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Microbial Reefs in the Black Sea Fueled by Anaerobic Oxidation of Methane

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Massive microbial mats covering up to 4-meter-high carbonate buildups prosper at methane seeps in anoxic waters of the northwestern Black Sea shelf. Strong ^{13}C depletions indicate an incorporation of methane carbon into carbonates, bulk biomass, and specific lipids. The mats mainly consist of densely aggregated archaea (phylogenetic ANME-1 cluster) and sulfate-reducing bacteria (*Desulfosarcina/Desulfococcus* group). If incubated in vitro, these mats perform anaerobic oxidation of methane coupled to sulfate reduction. Obviously, anaerobic microbial consortia can generate both carbonate precipitation and substantial biomass accumulation, which has implications for our understanding of carbon cycling during earlier periods of Earth's history.

Until recently, it was believed that only aerobic bacteria, depending on oxygen as an electron acceptor, build up substantial biomass from methane carbon in natural habitats (*1*). Because biogenic methane is strongly depleted in ^{13}C, a worldwide negative excursion in the isotopic signature of organic matter around 2.7 Ga (1 Ga = 10^9 years) ago was taken as an argument for methanotrophy and, consequently, an early oxygenation of the Earth's atmosphere (*2*). However, recent investigations have shown the existence of methane-consuming associations of archaea and sulfate-reducing bacteria (SRB) in anoxic marine sediments (*3*, *4*).

Microorganisms capable of anaerobic growth on methane have not been cultivated so far, and the biochemical pathway of the anaerobic oxidation of methane (AOM) remains speculative. Analyses of depth profiles and radiotracer studies in marine sediments (*5*, *6*) as well as molecular (*7–10*) and petrographic studies (*11*) argue for AOM (*12*) as a crucial process that channels ^{13}C-depleted methane carbon into carbonate and microbial biomass. Through AOM mediated by consortia of archaea and SRB, methane is oxidized with equimolar amounts of sulfate, yielding carbonate and sulfide, respectively (*13*). Generation of alkalinity favors the precipitation of methane-derived bicarbonate according to the following net reaction:

$$CH_4 + SO_4^{2-} + Ca^{2+} \rightarrow CaCO_3 + H_2S + H_2O$$

Here, we provide evidence that vast amounts of microbial biomass may accumulate in an anoxic marine environment because of the use of methane as an electron donor for sulfate reduction (SR) and as an organic carbon source for cell synthesis.

In the northwestern Black Sea, hundreds of active gas seeps occur along the shelf edge west of the Crimea peninsula at water depths between 35 and 800 m (*14*). At some of the shallow Crimean seeps, microbial mats were found associated with isotopically light carbonates. Aspects of the microbiology, sedimentology, mineralogy, and selected biomarker properties of these deposits were recently described (*11*, *15–17*). We explored the seeps on the lower Crimean shelf with the manned submersible *JAGO* from aboard the Russian R/V *Professor Logachev*. During dives to a seep area at 44°46′N, 31°60′E, we discovered a reef consisting of up to 4-m-high and 1-m-wide microbial structures projecting into permanently anoxic bottom water at a depth around 230 m (Fig. 1A). These buildups are formed by up to 10-cm-thick microbial mats that are internally stabilized by carbonate precipitates (Fig. 1A).

From holes in these structures, streams of gas bubbles emanate into the water column (Fig. 1A). The gas contains about 95% methane [see supporting online material (SOM)]. In cross section, the outside of the soft mat has a dark gray to black color (Fig. 1B). Inside the structure, most of the mat is pink to brownish. The interior rigid parts are porous carbonates (aragonite and calcite with up to 14% $MgCO_3$). Much of the structures consists of interconnected, irregularly distributed cavities and channels filled with seawater and gases. Apparently, the cavernous structure of these precipitates enables methane and sulfate to be transported and distributed throughout the massive mats. Smaller microbial structures and nodules from nearby areas were of the same morphology, with compact mat enclosing calcified parts and cavities. Obviously, the microorganisms do not grow on preformed carbonates but induce and shape their formation. Stable carbon isotope analyses of the carbonates yielded $\delta^{13}C$ values ranging from −25.5 to −32.2 per mil (‰) [for methods, see (*11*)]. Compared with the $\delta^{13}C$ values of dissolved inorganic carbon in the Black Sea water column from +0.8‰ at surface to −6.3‰ at depth (*18*), these values indicate that a major portion of the carbonate originates from the oxidation of

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methane. The methane seeping from the microbial structures and from nearby sediment pockmarks is of biogenic origin, as indicated by $\delta^{13}C$ values of −62.4 to −68.3‰ ($n = 6$) (SOM) and most probably evolves from a deeper sedimentary source.

Samples of living microbial mat were retrieved to investigate their microbiological and chemical composition as well as their catabolic activity. A short-term labeling experiment with added [^{14}C]methane and [^{35}S]sulfate (SOM) was performed directly after sampling. Pieces of mat incubated for 5 days in bottom water amended with methane (1.8 mM) exhibited AOM to carbon dioxide at a rate of 18 (±12) μmol (gram dry weight)$^{-1}$ d^{-1} and SR at a rate of 19 (±1) μmol (gram dry weight)$^{-1}$ d^{-1} (±SD; $n = 3$). In controls without mat samples, AOM and SR were below the detection limit. Results are in agreement with a stoichiometry of 1:1 between AOM and SR, as in experiments with sediment samples from a gas hydrate area (*13*). Methane-dependent SR to sulfide was also shown by chemical quantification with mat samples immediately incubated with and without methane, respectively (incubation time, 60 days). SR rates under an atmosphere of methane were 34 (±5.8) μmol (gram dry weight)$^{-1}$ d^{-1} (±SD; $n = 6$), whereas rates under nitrogen were 2.8 (±0.55) μmol (gram dry weight)$^{-1}$ d^{-1} (±SD; $n = 3$), probably because of gradual decay of biomass. Chemical quantification in another, long-term incubation experiment (166 days) again corroborated the 1:1 stoichiometry between AOM and SR (*13*). All these experiments indicate that methane is the main or only electron donor that accounts for SR and hence the buildup of the massive mat and carbonate structures in the investigated region of the Black Sea.

In extracts from homogenized mat samples, we observed isoprene-based constituents of archaeal lipids and hydrocarbons, such as archaeol, crocetane, and C_{40} isoprenoids (acyclic, mono-, and dicyclic biphytanes) (Table 1) [see SOM (fig. S1)]. A second compound cluster encompassed nonisoprenoid, linear, and monomethyl-branched carbon skeletons of presumably bacterial origin. Most prominent among these structures are ω-3 monomethylated (*anteiso*-) C_{15} carbon chains bound in glycerol esters and glycerol diethers. No such lipids were found in a surface sediment of a nearby non-seep area, whereas similar biomarker patterns typically occur at modern and fossil methane seeps and were consistently related to contributions from methane-consuming archaea and associated SRB (Table 1). Indeed, strong ^{13}C depletions of the bulk biomass (−72.2‰) and of the bacterial and archaeal lipids there (Table 1) indicate that the mat microbiota must have incorporated methane-derived carbon into their biomass. Hence, archaeal AOM and bacterial SR are relevant processes fueling biomass production and reef formation in this permanently anaerobic seep environment.

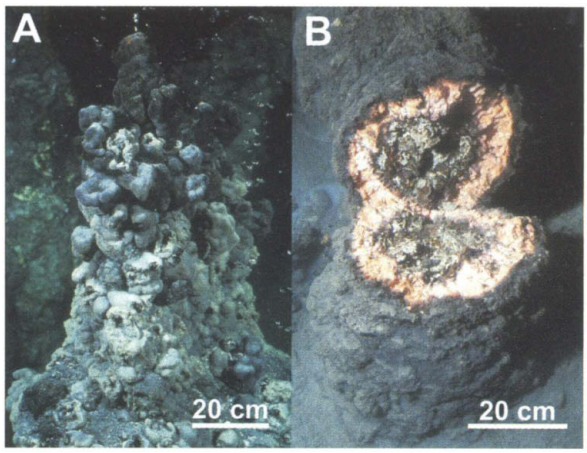

Fig. 1. Image of microbial reef structures (as seen from the submersible). (**A**) Tip of a chimney-like structure. Free gas emanates in constant streams from the microbial structures into the anoxic seawater. (**B**) Broken structure of about 1 m height. The surface of the structure consists of gray-black microbial mat; the interior of the massive mat is pink. The greenish-gray inner part of the structure consists of porous carbonate, which encloses microbial mats and forms irregular cavities.

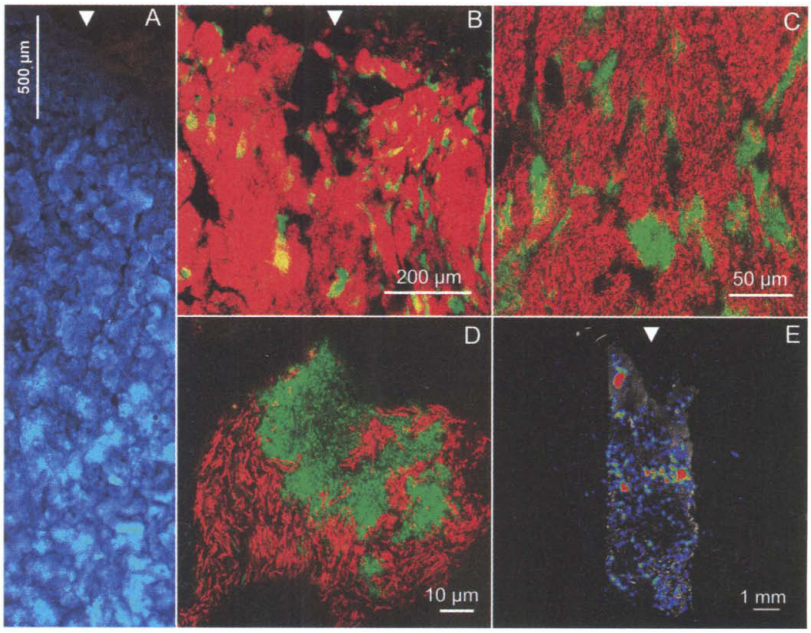

Fig. 2. Fluorescence image showing a thin section of the pink mat. Scale bars indicate different microscopic magnifications. The arrow marks the outside of the microbial mat. (**A**) Thin section of mat stained with 4′,6-diamidino-2-phenylindole (see SOM). (**B**) Archaea of the cluster ANME-1 were targeted with a red-fluorescent group-specific oligonucleotide probe. SRB were targeted with a probe specific for a cluster of δ-proteobacteria in the *Desulfosarcina/Desulfococcus* group and fluoresce green. These two populations comprise the bulk biomass in the microbial mat. (**C**) Microcolonies of SRB are surrounded by bulk ANME-1 cell clusters. (**D**) ANME-1 cells have a unique rectangular shape; SRB are small coccoid cells. Single SRB cells are dispersed throughout the ANME-1 cell clusters. (**E**) β-imager micrograph of a thin section of mat incubated with $^{14}CH_4$ (see SOM). The micrograph shows incorporated radioactivity of the mat after acidification. Green and red areas indicate ^{14}C uptake.

To directly trace the uptake and transformation of methane into organic and inorganic matter, we incubated 1-cm-thick pieces of microbial mat with radioactive methane ($^{14}CH_4$) and analyzed thin sections with a β-microimager that provides two-dimensional images of radioactivity distribution (see SOM). Incorporation of radiotracer into the solid phase was recorded throughout the sections, which demonstrates that methane is readily assimilated into the microbial mat (Fig. 2E). Upon acidification of the thin sections, about 25% of the ^{14}C radioactivity was lost. Obviously, a fraction of the methane carbon had been precipitated as carbonate. This approach provides direct laboratory evidence for methane-fueled calcification and for the microbially mediated formation of carbonate structures.

Epifluorescence microscopy of sections of microbial mat revealed dense aggregations of archaea and bacteria (Fig. 2) (see SOM). The mats are penetrated by systems of microchannels that may enable advective exchange with

Table 1. Selected biomarkers, their $\delta^{13}C$ values, and most likely biological sources in the Black Sea microbial mat. The reference column cites previous reports on these compounds in other methane-related environments. For additional information see SOM.

Compound	$\delta^{13}C$ (‰)	Source	Reference
Crocetane (2,6,11,15-tetramethylhexadecane)	−94.7 ± 0.7	Archaea (?)	(*3, 10, 15, 19, 24–27*)
2,6,10,15,19-Pentamethylicosane	−95.6 ±0.1	Archaea Methanosarcinales	(*3, 10, 15, 19, 24–27*)
Archaeol (2,3-di-*O*-phytanyl-*sn*-glycerol)	−87.9 ± 0.6	Archaea	(*3, 8, 10, 19, 24–26, 28*)
sn-2-Hydroxyarchaeol (2-*O*-3-hydroxyphytanyl-3-*O*-phytanyl-*sn*-glycerol)	−90.0 ± 1.0	Archaea Methanosarcinales	(*3, 8, 10, 19, 28*)
Biphytane (from tetraether after HI/$LiAlH_4$ cleavage)	−91.6 ± 0.6	Archaea	(*15*)
n-Tricos-10-ene	−90.9 ± 0.3	Unknown (bacteria?)	(*27*)
Anteiso-pentadecanoic acid (12-methyltetradecanoic acid)	−83.9 ± 0.1	SRB	(*3, 10, 19, 26, 28*)
1,2-di-*O*-12-Methyltetradecyl-*sn*-glycerol	−89.5 ± 0.5	SRB	(*19, 28*)

the surrounding seawater (Fig. 2A). The microchannels radiate out from the inner calcified part of the mat system and make up between 20 and 40% of the bulk mat volume. Fluorescence in situ hybridization (see SOM) shows that the mat biomass is dominated by one archaeal population comprising at least 70% of the mat biomass and belonging to the cluster ANME-1 (Fig. 2, B, C, and D). ANME-1 is only distantly related to the Methanosarcinales and the ANME-2 cluster, which forms consortia with SRB and is known to be capable of AOM (*3*, *4*, *6*). Nevertheless, the microbial mat dominated by ANME-1 comprises a similar pattern of the ^{13}C-depleted archaeal biomarkers crocetane, pentamethylicosane, pentamethylicosenes, archaeol, and *sn*-2-hydroxyarchaeol [see SOM (fig. S1)], as hydrate ridge sediments (*3*, *7*), in which archaea of the ANME-2 cluster prevail (*3*). The most abundant bacterial population in the mats from the Black Sea belongs to the *Desulfosarcina/Desulfococcus* group, the same taxon of SRB as found in the ANME-2/SRB consortium (*3*).

ANME-1 cells have a cylindrical shape and are autofluorescent under ultraviolet light, a feature typical for methanogenic archaea containing coenzyme F_{420}. Their length is about 3.5 μm and their diameter is about 0.6 μm (biovolume, about 1 μm^3). The coccoid SRB (cell diameter, about 0.6 μm; biovolume, about 0.1 μm^3) occur in larger clusters of 10 to 50 μm diameter (Fig. 2, B and C). Smaller clusters and single cells of SRB are dispersed throughout the bulk ANME-1 biomass (Fig. 2D). A 1-cm^3 mat contains about 10^{12} cells (see SOM), which corresponds to about 25 mg of carbon.

The growth yield of the mat community is currently unknown. Prokaryotes deriving their energy from dissimilatory SR generally have low growth yields. Pure cultures of SRB growing on conventional substrates (for example, organic acids) usually convert only one-tenth of the totally consumed organic compounds into cell mass, but the free energy gain obtained (for example, on lactate, ΔG around −100 kJ per mol of sulfate) remains higher than that of AOM. Even with high methane partial pressure as at the active seeps studied (up to about 20 atm), the ΔG should not exceed −40 kJ per mol of methane oxidized (see SOM). Hence, an even lower growth yield than for conventional cultures of SRB must be expected for the Black Sea mats. Consequently, the amount of methane oxidized for the buildup and maintenance of the existing mat structures should exceed their organic carbon content by more than an order of magnitude.

Microbial mats of the size currently observed are rarely found in oxic environments. This may reflect the absence of metazoan grazing and other mortality factors in the permanently anoxic and sulfidic environment of the Black Sea. There is increasing evidence that the capacity for AOM is present in several deep-branching clades of archaea (*19*), which indicates an early evolutionary origin, just as for methanogenesis.

AOM may have influenced the carbon isotope record of the Archaean (*20*). Moreover, recent studies suggest that SR has already been prolific 3.5 Ga ago (*21*), close to the first occurrence of microfossils (3.5 Ga ago) (*22*) and the first isotopic traces of bioorganic carbon cycling (3.8 Ga ago) (*23*). Hence, AOM may have represented an important link in the biological cycling of carbon in an anoxic biosphere. Even in the absence of free oxygen, methane formed by anaerobic degradation (fermentation and methanogenesis) of organic matter could have been recycled via AOM, leading to substrates (inorganic carbon and sulfide) for anoxygenic photosynthesis of new biomass. In this respect, the microbial reefs discovered at Black Sea methane seeps suggest how large parts of the ancient ocean might have looked when oxygen was a trace element in the atmosphere, long before the onset of metazoan evolution.

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Supporting Online Material

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Materials and Methods

Fig. S1

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