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EXPERIMENTAL ARTICLES

Sulfate-Reducing Bacteria in Gypsum Karst Lakes of Northern Lithuania

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Abstract—Microbiological studies were performed in three small gypsum karst lakes in northern Lithuania, most typical of the region. Samples were taken in different seasons of 2001. The conditions for microbial growth in the lakes are determined by elevated content of salts (from 0.5 to 2.0 g/l), dominated by SO_4^{2-} and Ca^{2+} ions (up to 1.4 and 0.6 g/l, respectively). The elevated sulfate concentration is favorable for sulfate-reducing bacteria (SRBs). Summer and winter stratification gives rise to anaerobic water layers enriched in products of anaerobic degradation: H_2S and CH_4. The lakes under study contain abundant SRBs not only in bottom sediments (from 10^3 to 10^7 cells/dm^3) but also in the water column (from 10^2 to 10^6 cells/ml). The characteristic spatial and temporal variations in the rate of sulfate reduction were noted. The highest rates of this process were recorded in summer: 0.95–2.60 mg S^{2-}/dm^3 per day in bottom sediments and up to 0.49 mg S^{2-}/l per day in the water column. The maximum values (up to 11.36 mg S^{2-}/dm^3) were noted in areas where bottom sediments were enriched in plankton debris. Molecular analysis of conservative sequences of the gene for 16S rRNA in sulfate-reducing microorganisms grown on lactate allowed them to be identified as *Desulfovibrio desulfuricans*.

Key words: gypsum karst lakes, sulfate-reducing bacteria, sulfate reduction rate, *Desulfovibrio desulfuricans.*

The Upper Devonian rocks in the karst region of northern Lithuania are covered only by thin Quaternary deposits. Intense karst events resulting from leaching give rise to holes and funnel sinks. In areas most prone to karst processes, their density reaches hundreds per square kilometer. Later, some of them give rise to small (0.01–4.0 ha) but relatively deep (5–10 m) water reservoirs [1, 2].

The main environmental factor in these lakes is elevated mineralization of water. Depending on weather and hydrological conditions, the total content of dissolved salts varies from 0.5 to 2 g/l. The concentrations of predominant ions, SO_4^{2-} and Ca^{2+}, reach 1.4 and 0.6 g/l, respectively. The environmental conditions vary during the growing season; in summer, however, the water column usually undergoes rapid stratification, so that the lower metalimnion becomes entirely devoid of oxygen, and reduction products start accumulating there. It is known that elevated sulfate concentrations in such karst and meromictic lakes are favorable for growth of sulfate-reducing bacteria (SRB) and production of hydrogen sulfide, if organic matter is present, as well. Under certain conditions, in the chemocline zone, they favor intense growth of sulfur-oxidizing bacteria [3, 4].

Much consideration is given to sulfate-reducing bacteria, particularly, their activity in various water reservoirs. In recent years, molecular identification methods have been applied to understanding the role of some SRB groups in water bodies of different types. It is known that SRBs of the genus *Desulfobacter* dominate in marine sediments, whereas *Desulfobulbus* spp. are active in both marine and freshwater low-sulfate sediments, being able to utilize other electron acceptors [5]. Nevertheless, bacteria of the genus *Desulfovibrio* are not the most abundant group in anaerobic bottom deposits [6], although they take an active part in corrosion [7] and even reduce toxic metals, e.g., uranium, converting them to poorly soluble compounds [8].

The goals of this work were to (1) identify the most abundant SRB species with the use of up-to-date molecular methods and (2) evaluate sulfate reduction rates in typical lakes of the gypsum karst region in northern Lithuania.

MATERIALS AND METHODS

The study was performed in 2001 in three typical small lakes in the active karst region near the village of Kirkilu (Fig. 1). The largest of the three, Kirkilu, has an area of about 4 ha and a very intricate shape. The lake was formed by merging of several karst holes over several centuries. The depth of the lake reaches 7 m in some places, the mean depth being 2 m. The other two lakes, Ramunelis and Katilnicha, are much smaller; their areas are no more than 0.1 ha. The three lakes are so close to each other that they often merge during spring floods. They are fed mainly by groundwater and surface inflow. Their surfaces are protected to a signifi-

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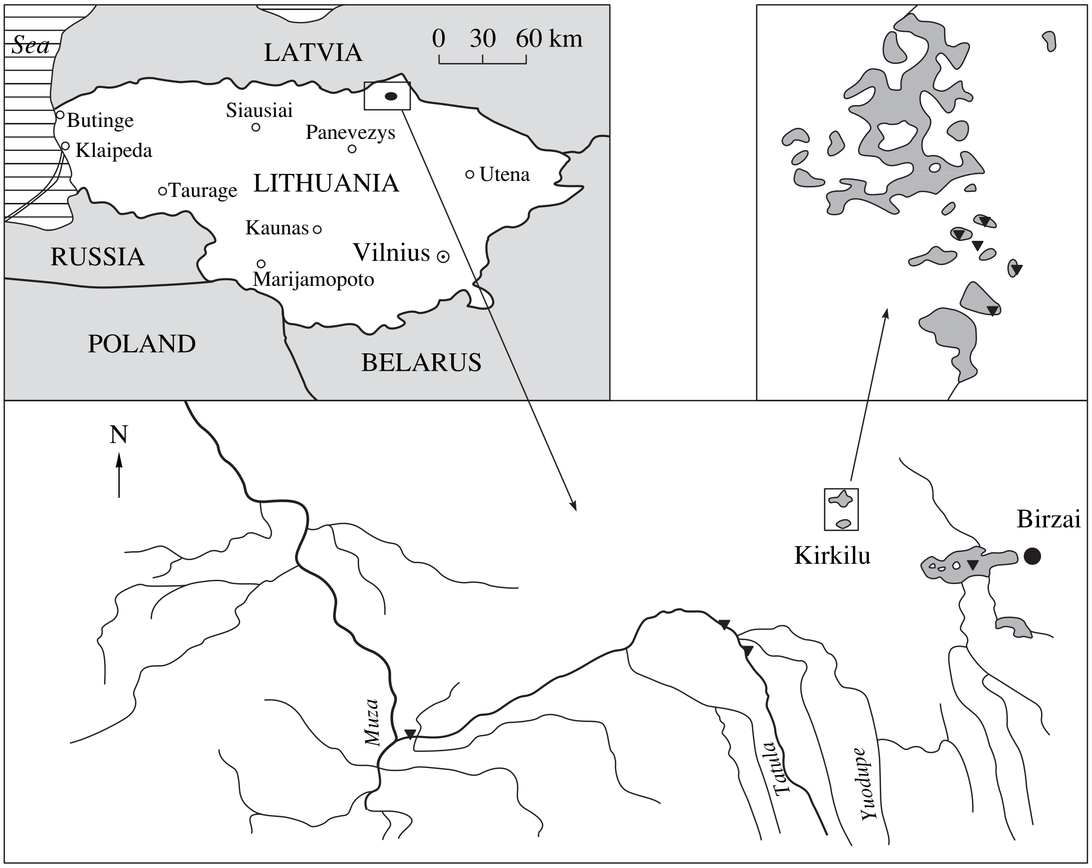

Fig. 1. Location of lakes in the gypsum karst region in northern Lithuania.

cant extent from wind and sunlight by steep shores, covered by bushes and trees. The study on Lake Kirkilu was performed in different seasons: in March, June, August, and October; on Lakes Katilnicha and Ramunelis, it was performed in June and August.

Water transparency was measured with the Secchi disk, and the main physicochemical parameters of water (temperature, pH, salinity, and electrical conductivity) were measured with a WTW MultiLine F/Set3 field meter equipped with selective electrodes. Dissolved oxygen was assayed by the Winkler method, and hydrogen sulfide, by colorimetry with dimethyl-1,4-phenylenediamine dihydrochloride using a Merck Microquant visual meter. The rate of sulfate reduction was determined by a radioisotope method using $Na_2{}^{35}SO_4$ [9]. To account for SRBs from various layers of water and bottom sediments and to obtain enrichment cultures for identification, samples were inoculated into a lactate-supplemented Postgate medium [10].

Genomic DNA was isolated from fresh SRB cultures after cultivation in liquid medium, using Genomic DNA Purification Kit K0512 (Fermentas, Lithuania). The isolation was carried out according to the manufacturer's recommendations.

As SRBs are subdivided into six phylogenetic subgroups, a PCR test for 16S rDNA was performed with six specific primers purchased from Fermentas [11]. Amplification medium: deionized water, 30.55 μl; primer I, 1 μl; primer II, 1 μl; $MgCl_2$, 8 μl; 2.5 μM dNTP, 4 μl; reaction buffer, 5 μl; BSA, 0.2 μl; *Taq* polymerase, 5 U; total volume 49 μl; DNA sample solution, 1 μl. The reaction was carried out in an Eppendorf Mastercycler. All reagents were purchased from Fermentas. The resulting amplicons were cloned into the TOPO TA cloning vector, having marker genes for kanamycin and ampicillin resistance. Plasmid DNA with the cloned 16S rRNA gene was isolated and purified with a kit for isolation and sequencing purchased from Bio-Rad. Sequencing was performed by the thermal denaturation method on an automated DNA sequencer.

RESULTS

In 2001, the environmental conditions in Lakes Kirkilu, Katilnicha, and Ramunelis varied from season to season. In particular, the most dramatic water stratification according to all indices studied was noted in the ice-cover season and in the summer (Table 1). Elevated mineralization of water, particularly in bottom layers, prolongs the stratification period. For this reason,

Table 1. Physicochemical indices of water of Lake Kirkilu in 2001

Date of analysis	Depth, m	Temperature, °C	pH	*Eh*, mV	O_2, mg/l	H_2S, mg/l	CH_4, µl/l	Total salt content, mg/l
March 7	0	1.2	7.34	–	1.60	0.5	104.30	100
	1	4.1	7.27	–	0	1.0	236.50	700
	2	5.4	7.27	–	0.32	0.3	237.71	900
	3	6.4	7.19	–	0.32	0.3	150.39	1000
	4	6.6	7.28	–	0	0.5	201.33	1000
	5	6.7	7.32	–	0	3.5	1576.66	1000
	5.5	6.8	7.32	–	–	4.0	2413.50	1100
April 24	0	14.5	7.50	+466	9.95	0	37.20	0
	1	11.8	7.30	+453	6.70	0	139.50	500
	2	9.0	7.16	+393	1.55	0	201.30	1000
	3	8.4	7.11	+393	0.85	0	215.90	1000
	4	8.2	7.13	+373	0.60	0	203.70	1100
	5	8.1	7.14	+370	0.32	0.1	195.30	1100
	6	8.1	7.08	+198	0.38	–	232.90	1100
	6.5	–	–	+28	–	0.3	–	–
June 20	0	18.8	7.92	+416	8.96	0	20.90	900
	1	16.8	7.76	+440	13.6	0	25.90	1000
	2	14.2	7.26	+451	9.92	0	14.00	1000
	3	11.2	7.09	+466	6.4	0	30.80	1100
	4	10.0	7.06	+443	5.6	0	33.20	1100
	5	9.1	7.03	+130	2.4	0.05	75.70	1100
	6	9.0	7.03	+32	0.96	0.2	117.60	1100
August 21	0	26.6	7.60	+352	9.28	0	54.82	500
	1	22.9	7.60	+387	6.70	0	22.32	500
	2	18.3	7.12	+433	3.20	0	7.28	900
	3	15.4	7.15	+384	1.82	0	0	1100
	4	13.9	7.14	+126	0.64	0.2	109.15	1000
	5	12.4	7.03	–23	0	3.0	548.19	1100
	5.7	10.7	7.14	–70	–	3.5	970.25	1100
October 29	0	6.6	7.77	+437	8.96	0	31.53	500
	1	6.6	7.77	+435	10.24	0	26.68	500
	2	6.8	7.59	+355	4.64	0.3	45.60	500
	3	8.9	7.15	+23	0	0.7	254.69	700
	4	10.9	7.05	–111	–	4.0	945.99	1000
	4.5	10.6	7.09	–76	–	4.0	1673.68	1100

anaerobic conditions persisted in the hypolimnion of the lake until the end of October, and the concentrations of reduced products of final stages of anaerobic degradation (hydrogen sulfide and methane) reached their maximum values over the growing season. It should be noted that the water level varied noticeably from season to season in all lakes depending on the weather, the magnitude reaching 1 m. Therefore, it is a matter of convention that the maximum depth at sampling sites reached 6–7 m.

Oxygen concentration and the presence of hydrogen sulfide were crucial for SRB development. In the ice-cover season (March), relatively small amounts of hydrogen sulfide spread throughout the water column of Lake Kirkilu, whereas oxygen was virtually absent. During summer stratification, oxygen deficiency was recorded starting from a depth of 2 m in Lakes Ramunelis and Katilnicha and in bottom water layers of Lake Kirkilu. Hydrogen sulfide was also present there at concentrations 0.2–4.5 mg/l (Fig. 2). It is of interest that the

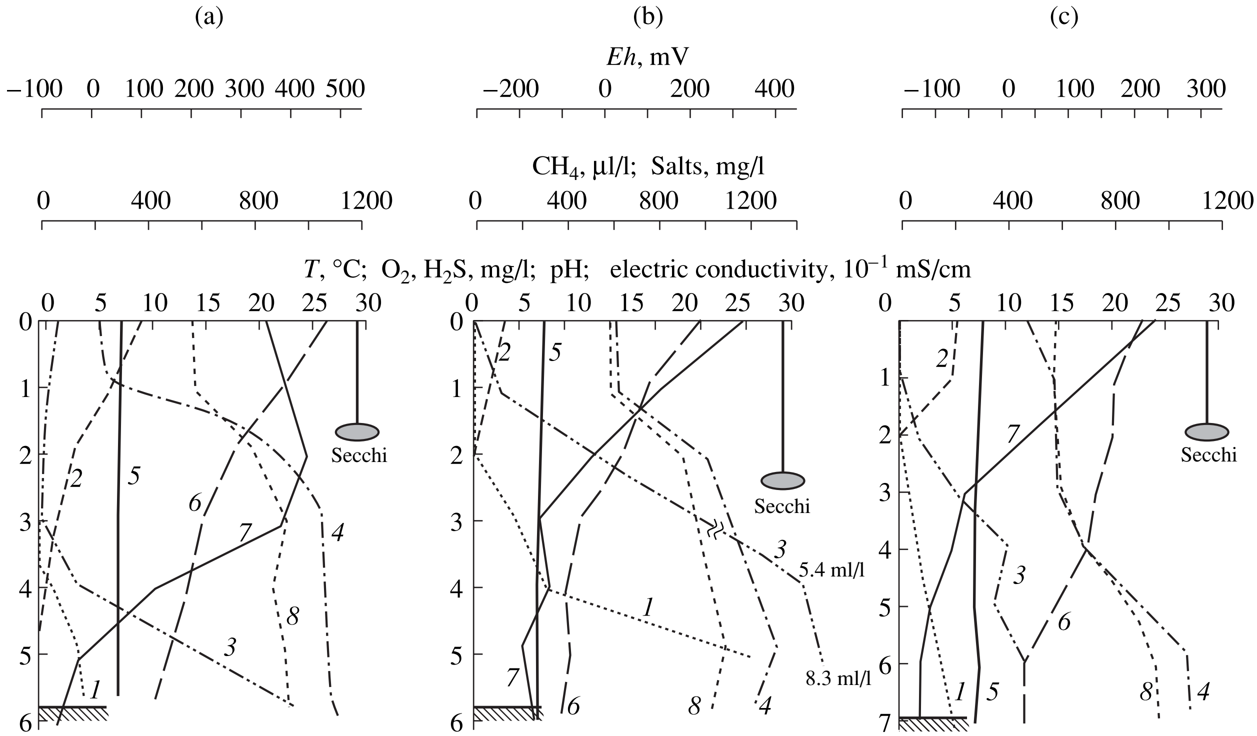

Fig. 2. Vertical profile of physicochemical indices in the water columns of Lakes (a) Kirkilu, (b) Ramunelis, and (c) Katilnicha during summer stratification (August 21, 2001): *1*, H_2S; *2*, O_2; *3*, CH_4; *4*, salts; *5*, pH; *6*, temperature; *7*, Eh; *8*, electric conductivity.

maximum hydrogen sulfide concentrations (of up to 30 mg/l) were recorded in late August in bottom layers of the nearby Lake Ramunelis, which is smaller in size.

Studies of the rate of sulfate reduction and occurrence of SRBs in various water layers and in the upper layer of bottom sediments were performed in 2001 with regard to potential formation of temporal and spatial optima of redox conditions in each lake. In this context, together with other factors (relatively warm water and supply of readily consumable organic matter), the most favorable conditions for sulfate reduction emerged in the second half of the active vegetation period. In the deepest parts of the lakes, the rate of the process in reduced sediments reached 0.95–2.60 mg S^{2-}/dm^3 per day (Table 2). Note that, at the same time, some littoral areas of Lake Kirkilu, where bottom sediments were enriched in plankton and plant debris, were characterized by a higher sulfate reduction rate (of up to 11.36 mg S^{2-}/dm^3 per day). However, this local phenomenon cannot be of great biogeochemical significance for sulfur circulation in karst lakes. Generally, bottom sediments of Lake Kirkilu with strictly reducing conditions were characterized by high concentrations of acid-soluble sulfides: 120–496 mg S^{2-}/dm^3 per day [12].

The rate of sulfate reduction in the water column was tenfold lower than in bottom sediments of the same water and mud volumes. In some cases, this rate reached 0.49 mg S^{2-}/l per day, being higher in anaerobic benthic water layers. [The process was slower at the redox boundary, in the uppermost layers of the hydrogen sulfide zone, forming at different depths in the karst lakes under study (Fig. 2). For example, sulfate reduction in Lake Ramunelis occurred at rates of up to 0.04 mg S^{2-}/l per day at a depth as little as 1 m.] Bacterial reduction of sulfates occurred more slowly in autumn (October), reaching 0.12–0.15 mg S^{2-}/l per day.

It should be noted that the presence of SRBs in the water column is also a characteristic feature of the karst lakes studied. Their density in various water layers varied during summer stratification from 10^3 to 10^4 cells/ml. It is of special interest that, in the ice-cover season (March), SRBs occurred throughout the water column of Lake Kirkilu, their population reaching 10^2–10^3 cells/ml.

Concentrations of SRBs in bottom sediments were one or two orders of magnitude higher, varying from season to season in the range 10^4–10^6 cells/cm^3 (in upper layers). The highest SRB density, up to 10^7 cells/cm^3, was noted in autumn in bottom sediments of Lake Kirkilu.

Microscopic examination of characteristic black SRB colonies from water and mud samples from the lakes under study revealed large bent rods from 0.5–1.0 × 3–5 µm in length. According to morphological features, the predominant SRB from enrichment cultures corresponded to *Desulfovibrio desulfuricans*. Further identification was performed by molecular methods. Taking into account the fact that SRBs are genetically subdivided into six physiological subgroups, we performed PCR tests with six specific primers. For this purpose, DNA was isolated from enrichment cultures of SRBs from water and mud samples taken from all the lakes under study. In all experiments, amplicons were obtained only with the primer for subgroup 6 (Fig. 3), which includes the genera *Desulfovibrio* and *Desulfomicrobium* [11].

With regard to this fact and the morphological similarity of microorganisms in all enrichment cultures, further identification was performed with the culture isolated from bottom sediments of a typical karst water body, Lake Kirkilu. Amplified 16S rDNA fragments were cloned and sequenced. Fragments with certain nucleotide sequences were assembled with the DNASTAR program. These sequences were compared with those stored at the National Center for Biotechnology Information (USA) (www.ncbi.nlm.nih.gov) using the BLAST program. A comparison of sequences of the gene for 16S rRNA (709 bp) showed that the sequences in the SRBs perfectly matched the sequences of *Desulfovibrio desulfuricans* stored in the gene bank (accession no. AF354664). According to our results, it is exactly this species that is predominant among karst lake SRBs cultivated in the presence of lactate.

Table 2. Population of SRBs and sulfate reduction rate in water and bottom sediments of karst lakes in 2001

Lake	Date	Depth, m	SRB, cells/cm^3	Rate of sulfate-reduction, mg S^{2-}/dm^3 per day
Kirkilu	March 9	0	10^2	–
		1	10^2	–
		2	10^3	–
		3	10^2	–
		4	10^2	–
		5	10^2	–
		5.5	10^2	–
		bottom sediments	10^6	–
Kirkilu	June 21	5.5	10^2	–
		bottom sediments	10^3	–
Kirkilu	August 23	4.0	–	0.38
		5.5	10^4	0.49
		bottom sediments	10^5	1.35
Kirkilu	October 29	3	10^4	0.12
		4	10^4	0.15
		5	10^6	0.14
		bottom sediments	10^7	1.60
Ramunelis	June 21	4	10^2	–
		bottom sediments	10^3	–
Ramunelis	August 23	1	–	0.04
		4	10^3	–
		5.5	10^4	0.26
		bottom sediments	10^6	0.95
Katilnicha	June 21	7	10^2	–
Katilnicha	August 23	4	–	0.21
		6.5	10^3	0.29
		bottom sediments	10^4	2.60

Note: "–" means not studied.

DISCUSSION

Environmental conditions in lakes of the gypsum karst region in northern Lithuania are favorable for sulfate-reducing bacteria, first of all, because of high sulfate contents. Our studies revealed their abundance not only in bottom sediments but also in the water column. These bacteria are most abundant during the winter and summer stratifications, when microaerophilic and anaerobic conditions reach their maximum in the annual cycle. Obviously, high mineralization of water greatly affects the gas conditions in lower water layers and prolongs stratification. Stably high contents of total dissolved salts (up to 1100 mg/l) in bottom layers were recorded in all seasons of the study. This fact suggests that the karst lakes are partially meromictic, but this suggestion has not been confirmed by comprehensive hydrological studies; moreover, the water bodies have been reported to be dimictic [13].

Nevertheless, it is obvious that the circannual rhythm of the gas conditions in these water bodies depend, to a marked extent, on the habit and direction of microbiological processes in both water column and bottom sediments. The vital activity of sulfate-reducing bacteria results in accumulation of hydrogen sulfide at concentrations reaching 4–5 mg/l in the lowermost layers. In some cases, its concentration reached 30 mg/l. However, in spite of SRB abundance, their activity in bottom sediments was not so high, matching the values determined in other Lithuanian lakes [12]. The mean rate of hydrogen sulfide formation (1.32 mg S^{2-}/l per day) was close to that detected in bottom sediments of mesotrophic and subsaline basins and in some meromictic lakes [14–16].

It is likely that the rate of sulfate reduction in lakes of the region under study is determined primarily by the structure and concentration of organic matter, rather than high sulfate concentrations. Fallen leaves from shores covered by bushes and trees provide poorly degradable organic substances (lignin, plant gums, and wax), which take a longer time to be converted to

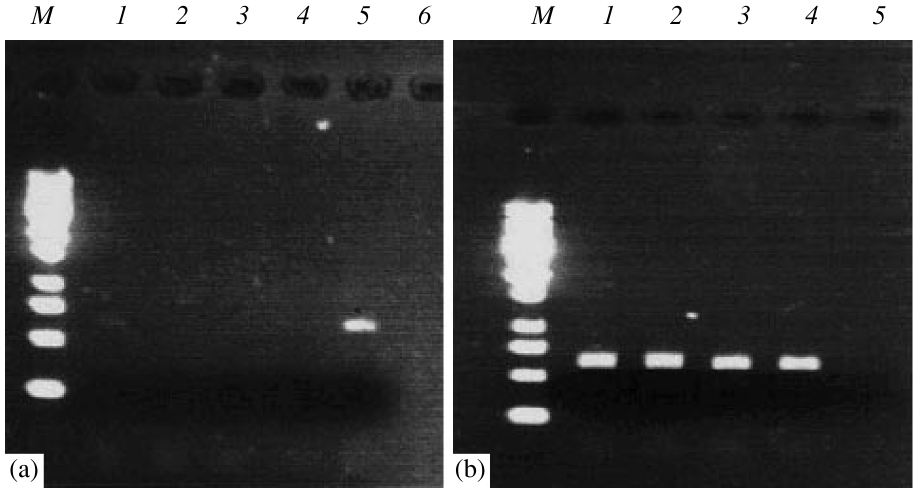

Fig. 3. Electrophoretic analysis of PCR amplicons of the 16S rRNA gene of SRBs. Panel a: test with six specific primers. Lanes: *M*, DNA length marker; *1, 2, 3, 4, 6,* negative results with primer pairs I, II, III, IV, and V, respectively; Lane *5*, amplicon with primer pair VI. SRBs were sampled from bottom sediments of Lake Kirkilu. Panel b: test with primer pair VI. Lanes: *M*, DNA length marker; *1, 2, 3, 4,* amplicons obtained with DNA of SRBs sampled from bottom sediments and various water levels in Lakes Ramunelis and Katilnicha; *5*, blank test.

readily consumable substrates (organic acids). Other terminal anaerobic processes also compete for the substrate, and this competition may decrease the rate of sulfate reduction. Earlier studies showed that the rate of methane production in bottom sediments of karst lakes was as high as 7 ml/dm^3 per day during summer stratification [17].

However, wherever conditions permit, SRBs reach their potential, converting sulfate at a rate of 11.36 mg S^{2-}/l per day. Areas with such rates of this process were noted in Lake Kirkilu, where bottom sediments were rich in plankton debris and aquatic plant remnants. High rates were also noted in other karst lakes [18].

The SRB population and bacterial sulfate reduction in anaerobic layers of the water column and at the redox zone boundaries was a specific feature of the karst lakes under study, and the rate of hydrogen sulfide production (up to 0.49 mg S^{2-}/l per day) was close to or higher than that of bottom sediments of other Lithuanian lakes [12]. Together with other reduction products, hydrogen sulfide provided conditions favorable for growth of various photo- and chemotrophs. Penetration of light into anaerobic water layers favored the formation of ecozones characterized by intense growth of anoxigenic phototrophic bacteria [19]. Earlier studies showed that, in certain periods, the photosynthesis rate in the anaerobic hypolimnion reached the level of 200 mgC/m^3 per day, nearly twice that near the surface (130 mgC/m^3 per day) [20].

Molecular methods are gaining increasing acceptance. When applied to identification of SRBs, they indicate that *D. desulfuricans* is one of the most widespread species in water bodies of the gypsum karst region in northern Lithuania. These bacteria are best studied among other SRB species because of their wide occurrence in various econiches and simplicity of their cultivation and isolation.

Sulfur-reducing bacteria use a wide range of organic substances as electron donors, although some species are strictly specific in utilizing certain organic substrates. As many SRB species grow on lactate, this substrate was used in enrichment cultivation, as recommended by Postgate. Probably, use of a wider range of organic substrates for SRB enrichment or *in situ* identification would reveal broader species diversity.

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