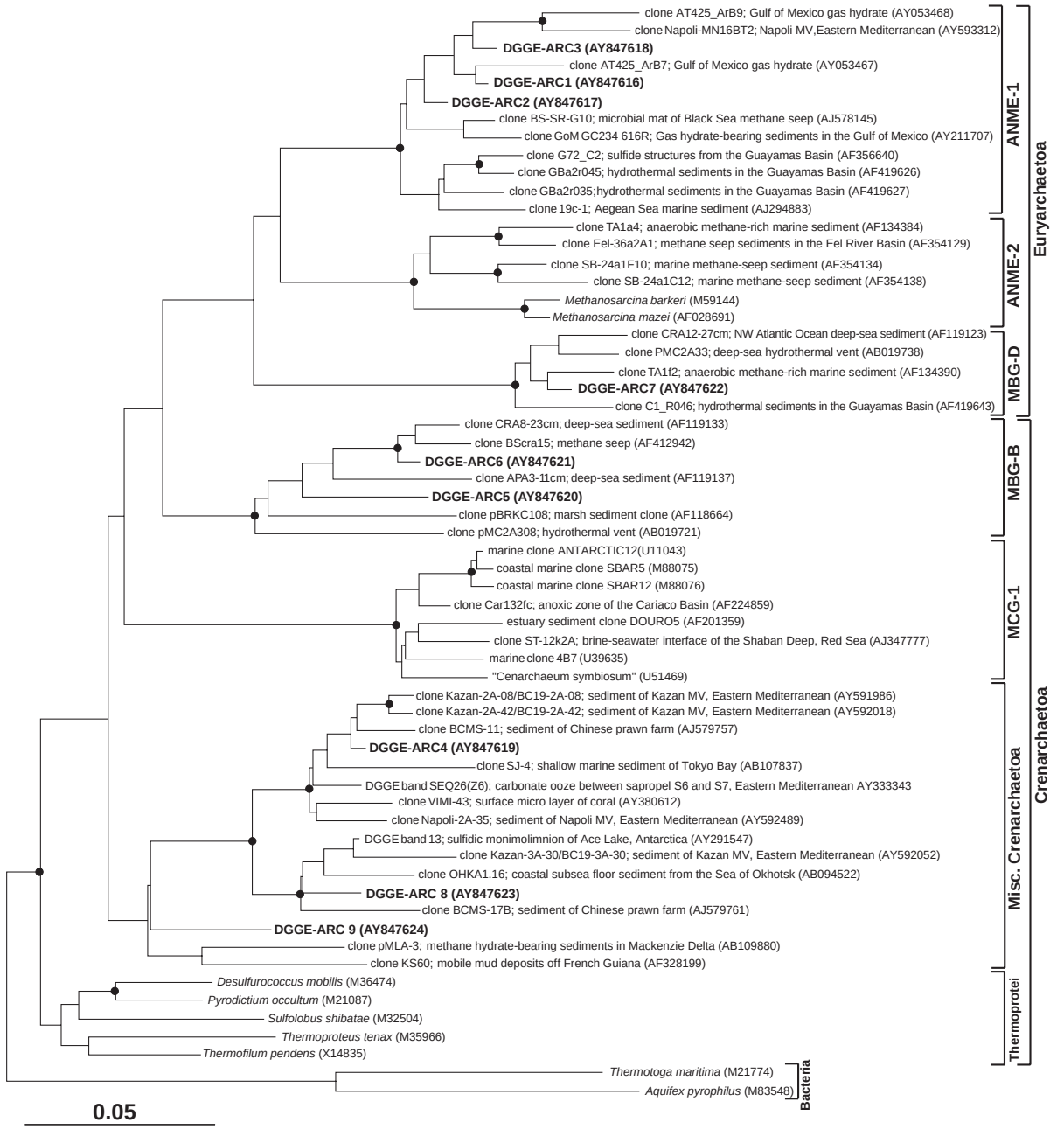


Fig. 9. Phylogenetic affiliation of Archaea present in the carbonate crusts and mud breccias as reveled by comparative analysis of 16S rRNA gene sequences from bands and those stored in public databases. Bootstrap values are based on 1000 replicates; only values between 90% and 100% are given and indicated by a solid dot on the branches. Sequences determined in this study are shown in bold and refer to the number of the band excised from the gel (see Fig. 8). The scale-bar represents 5% sequence divergence. Abbreviations used to indicate the different clusters in the tree: ANME-1 and ANME-2 are two groups of putative anaerobic methane-oxidizing *Archaea*; MBG-D is marine benthic group D; MBG-B is marine benthic group B; MCG-1 is marine crenarchaeotal group-1.

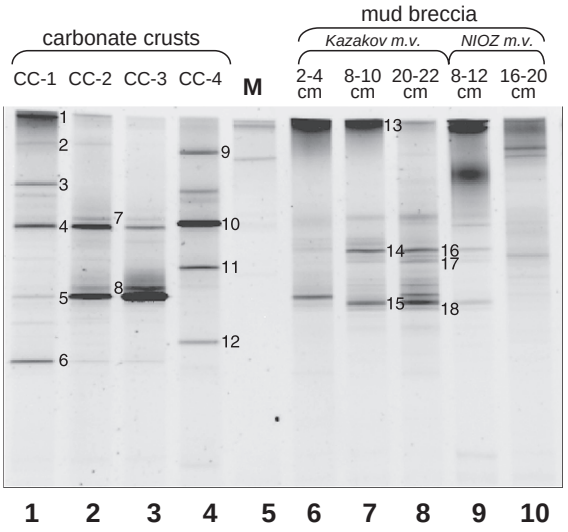


Fig. 10. DGGE analysis of DNA fragments obtained after PCR-amplification with primers specific for the 16S rRNA genes of bacteria and genomic DNA extracted from the carbonate crusts and mud breccias (for detail on the samples, see Table 2). Lane 5 represents marker fragments. Bands that were excised and sequenced are numbered; band no. 1 is DGGE-BAC1, band no. 2 is DGGE-BAC2, etc. (see Fig. 11 for their phylogenetic position).

revealed archaea related to the ANME-1 cluster (Fig. 9). Although this does not prove that archaea from the ANME-2 group are absent, it shows that archaea belonging to the ANME-1 cluster are far more dominant. Both DGGE of PCR amplified archaeal 16S rDNA (Fig. 8) and the archaeal biomarker composition (Figs. 3 and 5) show a clear distinction between carbonate crusts CC-1/CC-4 and CC-2/CC-3. Although the DGGE-ARC1 and -ARC2 sequences are relatively closely related (ca. 95%) and both fall in the ANME-1 cluster (Fig. 9), the archaeal lipid composition of crusts CC-1/CC-4 and CC-2/CC-3 is quite different. Archaea present in carbonates CC-1/CC-4 predominantly biosynthesize PMEs and archaeol, while archaea in crusts CC-2/CC-3 produce primarily PMI and, in addition, to archaeol also hydroxyarchaeol. All carbonates contain abundant GDGTs and show the typical pattern (Fig. 6) distinctive for AOM (cf. Pancost et al., 2001a,b; Wakeham et al., 2003).

Recent observations of archaeal membrane lipid patterns have typified distinct anaerobic methanotrophic consortia in AOM-driven carbonate reefs with living microbial mats (Blumenberg et al., 2004). It was shown that microbial communities dominated by archaea from the ANME-1 cluster biosynthesize cyclic GDGTs, archaeol and PMI with relatively low amounts of PMEs, whereas ANME-2 archaea are characterized by the absence of GDGTs, *sn*-2-hydroxyarchaeol in access to archaeol, abundant PMEs relative to PMI and the presence of crocetane and crocetenes (Blumenberg et al., 2004). The tetra- and pentaunsaturated PME isomers were initially thought to be indicative for members of the *Methanosarcinales*-related ANME-2 cluster (Sprott et al., 1990, 1993; Hinrichs et al., 1999) but the data of Blumenberg et al. (2004) do not comply with this. The archaeal lipid distribution in our carbonates is generally consistent with the classification of Blumenberg et al. (2004): GDGTs are relatively abundant, archaeol dominates over the hydroxyarchaeol isomers, and crocetane is absent, all in line with the predominance of ANME-1 archaea. However, despite that the archaea in the carbonate crusts all belong to the ANME-1 cluster, a substantial variation in the amounts of PMI relative to PMEs is observed (Fig. 4), indicating that this characteristic should be used cautiously.

In comparison with the carbonate crusts, the mud breccias revealed phylogenetically more diverse archaeal assemblages (Figs. 8 and 9). The mud breccias from the Kazakov MV are characterized by a similar suite of archaeal biomarkers as crusts CC-1 and CC-4, suggesting similar archaeal populations. This was confirmed by DGGE of archaeal 16S rDNA fragments, revealing the predominance of the DGGE-ARC1 sequence, which also characterizes the archaeal community in the crusts CC-1 and CC-4 (Fig. 8). Another, but less dominant, sequence (DGGE-ARC3) is phylogenetically closely affiliated with sequences DGGE-ARC2 and -ARC1 found in the carbonates. Sequence DGGE-ARC4, only obtained from the lowermost mud breccia (20–22 cm), is affiliated with the Miscellaneous Crenarchaeota Group of the *Crenarchaeota*. This group of *Crenarchaeota* is comprised of sequences found predominantly in recent sediments (e.g. Inagaki et al., 2003) and mud breccias from the Eastern Mediterranean MVs (Heijs et al., unpublished). In contrast to the Kazakov MV, the mud breccias from the NIOZ

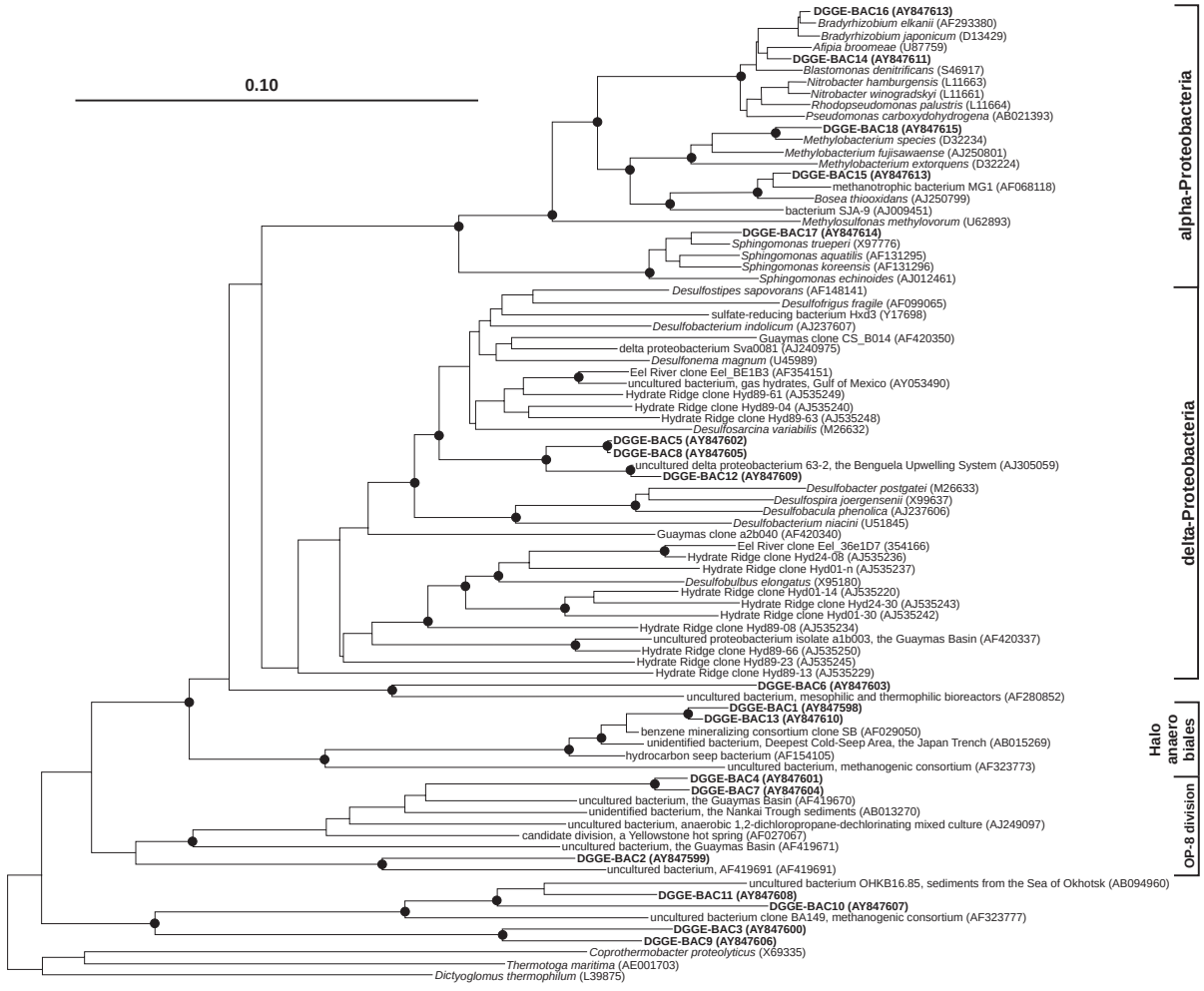


Fig. 11. Phylogenetic affiliation of bacteria present in the carbonate crusts and mud breccias as reveled by comparative analysis of 16S rRNA gene sequences from DGGE bands and those stored in public databases. Bootstrap values are based on 1000 replicates; only values between 90% and 100% are given and indicated by a solid dot on the branches. Sequences determined in this study are shown in bold and refer to the number of the band excised from the gel (see Fig. 10). The accession numbers are given in parentheses. The scale-bar represents 10% sequence divergence.

MV do not contain sequences affiliated with AOM archaeal groups. They are characterized by a phylogenetically more diverse archaeal community with members within the *Euryarchaeota*, i.e. Marine Benthic Group D (DGGE-ARC7) and *Crenarchaeota*, including Marine Benthic Group B (DGGE-ARC5 and -ARC6) and the Miscellaneous Crenarchaeota Group (DGGE-ARC4, -ARC8, and -ARC9). Such a variety occurs only in the interval 16–20 cm, below carbonate CC-4 (Figs. 8 and 9). The presence of these crenarchaeotal groups is indicative of sedimentary archaea which is most likely not involved in the anaerobic methanotrophy. The GDGT patterns of these sections of the mud breccias are similar to those in particulate organic matter from the water column derived from pelagic crenarchaeota (e.g. Sinninghe Damsté et al., 2002b; Wakeham et al., 2003). The absence of any archaeal sequences affiliated with marine pelagic crenarchaeota in the mud breccias (Fig. 9) indicates that these GDGTs are likely not derived from living archaeal cells. Crenarchaeol (XXII) and the other

GDGTs are most likely indigenous to the erupted mud breccia, i.e. they were transported by mud fluid from the subsurface, resulting in a background signal of GDGTs characteristic for pelagic crenarchaeota. This hypothesis is supported by the fact that different mud flows on top of each other may have quite different pelagic crenarchaeotal GDGTs patterns (Stadnitskaia et al., unpublished results). If AOM is taking place, the archaea are producing their characteristic GDGTs, overriding the background signal (Fig. 6). In the carbonates the purest AOM signal is found.

3.4.2. Sulfate-reducing bacteria

The presence of SRB was mainly detected in the carbonate crusts. Sequences DGGE-BAC5 and -BAC8 grouped within the SRB cluster (Fig. 11) and are detected in the carbonate crusts CC-1, CC-2 and CC-3 in association with the occurrence ^{13}C-depleted non-isoprenoidal diethers (Fig. 5), previously inferred as biomarkers of SRB (Pancost et al., 2001a; Werne et al., 2002). Carbonate crust CC-4 reveals relatively low quantities of ^{13}C-depleted non-isoprenoidal DGDs. However, the presence of sequence DGGE-BAC12 in the crust CC-4 confirms the presence of another species of SRB (Fig. 11).

Previous studies have demonstrated that archaea from both the ANME-1 and ANME-2 groups co-occur with members of the *Desulfosarcina/Desulfococcus* phylogenetic cluster (Boetius et al., 2000; Orphan et al., 2001a, 2002; Michaelis et al., 2002). The presence of other groups of SRB from seep-related sediments was recently shown in the Cascadia Margin of Oregon (Knittel et al., 2003). None of the sequences of SRB found in the carbonates belong to these phylogenetic clusters of SRB known to participate in AOM. The sequences, DGGE-BAC5, -BAC8, and -BAC12, form a separate cluster within the Desulfobacteraceae family which possess though *Desulfosarcina/Desulfococcus* group, a potential syntrophic archaeal partner in AOM consortia as it was previously inferred (Boetius et al., 2000). This indicates that archaea performing AOM in the carbonate crusts are most likely not limited to the known SRB partners but that the SRB diversity is larger than previously anticipated. FISH microscopy should confirm if the new SRB identified here form indeed synthrophic communities as observed previously (Boetius et al., 2000). The positive correlation between the relative abundance of SRB lipid biomarkers, their carbon isotopic signatures, and the presence of specific SRB-related DGGE bands in the carbonates provides additional evidence to attribute the observed non-isoprenoidal DGDs to SRB involved in AOM microbial communities.

3.4.3. Other bacterial assemblages

Sequences belonging to the benzene-mineralizing consortia (Phelps et al., 1998) within the Haloanaerobiales group were only found in the carbonate CC-1 and the mud breccia of the Kazakov MV. The presence of these bacteria is likely related to the abundance of allochthonous aromatic compounds detected in the hydrocarbon gas mixtures and in the lipid extracts of these sediments (Stadnitskaia et al., unpublished data).

The presence of sequences in the mud breccias of the Kazakov and NIOZ MVs (sequences DGGE-BAC18 and -BAC15) that are phylogenetically related to methylotrophic and methanotrophic bacteria in the α-proteobacteria (Fig. 11) is remarkable. This seems to indicate the presence of aerobic bacteria (all cultured bacteria falling in this cluster are aerobes) in an anoxic ecological niche. Alternatively, and perhaps more likely, these findings indicate the existence of microbes in this phylogenetic cluster that are anaerobic. The presence of ^{13}C-depleted bishomohopan-32-ol in the same sediment intervals where these sequences were detected might indicate that this hopanoid is derived from these bacteria (methanotrophs are known to biosynthesize hopanoids; Rohmer et al., 1992) and that they are actively involved in methane cycling. However, we were not able to detect intact bacteriohopanepolyols in these sediments. Hopanoids have recently been detected in strictly anaerobic bacteria (Sinninghe Damsté et al., 2004), so this finding is not inconsistent with the hypothesis that the phylogenetic cluster of methanotrophic bacteria in the α-proteobacteria may contain anaerobes.

The other detected bacterial sequences (Fig. 11) cannot be directly related to a metabolic reaction or ecological niche. A good example is the presence in the CC-1, CC-2, and CC-3 carbonate crusts of three

sequences (DGGE-BAC2, -BAC4, and -BAC7) affiliated with bacterial candidate division OP8. Related sequences of this bacterial division were first detected in a hot spring in Yellowstone National Park, rich in reduced iron, sulfide, CO_2, and hydrogen (Hugenholtz et al., 1998). Subsequently, related sequences were also obtained from a hydrocarbon-contaminated soil under methanogenic conditions (Dojka et al., 1998) and from the anoxic water column of the Cariaco Basin (Madrid et al., 2001). In all these settings, the representatives of OP8 candidate division co-occurred with various archaea. Although we have no carbon isotopic evidence that implies the involvement of these bacteria in AOM processes, their restricted occurrence in the crusts may indicate an ecologically significant but as yet unknown role for these bacteria. Another example is the sequence (DGGE-BAC16), closely affiliated with nitrogen-fixing bacteria (*Bradyrhizobium* species; Fig. 11), in the mud breccias of the Kazakov MV. *B. japonicum* biosynthesizes gammacerane derivatives (Bravo et al., 2001) and, thus, may be the biological source for tetrahymanol, but its biogeochemical role in this setting remains to be understood.

3.5. AOM processes and the formation of carbonates

A distinctive AOM signal, revealed by the GDGT composition and concentration (Fig. 6) and the presence of other ^{13}C-depleted archaeal and bacterial biomarkers (Table 2), is evident for all carbonate crusts. In contrast, none of the mud breccias hosting the carbonate crusts are characterized by a strong AOM signal (e.g. Fig. 6). This is strong evidence that AOM performed by the microbial community of archaea and SRB is indeed directly or indirectly responsible for the precipitation of carbonate. Furthermore, it reveals that AOM is the predominant microbial process within the carbonate crusts, inducing their formation. This is consistent with the 16S rDNA sequence data which revealed ANME-1 archaeal sequences only within the carbonate crusts. By contrast, the mud breccia below the crust CC-4 shows phylogenetically diverse archaeal populations which are not at all affiliated with methanotrophic archaea (Figs. 8 and 9). Most probably, the methanotrophic archaea are present in much lower numbers relative to other archaea in the mud breccia, consistent with the assumption of strongly reduced rates of AOM.

The carbon isotopic compositions of archaeal and bacterial biomarkers in both the carbonates and the mud breccia from the Kazakov MV show extremely depleted $\delta^{13}C$ values (Table 2), which is consistent with the data reported for various modern and ancient methane venting areas where AOM took or is taking place (Peckmann et al., 1999a,b, 2002; Thiel et al., 1999, 2001; Elvert et al., 1999; Hinrichs et al., 2000a,b; Pancost et al., 2000, 2001a,b; Michaelis et al., 2002; Teske et al., 2002; Zhang et al., 2002, 2003; Werne et al., 2002). PMI is enriched in ^{13}C relative to archaeol in both the mud breccia and the carbonate crusts. On the other hand, hydroxyarchaeol shows considerable isotopic variation. In the mud breccia it is enriched in ^{13}C relative to archaeol (by ca. 6‰) but depleted in carbonate crust CC3 (by ca.10‰). This is consistent with observations made in Eastern Mediterranean mud volcanic sediments and carbonate crusts (Pancost et al., 2001b). Mud breccias from the Kazakov MV show $\delta^{13}C$ values of archaeal and SRB biomarkers of ca. 10–20‰ enriched relative to those of the same lipids in the carbonate crusts from the other MVs. This enrichment in ^{13}C is likely related to the enrichment in ^{13}C of the substrate methane (Kazakov MV: $\delta^{13}C_{CH4}\sim-56$‰; Odessa MV: $\delta^{13}C_{CH4}\sim-63$‰; and NIOZ MV: $\delta^{13}C_{CH4}\sim-68$‰). This indicates that $\delta^{13}C$ value of microbial lipids is determined in part by the isotopic composition of methane.

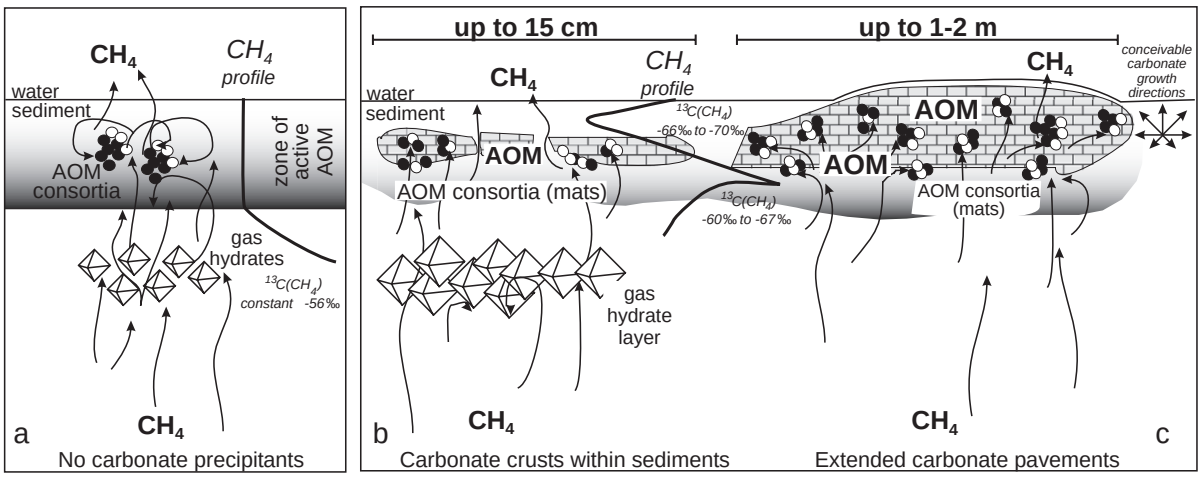
It is known, that authigenic carbonates derive their carbon from the pore water ΣCO_2 pool (Suess and Whiticar, 1989). Since the isotopic signal of methane in both the NIOZ and the Odessa MVs is ^{13}C-depleted, the bicarbonate produced via AOM from this methane is also ^{13}C-depleted. Therefore, the similarities between the $\delta^{13}C$ values of the methane, $\delta^{13}C$ values of carbonates from the NIOZ and the Odessa MVs (−41‰ and −44‰, respectively; Mazzini et al., 2002), and $\delta^{13}C$ values of the archaeal lipids in the carbonate crusts (Table 2) probably indicate that carbonate precipitation at these sites occurred under relatively constant biogeochemical environments.

Methane concentrations in the NIOZ (TTR-11 BS-325G) and in the Odessa (TTR-11 BS-336G) MVs were highest just below the carbonate crust levels and

then tend to decrease within the carbonate interval and just above the crust, indicating its consumption (Fig. 7b). Most likely, neoformed carbonates serve as a trap for migrated methane, thus partially reducing its diffusion into the water column. This could explain the settlement of microbial mats at the base of and within the carbonate crusts. Consequently, at sites where authigenic carbonate layers have already been formed (i.e. the NIOZ and the Odessa MVs), AOM occurs predominantly just below or/and within the carbonates.

The data obtained suggest that the studied locations within the NIOZ, the Odessa and the Kazakov MVs were characterized by different intensity and duration of AOM processes. The recovered sedimentary section from the Kazakov MV (TTR-11 BS-331G) was characterized by the absence of overlaying pelagic sediments, indicating that the sampling site was located on a relatively recent mud flow. The presence of only millimeter-sized gas hydrates suggests inherent limited scale methane diffusion from the subsurface. Low concentrations of GDGTs and the absence of AOM-induced authigenic carbonates suggest that microbial processes in this setting, in particular AOM, are at the "initial stage" of their development (Fig. 12a). Fig. 7a shows that, in spite of the relatively low GDGT content in the mud breccias, the distribution of GDGTs is similar to that of the carbonate crust CC-2 (Fig. 6). The distinctive concave-up shape of the methane concentration profile relates to the sharp decline in GDGT concentrations at the same depth interval (Fig. 7a). This is consistent with the decrease in concentration of SRB and other archaeal lipids (non-isoprenoidal and isoprenoidal DGDs and PMEs). The DGGE patterns for archaeal 16S rDNA from the uppermost 10 cm (Fig. 8) showed the predominance of sequences that are affiliated with the putative methanotrophic archaeal group ANME-1.

Compared with the site from the Kazakov MV (TTR-11 BS-331G), the recovered sedimentary sequences from the NIOZ (TTR-11 BS-325G) and the Odessa (TTR-11 BS-336G) MVs indicate longer methane seepage duration. The presence of pelagic Unit-1 and Unit-2 (Degens and Ross, 1972) at the Odessa MV (TTR-11 BS-336G) indicates that the sampling site was located on a relatively old mud flow, which is in agreement with the presence of a



Kazakov mud volcano (*TTR-11 BS-331G*)

Odessa mud volcano (*TTR-11 BS-336G*)
NIOZ mud volcano (central crater) (*TTR-11 BS-325G*)

NIOZ mud volcano (northern periphery of the crater) (*TTR-11 BS-328G*)

Fig. 12. Conceptual model showing probable zone of active AOM and related carbonate formation.

massive gas hydrate layer, indicating excess of methane. The presence of an AOM-related carbonate crusts and associated microbial mats in both the Odessa and NIOZ MVs indicate "long-term" AOM processes and therefore "long-term" methane availability. This might also designate the "next stage" of AOM-resulted diagenetic alterations followed after the "initial stage" observed in the mud breccia of the Kazakov MV (Fig. 12b).

The recovered sedimentary section from the central part of the crater of the NIOZ MV (TTR-11 BS-325G) consists of two different mud breccia intervals, and the carbonate crusts CC-4 found in between (Kenyon et al., 2002). The formation of this crust may have started prior to the second mud breccia effusion. The presence of microbial mats at the base and inside the carbonate crust, however, indicates still on-going microbial processes, resulting in crust development below the seafloor. In the Odessa MV, carbonate crust CC-1 with pink microbial mats is found at the same depth interval as carbonate CC-4. Carbonate crusts CC-2 and CC-3, which are parts of extensive carbonate pavements at the northern edge of the crater of the NIOZ MV (TTR-11 BS-328G), are also intensively colonized by microbes. Underwater camera-surveys revealed that these pavements are covered by pelagic sediments, forming a positive seafloor relief (Kenyon et al., 2002) which implies their formation below the seafloor (Fig. 12c). The presence of authigenic carbonates at the subsurface is common for the sediments of the Sorokin Trough and forms an indirect indication of past or present AOM-active horizon.

The strongest AOM signal was detected within carbonates but not in the underlying or covering sediments. This suggests that carbonate was precipitated within the sediments below the seafloor surface, and that putative AOM consortia become predominantly concentrated within the carbonates inducing their growth. The carbonate growth direction possibly follows the pathways of the migrated methane, which is mainly laterally and/or upwards. Initially, carbonate precipitation has to correspond to intervals with high AOM rates, i.e. where methane and sulfate are abundant (Fig. 12). After precipitation, carbonates may form a good growth substrate for microorganisms due to their large surface and porosity. The upward growth of carbonates in the Black Sea can be explained by partial diffusion of fluids through already formed carbonates. This is demonstrated by the abundant presence of scattered microbial communities within the carbonates investigated (see Materials and Methods). Besides, extensive carbonate pavements at the periphery of the NIOZ MV crater form a positive seafloor relief which might indirectly denote their partial upward accretion. On the other hand, we do not reject the idea that in general carbonates can also grow downwards, which might seem more logical. Serving as a trap for the upward migrated methane, carbonate formation may lead the growth of the carbonates downward. However, downward carbonate accretion could be bounded by the methane/sulfate interface.

The precipitation of carbonates via AOM and their upward growth in the marine environment in the presence of an anoxic/oxic interface will be restricted, because both methanotrophic archaea and SRB are obligate anaerobes. Furthermore, the interaction of sulfide-rich pore fluids, produced due to AOM, with oxygenated seawater results in locally acidic environments (Paull and Neumann, 1987) which may dissolve carbonates at the sediment/water interface. The deep Black Sea environment is strictly anoxic. Actual and active gas seepage into the water column (Michaelis et al., 2002) and active mud volcanism (Ivanov et al., 1992, 1996, 1998; Kenyon et al., 2002) were widely documented within the basin. The finding of 4-m high carbonate build-ups covered with massive microbial mats in the anoxic waters of the northwestern Black Sea shelf (Michaelis et al., 2002) can be considered as an example of possible upward carbonate accretion due to AOM. The formation of such build-ups above the seafloor is most probably only possible in an anoxic water column. In an oxygenated water column, AOM-related carbonates of any shape and morphology will be formed within the oxygen-free intervals below the sea surface. Interestingly, until now no "chimney" shaped carbonate build-ups in the seepage areas of the deep (>1000 m) Black Sea (Woodside et al., 1997; Kenyon et al., 2002) were reported. Probably, the widely observed extensive carbonate pavements in deep-sea fluid venting areas of the Central and the

North-eastern parts of the Black Sea might represent an "equivalent" to the shallow-depth "carbonate-chimney-forest" discovered in the Western shelf of the basin (Michaelis et al., 2002). Such extended carbonate edifices are a characteristic feature related to methane venting in the Sorokin Trough.

4. Conclusions

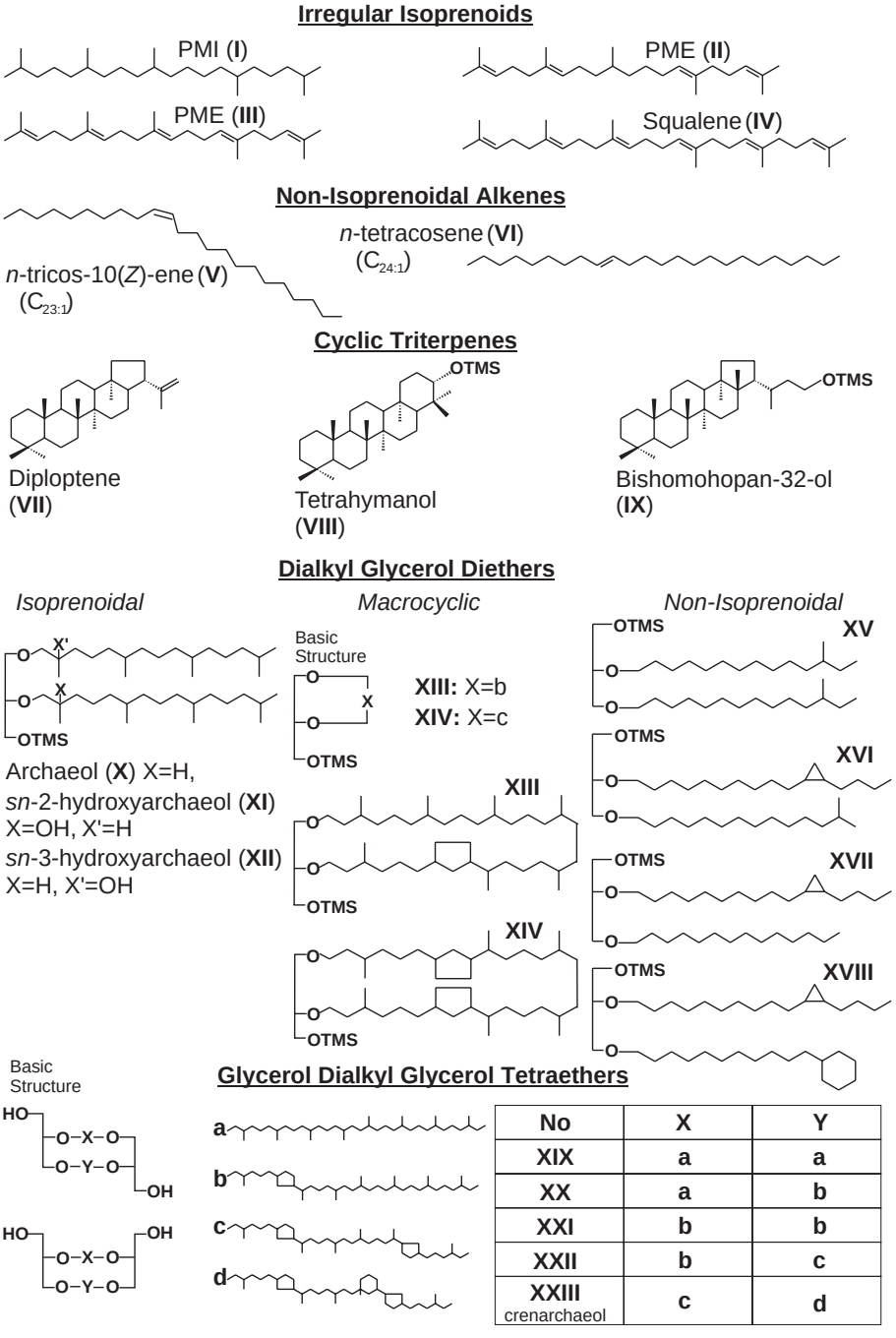
Methanotrophic microbial mats associated with carbonates were for the first time found in the deep waters (>1000 m) of the Black Sea. The combination of methane measurements, biomarker and compound-specific isotopic analysis accompanied with a survey of 16S rRNA gene sequences was performed for four methane-derived carbonates with microbial mats and mud breccias collected from the NIOZ, the Odessa and from the Kazakov MVs. Specific lipids for archaea and SRB show a good correlation between their carbon isotopic signatures and the $\delta^{13}C$ values of methane, indicating that methane is the dominant carbon source used by microorganisms for their microbial mass. Biomarker and phylogenetic studies of methane-related carbonate crusts and mud breccias show diverse bacterial and archaeal communities, suggesting a wide range of, most probably, novel groups of microorganisms that are directly or indirectly involved in anaerobic methanotrophy. The comparative analysis of archaeal sequences indicate that AOM in these settings is performed predominantly by archaeal populations affiliated with the ANME-1 group. The identified sequences do not belong to the phylogenetic clusters of known methanogenic archaea. A distinction of archaeal strains within the ANME-1 group was detected in carbonates and mud breccia. In combination with biomarker data a clear difference in archaeal assemblages was revealed within the carbonate crusts CC-1/CC-4 and the mud breccias from the Kazakov MV and carbonates CC-2/CC-3. Archaea retrieved from carbonates CC-1/CC-4 and mud breccias from the Kazakov MV predominantly biosynthesise PMEs, and archaea from crusts CC-2/CC-3 mainly produce the PMI. The existence of putative archaea/SRB consortia in the carbonate crusts is suggested by 16S rDNA and biomarker data. However, the identified SRB strains are different from those currently known in archaea/SRB AOM consortia, thus forming a new cluster of SRB within the Desulfobacteraceae family. This could indicate that archaea performing AOM are not limited to the currently known SRB partners.

Our data reveal a different intensity and duration of AOM, most probably forced by intensity of local seepage activity, pathways, and migrated products. Mud breccia from the Kazakov MV, where carbonates were not (yet) formed, uniquely demonstrated the "initial stage" of AOM. In contrast, the strongest signal of on-going AOM was obtained from carbonate crusts CC-1, CC-2 and CC-3, indicating their present formation via anaerobic methanotrophy. The methane, biomarker and 16S rRNA gene sequences data reveal that in these locations AOM processes are most active within the neoformed carbonates and are substantially reduced in the hosting mud breccias. It indicates that in these particular settings carbonate formation within the AOM zone started within the sediment corresponding to the intervals with high AOM rates, i.e. where methane and sulphate are abundant. The carbonate growth direction probably follows the pathways of migrated methane (that is, mainly laterally or/and upwards) due to the presence of anoxic environments in the Black Sea water column.

Acknowledgments

We acknowledge M. Kienhuis, E. Panoto and W.I.C. Rijpstra for technical and analytical assistance. We thank Drs. S. Schouten, M. van der Meer and J. Werne for helpful discussions and the referees, Prof. A. Boetius and Prof. C. Zhang, for their helpful comments. We are grateful to people from the UNESCO-MSU Center for Marine Geosciences from the Moscow State University (Russia) and to the captain, crew and shipboard scientific party of the Russian R/V *Professor Logachev*. This work was supported by a fellowship of the President of the Russian Federation and by a grant from the Huygens Programme of the Netherlands Organization for International Cooperation in Higher Education (Nuffic).

Appendix A



No	X	Y
XIX	a	a
XX	a	b
XXI	b	b
XXII	b	c
XXIII crenarchaeol	c	d

References

Aloisi, G., Pierre, C., Rouchyb, J.-M., Foucher, J.-P., and the MEDINAUT Scientific Party, 2000. Methane-related authigenic carbonates of eastern Mediterranean Sea mud volcanoes and their possible relation to gas hydrate destabilization. Earth Planet. Sci. Lett. 184, 321–338.

Aloisi, G., Bouloubassi, I., Heijs, S.K., Pancost, R.D., Pierre, C., Sinninghe Damsté, J.S., Gottschal, J.C., Forney, L.J., Rouchy, J.-M., 2002. CH_4-consuming microorganisms and the formation of carbonate crusts at cold seeps. Earth Planet. Sci. Lett. 203, 195–203.

Alperin, M.J., Reeburgh, W.S., 1984. Geochemical observations supporting anaerobic methane oxidation. In: Crawford, R.L., Hanson, R.S. (Eds.), Microbial Growth on C-1 Compounds. American Society of Microbiology, pp. 282–298.

Alperin, M.J., Reeburgh, W.S., 1985. Inhibition experiments on anoxic marine sediments suggests methane is not directly oxidized by sulfate-reducing bacteria. Book Monograph: 7th International Symposium on Environmental Biogeochemistry, p. 6.

Barnes, R.O., Goldberg, E.D., 1976. Methane production and consumption in anoxic marine sediments. Geology 4, 297–300.

Blumenberg, M., Seifert, R., Reitner, J., Pape, T., Michelis, W., 2004. Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. PNAS 101, 11111–11116.

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieske, A., Amann, R., Jørgensen, B.B., Witte, U., Pfannkuche, O., 2000. A marine anaerobic consortium apparently mediating anaerobic oxidation of methane. Nature 407, 623–626.

Bolshakov, A.M., Egorov, A.V., 1987. Using of phase-equilibrium degassing method for gasometric studies. Oceanology 27, 861–862.

Bouriak, S.V., Akhmetzhanov, A.M., 1998. Origin of gas hydrate accumulations on the continental slope of the Crimea from geophysical studies. In: Henriet, J.-P., Mienert, J. (Eds.), Gas Hydrates: Relevance to World Margin Stability and Climatic Change. Special Publications, vol. 137. Geological Society, London, pp. 215–222.

Bravo, J.-M., Perzl, M., Härtner, T., Kannenberg, E.L., Rohmer, M., 2001. Novel methylated triterpenoids of the gammacerane series from the nitrogen-fixing bacterium *Bradyrhizobium japonicum* USDA 110. Eur. J. Biochem. 268, 1323–1331.

Coolen, M.J.L., Hopmans, E.C., Rijpstra, W.I.C., Muyzer, G., Schouten, S., Volkman, J.K., Sinninghe Damsté, J.S., 2004. Evolution of the methane cycle in Ace Lake (Antarctica) during the Holocene: response of methanogens and methanotrophs to environmental change. Org. Geochem. 35, 1151–1167.

Comita, P.B., Gagosian, R.B., Pang, H., Costello, C.E., 1984. Structural elucidation of a unique macrocyclic membrane lipid from a new, extremely thermophilic, deep-sea hydrothermal vent archaebacterium, *Methanococcus jannaschii*. J. Biol. Chem. 259, 15234–15241.

De Rosa, M., Gambacorta, A., 1988. The lipids of archaebacteria. Prog. Lipid Res. 27, 153–175.

De Rosa, M., Gambacorta, A., Nicolaus, B., Chappe, B., Albrecht, P., 1983. Isoprenoid ethers: backbone of complex lipids of the archaebacterium *Sulfolobus solfataricus*. Biochim. Biophys. Acta 753, 249–256.

De Rosa, M., Trincone, A., Nicolaus, B., Gambacorta, A., 1991. Archaebacteria: lipids, membrane structures, and adaptation to environmental stresses. In: Di Prisco, G. (Ed.), Life Under Extreme Conditions. Springer-Verlag, Berlin, pp. 61–87.

Degens, E.T., Ross, D.A., 1972. Chronology of the Black Sea over the last 25,000 years. Chem. Geol. 10, 1–16.

Dojka, M.A., Hugenholtz, P., Haack, S.K., Pace, N.R., 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64, 3869–3877.

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

Elvert, M., Suess, E., Greinert, J., Whiticar, M.J., 2000. Archaea mediating anaerobic methane oxidation in deep-sea sediments at cold seeps of the eastern Aleutian subduction zone. Org. Geochem. 31, 1175–1187.

Ferrante, G., Ekiel, I., Patel, G.B., Sprott, G.D., 1988. A novel core lipid isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6. Biochim. Biophys. Acta 936, 173–182.

Hinrichs, K.-U., Hayes, J.M., Sylva, S.P., Brewer, P.G., De Long, E.F., 1999. Methane-consuming archaebacteria in marine sediments. Nature 398, 802–805.

Hinrichs, K.-U., Pancost, R.D., Summons, R.E., Sprott, G.D., Sylva, S.P., Sinninghe Damsté, J.S., Hayes, J.M., 2000a (May 26). Mass spectra of *sn*-2-hydroxyarchaeol, a polar lipid biomarker for anaerobic methanotrophy. Geochem. Geophys. Geosyst. 1.

Hinrichs, K.-U., Summons, R.E., Orphan, V., Sylva, S.P., Hayes, J.M., 2000b. Molecular and isotopic analysis of anaerobic methane-oxidizing communities in marine sediments. Org. Geochem. 31, 1685–1701.

Hoefs, M.J.L., Schouten, S., de Leeuw, J.W., King, L.L., Wakeham, S.G., Sinninghe Damsté, J.S., 1997. Ether lipids of planktonic archaea in the marine water column. Appl. Environ. Microbiol. 63, 3090–3095.

Hoehler, T.M., Alperin, M.J., 1996. Anaerobic methane oxidation by a methanogen-sulfate reducer consortium: geochemical evidence and biochemical considerations. In: Lidstrom, M.E., Tabita, R.F. (Eds.), Microbial Growth on C-1 Compounds. Kluwer, Dordrecht, The Netherlands, pp. 326–333.

Hoehler, T.M., Alperin, M.J., Albert, D.B., Martens, C.S., 1994. Field and laboratory studies of methane oxidation in an anoxic marine sediment: evidence for a methanogen-sulfate reducer consortium. Glob. Biogeochem. Cycles 8, 451–463.

Holzer, G., Oro, J., Tornabene, T.G., 1979. Gas chromatographic–mass spectrometric analysis of neutral lipids from methanogenic and thermoacidophilic bacteria. J. Chromatogr. 186, 795–809.

Hopmans, E.C., Schouten, S., Pancost, R.D., Van der Meer, M.T.J., Sinninghe Damsté, J.S., 2000. Analysis of intact tetraether lipids in archaeal cell material and sediments by high performance

liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Rapid Commun. Mass Spectrom. 14, 585–589.

Hovland, M., Talbout, M., Qvale, H., Olausson, S., Aasberg, L., 1987. Methane related carbonate cements in pockmarks of the North Sea. J. Sediment. Petrol. 57, 881–892.

Hugenholtz, P., Pitulle, C., Hershberger, K.L., Pace, N.R., 1998. Novel division level bacterial diversity in a Yellowstone Hot Spring. J. Bacteriol. 180, 366–376.

Inagaki, F., Suzuki, M., Takai, K., Oida, H., Sakamoto, T., Aoki, K., Nealson, K.H., Horikoshi, K., 2003. Microbial communities associated with geological horizons in coastal subseafloor sediments from the Sea of Okhotsk. Appl. Environ. Microbiol. 69, 7224–7235.

Ivanov, M.K., Limonov, A.F., Woodside, J.M. (Eds.), 1992. Geological and Geophysical Investigations in the Mediterranean and Black Seas: Initial Results of the "Training-Through-Research" Cruise of R/V Gelendzhik in the Eastern Mediterranean and the Black Sea (June/July 1991), UNESCO Rep. Mar. Sci. vol. 56, pp. 208.

Ivanov, M.K., Limonov, A., van Weering, T., 1996. Comparative characteristic of the Black Sea and Mediterranean Ridge mud volcanoes. Mar. Geol. 132, 253–271.

Ivanov, M.K., Limonov, A.F., Woodside, J.M., 1998. Extensive deep fluid flux through the see floor on the Crimean continental margin (Black Sea). In: Henriet, J.-P., Mienert, J. (Eds.), Gas Hydrates: Relevance to World Margin Stability and Climatic Change. Special Publications vol. 137. Geological Society, London, pp. 195–213.

Kenneth, E.P., Moldovan, J.M., 1993. The Biomarker Guide: Interpreting Molecular Fossils in Petroleum and Ancient Sediments. Prentice-Hall, Inc., A Simon and Schuster Company, Englewood Cliffs, New Jersey, p. 363.

Kenyon, N.H., Ivanov, M.K., Akhmetzhanov, A.M., Akhmanov, G.G. (Eds.), 2002. Geological Processes in the Mediterranean and Black Seas and North East Atlantic Preliminary Results of Investigations During the TTR-11 Cruise of RV Professor Logachev, July–September, 2001, Intergovernmental Oceanographic Commission Technical Series vol. 62, p. 114.

Kleemann, G., Poralla, K., Englert, G., Kjösen, H., Liaaen-Jensen, S., Neunlist, S., Rohmer, M., 1990. Tetrahymanol from the phototrophic bacterium *Rhodopseudomonas palustris*: first report of a gammacerane triterpene from a prokaryote. J. Gen. Microbiol. 136, 2551–2553.

Knittel, K., Boetius, A., Eilers, A.L.H., Lochte, K., Linke, O.P.P., 2003. Activity, distribution, and diversity of sulfate reducers and other bacteria in sediments above gas hydrate (Cascadia Margin, Oregon). Geomicrobiol. J. 20, 269–294.

Koga, Y., Akagawa-Matsushita, M., Ohga, M., Nishihara, M., 1993. Taxonomic significance of the distribution of component parts of polar ether lipids in methanogens. Syst. Appl. Microbiol. 16, 342–351.

Kohnen, M.E.L., Schouten, S., Sinninghe Damsté, J.S., de Leeuw, J.W., Merrit, D., Hayes, J.M., 1992. Recognition of paleobiochemicals by a combined molecular sulfur and isotope geochemical approach. Science 256, 358–362.

Kovalenko, O., Belenkaia, I., 2002. Methane-induced precipitation of authigenic carbonates in mud volcano deposits of the Black Sea. Geosphere/Biosphere/Hydrosphere Coupling Processes, Fluid Escape Structures and Tectonics at Continental Margins and Ocean Ridges. Intergovernmental Oceanographic Commission Workshop Report vol. 183, pp. 14–15.

Langworthy, T.A., 1985. Lipids of archaebacteria. In: Woese, C.R., Wolfe, R.S. (Eds.), The Bacteria VII. Academic Press, Inc., London, UK, pp. 459–497.

Madrid, V.M., Taylor, G.T., Scranton, M.I., Chistoserdov, A.Y., 2001. Phylogenetic diversity of bacterial and archaeal communities in the anoxic zone of the Cariaco Basin. Appl. Environ. Microbiol. 67, 1663–1674.

Mallory, F.B., Gordon, J.T., Conner, R.L., 1963. The isolation of a pentacyclic triterpenoid alcohol from a protozoon. J. Am. Chem. Soc. 85, 1362–1363.

Martens, C.S., Berner, R.A., 1974. Methane production in the interstitial waters of sulfate-depleted marine sediments. Science 185, 1167–1169.

Mazzini, A., Cronin, B.T., Parnell, J., 2002. Carbonate crust stratigraphy from the Black Sea. Geosphere/Biosphere/Hydrosphere Coupling Processes, Fluid Escape Structures and Tectonics at Continental Margins and Ocean Ridges. Intergovernmental Oceanographic Commission Workshop Report vol. 183, pp. 15–16.

Michaelis, W., Seifert, R., Nauhaus, K., Treude, T., Thiel, V., Blumenberg, M., Knittle, K., Gieseke, A., Peterknecht, K., Pape, T., Boetius, A., Amann, R., Jorgensen, B.B., Widdel, F., Peckmann, J., Pimenov, N.V., Gulin, M.B., 2002. Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. Science 297, 1013–1015.

Muyzer, G., De Waal, E.D., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Nishihara, M., Koga, Y., 1991. Hydroxyarchaetidylserine and hydroxyarchaetidyl-*myo*-inositol in *Methanosarcina barkeri*: polar lipids with a new ether core portion. Biochim. Biophys. Acta 1082, 211–217.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D., DeLong, E.F., 2001a. Methane-consuming archaea revealed by directly coupled isotopic and phylogenic analysis. Science 293, 484–487.

Orphan, V.J., Hinrichs, K.-U., Ussler III, W., Paull, C.K., Taylor, L.T., Sylva, S.P., Hayes, J.M., Delong, E.F., 2001b. Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. Appl. Environ. Microbiol. 67, 1922–1934.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D., DeLong, E.F., 2002. Multiple archael groups mediate methane oxidation in anoxic cold seep sediments. Proc. Natl. Acad. Sci. 99, 7663–7668.

Øvreås, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-

amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63, 3367–3373.

Pancost, R.D., Sinninghe Damsté, J.S., De Lint, S., van der Maarel, M.J.E.C., and the Medinaut Shipboard Scientific Party, 2000. Biomarker evidence for widespread anaerobic methane oxidation in Mediterranean sediments by a consortium of methanogenic archaea and bacteria. Appl. Environ. Microbiol. 66, 1126–1132.

Pancost, R.D., Bouloubassi, I., Aloisi, G., and the Medinaut Shipboard Scientific Party, 2001a. Three series of non-isoprenoidal dialkyl glycerol diethers in cold-seeps carbonate crusts. Org. Geochem. 32, 695–707.

Pancost, R.D., Hopmans, E.C., and the Medinaut Shipboard Scientific Party, 2001b. Archaeal lipids in mediterranean cold-seeps: molecular proxies for anaerobic methane oxidation. Geochim. Cosmochim. Acta 65, 1611–1627.

Paull, C.K., Neumann, A.C., 1987. Continental margin brine seeps: their geological consequences. Geology 15, 545–548.

Peckmann, J., Thiel, V., Michaelis, W., Clari, P., Gaillard, C., Martire, L., Reitner, J., 1999. Cold seep deposits of Beauvoisin (Oxfordian; southeastern France) and Marmorito (Miocene; northern Italy): microbially induced authigenic carbonates. Int. J. Earth Sci. 88, 60–75.

Peckmann, J., Walliser, O.H., Reigel, W., Reitner, J., 1999. Signatures of hydrocarbon venting in a middle Devonian carbonate mound (Hollard Mound) at the Hamar Laghdat (Antiatlas Morocco). Facies 40, 281–296.

Peckmann, J., Reimer, A., Luth, U., Luth, C., Hansen, B.T., Heinicke, C., Hoefs, J., Reitner, J., 2001. Methane-derived carbonates and authigenic pyrite from the northwestern Black Sea. Mar. Geol. 177, 129–150.

Peckmann, J., Goedert, J.L., Thiel, V., Michaelis, W., Reitner, J., 2002. A comprehensive approach to the study of methane-seep deposits from the Lincoln Creek Formation, western Washington State, USA. Sedimentology 49, 855–873.

Phelps, C.D., Kerkhof, L.J., Young, L.Y., 1998. Molecular characterization of a sulfate-reducing consortium which mineralizes benzene. FEMS Microbiol. Ecol. 27, 269–279.

Reeburgh, W.S., 1976. Methane consumption in Cariaco Trench waters and sediments. Earth Planet. Sci. Lett. 28, 337–344.

Rice, D.D., Claypool, G.E., 1981. Generation, accumulation and resource potential of biogenic gas. AAPG Bull. 65, 5–21.

Ritger, S., Carson, B., Suess, E., 1987. Methane-derived authigenic carbonates formed by subduction-induced pore-water expulsion along the Oregon/Washington margin. Geol. Soc. Amer. Bull. 98, 147–156.

Roberts, H.H., Aharon, P., 1994. Hydrocarbon-derived carbonate buildups of the northern Gulf of Mexico continental slope: a review of submersible investigations. Geo Mar. Lett. 14, 135–148.

Rohmer, M., Bouvier-Nave, P., Ourisson, G., 1984. Distribution of hopanoid triterpens in prokaryotes. J. Gen. Microbiol. 130, 1137–1150.

Rohmer, M., Bissert, P., Neunlist, S., 1992. The hopanoids, prokaryotic triterpenoids and precursors of ubiquitous molecular fossils. In: Moldowan, J.M., Albrecht, M., Philp, R.P. (Eds.), Biological Markers in Sediments and Petroleum. Prentice Hall, London, pp. 1–17.

Schäfer, H., Muyzer, G., 2001. Denaturing gradient gel electrophoresis in marine microbial ecology. In: Paul, J.H. (Ed.), Methods in Microbiology, Marine Microbiology, vol. 30. Academic Press, pp. 425–468.

Schoell, M., Schouten, S., Sinninghe Damsté, J.S., de Leeuw, J.W., Summons, R.E., 1994. A molecular organic carbon isotope record of Miocene climate changes. Science 263, 1122–1125.

Schouten, S., Van der Maarel, M.J.E.C., Huber, R., Sinninghe Damsté, J.S., 1997. 2,6,10,15,19-Pentamethylicosenes in *Methanolobus bombayensis*, a marine methanogenic archaeon, and in *Methanosarcina mazei*. Org. Geochem. 26, 409–414.

Schouten, S., Hoefs, M.J.L., Koopmans, M.P., Bosch, H.-J., Sinninghe Damsté, J.S., 1998. Structural characterization, occurrence and fate of archaeal ether-bound acyclic and cyclic biphytanes and corresponding diols in sediments. Org. Geochem. 29, 1305–1319.

Schouten, S., Hopmans, E.C., Pancost, R.D., Sinninghe Damsté, J.S., 2000. Widespread occurrence of structurally diverse tetraether membrane lipids: evidence for the ubiquitous presence of low-temperature relatives of hyperthermophiles. Proc. Natl. Acad. Sci. U. S. A. 97, 14421–14426.

Schouten, S., Wakeham, S.G., Sinninghe Damsté, J.S., 2001. Evidence for anaerobic methane oxidation by archaea in euxinic waters of the Black Sea. Org. Geochem. 32, 1277–1281.

Schouten, S., Wakeham, S.G., Hopmans, E.C., Sinninghe Damsté, J.S., 2003. Biogeochemical evidence that thermophilic Archaea mediate the anaerobic oxidation of methane. Appl. Environ. Microbiol. 69, 1680–1686.

Sinninghe Damsté, J.S., Schouten, S., Van Vliet, N.H., Huber, R., Geenevasen, J.A.J., 1997. A polyunsaturated irregular acyclic C_{25} isoprenoid in a methanogenic archaeon. Tetrahedron Lett. 38, 6881–6884.

Sinninghe Damsté, J.S., Schouten, S., Hopmans, E.C., Van Duin, A.C.T., Geenevasen, J.A.J., 2002a. Crenarchaeol: the characteristic core glycerol dibiphytanyl glycerol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota. J. Lipid Res. 43, 1641–1651.

Sinninghe Damsté, J.S., Rijpstra, W.I.C., Hopmans, E.C., Prahl, F.G., Wakeham, S.G., Schouten, S., 2002b. Distribution of membrane lipids of planktonic *Crenarchaeota* in the Arabian Sea. Appl. Environ. Microbiol. 68, 2997–3002.

Sinninghe Damsté, J.S., Rijpstra, W.I.C., Schouten, S., Fuerst, J.A., Jetten, M.S.M., Strous, M., 2004. The occurrence of hopanoids in planctomycetes: implications for the sedimentary biomarker record. Org. Geochem. 35, 561–566.

Sprott, G.D., Ekiel, I., Dicaire, C., 1990. Novel, acid-labile, hydroxydiether lipid cores in methanogenic bacteria. J. Biol. Chem. 265, 13735–13740.

Sprott, G.D., Dicaire, C.J., Choquet, C.G., Patel, G.B., Ekiel, I., 1993. Hydroxydiether lipid structures in *Methanosarcina* spp. and *Methanococcus voltae*. Appl. Environ. Microbiol. 59, 912–914.

Stadnitskaia, A., Baas, M., Ivanov, M.K., Van Weering, T.C.E., Sinninghe Damsté, J.S., 2003. Novel archaeal macrocyclic diether core membrane lipids in a methane-derived carbonate crust from a mud volcano in the Sorokin Trough, NE Black Sea. Archaea 1, 165–173.

Stahl, D.A., Amann, R., 1991. Development and application of nucleic acid probes. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics, John Wiley & Sons Ltd., Chichester, England, pp. 205–248.

Stakes, D.S., Orange, D., Paduan, J.B., Salamy, K.A., Maher, N., 1999. Cold-seeps and authigenic carbonate formation in Monterey Bay, California. Mar. Geol. 159, 93–109.

Summons, R.E., Jahnke, L.L., Hope, J.M., Logan, G.A., 1999. 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. Nature 400, 554–557.

Suess, E., Whiticar, M.J., 1989. Methane-derived CO2 in pore fluids expelled from the Oregon subduction zone. Paleogeogr. Paleoclimatol. Paleoecol. 71, 119–136.

Teske, A., Hinrichs, K.-U., Edgcomb, V., De Vera Gomez, A., Kysela, D., Sylva, S.P., Sogin, M.L., Jannasch, H.W., 2002. Microbial diversity of hydrothermal sediments in the Guaymas basin: evidence for anaerobic methanotrophic communities. Appl. Environ. Microbiol. 68, 1994–2007.

Thiel, V., Peckmann, J., Seifert, R., Wehrung, P., Reitner, J., Michaelis, W., 1999. Highly isotopically depleted isoprenoids: molecular markers for ancient methane venting. Geochim. Cosmochim. Acta 63, 3959–3966.

Thiel, V., Peckman, J., Richnow, H.H., Luth, U., Reitner, J., Michaelis, W., 2001. Molecular signals for anaerobic methane oxidation in Black Sea seep carbonates and microbial mat. Mar. Chem. 73, 97–112.

Thiel, V., Blumenberg, M., Pape, T., Seifert, R., Michaelis, W., 2003. Unexpected occurrence of hopanoids at gas seeps in the Black Sea. Org. Geochem. 34, 81–87.

Tornabene, T.G., Langworthy, T.A., Holzer, G., Oro, J., 1979. Squalanes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic “archaebacteria”. J. Mol. Evol. 13, 73–83.

Valentine, D.L., Reeburgh, W.S., 2000. New perspectives on anaerobic methane oxidation. Environ. Microbiol. 2, 477–484.

Vetriani, C., Jannasch, H.W., MacGregor, B.J., Stahl, D.A., Reysenbach, A.-L., 1999. Population structure and phylogenetic characterization of marine benthic archaea in deep-see sediments. Appl. Environ. Microbiol. 65, 4375–4384.

Von Rad, U., Rösch, H., Berner, U., Geyh, M., Marchig, V., Schulz, H., 1996. Authigenic carbonates derived from oxidized methane vented from Makran accretionary prism off Pakistan. Mar. Geol. 136, 55–77.

Wakeham, S.G., 1990. Algal and bacterial hydrocarbons in particulate organic matter and interfacial sediments of the Cariaco Trench. Geochim. Cosmochim. Acta 54, 1325–1336.

Wakeham, S.G., Lewis, C.M., Hopmans, E.C., Schouten, S., Sinninghe Damsté, J.S., 2003. Archaea mediate anaerobic oxidation of methane in deep euxinic waters of the Black Sea. Geochim. Cosmochim. Acta 67, 1359–1374.

Werne, J.P., Baas, M., Sinninghe Damsté, J.S., 2002. Molecular isotopic tracing of carbon flow and trophic relationships in a methane-supported benthic microbial community. Limnol. Oceanogr. 47, 1694–1701.

Woodside, J.M., Ivanov, M.K., Limonov, A.F. (Eds.), 1997. Neotectonics and fluid flow through seafloor sediments in the Eastern Mediterranean and Black Seas: Part II. Black Sea. Intergovernmental Oceanographic Commission Technical Series vol. 48, pp. 225 Preliminary results of geological and geophysical investigations during the ANAXPROBE/TTR-6 cruise of R/V Gelendzhik, July–August 1996.

Wuchter, C., Schouten, S., Boschker, H.T.S, Sinninghe Damsté, J.S., 2003. Bicarbonate uptake by marine Crenarchaeota. FEMS Microbiol. Lett. 219, 203–207.

Zender, A.J.B., Brock, T.D., 1979. Methane formation and methane oxidation by methanogenic bacteria. J. Bacteriol. 137, 420–432.

Zhang, C.L., Li, Y., Wall, J.D., Larsen, L., Sassen, R., Huang, Y., Wang, Y., Peacock, A., White, D.C., Horita, J., Cole, D.R., 2002. Lipid and carbon isotopic evidence of methane-oxidizing and sulfate-reducing bacteria in association with gas hydrates from the Gulf of Mexico. Geology 30, 239–242.

Zhang, C.L., Pancost, R.D., Sassen, R., Qian, Y., Macko, S.A., 2003. Archaeal lipid biomarkers and isotopic evidence of anaerobic methane oxidation associated with gas hydrates in the Gulf of Mexico. Org. Geochem. 34, 827–836.