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Aerobic and anaerobic methanotrophs in the Black Sea water column

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Summary

Inputs of CH_4 from sediments, including methane seeps on the continental margin and methane-rich mud volcanoes on the abyssal plain, make the Black Sea the world's largest surface water reservoir of dissolved methane and drive a high rate of aerobic and anaerobic oxidation of methane in the water column. Here we present the first combined organic geochemical and molecular ecology data on a water column profile of the western Black Sea. We show that aerobic methanotrophs type I are responsible for methane oxidation in the oxic water column and ANME-1- and ANME-2-related organisms for anaerobic methane oxidation. The occurrence of methanotrophs type I cells in the anoxic zone suggests that inactive cells settle to deeper waters. Molecular and biomarker results suggest that a clear distinction between the occurrence of ANME-1- and ANME-2-related lineages exists, i.e. ANME-1-related organisms are responsible for anaerobic methane oxidation below 600 m water depth, whereas ANME-2-related organisms are responsible for this process in the anoxic water column above approximately 600 m water depth.

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Introduction

Methane is a radiatively active gas which has increased in concentration in the atmosphere from 850 p.p.b. before industrialization to a level of 1.7 p.p.m. and continues to increase at approximately 1% per year (Rasmussen and Khalil, 1984; Cicerone and Oremland, 1988). Although the methane concentration in the atmosphere is small compared with CO_2 (360 p.p.m.), methane's impact as a greenhouse gas is about 24 times higher (IPPC, 2001). The ocean generally contributes only a small amount of ~5–20 Tg methane (2% of the global budget) to the atmosphere per year (Cicerone and Oremland, 1988) due to very effective aerobic and anaerobic methane oxidation processes in sediments and the water column (Reeburgh, 1996; 2003). However, if CH_4 release from marine gas hydrates and seeps on continental margins of the ocean were to overwhelm oxidation, a major alteration of global climate might occur (Katz *et al*., 1999; Dickens, 2001).

Methanotrophic communities in aquatic environments play a major role in the biogeochemical cycle of methane. An increasing number of studies show that methane is oxidized anaerobically in sediments, especially in methane-rich sediments associated with methane hydrates and methane seeps (see reviews by Reeburgh *et al*., 1991; Hinrichs and Boetius, 2002), as well as in euxinic water columns (Ward *et al*., 1987; Reeburgh *et al*., 1991). Zehnder and Brock (1979; 1980) first proposed that anaerobic oxidation of methane (AOM) might be carried out by a consortium of different groups of bacteria. There is now compelling biomarker, isotopic and microbiological evidence (e.g. Hinrichs *et al*., 1999; Boetius *et al*., 2000; Pancost *et al*., 2000; Michaelis *et al*., 2002; Nauhaus *et al*., 2002; Orphan *et al*., 2002) for a syntrophy between methane-oxidizing Archaea and sulfate-reducing bacteria. However, some methanotrophic Archaea may oxidize CH_4 without being tightly coupled to a syntrophic partner (Valentine, 2002). While methane-rich sediments often have been the focus of studies related to AOM, studies in the water column combining biomarker, isotopic and molecular ecology investigations are scarce and only one report from a freshwater environment exists (Eller *et al*., 2005).

Inputs of CH_4 from sediments, including methane seeps on the continental margin (Luth *et al*., 1999; Kruglyakova *et al*., 2002; Schubert *et al*., 2006) and methane-rich mud volcanoes on the abyssal plain of the Black Sea (Ivanov *et al*., 1998; Kruglyakova *et al*., 2002; Schubert *et al*., 2006), make the Black Sea the world's largest surface water reservoir of dissolved methane. With methane concentrations of up to 12 μM and rates of AOM in the anoxic zone averaging 2 nM day^{-1} and consuming > 99% of that CH_4 (Reeburgh *et al*., 1991), the Black Sea is an ideal environment to study the organisms responsible for pelagic methane oxidation. The main question that still needs to be solved is which organisms are responsible for anaerobic and aerobic methane oxidation in the Black Sea water column. Studies of membrane lipids in suspended particulate material from the anoxic water column revealed that methanotrophic Archaea are involved in AOM. These lipid biomarkers were strongly depleted in ^{13}C indicating the use of methane as a substrate (Schouten *et al*., 2001; Wakeham *et al*., 2003). However, there is to date only one published molecular biological report – a single sample at 305 m depth (Vetriani *et al*., 2003) – that has identified a phylotype related to the Archaea that are most likely to be involved in AOM in the water column of the Black Sea. Additionally, Durisch-Kaiser and colleagues (2005) used fluorescence *in situ* hybridization (FISH) methods to detect methanotrophic communities connected to methane seep sites in the northern Black Sea.

Here, we used an integrated microbiological and biogeochemical approach [16S ribosomal DNA (rDNA) and lipid biomarkers and their stable isotopic fingerprints] to investigate the prokaryotes involved in the aerobic and anaerobic oxidation of methane in the waters of the western gyre of the Black Sea.

Results

Oxygen and methane concentrations, isotopic composition of methane in the water column

The Black Sea is separated in an uppermost oxic to suboxic (0–200 m, depending on location) (Sorokin, 2002) and a lowermost anoxic and sulfidic water column as described previously by White and colleagues (1989) and Friederich and colleagues (1990). At station 7605 oxygen concentrations of up to 220 μM were measured from the surface down to 55 m. Below 55 m, oxygen concentrations sharply decreased and were below the detection limit (1.5 μM) from 90 m to 2130 m. Based on the oxygen concentrations, the chemocline was defined to 75–130 m.

Methane concentrations in the aerobic water column varied from 20 nM at the surface to 50 nM at 100 m and increased to ~11 μM by 500 m depth (Fig. 1). Although we have no data between 100 and 500 m, we assume that methane concentrations rise rapidly just below the chemocline (> 130 m) in the anoxic zone as described earlier by Reeburgh and colleagues (1991) and Schubert and colleagues (2006).

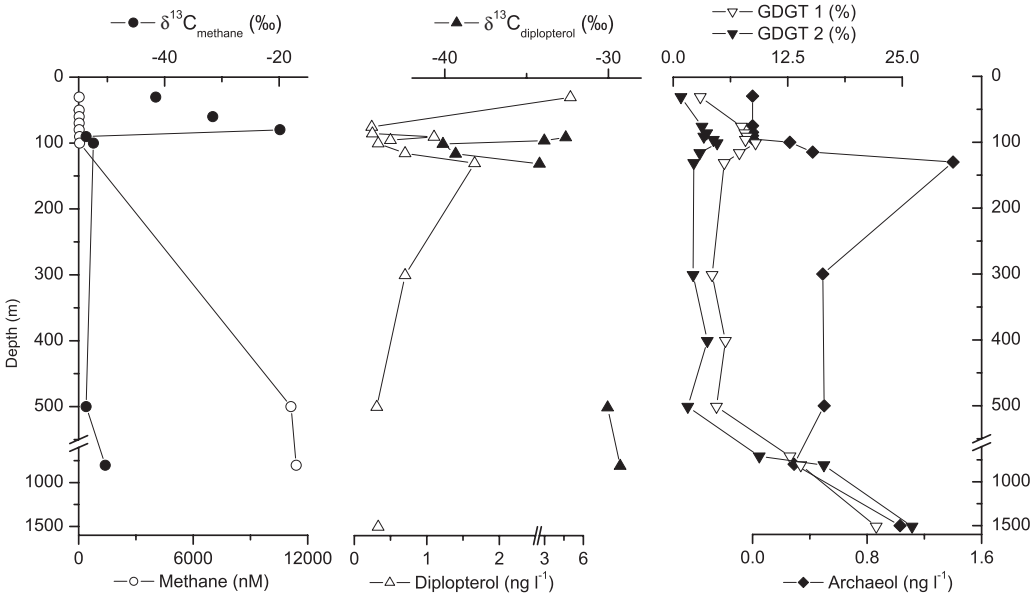

Fig. 1. Concentration (nM) and isotopic composition (‰ VPDB) of the dissolved methane in the Black Sea water column (compilation of stations 7605 and 7620). Methane concentrations increase drastically in the anoxic layer of the Black Sea water column. Diplopterol concentrations (ng l^{-1}) and the carbon isotopic composition of diplopterol (‰) in filtered particles. Monocyclic and bicyclic glycerol dialkyl glycerol tetraethers (GDGTs) in per cent of total GDGTs. Please note the relative increase of both compounds below 500 m water depth. Concentrations of archaeol (ng l^{-1}) in filtered particulate matter. Note that this compound is only detected in the lower chemocline and in the anoxic water column.

$\delta^{13}C_{CH_4}$ values in the suboxic (75–100 m) and anoxic zones varied from around –47 to –54‰ and the sample at 30 m water depth had a $\delta^{13}C_{CH_4}$ value of –42‰ (Fig. 1) close to the relatively constant global value of –47‰ for atmospheric methane (Stevens and Rust, 1982). In the suboxic zone, however, a sudden increase in $\delta^{13}C_{CH_4}$ values from –54‰ to up to –20‰ was observed. The same trend, although less pronounced (–56‰ to –51‰), was observed in the chemocline at a station located further north on the north-western shelf (data not shown).

Abundance of prokaryotes

The quantitative, real-time polymerase chain reaction (QPCR) analysis of the water column samples exhibited almost identical 16S rDNA copy numbers of total prokaryotes and Bacteria (not shown). Generally, the bacterial 16S rDNA copy numbers were at least one order of magnitude higher than the archaeal 16S rDNA copy numbers throughout the water column. Bacterial 16S rDNA copy numbers were between 1.9×10^4 and 7.1×10^4 copies ml^{-1} in the upper 80 m, whereas the copy numbers of Archaea were less than 1.8×10^3 copies ml^{-1} (Fig. 2A and C). At the upper anoxic boundary around 90 m the 16S rDNA copy numbers increased significantly, both for Bacteria (up to 2×10^5 copies ml^{-1}) and for Archaea (up to 2.3×10^4 copies ml^{-1}; Fig. 2A and C). In the layer at 80–95 m Archaea represented about 10% of the total prokaryotic community. Below the suboxic zone down to 300 m, bacterial numbers were also quite high with up to 9.5×10^4 16S rDNA copies ml^{-1}. The vertical distribution of Archaea showed a broad peak down to 300 m with a maximum at the sulfide upper boundary at 120 m where they constitute the highest relative abundance of prokaryotes (data not shown). Below 300 m, the 16S rDNA copy numbers of Bacteria and Archaea were about 4×10^4 copies ml^{-1} and $2–3 \times 10^2$ copies ml^{-1} respectively. Overall, the relative abundance of Archaea was higher in the oxic zone, in the suboxic zone and down to 300 m (between 1% and 19% of total prokaryotes) and less than 0.5% in the water column below 300 m.

Our PCR-denaturing gradient gel electrophoresis (DGGE) method to detect aerobic methanotrophic bacteria revealed the presence of three phylotypes (called BS7605/7620 Mph1_DGGE-1 to -3; Fig. 3) falling into the methanotroph type I cluster (Fig. 4). The occurrence of these sequences was limited to the deeper suboxic to anoxic water column between 95 and 300 m. At 30 and 75 m no copies could be detected, whereas at 95 m the highest relative contribution to the total bacterial community was reached (i.e. 4.2% methanotrophs type I; Fig. 2B). This relative contribution decreased substantially

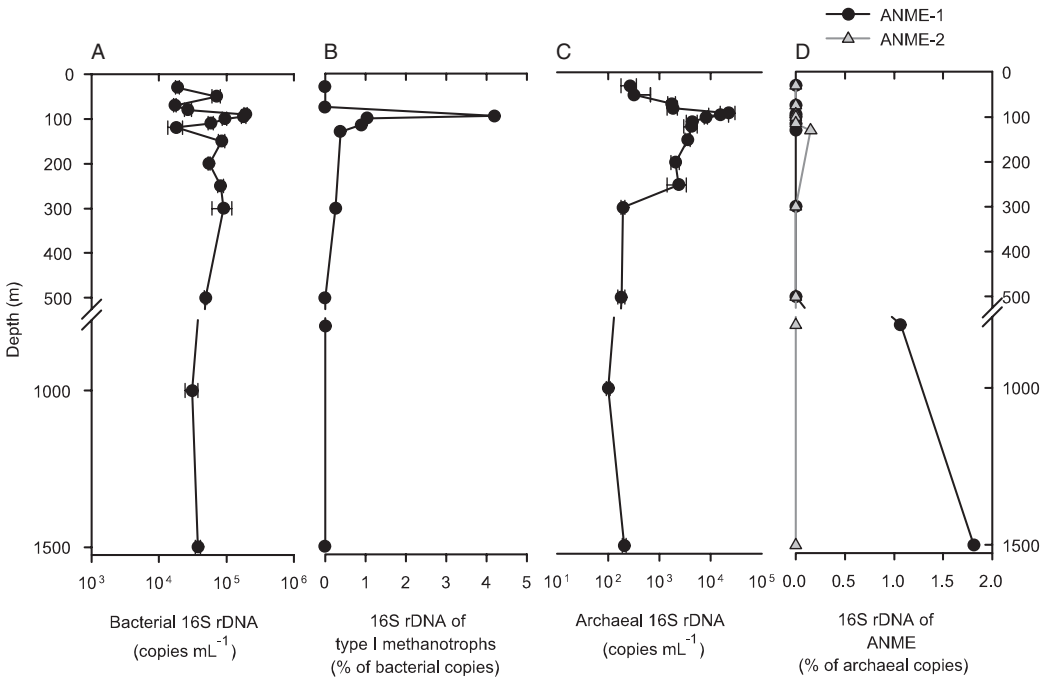

Fig. 2. Quantitative analysis of Bacteria, Archaea and prokaryotes involved in the aerobic (type I methanotrophic bacteria) and anaerobic (archaea of the ANME-1 and ANME-2 lineages) oxidation of methane.
A. 16S rRNA gene copy numbers of the domain Bacteria.
B. Abundance of type I methanotrophic bacteria (Methylococcaceae).
C. 16S rRNA gene copy numbers of the domain Archaea.
D. Abundance of ANME-1- and ANME-2-related phylotypes.

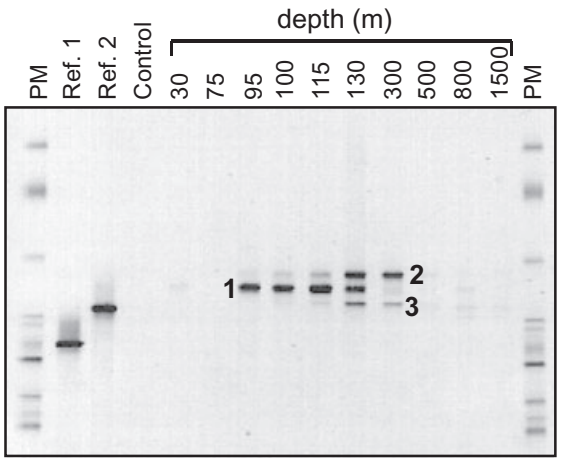

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) with 16S rDNA amplicons of type I aerobic methanotrophic bacteria (Methylococcaceae) from the Black Sea water column (Station 7605 and 7620). Sequenced DGGE fragments are numbered 1–3. For comparative analysis, PCR products of reference strains (*Methylomonas* sp. 1 and 2), and a position marker (PM) were separated by DGGE along with the sediment samples. A PCR with 15 ng of genomic DNA of *E. coli* was performed as a control for the specificity of the reactions.

to 0.2–0.3% at 130 and 300 m (Fig. 2B). Sequences of type II methanotrophs were not detected in any of the samples.

Polymerase chain reaction with a general archaeal primer followed by DGGE and sequencing of excised bands indicated that archaeal cells in the water column of the Black Sea were dominated by marine Crenarchaeota, whereas Euryarchaeota only played a minor role (data not shown). Archaea falling into the ANME-1 and ANME-2 phylogenetic clusters are held responsible for AOM in sediments from various locations (Boetius *et al*., 2000; Michaelis *et al*., 2002; Orphan *et al*., 2002) and belong to the Euryarchaeota. To increase the detection limit for ANME-related sequences a specific PCR presumably targeting only the 16S rDNA of the sequences of the ANME-1 and -2 lineages known from the National Centre for Biotechnology Information (NCBI) database was performed. Denaturing gradient gel electrophoresis analysis (Fig. 5) revealed an ANME-2-related sequence (DGGE-1; Fig. 6) at 130 m but not in the deeper water column. ANME-1-related sequences (DGGE-2 through -4; Fig. 6) were only detected at and below 800 m in the water column. In addition to the seven sequenced DGGE fragments (denoted with rectangles in Fig. 5) which represented four unique phylotypes clearly affiliated with the ANME lineages (Figs 5 and 6), 17 additional DGGE fragments were sequenced. The latter 17 sequences were all euryarchaeotal phylotypes but only distantly related (85–95% similarity) to the ANME lineages (data not shown). This was surprising because, in contrast to Thomsen and colleagues (2001) who used a universal prokaryotic reverse primer 907r in combination with the ANME-specific primer ANMEF, we performed our 'ANME'-specific PCR with primer ANMEF (called ANME-111f in our study) in combination with the general archaeal reverse primer ARC915r. Nonetheless, the broader specificity of the primers increased the chance that most of the ANME-related sequences were selected during QPCR. This primer combination was then also used for the QPCR for 16S rDNA of the ANME lineages. Based on the knowledge of the total number of archaeal copies as revealed by QPCR and the integrated intensities of the ANME-DGGE fragments (which varied between 3% and 35% of the total pixel density of the DGGE fragments in Fig. 5), we then quantified the relative abundance of ANME lineages. A similar semi-quantitative approach has been applied in previous studies (e.g. Casamayor *et al*., 2001). The ANME-related sequences were found not to be numerically important members of the archaeal community as ANME-1-related sequences comprised only 1.8% of total archaeal cells at 1500 m (assuming one to two copies per genome to express cell numbers) (Klappenbach *et al*., 2001; Fig. 2D). This number is very close to ANME-1 direct cell counts using FISH (Durisch-Kaiser *et al*., 2005). The sequence falling into the ANME-2 cluster (130 m) represented only 0.2% of the archaeal community.

Lipid biomarkers for methane oxidizers in the water column

Lipids indicative for methane-oxidizing bacteria and archaea were investigated at site 7605. In our samples, the highest diplopterol concentration presumably related to cyanobacteria was found at 30 m depth (5.0 ng l^{-1}; Fig. 1). Concentrations of diplopterol were lower (0.2–1.1 ng l^{-1}) at the chemocline and then increased to concentration of 1.7 ng l^{-1} at 130 m, followed by a decrease towards the sea floor. $\delta^{13}C$ values of diplopterol varied from –29.3‰ to –34.2‰ with the exception of the water column samples at 100 and 115 m where values were ^{13}C-depleted by approximately 5‰ with –40.1‰ and –39.3‰ respectively (Fig. 1).

Archaeol and *sn*-2-hydroxyarchaeol, two glycerol-ethers indicative of methanogenic archaea (Koga *et al*., 1998), were also detected but only in the anoxic zone. Concentrations for archaeol were low and varied from 0.3 to 1.5 ng l^{-1} (Fig. 1). *Sn*-2-hydroxyarchaeol could only be detected at a depth of 130 m (~0.1 ng l^{-1}). Due to the very low abundance of these compounds, the determination of the carbon isotopic composition was not possible.

All water column samples contained up to five different glycerol diphytanyl glycerol tetraethers (GDGTs). These archaeal membrane lipids can be derived from (hyper)-thermophilic and mesophilic crenarchaeota (Koga *et al*.,

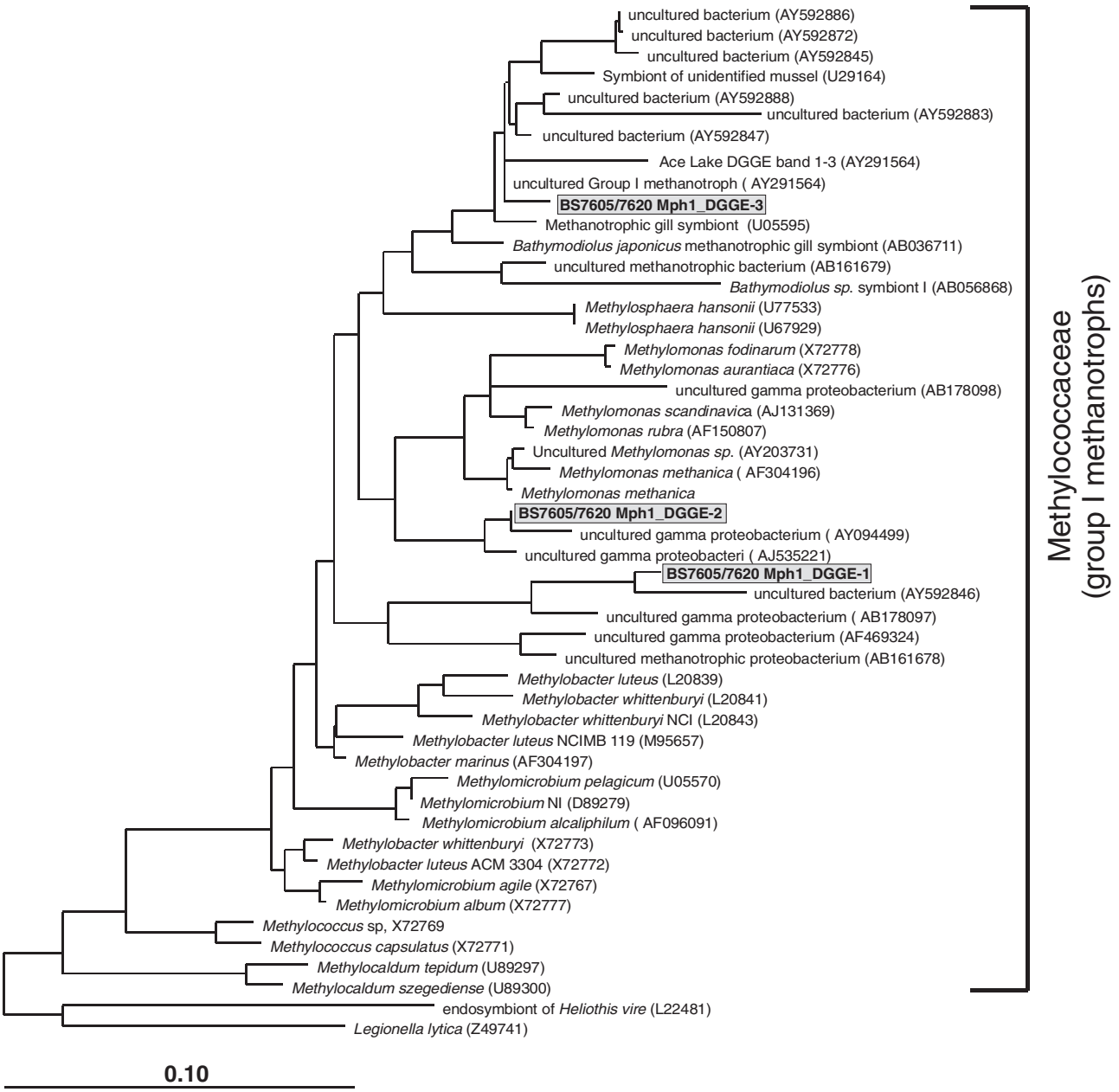

Fig. 4. Phylogenetic tree showing relationships of 16S rRNA genes of Methylococcaceae obtained from the Black Sea chemocline (text in grey rectangles) to selected cultured and environmental sequences of Methylococcaceae in the NCBI database. Two sequences of the γ-Proteobacteria subdivision (Accession No. Z49741 and L22481) were used as an outgroup.

1998; Sinninghe Damsté *et al*., 2002) and methanotrophic archaea (Pancost *et al*., 2001; Wakeham *et al*., 2003). The 30 m sample contained a relatively high abundance of GDGT-0, a non-specific archaeal GDGT, and crenarchaeol, common in pelagic marine crenarchaeota (Sinninghe Damsté *et al*., 2002; Wuchter *et al*., 2005). Glycerol diphytanyl glycerol tetraether distributions with unusually high relative abundances of GDGT-1 and -2 (i.e. possessing one and two cyclopentane rings), likely derived from archaea involved in AOM (Pancost *et al*., 2001), were observed below 700 m (Fig. 1) in agreement with previous results (Wakeham *et al*., 2003).

Discussion

Although AOM was first suggested to occur in sediments about 30 years ago (Reeburgh, 1976; 1980; Iversen and Blackburn, 1981), the type organisms responsible for this process in sediments were only recently identified (e.g. Hinrichs *et al*., 1999; Boetius *et al*., 2000). Whereas the

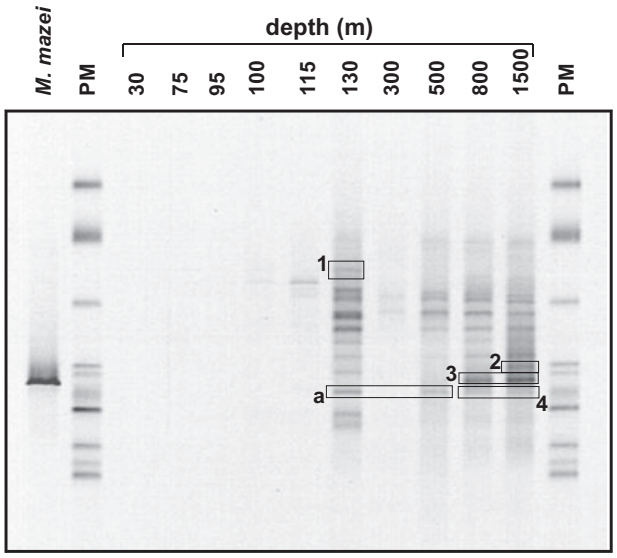

Fig. 5. Denaturing gradient gel electrophoresis (DGGE) with euryarchaeal 16S rRNA genes amplicons obtained from the Black Sea water column. The amplicons were generated with primers ANME-111f and GC-ARC915r. In total, 24 predominant DGGE fragments were sequenced and only the seven sequences found to be phylogenetically affiliated with the ANME-1 and ANME-2 lineages, assigned to methanotrophic archaea, and of which four appeared to be unique phylotypes are marked with a rectangle and numbered 1–4. The fragment 'a' melted at the same position as fragment 4 but its closest relative (98% sequence similarity) was an uncultured euryarchaeote associated with corals (AY380683). For comparative analysis, PCR products of a reference strain *Methanosarcina mazei* and position marker (PM) were separated by DGGE along with the water samples.

number of locations where AOM is known to occur in sediments is growing and more type organisms involved in this process have been suggested by means of lipid biomarkers and molecular biological tools, little is known about the community responsible for AOM in the water column. Beside a publication where microbial communities are described in the anoxic water column of the Cariaco basin (Madrid *et al*., 2001), there is only one publication (Vetriani *et al*., 2003) in which the archaeal diversity was studied in the upper 500 m of the Black Sea water column. We used a combination of molecular and organic geochemical tools to reveal which types of organisms are responsible for aerobic oxidation (mediated by bacteria) and, even more important in the Black Sea, anaerobic oxidation (mediated by archaea) of methane.

Methane oxidation in the oxic water column

Samples that were taken down to 75 m water depth are considered to be located in the fully oxic water column. Methane concentrations in this zone were rather low with values around 20 nM. The absence of archaeol and *sn*-2-hydroxyarchaeol suggests that neither methanogens nor methanotrophic archaea are present in the well-oxygenated water column (Fig. 1). Diplopterol, a constituent of aerobic bacteria and especially enriched in methylotrophic bacteria (Rohmer *et al*., 1984), was present throughout the oxic water column but showed its highest concentration at 30 m depth, where it most likely is related to cyanobacteria cells and not to methanotrophs. Due to the oxic conditions, it can be excluded that diplopterol is derived from anaerobic organisms, notably anammox bacteria, as described before (Sinninghe Damsté *et al*., 2004).

Low methane oxidation rates (10^{-3} nM day^{-1}) have previously been measured in the oxic Black Sea water column (Reeburgh *et al*., 1991; Durisch-Kaiser *et al*., 2005). However, PCR-DGGE did not detect methanotrophic bacteria at 30 and 75 m and their copy numbers were below the QPCR detection limit (less than ~200 copies l^{-1}). As methane oxidation does actually occur in the oxic water column, although at a very low rate, it should have been possible to detect the responsible organisms. Therefore, it is likely that the primers used in this study have not detected all existing aerobic methanotrophic bacteria.

Methane oxidation at the chemocline

The chemocline is one of the most important features in the Black Sea water column dividing the oxic from the anoxic water layer and acting as a zone of several biogeochemical transformations (see Sorokin, 2002 for an overview). High bacterial cell numbers in this zone (up to 2×10^5 copies ml^{-1}) in our study confirm results by Manske and colleagues (2005) and underline the involvement of microorganisms in these transformation reactions including methane oxidation.

The substantial change in isotopic composition of the dissolved methane at the chemocline underlines the importance of this zone for methane transformation (Fig. 1). The dramatic change in $\delta^{13}C_{CH_4}$ values from –54‰ just below the chemocline to up to –20‰ in the chemocline, clearly shows the involvement of microorganisms in methane oxidation, i.e. a biological fractionation where $^{12}C_{CH_4}$ is preferred over $^{13}C_{CH_4}$ (Barker and Fritz, 1981). The heaviest $\delta^{13}C_{CH_4}$ value is observed at 80 m where oxygen concentrations are below 3 μM and very low diffusion coefficients (k_z values are below 1×10^{-6} m^2 s^{-1}) results in slow methane transport from below leading to low methane concentrations (20 nM). The ^{13}C enrichment of the dissolved methane at 80 m is therefore related to the preferential microbial uptake of $^{12}C_{CH_4}$ leaving isotopically heavy methane behind. This preferential uptake of isotopically light methane also takes place in the deeper water layers but the high and constant methane flux from below hides this process.

Sequence analysis revealed that phylotypes BS7605/7620 Mph1_DGGE-1 to -3 belonged to the type I

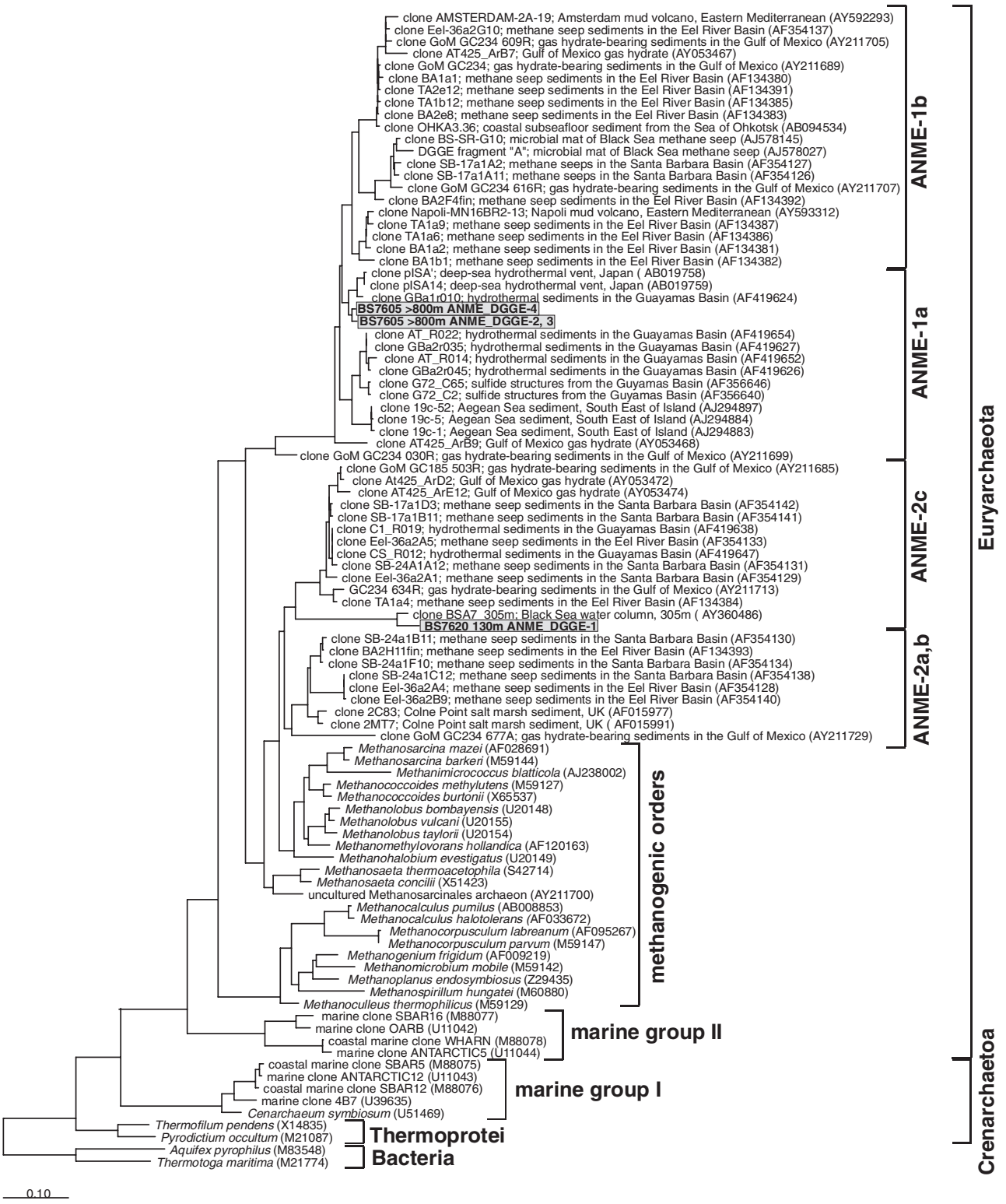

Fig. 6. Phylogenetic tree showing relationships of ANME-related 16S rRNA genes obtained from the anaerobic and sulfidic part of the water column of the Black Sea (text in grey rectangles) to selected environmental sequences of ANME-1 and ANME-2 lineages as well as cultured methanogens, environmental sequences of marine group II Euryarchaeota, and marine group I Crenarchaetoa in the NCBI database. Sequences of Thermoprotei and Bacteria were used as an outgroup.

methanotrophs (Methylococaceae; Fig. 4) and their closest relatives were *Methylosphaera hansonii* (Bowman *et al*., 1997). This methanotroph has been isolated from the suboxic water layer from the stratified, saline Ace Lake (Antarctica; Bowman *et al*., 1997) which resembles the water column of the Black Sea by the presence of high sulfide concentrations in the lower chemocline.

It is questionable whether the detected methanotrophic 'aerobic' cells at 130 m are really active as no oxygen, a prerequisite for the enzyme monooxygenase involved in aerobic methanotrophy, is present at this depth. It is therefore assumed that cells at this depth are barely functioning or that inactive cells are sinking from above. At 95 m the oxygen sensor showed an oxygen concentration of 1 μM which is the detection limit of the sensor. Measuring oxygen below 1 μM is not possible with currently available sensors and even Winkler determinations fail at these concentrations. It is therefore not possible to say whether oxygen is really present or not. Another explanation for the occurrence of methanotrophs in high amounts at this depth would be that a lateral intrusion of oxygen was just sufficient to support this community. Nevertheless, methanotrophy and other redox reactions kept the oxygen concentrations at very low, almost undetectable values.

In the lower part below hundred metres a correspondence in shapes of the profiles of the abundance of methanotrophs and diplopterol concentrations suggests that methanotrophs were a major source for diplopterol within these layers. This is supported by the isotopic composition of diplopterol at 130 m water depth which was about 3‰ depleted compared with the values at 90, 500 and 800 m water depth, suggesting that some of the diplopterol-producing organisms used isotopically light methane for biomass production. Based on the isotopic composition, the real zone of methane oxidation where diplopterol shows very light carbon isotopic values seems to be at 100 and 115 m water depth. Here, the ^{13}C values reached –39.3‰ and –40.1‰ and were therefore up to 10 per mil lighter than above and below the chemocline. Nevertheless, it cannot be excluded that chemoautotrophic bacteria use ^{13}C-depleted dissolved inorganic carbon (DIC) from these water depths (cf. Freeman *et al*., 1994).

In conclusion, the phylogenetic position of our methanotrophic sequences as well as the concomitant presence of lipid biomarkers with depleted isotopic signatures indicative for a methanotrophic metabolism all strongly suggests that methanotrophic bacteria are responsible for methane oxidation through the chemocline down to 115 m.

Methane oxidation in the anoxic water column

Compounds of archaeal origin that have been found associated with methane seep sediments include acyclic isoprenoids 2,6,11,15-tetramethylhexadecane (crocetane), 2,6,10,15,19-pentamethylicosane (PMI) and unsaturated PMI analogues, 3,7,11,15-tetramethylhexadecanol (phytanol), archaeol, *sn*-2-hydroxyarchaeol and GDGTs (see Hinrichs and Boetius, 2002 for an overview). We have concentrated our work on the last three compounds as they have also been detected in some cultured archaea, although methanogens. Archaeol and *sn*-2-hydroxyarchaeol have been described for methanogens by Koga and co-workers (e.g. Koga *et al*., 1993; 1998) with *sn*-2-hydroxyarchaeol being more specific than archaeol, i.e. it does not occur in all investigated methanogens but is restricted to some species.

Both biomarkers are related to processes occurring in the anoxic water column as underlined by the fact that they were not detected in the oxic zone (above 95 m). Methanogenesis in the water column is unlikely as sulfate reducers, which are abundant and active in the Black Sea water column (Albert *et al*., 1995; F.S. Lucas and C.J. Schubert, unpublished), would out-compete methanogens for substrate (see also Reeburgh *et al*., 1991). This strengthens the argument that in our study archaeol and *sn*-2-hydroxyarchaeol are derived from methanotrophic and not from methanogenic archaea.

A phylotype of the ANME-2c cluster recovered from the base of the chemocline at 130 m depth is closely related to the clone found by Vetriani and colleagues (2003) at 305 m in the Black Sea water column. Additionally, the incidence of *sn*-2-hydroxyarchaeol detected only at 130 m underlines the occurrence of the ANME-2 phylotype as high amounts of *sn*-2-hydroxyarchaeol relative to archaeol were found in organisms belonging to the ANME-2 lineages (Blumenberg *et al*., 2004; Pape *et al*., 2005).

Another group of organic molecules (GDGTs), the core membrane lipids of diverse taxa of Archaea, has been detected in sediments and water columns where AOM occurs (Pancost *et al*., 2001; Schouten *et al*., 2001; Wakeham *et al*., 2003). Whereas the relative contribution of GDGT-1 and -2 to total GDGTs was below 9% down to 500 m it strongly increased at and below 700 m to up to 26% at 1500 m; a typical distribution for environments where AOM is mediated by archaea (e.g. Pancost *et al*., 2001; Wakeham *et al*., 2003). The co-occurrence of this typical GDGT distribution with ANME-1 phylotypes in Black Sea deep waters agrees with findings by Blumenberg and colleagues (2004) who showed that in a carbonate reef related to AOM, ANME-1 phylotypes contained GDGT-1 and -2, whereas ANME-2 phylotypes lacked these molecules. Combining our biomarker and DGGE results it seems that we are dealing with two distinct communities living in different water depths. As our sample resolution is not high enough (one biomarker sample each at 500 and 700 m) we can only estimate that one community (ANME-2) lives above approximately 600 m

and the second community (ANME-1) below 600 m. This is supported by the finding that three out of four sequences were affiliated with the ANME-1a group (closely related to strains from deep sea hydrothermal vents off Japan and sediments in the Guayamas Basin) (Takai and Horikoshi, 1999; Teske *et al*., 2002) occurring only at depths greater than 800 m (Figs 2, 5 and 6).

The occurrence of two different communities involved in AOM as suggested here by biomarker data and molecular (DGGE and sequencing) work is in agreement with earlier interpretations of biomarker results from a central Black Sea station (Wakeham *et al*., 2003) where GDGT-1 and -2 were ^{13}C depleted down to –67‰ clearly indicating that these compounds derived from organisms involved in AOM.

Cell-specific methane oxidation rates of AOM consortia varied from 0.13 to 0.28 fmol day^{-1} in an *in vitro* study of an ANME-1 dominated bacterial mat from the Black Sea (Nauhaus *et al*., 2005), sediments at a North Sea seep site (Niemann *et al*., 2005) and a cold seep at Hydrate Ridge (Boetius *et al*., 2000; Treude *et al*., 2003). In a rough calculation we estimated, based on the number of ANME-1 and ANME-2 cells and a methane oxidation rate of about 2 nM day^{-1} (Reeburgh *et al*., 1991), a more than 1000 times higher cell-specific oxidation rate. This unrealistic high number shows that there must be other methane-oxidizing archaea in the water column that were not detected by our approach. In contrast, to findings by Blumenberg and colleagues (2004) and Pape and colleagues (2005) suggesting ANME-1 to be linked to moderate and ANME-2 to high methane partial pressure we find ANME-1-related organisms in the deep water of the Black Sea with methane concentrations (~11 μM) and ANME-2-related organisms where much lower methane concentrations (~50 nM) prevail. As the former study was performed in sediments at a seep location the results might not be directly comparable to our Black Sea water column results. In any case, higher resolution studies are necessary to really identify the water depth where the communities get divided by whatever process or better environmental conditions like, for instance, methane concentrations.

In conclusion, we present here the first combined biomarker and molecular ecology study of the Black Sea water column with respect to methane biogeochemistry. The decreasing concentration and isotopic composition of methane showed that the chemocline plays a key role in methane transformation, although methane oxidation rates are highest in the anoxic water column. Processes in the chemocline seem strongly regulated by the diffusion of methane from the deeper water column. In the uppermost water column down to 75 m we could not detect any methanotrophs with the selected gene probes, although methane oxidation rates suggest that, while low, oxidation takes place. It seems that none of the available gene probes was able to detect the responsible organisms. In the chemocline from 95 m down to 130 m cell counts using specific gene probes revealed that methanotrophic cells belong to type I methanotrophs (Methylococaceae) representing 0.3–4% (mainly below 1%) of total bacterial cells.

In the anoxic water column four sequences were phylogenetically affiliated with the ANME-1 and ANME-2 lineages, assigned previously to methanotrophic archaea. ANME-1 cells were only found below 600 m with increasing amounts (up to 2% of total archaeal cells) towards the bottom of the Black Sea. ANME-2 cells were only found at 130 m in low amounts. It seems that two distinct communities responsible for AOM in the anoxic water column of the Black Sea are acting at different water layers, a result that is also confirmed by the biomarker distribution over the water column. Unrealistic high specific cell rates suggest that there must be other methane-oxidizing communities living in the anoxic water column still to be discovered.

Experimental procedures

Sampling

During cruise M51-4 of the German research vessel *R/V Meteor* in December 2001, the water column of the Black Sea was sampled at stations 7605 (42 30,71′N, 30 14,69′E) and 7620 (42 55,56′N, 30 03,65′E) located in the western central basin. Oxygen profiles were recorded using a CTD system equipped with an oxygen sensor that was calibrated by manual Winkler titration (in duplicate) of 100 ml water samples collected from four depths throughout the water column for each station. Water samples were taken with a rosette system equipped with 10 l Niskin bottles. Suspended particulate organic matter for lipid analyses was collected from specific water depths (30, 75, 85, 90, 95, 100, 115, 130, 300, 500, 800 and 1500 m) by *in situ* pump filtration of up to 1000 l of water through 142-mm-diameter glass fibre filters (GFF; nominal pore size 0.7 μm, pre-combusted at 370°C). Filters were kept frozen (–20°C) until extraction. Additionally for the molecular biological analysis particulate organic matter (POM) from 3 to 5 l of seawater has been filtered on Sterivex filters.

Methane concentration and isotopic composition

For methane measurements, 120 ml serum bottles were filled from the Niskin bottle directly after the rosette came on board. The water samples were poisoned with NaOH pellets, crimped immediately with a butyl-rubber stopper, and kept in the dark at 4–8°C. In the ship laboratory, a 20 ml helium headspace was introduced and equilibration between both phases was achieved at 25°C. Quantification of methane was accomplished by injecting 1–5 ml of headspace from the serum vials into a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector. Injector

temperature was 200°C and the detector was at 225°C. The column, 6′ × 1/8′ stainless steel packed with Poropak Q (80/100 mesh), was maintained at 40°C. The carrier gas was N_2 flowing at 25 ml min^{-1}, and the retention time for CH_4 was about 0.7 min. Peak areas were quantified with an HP 3396 Series II electronic integrator. Known amounts of standard gas (Scotty, Supelco) were injected in quadruplicate and served for quantification. Analytical precision was ±5%.

The methane isotopic composition ($\delta^{13}C_{CH_4}$ against VPDB) on water samples was determined by a method described earlier (Sansone *et al*., 1997).

Extraction of total DNA

At MPI (Max Planck Institute for Marine Microbiology) total DNA for the quantification of total prokaryotes, Bacteria and total Archaea was extracted from Sterivex filters following the procedure described by Massana and colleagues (1997). DNA was purified using Microcon YM-100 filters (Millipore, Bedford, MA, USA) and quantified with a NanoDrop ND-1000 UV/Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

At NIOZ (Royal Netherlands Institute for Sea Research) total DNA for quantitative PCR of methanotrophs, ANME-1 and ANME-2 Archaea, DGGE, and phylogenetic analyses was extracted from a section (2 cm diameter) of each GFF filter using the UltraClean Soil DNA Kit following the descriptions of the manufacturer (Mobio, Carlsbad, CA, USA). The sections of the filters used for DNA extraction contained POM from 4 to 7 l of filtered water. Prior to extraction, the filters were sliced with a sterile scalpel in order to enhance the extraction efficiency. The total DNA extract for each POM sample was quantified with the fluorescent dye PicoGreen (MoBiTec, Göttingen, Germany).

Quantitative real-time PCR (QPCR)

Quantification of total prokaryotes, Bacteria and Archaea based on 16S rDNA extracted from the Sterivex filters was performed using an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA, USA). Total prokaryotes were quantified using probe Uni516F 5′FAM-TGYCAGCMGCC GCGGTAAHACVNRS-TAMRA3′ and primer sets UNI 340F 5′-CCTACGGGRBGCASCAG-3′ and UNI 806R 5′-GGAC TACNNGGGTATCTAAT-3′ and PCR conditions described by Takai and Horikoshi (2000). Archaea were quantified using probe Arch 516F 5′FAM-TGYCAGCCGCCGCGGTAAHAC CVGC-TAMRA3′ and primer sets ARCH 349F 5′-GYGCAS CAGKCGMGAAW-3′ and ARCH 806R 5′-GGACTACV SGGGTATCTAAT-3′ and PCR conditions described by Takai and Horikoshi (2000). Bacteria were quantified using probe 5′FAM- CGTATTACCGCGGCTGCTGGCAC-TAMRA3′ and primer set 331F 5′-TCCTACGGGAGGCAGCAGT-3′ and 797R 5′-GGACTACCAGGGTATCTAATCCTGTT-3′ and PCR conditions described by Nadkarni and colleagues (2002). Quantitative real-time PCR Master Mix (Eurogentec, Seraing, Belgium) was used for all assays. Polymerase chain reaction products obtained from DNA of pure culture cell extracts (*Geobacter metallireducens* – for total prokaryotes and Bacteria and *Methanohalobium* sp. – for total Archaea) were used as DNA quantification standards. Real-time PCR runs were analysed using sequence detection software (SDS) of Applied Biosystems. The amplification efficiency of each real-time PCR reaction was verified with a software LinRegPCR v.7.2 (Ramakers *et al*., 2003).

The copy numbers of 16S rDNA of type I methane-oxidizing bacteria (AOB) and 16S rDNA affiliated with the ANME-1 and ANME-2 lineages were determined using an iCycler system (Bio-Rad). A total of 40 cycles were run with PCR conditions and reagents as described previously (Coolen *et al*., 2006) but with annealing temperatures and primer combinations as follows: for the quantification of 16S rDNA of type I AOB the primer set MphI-703r (*Escherichia coli* positions 686–703; 5′-GGT GTT CCT TCA GAT CTC-3′; Coolen *et al*., 2004) and Bac341r (*E. coli* positions 341–357; 5′-CCT ACG GGA GGC AGC AG-3′; Muyzer *et al*., 1993) was used and the annealing temperature was set to 59°C. Archaeal phylotypes of the ANME-1 and 2 lineages were quantified using the primers ANME111f (*E. coli* positions 111–128; 5′-GGC TCA GTA ACA CGT GGA-3′ similar sequence as the previously described primer ANMEF; Thomsen *et al*., 2001) and the general archaeal primer ARC915r (*E. coli* positions 915–934; 5′-GTG CTC CCC CGC CAA TTC CT-3′; Stahl and Amann, 1991) with the annealing temperature set to 64°C. Fluorescently measured (Picogreen, Molecular Probes) exact concentrations (10 ng) of template DNA from the filtered samples was added to the reaction mixtures. Accumulation of newly amplified double-stranded gene products was followed online as the increase in fluorescence due to the binding of the fluorescent dye SYBRgreen (Molecular Probes). Calibration of the samples was performed with known copies (between 10^{-2} and 10^{7}) of *Methylomonas sp.1* (16S rDNA of type I methanotrophs) or *Methanosarcina mazei* (ANME 16S rDNA). As a control of the specificity of the QPCR, the runs were repeated with only 32 cycles so that most amplicons reached the threshold cycle. In addition, 1 μl of the first reaction with 32 cycles was added to a fresh mixture of PCR ingredients and run for 15 cycles but this time with primers including a 40-bp-long GC clamp (Muyzer *et al*., 1993) attached to Bac341f or ARC915r to allow subsequent DGGE analysis. Aliquots of these QPCR products were run on an agarose gel in order to identify unspecific PCR products such as primer dimers or fragments with unexpected fragment lengths. Sequence analysis (Figs 4 and 6) of the excised DGGE fragments revealed the diversity of the amplicons generated by QPCR.

Denaturing gradient gel electrophoresis (DGGE) and sequence analysis

Polymerase chain reaction products of type I methanotrophs and phylotypes of the ANME-1 and -2 lineages were separated by DGGE (Muyzer *et al*., 1993). The gels contained a linear gradient of denaturant of 20–70% for 16S rDNA fragments of type I methanotrophs and 30–60% for the ANME 16S rDNA fragments. All steps performed prior, during and after DGGE including sequence analysis of excised DGGE fragments have been described previously (Coolen *et al*., 2006).

Phylogenetic analysis

Sequence data were compiled by using ARB software (Ludwig *et al*., 2004) and aligned with complete length sequences

of closest relatives obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/) using the ARB FastAligner utility. Using ARB, the phylogenetic trees shown in Figs 4 and 6 were first generated with the aligned, almost complete length sequences of closest relatives from the NCBI database using the neighbour-joining method (Saitou and Nei, 1987) and the Jukes and Cantor correction (Jukes and Cantor, 1969). Then the short aligned DGGE sequences were added to the trees using the maximum parsimony option implemented in ARB. Sequences obtained in this study have been deposited in the NCBI sequence database under Accession No. DQ507395 to DQ507400.

Lipid biomarker analysis

The GFFs were extracted for 24 h in a Soxhlet apparatus to obtain the total lipid extracts. Aliquots of the total extracts were saponified after addition of internal standards and separated into fatty acid and neutral (non-saponifiable) lipid fractions. The fatty acid fractions were methylated (BF3-MeOH, Sigma) and the neutral fractions were silylated (BSTFA, Sigma). Both fractions were analysed by gas chromatography and gas chromatography-mass spectrometry for the quantification and identification of biomarkers respectively. The chromatographic column in both systems was a DB5 (50 m, 0.25 mm, 25 μm) and the temperature programme was 80°C (1 min), 20°C min^{-1} to 130°C, and 4°C min^{-1} to 320°C (20 min). The isotopic composition ($\delta^{13}C$ against VPDB) of compounds of the neutral fraction was measured with a GC connected via a combustion interface to a mass spectrometer. $\delta^{13}C$ values were corrected for the carbon atoms introduced during derivatization.

Aliquots of total extracts were analysed for intact GDGTs by high-performance liquid chromatography-atmospheric pressure-positive ion chemical ionization mass chromatography as described previously (HPLC/APCI-MS) (Hopmans *et al*., 2000).

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