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Community Shift from Phototrophic to Chemotrophic Sulfide Oxidation following Anoxic Holomixis in a Stratified Seawater Lake

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Most stratified sulfidic holomictic lakes become oxygenated after annual turnover. In contrast, Lake Rogoznica, on the eastern Adriatic coast, has been observed to undergo a period of water column anoxia after water layer mixing and establishment of holomictic conditions. Although Lake Rogoznica's chemistry and hydrography have been studied extensively, it is unclear how the microbial communities typically inhabiting the oxic epilimnion and a sulfidic hypolimnion respond to such a drastic shift in redox conditions. We investigated the impact of anoxic holomixis on microbial diversity and microbially mediated sulfur cycling in Lake Rogoznica with an array of culture-independent microbiological methods. Our data suggest a tight coupling between the lake's chemistry and occurring microorganisms. During stratification, anoxygenic phototrophic sulfur bacteria were dominant at the chemocline and in the hypolimnion. After an anoxic mixing event, the anoxygenic phototrophic sulfur bacteria entirely disappeared, and the homogeneous, anoxic water column was dominated by a bloom of gammaproteobacterial sulfur oxidizers related to the GSO/SUP05 clade. This study is the first report of a community shift from phototrophic to chemotrophic sulfide oxidizers as a response to anoxic holomictic conditions in a seasonally stratified seawater lake.

Stratified sulfidic lakes offer a physically and chemically well-defined environment for the development of complex yet stable microbial communities along light, oxygen, and sulfide gradients (1–4). Their water column is divided into an oxic surface layer, the epilimnion, and an anoxic bottom layer, the hypolimnion (5). The interface between these layers is the chemocline. It is the zone of highest chemical reactivity in the lake, typically accompanied by elevated microbial activity. In the anoxic hypolimnion and the underlying sediments, sulfate reduction plays a key role in organic matter remineralization (e.g., see references 3 and 6), resulting in the production of hydrogen sulfide. Where sulfide and light are present but oxygen is absent, anoxygenic phototrophic green sulfur bacteria (GSB) and purple sulfur bacteria (PSB) oxidize sulfide and form extensive blooms with cell densities of up to 10^7 cells ml^{-1} (1, 3, 7–10). Chemotrophic sulfur oxidizers (e.g., *Alphaproteobacteria*, *Gammaproteobacteria*, and *Epsilonproteobacteria*) often occur in layers above or together with GSB and PSB at the chemocline (3, 9, 11–13). In the oxic, sulfide-free epilimnion, the microbial community composition is distinct from those in underlying anoxic waters (10, 13, 14).

Lake Rogoznica is a seasonally stratified seawater lake on the Croatian Adriatic coast. Its hydrography and chemistry have been studied extensively throughout the last 2 decades. The epilimnion is similar to waters of the adjacent Adriatic Sea in temperature and salinity, while the hypolimnion is highly enriched in nutrients (NH_4^+, up to 150 μM; PO_4^{3-}, up to 22 μM; SiO_4^{4-}, up to 400 μM) and dissolved organic carbon (DOC) (up to 6 mg $liter^{-1}$) (15–18). This is assumed to be a consequence of extensive organic matter remineralization (19, 20) coupled to microbial sulfate reduction in anoxic lake sediments and the hypolimnion (21), where sulfide concentrations reach up to 5 mmol $liter^{-1}$ (15, 17, 19, 21, 22). The depth of the chemocline increases from winter to summer months (15). In autumn (October and November), surface cooling usually promotes diffusive water column mixing, leading to at least partial oxygenation of the hypolimnion (18). However, in some years (e.g., 1947, 1997, and 2011), meteorological conditions caused a temperature-driven turnover, resulting in complete water layer mixing and anoxia in the entire water column. A milky turbidity caused by the formation of colloidal elemental sulfur (S^0) along with mass mortality of pelagic and benthic eukaryotes during anoxic holomixis have been reported (see references 15 and 19 and references therein). This phenomenon (see Fig. S1 in the supplemental material) gave rise to Lake Rogoznica's vernacular name, "zmajevo oko" ("dragon's eye").

While the diversity of benthic metazoans and zoo- and phytoplankton communities in Lake Rogoznica has been well investigated (e.g., see references 16, 19, 23, and 24), only limited data on the lake's microbial diversity are available (25, 26). Previous studies proposed the cooccurrence of PSB-mediated phototrophic sulfur oxidation (27), chemotrophic sulfur oxidation, and sulfate reduction (21) under stratified conditions in Lake Rogoznica. Moreover, an increase in the abundance of PSB during and after anoxic holomixis in 1997 was reported (25), but no further details

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are available. Therefore, we performed the first thorough characterization of the microbial diversity in Lake Rogoznica under both stratified and mixed conditions. We hypothesized that during stratification, the microbial community in the epilimnion resembles communities in the photic zone of the adjacent Adriatic Sea, with *Synechococcus-Prochlorococcus*-type *Cyanobacteria* and SAR11 clade-related *Alphaproteobacteria* being the dominant bacterioplankton (26, 28, 29). In anoxic waters and underlying sediments, a microbial community with sulfate-reducing potential was predicted based on observed sulfur isotope fractionation values (21). The effect of anoxic holomixis and S^0 accumulation on the diversity and distribution of photo- and chemotrophic sulfur-oxidizing prokaryotes (SOP) in Lake Rogoznica was of particular interest, as this phenomenon has thus far been described only for oceanic redoxclines (e.g., off the coast of Namibia [30]), where phototrophic sulfide oxidation is of no significance.

We investigated microbial diversity and community composition via the 16S rRNA approach, by performing 16S rRNA gene (Sanger and 454 pyrotag) sequencing and catalyzed reporter deposition-*in situ* fluorescence hybridization (CARD-FISH). SOP diversity was further examined by amplification and sequencing of the sulfate thiohydrolase-encoding gene, *soxB*. The physicochemical parameters (water temperature; salinity; pH; and oxygen, reduced sulfur species [RSS], DOC, and particulate organic carbon [POC] concentrations) of Lake Rogoznica were monitored in parallel with microbiological sampling, allowing us to identify links between microbial community composition and the chemical environment.

MATERIALS AND METHODS

Sampling location and sample collection. Lake Rogoznica (43°32′N, 15°58′E) is a small (~10,300-m^2 surface area), 15-m-deep, seasonally stratified seawater lake located between high limestone cliffs on the Dalmatian peninsula Gradina (see Fig. S1 in the supplemental material), at an ~100-m distance from the Adriatic Sea. No riverine input and no surface connection to the open sea are present, but water is exchanged with the Adriatic Sea by subsurface circulation through the underlying porous karst (18). Between June 2011 and May 2012, we undertook eight sampling campaigns, collecting samples for microbial community analysis and chemical and hydrographical characterization. During each sampling campaign, water from six to eight depths (surface [~30 to 50 cm], 5 m, 7 m, 9 m, 10 m, 11 m, 12 m, and 13 m) (see Table S1 in the supplemental material) was collected with horizontally lowered, 5-liter Niskin bottles (General Oceanics, USA). An additional sample was obtained on 30 September 2013 at a 10-m water depth.

Hydrographical and chemical data. A HQ40D multimeter probe (Hach Lange, Germany) was used to record dissolved oxygen (O_2) concentrations and hydrographical data (temperature, salinity, and pH) during sampling. Total RSS concentrations (here defined as compounds that contain sulfur in oxidation states −2 and 0) were determined by using cathodic stripping linear sweep voltammetry (CSLSV) on unfiltered water samples (see reference 22 and references therein). POC (size fraction, >0.7 μm) and DOC (size fraction, <0.7 μm) concentrations were determined by a high-temperature catalytic oxidation (HTCO) method (31) with a TOC-VCPH-5000 solid-sample total organic carbon (TOC) analyzer (Shimadzu, Japan) coupled to an SSM-5000A solid-sample combustion unit (Shimadzu, Japan), according to protocols described previously (32, 33).

DNA isolation. For DNA-based microbial diversity analysis, 120 ml water was filtered onto polycarbonate membrane filters (0.2-μm pore size; Whatman, United Kingdom) immediately after sampling. Filters were stored at −80°C in ~1 ml sucrose buffer (40 mmol $liter^{-1}$ EDTA, 50 mmol $liter^{-1}$ Tris-HCl, and 0.75 mol $liter^{-1}$ sucrose) in 2.2-ml sample tubes (Eppendorf, Germany). DNA was extracted according to a phenol-chloroform procedure (34) and stored at −20°C. Further details are given in the supplemental material.

16S rRNA gene clone libraries. For initial bacterial diversity analysis, 16S rRNA gene amplification, cloning, and Sanger sequencing were performed. Bacterial 16S rRNA genes were amplified with general bacterial primers GM3F and GM4R (35) (see Table S2 in the supplemental material) for samples from three to six water depths retrieved on 20 June 2011, 9 October 2011, and 8 May 2012. Four replicated PCRs with 30 amplification cycles were performed for every sample. PCR and cycling conditions are given in the supplemental material. After amplification, replicate PCR products were pooled and purified with the PureLink PCR purification kit (Invitrogen, USA), according to the manufacturer's instructions, and eluted in 30 μl deionized water. Purified PCR products were cloned by using the pGEM-T vector system (Promega, USA) or the TA cloning kit (Invitrogen, USA) and subcloning-efficiency DH5α competent cells according to the manufacturer's instructions. Randomly selected clones were sent for commercial Sanger sequencing (Macrogen, The Netherlands).

Retrieved sequences were quality trimmed with the Sequencher version 4.6.1 software package (GeneCodes Corporation, USA), and phylogenetic analysis was performed with the ARB software package (36). Sequences were aligned against the SILVA 16S rRNA small-subunit (SSU) reference database (release 115) with the SINA (SILVA Incremental) aligner (37). Nearly full-length 16S rRNA gene sequences from this study and reference sequences from the SILVA database were used for phylogenetic reconstruction with the ARB RAxML maximum likelihood method (38). Partial 16S rRNA gene sequences were added to the tree by using maximum parsimony criteria while disabling changes to the general tree topology. Sequences were grouped into operational taxonomic units (OTUs) based on 98% sequence identity (SI).

16S rRNA gene 454 pyrosequencing. To obtain a more-in-depth picture of 16S rRNA gene diversity, DNA samples from three water depths (surface, 10 m, and 13 m) on three sampling dates (20 June 2011, 9 October 2011, and 8 May 2012) were sent to the MR DNA Molecular Research Laboratory (TX, USA) for amplification (30 amplification cycles) and 454 pyrosequencing of bacterial 16S rRNA genes. Tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed with the Roche 454 FLX Titanium system (39). Retrieved 454 pyrosequence reads were adapter and quality clipped with the "sff_extract-c" option of the COMAV Bioinformatics tool sff_extract (http://bioinf.comav.upv.es/sff_extract) and analyzed with the Next Generation Sequencing pipeline of the SILVA Database Project (40). Further details on gene amplification, 454 pyrosequencing, and pyrosequence analysis are given in the supplemental material. Descriptive analysis statistics are provided in Table S3 in the supplemental material.

Total cell counts and CARD-FISH analyses. Samples designated for microscopic analyses were fixed by the addition of a formaldehyde solution (final concentration, 2%) and subsequent incubation at 4°C for up to 12 h. After fixation, sample aliquots of 10 to 100 ml were filtered onto polycarbonate membrane filters (pore size, 0.2 μm, type GTTP; Millipore, Germany), air dried, and stored at −20°C. Total cell counts (TCC) were determined by epifluorescence microscopy on agarose-embedded filter sections after 4′,6-diamidino-2-phenylindole (DAPI) or SYBR green I staining (41). Abundances of several microbial taxa selected based on 16S rRNA gene diversity results were determined by CARD-FISH on agarose-embedded filters (42). The detection limit for CARD-FISH, determined by the quantification of hybridization signals with the Non388 probe (false-positive signals), was 0.25%. Applied oligonucleotide probes are listed in Table S4 in the supplemental material.

Oligonucleotide probe design. A specific oligonucleotide probe targeting the gammaproteobacterial sulfur oxidizers related to the GSO/SUP05 clade present in Lake Rogoznica (see Fig. S2 in the supplemental material) was designed based on 16S rRNA gene clone sequences and pyrosequences with the help of the ARB software package (36). The new

probe was named S-*-GSO-LR-0183-a-A-20 (abbreviated GSO-LR183) (see Table S4 in the supplemental material) according to guidelines reported previously (43). The specificity of the probe was evaluated with the ARB PROBE_MATCH tool. The expected half-dissociation temperature was calculated *in silico* with mathFISH (44) and evaluated by *in situ* hybridization at 46°C over a formamide range of 25 to 40% with environmental samples from Lake Rogoznica. The optimal formamide concentration for hybridization at 46°C was determined to be 30%.

Sorting, identification, and analysis of PSB cells. For definite phylogenetic identification and characterization of large PSB cells, an additional sample from the chemocline was collected on 30 September 2013. The sample was preserved by the addition of glycerol (12% final concentration), to avoid cell damage and lysis upon freezing, and stored at −20°C.

For 16S rRNA gene amplification and sequencing, PSB cells were sorted from a DAPI-stained aliquot of the glycerol-preserved chemocline sample with a MoFlo flow cytometer (Beckman Coulter GmbH, Germany) based on size, pigmentation, and DAPI staining, as described in the supplemental material. 16S rRNA gene amplification was performed with 30 cycles in three replicate PCRs, with each mixture containing ~100 sorted cells. The purified PCR product (PureLink PCR purification kit; Invitrogen, USA) was used as the direct template for Sanger sequencing with a Sequencer 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Obtained sequences were quality checked, and a consensus sequence was generated with the Sequencher version 4.6.1 software package (GeneCodes Corporation, USA).

To determine the nature of inclusions in PSB cells, Raman spectroscopy and Nile Red staining were applied. For Raman spectroscopy, aliquots of the glycerol-preserved chemocline sample were mounted between two glass coverslips and sealed with electrical tape to prevent drying. Measurements were carried out with an Ntegra Spectra confocal spectrometer (NT-MDT, Zelenograd, Russian Federation) coupled to an inverted Olympus IX71 microscope. The spatial resolution of the measurements was 250 to 300 μm, and Raman spectra were recorded between 0 and 4,500 cm^{-1} with a spectral resolution of 0.2 cm^{-1} (described in more detail in the supplemental material). Full-spectrum maps of the measured cells were taken at 1-μm *z*-intervals. Spectra were analyzed by using Nova_Px 3.1.0.0 software (NT-MDT, Zelenograd, Russian Federation). For easier comparison, all spectra were normalized to an arbitrary unit of intensity on the *y* axis, and noisy spectra were smoothed by using a Gaussian function with a tau value of 3. Raman maps were first background corrected and brightness/contrast adjusted, and *z*-scans were then assembled by autoaligning and autoblending in Adobe Photoshop CS2. Analytical-grade (purity, 99%) elemental sulfur (Roth GmbH) was used as a standard.

For Nile Red staining and detection of lipophilic inclusions, an aliquot of the glycerol-preserved sample was mixed 1:1 with a Nile Red solution (1 μl Nile Red stock solution [1 mg ml^{-1} of Nile Red in dimethyl sulfoxide {DMSO}] in 200 μl phosphate-buffered saline [PBS]) and mounted onto a glass slide. Stained cells were visualized under a 1,000-fold magnification on an AxioSkop 2 Mot Plus epifluorescence microscope equipped with a fluorescein isothiocyanate (FITC) filter set (Carl Zeiss, Germany).

***soxB* gene diversity.** Diversity of the sulfur oxidation marker gene *soxB*, encoding sulfate thiohydrolase, was investigated by gene amplification, cloning, and sequencing. *soxB* gene sequences were successfully obtained from surface samples obtained on 20 June 2011 and 9 October 2011 and from a 10-m sample obtained on 9 October 2011. Gene amplification was performed by two-step PCR (45) with gene-specific primers soxB432F and soxB1446R (see Table S2 in the supplemental material) in a total of 35 amplification cycles, as described previously (45) (see methods in the supplemental material). PCR products were purified by the excision of desired bands (~1,000 bp) from a SYBR green I (Invitrogen, Germany)-stained 1% agarose gel. Gel-purified PCR products were cloned with the TOPO TA cloning kit (Invitrogen, Germany), according to the manufacturer's instructions, and vector inserts were Sanger sequenced by using a Sequencer 3130xl genetic analyzer (Applied Biosystems, USA). Again, the Sequencher version 4.6.1 software package was used for quality trimming and generation of consensus sequences.

About 500 publically available, full-length *soxB* gene sequences from isolates and environmental samples were aligned with the Fourier transformation-based multiple-sequence alignment tool MAFFT v6.864 (46) (http://www.ebi.ac.uk/Tools/msa/mafft/) and used for reconstruction of a phylogenetic guide tree with the ARB RAxML maximum likelihood method (38). Sequences generated in this study were added to the guide tree by using maximum parsimony criteria, without allowing changes to the overall tree topology (see Fig. S3 in the supplemental material). *soxB* gene nucleotide sequences were grouped into OTUs based on a 94% similarity cutoff, as inferred from previous studies of *soxB* gene sequence divergence in closely related strains (45, 47).

Nucleotide sequence accession numbers. Nucleotide sequences from this study have been deposited in the NCBI nucleotide database under GenBank accession numbers KM270117 to KM270490. The 16S rRNA gene pyrotag sequences have been deposited in the ENA Sequence Read Archive under study accession number PRJEB6873/ERP006515.

RESULTS

Chemical and hydrographical characteristics of Lake Rogoznica. In parallel with microbiological samplings between June 2011 and May 2012, we measured basic hydrographic parameters (salinity, temperature, and pH) and POC, DOC, RSS, and O_2 concentrations in the water column of Lake Rogoznica (Fig. 1; see also Table S5 in the supplemental material). Hydrographical profiles as well as dissolved O_2, RSS, and organic carbon concentrations were well in line with previously reported values from the respective seasons in the 1990s and 2000s (15, 21, 22). Water column stratification was stable throughout the spring and summer of 2011. Water layer mixing, resulting in the formation of a chemically homogeneous water column, took place in early October 2011. Holomixis lasted until mid-November, when stratification was reestablished and persisted until the end of our sampling series in May 2012 (Fig. 1). As in previous years, the depth of the chemocline migrated with changing seasons (15). It was located close to the lake bottom (below 8 m) during summer months (June 2011 to September 2011 and May 2012) and reestablished at shallower depths (4 to 7 m) after holomixis in October 2011, where it remained during the winter and spring months (November 2011 to March 2012).

During stratified periods, concentrations of RSS, present mainly in the form of sulfide (15, 18, 21, 22), were highest in the anoxic hypolimnion and higher during the summer than during the winter and spring months (Fig. 1; see also Table S5 in the supplemental material). The highest total RSS concentration was 3.75 mmol $liter^{-1}$, recorded on 1 September 2011 at a 13-m depth (see Table S5 in the supplemental material). Above the chemocline, where RSS are present mainly in the form of organosulfur compounds and elemental sulfur (15, 18, 21, 22), RSS concentrations were at the detection limit. O_2 concentrations increased above the chemocline, and O_2 saturation at the surface was at or above 100% at all times during stratification (data not shown). In line with data from previous reports (15, 17, 26), DOC and POC concentrations were generally higher at and below the chemocline, with POC concentrations peaking at 0.5 to 1 m below the chemocline depth (Fig. 1). After holomixis in October 2011, oxygen was almost depleted (0.02 to 0.13 mg $liter^{-1}$) throughout the water column, with microoxic conditions prevailing only at the surface (~1 m). During this period, RSS were present in the form of elemental sulfur (15, 16, 22), with low (0.02 to 0.40 μmol li-

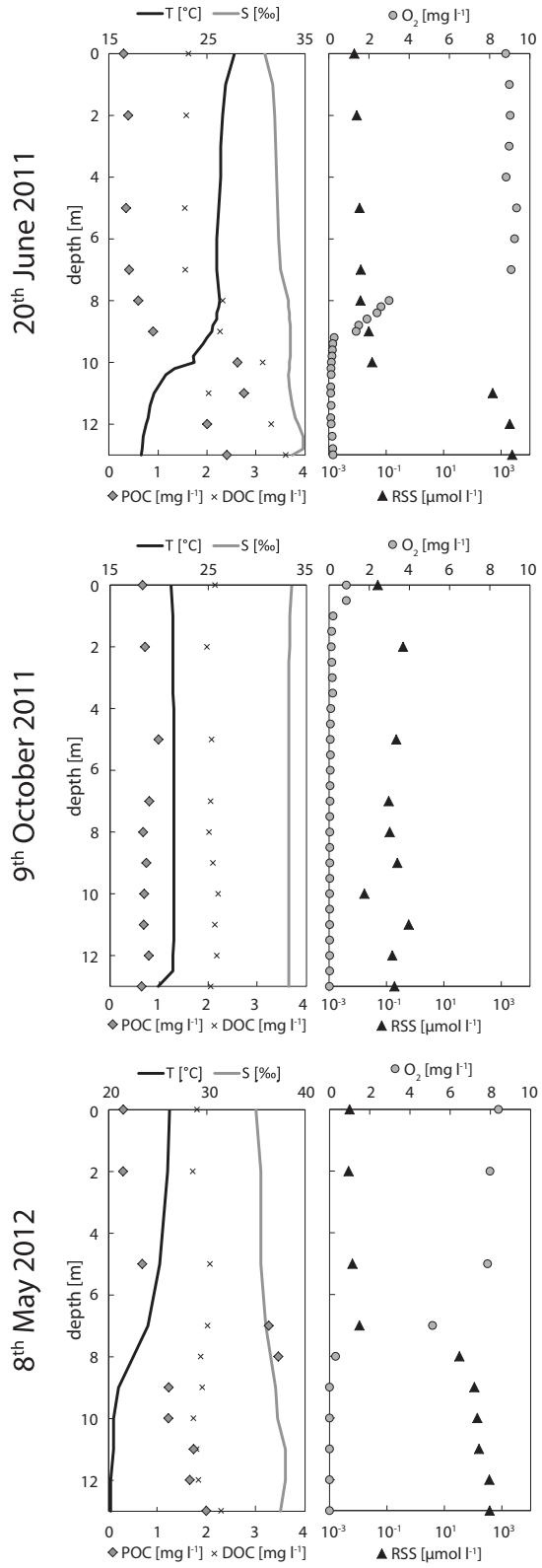

FIG 1 Hydrographical and chemical profiles of Lake Rogoznica's water column on 20 June 2011, 9 October 2011, and 8 May 2012. (Left) Temperature (T) (°C) and salinity (S) (‰) (top *x* axis) data and particulate organic carbon (POC) concentration (mg liter^{-1}) (bottom *x* axis). (Right) Dissolved oxygen (O_2) concentration (mg liter^{-1}) (top *x* axis) and total reduced sulfur species (RSS) concentration (μmol liter^{-1}) (bottom *x* axis) on a logarithmic scale.

ter^{-1}) but detectable concentrations in the entire water column (Fig. 1). DOC and POC concentrations varied only slightly with depth (Fig. 1).

Microbial diversity in Lake Rogoznica. To obtain insights into the microbial diversity of Lake Rogoznica, we constructed bacterial 16S rRNA gene clone libraries from water samples retrieved at several depths on 20 June 2011, 9 October 2011 (anoxic holomixis), and 8 May 2012. A total of 374 sequences from 15 samples were retrieved.

In epilimnion samples (0- to 7-m depth in June 2011 and May 2012), SAR11 clade-related *Alphaproteobacteria* were the most frequently retrieved sequence type. The remaining sequences from epilimnion samples were affiliated with diverse *Alphaproteobacteria*, *Bacteroidetes*, and *Cyanobacteria* (Fig. 2). In samples from the chemocline and the hypolimnion from June 2011 and May 2012, sequences related to uncultured GSB (phylum *Chlorobi*) were most numerous (up to 97%). Phylogenetic reconstruction revealed that the majority of GSB sequences (114 out of 118) were closely related to *Prosthecochloris vibrioformis* (87 to 96% SI) (Fig. 2). Although PSB (*Chromatiales*) were previously described as the dominant phototrophic sulfur oxidizers in Lake Rogoznica (27), only three *Chromatiales*-related sequences were obtained from chemocline and hypolimnion samples. They formed a single OTU closely related to *Halochromatium roseum* (99 to 100% SI) (48) (Fig. 2). In samples from all depths collected during anoxic holomixis (9 October 2011), sequences related to the GSO/SUP05 clade of *Gammaproteobacteria* were most frequently retrieved (86%) (Fig. 2). Furthermore, sequences related to other thiotrophic *Gammaproteobacteria* and *Epsilonproteobacteria* were recovered (Fig. 2).

Based on the 16S rRNA gene clone library results, we selected nine representative samples from the epilimnion (surface samples from 20 June 2011 and 8 May 2012), the chemocline (samples from 20 June 2011 and 8 May 2012 at a 10-m depth), the hypolimnion (samples from 20 June 2011 and 8 May 2012 at a 13-m depth), and the anoxic holomictic event (samples from 9 October 2011 at surface and 10-m and 13-m depths) for 454 pyrosequencing. The significantly increased amount of sequence data (154,152 reads) obtained by this high-throughput method provided us with a more complete overview of the diversity within and between the distinct microbial communities present in Lake Rogoznica.

The epilimnion sample from 8 May 2012 was dominated by SAR11 clade-related 16S rRNA gene pyrosequences (85%). In contrast, SAR11 clade-related reads accounted for only 37% of the pyrosequences retrieved in the epilimnion sample from 20 June 2011, where reads affiliated with *Roseobacter* clade *Alphaproteobacteria* (6%), *Synechococcus*-related *Cyanobacteria* (18%), *Flavobacteriaceae*-related *Bacteroidetes* (7%), and algal chloroplasts (9%) were also frequently detected (Fig. 3). Pyrosequence diversity in both chemocline samples (20 June 2011 and 8 May 2012 at a 10-m depth) was low, and the majority of retrieved sequences (81% and 96%, respectively) were related to GSB (Fig. 3). GSB sequences (88%) were almost exclusively retrieved from the hypolimnion sample from 8 May 2012, while the pyrosequences retrieved from the hypolimnion sample from 20 June 2011 were very diverse (Fig. 3). Reads related to SAR11 clade *Alphaproteobacteria* (29%), thiotrophic *Epsilonproteobacteria* (*Arcobacter*, *Sulfurimonas*, and *Sulfurovum*) (16%), and diverse *Bacteroidetes* (8%) were frequently detected. In all four chemocline and hypolimnion samples, very few reads related to the *Halochromatiaceae* detected in

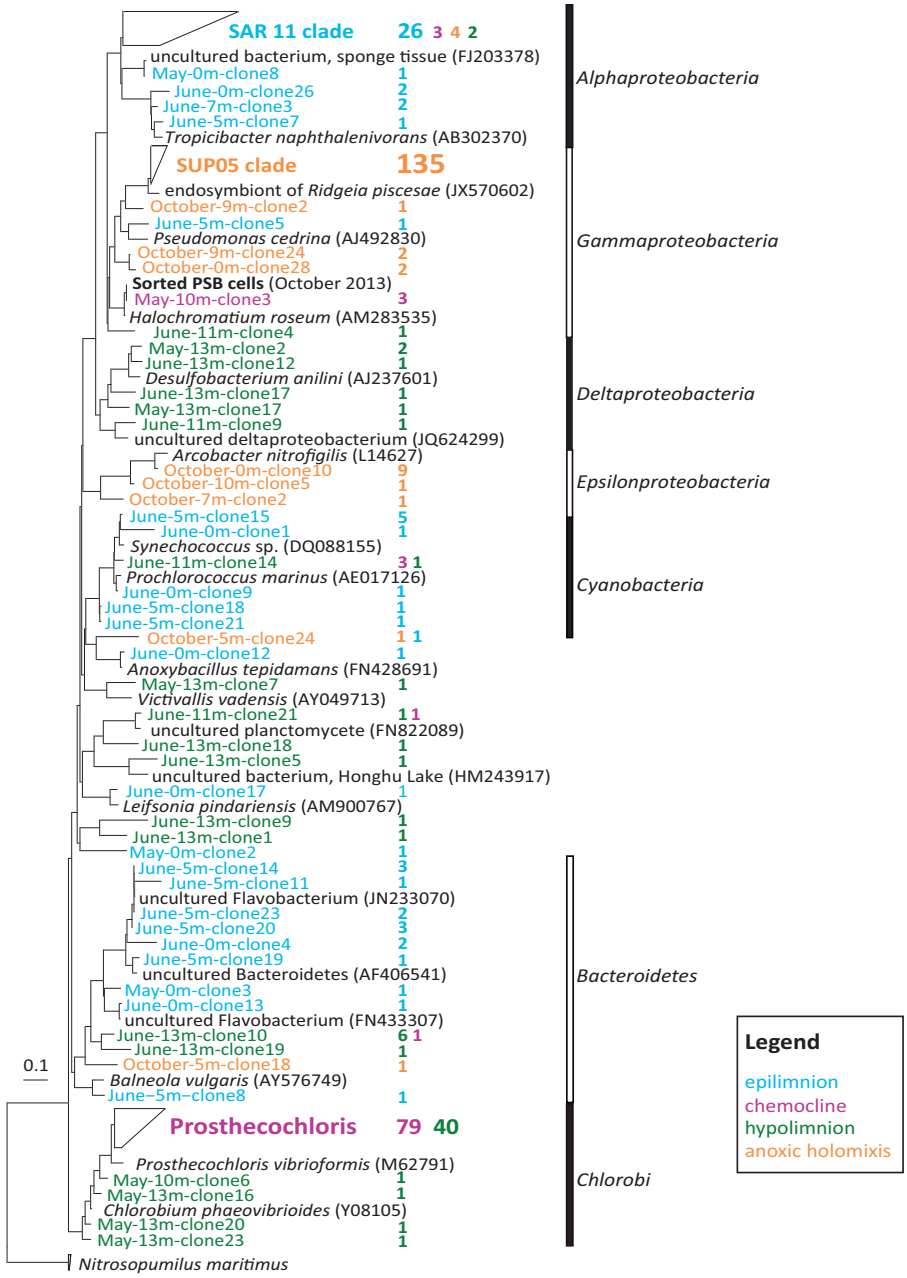

FIG 2 Phylogenetic tree illustrating 16S rRNA gene diversity in clone libraries from samples collected from the epilimnion, chemocline, and hypolimnion on 20 June 2011 and 8 May 2012 and during anoxic holomixis on 9 October 2011. Each OTU (grouped at 98% SI) is represented by one selected sequence, while the number and origin of the clone in one OTU are depicted next to the representative sequence. GenBank accession numbers are shown in parentheses.

the clone libraries or other PSB (<1%) were retrieved. In samples from the anoxic holomixis (9 October 2011), pyrosequences affiliated with the GSO/SUP05 clade-related *Gammaproteobacteria* made up the majority of retrieved pyrosequences at all water depths (57 to 87%). The remaining 16S rRNA gene pyrosequence reads were affiliated mainly with SAR11 and *Roseobacter* clade *Alphaproteobacteria* (14 to 17%), *Cyanobacteria* (3 to 4%), *Epsilonproteobacteria* (4%), and *Bacteroidetes* (3 to 4%) (Fig. 3).

Bacterial abundance in Lake Rogoznica through an annual cycle. After identifying the dominant bacterial taxa in representative water layers and seasons in Lake Rogoznica by 16S rRNA gene sequencing, we identified and quantified microorganisms by total cell counts (TCC) and CARD-FISH in circa monthly samples for the sampling period between June 2011 and May 2012 (Fig. 4; see also Table S6 in the supplemental material).

During stratified periods, TCC ranged between 2×10^6 and 9×10^6 cells ml^{-1}. The TCC was lowest at the lake's surface and increased with depth, peaking below the chemocline along with the POC concentration (Fig. 1). SAR11 and *Roseobacter* clade *Alphaproteobacteria* represented the largest fraction of cells in epilimnion waters throughout the period of stratification (up to 54% of all cells on 8 May 2012) (see Table S6 in the supplemental

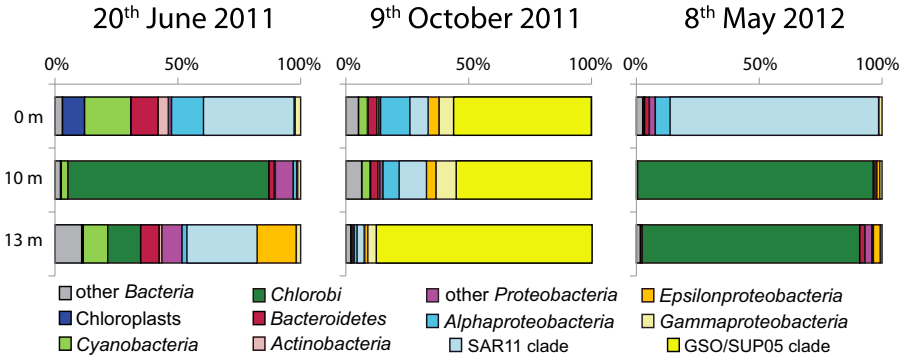

FIG 3 Diversity of 16S rRNA genes in 454 pyrotags from different water layers sampled on 20 June 2011, 9 October 2011, and 8 May 2012.

material), followed in abundance by *Bacteroidetes* (up to 16%) and *Gammaproteobacteria* (up to 14%) (see Table S6 in the supplemental material). *Cyanobacteria* were abundant in epilimnion waters during the spring and summer months (March to September), where they accounted for 7 to 24% of all cells (Fig. 4), while almost no *Cyanobacteria* were detected during winter (see Table S6 in the supplemental material). At the chemocline and in the hypolimnion, GSB (*Chlorobi*) accounted for the great majority (up to 89%) of detected cells. These small, rod-shaped cells (~3 by 1 μm) formed long, nonbranching chains, as has been observed for many *Chlorobi* and *Prosthecochloris* species (49). There were comparatively few PSB (1 to 3%) (Fig. 4; see also Table S6 in the supplemental material), but individual, spherical cells (~5 μm in diameter) had an ~28-times-higher volume than GSB cells and therefore accounted for a large fraction of the biovolume (27 to 83%) in some samples. Besides phototrophic SOP, *Deltaproteobacteria* and *Bacteroidetes* were abundant in hypolimnion samples, while numbers of alpha- and gammaproteobacterial cells were generally low (see Table S6 in the supplemental material).

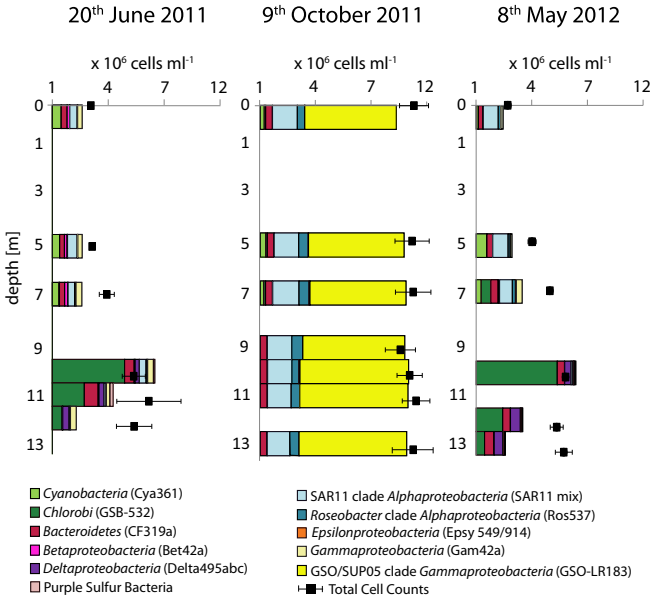

FIG 4 Total cell counts (TCC) and abundance of different bacterial taxa in cells ml^{-1} in samples retrieved on 20 June 2011, 9 October 2011, and 8 May 2012, as determined by CARD-FISH. The oligonucleotide probe used for hybridization is given in parentheses next to the taxon name.

During total anoxia, TCC were comparable at all water depths (~1×10^7 cells ml^{-1}) and 1.2- to 4.9-fold higher than TCC in any water layer during stratification (Fig. 1). The distribution of different bacterial taxa during anoxic holomixis was uniform throughout all water depths (Fig. 4). Numbers of *Cyanobacteria*, *Deltaproteobacteria*, and *Betaproteobacteria* decreased in comparison to their absolute abundances during stratification. No GSB or PSB cells were detected at any water depth during anoxic holomixis. SAR11 and *Roseobacter* clade *Alphaproteobacteria* and *Bacteroidetes* were evenly distributed throughout the water column and showed a slightly increased abundance compared to abundances under stratified conditions. A strong increase in absolute cell numbers throughout the water column was observed only for *Gammaproteobacteria* (Fig. 4).

The GSO/SUP05 clade-related *Gammaproteobacteria* were likely undercovered by the phylum-specific 23S rRNA-targeting oligonucleotide probe Gam42a, which does not hybridize with all members of the GSO/SUP05 clade, as previously reported by Glaubitz and colleagues (50). Two central mismatches at the target position of the Gam42a probe were detected in a reconstructed 23S rRNA gene sequence from a SUP05 metagenome and the 23S rRNA gene sequence from the closely related endosymbiotic bacterium "*Candidatus* Ruthia magnifica." Multiple oligonucleotide probes targeting specific populations within the GSO/SUP05 cluster have been reported (e.g., see references 30 and 50). *In silico* analyses revealed that the oligonucleotide probes reported previously by Glaubitz and colleagues (50) do not target the majority of the GSO/SUP05 clade-related sequences retrieved from Lake Rogoznica (see Fig. S2 in the supplemental material), while *in situ* hybridizations with probe GSO477 (30) yielded no results. Therefore, a new 16S rRNA-targeting oligonucleotide probe (GSO-LR183) was designed, specifically targeting a cluster with 87% 16S rRNA sequence identity, which harbors the majority (70%) of GSO/SUP05 clade-related sequences obtained in this study (see Fig. S2 in the supplemental material). In all samples collected during anoxic holomixis (9 October 2011), >58% of cells hybridized with the new GSO-LR183 probe, confirming the dominance of GSO/SUP05 clade-related *Gammaproteobacteria* (Fig. 4). Also, in samples collected shortly after reestablishment of stratification on 7 November 2011, up to 36% of cells hybridized with the GSO-LR183 probe. In contrast, no GSO-LR183-positive cells were de-

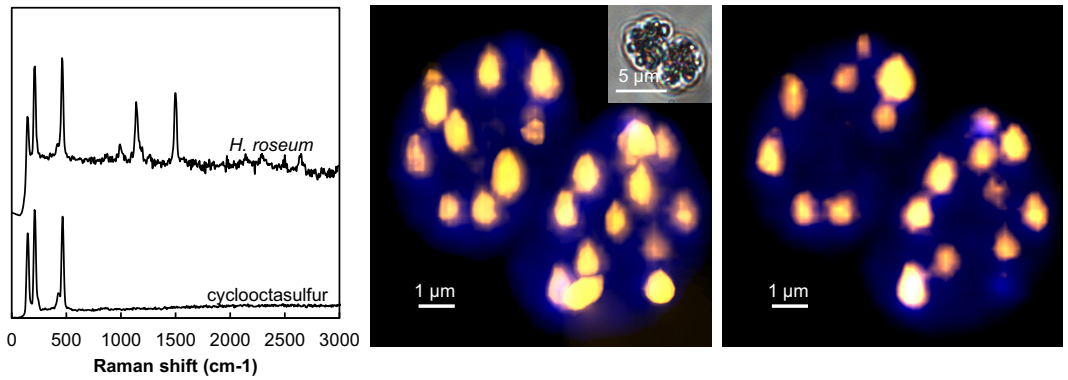

FIG 5 (Left) Raman spectrum of a sulfur inclusion in a *Halochromatium* cell with characteristic S^0 peaks at 186, 219, and 473 cm^{-1} along with carotenoid peaks at 1,008, 1,157, and 1,535 cm^{-1} originating from membrane-associated photopigments, compared to a cyclooctasulfur (S_8) standard. (Middle) Accumulated full-spectrum Raman maps recorded at 1-μm-depth intervals with 0.19-μm spatial resolution showing S^0 inside two *Halochromatium* cells and the corresponding optical microscope image. (Right) Cross section of the *Halochromatium* cell showing S^0 localized along the cell membrane and a hollow interior. Sulfur is shown in yellow, and background fluorescence of the cells is shown in blue.

tected in samples obtained prior to anoxic holomixis (1 September 2011) or after stratification was fully reestablished (13 December 2011).

Identification and investigation of PSB cells. While only a few PSB 16S rRNA sequences were retrieved in 16S rRNA gene libraries and 454 pyrotag sequences, the large cells comprised a very large fraction (up to 83%) of the biovolume in anoxic waters. To perform a definite phylogenetic identification, we amplified and sequenced the 16S rRNA genes from sorted PSB cells collected during a sampling trip on 30 September 2013. The obtained consensus 16S rRNA gene sequence was >99% identical to sequences from our clone libraries and the type strain of *Halochromatium roseum* (Fig. 2). Unlike the species description of *Halochromatium roseum*, no cell chains were observed in our samples, and individual cells did not appear rod shaped but appeared spherical. These PSB cells contained a large number of refractive inclusions. Using confocal Raman spectroscopy, we identified these inclusions as being elemental sulfur (S^0) (Fig. 5). Furthermore, full-spectrum Raman scans of single PSB cells resolved individual granules of S^0 stored only along the cell periphery (Fig. 5). The high confocality (~250 nm) of the Raman microscope allowed mapping of the occurrence of the S^0 peak at 473 cm^{-1} with respect to the background fluorescence of the cell in several different depth planes. In a cross section of the cell (Fig. 5), sulfur granules appeared localized close to the cell membrane surrounding a hollow interior. We speculate that the cells might contain a gas vacuole, as has been described for the type strain of *Halochromatium roseum* (48). Although many species of *Chromatiaceae*, including another *Halochromatium* species (51), are known to also store organic carbon compounds, no characteristic peaks of the known carbon storage compounds glycogen and polyhydroxybutyrate (PHB) (52, 53) were detected in the investigated sample by Raman spectroscopy under the applied measuring conditions. Additional experiments with Nile Red staining confirmed the absence of lipophilic carbon storage compounds in PSB cells sampled on 30 September 2013.

***soxB* gene diversity.** To confirm the sulfur oxidation potential of microorganisms other than GSB and PSB in Lake Rogoznica, we amplified and sequenced *soxB* genes from a sample collected in the epilimnion during stratification (surface sample from 20 June 2011) and from two samples collected during anoxic holomixis (surface and 10-m samples from 9 October 2011). In total, 266 *soxB* gene sequences were retrieved and grouped into 27 OTUs based on a 94% sequence identity cutoff.

The majority of *soxB* gene sequences retrieved from the epilimnion sample and several sequences from anoxic holomixis samples were affiliated with diverse uncultured and cultured *Alphaproteobacteria*, more specifically with environmental sequences of *Roseobacter* clade relatives retrieved from sulfidic coastal sediments (54) (see Fig. S3 in the supplemental material). In contrast, the majority (58%) of *soxB* gene sequences retrieved from anoxic holomixis samples were closely related to sequences of uncultured GSO/SUP05 clade *Gammaproteobacteria* and the sulfur-oxidizing clam endosymbiont "*Candidatus* Vesicomyosocius okutanii" (see Fig. S3 in the supplemental material), while no *soxB* gene sequences from the epilimnion sample were affiliated with this group. It is important to note that the lack of epsilonproteobacterial *soxB* gene sequences is possibly related to a primer mismatch between the epsilonproteobacterial *soxB* gene target site and the 3′ end of the applied primers, which significantly affects PCR efficiency (47).

DISCUSSION

Patterns of microbial diversity in Lake Rogoznica relate well to the recorded hydrographical and chemical conditions of the stratified water column throughout the whole sampling period (March 2011 to May 2012). For most of the sampling dates, the microbial communities in Lake Rogoznica can be divided into epilimnion and chemocline/hypolimnion populations.

As expected, the microbial diversity in the epilimnion resembled the diversity of the central Adriatic Sea, with *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* being the most abundant bacterial groups (28, 29). The abundance of *Roseobacter* clade *Alphaproteobacteria* and the retrieval of *soxB* genes from these organisms indicate that RSS oxidation might occur in the oxic epilimnion.

The chemocline and hypolimnion populations were dominated by anoxygenic phototrophic SOP (*Prosthecochloris* and *Halochromatium*) (48, 55). In contrast to previous reports of abundant PSB at the chemocline (27) and regardless of the high

biovolume of *Halochromatium*-related PSB in some samples, GSB (*Chlorobi*) were the most abundant anoxygenic phototrophs in Lake Rogoznica during stratified periods in 2011 and 2012. This might reflect a temporal shift, as anoxygenic phototrophic SOPs annually diminish and reestablish during and after holomixis. However, the abundance of GSB in the chemocline sample collected on 30 September 2013 (data not shown) points toward a generally high abundance of GSB in Lake Rogoznica. It is possible that the previous description of PSB dominance, based on microscopy and water column coloration (25), was biased toward the ~28-times-larger cells of the *Halochromatium*-related PSB. Regardless, anoxygenic phototrophic SOP account for the bulk of the bacterial biomass at and below the chemocline. The GSB dominated in numbers and total biovolume (10^6 to 10^7 μm^3 ml^{-1}). However, single *Halochromatium*-related PSB cells were significantly larger and contained abundant S^0 inclusions (Fig. 5), reflecting sulfide oxidation activity. PSB can oxidize stored S^0 under microoxic, dark conditions or reduce it with organic carbon compounds to gain energy (53). These processes may provide PSB with advantages in competition with the obligatory photolithotrophic GSB (49). Although no organic carbon storage compounds were found by Raman spectroscopy or Nile Red staining in the investigated *Halochromatium* cells, it cannot be excluded that such storage compounds can accumulate under certain conditions or that external organic carbon sources are utilized for S^0 reduction. Such a metabolic flexibility would allow *Halochromatium* to maintain activity under the fluctuating oxygen, sulfide, and light conditions occurring in Lake Rogoznica, where chemocline localization can change up to 1 m in depth between day and night (I. Ciglenečki, unpublished data). In addition, a central gas vesicle may be used by these nonmotile bacteria to adjust buoyancy during S^0 accumulation or reduction (56) and position themselves with respect to sulfide, oxygen, and/or light gradients in the stratified lake. It is known from other studies that even numerically small populations of anoxygenic phototrophic SOP that account for a significant part, if not the majority, of the total biovolume can be responsible for the majority of the overall phototrophic CO_2 fixation in stratified lakes (57, 58). However, no conclusions on the relative contribution of GSB versus PSB to overall phototrophic sulfide oxidation or carbon fixation can be made based solely on their abundance, biovolume, or cell structure.

The abundance and diversity of *Deltaproteobacteria* in hypolimnion waters support findings reported previously by Kamyshny and colleagues (21), suggesting that microbial sulfate reduction in the hypolimnion and lake sediments is the principal sulfide source in Lake Rogoznica. Interestingly, the abundance of *Deltaproteobacteria* in the hypolimnion is comparable throughout seasons (see Table S6 in the supplemental material), while RSS concentrations are increased during the summer months (see Table S5 in the supplemental material). Higher water temperatures, higher productivity in the epilimnion, and greater export of cells and organic matter from the surface (15, 20) could facilitate increased sulfate reduction rates in the anoxic bottom layers and sediments during the summer months. However, epilimnion production, organic matter export to the hypolimnion, and sulfate reduction rates in anoxic sediments and waters remain to be quantified to test this hypothesis.

Community shift from phototrophic to chemotrophic sulfur oxidation during anoxic holomixis. Anoxic holomixis in October 2011 led to a drastic community shift in Lake Rogoznica. Community shifts during holomixis have been reported for other stratified lakes (10, 59). Most lakes are oxygenated during holomixis, and an epilimnion-like community is usually observed (59–61). However, during a previous anoxic holomixis in Lake Rogoznica (25) and in a similar karst lake in Spain (Lake Cìso) (7), a dominance of phototrophic SOP was reported. In contrast to those results, we detected a bloom of chemotrophic GSO/SUP05 clade-related *Gammaproteobacteria* during anoxic holomixis in October 2011. These microorganisms were not detected in any water layer during stratification. Furthermore, a complete collapse of the anoxygenic phototrophic SOP community was observed.

Members of the GSO/SUP05 clade-related *Gammaproteobacteria* are involved in chemotrophic sulfur oxidation in diverse marine systems with chemical conditions similar to those reported for Lake Rogoznica during anoxic holomixis. Such systems include anoxic fjords and inlets (62, 63), pelagic redoxclines and oxygen-minimum zones (30, 50, 64), hydrothermal plumes (65), low-temperature hydrothermal fluids (66), and Antarctic lakes (67). This is the first report of the abundance of this marine clade in a stratified seawater lake from a temperate region. The factors favoring the sudden bloom of GSO/SUP05 clade-related SOP in Lake Rogoznica and causing the disappearance of anoxygenic phototrophic SOP during anoxic holomixis are not entirely clear. It is possible that the relatively low RSS concentrations (~1 μmol $liter^{-1}$) after holomixis were insufficient to sustain the large GSB and PSB populations. Moreover, the formation of colloidal S^0 in the lake's water column after perturbation might have hampered light penetration and photosynthesis. Various chemotrophs could have profited from the disappearance of anoxygenic phototrophic SOP. A higher affinity for sulfide might be the key advantage for GSO/SUP05 clade-related *Gammaproteobacteria* in competition with *Epsilonproteobacteria*, which are more common in high-sulfide/high-flux environments (68, 69). Furthermore, the GSO/SUP05 clade is metabolically versatile, with the capacity to utilize multiple electron donors and perhaps even organic carbon sources (70–72). GSO/SUP05 clade-related *Gammaproteobacteria* are frequently found in microoxic environments (30, 50, 62–67), and sulfide oxidation under microaerobic as well as denitrifying conditions is possible (72). Taking into account the relatively high DOC and POC concentrations in Lake Rogoznica, the ability to utilize organic carbon compounds could also have served members of the GSO/SUP05 clade as a competitive advantage over other chemotrophic SOP.

Conclusion. This study represents the first comprehensive report on microbial diversity in Lake Rogoznica and includes the quantification of bacterial populations under stratified and anoxic holomictic conditions. Microbial diversity and water column chemistry are tightly coupled. Microorganisms are largely responsible for both oxygen and sulfide production and consumption in Lake Rogoznica, while water temperature, light availability, and temperature-driven turnover affect microbial activity and distribution. The shift from predominantly phototrophic sulfur oxidation to almost exclusively chemotrophic sulfur oxidation and the bloom of GSO/SUP05 clade-related *Gammaproteobacteria* in response to anoxic holomixis are described here for the first time. The similarities of chemotrophic SOP communities in Lake Rogoznica to those in oxygen-deficient pelagic waters and mildly sulfidic hydrothermal

environments underline the habitat-independent community structuring effect of sulfide concentrations.

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