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EXPERIMENTAL ARTICLES

Sulfate Reduction and Methanogenesis in the Shira and Shunet Meromictic Lakes (Khakasia, Russia)

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Abstract—The biogeochemical and molecular biological study of the chemocline and sediments of saline meromictic lakes Shira and Shunet (Khakasia, Russia) was performed. A marked increase in the rates of sulfate reduction and methanogenesis was revealed at the medium depths of the chemocline. The rates of these processes in the bottom sediments decreased with depth. The numbers of the members of domains *Bacteria, Archaea*, and of sulfate-reducing bacteria (SRB) were determined by fluorescence in situ hybridization with rRNA specific oligonucleotide probes labeled with horseradish peroxidase and subsequent tyramide signal amplification. In the chemocline, both the total microbial numbers and those of *Bacteria* were shown to increase with depth. The archaea and SRB were present in almost equal numbers. In the lake sediments, a drastic decrease in microbial numbers with depth was revealed. SRB were found to prevail in the upper sediment layer and archaea in the lower one. This finding correlated with the measured rates of sulfate reduction and methanogenesis.

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The stratified water column of meromictic reservoirs contains an aerobic and an anaerobic zone; the chemocline is the transition zone between them. The stable anaerobic conditions below the chemocline make meromictic reservoirs a convenient model for the study of methanogenesis (MG) and sulfate reduction (SR), the key processes in the carbon and sulfur cycles [1].

Lakes Shira and Shunet, saline meromictic lakes with a pronounced sulfide-rich zone are the best known in the Khakasia. Lake Shira is a Siberian balneological resort and a popular object of microbiological research [2–5]. Lake Shunet, although known as a source of natural salt and medical mud, is poorly studied from the point of view of microbial ecology. Data concerning in situ microbial numbers in these lakes are also scarce. Radioisotope studies of the sediments and water column in summer and in winter revealed the highest rates of MG and SR in the chemocline, in the near–bottom water, and in the sediments [3, 4]. The quantitative study of the vertical distribution of various bacterial groups, especially of sulfate-reducing bacteria (SRB) and methanogenic archaea (MA), in the chemocline and in the lake sediments is therefore important.

Molecular methods, which do not require cultivation on elective media for the identification and quantitative determination of various microbial groups, have become popular in microbial ecology. Fluorescence in situ hybridization (FISH) is one of the most promising molecular methods; it provides the most complete information concerning the quantitative in situ composition of microbial communities [6]. Hybridization with oligonucleotide probes labeled with horseradish peroxidase and subsequent signal amplification with tyramide solution (FISH-TSA) is a modification of the classical FISH method. FISH-TSA enhanced the fluorescent signal of the cell hybridized with the probe; this method is therefore more sensitive [7].

The goal of the present work was to determine the rates of SR and MG in the chemocline and bottom sediments of the Shira and Shunet saline meromictic lakes by radioisotope methods and to measure the numbers of the members of domains *Bacteria, Archaea*, and SRB in the same habitats by FISH-TSA optimized for highly mineralized samples.

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MATERIALS AND METHODS

Saline meromictic lakes Shira and Shunet (Khakasia, Russia) were the objects of investigation. Lake Shira (90°14′ E, 54°30′ N) with an area of 35.9 km^2 is located 10 km northeast of Shira town. Its average depth is 11.2 m, with a maximum depth of 23 m. Its water is of the sulfate–chloride–sodium–potassium hydrochemical type. Lake Shunet, with an area of 0.47 km^2 is located 19 km northeast of Shira town. Its maximal depth is 6.23 m. Its water is of the chloride–sulfate–sodium–magnesium hydrochemical type [2].

The main physicochemical parameters were determined using a Hydrolab Data Sonde 4a submerged multichannel analyzer (Hydrolab, United States) equipped with temperature, salinity, turbidity, pH, and Eh sensors. Methane content in the samples was determined using a Chrom-5 gas chromatograph equipped with a flame ionization detector by the phase-equilibrium degassing method. Oxygen and sulfide were determined using the Aquamerk (Merck, Germany) standard reagents kit.

The rates of SR and MG were determined by the radioisotope method with the following labeled compounds: $Na_2^{35}SO_4$, $^{14}CH_3COONa$, and $NaH^{14}CO_3$ [3]. The rate of methanogenesis comprises the rates of aceticlastic and hydrogenotrophic methanogenesis.

Water and sediment samples were taken in June 2002. Water samples were taken with a 1.2 l glass Niskin bathometer, fixed with 96% ethanol (to a final ethanol concentration of 20%), and stored at +4°C. The bottom sediments were sampled with a limnological sampler (stratometer) with a plastic tube 6 cm in diameter. The samples were fixed with 96% ethanol (1 : 1 vol/vol) and stored at –20°C. The absolutely dry mass (adm) of the sediments was determined after over-drying at 105°C.

Peroxidase-labeled oligonucleotide probes specific to the members of domains *Bacteria* (EUB338) [6] and *Archaea* (ARCH915) [9] and to the families *Desulfobacteriaceae* (SRB385Db) [10] and *Desulfovibrionaceae/Desulfobulbaceae* (SRB385) [6] were used for hybridization. Peroxidase-labeled probes SRB385Db and SRB385 were used together with competitive unlabeled probes SRB385Db and SRB385. All the probes were designed by MWG-Biotech AG (Germany).

Hybridization conditions for highly mineralized water and sediment samples were adjusted on the basis of the previously developed FISH-TSA techniques [7, 11–14]. Prior to hybridization, the water samples were sonicated for 10 min in a device equipped with a water bath (SKAN AG, Switzerland). The cells were concentrated on black polycarbonate membrane filters (25 mm diameter, 0.2 μm pore size, type GTBP2500, Millipore, Ireland) placed on top of nitrocellulose filters (25 mm diameter, 0.45 μm pore size, Millipore, Ireland). Filtration under vacuum (–550 mbar) was used for the concentration. The filters were then washed with a mixture of phosphate buffer [6] and ethanol (1 : 1 vol/vol) under vacuum (1 min at 20°C), air-dried, cut into sections, and covered with 0.2% aqueous agarose solution (Invitrogen Life Technologies Ltd., Scotland). After dehydration in 96% ethanol (1 min at 20°C), the filters were air-dried [7]. To each section of the filter, 10 μl of 0.05% solution of 76200 U mg^{-1} lysozyme (Fluka Chemika, Switzerland) in 0.1 M Tris–HCl buffer, pH 7.2–7.5) and 5 mM EDTA (pH 8.0) were applied; the filters were then incubated for 30 min at 37°C, washed with distilled water and 96% ethanol (1 min at 20°C), and air-dried.

For water samples, a modified hybridization buffer [7] with a blocking agent (Boehringer, Germany) and appropriate comcentrations of formamide (Qbiogene, France) was used. The blocking reagent solution in 1 M Tris–HCl buffer was prepared separately and added to the hybridization buffer to 0.2% final concentration. Formamide final concentrations were 40% for the EUB338 probe and 30% for ARCH915, SRB385, and SRB385Db [11]. The hybridization buffer was applied to filter surface and 1 μl of the labeled probe solution (20 ng μl^{-1}) was added. For SRB determination, 1 μl of the labeled probe was added together with 1 μl of the competitive unlabeled probe in order to prevent nonspecific binding. The total volume of the buffer and probe was 10 μl. Hybridization was performed at 37°C for two hours.

After hybridization, the filters were washed with distilled water, placed into test tubes with washing buffer [12], and incubated for 10 min at 37°C. The filters were then washed with distilled water and placed on microscope slides; excessive liquid was removed with filter paper. Each filter section was overlayed with 10 μl of TNT buffer [13], incubated for 15 min at 20°C, washed with distilled water, and treated with 10 μl of freshly prepared solution of tyramide bound to Cy3 fluorescent dye (NEN Life Science Products, United States). The filters were incubated in the dark for 5 min, washed in TNT buffer for 15 min at 20°C, and air-dried. The working tyramide solution was prepared by dilution of the tyramide stock solution with the amplification buffer (1 : 20 vol/vol). The total SRB number was determined as the number of cells revealed after hybridization with SRB385 and SRB385Db probes.

Hybridization of sediment samples was performed on microscope slides. One ml of the fixed sample was centrifuged, the supernatant was removed, and the pellet was resuspended in the mixture of phosphate buffer [6] and ethanol (1 : 1 vol/vol). The samples were sonicated at 20% output level of a Branson Sonifier 250 (United States) for 10 sec and diluted with a 0.1% sodium pyrophosphate solution. Microscope slides were covered with 0.1% aqueous gelatin solution with 0.01% $KCr(SO_4)_2$, and 10 μl of the suspension was uniformly spread in the well of the slide. The slides were treated with ethanol (50, 80, and 96%, 3 min in each). The hybridization buffer [14] was used; the concentra-

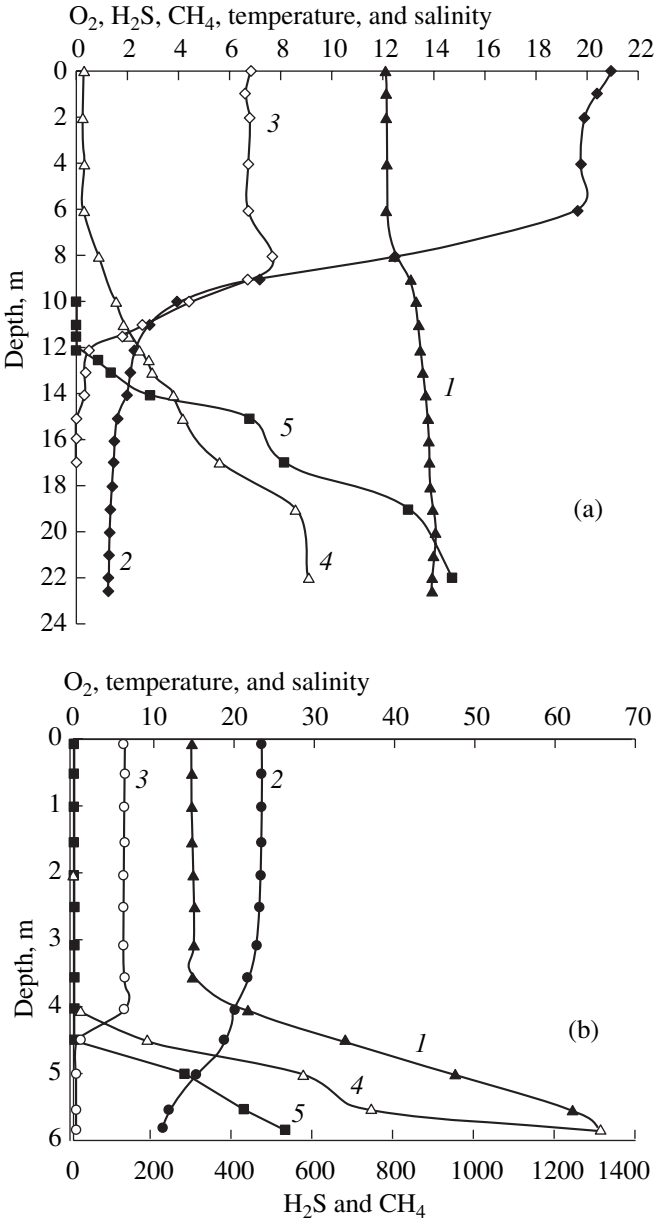

Fig. 1. Physicochemical parameters of the water column of saline meromictic lakes Shira (a) and Shunet (b). The curves represent salinity (*1*) ‰; temperature (*2*) °C; concentrations of O_2 (*3*) mg l^{-1}; CH_4 (*4*) μl l^{-1}; and H_2S (*5*) mg l^{-1}.

tions of formamide and blocking reagent were the same as for water samples. All the subsequent procedures, including hybridization, washing, and incubation in TNT buffer and tyramide solution, were performed as described above.

The total microbial numbers were determined by staining with DAPI (4',6'-diamidyno-2-phenylindole), a universal DNA-specific dye. After hybridization, aqueous DAPI solution (0.5 ng μl^{-1}) was applied to filter sections or to wells on a microscope slide; after 15 min incubation at 20°C in the dark, the solution was removed with distilled water and the filters (slides) were air-dried.

The filters (slides) were overlayed with a mixture (4 : 1 vol/vol) of Citifluor (AF1, Citifluor, United Kingdom) and Vectashield (Vector Laboratories Inc., Canada). The cells were counted in 20–40 microscope fields under an epifluorescence microscope (Zeiss, Germany) at 100× magnification. DAPI-stained cells and the cells hybridized with probes were counted using the filters no. 02 and HQ-Cy3 (AHF, Analysentechnik, Germany). Microbial numbers were expressed as cell numbers per 1 ml of the water or per 1 g of sediment adm.

RESULTS

Physicochemical characterization of Lake Shira. During the period of sampling, the chemocline in Lake Shira was at the depth of 11–13 m. Below 13 m, oxygen was absent; sulfide concentration increased with depth and reached 12–15 mg l^{-1} in the near-bottom water. Methane concentration in the anaerobic zone also increased to 9 μl l^{-1}. A slight increase in salinity from 13 to 15‰ was recorded. In the depth interval from 6 to 11 m, the water temperature decreased sharply from 19.7 to 2.9°C (Fig. 1a).

Methane content in the bottom sediments was higher than in the near-bottom water; it increased with depth and peaked (60 μl dm^{-3}) in the 30–35 cm sediment layer.

Rates of sulfate reduction and methanogenesis in Lake Shira chemocline and sediments. A sharp increase in SR (to 10.8 μg S^{-1} day^{-1}) was revealed in the chemocline at 12.5 m depth. The rates of SR and MG decreased at greater depths and increased again in the near-bottom water.

The measured rates of SR and MG in Lake Shira sediments are presented in Table 1. The rates of SR and MG in the sediments were higher than in the chemocline. The highest SR rate (248.7 μg S l^{-1} day^{-1}) was revealed in the upper 2 cm of the sediments; it decreased in the lower layers. The zone of the highest MG rate (1.75 μl CH_4 l^{-1} day^{-1}) was revealed immediately below the zone of SR, at 3–5 cm. Intense methanogenesis also occurred in the 10–25 cm sediment layer.

Microbial numbers in the chemocline and sediments of Lake Shira. In the chemocline of Lake Shira, both the total number of DAPI-staining microorganisms and the number of Bacteria determined by FISH-TSA were shown to increase with depth. Two peaks of bacterial numbers were revealed at 12.5 and 15 m. Bacteria constituted 33–62% of the total microbial numbers. The numbers of SRB were 16–42% of the total bacterial numbers. The representatives of *Desulfobacteriaceae* revealed with SRB385Db predominated at the intermediate depths of the chemocline (11.5–14 m). At 11 and 15 m, their numbers were equal to those of

Desulfovibrionaceae/Desulfobulbaceae (revealed with SRB385). Archaeal numbers constituted up to 25% of the total microbial numbers. Both the numbers and the depth profiles of SRB and archaea were similar (Fig. 2b).

The data on microbial numbers in the sediments of Lake Shira are presented in Table 2. The numbers of every group investigated decreased with depth. Bacterial numbers exceeded archaeal ones. The highest ratio of SRB was revealed in the upper sediment layer (0–2 cm); it decreased sharply at 2–5 cm. No active SRB were found at 30–35 cm. In the lower sediment layers, archaea prevailed over SRB. Similar to the chemocline, *Desulfobacteriaceae* were the predominant group of SRB. The numbers of all the studied microbial groups in the sediments was higher than in the chemocline.

Physicochemical characterization of Lake Shunet. In Lake Shunet, the chemocline was located at 4.5–5 m. The highest concentration of dissolved oxygen (6 mg l^{-1}) was detected at 4 m. Salinity changed sharply (from 15 to 65.6 ‰) in the depth interval from 3.54 to 5.86 m. Sulfide was detected at 4.75 m; its concentration increased with depth and reached 530 mg l^{-1} in the near-bottom water; i.e., it was 36 times as high as in Lake Shira. Methane was present at 4 m and deeper; its concentration in the near-bottom water was 140 times higher than in Lake Shira (Fig 1b).

In Lake Shunet, unlike Lake Shira, methane concentration in the upper sediment layer was lower than in the near-bottom water; maximal CH_4 concentration (1.4 ml dm^{-3}) was revealed at the depth of 8 cm.

Table 1. Rates of sulfate reduction and methanogenesis in the bottom sediments of saline meromictic lakes Shira and Shunet determined by radioisotope methods

Depth, cm	Process rate	
	Sulfate reduction, μg S $l^{-1}day^{-1}$	Methanogenesis, μl CH_4 $l^{-1}day^{-1}$
	Lake Shira	
0–2	248.65	0.68
2–5	77.77	1.75
5–10	20.17	0.12
10–15	9.71	0.29
20–25	7.66	0.28
25–30	2.78	0.18
30–35	0.79	0.026
	Lake Shunet	
0–3	25.43	21.73
5–10	4.22	12.90
10–15	1.57	7.19

Rates of sulfate reduction and methanogenesis in Lake Shunet chemocline and sediments. The rates of SR and MG increased slightly in the chemocline of Lake Shunet at 4.75 m (Fig 3a). The rates of both processes in the sediment decreased with depth (Table 1). Unlike SR rates, MG rates in the sediments were higher than in the water. The rate of methanogenesis in Lake Shunet chemocline and sediments was at least an order of magnitude higher than in Lake Shira. Sulfate reduc-

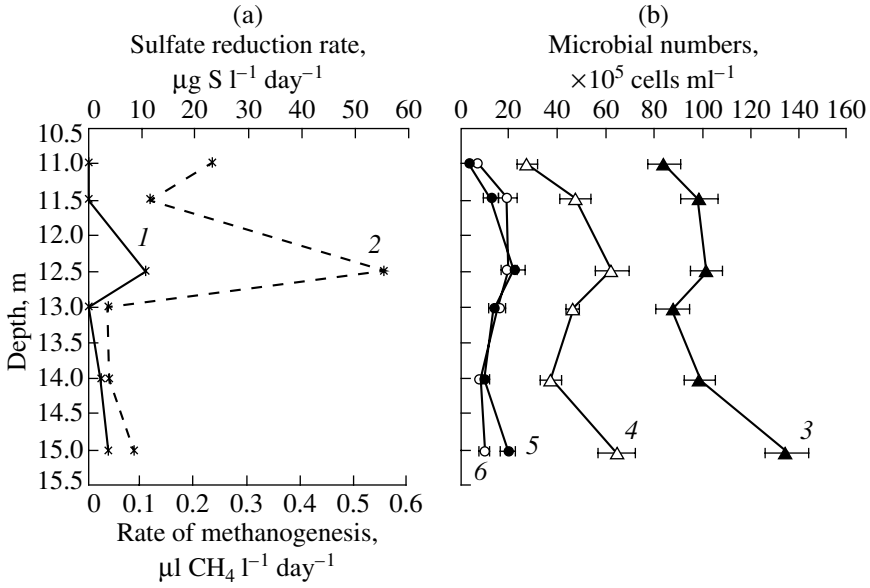

Fig. 2. Rates of sulfate reduction (*1*) and methanogenesis (*2*) determined by the radioisotope method (a) and microbial numbers determined by FISH-TSA (b) in the chemocline of Lake Shira. The curves represent total microbial numbers (*3*), the numbers of *Bacteria* (*4*), *Archaea* (*5*), and SRB (*6*).

Table 2. Microbial numbers in the bottom sediments of saline meromictic lakes Shira and Shunet determined by FISH-TSA

Depth, cm	Microbial numbers, $\times 10^8$ cells g^{-1} adm sediment				SRB : archaea ratio
	Total numbers (DAPI)	Bacteria (EUB338)	SRB (SRB385 + SRB385Db)	Archaea (ARCH915)	
Lake Shira					
0–2	307 ± 15	124.6 ± 6.2	108.6 ± 4.4	80.2 ± 4	1 : 0.74
2–5	70.4 ± 3.5	19.6 ± 1	3.9 ± 0.3	3.2 ± 0.3	1 : 0.82
5–10	51.5 ± 2.6	9.6 ± 1	2.05 ± 0.15	2.5 ± 0.25	1 : 1.22
10–15	16.4 ± 0.8	1.9 ± 0.2	0.13 ± 0.008	0.15 ± 0.015	1 : 1.15
20–25	8.5 ± 0.4	0.7 ± 0.07	0.08 ± 0.005	0.08 ± 0.008	1 : 1
25–30	6.4 ± 0.3	0.3 ± 0.03	0.01 ± 0.001	0.05 ± 0.005	1 : 5
30–35	3.4 ± 0.2	0.3 ± 0.03	0	0.03 ± 0.003	–
Lake Shunet					
0–3	361 ± 18	154.5 ± 7.7	133 ± 6.6	46 ± 2.3	1 : 0.35
5–10	104.3 ± 5	15.8 ± 0.8	1.34 ± 0.12	2.3 ± 0.2	1 : 1.72

tion rate in the chemocline of Lake Shunet was higher than in Lake Shira; the SR rate in the sediments was, however, lower than in Lake Shira.

Microbial numbers in the chemocline and sediments of Lake Shunet. Both the total numbers of microorganisms and the numbers of *Bacteria* in the chemocline of Lake Shunet increased with depth. Archaeal numbers did not change greatly with depth; they exceeded the numbers of bacteria in the upper horizon of the chemocline (Fig. 3b). Bacteria and archaea together comprised 86–97% of the total microbial numbers. The numbers of SRB increased with depth; the members of the family *Desulfobacteriaceae* were predominant. The number of microorganisms in the chemocline was higher in Lake Shunet than in Lake Shira.

The data on microbial numbers in Lake Shunet sediments are presented in Table 2. Only two sediment samples from depths up to 10 cm were analyzed. The numbers of all microbial groups were found to decrease sharply at 3 cm depth. In the sediments of Lake Shunet, similar to Lake Shira, bacteria were more numerous

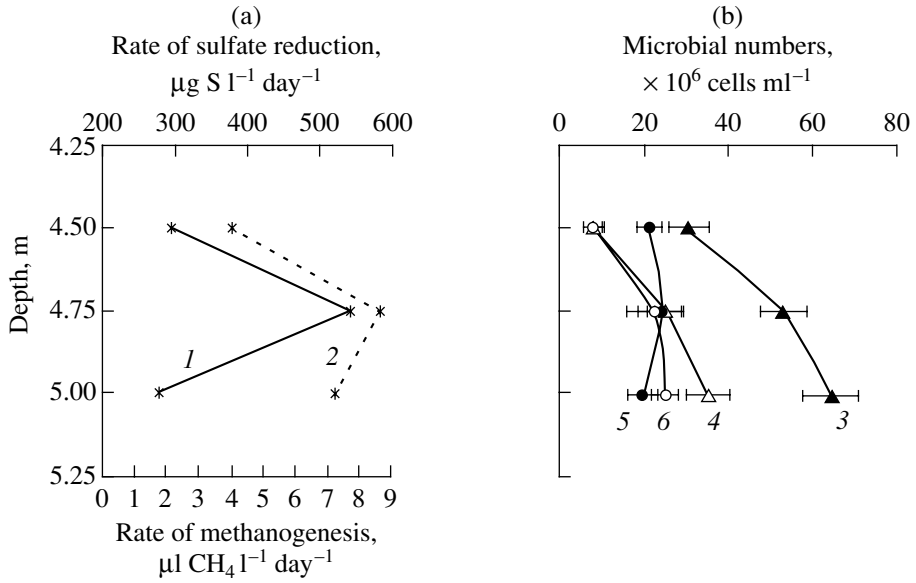

Fig. 3. Rates of sulfate reduction (*1*) and methanogenesis (*2*) determined by the radioisotope method (a) and microbial numbers determined by FISH-TSA (b) in the chemocline of Lake Shunet. The curves represent total microbial numbers (*3*), and the numbers of *Bacteria* (*4*), *Archaea* (*5*), and SRB (*6*).

than archaea. Sulfate reducers predominated in the upper sediment layer and archaea in the lower one. *Desulfobacteriaceae* were predominant in the sediments. The number of microorganisms was higher in the sediments than in the chemocline.

DISCUSSION

The water columns of the lakes under investigation are permanently stratified due to the sharp salinity gradient in Lake Shunet and a weak salinity gradient combined with a temperature gradient in Lake Shira. Various physiological groups of microorganisms coexist in a narrow transition zone (chemocline). They derive energy from aerobic or anaerobic respiration, photosynthesis, and fermentation. The in situ study of the quantitative microbial composition in this zone was therefore of interest.

High concentrations of H_2S and CH_4, together with intense SR and MG in the water column and sediments of these lakes, indicated that SRB and MA were numerous. The relationships between SRB and MA in marine and freshwater environments are well studied. In marine environments with high sulfate levels, SRB displace MA in the course of competition for H_2 and acetate [1]. Sulfate concentration in the lakes under investigation was several times higher than in seawater [4]. The predominance of SRB over MA was therefore expected.

Probes developed for detection of *Desulfovibrionaceae* (SRB385) and *Desulfobacteriaceae* (SRB385Db) were used in the present work for the quantitative determination of SRB [6, 10]. Although developed more than ten years ago, these probes are still used for SRB enumeration in aquatic environments [15, 16]. However, comparison of the probe sequences with the known 16S rRNA sequences of cultured and uncultured *Bacteria* revealed that these probes were not strictly specific to the two SRB families; they can also hybridize with some bacteria incapable of sulfate reduction. On the other hand, considering the physicochemical characteristics of the lakes and the high rates of sulfate reduction, we suggest that sulfate-reducers predominated among the bacteria revealed with the SRB385 and SRB385Db probes. We may have obtained somewhat overestimated SRB numbers; we believe, however, that the tendencies in the SRB numbers can be compared to SR rates in the chemocline and water column of the lakes under investigation.

The total numbers of Archaea were determined with a strictly specific ARCH915 probe. High methane content and intense methanogenesis in the sediments and water column of the lake indicated the predominance of MA among the representatives of the *Archaea* domain. Mesophilic nonmethanogenic archaea, including *Crenarachaeota*, were probably also present [17]. Their presence, however, was not revealed in all aquatic environments. For instance, all the archaea revealed by the ARCH915 probe in the study of MA vertical distribution in the anaerobic sediments of the monomictic Lake Rotsee belonged to the kingdom *Euryarchaeota* [18]. Hybridization of the samples with the *Crenarchaeota*-specific Cren499 probe revealed no positive signal [18].

The numbers of SRB and archaea in the chemocline and sediments of the lakes obtained by FISH-TSA were generally in correlation with the rates of SR and MG determined by the radioisotope method. The slight increase in microbial numbers in the intermediate depths of the chemocline (12.5 and 4.75 m for Lake Shira and Lake Shunet, respectively) correlated with the maximal rates of the processes. In Lake Shunet, with its higher content of sulfate and organic matter in the water column [4], SR and MG rates, total microbial numbers, and bacterial and archaeal numbers were all an order of magnitude higher than in Lake Shira. Both SR rates and SRB numbers were higher in the upper sediment horizon, and MG rates and archaeal numbers were higher in the lower one. In Lake Shira, both the microbial numbers and the rates of the processes were higher in the sediments than in the chemocline. In Lake Shunet, SRB numbers in the sediments were higher and SR rates lower than in the chemocline. High amounts of H_2S, up to 500 mg l^{-1} in the near-bottom water accumulate in this lake by midsummer; high sulfide levels can suppress sulfate reduction, albeit without hampering the viability of bacteria. Suppressed SR makes more substrates available to MA. This is a possible explanation for the higher MG rates and archaeal numbers in the sediments of Lake Shunet compared to the chemocline.

Thus, the data on bacterial (including SRB) and archaeal numbers determined by FISH-TSA demonstrate an in situ quantitative relationship between these microbial groups and exhibit correlations with the physicochemical characteristics of the lakes and with SR and MG rates. Together with the traditional methods of measuring microbial activity rates, the use of the highly specific probes for certain physiological and phylogenetic microbial groups enables FISH-TSA to reveal the structure and interactions of microbial communities.

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