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**EXPERIMENTAL ARTICLES**

# Microbiological Processes of the Carbon and Sulfur Cycles at Cold Methane Seeps of the North Atlantic

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**Abstract**—Functioning of microbial communities in surface sediments of the Haakon Mosby underwater mud volcano (lat. 72°N) and in gas seepage fields of the Vestnesa Ridge was investigated using *Mir-1* and *Mir-2* deep-sea submersibles during the 40th voyage of the research vessel *Academician Mstislav Keldysh*. Large areas of sedimentary deposits of the Haakon Mosby mud volcano (HMMV) and pockmarks of the Vestnesa Ridge (VR) are covered with bacterial mats 0.1 to 0.5 cm thick. The microbial community making up bacterial mats of the HMMV was dominated by large filamentous bacteria with filaments measuring up to 100 μm in length and 2 to 8 μm in width. The occurrence of rosettes allowed the observed filamentous bacteria to be referred to the morphologically similar genera *Leucothrix* or *Thiothrix*. Three morphological types of filamentous bacteria were identified in bacterial mats covering VR pockmarks. Filaments of type one are morphologically similar with representatives of the genera *Thioploca* or *Desmanthos.* Type two filaments had numerous inclusions of sulfur and resembled representatives of the genus *Thiothrix*. The third morphological type was constituted by single filaments made up of tightly connected disk-like cells and can be assigned to the genus *Beggiatoa.* The rates of methane oxidation (up to 1570 μl C/(dm$^3$ day)) and sulfate reduction (up to 17 mg S/(dm$^3$day)) measured in the surface sediments of HMMV and VR were close to the maximum rates of these processes observed in heavily polluted regions of the northwestern shelf of the Black Sea. High rates of microbiological processes correlated with the high number of bacteria. The rate of methane production in sediments studied was notably lower and ranged from 0.1 to 3.5 μ $CH_4$/(dm$^3$ day). Large areas of the HMMV caldera were populated by pogonophoras, represented by the two species *Sclerolinum* sp. and *Oligobrachia* sp. The mass development of *Sclerolinum* sp. in the HMMV caldera was by the activity of aerobic methane-oxidizing bacteria localized inside the cells of these animals. Bacterial cells were also found in the trophosome tissue of *Oligobrachia* sp., but in cells of these bacteria, we did not observe the membrane structures typical of methanotrophs. The localization pattern of pogonophoras on the surface of reduced sediments suggests that the predominant bacteria in *Oligobrachia* tissues are sulfur-oxidizing endosymbionts.

*Key words*: methane seeps, bacterial mats, filamentous bacteria, sulfate reduction, methanogenesis, methane oxidation, pogonophoras, methanotrophic endosymbiotic bacteria

Over the last decade, numerous seeps of gas, with methane as the main component, were discovered on continental margins of seas and oceans [1, 2]. The characteristic pattern of the seafloor relief (pockmarks, which are crater-like depressions in the seafloor surface) observed in zones containing hydrocarbon seeps, as well as geophysical and geochemical anomalies in the vertical sediment distribution, are a good clue in the search for methane seeps.

Detailed geochemical and biological explorations undertaken in zones of cold methane seeps of various types resulted in the discovery of specific benthic communities with a food chain based on the production of organic matter by methanotrophic and chemoautotrophic bacteria [3, 4]. In several cases, the surface of bottom deposits in the region of pockmarks is covered with bacterial mats having white or yellow color and formed by filamentous bacteria that morphologically resemble *Beggiatoa* [4]. The tissue of the predominant fauna representatives of benthic communities at cold methane seeps was shown to contain methane-oxidizing and chemoautotrophic (sulfur-oxidizing) endosymbiotic bacteria, which allowed such atypical substrates as methane, hydrogen sulfide, thiosulfate and carbon dioxide to be involved in the animal's nutritional and energy metabolism [5, 6].

In 1995, a joint Norwegian-American geophysical expedition aboard the research vessel *Haakon Mosby* discovered on the seafloor of the Norwegian Sea a gashydrate methane-bearing field associated with a mud volcano with the diameter of about 1 km. Explorations of this volcano, named Haakon Mosby, were continued in 1996 by a Norwegian-American-Russian expedition aboard the research vessel *Professor*

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*Logachev* [7, 8]. Surface sediments of the Haakon Mosby Mud Volcano (HMMV) were found to host abundant populations of microorganisms (up to $10^{10}$ cells/$dm^3$). This, along with high rates of dark carbon dioxide fixation, sulfate reduction and methane oxidation, which is unusual for deep-sea sediments, was an evidence for active transformation of organic matter (OM) underway in HMMV bottom sediments. [9].

Slopes of this mud volcano were found to be inhabited by a large number of tube worms, or pogonophoras, represented by the two species *Oligobrachia* sp. and *Sclerolinum* sp. It was found that $^{14}CH_4$ was oxidized in tissues of the latter species, pointing to the presence of symbiotrophic methane-oxidizing bacteria [9].

Despite the preceding fairly detailed studies of HMMV, many questions relating to functioning of the microbial community were not answered. In particular, because the sediments were so highly gas-saturated, the uppermost and most active layer (0–10 cm) was obtained only from the peripheral zone of the HMMV caldera. It was not possible to determine the origin of the large white spots on the surface of bottom sediments seen on photographs that were taken with a camera mounted on a towed apparatus. The tissues of pogonophoras, usually hosting endosymbiotic bacteria, at that point were not studied either. With the objective to address these problems, we took part in an international expedition aboard the research vessel *Academician Mstislav Keldysh* in 1998. In addition to HMMV, microbiological and biogeochemical studies during this expedition were conducted on the Vestnesa Ridge extending over the continental slope westwards of Spitzbergen, where seafloor mapping by side-scanning sonar revealed pockmark-looking structures [10].

The primary goal of this study was to investigate the functioning of microbial communities in surface sediments of methane seeps of the Norwegian Sea (lat. 72° and 76° N) and the effect these communities have on benthic fauna development in sites of gas seepage.

## MATERIALS AND METHODS

The samples were collected during the 40th voyage of the research vessel *Academician Mstislav Keldysh*. Figure 1 shows the location of the Haakon Mosby mud volcano (HMMV) and methane seeps on the Vestnesa.

Samples of rock were collected with a tube or with a box-corer sampler manipulated from the *Mir-1* and *Mir-2* deep-sea submersibles (DSS). Samples of benthic fauna were collected with a slab gun (a tool resembling a vacuum cleaner) mounted on *Mir-2*.

To enumerate microorganisms, samples of bottom sediments were fixed with glutaraldehyde (0.5 ml of 25% GA per 5 ml of sediment). Then samples were brought to the laboratory where cells were desorbed from sediment particles by applying sonication using an UZDN-2T apparatus (2–10 s in a pulsed mode, a 0.015 A, 22 kHz). DAPI was used for fluorescence staining [11], and bacterial cells were enumerated in a LUMAM-3 luminescent microscope.

The population density of different physiological groups of microorganisms was determined by the method of serial dilutions on selective nutrient media prepared on seawater. For enumerating methanotrophic bacteria, mineral medium was used with the addition of 5% of methane to the gas phase [12]. Sulfur-oxidizing bacteria were enumerated on mineral medium with the addition of thiosulfate (1 g/l), methanogens on the medium with $H_2$ and $CO_2$ [13], and sulfate reducers on the medium containing acetate [13]. Growth of methanotrophic bacteria was determined chromatographically by $CO_2$ formation; the growth of methane producers also chromatographically by $CH_4$ formation; the growth of sulfate reducers–by a change of nutrient medium color caused by the formation of ferrous sulfide; and the growth of sulfur-oxidizing bacteria by medium acidification and thiosulfate content depletion.

Rates of microbial processes were determined radioisotopically using substrates labeled with $^{14}C$ and $^{35}S$. Immediately after lifting aboard, 3-ml portions of sediment were placed into 5-ml plastic syringes with cut nozzles and sealed with a gas-tight butyl rubber stopper. The appropriate labeled substrate (0.2 ml) was introduced through the stopper and samples were incubated in a refrigerator at a temperature of 1–3°C for 1–2 days. Upon completion of the incubation, the samples were fixed with 0.5 ml of 2 M KOH solution and transported to the mainland laboratory. Further sample treatment followed the method described elsewhere [14, 15]. The rate of methane oxidation was determined with the use of $^{14}C$-methane dissolved in gas-free distilled water that was introduced at a rate of 2 μCi per sediment samples. The rate of sulfate reduction was determined with $^{35}S$-sulfate (10 μCi per sample); methane production with using $^{14}C$-bicarbonate (10 μCi per sample) and $^{14}C$-acetate labeled at the second position (10 μCi per sample); utilization of glucose with evenly labeled $^{14}C$-glucose (2 μCi per sample); and $CO_2$-assimilation with $^{14}C$-bicarbonate (10 μCi per sample). Sediment samples fixed with KOH and stored in a refrigerator for 6 h prior to the addition of the labeled substrate served as controls.

Silt water was squeezed using a pneumopress. Alkalinity determination (by titration) and the ammonium content assays (using the Nessler reagent) were carried out by G.A. Pavlova (the researcher of the Institute of Oceanology, Russian Academy of Sciences) directly aboard the ship. Sulfates were determined on a Biotronik chromatograph with a flame-ionization detector. The content of methane was measured by the method of gas chromatography on board the ship by A.V. Egorov (the researcher of the Institute of Oceanology, RAS).

Specimens of bacterial mat and animal tissue for scanning electron microscopy studies were prepared

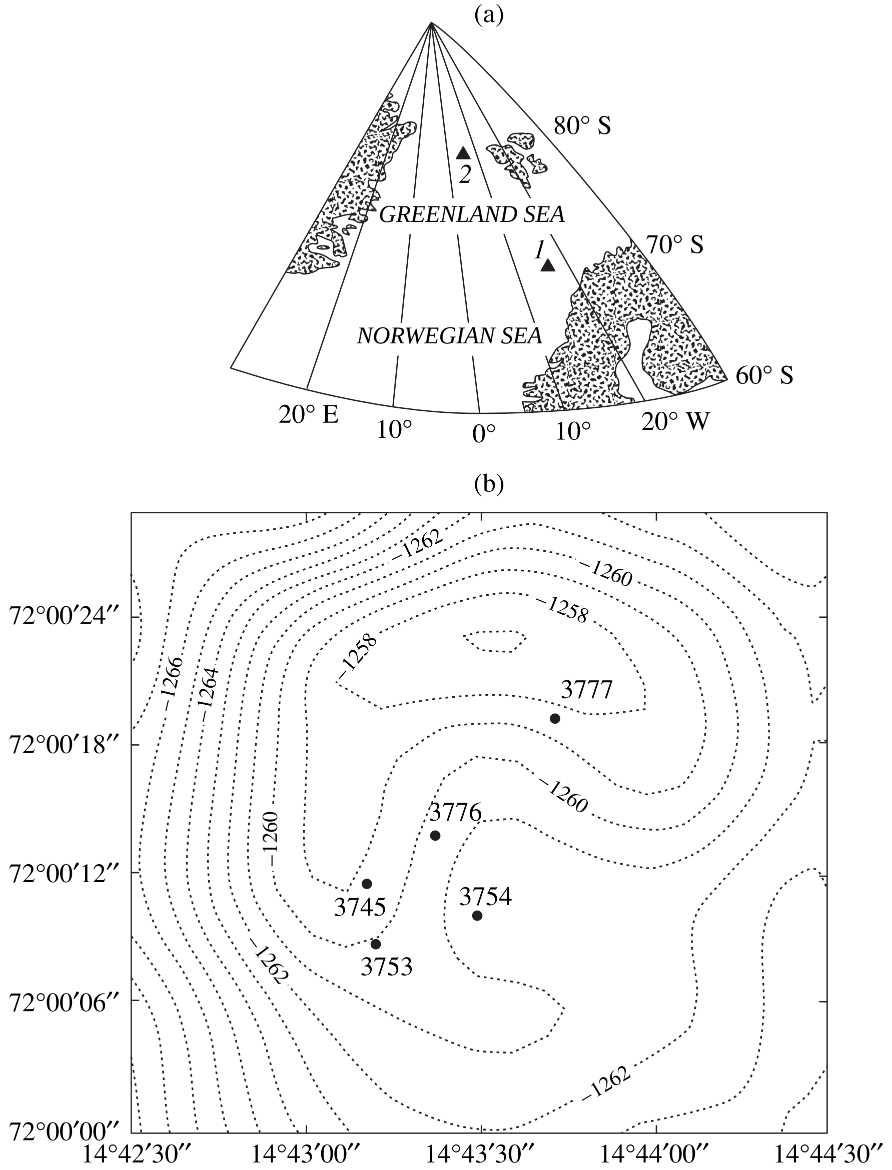

**Fig. 1.** (a) The geographic locations of (*1*) the Haakon Mosby mud volcano and (*2*) pockmarks on the Vestnesa Ridge; and (b) a location chart of stations where samples of HMMV surface sediments were obtained with geological samplers available on the deep-sea submersibles *Mir-1* and *Mir-2*.

aboard the ship. Samples of animal tissue were fixed with glutaraldehyde (4 h at 4°C), kept in 2% $OsO_4$ vapor for 4 h at 20°C, and dehydrated with ethanol. Specimens for scanning electron microscopy were dried according to the temperature critical point method and examined in a Hitachi S 405a scanning electron microscope (Japan) at an accelerating voltage of 15 kV.

Ultrathin sections were cut on an LKB-III ultramicrotome with subsequent lead citrate staining according to Reynolds [16] and examined in a JEM-100C microscope (Japan) at an accelerating voltage of 80 kV.

## RESULTS

**A brief description of the exploration region.** The Haakon Mosby undersea mud volcano (72°00′ N, 14°44′ E) is located on the eastern continental slope of the Norwegian Sea southwest of Medvezhi Island at a depth of 1270 m. On the seafloor, HMMV looks like a round flat disk with a diameter of about 1 km surrounded by a ring-like set of hills with a height of 3–6 m [7]. Detailed morphological description of its caldera was given by Bogdanov *et al.*, who carried out oceanographic observations of the volcano from DSS *Mir* [17]. Based on videos and direct observations from DSS *Mir*, it was shown that no less than 20 ha of the

**Table 1.** Chemical composition of silt waters from surface sediments in the caldera of HMMV and pockmarks on the Vestnesa Ridge

| Station no. | Horizon, cm | Eh, mV | Alk, mg-equiv/l | N-$NH_4^+$, mg/l | $SO_4^{2-}$, mg/l | $CH_4$, mg/dm$^3$ |
|---|---|---|---|---|---|---|
| Haakon Mosby mud volcano | | | | | | |
| 3745M1-3 | 0–2 | –20 | 11.7 | 4.7 | 1560 | 3.0 |
| | 3–5 | –250 | 18.5 | 18.5 | 1050 | >50 |
| | 5–8 | –250 | 21.3 | 27.5 | 750 | >50 |
| 3753-M2-2 | 0–20 | –250 | 38.9 | 33.8 | 1250 | >50 |
| 3754-M1-5 | 0–1 | 20 | 19.0 | 17.0 | 1000 | 3.5 |
| | 5–10 | –200 | 30.0 | 34.5 | 145 | >50 |
| | 10–20 | –250 | 14.0 | 21.5 | 435 | >50 |
| 3776-M1-1 | 0–5 | –280 | 22.1 | 42.5 | 85 | >50 |
| | 5–10 | –295 | 22.8 | 45.5 | 25 | >50 |
| | 10–20 | –300 | 25.9 | 42.0 | 15 | >50 |
| 3777-M2-1 | 0–1 | –200 | 17.5 | 25.0 | 1530 | >50 |
| Pockmarks on the Vestnesa Ridge | | | | | | |
| 379M1-1 | 0–0.5 | 100 | 6.9 | – | 2015 | 3 |
| | 1–3 | –200 | 12.7 | 16.5 | 1540 | >50 |
| | 3–5 | –200 | 18.1 | 19.7 | 1540 | >50 |
| 3795-M1-2 | 0–2 | 180 | 4.7 | 4.1 | 1990 | 2 |
| | 3–6 | –100 | 18.8 | 7.5 | 1280 | 15 |
| 3802 | 0–1 | –50 | 12.7 | 9.8 | 1950 | 3.0 |
| | 1–3 | –220 | 22.5 | 15.4 | 1460 | >50 |
| | 6–8 | –230 | 25.0 | 8.7 | 865 | >50 |
| | 15–20 | –215 | 21.0 | 4.5 | 760 | >50 |
| Hemipelagic Holocene sediments outside the caldera (background) | | | | | | |
| 3751 | 0–10 | 350 | 4.01 | 3.5 | 2650 | 0.0007 |
| 3752 | 0–5 | 350 | 3.67 | 2.9 | 2510 | 0.0006 |

caldera seafloor was covered with bacterial mats [17, 18]. The mats grew in patches and, depending on their location, covered from 5 to 80–90% of the seafloor bottom deposits. An important observation was that, on the microrelief scale, bacterial mats could be clearly associated with local depressions in the seafloor. Underlying the mats, there were black, strongly reduced, gas-saturated deposits giving off a pungent odor of hydrogen sulfide.

The dives of *Mir* submersibles to the pockmark field hypothesized on the basis of geophysical studies confirmed its presence on the Vestnesa. The major part of the investigated area is a plane covered with highly bioturbulated sediments. There were also a few elevations with a fairly hilly relief. Some of these seafloor regions had small depressions up to a few meters in the diameter covered with bacterial mats. Samples of benthic water taken from this zone showed increased concentration of methane. Based on the reports of the observers and video recording of the dives, it can be concluded that, in color and their pattern of occurrence at local seafloor depressions, the bacterial mats of the Vestnesa pockmarks resemble those of HMMV. Beneath bacterial mats, there were black strongly reduced hydrogen sulfide silts with high concentrations of methane (more than 50 ml/l) and unsaturated homologs of methane, indicating the discharge of subsurface hydrocarbons.

**The chemical composition of silt water.** Silt water in samples collected from under bacterial mats had high alkalinity (up to 38 mg-equiv/l, in the layer 0–10 cm) and high concentrations of ammonium (up to 45 $\mu$g N-$NH_4^+$/l) and methane (Table 1). In most of the obtained silt columns, the reliable measurements of the methane content were possible only in the top 0.5 cm, giving the value of 3 ml/kg sediment. The lower sediment layers were gas-saturated and contained more than 50 ml/l of methane. By contrast, the concentration of the sulfate-ion in silt waters declined sharply from the surface downward in the sediment profile. Compared to benthic waters, where the sulfate-ion concentrations varied between 2.67 and 2.7 g/l [19], it did not exceed 1.5 g/l in the topmost 10 cm of the sediment under bacterial mats.

**Table 2.** Rates of microbiological processes in surface sediments in the caldera of HMMV and pockmarks on the Vestnesa Ridge

| Station no. | Horizon, cm | Glucose utilization, nmol C/(dm$^3$ day) | $CO_2$-assimilation, μg C/(dm$^3$ day) | $SO_4^{2-}$-reduction, μg S/(dm$^3$ day) | $CH_4$-oxidation*, μl/(dm$^3$ day) | $CH_4$-generation, μl/(dm$^3$ day) |
|---|---|---|---|---|---|---|
| Haakon Mosby mud volcano | | | | | | |
| 3745-M1-3 | 0–2 | 310 | 985 | 4090 | 184(23) | n. o. |
| | 3–5 | 360 | 852 | 850 | 1570(15) | 0.15 |
| | 5–8 | 125 | 548 | 155 | 115(8) | 1.25 |
| 3753-M2-2 | 0–20 | 840 | 1530 | 5390 | 275(29) | 2.40 |
| 3754-M1-5 | 0–1 | 770 | 1850 | 13390 | 325(30) | n. o. |
| | 5–10 | 720 | 780 | 1145 | 732(17) | 3.50 |
| | 10–20 | 335 | 605 | 135 | 1065(23) | 2.15 |
| 3776-M1-1 | 0–5 | 385 | 395 | 4510 | 845(30) | 0.10 |
| 3777-M2-1 | 0–1 | 1490 | 1660 | 1020 | 260(26) | 0.35 |
| Pockmarks on the Vestnesa Ridge | | | | | | |
| 3795-M1-1 | 0–0.5 | 145 | 1350 | 24 | 14(29) | – |
| | 1–3 | 530 | 110 | 405 | 276(28) | 0.75 |
| | 3–5 | 170 | – | – | 250(26) | 1.00 |
| 3795-M1-2 | 0–2 | 720 | 510 | 2.5 | 430(27) | – |
| | 3–6 | 150 | 1220 | 100 | 540(18) | 2.80 |
| 3802 | 0–1 | 790 | 2080 | 4670 | 670(35) | – |
| | 1–3 | 890 | 445 | 17370 | 850(21) | – |
| | 6–8 | 910 | 230 | 825 | 150(33) | 1.95 |
| | 15–20 | 85 | 11 | 2180 | 180(12) | 3.25 |
| Hemipelagic Holocene sediments outside caldera (the background) | | | | | | |
| 3751 | 0–10 | 25 | 5 | n. o. | 0.002(25) | n. o. |
| 3752 | 0–5 | 50 | 3 | n. o. | 0.003(18) | n. o. |

Note: "–" means "not determined," and "n. o." means "not observed."

*Indicated in brackets is the percent of methane included into the acid-resistant fraction (bacterial cell mass + organic metabolites).

The concentration of sulfates further decreased down the silt column (Table 1; st. 3745-M1, st. 3802-M1).

In the caldera periphery and outside it, we observed oxidized surface sediments with a low methane content of less than 5 μl/kg. No bacterial mats occurred on the surface of such sediments. The low alkalinity of pore waters and their low ammonium content against the backdrop of high concentrations of sulfates (about 2.5 g/l) were indicative of sluggish diagenetic processes in sedimentary deposits in the caldera periphery and beyond its range (Table 1).

### *Rates of Microbial Processes*

**1. Sediments under bacterial mats.** Given in Table 2 are rates of microbial processes determined in surface sediments of the HMMV caldera and pockmarks of the Vestnesa Ridge. The highest intensities of microbial processes were observed in the topmost 20 cm of sediments. Compared to the data obtained in 1996, the rates of microbial processes in surface sediments sampled with the help of deep-sea submersibles turned out to be greater by several orders of magnitude [9]. It is evident that the most active part of surface sediments is lost as samples are collected and lifted aboard the ship using regular geological samplers.

The determined rates of glucose consumption (up to 1.49 mmol C/(dm$^3$ day)) and dark $CO_2$-assimilation (2.08 mg C/(dm$^3$ day)), which reflect the overall activity of microbial processes, are quite high for hemipelagic sediments and comparable to rates of these processes in shallow shelf sediments with high concentrations of labile organic matter (Table 2). The rates of methane oxidation (reaching 1570 μl C/(dm$^3$day)) and sulfate reduction (up to 17 mg S/(dm$^3$day)) measured in the surface sediments of HMMV and pockmarks of the Vestnesa Ridge (Table 2) were close to the maximum rates of these processes observed in badly polluted regions of the northwestern shelf of the Black Sea [20].

The rate of methane production (Table 2) in sediments studied was much lower and ranged from 0.1 to 3.5 μl $CH_4$/(dm$^3$day).

The gas-saturated surface sediments covered by the bacterial mat are, therefore, characterized by extremely

**Table 3.** Abundance of microorganisms in surface sediments in the caldera of HMMV and pockmarks on the Vestnesa Ridge

| Station no. | Horizon, cm | Total number, $10^5$ cells/cm$^3$ | Numbers of different groups of bacteria, cells/cm$^3$ | | | |
|---|---|---|---|---|---|---|
| | | | sulfate reducers | methanogens | sulfur oxidizers | methanotrophs |
| Haakon Mosby mud volcano | | | | | | |
| 3745-M1-3 | 0–2 | 1500 | 1000 | n.o. | 100 000 | 1000000 |
| | 3–5 | 950 | 1000000 | 100 | 100 | 1000 |
| | 5–8 | 1800 | 100000 | 100 | – | – |
| 3753-M2-2 | 0–20 | 200 | 10000000 | 1000 | 100 | 1000 |
| 3754-M1-5 | 0–1 | 1000 | 100000 | 10 | 1000000 | 1000000 |
| | 5–10 | 150 | 100000 | 100 | – | – |
| | 10–20 | 25 | 1000 | 10000 | – | – |
| 3776-M1-1 | 0–5 | 100 | 10000 | 100 | 1000 | 100000 |
| 3777-M2-1 | 0–1 | 1250 | 10000 | 10 | 10000 | 100000 |
| Pockmarks on the Vestnesa Ridge | | | | | | |
| 3795-M1-1 | 0–0.5 | 15 | 10 | n.o. | 100 | 1000 |
| | 1–3 | 85 | 1000 | 10 | 1000 | 10000 |
| | 3–5 | 15 | 10 | 1000 | – | – |
| 3795-M1-2 | 0–2 | 30 | n.o. | n.o. | 100 | 100 |
| | 3–6 | 50 | 100 | 10 | 1000 | 1000 |
| 3802 | 0–1 | 1500 | 1000 | 10 | 1000000 | 1000000 |
| | 1–3 | 1200 | 1000000 | 100 | – | – |
| | 6–8 | 650 | 10000 | 10000 | – | – |
| | 15–20 | 250 | 10000 | 100 | – | – |

Note: "–" means "not determined" and "n. o." means "not observed."

high rates of glucose consumption, carbon dioxide assimilation, methane oxidation and sulfate reduction, but show relatively low rates of methane formation.

**2. Tissues of pogonophoras.** It was shown elsewhere that the tissue of the tube worm *Screlolinum* sp. oxidizes $^{14}$C-methane at a rate of 2 ng C/(mg protein h) [9]. This finding was confirmed by our new experiments with tissues of *Screlolinum* sp. We failed, however, to detect methane oxidation in tissues of a less abundant tube worm, *Oligobrachia* sp. Unfortunately, lacking enough material, we were not able to study the capacity of pogonophoras to assimilate $^{14}CO_2$.

### *Abundance of Microorganisms*

The total number of microorganisms in surface sediments of the methane seep regions studied could be as high as $10^8$ cells/cm$^3$, which confirms the high rates of microbial processes observed (Table 3). On selective nutrient media, high numbers of methanotrophic and sulfur-oxidizing bacteria (up to $10^6$ cells/cm$^3$) were found in the mildly reduced surface layer of sediment (0–0.5 cm). The maximal numbers of sulfate reducers (up to $10^7$ cells/cm$^3$) and methanogens (up to $10^4$ cells/cm$^3$) were observed at a somewhat greater depth of 3–10 cm.

### *Electron Microscopic Studies*

**1. Bacterial mats.** Dive video records and reports of the observers left no doubt that the white spots on the surface of HMMV were in fact bacterial mats with a thickness of 0.1 to 0.5 cm. Shown in Fig. 2 are micrographs of bacterial cells in these mats obtained with a scanning electron microscope. The community of microorganisms composing bacterial mats was manifestly dominated by large filamentous bacteria with filaments measuring up to 100 μm in length and between 2 and 8 μm in width. Occasionally, the filaments were connected into rosettes (Fig. 2b). The occurrence of rosettes is an important taxonomic feature, allowing the obtained filamentous bacteria to be referred to the morphologically similar genera *Leucothrix* (nonsulfur filamentous bacteria) and *Thiothrix* (colorless filamentous sulfur bacteria). The community also included short rods and cocci in numbers notably smaller than that of the filamentous forms.

Three morphological types of filamentous bacteria were identified in bacterial mats covering pockmarks of the Vestnesa Ridge (Fig. 3). The first morphological type (Fig. 3a) was represented by long filaments joined in bundles. The thickness of cells along the entire filament was constant (6–7 μm, Fig. 3b). The filaments

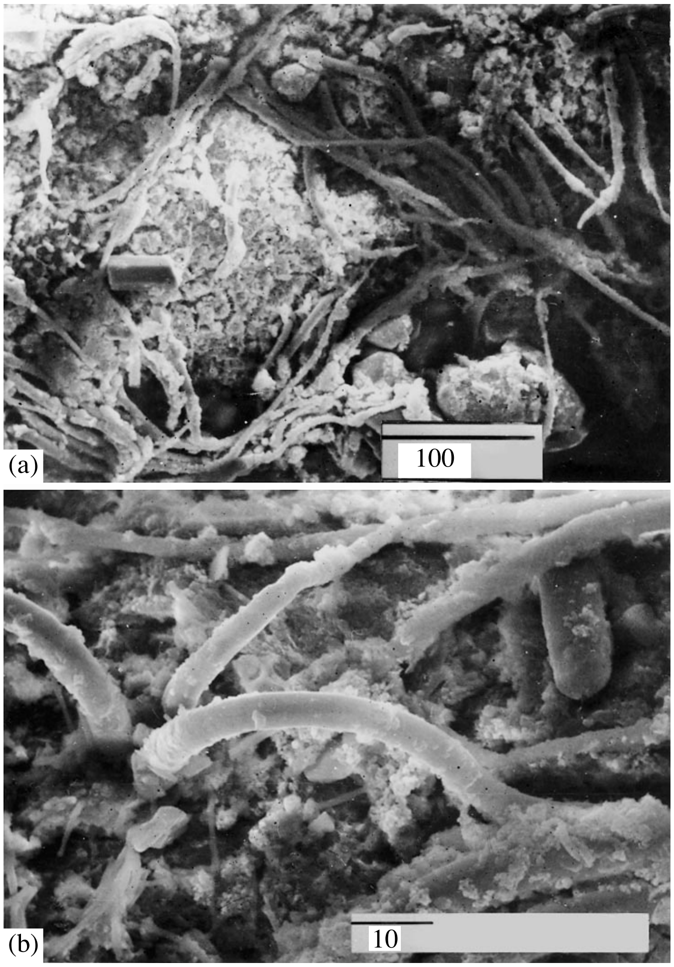

**Fig. 2.** Large filamentous bacteria that are predominant in bacterial mats on the surface of sedimentary deposits of HMMV: (a) accumulation of filaments, bar, 100 µm; and (b) rosettes at the base of filaments, bar, 10 µm.

adhered tightly to one another near the base, whereas on the other side they were usually free and spread. Individual cells in filaments were separated by readily visualized intercellular septa. The length of cells ranged from 4 to 8 µm, and in longer cells, one can see incomplete (indistinct) intercellular septa (Fig. 3c). Filaments of the first type are morphologically similar with representatives of the genera *Thioploca* or *Desmanthos* [21]. The second morphological type was represented by smooth filaments of variable thickness (2.5–6.5µm). Examination of such cells in a light microscope revealed numerous inclusions of sulfur, suggesting these cells were representatives of the genus *Thiothrix* (Fig. 3d). The third morphological type was represented by single filaments made up of tightly connected disk-like cells (Fig. 3e). A considerable part of filaments was coated with polymeric material that included mineral particles. The diameter of individual cells was 5–6 µm, and the disk thickness measured 0.7–0.9 µm (Fig. 3f). This morphological type can, presumably, be referred to the genus *Beggiatoa*.

**2. Tissues of pogonophoras.** Shown in Fig. 4 are results of electron microscopic studies of fine sections of tissue of the pogonophora *Sclerolinum* sp. The pogonophora's tissue was found to contain prokaryotic cells with intracellular membrane structures characteristic of methane-oxidizing bacteria (Figs. 4a, 4b). In the sections we examined, these bacterial cells were patently predominant.

Prokaryotic cells were also observed in tissues of the pogonophora *Oligobrachia* sp. (Fig. 4c), but its bacterial endosymbionts had no intracellular membranes.

## DISCUSSION

Bacterial mats, mostly composed of filamentous bacteria of the genera *Beggiatoa*, *Thiothrix*, and *Leucothrix*, were discovered in the peripheral zones of deep-sea hydrothermal vents [22] and methane seeps [4]. It was experimentally shown that filamentous sulfur bacteria morphologically resembling *Beggiatoa* and forming mats a few centimeters thick over sediments and

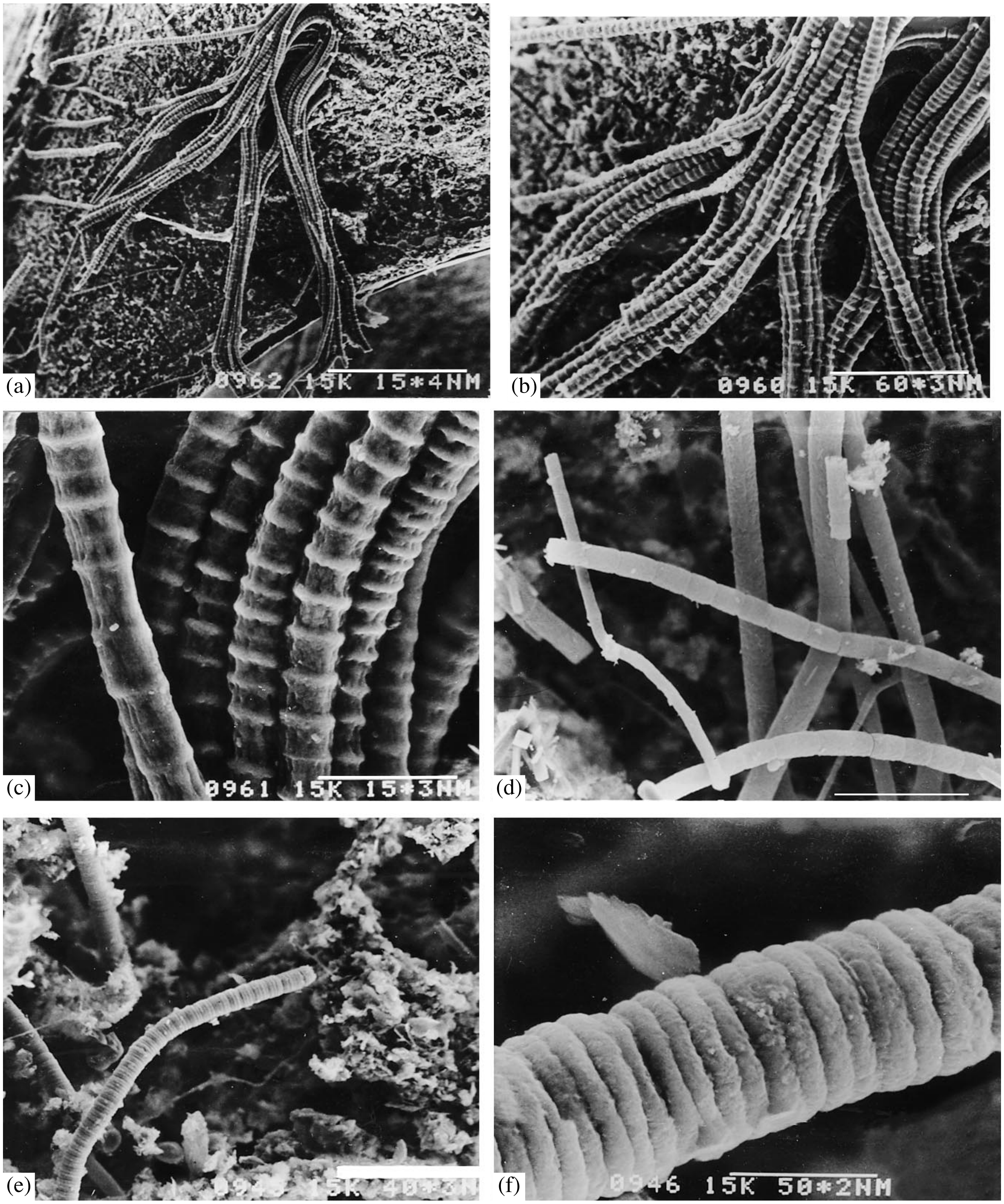

**Fig. 3.** Morphological types of filamentous bacteria found in bacterial mats on the surface of sedimentary deposits in pockmarks of the Vestnesa Ridge: (a) long filaments adhered into bundles, bar, 150 μm; (b) individual cells making up filaments, bar, 60 μm; (c) intercellular septa, bar, 15 μm; (d) smooth filaments with width varying between 2.5 and 6.5 μm, bar, 30 μm; (e) single filaments consisting of tightly connected disk-like cells, bar, 40 μm; (f) the diameter of individual cells is 5–6 μm, disk thickness is 0.7–0.9 μm, bar, 5 μm.

hydrothermal constructions in the Guaymas Basin (the California Bay) were capable of lithoautotropic growth [23]. Chemolithoheterotrophic growth with utilization of reduced sulfur compounds and carbon dioxide fixation was shown in *Thiothrix ramosa* isolated from a hydrogen sulfide vent in Florida [24]. It was also shown that the nonsulfur bacterium *Leucothrix thiophila*, morphologically similar in having rosettes to bacteria of the genus *Thiothrix*, were capable of lithoheterotrophic growth based on the utilization of reduced sulfur compounds as an electron donor [25].

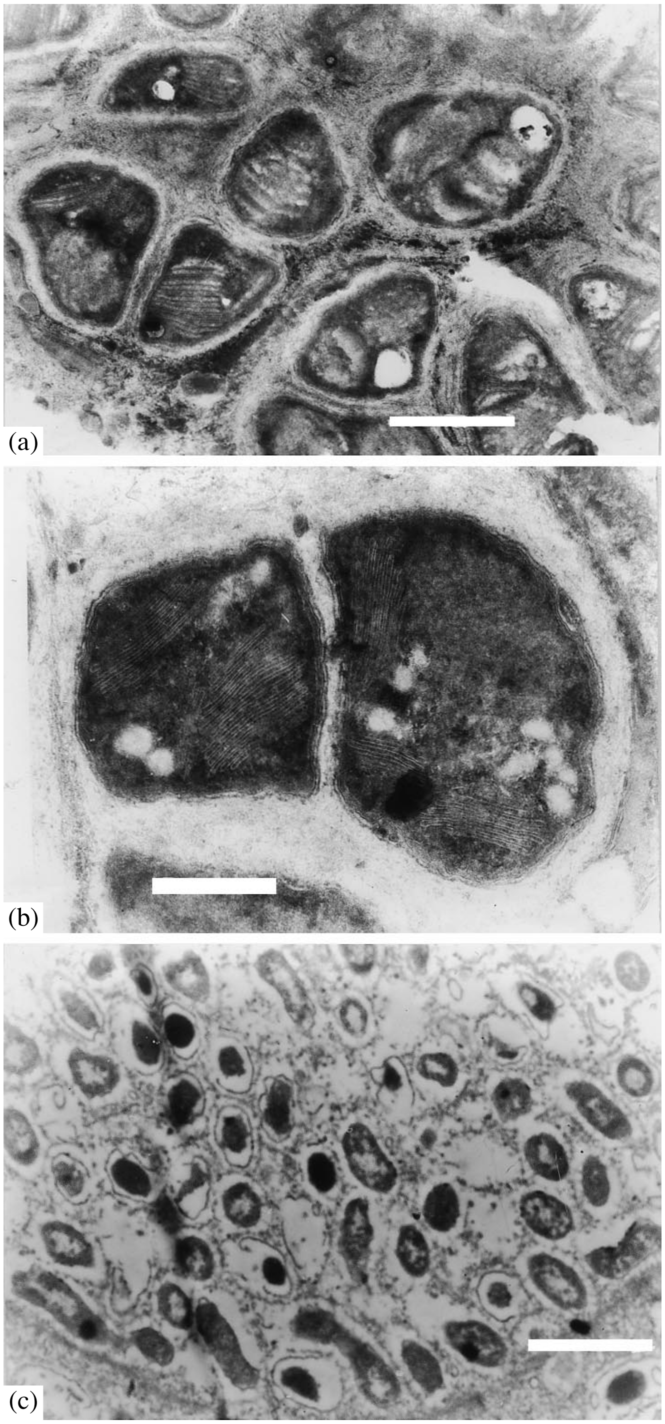

**Fig. 4.** Bacterial endosymbionts in trophosomes of pogonophoras: (a) and (b) ultrathin sections of the trophosome tissue of *Sclerolinum* sp.; one can readily see membrane structures in bacterial cells, bars, (a) 0.6 μm, and (b) 0.3 μm; (c) ultrathin sections of the trophosome tissue of *Oligobrachia* sp., bar, 4 μm.

By sampling surface sediments covered by bacterial mats with a slab gun or a box-corer from *Mir* submersibles, such sediments were found to be strongly reduced and saturated with free hydrogen sulfide. It is, therefore, beyond any doubt that the development of filamentous bacteria is strongly influenced by reduced sulfur compounds diffusing from sediment. In the marginal part of the caldera with low methane content, the oxidized sediment layer within the silt column measured 2–10 cm, and no bacterial mats were present on the surface of bottom sediments.

The obtained data on vertical distributions of alkalinity and contents of ammonium and sulfate combined with extremely high numbers of microorganisms and rates of sulfate reduction, glucose decomposition, and carbon dioxide assimilation are clear evidence of active processes of organic matter degradation in surface sediments of HMMV and Vestnesa Ridge pockmarks (Tables 2, 3). Considerable enrichment of the sulfate with heavy $^{34}S$ isotope ($\delta^{34}S$ as high as 30.1‰) is still more evidence of a high rate of sulfate reduction in surface sediments of HMMV [19].

The results of our study show that in methane-containing sediments of the caldera, active degradation of organic matter takes place with the formation of significant amounts of hydrogen sulfide through bacterial reduction of sulfates. The generated reduced sulfur compounds are utilized by microorganisms forming bacterial mats on the sediment surface.

The organic matter in sediments under bacterial mats turned out to be lighter than that of phytoplankton origin ($\delta^{13}C_{org}$ = –27.3 and –34.4‰, [19]). Such a considerable lightening of organic matter in HMMV and Vestnesa Ridge surface sediments might be due to active bacterial processes of methane oxidation ($\delta^{13}C\text{-}CH_4$ = –60‰; according to [8, 19]), which result in the production of carbon dioxide and in the formation of additional organic matter represented by cell mass of bacteria and their exometabolites (Table 2). The extremely high rates of methane oxidation (up to 1.5 ml/($dm^3$ day) in surface sediments of the areas studied bear witness to the hypothesis that, in the zone of methane seeps, methane serves as an important source of organic matter and carbon dioxide. The number of methane-oxidizing bacteria in oxidized surface sediments of HMMV was as high as $10^6$ cells/$cm^3$ (Table 3). According to a private communication by Yu.A. Trotsenko (Institute of Biochemistry and Physiology of Microorganisms, Pushchino), enrichment cultures of methanotrophs were isolated from surface sediments of HMMV. However, the fact to be given special attention is that the highest rates of methane oxidation were observed not in the topmost 1–2 cm of the sediment, where active aerobic oxidation of methane is possible with participation of methanotrophic bacteria, but rather in the layer of strongly reduced sediment at a depth of 3–20 cm. Unfortunately, the mechanisms of anaerobic methane oxidation are not yet clear. Based on the vertical profiles of methane and sulfate concentration in sediments, several workers suggested that the oxidation of methane with oxygen of sulfates might follow the equation $CH_4 + SO_4^{2-} \longrightarrow HCO_3^- + HS^- + H_2O$ [26]. However, it was shown by thermodynamic calculations that this happens to be an energy-requiring rather than an energy-yielding reaction [27] and is, therefore, not energetically favorable for bacteria. Given the high rate of anaerobic oxidation of methane observed in reduced HMMV sediments and that 8–35% of carbon contained in utilized methane (Table 2) is incorporated into organic matter (exometabolites + biomass), the bacterial oxidation of methane at the expense of sulfates does not seem likely. About 20 years ago, it was shown that, under an excess of methane in the culture medium, some part of methane could be anaerobically oxidized by pure cultures of methanogens to $CO_2$ by a reaction reverse to that of methane formation: $CH_4 + 2H_2O \longrightarrow CO_2 + 4H^+$- [28]. It was also established that the use of water as a terminal electron acceptor is thermodynamically possible at high concentrations of methane and low content of $H_2$ [26].

The previously shown occurrence of methanogens in HMMV sediments [9] was fully substantiated in this study of surface sediments. The number of these microorganisms in a medium with $H_2$ and $CO_2$ amounted to $10^3$–$10^4$ cells/$cm^3$, even though the rate of methane formation was low and did not exceed 6 μl $CH_4$/($dm^3$ day). It is possible that, in methane saturated sediments of HMMV, the methanogenic bacteria do not form but rather oxidize methane with the production of hydrogen, which is then utilized in sulfate reduction by sulfate-reducing microorganisms. This mechanism of methane oxidation involving a consortium of methanogenic and sulfate-reducing bacteria was suggested by Hoehler *et al.* [29] on the basis of field and laboratory studies of methane oxidation in anaerobic silts.

It follows that active oxidation of methane with the formation of $CO_2$ and production of organic matter in surface sediments of HMMV and Vestnesa Ridge pockmarks proceeds both under aerobic conditions through the action of methanotrophic bacteria and in the anoxic zone with the participation of a microbial consortium, presumably composed of methanogens and sulfate reducers.

Large areas in the HMMV caldera are populated by pogonophoras, represented by the two species *Sclerolinum* sp., and *Oligobrachia* sp., and forming enormous colonies that spread over tens of square meters. It is an important fact that these two species of pogonophoras do not mix in areas of their abundance: the wider occurring species *Sclerolinum* sp. develops on surface-oxidized caldera sediments, whereas bushes of *Oligobrachia* can be observed on more reduced sediments. It was already established in the first voyage of the research vessel *Professor Logachev* that tissues of the pogonophoras *Sclerolinum* sp. oxidized methane at a rate of 2 ng C/(mg protein) [9]. The isotopic composition of organic carbon in the pogonophora tissue ($\delta^{13}C$ = –48.3 and –52.4‰ [19]) suggests that methane serves as the primary source of carbon used to form organic components of cells of these animals. This hypothesis is also confirmed by the results of electron microscopic studies of tissue sections of the pogonophora *Sclerolinum* sp., which revealed the presence of endosymbiotic bacteria with a distinct system of intracellular membranes (Figs. 4a, 4b). It is beyond any doubt that the mass development of the pogonophora *Sclerolinum* sp. in the HMMV caldera is determined by the activity of aerobic methane-oxidizing bacteria localized inside cells of these animals. According to the estimates based on observations from *Mir* submersibles, the biomass of the pogonophora *Sclerolinum* sp. in areas of their proliferation can be as high as 435 g/m$^2$, and so these animals make up a considerable portion of the total organic matter in the HMMV region. The biomass of the pogonophora *Oligobrachia* sp. is somewhat lower, 350 g/m$^2$ and, as noted above, these animals tend to develop on more reduced sediments [19].

Electron microscopic studies of cell sections of *Oligobrachia* tissues also revealed the presence of bacterial endosymbionts (Fig. 4c). However, no typical membrane structures were observed inside cells of the endosymbiotic bacteria. We also failed to detect the oxidation of methane in tissue of these animals. The localization pattern of the pogonophora on the surface of reduced sediments suggests that the predominant bacteria in *Oligobrachia* tissues are sulfur-oxidizing endosymbionts, responsible for the synthesis of the required organic compounds from carbon dioxide at the expense of energy of reduced sulfur compounds supplied from bottom sediments.

No mass accumulation of pogonophoras as part of the benthic community was observed in areas of gas seeps on the Vestnesa Ridge. This can, probably, be explained by a small area of methane fields. It was already noted that *Sclerolinum* sp., the most abundant pogonophora species in the HMMV caldera, proliferated on sediments with an oxidized surface (2–10 cm deep). No such sediments were observed in the pockmark zone of the Vestnesa Ridge, where the diameter of craters bearing signs of methane seepage never exceeded a few meters. The sediments in the crater zone, covered with bacterial mat, contained high concentrations of hydrogen sulfide in the surface horizons, which, apparently, prevented the development of the pogonophora *Sclerolinum* sp. At the same time, in the area of bacterial mats, accumulations of small gastropodas (up to 1 cm) and benthic fish were noted; and the light isotopic composition of carbon contained in their tissues [19] indicates that the biomass of methanotrophic and chemoautotrophic bacteria constitutes an important component of their diet.

## CONCLUSION

The results of our studies of deep-sea methane seeps of the North Atlantic support the claim that perpetually low temperatures (around 0°C) pose no obstacle for the development of complex benthic communities observed at sites of gas seepage. The trophic chain of such a community is based on production of methanotrophic and chemoautotrophic bacteria. It is not yet clear if the production of organic matter by aerobic methane-oxidizing bacteria serves as a trigger for the development of the benthic community peculiar to cold methane seeps. A constant inflow of gas, observed in seep sediments, seems likely to render them anoxic. The oxidation of methane is carried out under anaerobic conditions by a consortium of methanogens and sulfate reducers. The resulting hydrogen sulfide sustains the development of bacterial mats on the surface of bottom sediments in pockmarks. The extremely high rates of glucose utilization, dark assimilation of carbon dioxide, methane oxidation, and sulfate reduction that were observed in sediments in the HMMV caldera and in Vestnesa Ridge pockmarks suggest high coordination in the multifunctional microbial community of cold methane seeps, carrying out organic matter production at the expense of methane and reduced sulfur compounds.

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