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Evidence of Intense Archaeal and Bacterial Methanotrophic Activity in the Black Sea Water Column

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In the northwestern Black Sea, methane oxidation rates reveal that above shallow and deep gas seeps methane is removed from the water column as efficiently as it is at sites located off seeps. Hence, seeps should not have a significant impact on the estimated annual flux of ~4.1×10^9 mol methane to the atmosphere [W. S. Reeburgh, B. B. Ward, S. C. Wahlen, K. A. Sandbeck, K. A. Kilatrick, and L. J. Kerkhof, Deep-Sea Res. 38(Suppl. 2):S1189–S1210, 1991]. Both the stable carbon isotopic composition of dissolved methane and the microbial community structure analyzed by fluorescent in situ hybridization provide strong evidence that microbially mediated methane oxidation occurs. At the shelf, strong isotope fractionation was observed above high-intensity seeps. This effect was attributed to bacterial type I and II methanotrophs, which on average accounted for 2.5% of the DAPI (4′,6′-diamidino-2-phenylindole)-stained cells in the whole oxic water column. At deep sites, in the oxic-anoxic transition zone, strong isotopic fractionation of methane overlapped with an increased abundance of *Archaea* and *Bacteria*, indicating that these organisms are involved in the oxidation of methane. In underlying anoxic water, we successfully identified the archaeal methanotrophs ANME-1 and ANME-2, each of which accounted for 3 to 4% of the total cell counts. ANME-1 and ANME-2 appear as single cells in anoxic water, compared to the sediment, where they may form cell aggregates with sulfate-reducing bacteria (A. Boetius, K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Giesecke, R. Amann, B. B. Jørgensen, U. Witte, and O. Pfannkuche, Nature 407:623–626, 2000; V. J. Orphan, C. H. House, K.-U. Hinrichs, K. D. McKeegan, and E. F. DeLong, Proc. Natl. Acad. Sci. USA 99:7663–7668, 2002).

Methane is the most abundant hydrocarbon in the atmosphere and, as an aggressive greenhouse gas, plays an important role in the Earth's radiative balance (33; http://www.ipcc.ch). Anaerobic sediments are the major source of methane in the marine environment and contain large deposits of methane gas hydrates (23). Thus, our recent climate would change dramatically if the ocean released the methane stored as gas hydrates in sediments into the atmosphere (23). However, through anaerobic oxidation, methane is >98% oxidized microbially, which prevents large fluxes of methane from the sediments to the atmosphere (35).

In sediments, anaerobic oxidation of methane occurs syntrophically with sulfate reduction (6, 44, 46). Boetius et al. (6) have described a methanotrophic consortium of *Archaea* (ANME-2) and sulfate-reducing bacteria (SRB). Methantrophic ANME-1 cells were found to also form monopecific archaeal cell aggregates or loose consortia with SRB (26, 29). Several studies have documented that microbial oxidation of methane occurs in the anaerobic water column (15, 20, 34, 37, 45), suggesting that this may be another significant sink for methane in oceanic systems. Measurements of the isotopic carbon composition of water column methane (41) and of archaeal biomarkers (39, 49) suggest that archaeal methanotrophs are mainly responsible for methane removal in the anaerobic water column. Oxidation of methane in the aerobic water column has received similar amounts of attention compared to anaerobic oxidation, although for the most part the concentrations are low in the oxic fraction (16, 34, 36, 45, 50).

It is interesting that although microbial oxidation of methane occurs in the whole water column, the microbial community mediating this pathway has scarcely been investigated. Using immunofluorescence, Gal'chenko et al. (14) showed that *Methylobacter*, *Methylocystis*, *Methylomonas*, *Methylosinus*, and *Methylococcus* are the dominant genera that mediate the aerobic oxidation of methane in the Black Sea water column. In a more recent study, Vetriani et al. (47) identified methanotrophic *Archaea* that are related to the phylotypes of the ANME groups from one anaerobic water sample from the Black Sea using terminal restriction fragment length polymorphism. Using other methods, such as fluorescent in situ hybridization (FISH), allows species-specific quantitative detection of methanotrophic organisms.

The Black Sea is the world's largest meromictic marine basin having an anoxic water column below a permanent halocline; today it is at a depth of 90 to 170 m (41). The water column methane concentrations are fairly stable below the halocline (~11 μM) and generally decrease to 1 to 4 nM in surface water (34). In the northwestern Black Sea, there are numerous shallow seeps (depths, 35 to 800 m), which emit methane. Also, off the shelf (>1,500 m), deep active seeps have been detected (8, 17, 38). These seeps contribute massive but unknown amounts of methane to the Black Sea water column. Because no water column microbiological studies have ever been conducted for gas seeps, it is not known how intense methane release triggers oxidation by microbes or determines the distribution of methanotrophs in the water column.

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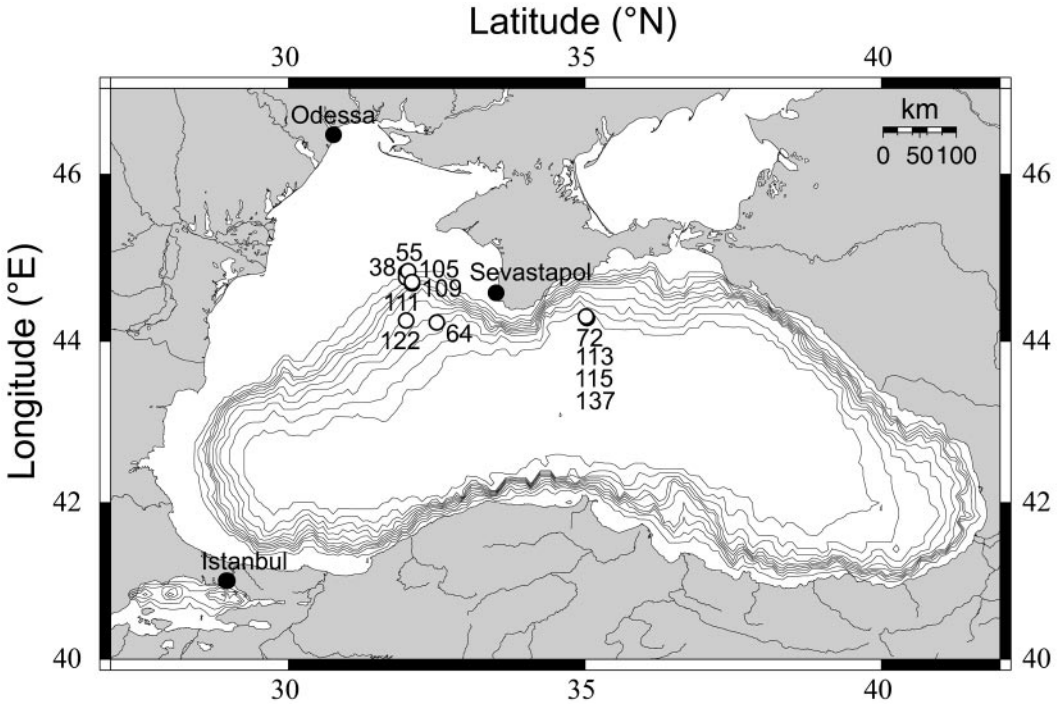

FIG. 1. Black Sea map showing the sampling stations from the CRIMEA cruises in 2003 and 2004. Sites CTD-38, -55, -64, and -72 were sampled during the CRIMEA cruise in 2003, and sites CTD-105, -109, -111, -122, and -137 were sampled during the CRIMEA cruise in 2004.

In this study, samples were collected from the oxic and anoxic portions of the water column of the northwestern Black Sea (Fig. 1). Water column methane concentrations, the oxidation rates of methane, and the stable carbon isotopic composition of methane were used to describe transformation of methane above high-intensity methane seeps compared to that at nonseep (reference) sites. Furthermore, this study was designed to characterize the aerobic and anaerobic methanotrophic microbial communities in the seep-influenced and reference water columns. We also related the microbial community composition to methane concentrations and isotopic signatures in order to identify groups of microorganisms responsible for the removal of methane in the Black Sea water column.

MATERIALS AND METHODS

Sampling. Water samples were collected with the R/V *Professor Vodyanitsky* on two expeditions to the northwestern Black Sea in June 2003 and 2004 (Fig. 1). Samples were collected at shallow and deep plume sites, as well as shallow and deep reference sites (Table 1). The deep plume site sampled in 2003 was located close to the newly identified Vodyanitsky mud volcano (38). For chemical and microbiological analyses, water was collected with a rosette sampler equipped with gas-tight water bottles.

Methane concentration and stable ^{13}C isotopic ratio. Water samples were withdrawn immediately after collection using gas-tight bottles (2 liters), and methane concentrations were measured using a modification of the vacuum degassing method described by Lammers and Suess (25) and Rehder et al. (36). Water samples (1,600 ml) were injected into preevacuated 2,200-ml glass bottles, which led to almost quantitative degassing. The gas phase was subsequently recompressed to atmospheric pressure, and the methane concentration in the extracted gas was determined by gas chromatography. A Shimadzu GC14A gas chromatograph equipped with a flame ionization detector was used in connection with a Shimadzu CR6A integrator. Nitrogen was used as the carrier gas, and separation was performed using a 4-m 1/8′ SS column packed with Porapack Q (50/80 mesh) running isothermally at 50°C. The analytical precision was 1.5 to 2%.

For stable carbon isotope measurement of methane, water was transferred in duplicate into 120-ml crimp seal bottles that were capped with gas-tight butyl rubber stoppers. Samples were poisoned by adding 50 μl of a 50 mM $HgCl_2$ solution and stored upside down for onshore analysis. The methane isotopic carbon signature was measured with an Isoprime isotope ratio mass spectrometer linked to a trace gas preconcentrator (GV Instruments). Oxidation of methane to carbon dioxide was performed with copper oxide (CuO) at 950°C. Isotopic compositions are reported in δ notation relative to PeeDee Belemnite (VPDB) (IAOA, Vienna, Austria). The ^{13}C isotope ratios of the samples were determined by comparison with a 1% CH_4 lab standard having a known isotopic composition relative to the VPDB standard. The reproducibility of the analysis was typically ±0.8‰, and at methane concentrations of <1 μM it was ~2‰.

Total organic carbon concentration. Water samples (10 ml) were collected with acid-rinsed PP tubes, acidified with 25% phosphoric acid to a pH of approximately 3, and stored capped at 4°C. After 5 min of sparging, the total organic carbon concentrations were determined by high-temperature catalytic oxidation with a Shimadzu 5050A analyzer (3).

TABLE 1. Sampling stations and their geographic positions, depths, and site characteristics from the CRIMEA cruises in 2003 and 2004

Site	Geographic position	Depth (m)	Description
CTD-38	44° 50′N, 31° 59′E	92	Seep
CTD-72	44° 17′N, 35° 02′E	1,985	Seep
CTD-105	44° 46′N, 31° 59′E	192	Seep
CTD-109	44° 42′N, 32° 05′E	606	Seep
CTD-115	44° 17′N, 35° 02′E	2,043	Seep
CTD-137	44° 17′N, 35° 02′E	2,088	Seep
CTD-55	44° 51′N, 32° 01′E	76	Reference
CTD-64	44° 14′N, 32° 30′E	1,685	Reference
CTD-111	44° 43′N, 32° 05′E	616	Reference
CTD-113	44° 18′N, 35° 01′E	2,051	Reference[a]
CTD-122	44° 45′N, 31° 59′E	265	Reference

[a] This reference site was located near a methane plume (Fig. 1).

Methane oxidation rate. Water samples were transferred bubble-free in triplicate to crimp seal bottles (20 ml) that were sealed gas tight. For determination of water column methane oxidation rates, one control sample for each triplicate sample was killed with concentrated formaldehyde, and then all samples were labeled by using tritiated methane ([^{3}H]methane), as described by Valentine et al. (45). The samples were incubated for 4 days in the dark and at an in situ water temperature imitating natural conditions.

After incubation, 5 ml of each incubated water sample was mixed with 10 ml of scintillation cocktail (Ultima Gold; Packard) and counted using the external standard ratio technique (Tri-Carb; 1600 CA; Packard) to determine the total amount of tracer added. Subsequently, the remaining water samples were kept uncovered for 24 h and bubbled with nitrogen to remove all unreacted tritiated methane which was not incorporated. A 5-ml aliquot of each bubbled water sample was again mixed with 10 ml scintillation cocktail and counted to obtain oxidation rates.

Analyses of bacterial abundance and community structure by FISH. Water samples were collected in acid-cleaned 100-ml high-density polyethylene bottles, preserved with concentrated formaldehyde, and stored in the dark at 4°C until filtration. From each preserved sample, 20- and 50-ml portions were filtered onto polycarbonate filter (pore size, 0.2 μm; diameter, 25 mm; Millipore) and stored frozen at −20°C. Bacterial abundance was determined by epifluorescence microscopy (Zeiss HBO50 Axioscope; magnification, ×1,000) of DAPI (4′,6-diamidino-2-phenylindole)-stained cells (32). FISH was performed on the filters using the protocol of Pernthaler et al. (30) and Orphan et al. (28, 29). Microscopy for FISH was also performed with a Zeiss Axioscope microscope equipped with filters for Cy3 and DAPI.

The following oligonucleotide probes (MWG Biotech AG) were used to describe the microbial communities: EUB338 (16S rRNA; positions 338 to 355) for members of the domain *Bacteria* (1), MG84/705 for type I methanotrophic bacteria (16S rRNA; positions 84 to 101 and positions 705 to 722) and Ma 450 for type II methanotrophic bacteria (16S rRNA; positions 450 to 469) belonging to the domain *Bacteria* (12), SRB 385 (16S rRNA; positions 385 to 402) for members of the cluster of sulfate-reducing bacteria belonging to the domain *Bacteria* (1), AR 915 (16S rRNA; positions 915 to 934) for members of the domain *Archaea* (42), ANME-1 862 (16S rRNA; positions 862 to 880) for members of the ANME-1 archaeal lineage, and Eel MS 932 (16S rRNA; positions 932 to 949) for members of the ANME-2 group (both ANME-1 and ANME-2 are related to the *Methanosarcinales* [6, 28]). Probes were labeled with the indocarbocyanine fluorescence dye Cy3 and fluorescein (MWG).

RESULTS AND DISCUSSION

The Black Sea water column is divided into a small oxic portion (5.3×10^{16} liters) and a much larger anoxic portion (4.8×10^{17} liters) (34, 41), which altogether contain approximately 96 Tg methane (9). Using methane dynamics, it was estimated that the vast anoxic body of water produces about 62.9×10^{10} mol methane year^{-1}; however, according to recent predictions the methane consumption may be even greater (21). These predictions, combined with studies of pore water methane concentrations (22), benthic nutrient fluxes (13), and water column methanogenesis (19), led Schubert et al. (40) to propose that the amount of methane released from sediments and internal production by methanogenesis is negligible compared to the amount of methane released from seeps with variable intensities into the Black Sea water. There have been studies that dealt with the fate of methane originating from seeps in seawater (10, 11). However, essentially nothing is known about water column microbial processes driven by seep-derived methane. Measurements have generally been obtained at sites located far from seeps (15, 34, 37), and what role microbes play at locations where there is intense gas release still needs to be determined. This study was designed to obtain direct evidence of the microbial communities which consume methane in areas where there is massive gas release compared to reference sites.

High-intensity seep and reference stations in deep water. On both expeditions, the methane gas released at the Vodyanitsky mud volcano discovered in 2003 (deep plume sites) was recorded (Table 1). The average methane concentrations in the anoxic water column above the plume were 0.1 to 12.5 μM and were as high as the values for the nonseep reference sites (0.3 to 13.6 μM) (Fig. 2) or similar to values reported previously (15, 34). Within the chemocline (depth, 80 to 100 m) the concentrations decreased further, to 84 nM methane above seeps and to 56 nM at reference sites (Fig. 2), indicating that in the whole anoxic and suboxic water column the oxidation of methane efficiently consumes the bulk of the methane present. It appears that at seep sites elevated methane concentrations can be detected only very close to the sediments where gas in form of bubbles enters the water column (7). In the Black Sea, two factors, methane consumption rates and advective mixing, may help explain why seeps do not leave a traceable imprint of elevated methane concentrations in the associated water column. In addition, in the deep anaerobic water column, the background methane concentrations are very high (~12 μM), which prevents incoming methane bubbles from leaving an additional signal in the seep-related water column. If the incoming dissolved methane is rapidly removed by microbial activity while the mixing in of methane from seeps is continuous, the net water column concentrations remain constant. Therefore, we concluded that methane turnover must be much faster above seeps than at reference sites not influenced by seeps.

In anoxic water the average methane oxidation rate increased from 0.03 nM day^{-1} below the chemocline to a maximum of 3.1 nM day^{-1} at greater depths (Fig. 2). In comparison, at deep reference sites the rates were only 0.03 to 1.2 nM day^{-1}. Indeed, above plumes the average methane oxidation rates (from water containing 10 to 12.5 μM methane) were higher than the average rates for reference sites by a factor of about 1.6 (Fig. 2). However, it is interesting that microbial oxidation had no visible impact on the isotopic signature of methane at these depths. The methane isotopic carbon ratios (δ^{13}C) of samples ranged from −60 to −57‰ at seep sites and from −57 to −55‰ at off-seep locations (Fig. 3). The δ^{13}C of dissolved methane may not have changed in the deep water column (Fig. 3), because continuous flux from active seeps kept the methane turnover in balance, leaving the methane pool with a steady isotope composition. At intermediate depths (approximately 500 m to the chemocline), microbial activity appeared to act similarly above seep and reference sites. At this range of depths, the methane concentration decreased by 98% to ~250 nM at the top of the anoxic zone (Fig. 2). In addition, we observed that particularly at the oxic-anoxic interface the δ^{13}C of dissolved methane increased sharply, changing from −60 to −44‰ at seep sites and from −55 to −42‰ at reference sites (Fig. 3). Finally, at the sea surface at reference locations, the δ^{13}C value for methane was −46‰, which closely matched the isotopic carbon signature of atmospheric methane (43). Overall, isotopic enrichment of up to 16‰ associated with an enrichment factor (51) of 1.016 at seeps and with an enrichment factor of only 1.008 at off-seep sites has been observed below and in the chemocline. Previous studies also documented that microbial methane oxidation is the only plausible explanation for pronounced isotope frac-

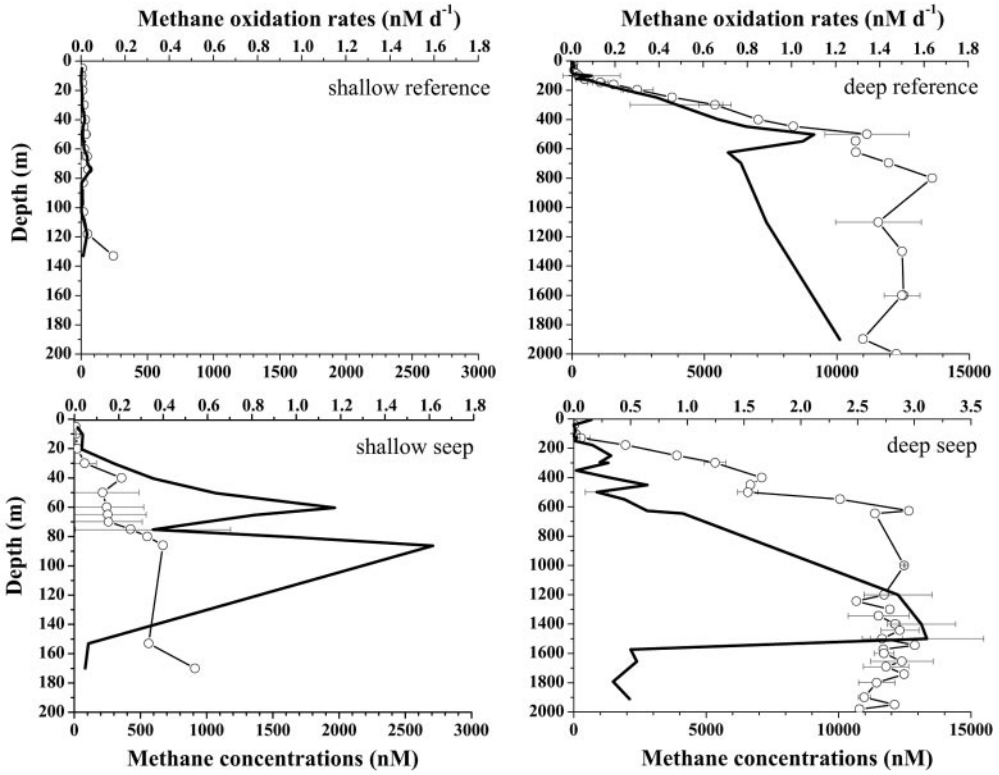

FIG. 2. Average methane concentrations and methane oxidation rates and standard deviations for shallow seep and reference sites, as well as deep seep and deep reference sites. The open circles indicate methane concentrations, and the thick lines indicate methane oxidation rates.

tionation in a very defined region (2, 51). In comparison, mixing processes could not lead to this enrichment due to the missing heavy (^{13}C-enriched) methane source at these relatively shallow depths (Fig. 3). Furthermore, this zone overlaps an area in which there is minimal gas diffusion (38), which allows strong methane depletion in water due to microbial activity.

For the most part, the water column methane oxidation rates measured in this study for anoxic water are comparable to previously published data (15, 31, 34, 37); the exceptions were the elevated rates (~3 nM day^{-1}) measured close to seeps (Fig. 2). Interestingly, although we included these higher rates in our calculation that resulted in annual oxidation of 11.2×10^{10} mol methane in anoxic water, our value is still lower than the value estimated by Reeburgh et al. (34) by a factor of 2.6. We utilized tritiated methane for rate measurements, whereas Reeburgh et al. used ^{14}C-labeled and tritiated methane. These authors even found that rates obtained by using the ^{14}C technique were higher (~1.6-fold) than the other rates, which can explain the notable difference in annual methane consumption values. Overall, due to measurement of efficient methane removal above seeps, we concluded that if the permanent stratification separating anoxic water and oxic water continues to persist in the Black Sea, the methane released from seeps will remain below the chemocline and will be efficiently oxidized by microbes.

High-intensity seep and reference sites in the shallow water column. Along the northwestern shelf and slope numerous shallow seeps emit mainly methane into the water column (18, 38). In 2004 alone, we discovered more than 1,700 new seeps (http://www.crimea-info.org). On both cruises, at selected seep sites (Table 1 and Fig. 1), we detected an excess of methane in the whole water column, and on average the concentrations were 10 times higher (11 to 912 nM) than the concentrations at the shallow reference sites (8 to 86 nM) (36) (Fig. 2). The methane concentrations at shallow reference sites corresponded well with the concentrations described previously (20, 34, 37); however, no comparisons are available for seep sites. At shallow seeps, strongly elevated methane concentrations stimulated microbial activity and resulted in methane oxidation rates (0.02 to 1.6 nM day^{-1}) that were 38-fold higher than those observed at shallow sites located off seeps (0.001 to 0.05 nM day^{-1}) (Fig. 2). If we included oxidation rates measured in the oxic layer at all deep-water sites (0.001 nM day^{-1} to a maximum of 0.2 nM day^{-1}), we still found a 30-fold difference. The highest oxidation rates measured were 1.6 nM day^{-1}, and these rates were obtained for water located close to shallow seeps. These values also compare well with rates determined for anoxic deep water (Fig. 2).

On an annual basis, water column methanotrophic activity in the oxic layer of the Black Sea consumed 2.6×10^9 mol methane year^{-1}, which is 137-fold higher than the value estimated by Reeburgh et al. (34), who did not take seep locations into account. Furthermore, elevated methane oxidation rates above seeps are the sole reason why the surface water methane concentrations (~11 nM) are almost as low as the concentrations at reference sites (~8 nM) (Fig. 2). At shallow seeps, along with high methanotrophic activity, we observed that methane became strongly enriched in heavy carbon, showing the highest enrichment factor (1.018) for all sites. For the 80-m water

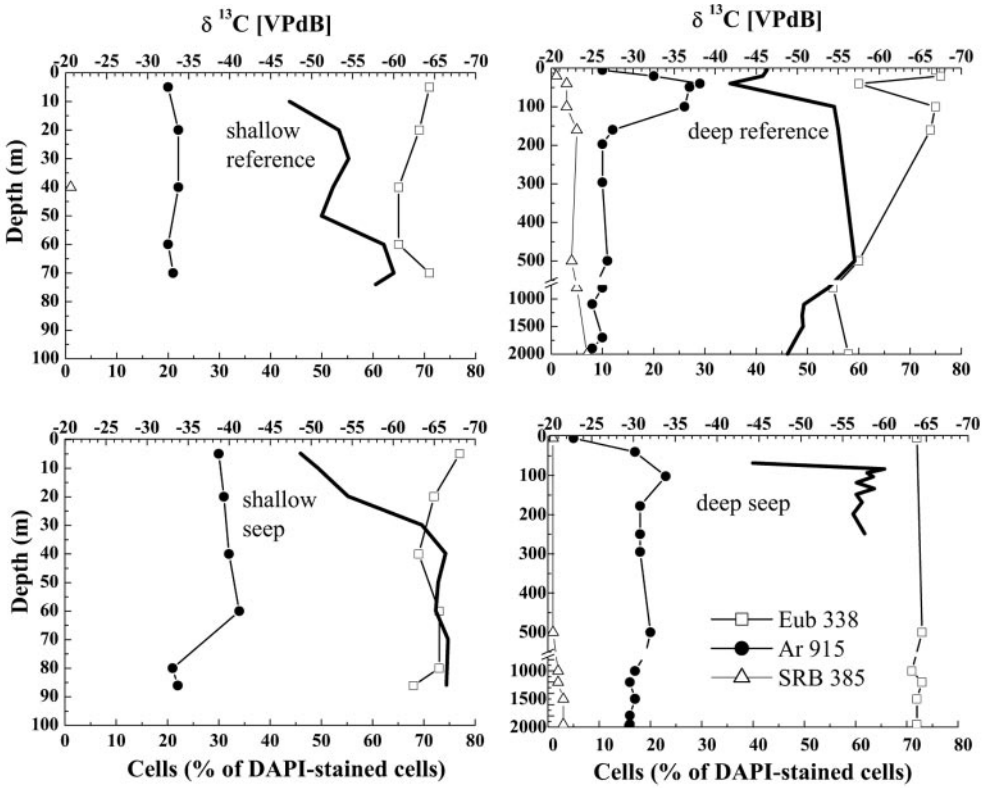

FIG. 3. Average percentages of *Bacteria* (probe EUB 338), *Archaea* (AR 915), and sulfate-reducing bacteria (SRB 385) in all DAPI-stained cells and stable carbon isotopic ratios of dissolved methane (δ^{13}C [VPDB]) for shallow seep and reference sites, as well as deep seep and deep reference sites. All values are average percentages for DAPI-stained cells counted for each individual sample. Stable carbon isotopic compositions are indicated by thick lines. The 16S rRNA-targeted oligonucleotide probes are described in Materials and Methods.

column (sediment to surface water) the isotopic ratio of methane changed from −67 ‰ to −49‰, which supported the hypothesis that there was intense methane oxidation in the shallow water column (Fig. 3). In comparison, under reference conditions, the stable carbon isotopic composition of methane was −58‰ in bottom water. The isotopic value increased to −47‰ toward the sea surface, corresponding to an enrichment factor of only 1.011 and suggesting that the methane removal rates were lower than those at seep sites (Fig. 3).

Black Sea surface water is 3 to 5% supersaturated relative to atmospheric equilibrium concentrations (52). Consequently, a flux of methane from the body of water to the atmosphere can be measured (38). According to our results, the magnitude of this flux of methane appears to be the same at seep and reference locations. We mentioned above that the shallow water column at sites with active seeps receives larger amounts of methane than the water column at reference sites receives; however, based on methane concentrations, 91% of the methane and 98% of the methane, respectively, are oxidized on the shelf. This finding corresponds well with the fact that sea surface methane concentrations were similarly low at all locations (Fig. 2). Therefore, methanotrophy seems to also play a key role in methane turnover in the shallow water column on the shelf and regulates methane release into the atmosphere.

Microbial community in deep anoxic water. The levels of prokaryotes (identified by DAPI staining) in anoxic deep water were similar at seep (1.16×10^8 to 3.69×10^8 cells liter^{-1}) and reference (2.11×10^8 to 4.47×10^8 cells liter^{-1}) sites (Fig. 4) and comparable to the levels obtained in other studies of the Black Sea water column (15, 31). Cell concentrations (global average, $\sim 3 \times 10^8$ cells liter^{-1}) were only partially coupled to the availability of organic carbon ($r = 0.4$, $n = 19$). Total organic carbon, the main energy source of water column microbes, was present at high concentrations at off-slope locations (127 to 158 μM C at seep sites and 139 to 189 μM C at references sites) (Fig. 4) but did not result in higher concentrations of cells, as observed for other systems (4). We assume that the organic matter pool is characterized by lower bioavailability and is composed of a mixture of diagenetically reworked allochthonous matter from riverine input and autochthonous substances produced by internal primary production (13).

Of all the microorganisms inhabiting the Black Sea anoxic water column, the *Bacteria* are the most important group (Fig. 3), as also found by Pimenov et al. (31). In water influenced by seeps, the presence of *Bacteria* did not vary with depth (71 to 73%). Far from active seep sites, particularly in the oxic-anoxic transition zone, the abundance of *Bacteria* decreased (from 75 to 55%) parallel to ^{13}C enrichment and an increase in the abundance of *Archaea* (Fig. 3). This inverse pattern for cellular abundance obviously implies that *Archaea* dominantly mediate the anaerobic oxidation of methane. *Bacteria* may also contribute to this pathway, but to date nothing is known about which organisms could be responsible for this.

It is well known from sediments that *Archaea* related to the

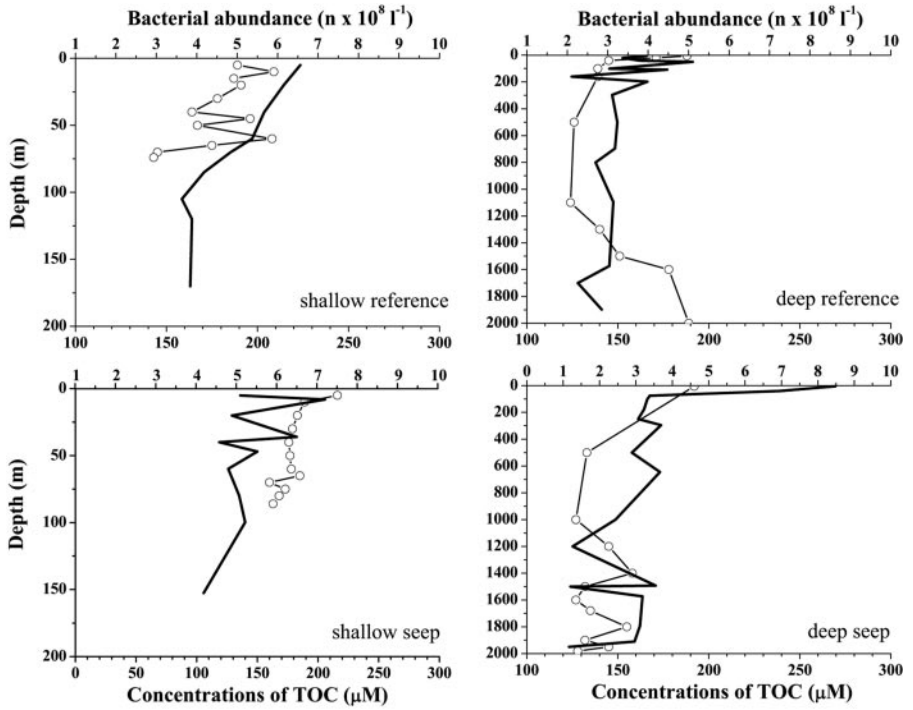

FIG. 4. Concentrations of total organic carbon (TOC) and average numbers of total cell counts based on DAPI staining for shallow seep and reference sites, as well as deep seep and deep reference sites. The open circles indicate total organic carbon concentrations, and the thick lines indicate cell numbers.

Methanosarcinales mediate the oxidation of methane in seafloor sediments (6, 28). The discovery of a ^{13}C-depleted archaeon-derived biomarker provided the first strong evidence for archaeal methanotrophy in euxinic water of the Black Sea (39, 48). In this study, we identified *Archaea* in the whole anoxic water column; interestingly, the highest proportions were in the chemocline (20 to 30%), and then at greater depths the proportion decreased to 10 to 17% (Fig. 3). A similar trend was observed by Pimenov et al. (31). As mentioned above, at the deep seep and reference sites, an increased proportion of *Archaea* perfectly overlapped with a pronounced shift in the carbon isotopic ratio of dissolved methane (Fig. 3). Strong methane oxidation obviously drives the observed isotope fractionation at this depth, and *Archaea* must be partially responsible for this (Fig. 2). Interestingly, we found ~5% more *Archaea* close to seeps than at off-seep sites. We suspect that greater availability of nutritional sources, such as methane, results not only in higher oxidation rates but also in increased levels of cells.

Vetriani et al. (47) described *Archaea* from anoxic Black Sea water which are related to the phylotypes of the ANME groups. In this study, we successfully identified for the first time both archaeal ANME-1 and ANME-2 cells in seawater obtained from seep and reference sites by using the FISH technique (Fig. 5). ANME-1 cells were present at similar proportions (~3%) in seep and reference water samples containing 10 to 12.5 μM methane, whereas ANME-2 cells accounted for fewer (~1%) of all DAPI-stained cells. Water temperatures of 8 to 9°C, which are typical for the deep Black Sea, may favor ANME-1 bacteria over ANME-2 bacteria, which were recently found to be better adapted to cold temperatures than ANME-1 bacteria (27). Therefore, if global warming results in a change in the deep-water temperature, not only would gas hydrate stability and methane availability be affected, but communities mediating the anaerobic oxidation of methane would also be affected. Overall, methanotrophic cells were living as single organisms in anoxic water. We were not able to detect any formation of aggregates with SRB. Hence, it is still not known if ANME-1 or ANME-2 cooccur with SRB in the water column, as reported for Hydrate Ridge and Black Sea sediments (6, 26). Proportions of SRB of 3 to 7% in anoxic water (Fig. 3) would allow the formation of cell consortia that favor the enzymatic mechanisms (6, 28).

Microbial community in shallow oxic water. In general, the microbial levels were found to be higher in oxic water on the shelf (3.84×10^8 to 7.20×10^8 cells liter^{-1}) and in the uppermost oxic water at the deep stations (3.04×10^8 to 8.48×10^8 cells liter^{-1}) than in deep anoxic water (Fig. 4). We attribute the higher numbers of cells to increased concentrations of bioavailable organic matter (163 to 216 μM C at seep sites and 143 to 209 μM C at reference sites [Fig. 4]) supplied by riverine inflow from the close Dniepr Delta and elevated primary production (13). In the whole microbial community living in the uppermost oxic layer of the Black Sea, *Bacteria* dominated. These organisms accounted for 65 to 77% of the organisms at seep and reference sites (Fig. 3). High levels of archaeal cells were also present, but these organisms accounted for only 20 to 34% of the total cell counts depending on the location (Fig. 3) (see below).

On the shelf, methane concentrations decrease in the water during the relatively short ascent from the seafloor to the sea surface. As mentioned above, the accompanying isotopic frac-

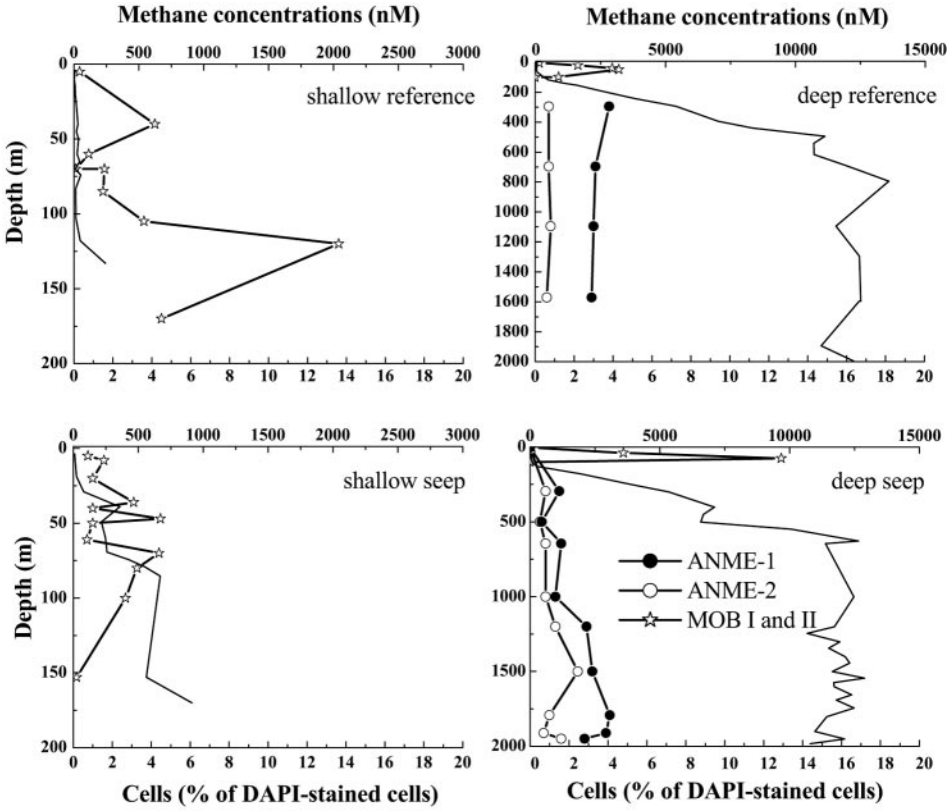

FIG. 5. Average percentages of aerobic type I and II methanotrophs (MOB I and II) and of anaerobic ANME-1 and ANME-2 cells in all DAPI-stained cells sampled at shallow seep and reference sites, as well as at deep seep and deep reference sites. The thick lines indicate methane concentrations.

tionation of dissolved methane provides a strong argument that it is oxidized by the microbial community inhabiting shelf water. Previously published data clearly demonstrate that *Bacteria* mediate methane oxidation in the oxygenated water column (14). In this study, based on the dominance of *Bacteria*, we concluded that this group of microorganisms is the group that is most responsible for aerobic methane oxidation. However, we should not neglect the fact that close to shallow seeps, the proportions of archaeal cells were on average 10% higher than the proportions at reference sites (Fig. 3). The increased abundance may imply that *Archaea* are involved in aerobic methane oxidation. Unfortunately, so far no members of the domain *Archaea* that could contribute to this pathway under oxic conditions are known.

As mentioned above, *Bacteria* are the most abundant group living in the aerobic Black Sea water column (Fig. 3), and we found that these organsisms include aerobic type I and II methanotrophs. This study also showed that type I cells were dominant and accounted for up to ~90% of the total number of aerobic methanotrophs identified (data not shown). Gal'chencko et al. (14) documented for the first time the presence of these cells by immunofluorescence. However, in contrast to our work, these authors concluded that type I and II methanotrophs are equally represented in surface water; they also found that these organisms accounted for 10% of the total cells. We found that the proportion of methanotrophic bacteria in oxic water was variable depending on the site (0.2 to 13.6%), and interestingly, the highest numbers were found at the deep seep sites (12.9%) and shallow reference sites (13.6%) (Fig. 5). We propose that other factors, such as grazing pressure, certainly the availability of oxygen and inorganic nitrogen (5), and possibly the concentrations of trace metals, such as copper, which controls methane oxidation (24), determine the water column distribution. We tested this hypothesis, however, and found that >90% of copper is bound in particles (unpublished results). The remaining dilute amount of dissolved copper may be particularly hard for free-living microorganisms to consume. Therefore, understanding the control mechanisms of methanotrophic activity could greatly increase our understanding of methane turnover in aquatic systems.

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