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EXPERIMENTAL ARTICLES

Geochemical Peculiarities of the Carbonate Constructions Formed during Microbial Oxidation of Methane under Anaerobic Conditions

A. Yu. Lein*, M. V. Ivanov, N. V. Pimenov**, and M. B. Gulin*****
**Shirshov Institute of Oceanology, Russian Academy of Sciences, pr. Nakhimovskii 36, Moscow, 117851 Russia*
***Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
****Institute of Biology of Southern Seas, National Academy of Sciences of Ukraine, pr. Nakhimova 2, Sevastopol, Ukraine*

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Abstract—The aragonite constructions of the Black Sea are formed in a stable anaerobic zone and are a perfect object to study the natural mechanism of anaerobic methane oxidation. The most probable pathway of methane oxidation is its methanogen-mediated reaction with bicarbonates, dissolved in seawater, with the formation of water and acetate, which is then consumed by other components of the anaerobic community. Comparison of the $\delta^{13}C$ values of carbonate minerals and organic matter once more demonstrated that the formation of the organic matter of biomass is accompanied by intense fractionation of carbon isotopes, as a result of which the total organic matter of biomass acquires an extremely light isotopic composition, characterized by $\delta^{13}C$ values as low as –83.8‰.

Key words: carbonate constructions, isotopic composition anaerobic methane oxidation.

Autigenic carbonate formation with the involvement of carbon dioxide formed by microorganisms is widespread in modern marine reservoirs. In reduced sediments of the shelf and of the continental slope, protodolomite and magnesian calcite are formed with the involvement of carbon dioxide produced during anaerobic decomposition of organic matter in the course of sulfate reduction [1]. The formation of autigenic carbonates of this type, as distinct from sedimentary and shell carbonates, involves carbon dioxide with a lighter isotopic composition of carbon, which is derived from the organic matter of bottom sediments.

Methane-derived carbonates, formed in the microbial processes of aerobic and anaerobic methane oxidation, have a quite different, much lighter isotopic composition [2].

An example of large agglomerations of autigenic carbonates formed during aerobic oxidation of methane is carbonate plates complicating the bottom surface of the northwestern shelf of the Black Sea at depths from 60 to 170 m [3]. The only known example of carbonate constructions formed under anaerobic conditions are coral-like constructions on the slopes of the Dnepr canyon at depths of 170 to 600 m in the hydrogen-sulfide zone of the Black Sea [4]; these constructions were discovered in 1989.

One such construction (44°41.7' N; 31°40.7' E; depth, 226 m) was lifted with a sampler of a *Bentos-300* submersible laboratory (*Bentos-300* SL) and was studied in detail at the Institute of Microbiology, Russian Academy of Sciences (RAS) [4].

The thick slimy microbial mat, covering the surface of the construction, was found to contain morphologically diverse microorganisms, and the extremely light isotopic composition of the carbon of both carbonate minerals and organic matter allowed a suggestion to be made that the coral-like constructions are formed at the expense of microbial oxidation of methane [4].

Over the last decade, several investigations were undertaken to elucidate the microorganisms (or their communities) responsible for methane oxidation and the formation of carbonate constructions in this anaerobic zone of the Black Sea. Analysis of electron micrographs of microbial mats from the surface of the carbonate construction taken from a depth of 226 m revealed rarely occurring microbial cells that possessed well-developed systems of intracytoplasmic membranes similar to those of aerobic methylotrophic bacteria. Inoculation of media for cultivation of aerobic methanotrophs with mat samples resulted in the development of these microorganisms; immunofluorescent methods demonstrated representatives of the genera *Methylobacter* and *Methylomonas* to be predominant among methanotrophs [4].

Different results were obtained by a team of researchers from the Institute of Microbiology, RAS and Institute of Biochemistry and Physiology of Microorganisms, RAS [5] during the study of another bacterial mat from the surface of a carbonate construction lifted with a trawl from a depth of 188–195 m in the course of an expedition in 1994. A radioisotopic test for aerobic oxidation of $^{14}CH_4$ produced a negative result. Cellular extracts exhibited no activity of methane monooxygenase or of enzymes of C_1 compound oxidation typical of aerobic methylotrophic bacteria. At the same time, radioisotopic experiments showed active occurrence in the mat of anaerobic methane oxidation, methanogenesis (by the reaction $CO_2 + H_2 \longrightarrow CH_4 + 2H_2O$), and sulfate reduction.

Light and electron microscopy revealed large filamentous bacteria morphologically similar to *Methanosaeta* [5]. Summing up the data obtained resulted in a conclusion that anaerobic methane oxidation in the bacterial mat involves anaerobic methanogens and follows the reaction reverse to methane formation that was first suggested by Zehnder and Brock [6, 7]: $CH_4 + H_2O \longrightarrow CO_2 + H_2$.

One more convincing proof of the presence of methanogens in the mats from carbonate constructions lifted from a depth of 188–195 m was provided by molecular-biological investigations, which yielded clone Bseual 2, which is an archaeal phylotype clustering with the voluminous ANME-1 group, remotely but specifically related to methanogens of the orders *Methanosarcinales* and *Methanomicrobiales* [8].

However, molecular-biological investigations also revealed in the same mat samples genes coding for enzymes specific for aerobic methanotrophs [8]. Thus, microbiological, biochemical, and molecular-biological methods used by the authors of three works carried out over the last decade, i.e., after the discovery of the carbonate constructions in the anaerobic zone of the Black Sea, failed to give an unequivocal answer to the question as to what microorganisms play the major role in the methane oxidation to CO_2 and in the formation of carbonate constructions in the Black Sea.

Moreover, the above-cited works did not take into account the possible involvement of sulfate-reducing bacteria in anaerobic methane oxidation and in the formation of carbonate constructions, although some authors [9] consider feasible anaerobic methane oxidation by these bacteria according to the reaction $CH_4 + SO_4^{2-} \longrightarrow H_2S + CO_2$. It should be noted that microbial sulfate reduction is known to lead to medium alkalization, which is to favor precipitation of calcium carbonate.

The aim of the present work was to study in detail microbiological, mineralogical, and geochemical features of the carbonate constructions at the active methane seeps in the anaerobic zone of the Black Sea with the final goal of elucidating the mechanism of their formation.

MATERIALS AND METHODS

The materials for investigations were collected during the expedition on board the *Bentos-300* SL (December 1990) and during the 44th and 45th voyages of the research vessel *Professor Vodyanitskii* in October 1993 and July 1994. During the first expedition on *Bentos-300* SL, the constructions at the bottom were described based on visual observations and video tape recording; sampling was performed using a hydraulic ground sampler. During the voyages of the research vessel, carbonate constructions and bacterial mats were sampled with a bottom trawl (mesh size, 0.5 cm) in the zone of transition from open shelf to continental slope, at depths of 130–600 m (Fig. 1).

Investigation of the carbonate constructions employed traditional methods of light microscopy (analysis of sections, immersion specimens, stained specimens, etc.).

The morphology of minerals was studied using a Tesla-B-300 scanning electron microscope. The chemical composition of the carbonate constructions was determined by X-ray-fluorescence analysis (Philips PW 1600). The content of organic carbon (C_{org}) was determined on an AH-759 express analyzer.

To determine the isotopic composition of the carbon and oxygen of carbonates, the samples were decomposed with dehydrated orthophosphoric acid on a circular vacuum device and purified by the cryogenic method [10]. C_{org} was oxidized to CO_2, which was then purified and trapped in sealed glass ampules. The isotopic composition of carbon was determined on a MI-1201V mass spectrometer equipped with an SNG-3 three-channeled system of gas admission. The isotopic composition of oxygen was determined on a Micromass-602 D mass spectrometer (United Kingdom). The accuracy of measurements was ±0.2%. The $\delta^{13}C$ and $\delta^{18}O$ data are presented in relation to the PDB standard. The absolute age of the carbonate carbon was determined by the radiocarbon method described in [11]. The radioactive strontium ^{90}Sr was determined according to [12].

Geological Situation

Active seepages of gas into the water column in the region studied were first discovered in 1989 [13]. This region is situated in the marginal zone of the continental platform and, in part, in the zone of transition to the Black Sea oceanic-type depression. The region represents a continuation of the Odessa tectonic zone.

The structural–morphological peculiarities of the seafloor in the zone of seeps were studied in most detail in December 1990 using a *Bentos-300* SL. The main gas seeps occur at the top of the ridge-type flattened elevations complicating the walls of the Dnepr canyon

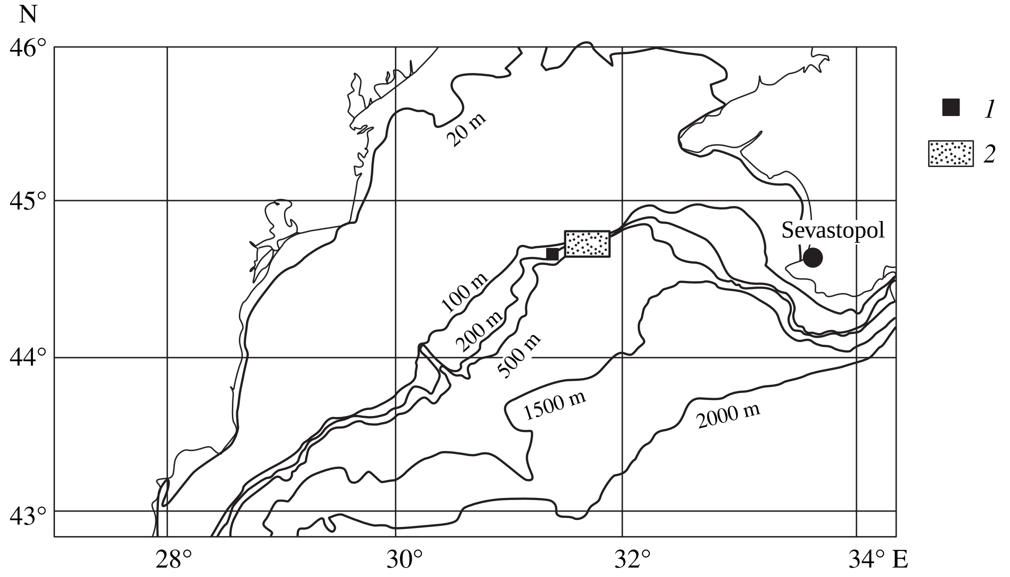

Fig. 1. Zone of methane seeps in the Dnepr canyon (northwestern part of the Black Sea) at depth from 60 to 590 m: *1*, the site of sampling from *Bentos-300* SL in December 1990; *2*, the site of sampling by trawling from board research vessel *Professor Vodyanitskii* in October 1993 and July 1994.

at various depths. In the Dnepr canyon, modern sediments are eroded, exposing sediments of Neoeuxine age [14]. Visual observation of the gas emission pattern, performed from the *Bentos-300* SL, showed that the period of gas accumulation in the "gas chamber" lasts 90 to 120 min. Gas bubbling takes 30–60 s and occurs in an accelerating tempo, first as separate 10-mm bubbles and then as a continuous flow, which gradually damps [3]. Observations from *Bentos-300* SL resulted in the first description of carbonate constructions, light-colored bacterial mats, and black "sulfide" spots on the bottom [4].

On the methane seep field, at a depth of 60 m, carbonate tablets were found, which were 20–30 cm in diameter and less than 2 cm thick and had a central channel 1–2 cm in diameter. Gas flows emerged from the bottom from carbonate-cemented pelite–aleurite terrigenous sediments of Neoeuxine age (determined from the presence of *Dreissena rostriformis* fauna). Near gas seeps, the content of $CaCO_3$ in the sediments reached 45%, whereas normal Neoeuxine sediments contain 8–11% carbonate. Gas from a sample of near-bottom water contained 50% methane, 30–40% N_2, and 10% O_2. Thus, in the aerobic shallow-water shelf zone, autigenic carbonate cemented the sediment without forming elevated constructions. In the oxygen–hydrogen sulfide interface zone, at depths of 110–130 m, the surface carbonate formations increased in their area and reached 10 cm in height [3, 4].

At depths of 160 to 240 m, in the upper part of the Dnepr canyon, somewhat to the east of its axial zone, on the surface of clayey sediment covering the top and slopes of a flattened ridge, carbonate constructions of various morphology were found [4]. At depths of 160–190 m, flat tabular constructions with an area of 3 m^2 and a height of 40–60 cm occurred, as well as carbonate plates several centimeters thick. At depths greater than 190 m, the morphology of the constructions was more diverse. Here, vertically growing coral-like and tree-like constructions were found, which sometimes reached 3.5 m in height and 1.5 m in diameter. These constructions emerge from local dish-shaped or bowl-shaped depressions in the sediments.

The tops of coral-like and tree-like constructions are crowned with rosette-like or sponge-like black hollow excrescences. Pressing on these excrescences resulted in emission of gas bubbles; disturbing of the black outer cover revealed a bright pink jelly-like mass [3].

During the work on the *Bentos-300* SL in 1990 and as a result of trawling during the expeditions in 1993 and 1994, carbonate constructions with thick (up to 5 cm) microbial mats were lifted from the hydrogen-sulfide zone of the sea [5].

RESULTS

Petrography, Mineralogy, and Chemical Composition of the Carbonate Constructions

Among the samples of carbonate constructions that we studied (Table 1), two major morphological types can be distinguished: (I) tabular, often laminated, dark-gray or gray, sometimes with low tree-like projections

Table 1. Sampling stations and their locations

Date of sampling	Station nos. and sampling devices	Depth, m	Location	Sample description
Dec. 7, 1990	1, 2, *Bentos-300* SL, bucket	226	44°41.7′ N 31°40.7′ E	Coral-like constructions and fragments of microbial mats
Oct. 15, 1993	5176, trawl	178–198	from 44°46.68′ N 31°57.95′ E to 44°46.66′ N 31°59.21′ E	Fragments of plates and coral-like constructions without mats and samples of mats
June 22, 1994	5212, 5213, trawl	180–200	from 44°51.24′ N 32°03.43′ E to 44°46.62′ N 31°58.67′ E	A large fragment of a carbonate plate
June 24, 1994	5224, trawl	230–260	from 44°46.50′ N 31°59.57′ E to 44°46.35′ N 31°59.99′ E	Fragments of plates and coral-like constructions
June 24, 1994	5228, trawl	590	from 44°39.48′ N 31°46.50′ E to 44°39.55′ N 31°47.57′ E	Fragments of a coral-like construction and black microbial mat

whose central part exhibits a pronounced zone of hollow channels (Fig. 2a), and (II) coral-like (Fig. 2b), with abundant residues of laminated bacterial mats, whose color changes from bright pink in the internal part of the mat adjoining the carbonate carcass to greenish-gray and black in the outer layer of the mat (Fig. 2c). The color of the mat often determines the color of the construction.

In tabular constructions, finely crystalline calcite prevails, which cements the terrigenous sedimentary material (Fig. 3a): detrital quarts, feldspar, micaceous and clayey minerals, diatom shells, coal-like wood residues. In the microcrystalline carbonate cement, finely crystalline framboidal pyrite also occurs. In the upper parts of tabular constructions, along with microcrystalline calcite, microcrystalline aragonite appears. Aragonite was identified from the refraction value ($n_p = 1.48$; for calcite, $n_p = 1.530$) and from the acquisition of lilac color upon boiling with $Co(NO_3)_3$ (the Meigen reaction). The size of calcite and aragonite crystals from the microcrystalline cement does not exceed 30 μm.

The chemical composition of tabular constructions is characterized by broad variations in the carbonate content (from 33 to 60%) and by the presence of terrigenous silicate and aluminosilicate minerals and detrital quarts. Microcrystalline carbonate from the plate bases contains 7 to 10% $MgCO_3$ and less than 0.2% $SrCO_3$; i.e., microcrystalline carbonate is mainly represented by Mg-calcite. The content of C_{org} varies from 0.37 to 0.58%.

Coral-like constructions virtually consist of monomineral aragonite (Table 2). Their content of compounds insoluble in 7% CH_3COOH is 0.6–2.5%. Aragonite is represented by oolitic and globular aggregates with a radial structure (Fig. 3b) and columnar aggregates consisting of prismatic, pyramidal, and needle-shaped crystals (Figs. 3c, 3d). Twin and trilling crystal penetration is typical.

The aragonite of coral-like constructions contains less than 0.3% $MgCO_3$, 0.5–1.5% $SrCO_3$, and 1.13–1.63% C_{org}. The high content of strontium in coral-like constructions can be explained by the preferential

Table 2. Chemical composition of carbonate constructions (according to [15, 16], extended)

Component	Station 5176, depth 178–198 m		Station 1, depth 226 m	
	tabular construction	coral-like construction	coral-like construction	
			top	bottom
SiO_2	56.79	1.86	0.97	0.84
TiO_2	0.22	0.01	0.03	0.04
Al_2O_3	2.30	0.32	0.04	0.00
Fe_2O_3	0.79	0.24	0.20	0.17
MnO	0.03	0.05	0.03	0.01
MgO	2.40	1.04	0.04	0.01
CaO	28.57	50.95	53.73	54.87
SrO	0.03	1.02	1.10	1.12
Na_2O	0.36	0.67	0.16	0.00
K_2O	0.78	0.09	0.03	0.04
P_2O_5	0.11	0.17	0.14	0.11
n.n.n.	7.55	44.02	43.33	43.15
Total	99.93	100.44	99.80	100.36

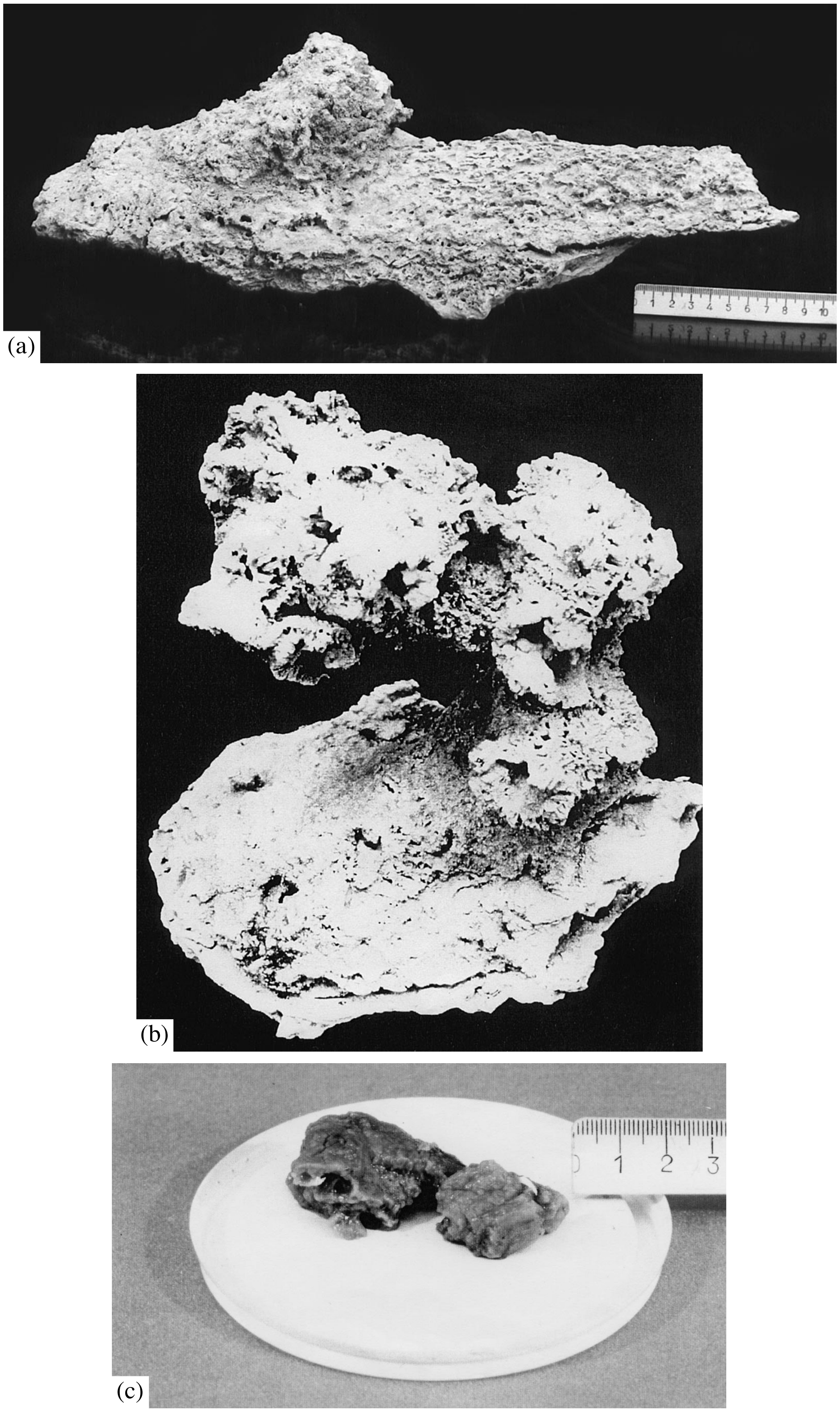

Fig. 2. Morphology of autigenic constructions form methane seep fields in the Dnepr canyon. (a) A fragment of a carbonate plate consisting of exposed sediments of Neoeuxine (Lacustrine) age, containing *Dreissena rostriformis* fauna cemented by microcrystalline Mg-calcite. The bottom part of the plate was ingrown into the sediment. The plate is complicated by a small aragonite superstructure with numerous hollow channels. Depth, 180–200 m. (b) Coral-like aragonite construction, 30 cm in height, on a small tabular pedestal consisting of sediment cemented by microcrystalline Mg-calcite at the base and by Mg-calcite and aragonite in the upper part of the plate. Depth, 230 m. (c) Microbial mats from coral-like aragonite constructions, up to 5 cm thick and consisting of an outer black and inner pink layers.

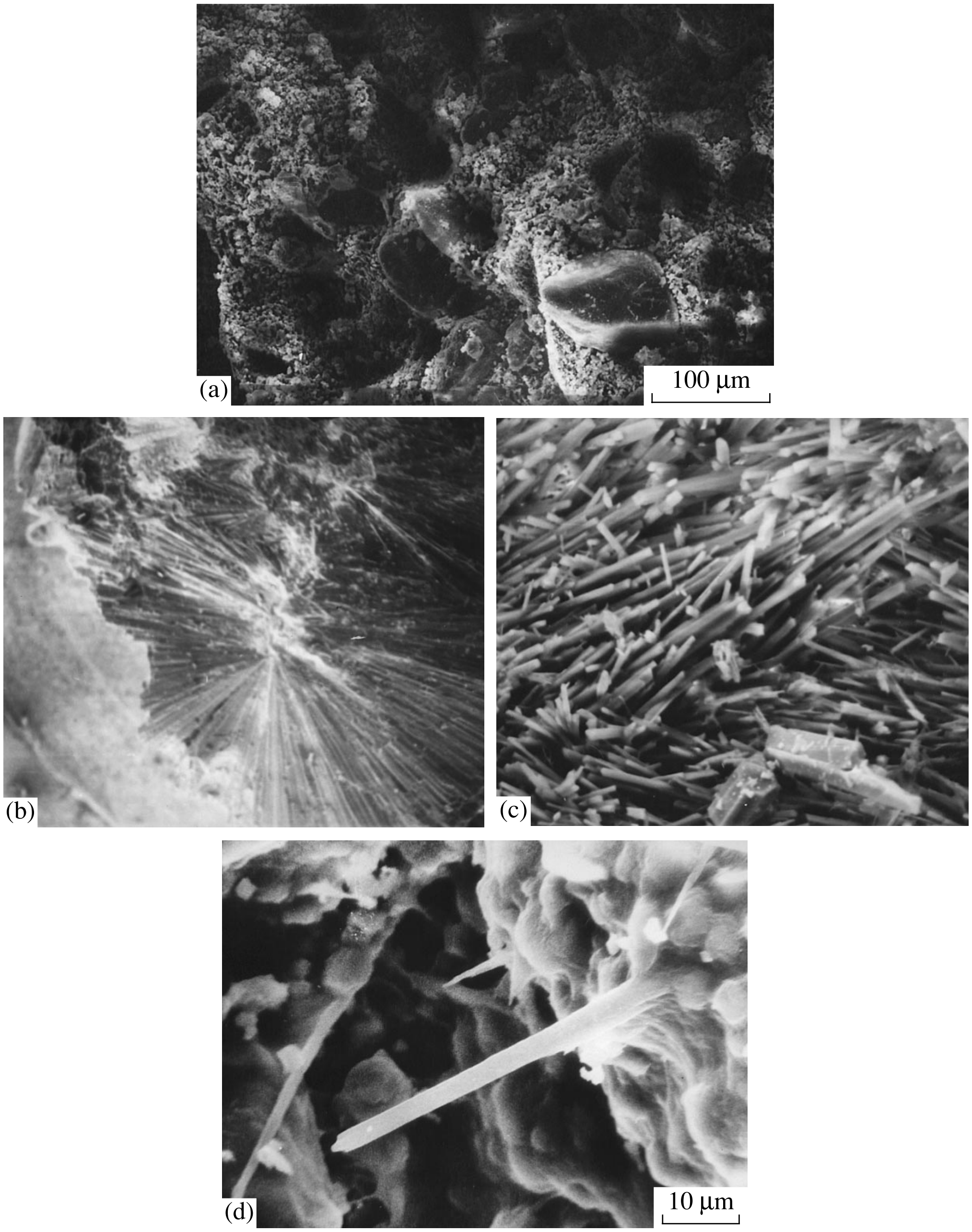

Fig. 3. Textural–structural features of autigenic carbonates: (a) microcrystalline Mg-calcite cementing grains of rounded terrigenous quartz in the plate; (b) a fragment of the coral-like top composed of radial aragonite and of the biomass of jelly-like microbial mat (on the left); (c) columnar crystals of aragonite; (d) structure of a fragment of microbial mat and non-decrystallized aragonite on the walls of a cavity in an aragonite construction; on the right, a mat fragment and an outgrowing aragonite "needle."

incorporation of strontianite into the crystalline structure of aragonite.

Magnesian calcite and aragonite are minerals typical of many known methane-derived carbonate constructions [15, 16]; however, it is for the Black Sea aragonite constructions that definitely pronounced coral-like morphology was described for the first time [4].

Isotopic Composition of the Carbon of Seep Methane and of the Autigenic Carbonates and Organic Matter of Carbonate Constructions and Bacterial Mats

Already the very first analyses of the isotopic composition of the carbonate minerals and the organic matter of the constructions revealed the anomalously light

Table 3. Isotopic composition of the carbon (δ^{13}C) and oxygen (δ^{18}O) of carbonate minerals and isotopic composition of the carbon of the organic matter of carbonate plates and constructions and of microbial mats

Sample no.	Site of sampling and sample description	$\delta^{13}C_{C_{org}}$, ‰	$\delta^{13}C_{CaCO_3}$, ‰	$\Delta = (\delta^{18}C_{C_{org}} - \delta^{13}C_{CaCO_3})$	$\delta^{18}O_{CaCO_3}$, ‰
Bentos-300 **SL, depth 226 m, coral-like construction**					
1	Carbonate plate of Mg-calcite at the base of an aragonite construction	–36.4	–34.6	–1.8	1.1
2	A fragment of an aragonite construction with bacterial mat on the surface	–69.2	–39.4	–29.8	0.4
3	Another fragment of a coral-like aragonite construction ($CaCO_3$, 91.6%; C_{org}, 1.13%)	–80.1	–37.9	–42.2	0.5
4	Pink bacterial mat from the construction surface	–75.6	–	–	–
5	Bright pink bacterial mat with aragonite crystals	–78.7	–46.4	–38.3	–
6	Grayish-pink bacterial mat with aragonite crystals	–83.8	–43.5	–40.3	–
7	Light-brown bacterial mat with aragonite crystals	–79.3	–46.1	–33.2	–
8	Pink bacterial mat with aragonite crystals	–80.2	–40.3	–39.9	–
St. 5176, trawling at a depth of 178–198 m, coral-like construction on a plate of Mg-calcite					
9	A plate fragment without mat	–33.4	–	–	–
10	Microbial mat fragment	–61.2	–	–	–
St. 5212–5213, trawling at a depth of 180–200 m, a fragment of a large carbonate plate					
11	Microcrystalline carbonate from the plate surface	–	–38.1	–	1.0
12	Microcrystalline carbonate from the plate middle part	–	–36.7	–	1.2
13	The same as no. 12	–50.9	–36.4	–14.5	1.1
14	Microcrystalline carbonate from the plate base	–	–29.3	–	1.2
15	The same as no. 14 but with *Dreissena* shell valves ingrown into the plate	–	–25.2	–	0.3
16	*Dreissena* shell valves purified of the sediment	–	–0.7	–	–2.8
St. 5224, trawling at a depth of 230–260 m, a coral-like construction					
17	Microcrystalline Mg-calcite with aragonite at the base of the construction	–	–33.8	–	1.1
18	The same as no. 17	–	–33.6	–	1.0
19	The same as nos. 16 and 17	–	–32.5	–	0.9
20	Aragonite of the coral-like construction	–	–37.7	–	1.2
21	The same as no. 20	–	–35.7	–	1.2
22	The same as no. 21	–	–40.0	–	0.4
23	Microcrystalline carbonate at the base of the aragonite construction	–	–33.6	–	0.9
24	Aragonite from the surface of the construction	–	–36.1	–	0.7
25	Aragonite from the construction interior, adjoining the gas channel	–	–40.4	–	0.4
26	The same as no. 25	–	–40.4	–	0.4
27	Intergrown globular aragonite aggregates from the interior of the construction near the central channel	–	–36.6	–	1.1
28	Globular aragonite aggregate (central part) on the surface of the construction	–	–36.0	–	0.6
29	Outer part of aragonite aggregates (no. 28)	–	–39.6	–	0.6
St. 5228, trawling at a depth of 590 m, a coral-like construction and microbial mat					
30	Aragonite from the construction ($CaCO_3$, 88,5%; C_{org}, 1.35%)	–76.0	–40.4	–35.6	0.4
31	Black bacterial mat	–77.2	–	–	–

Table 4. Content of ^{90}Sr in carbonate constructions, microbial mats, and surface sediments

Sample no.	Sample description	^{90}Sr, Bq/kg*
	St. 1, depth 226 m	
Bentos-300 SL	A fragment of coral-like construction	11.97 ± 1.68
	St. 5212, depth 180–200 m, carbonate plate	
1	0–5 mm layer, brown surface of the plate	1.65 ± 0.2
2	5–8 mm layer, gray surface layer	2.32 ± 0.2
3	15–18 mm layer, dark-gray base of the plate, submerged into sediment	1.27 ± 0.2
Sediment	Surface horizon of sediment	1.33 ± 0.2
	St. 5229, depth 487–593 m	
4	A fragment of a coral-like construction (top)	0.78 ± 0.2
5	Jelly-like black microbial mat	1.33 ± 0.2
6	Metamorphosed wood fragment	0.00
Sediment	Surface horizon of sediment	0.37 ± 0.10
Water	Near-bottom water at depths 100–600 m, northwestern part of the Black Sea	0.19 ± 0.002

* The analyses were performed by S.I. Arkhipova and N.Yu. Mirzoeva from the Institute of Biology of Southern Seas, National Academy of Sciences of Ukraine.

isotopic composition of the carbon of these compounds [4]. Therefore, in subsequent expeditions, much attention was paid to collecting samples of methane, carbonate minerals, and organic matter to obtain additional information on the isotopic composition of carbon.

The isotopic composition of the carbon of methane evolving in the form of bubble jets was studied in two samples, the first of which was collected 44°41.7′ N 31°40.7' E from a depth of 226 m and the second of which was collected 44°52.97′ N–31°52.59′ E from a depth of 65 m (Fig. 1). The δ^{13}C values were –58.2 and –64.4‰, respectively.

The lightest isotopic composition (δ^{13}C values from –40.3 to –46.4‰, Table 3, samples 5–8) proved to be characteristic of aragonite crystals and non-decrystallized carbonates incorporated into the slimy microbial mat, which, along with carbonates, contained more than 80% organic matter.

The δ^{13}C values of the carbonate of the aragonite constructions per se (purified of microbial mats) varied from –35.7 to –38.3‰; the value averaged over 12 aragonite samples was –38.3‰ (Table 3).

Magnesian calcite and microcrystalline carbonate minerals of the plates cementing the surface of bottom sediments were considerably less enriched with the ^{12}C isotope: the δ^{13}C values for ten samples of carbonate plates varied from –25.2 to –38.1‰ (Table 3). The reason for this is that the composition of plates includes not only the isotopically light autigenic carbonates but also an admixture of carbonates of sedimentary origin, whose δ^{13}C values in this region range from –2.0 to –10.0‰. The influence of the admixture of shell carbonate on the isotopic composition of the carbon of carbonate plates is demonstrated by the analysis of the plate from a depth of 180–200 m that was performed in layers (Table 3, samples 11–16). The δ^{13}C values of microcrystalline Mg-calcite from the bottom part of the plate were –29.3 and –25.2‰ (Table 3, samples 14 and 15). The δ^{13}C values of the mixed carbonate phases, occurring in the middle part of the plate and consisting of Mg-calcite and aragonite micrite, were –36.7 and –36.4‰ (Table 3, samples 12 and 13). The isotopically lightest carbonate composition (δ^{13}C = –38.1‰) was found in the surface layers of the plate (Table 3, sample 16).

The carbon of the organic matter of carbonate constructions and, in particular, of the microbial mats, was significantly depleted of the ^{13}C isotope as compared to carbonate minerals and background δ^{13}C values of the organic carbon of bottom sediments of the Black Sea northwestern part (from –23.4 to –25.6‰). An extremely light isotopic composition is characteristic of the C_{org} of microbial mats (δ^{13}C from –61.2 to –83.8‰, Table 3, samples 2–8; the δ^{13}C value averaged over seven samples is –76.7‰). The average δ^{13}C value for three samples of aragonite constructions (Table 3, samples 2, 3, 30) was –75.4‰. The C_{org} of the three samples of carbonate plates whose composition includes an

Table 5. Isotopic composition of carbon (δ^{13}C, ‰) of carbonate minerals and organic matter in samples from various depths

Depth range, m	δ$^{13}C_{CaCO_3}$		δ$^{13}C_{C_{org}}$	
	number of samples	δ^{13}C	number of samples	δ^{13}C
178–198	–	–	3	–44.5
180–200	5	–33.3	1	–50.9
220–260	20	–38.7	8	–72.9
590	1	–40.4	2	–76.6

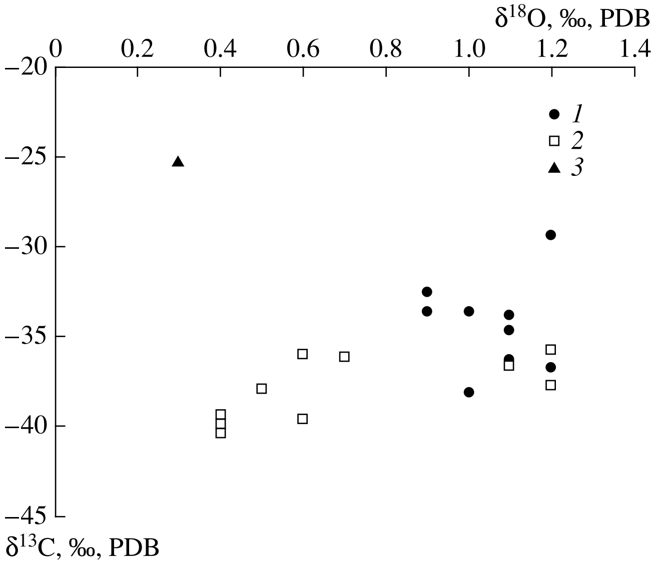

Fig. 4. Isotopic composition of carbon ($CaCO_3$ and C_{org}) and of the oxygen of autigenic carbonates: *1*, carbonate of tabular constructions; *2*, aragonite of coral-like constructions; *3*, Mg-calcite with an admixture of sedimentary shell material.

admixture of sedimentary organic matter exhibited the greatest variation of the $\delta^{13}C$ value (–33.4, –36.4, and –50.9‰, Table 3, samples 1, 9, 13).

Isotopic Composition of Carbonate Oxygen

As can be seen from data in Table 3, the isotopic composition of oxygen ($\delta^{18}O$, ‰) of various carbonate phases was rather similar: for samples of microcrystalline carbonate, $\delta^{18}O$ varied from 0.9 to 1.2‰ and for pure aragonite of coral-like constructions, from 0.2 to 0.8‰.

Distribution of ^{90}Sr

The distribution of ^{90}Sr in autigenic carbonates of plates, coral-like constructions, and mats was compared with ^{90}Sr concentrations in the upper layer of underlying sediments, near-bottom water, and metamorphosed wood (Table 4). Taking into account the decay of radioactive strontium in the course of sample preparation and analysis, all data were unified and dated March 1995. The greatest accumulation of ^{90}Sr was recorded in the top part of a coral-like construction (Table 4, sample from st. 1). In a carbonate plate (samples 1–3), the highest content of ^{90}Sr was found in the near-surface layer, with a regular decrease of ^{90}Sr content both toward the base of the plate and toward its surface.

In a coral-like construction from a depth of 487–593 m (st. 5228), the content of ^{90}Sr was 15-fold lower than in an analogous construction from a depth of 226 m. In microbial mats from the same depths (487–593 m, sample 5), the content of ^{90}Sr was higher because its accumulation by living matter is more active. At the same time, in wood (sample 6), the content of ^{90}Sr was at the level of background values (Table 4).

DISCUSSION

The absolute age of methane evolved from seeps in the northwestern part of the Black Sea is 3400–5100 years as determined from the content of the ^{14}C isotope in the carbonate constructions from a depth of 226 m [4]. It follows that the methane of the Black Sea seeps was formed in the clayey–sapropelite sediments aged 3500–7000 years.

The $\delta^{13}C$ values of the carbon of seep methane that we determined (–58.2 and –64.4‰) unequivocally prove the microbial origin of this methane and allow us to state that the main role in its formation was played by autotrophic methanogens producing methane according to the reaction (1):

$$CO_2 + 4H_2 = CH_4 + H_2O. \quad (1)$$

The conclusion about the leading role of autotrophic methanogenesis is based on the results of Ivanov *et al.* [19], who demonstrated that the fractionation of stable carbon isotopes during aceticlastic methanogenesis does not exceed 40–45‰, and, thus, the $\delta^{13}C$ value of aceticlastic methane cannot be lower than –50‰.

In our opinion, the most important point in our investigation is the obtained complex of data proving the existence in nature (by the example of the Black Sea hydrogen-sulfide zone) of a large-scale process of microbial methane oxidation under strictly anaerobic conditions.

The scale of this process is confirmed by the finding of numerous large coral-like constructions during visual observations from *Bentos-300* SL in 1988–1990 [3, 4] and by the results of demersal trawling during the voyages of the research vessel *Professor Vodyanitskii* in 1993–1994 [20].

The microbiological nature of the process of methane oxidation followed by sedimentation of methane-derived carbonates is unequivocally proved by the data from microbiological and molecular-biological investigations of the mats covering the carbonate constructions [4, 5, 8] and by the results of analysis of the distribution of stable carbon isotopes in carbonates and organic matter of microbial mats (Table 3).

The analysis of data presented in Table 3 allows certain interesting conclusions to be made concerning the nature of the microbial processes occurring during the anaerobic oxidation of methane:

(1) All 26 carbonate samples and all 12 samples of organic carbon of the constructions and mats are greatly enriched with the light ^{12}C isotope as compared to the carbonate and organic carbon of sedimentary origin. These data unequivocally prove that all carbon compounds of carbonate constructions contain the iso-

Table 6. Isotopic composition of oxygen ($\delta^{18}O$, ‰, PDB) of various compounds in the Black Sea

Compound	$\delta^{18}O$, ‰	Reference
Seawater from depths of 100–200 m	from –32.8 to –33.1	[26, 27]
Seawater from depths greater than 200 m	–31.5	[27]
Sulfate dissolved in seawater	from –15.5 to –20.0	[28]
Autigenic carbonates of plates and constructions	from 0.4 to 1.2	Our data (Table 3)
Bicarbonate of sediment waters	from –0.5 to –3.2	Our data

topically light carbon of methane. As can be seen from the data in Table 5, the isotopically lightest carbon compounds were found in the sample taken from the maximum depth, 590 m. In samples from lesser depths, carbonate minerals and organic matter carbon are less enriched with the ^{12}C isotope. Two hypotheses can be suggested to explain this regularity. First, under shallow-water conditions, the admixture of sedimentary carbonate and planktonogenic organic carbon may be significantly greater, and their $\delta^{13}C$ values are known to be less negative [17, 18]. Second, the above regularity may reflect the dependence of stable isotope fractionation on the rate of microbial oxidation and on the portion of methane consumed. It has been experimentally shown [19] that the lower the rate of a microbial process and the lesser the portion of the substrate consumed, the lighter isotopically are the products of microbial metabolism.

(2) In all samples of carbonate plates (Table 3, samples 1 and 13) and aragonite constructions (Table 3, samples 2, 3, 5–8, 30) for which the isotopic composition of carbonate and organic matter carbon has been analyzed, the organic matter was notably greater enriched with the light ^{12}C isotope. The maximum difference $[\Delta^{13}C = (\delta^{13}C_{org} - \delta^{13}C_{CaCO_3})]$ reaches –39.9 and –40.3‰ in samples of bacterial mats and –42.2‰ in aragonite constructions (Table 3). The relatively heavier isotopic composition of the carbonate carbon can have two explanations. The composition of carbonate constructions and, especially, that of carbonate plates may include, in addition to methane-derived carbonate, an admixture of sedimentary carbonate, having a heavier isotopic composition. However, in our opinion, the main reason for the difference between the isotopic compositions of carbonates and organic matter of mats and constructions is the biological fractionation of isotopes during the enzymatic processes of biomass synthesis in methane-oxidizing microorganisms from intermediate (methanol, formate) or final (carbon dioxide) products of methane oxidation. This supposition is in full agreement with the following issue.

(3) All samples of organic carbon of microbial mats and aragonite constructions (Table 3, samples 2–8, 30, and 31) are considerably enriched with the light ^{12}C isotope as compared with the carbon of the methane consumed (the methane CH_4 value averaged over two samples is $\delta^{13}C = –61.3‰$).

Thus, detailed analysis of the results of our study of the distribution of stable carbon isotopes in the carbonates and organic matter of constructions and mats leaves no place for doubt about the biological nature of the ^{12}C and ^{13}C isotope fractionation occurring during anaerobic oxidation of methane.

The question of the mechanism of anaerobic methane oxidation and of the role of various physiological groups of anaerobic microorganisms in this process has been actively debated in the microbiological and geochemical literature sine the pioneer works published by Zehnder and Brock in 1979 and 1980 [6, 7]. In laboratory experiments with pure cultures of methanogens, these researchers demonstrated that, upon the depletion of substrates (CO_2 and H_2) under conditions of a high concentration of methane, methanogens can drive the process of anaerobic methane oxidation according to the equation

$$CH_4 + 2H_2O = CH_4 + 4H_2. \qquad (2)$$

Since, in pure cultures, this process was very slow due to hydrogen accumulation, Alperin and Reeburgh [21] and then other researchers [9] put forward a hypothesis according to which, in natural anaerobic ecosystems, reaction (2) is accompanied by reaction (3), driven by sulfate-reducing bacteria:

$$4H_2 + SO_4^{2-} + H^+ = HS^- + 4H_2O. \qquad (3)$$

Formal summation of the equations (2) and (3) provides a description of the so-called sulfate-dependent methane oxidation (SDMO) by the equation (4):

$$CH_4 + SO_4^{2-} = HCO_3^- + HS^- + H_2O. \qquad (4)$$

Along with this mechanism of anaerobic methane oxidation by a consortium of methanogens and sulfate reducers, which is the one most commonly accepted in the modern literature, a hypothesis has been put forward assuming direct anaerobic oxidation of methane by sulfate reducers [22–24].

To substantiate the three discussed mechanisms of anaerobic methane oxidation (equations (2)–(4)), biologists use data on the distribution of various microbial groups and molecular-biological evidence for the presence of particular microorganisms in natural environments (microbial mats, carbonate sediments, bottom sediments), and geochemists invoke data on the in situ rates of processes measured with the use of radioactively labeled carbon and sulfur compounds and data on the distribution of stable isotopes of sulfur, carbon, oxygen, and hydrogen [9].

In our earlier publications on the biogeochemistry of the Black Sea [4, 5, 8, 15, 16], as well as in our present work and in the publications of other researchers [3, 20, 25], all of the above-mentioned methods and approaches have been used to this or that extent. This allows us to attempt a brief review of the problem of anaerobic methane oxidation as applied to the conditions existing in the strictly anaerobic zone of active methane seeps and carbonate constructions in the northwestern part of the Black Sea.

Microscopic examination of mat samples from the surface of constructions and inoculation of selective nutrient media revealed abundant and diverse microflora, including sulfate reducers, aerobic methanotrophs, and large filamentous microorganisms morphologically similar to methanogens [4, 5]. The presence of methanogens in the mats is confirmed by the results of molecular-biological investigations that revealed biomarkers characteristic of archaea [5, 25].

Experiments with radiolabeled compounds, carried out by Pimenov *et al.* [5], revealed in the mats active processes of sulfate reduction, aceticlastic and autotrophic methanogenesis, and anaerobic methane oxidation. All these data, together with the data on the isotopic composition of carbonates and organic matter of mats, obtained in the present work, unequivocally demonstrate the microbiological nature of anaerobic methane oxidation, but do not shed light on the mechanism of this process.

To establish the nature of the final electron acceptor in the process of anaerobic methane oxidation, we attempted using a new approach, namely a study of the isotopic composition of oxygen of the carbonate minerals formed in anaerobic oxidation of methane. Since the $\delta^{18}O$ values of potential terminal electron acceptors (seawater, see equation (2), and sulfate, see equation (3)) differ appreciably (Table 6), it could be expected that the $\delta^{18}O$ value of carbonates would help us determine which of the two compounds had provided its oxygen for methane oxidation.

However, as can be seen from the data in Table 6, the $\delta^{18}O$ values of carbonates considerably differed from the $\delta^{18}O$ values of both seawater and dissolved sulfate; this fact prevents these compounds from being considered as oxygen donors during anaerobic methane oxidation by equations (2) and (4).

At the same time, the $\delta^{18}O$ values of autigenic carbonates are rather close to the $\delta^{18}O$ value of bicarbonate dissolved in the water of the upper horizon of bottom sediments (Table 6). Thus, it follows that it is bicarbonate oxygen that could have been used in the anaerobic methane oxidation.

The feasibility of such a reaction was substantiated in a work by Valentine and Reeburgh [9], who believe that this reaction can proceed via methane oxidation to acetate and water (equation (5)) with the participation of aceticlastic methanogens and then via anaerobic oxidation of acetate with the participation of sulfate-reducing bacteria by equation (6):

$$CH_4 + HCO_3^- = CH_3COO^- + H_2O, \qquad (5)$$

$$CH_3COO^- + SO_4^{2-} = 2HCO_3^- + HS^-, \qquad (6)$$

$$CH_4 + SO_4^{2-} = 2HCO_3^- + HS^- + H_2O. \qquad (7)$$

Summation of equations (5) and (6) gives equation (7), which is realized by methanogens and sulfate reducers and which is outwardly identical to the overall equation (4) of the SDMO. However, the source of oxygen (HCO_3^-) and the intermediate product of methane oxidation (CH_3COO^-) are quite different.

It should be mentioned that the involvement of bicarbonate, which has a rather heavy isotopic composition of carbon ($\delta^{13}C = -2.1‰$), can explain the above-discussed considerable difference between the $\delta^{13}C$ values of autigenic carbonates and organic matter of the carbonate constructions (Tables 3 and 5). If reaction (6) indeed occurs in the natural environment, both the isotopically light carbon formed during methane oxidation and the isotopically heavy carbon of the seawater bicarbonate are to contribute to the carbon of the carbonate constructions.

The suggested mechanism of anaerobic methane oxidation requires additional studies that should involve both laboratory experiments and the search for additional evidence in natural ecosystems where the active anaerobic oxidation of methane takes place.

Of considerable interest is the investigation of the isotopic composition of acetate, which is, according to our data, permanently present in anaerobic sediments and microbial mats [5]. In the case of the operation of reaction (6), the isotopic composition of acetate carbon should be considerably enriched with the ^{12}C isotope.

To determine the rate of growth of the carbonate constructions, we used data on the distribution in the Black Sea of strontium-90, whose content in the Black Sea is known to have increased by an order of magnitude as a result of the Chernobyl disaster [3].

The most reliable data on the rate of growth of the carbonate constructions were obtained in the analysis of the ^{90}Sr content in various layers of a carbonate plate lifted from a depth of 180–220 m (Table 4). Maximum accumulation of ^{90}Sr in the 5–8 mm layer and decrease in its content observed toward the plate surface and base provides grounds to relate this maximum to the Chernobyl disaster. Then, the rate of growth of the carbonate plate should be 0.65 mm per year.

In coral-like constructions and, especially, in the mats covering them, the ^{90}Sr concentration is considerably higher than in the plates; the highest ^{90}Sr concentration was recorded in a construction from a depth of 226 m (Table 4).

The majority of methane-derived carbonates described in the literature were formed either at the interface of reduced and oxidized conditions (the surface of bottom sediments; upper horizons of bottom sediments) or in the upper horizons of reduced sediments. In both cases, methane oxidation may involve both aerobic and anaerobic microorganisms.

The aragonite constructions of the Black Sea that we studied are formed in a stable anaerobic zone and are a thus perfect object to investigate the natural mechanism of anaerobic methane oxidation.

Therefore, the analysis of the results of the detailed study of mineralogy and chemical composition of the carbonate constructions and of the distribution of stable carbon and oxygen isotopes in carbonate minerals and organic matter of the constructions and microbial mats allowed us to elucidate important details of the microbial processes of anaerobic methane oxidation.

The determined δ^{18}O values of carbonate minerals disagree with the existing hypotheses of methane oxidation either with the sulfate oxygen (with the formation of H_2S and CO_2) or with the water oxygen (with the formation of hydrogen and CO_2). The most feasible pathway of methane oxidation is its reaction with bicarbonate dissolved in seawater; this reaction is driven by aceticlastic methanogens and yields water and acetate, which is then consumed by other members of the anaerobic community, including sulfate reducers producing HS^- and HCO_3^-.

Comparison of the δ^{13}C values of carbonate minerals and organic matter not only confirms the leading role of the isotopically light methane carbon in the formation of all carbon-containing compounds of the carbonate constructions but also once more demonstrates that the formation of the organic matter of microbial biomass is accompanied by intense fractionation of carbon isotopes, as a result of which the total organic matter of biomass acquires an extremely light isotopic composition, characterized by δ^{13}C values as low as –83.8‰.

In light of these data, it is easily conceivable that the isotopically lightest components of the biomass of microorganisms in the mats (namely, lipids) may have still lower δ^{13}C values [25].

Considerable variation in the isotopic composition of the carbon of carbonates and organic matter of constructions and mats, revealed upon comparison of samples from different depths, can be explained by the variation in the proportion of admixed sedimentary carbonate and planktonogenic organic matter and/or by the variation in the rate of methane oxidation with depth.

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