Subsurface Microbial Methanotrophic Mats in the Black Sea†

Tina Treude,[1,2]* Katrin Knittel,[1] Martin Blumenberg,[3] Richard Seifert,[3] and Antje Boetius[1,4]

*Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen, Germany[1]; Alfred Wegener Institute
for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany[2]; Institute of
Biogeochemistry and Marine Chemistry, University of Hamburg, Bundesstrasse 55, 20146
Hamburg, Germany[3]; and International University of Bremen, Campusring 1,
28759 Bremen, Germany[4]*

**A nodule-shaped microbial mat was found subsurface in sediments of a gas seep in the anoxic Black Sea.
This mat was dominated by ANME-1 archaea and consumed methane and sulfate simultaneously. We propose
that such subsurface mats represent the initial stage of previously investigated microbial reefs.**

In permanently anoxic parts of the northwestern Black Sea, methanotrophic microbial reefs that are up to 4 m high and 1 m in diameter thrive above methane seeps characterized by strong emanation of gas bubbles (3, 9, 12). Similar microbial reef systems have recently been found in several areas of the Black Sea shelf (B. B. Jørgensen and scientific party, Poseidon cruise POS 317-3 2004). The surface reef structures consist of microbial mats that are up to 10 cm thick enclosing porous carbonates. Biomarker and stable isotope analyses have shown that the precipitation of the carbonate is associated with the anaerobic oxidation of methane (AOM) (13, 15). In this study, sediment push cores (diameter, 60 mm) were obtained by the submersible Jago (MPI Seewiesen, Germany) within 1 m of a gas-emanating microbial reef structure (water depth, 313 m; 44°44.202′N, 31°47.333′E; GHOSTDABS station 68). In two adjacent push cores an approximately 4-cm-thick subsurface mat was discovered below a sediment depth of 10 cm. The mat had a spherical nodule-like shape and was very soft; i.e., no carbonate incrustations were detected visually during sectioning and dissection. Below an outer black layer that was a few millimeters thick the mat appeared pinkish, similar to the mats growing on the reef structures. The two push cores were sampled by taking five subcores (diameter, 26 mm) (Fig. 1a) to identify the dominant microbial populations and to measure the rates of AOM and sulfate reduction (SR), the concentration and stable carbon isotope signatures of methane, and the concentration of sulfate at 1-cm intervals above, within, and below the mat. The microbial community of the mat was analyzed by examining one of the subcores by fluorescence in situ hybridization of sonicated samples (8) (Table 1). A major fraction (estimate, ≫50%) of the mat consisted of ANME-1 cells. No aggregates or single cells of ANME-2 archaea were detected by epifluorescence microscopy. rRNA slot blot hybridization was used to obtain a semiquantitative estimate of microbial abundance around and within the subsurface mat. RNA extraction, blotting, and hybridization using ^{33}P-labeled

oligonucleotide probes (Table 1) were performed as described previously (14). Quantitative rRNA slot blot hybridization confirmed that ANME-1 was the dominant group within the mat (up to 40% ANME-1 rRNA in the total rRNA) (Fig. 1b) and showed that this taxon accounted for 83% of total archaeal RNA in these layers (data not shown). Slot blot hybridization of sulfate-reducing bacteria (SRB) belonging to the *Desulfococcus-Desulfosarcina* cluster revealed a concentration of 16S rRNA in the mat that was similar to that in the surrounding sediments (Fig. 1b); the level increased only slightly, from 5% at the sediment surface to 11% of the total 16S rRNA within the mat (6 to 27% of the total bacterial rRNA [data not shown]). The AOM and SR rates were determined for two separate subcores by 24 h of incubation with $^{14}CH_4$ and $^{35}SO_4^{2-}$ radiotracers by using the whole-core injection method (6). Incubation was performed at the in situ temperature (9°C). AOM and SR samples were fixed, stored, and analyzed as described previously (16, 7). Concentrations and stable carbon isotope signatures of methane were analyzed by gas chromatography and gas chromatography-stable isotope mass spectrometry, respectively (12). Sulfate concentrations were determined for one subcore in the supernatant of sediment fixed with zinc acetate (20%, wt/wt) using nonsuppressed ion chromatography (5). The activity profiles measured reflected the distribution of the ANME-1-dominated mat. High AOM and SR activities were detected within the mat (Fig. 1c), and the data revealed a close 1:1 coupling between the two processes. The peaks of methanotrophic activity (AOM, 1.5 μmol cm^{-3} day^{-1} at 12 to 13 cm; SR, 1.8 μmol cm^{-3} day^{-1} at 11 to 12 cm) coincided with the center of the mat, where the steepest gradient in the pore water methane content (Fig. 1d) was measured. The methane concentration decreased from the atmospheric saturation level (1.3 mM) below the mat to <0.1 mM above the mat. Stable carbon isotope signatures of the methane profile generally indicated that there was methanotrophy because $\delta^{13}C$ values increased from −70.2 ‰ below the mat to −61.7‰ or less above the mat. However, most interestingly, the $\delta^{13}C$ value of methane directly underneath the AOM peak (−73.7 ‰) showed a minimum and was lower than the reported isotope signatures of methane seeping in this area (minimum, −68.3 ‰) (12). This may indicate that there was net methanogenic activity in this part of the mat, which we could

---

* Corresponding author. Mailing address: Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen, Germany. Phone: 49 421 2028630. Fax: 49 421 2028690. E-mail: ttreude@mpi-bremen.de.
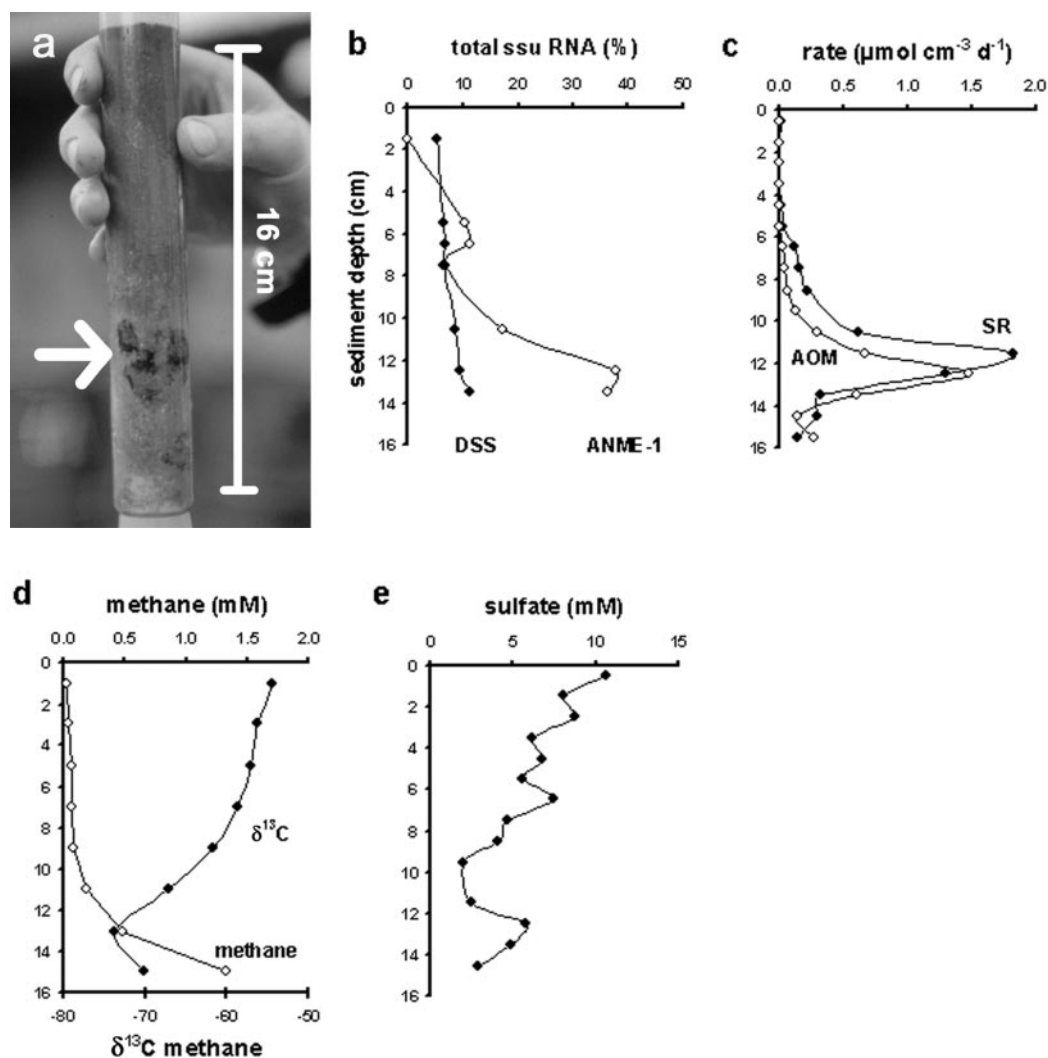
FIG. 1. Parameters measured in the sediment containing a subsurface microbial mat. (a) Position of the microbial mat (arrow) in one of the subcores; (b) percentages of archaeal ANME-1 and bacterial *Desulfococcus-Desulfosarcina* (DSS) rRNA in the total small-subunit (ssu) rRNA; (c) rates of AOM and SR; (d) concentrations and stable carbon isotope signatures of methane ($\delta^{13}C$ values; $n = 3$; standard errors, ± 1 ‰); (e) concentrations of sulfate.

not investigate further. The concentration of sulfate, the electron acceptor of AOM, decreased from 11 mM in the surface sediments to a minimum of 2 mM within the mat (Fig. 1e). In conclusion, the subsurface microbial mat studied in this inves-

tigation met all of the following characteristics of the reef mats described in previous studies (3, 12): (i) nodule-shaped growth, (ii) pinkish color with a blackened surface, (iii) simultaneous consumption of methane and sulfate under anoxic conditions,

TABLE 1. Oligonucleotide probes used for fluorescence in situ hybridization and slot blot hybridization in sediment-mat samples

Probe	Specificity	Sequence (5′ to 3′)	Position[a]	Slot blot denaturation temp (°C)	Formamide concn (%, vol/vol)[b]	Reference
Uni1390	All organisms	GACGGGCGGTGTGTACAA	1390–1407	44	Not used	17
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	338–355	54	35	1
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	915–935	56	35	2
ANME-2-538	ANME-2 cluster	GGC TAC CAC TCG GGC CGC	538–555	Not used	50	This study
ANME-1-350	ANME-1 cluster	AGT TTT CGC GCC TGA TGC	350–367	60	50	4
DSS658	*Desulfosarcina* spp./*Desulfococcus* spp. cluster	TCCACTTCCCTCTCCCAT	658–685	58[c]	60	11

[a] Position in the 16S rRNA of *Escherichia coli*.
[b] Formamide concentration in the hybridization buffer used in fluorescence in situ hybridization.
[c] Washing buffer containing 1× SSC and 0.1% sodium dodecyl sulfate was used (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).
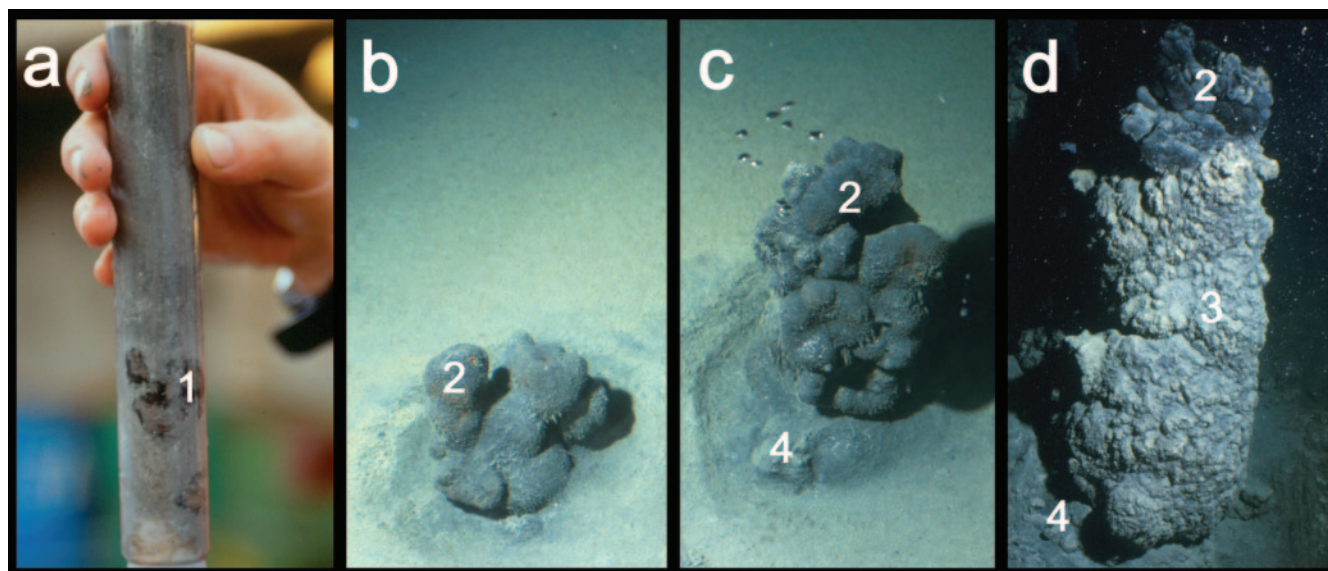
FIG. 2. Suggested stages of formation of methanotrophic microbial reefs in the anoxic zone observed at different locations in the study area. (a) Uncalcified subsurface microbial mat; (b) breakthrough of nodule-shaped microbial structures into the water column (height, approximately 20 cm); (c) advanced growth into the water column and formation of calcified basement (~40 cm); (d) chimney-like reef structures with a calcified inner core surrounded by microbial mat and soft nodule-shaped microbial structures on top (~150 cm). 1, subsurface nodule; 2, surface nodule; 3, surface reef structure; 4, carbonate basement. The picture sources were project MUMM (a) and project GHOSTDABS/Jago Team, MPI Seewiesen (b to d).

and (iv) dominance of methanotrophic ANME-1 archaea associated with the *Desulfococcus-Desulfosarcina* SRB. However, in contrast to the reef mats, the subsurface mat did not contain visually detectable carbonate precipitates. We therefore propose that the subsurface growth represents the preliminary stage of microbial reef formation. Most likely, the slowly growing communities of methanotrophic archaea accumulate around gas leakage pathways where sulfate is still available. The growth into a densely aggregated mat could even support gas trapping. It was observed visually during dives that surface reef nodules retain free gas in interior cavities, apparently causing an overpressure within the nodule (9). The formation of the reef (Fig. 2) may proceed by gradual calcification of the subsurface mat forming a basement around the gas channels and finally growth into the water column supported by the calcareous core. The direction of growth of the reef seems to be relatively vertical from the sediment into the water column since the nodule-like mats on top of the reefs represent the youngest structures, as confirmed by ^{90}Sr detections (9). In studies investigating methane seeps at the transition from oxic water to anoxic water of the Black Sea (water depth, 60 to 230 m), scientists reported a successive increase from bottom-level pancake-like carbonate cements with interior mat inclusions in the oxic zone to >1-m-high carbonate-mat chimneys extending into the water column of the anoxic zone (10, 13). The presence of oxygen in the water column evidently limits the vertical growth of the methanotrophic mats. One advantage of the reef-forming growth pattern within the anoxic zone would be maximized access to sulfate. Within the sediment, diffusion limits the supply of sulfate, but the upward growth into chimney-like structures along gas leakage pathways could facilitate the supply of sulfate from the surrounding water.

REFERENCES

1. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probing of whole cells with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, environmental studies in microbiology. J. Bacteriol. **172:**762–770.
3. **Blumenberg, M., R. Seifert, J. Reitner, T. Pape, and W. Michaelis.** 2004. Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. Proc. Natl. Acad. Sci. USA **101:**11111–11116.
4. **Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Giesecke, R. Amann, B. B. Jørgensen, U. Witte, and O. Pfannkuche.** 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature **407:**623–626.
5. **Ferdelman, T. G., C. Lee, S. Pantoja, J. Harder, B. M. Bebout, and H. Fossing.** 1997. Sulfate reduction and methanogenesis in a *Thioploca*-dominated sediment off the coast of Chile. Geochim. Cosmochim. Acta **61:**3065–3079.
6. **Jørgensen, B. B.** 1978. A comparison of methods for the quantification of bacterial sulphate reduction in coastal marine sediments. I. Measurements with radiotracer techniques. Geomicrobiol. J. **1:**11–27.
7. **Kallmeyer, J., T. G. Ferdelman, A. Weber, H. Fossing, and B. B. Jørgensen.** 2004. A cold chromium distillation procedure for radiolabeled sulfide applied to sulfate reduction measurements. Limnol. Oceanogr. Methods **2:**171–180.
8. **Knittel, K., T. Lösekann, A. Boetius, R. Kort, and R. Amann.** 2005. Diversity and distribution of methanotrophic archaea (ANME) at cold seeps. Appl. Environ. Microbiol. **71:**467–479.
9. **Lein, A. Y., M. V. Ivanov, N. V. Pimenov, and M. B. Gulin.** 2002. Geochemical characteristics of the carbonate constructions formed during microbial

oxidation of methane under anaerobic conditions. Mikrobiologiya **71:**89–103.

10. **Luth, C., U. Luth, V. Gebruck, and H. Thiel.** 1999. Methane seeps along the oxic/anoxic gradient in the Black Sea: manifestations, biogenic sediment compounds, and preliminary results on benthic ecology. Pubbl. Stn. Zool. Napoli Mar. Ecol. **20:**221–249.

11. **Manz, W., M. Eisenbrecher, T. R. Neu, and U. Szewzyk.** 1998. Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. **25:**43–61.

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

13. **Peckmann, J., A. Reimer, U. Luth, C. Luth, B. T. Hansen, C. Heincke, J.**

Hoefs, and J. Reitner. 2001. Methane-derived carbonates and authigenic pyrite from the northwestern Black Sea. Mar. Geol. **177:**129–150.

14. **Ravenschlag, K., K. Sahm, C. Knoblauch, B. B. Jørgensen, and R. Amann.** 2000. Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine Arctic sediments. Appl. Environ. Microbiol. **66:**3592–3602.

15. **Thiel, V., J. Peckmann, H. H. Richnow, U. Luth, J. Reitner, and W. Michaelis.** 2001. Molecular signals for anaerobic methane oxidation in Black Sea seep carbonates and microbial mat. Mar. Chem. **73:**91–112.

16. **Treude, T., A. Boetius, K. Knittel, K. Wallmann, and B. B. Jørgensen.** 2003. Anaerobic oxidation of methane above gas hydrates at Hydrate Ridge, NE Pacific Ocean. Mar. Ecol. Prog. Ser. **264:**1–14.

17. **Zheng, D., E. W. Alm, D. A. Stahl, and L. Raskin.** 1996. Characterization of universal small subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. **62:**4314–4317.