ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 2, pp. 247–252. © Pleiades Publishing, Ltd., 2010.
Original Russian Text © N.V. Pimenov, A.Yu. Kallistova, I.I. Rusanov, S.K. Yusupov, L. Montonen, G. Jurgens, U. Münster, A.N. Nozhevnikova, M.V. Ivanov, 2010, published in Mikrobiologiya, 2010, Vol. 79, No. 2, pp. 270–276.

EXPERIMENTAL ARTICLES

Methane Formation and Oxidation in the Meromictic Oligotrophic Lake Gek-Gel (Azerbaijan)

N. V. Pimenov[a,1], A. Yu. Kallistova[a], I. I. Rusanov[a], S. K. Yusupov[a], L. Montonen[b], G. Jurgens[b], U. Münster[c], A. N. Nozhevnikova[a], and M. V. Ivanov[a]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Octyabrya 7/2, 117312 Moscow, Russia*

[b] *Department of Applied Chemistry and Microbiology, University of Helsinki, P. O. Box 56, Biocenter 1A, Viikinkaari 9, FIN-00014 Helsinki, Finland*

[c] *Institute of Environmental Engineering and Biotechnology, Tampere University of Technology, P.O. Box 541, Korkeakoulunkatu 8, FIN-33101 Tampere, Finland*

Received September 29, 2009

Abstract—The production and oxidation of methane and diversity of culturable aerobic methanotrophic bacteria in the water column and upper sediments of the meromictic oligotrophic Lake Gek-Gel (Azerbaijan) were studied by radioisotope, molecular, and microbiological techniques. The rate of methane oxidation was low in the aerobic mixolimnion, increased in the chemocline, and peaked at the depth where oxygen was detected in the water column. Aerobic methanotrophic bacteria of type II belonging to the genus *Methylocystis* were identified in enrichment cultures obtained from the chemocline. Methane oxidation in the anaerobic water of the monimolimnion was much more intense than in the aerobic zone. However, below 29–30 m methane concentration increased and reached 68 μM at the bottom. The highest rate of methane oxidation under anaerobic conditions was revealed in the upper layer of bottom sediments. The rate of methane oxidation significantly exceeding that of methane production suggests a deep source of methane in this lake.

Key words: meromictic oligotrophic Lake Gek-Gel, methanogenesis, methane oxidation, methanotrophic bacteria.

DOI: 10.1134/S0026261710020177

From the point of view of microbial ecology, meromictic lakes are important and intriguing objects. Physicochemical stability of their water masses and relatively constant vertical stratification of microbial populations make them a convenient model for investigation of the structure of microbial communities. In meromictic lakes, microbial number and diversity usually increase in the chemocline at the boundary between the aerobic and anaerobic zones, with stable anaerobic conditions below. In the anaerobic zone of freshwater and saline meromictic lakes, free sulfide is usually present due to the activity of sulfate-reducing prokaryotes [1, 2].

High concentrations of methane resulting from the activity of methanogenic archaea are also common in the anaerobic monimolimnion. Usually, methane content decreases drastically in the chemocline (where the highest rates of aerobic methane oxidation occur) and remains low in the mixolimnion [3]. Aerobic methanotrophic bacteria consume a significant portion of methane formed in the anaerobic zone, thus returning carbon to the food chain and preventing methane emission into the atmosphere [4, 5]. In addition to aerobic methane oxidation, high rates of anaerobic methane oxidation were revealed in the monimolimnion and upper bottom sediments of stratified lakes with sufficiently high sulfate content [2, 6–8]. Most probably, anaerobic methane oxidation occurs via reverse methanogenesis with sulfate as an electron acceptor and is carried out by a consortium of methanotrophic archaea and sulfate-reducing bacteria [9, 10]. In the water column of the freshwater stratified Lake Plüβsee, the cells of anaerobic methanotrophic archaea were visualized by CARD-FISH [11]. Anaerobic methane oxidation not related to the activity of sulfate-reducing bacteria was revealed in the sediments of a freshwater channel [12]; the authors suggested that it was carried out by a consortium of methanotrophic archaea (ANME II) and denitrifying bacteria. Evidence was recently obtained that denitrifying bacteria are capable of anaerobic methane oxidation coupled to nitrate reduction to dinitrogen, not involving participation of archaea [13]. Thus, our present knowledge does not rule out anaerobic methane oxidation in anaerobic zones of reservoirs with low sulfate content.

[1] Corresponding author; e-mail: npimenov@mail.ru

The meromictic oligotrophic Lake Gek-Gel is located in the Caucasus Mountains (Azerbaijan) 1650 m above sea level. It was formed in the 12th century in the Ag-Su river valley after an earthquake. Its surface area is 1.25 km^2, and the maximum depth is 92 m. The lake is characterized by pronounced temperature and chemical stratification. Free sulfide is detected at depths exceeding 29–30 m; its concentration at the near-bottom horizons is as high as 4.0–4.5 mg l^{-1}. Sulfate concentration in the water column varies from 30 to 50 mg l^{-1}. Investigations of the physicochemical water parameters, photosynthesis, chemosynthesis, of number and diversity of heterotrophic, photosynthetic, iron-oxidizing, and sulfate-reducing bacteria, as well as of zooplankton, have been previously carried out in Lake Gek-Gel [14–17]. However, no data exist in the literature concerning the methane cycle in this lake.

The goal of the present work was investigation of methane content and rates of methanogenesis and methane oxidation in the water column and upper sediment layers of Lake Gek-Gel by biochemical methods, as well as the isolation of aerobic methanotrophic bacteria from enrichment cultures with subsequent identification by molecular techniques.

MATERIALS AND METHODS

Research on Lake Gek-Gel was carried out in September 2003 at a point with 72-m depth (40°24.706′ N, 46°19.696′ E).

The water was sampled with a 1-l glass bathometer. The upper sediments were collected with a limnological stratometer with a 4 × 40-cm Perspex tube. Determination of oxygen content by the Winkler method and of sulfide content by the standard reagent kit (Aquamerck, Germany), as well as collection of samples for gas chromatographic methane determination on a Chrom 5 chromatograph (Chzech Republic) with a flame ionization detector were carried out immediately after sampling.

The rates of methane production and oxidation in the water column and bottom sediments were measured by the radioisotope method. The substrates for hydrogenotrophic and aceticlastic methanogenesis were $NaH^{14}CO_3$ and methyl-labeled ^{14}C acetate. The rate of methane oxidation was determined with $^{14}CH_4$. Incubation of water samples with labeled substrates was carried out for 24 h in hermetically sealed, bubble-free 30-ml penicillin vials. The incubation temperature was maintained by submerging the vials suspended on nylon lines to the sampling depth.

Bottom sediment samples were placed in cut-off 5-ml plastic syringes, sealed with butyl rubber stoppers, and incubated for 24 h in a freezer at 5°C, close to in situ temperature.

Aquatic solutions of ^{14}C-bicarbonate, ^{14}C-acetate, and ^{14}C-methane (0.2 ml) were injected into the water and sediment samples to a final radioactivity of 10, 15, and 2 μCi, respectively. Sampling and incubation procedures for radioisotope determination of sulfate reduction rates (with $^{35}SO_4^{2-}$ at the final radioactivity of 20 μCi) in lake sediments were similar to those applied for the methane cycle processes.

Immediately after incubation, the samples were fixed with 1 ml of 2N NaOH and transferred to the stationary laboratory of the Winogradsky Institute of Microbiology. The samples were then treated as described earlier [18, 19].

Aerobic methanotrophic bacteria were cultivated in P liquid mineral medium [20]. The vials (20 ml) with 5 ml of sterile medium and 10% methane in the gas phase were inoculated with 5 ml of lake water sampled at a specified depth. The vials were incubated for two months at 20°C. The growth of methanotrophs was assayed as a decrease in methane concentration, which was determined on a Khrom 5 gas chromatograph. Enrichments demonstrating evidence of growth were transferred to fresh medium every three weeks.

The total DNA from enrichment cultures was isolated by the method with hexadecyltrimethylammonium bromide [21].

PCR amplification of the gene fragments encoding 16S rRNA was carried out with GC984F, 984F, and 1492R universal bacterial primers [22]. A DNA sample (1–3 μl) was added to 30 μl of the reaction mixture containing 0.75 μl of dNTP mixture (10 mM, Finnzymes, Finland), 1.2 μl of each primer (20 pmol l^{-1}), 3 μl 10× buffer for DyNAzyme™ II DNA polymerase (Finnzymes, Finland), and 0.45 μl of DyNAzyme™ II DNA polymerase (2 U μl^{-1}, Finnzymes, Finland). PCR was carried out in an Eppendorf Master Cycler Gradient thermocycler (Germany) according to the following program: (1) initial denaturation, 95°C, 4 min; (2) 38 cycles of denaturation (40 s, 94°C), annealing (1.5 min, 55°C), and elongation (2.5 min, 72°C); and (3) final elongation at 72°C for 15 min. PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.2 mg l^{-1}) and visualized with an UV transilluminator.

The PCR products obtained by amplification with GC984F and 1492R primers were separated by denaturing gel electrophoresis in 6% acrylamide gel with a linear gradient (from 30 to 60%) of denaturing agents (a mixture of ures and formamide). Electrophoresis was carried out at 60°C and 60 V for the first 15 min and at 150 V for the subsequent 225 min. A DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., United States) was used. After electrophoresis, the gels were stained with a water solution of GelStar DNA and RNA fluorescent stain (Cambrex Bio Science Rockland, Inc., United States) and visualized with a Clare Chemical Dark Reader transilluminator (United States). The bands containing DNA

fragments with specific nucleotide sequences were excised, and DNA was extracted and reamplified. The PCR products were purified and sequenced at the Haartman Institute (University of Helsinki, Finland).

The sequenced 16S rDNA sequences were compared to the sequences of various members of *Bacteria* in GenBank using the BLAST software package (http://www.ncbi.nlm.nih.gov/blast). The sequences were deposited to the EMBL database under accession numbers FM865636 and FM865637.

RESULTS

Physicochemical parameters affecting the distribution and activity of the microorganisms of the methane cycle. At the sampling period (the second decade of September 2003), the water column of Lake Gek-Gel was stratified. The thermocline was located in the metalimnion at 7–19 m (Fig. 1a). Oxygen was detected to a 29-m depth. Deeper, free sulfide was present; its concentration increased with depth, reaching 0.12 μmol/l. At the depth of 29 m, both oxygen (0.15 mg l^{-1}) and traces of sulfide (< 0.1 mg l^{-1}) were detected. Methane distribution in the water column was typical of meromictic lakes (Fig. 1a). Methane concentration in the oxygen-containing mixo- and metalimnion water was low (from 0.03 to 0.13 μM). The highest methane concentrations were found in the surface layers. This increase of methane content near the surface may result from the influx of methane-enriched river water. At depths over 27 m, methane concentration increased sharply; below 35 m and all the way to the bottom, its content changed from 53 to 68 μM. Methane concentration in the upper sediments was still higher, from 312 to 938 μmol dm^{-3}.

Rates of the microbial processes of the methane cycle. The profile of the rate of microbial methane oxidation (MO) in the water column exhibited a maximum at the depth of 27–31 m (Fig. 1b). Its comparison with the graph of methane content demonstrates that the 27 to 30-m water layer with the highest MO rate (18–25 nmol l^{-1} day^{-1}) corresponds to the zone of a sharp decrease in methane concentration from 41.3 to 0.05 μmol l^{-1}. In the anaerobic water column, the MO rate varied within a narrow range, from 7.8 to 13.5 nmol l^{-1} day^{-1}.

MO rate in the upper sediments was almost an order of magnitude higher than in the water column. The highest rates (670–760 nmol dm^{-3} day^{-1}) were found in subsurface sediments 5–12 cm deep.

Fig. 1b presents the profile of methanogenesis (MG) rates in the water column of Lake Gek-Gel. A certain increase in the rate of this process (1.5–3.4 nmol l^{-1} day^{-1} was found at the depths from 40 to 60 m. The methanogenesis rate in the sediments was higher, from 4.5 to 11.7 nmol dm^{-3} day^{-1} (Fig. 2). The rates of both hydrogenotrophic and aceticlastic methanogenesis were determined. Hydrogenotrophic methanogenesis contributed mainly to methane production both in the water column and in the bottom sediments. Aceticlastic methanogenesis was responsible for less than 5% of the total methane production.

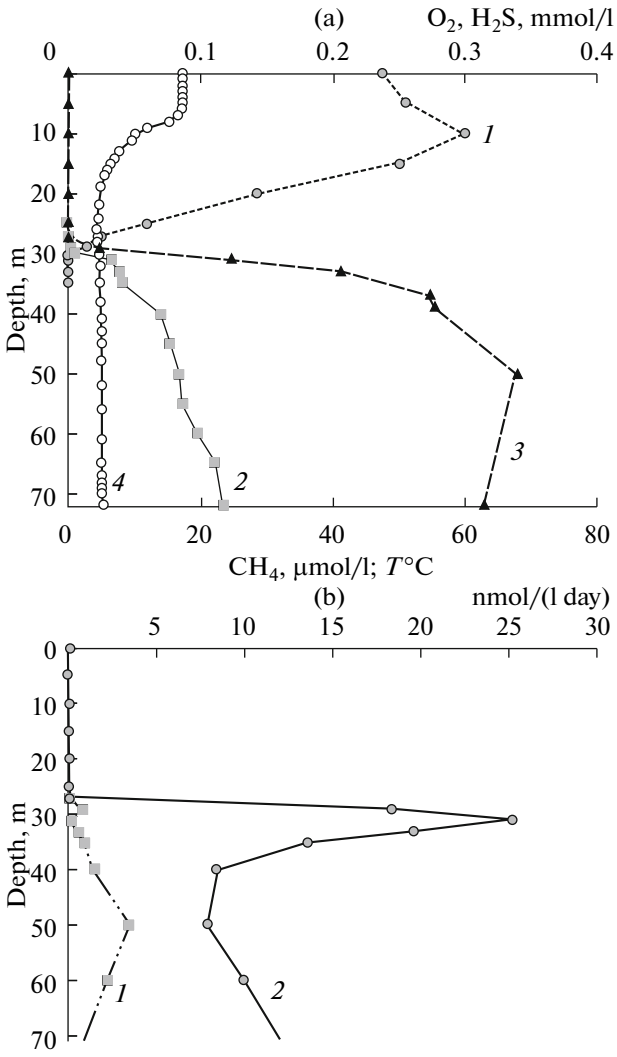

Fig. 1. Profiles of temperature (*4*), O_2 (*1*), H_2S (*2*), and CH_4 (*3*) in the water column of Lake Gek-Gel (a); rates of methane production (*1*) and oxidation (*2*) in the water column of Lake Gek-Gel (b).

Anaerobic methane oxidation (AME) is presently believed to involve a consortium of methanotrophic archaea and sulfate-reducing bacteria, in which archaea carry out methane transformation by a reverse methanogenesis pathway [9, 10]. Figure 3 shows the profiles of sulfate and the rates of sulfate reduction (SR). The highest rates of this process were found in the uppermost sediment horizons, where increased microbial activity probably results from decomposition of organic matter precipitating from the water column. Lower (at 5–12 cm), the AMO rate increased (Fig. 2), while the SR rate decreased several fold (Fig. 3).

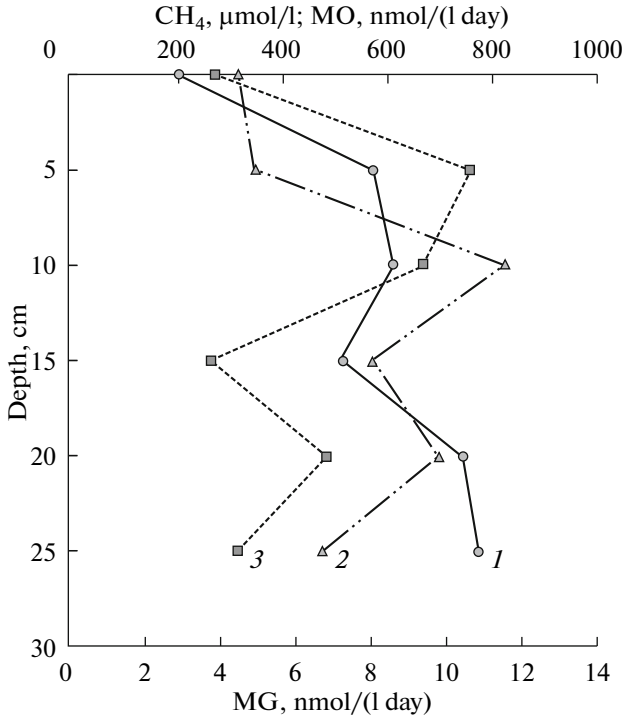

Fig. 2. Methane content, μmol l^{-1} (*1*) and rates of methane production (MG), nmol dm^{-3} day^{-1} (*2*) and methane oxidation (MO), nmol dm^{-3} day^{-1} (*3*) in the upper sediments of Lake Gek-Gel.

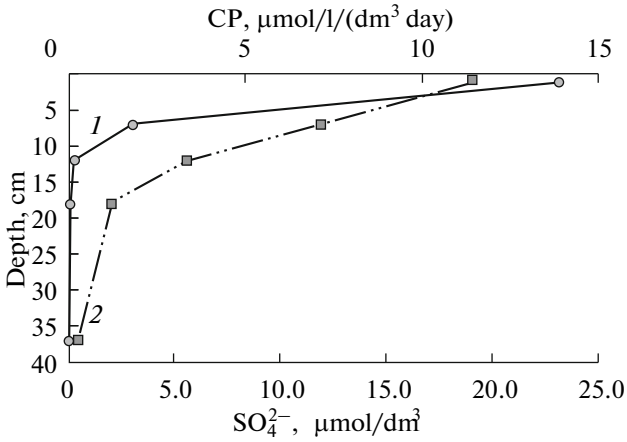

Fig. 3. Profiles of sulfate reduction (SR), μmol dm^{-3} day^{-1} (*1*) and SO_4^{2-} content, μmol dm^{-3} (*2*) in the upper sediments of Lake Gek-Gel.

Identification of aerobic methane-oxidizing microorganisms. The cells in enrichment cultures from the Lake Gek-Gel chemocline were morphologically uniform rounded rods. Only type II methanotrophs of the genus *Methylocystis* were identified in the cultures by DGGE. In enrichments from 25 and 27 m, methanotrophs closely related (100% similarity for 481-bp 16S rRNA gene fragment) to *Methylocystis* sp. B3 (DQ496232) and *M. rosea* SV97 (AJ414656). Methanotrophs isolated from a 29-m depth were related (99% similarity for 488-bp 16S rRNA gene fragment) to *Methylocystis* sp. clone m261 (DQ852351) and *M. parvus* clone pAMC269 (AF150805).

DISCUSSION

In meromictic lakes, methane concentration is known to increase below the chemocline due to the activity of methanogenic archaea. Lake Gek-Gel is not exceptional in this respect. The data on methane content in some meromictic lakes of different salinities and trophicities are presented in Table 1. Methane content in the anaerobic zone of the oligotrophic Lake Gek-Gel (up to 68 μM) is comparable to or higher than in some eutrophic lakes with high rates of microbial processes. For example, methane concentrations we observed in the brackish Lake Shunet (Khakassia) were about half of those in Lake Gek-Gel. High methane content correlates with a relatively high rate of methane oxidation, which was higher in Lake Gek-Gel than in some oligotrophic and even mesotrophic lakes (Table 1). Increased methane content in Lake Gek-Gel may result from the activity of methanogenic archaea that are responsible for the terminal stage of organic matter decomposition at low sulfate concentrations. In order to check this hypothesis, integral rates of methanogenesis, sulfate reduction, and methane oxidation were calculated for the water column and upper sediments of Lake Gek-Gel (Table 2). In spite of the low sulfate content, integral SR rate in the upper sediments was more than 200 times higher than the methanogenesis rate. At a depth of 20 cm, sulfate concentration decreases to 2 μmol dm^{-3} and the SR rate decreases almost to zero (Fig. 3). However, unlike most marine sediments, no tendency toward increased methanogenesis rates below the horizon of sulfate consumption was observed (Fig. 2). An integral rate of methane oxidation significantly exceeding the rate of methanogenesis both in the water column and sediments of Lake Gek-Gel (Table 2) suggest a deep origin of methane in this lake. Since the lake is located in a zone of seismic activity, inflow of abiogenic deep methane cannot be ruled out. Elucidation of the origin of methane in Lake Gek-Gel and of the reasons for its high concentration require investigation of the isotopic composition of methane carbon and hydrogen ($^{13}\delta C$, δD).

The maximum of aerobic methane oxidation at the upper boundary of the chemocline has been demonstrated for many meromictic lakes and results from high activity of aerobic methanotrophic bacteria. Methanotrophic bacteria were isolated from the water samples collected along the profile of the aerobic zone of Lake Gek-Gel. Enrichment cultures, however, were obtained only from the chemocline zone, with the highest activity (and probably abundance and diversity) of methanotrophs. Methanotrophic bacteria were

Table 1. Methane content (μmol l^{-1}) and rates of methanogenesis and methane oxidation (nmol l^{-1} day^{-1}) in the water column of some stratified lakes of different trophicities

Lake	Trophic status	CH_4 content	CH_4 production rate	CH_4 oxidation rate		Reference
				Aerobic	Anaerobic	
Freshwater						
Gek-Gel, meromictic	Oligotrophic	0.03–68	0.01–3.39	0.03–25.12	7.83–13.49	Our data
Lillsjon, stratified in summer	Oligotrophic	~0–31.5	ND*	~0.8–12.5	ND	[23]
Mårn, stratified in summer	Eutrophic	~0–55	ND	~0–833	~833–2083	[23]
Illersjön, stratified in summer	Eutrophic	~0–135	ND	0	~0–3250	[23]
Knaak, meromictic	Eutrophic	0–4000	720–1440	ND	ND	[24]
Brackish						
Ace Lake, meromictic	Oligotrophic	0.9–4930	~1.56–15.71	ND	ND	[25]
Mogil'noe, meromictic	Mesotrophic	0.01–2.28	0.85–29.24	0.03–4.06	0.65–83.13	[2]
Shira, meromictic	Mesotrophic	0.02–0.77	4.46–43.75	0.30–3.08	0.71–3.02	[8]
Shunet, meromictic	Eutrophic	0.08–34.15	1.20–9.4	0.42–1.24	3.84–167.41	[26]
Mono Lake, meromictic	Eutrophic	~2–68	ND	~0–160	~5–110	[7]

Note: ND, not determined.

isolated on a mineral medium suitable for cultivation of various species of neutrophilic type I and type II methanotrophs [27]. However, all methanotrophs identified in our enrichments belonged to type II and were phylogenetically related to the single genus *Methylocystis*. These data contradicted the results of other investigations of stratified lakes of various degrees of trophicity, which revealed significant predominance of type I over type II methanotrophs both in numbers and diversity [5, 7, 28]. In the eutrophic Lake Plüβsee (Germany), only type I methanotrophs were revealed by FISH [11]. Our results obtained by the cultivation method do not exclude the presence of other methanotrophic genera of both type I and type II, which either did not grow on the selective medium, were eliminated by transfers, or were quantitatively minor. Application of in situ methods (analysis of DNA isolated directly from water samples, and CARD-FISH) is required to determine the ecologically significant and numerously predominant species of methanotrophs in Lake Gek-Gel.

Table 2. Integral rates of microbial processes (μmol m^{-2} day^{-1}) in the water column and surface sediments of Lake Gek-Gel

Process	Water column	Bottom sediments*
Methane oxidation	0.5	124
Methane production	0.08	2.0
Sulfate reduction	0.05	458

* The calculation was carried out for the upper 25 cm of the sediment.

ACKNOWLEDGMENTS

The authors are grateful to Academician M.A. Salmanov, director of the Institute of Microbiology (National Academy of Sciences of Azerbaijan), for his help in organization of the expedition.

The work was supported by INTAC (project 01-2333), the Russian Academy of Sciences (project no. 3 on Russian–Finnish scientific exchange), and the Academy of Finland (project no. 214719).

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