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Green sulphur bacteria as a component of the photosynthetic plankton community in small dimictic humic lakes with an anoxic hypolimnion

Jatta Karhunen[1,*], Lauri Arvola[2], Sari Peura[1,3], Marja Tiirola[1]

[1]Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland
[2]Lammi Biological Station, University of Helsinki, Pääjärventie 320, 16900 Lammi, Finland
[3]Department of Microbiology and Immunology, University of Michigan, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109, USA

ABSTRACT: High bacteriochlorophyll (BChl) concentrations in the anoxic water layers of some humic lakes have indicated that green sulphur bacteria (GSB) may be ecologically significant. The abundance and spatial distribution of GSB were therefore addressed in 13 small humic lakes using fragment analysis and sequencing of PCR-amplified 16S rRNA genes. GSB were detected from lakes where the photosynthetically active radiation was at least 1.1 µE m^{-2} s^{-1} at the oxic–anoxic boundary layer. In these lakes, 13 to 42% of the 16S rRNA gene sequences of the anoxic water column were assigned to GSB. The spatial distribution of GSB was tightly correlated with the spectrophotometrically measured BChl concentration during the summer season. Maximum BChl concentrations were observed in the uppermost part of the anoxic water layer, covering most of the chlorophyll pigment in these lakes. The GSB of the humic lakes typically belonged to a phylogenetically homogenous group closely related to *Chlorobium clathratiforme*.

KEY WORDS: *Chlorobium* sp. · LH-PCR · Green sulphur bacteria · Humic lake · Boreal lake

INTRODUCTION

The anaerobic microbiology of small humic lakes in the boreal zone is still largely unexplored. The few microscopic (e.g. Baker et al. 1985, Arvola et al. 1992) and molecular (Taipale et al. 2009, 2011, Peura et al. 2012) studies suggest that the microbial communities in anoxic layers of these lakes are dominated by bacteria, which are distinct from bacteria typical for epilimnetic communities, such as green sulphur bacteria (GSB) and candidate phylum OD1. GSB are strictly anaerobic and obligatory phototrophic; thus, they require the presence of reduced sulphur compounds and the availability of light for growth (Van Gemerden & Mas 1995). Such habitat is provided by small humic lakes, where a combination of dark water colour and infrequent vernal mixing guarantees an optimal light and oxygen environment for these organisms.

GSB have a special light-capturing capability, which is based on very efficient light-harvesting pigments: bacteriochlorophylls (BChl) *c*, *d* or *e* together with BChl *a* and carotenoids (Olson 1998). Studies on the chlorophyll contents of photosynthetic microbial groups have shown that BChl types typical for GSB or multicellular filamentous green bacteria (MFGB) were abundant in most of the studied 24 North American kettle lakes (Vila et al. 1998), and in most of the 11 Spanish karstic dolines and coastal lagoons (Guerrero et al. 1987). As stated above, the environment in humic lakes also seemingly provides optimal growth conditions for GSB, but their prevalence in these lakes remains to be addressed. Further, analysis through chlorophyll content requires high-perfor-

*Email: jatta.karhunen@jyu.fi

mance liquid chromatography (HPLC) analysis and should also consider the phenotypical variation of the pigment types and their contents in different GSB species. We opted to use molecular methods based on the marker genes found only in the organisms in question.

Chlorobium, which belongs to green subset of the GSB, was previously found to constitute an important carbon source for the lake food web in the small boreal humic lake Mekkojärvi, and it was the dominating species in the bacterial community (Taipale et al. 2009). The present study aimed to discover whether this is true for humic lakes in general. The survey covered 13 steeply stratified small boreal lakes that vary in terms of humic matter concentration, thermal and chemical stratification, as well as in the availability of photosynthetically active radiation (PAR) in their water columns. The abundance of GSB was studied using length heterogeneity analysis (LH-PCR; Suzuki et al. 1998) of polymerase chain reaction (PCR)-amplified 16S rRNA genes, and we sequenced several amplicons to study their phylogenetic affiliation. The study was supported by BChl analysis, which was performed during the open water season and under the ice.

MATERIALS AND METHODS

The study lakes were located in the Evo forest area in southern Finland (an area of 48 km^2 61° 10′ to 61° 13′ N and 25° 5′ to 25° 12′ E). The lakes have an ice cover from approximately early November until the beginning of May. Spring mixing can be incomplete, but the autumnal overturn happens more regularly in many of the lakes in the area (Salonen et al. 1984). The study lakes were stratified with regard to the availability of light, temperature, oxygen and nutrients (Table 1). Each lake was sampled 1 to 4 times in 2009. Seasonal changes in the abundance of GSB were followed in lakes Alinen Mustajärvi, Halsjärvi and Mekkojärvi, and GSB communities under ice were studied in Alinen Mustajärvi, Halsjärvi, Nimetön and Horkkajärvi.

Water samples were taken with a Limnos water sampler (30 cm height, 2.1 l volume), at 0.5 m intervals from the oxic–anoxic boundary layer (oxygen saturation between 0 and 10 %) and at 1 m intervals from the other water layers. Methods for sample preparation, inorganic nutrient measurements and dissolved organic carbon (DOC) are explained in more detail in the supplement at www.int-res.com/articles/suppl/a068p267_supp.pdf. The light intensities at different depths were calculated according to Wetzel (1983).

Bacterial DNA samples for the LH-PCR analyses were prepared using a lysate technique (detailed in the supplement). LH-PCR analysis was done according to Taipale et al. (2009), using 27f (5′-AGA GTT TGA TCN TGG CTC AG-3′; Lane 1991) and 518r (5′-ATT ACC GCG GCT GCT GG-3′; Muyzer et al. 1993) as primers in the PCR reaction for amplification of the 16S rRNA genes of bacteria, except that the polymerase brand used was DreamTaq by Fermentas. The peak size 512 ± 1 bp was used as a specific biomarker for *Chlorobi* spp., since previous results from Mekkojärvi, one of the lakes sampled for this study, revealed that this distinct fragment length corresponded to the genus *Chlorobium* according sequence analysis (Taipale et al. 2009). Therefore, we propose that the relative proportion of the 512 ± 1

Table 1. Maximum depth, depth of the oxic epilimnion, dissolved organic carbon (DOC) concentration, water colour and sampling times for the study lakes. Sampling dates — 1: 6 to 9 April 2009; 2: 14 to 15 May 2009; 3: 13 to 27 July 2009; 4: 11 August 2009

Lake	Max. depth (m)	Depth of oxic epilimnion (m)	DOC (mg C l^{-1})	pH	Water colour (mg platinum l^{-1})	Sampling date
Alinen Mustajärvi	7	2	12.1	5.16	133	1, 2, 3, 4
Halsjärvi	6	2	6.8	6.23	130	1, 2, 3, 4
Mekkojärvi	4	1	25.1	5.59	388	2, 3, 4
Nimetön	10	2	22.2	5.28	269	1, 3
Tavilampi	7	2	13.3	5.79	155	3
Särkijärvi	3	1.5	14.1	6.33	206	3
Iso-Valkjärvi	7	4	7.6	5.55	65	3
Huhmari	9	1.5	10.7	6.24	139	3
Horkkajärvi	10	1.5	22.4	5.62	325	1, 3
Rieskalammi	5	1	26.5	6.24	459	3
Keskinen Rajajärvi	12	5.5	19.6	5.25	294	3
Ylinen Rajajärvi	6	4.5	24.2	4.96	336	3
Vähä-Keltajärvi	4	2	27.7	5.75	399	3

peak size among the bacterial 16S rRNA PCR-amplified gene products can be used as an estimator of the relative abundance of GSB, and hereafter this relative proportion of GSB in the LH-PCR analysis is expressed as LH-PCR$_{512}$.

To confirm the pertinence of the LH-PCR$_{512}$ biomarker and to study the diversity in the anaerobic microbial communities and *Chlorobium* spp. genotypes, clone libraries were constructed from lakes Alinen Mustajärvi, Halsjärvi, Mekkojärvi, Nimetön, Tavilammi, Särkijärvi, Iso-Valkjärvi and Huhmari from the depth of the maximum BChl concentration. DNA was extracted from freeze-dried water samples using MO BIO PowerSoil DNA isolation kit (MO BIO Laboratories). Amplification of the 16S rRNA gene was performed as described for LH-PCR, except that the reverse primer 907 R (5′-CCG TCA ATT CMT TTR AGT TT-3′; Amann et al. 1992) was used to amplify longer sequences of ~880 bp. Cloning and sequencing of the PCR products was performed as described by Taipale et al. (2009). From each sample, 24 clones were sequenced, adding up to a total of 192 sequences. The sequences have been deposited in the EMBL database under accession numbers HE793329–HE793375 and HF543675–HF543817 (details in the supplement).

For BChl determinations, 0.12 to 0.5 l sample from each water layer was filtered through a GF/C filter (45 mm diameter, Whatman) and measured as described by Arvola et al. (1992). Because different BChls (*c*, *d* and *e*) overlap greatly, but have different maximum absorptions (666, 655 and 654 nm, respectively) (Frigaard et al. 1996), we measured the combined estimate of BChl at the 654 nm wavelength. Spectral overlapping of chlorophyll *a* (chl *a*) and BChl peaks was corrected by a model based on the data from the water layers in which only one of the chlorophylls was present (Fig. S1 in the supplement at www.int-res.com/articles/suppl/a068p267_supp.pdf). The absorbance ratio of 665:654 nm (Arvola et al. 1992) indicated the boundary layer where the dominance of chl *a* shifted to BChl between oxic and anoxic water layers in these lakes.

Correlation between light intensity and BChl concentration, and correlation between LH-PCR$_{512}$ results and BChl concentration were tested using Spearman's rank correlation (PASW Statistics 18, Release Version 18.0.0, SPSS).

RESULTS AND DISCUSSION

LH-PCR$_{512}$ and sequencing provided consistent evidence of the significant *Chlorobium* spp. population (representing at least 10% the PCR-amplified 16S rRNA genes of the bacteria in any of the water layers) in 8 of the 13 study lakes. Supported by the strong correlation ($\rho = 0.827$ and $p < 0.001$) between LH-PCR$_{512}$ proportions and BChl concentrations, we were able to locate the maximum GSB abundance in the anoxic zone close to the oxic–anoxic boundary layer (Fig. 1). Based on the sequences of the bacterial 16S rRNA PCR-amplified genes, we estimated that the proportion of GSB varied between 14 and 38% of the bacterial community in this layer in the GSB-positive lakes.

The results showed that, although the light conditions in boreal humic lakes are poor, many of these lakes provide an ecological niche for GSB. The PAR intensity at the depth of the oxic–anoxic interface was higher in the lakes where GSB were abundant compared to in the lakes without GSB (1.1 to 24.2 µE m^{-2} s^{-1} and <0.1 µE m^{-2} s^{-1}, respectively; corresponding to 0.08 to 1.7% and <0.007% of the surface PAR, respectively). There was a clear relationship between PAR intensity at the oxic–anoxic interface and BChl concentration ($\rho = 0.813$ and $p < 0.001$).

In GSB-positive lakes the estimate of BChl covered up to 92% (in Särkijärvi) of the total lake chlorophyll, and even the lowest proportion of BChl was 58% (in Huhmari) (Fig. S2 in the supplement at www.int-res.com/articles/suppl/a068p267_supp.pdf). The concentration of BChl more than doubled in all the study lakes between May and August. In Alinen Mustajärvi, the BChl was saturated in July, while in Halsjärvi and Mekkojärvi, the concentration of BChl increased over the course of the whole summer season (data not shown).

In 3 out of the 4 lakes that were studied during winter (Alinen Mustajärvi, Halsjärvi and Nimetön), GSB communities were found under ice, although the PAR conditions under the ice and snow in mid-winter did not support the phototrophic growth of GSB. The highest LH-PCR$_{512}$ proportions were observed in the upper hypolimnion and BChl densities close to the bottom (Fig. S3 in the supplement at www.int-res.com/articles/suppl/a068p267_supp.pdf), indicating increasing abundance of other bacterial groups closer to the sediment, which decreased the relative abundance of GSB. The primary reason for the highest winter BChl concentrations near the bottom could be the aeration of the water column during autumnal mixing, leading to death of the majority of the GSB cells in the water column, except at the very bottom. Another possible explanation for the occurrence in deeper waters during winter could be cell sinking due to the loss of turbulence. Consistent with our

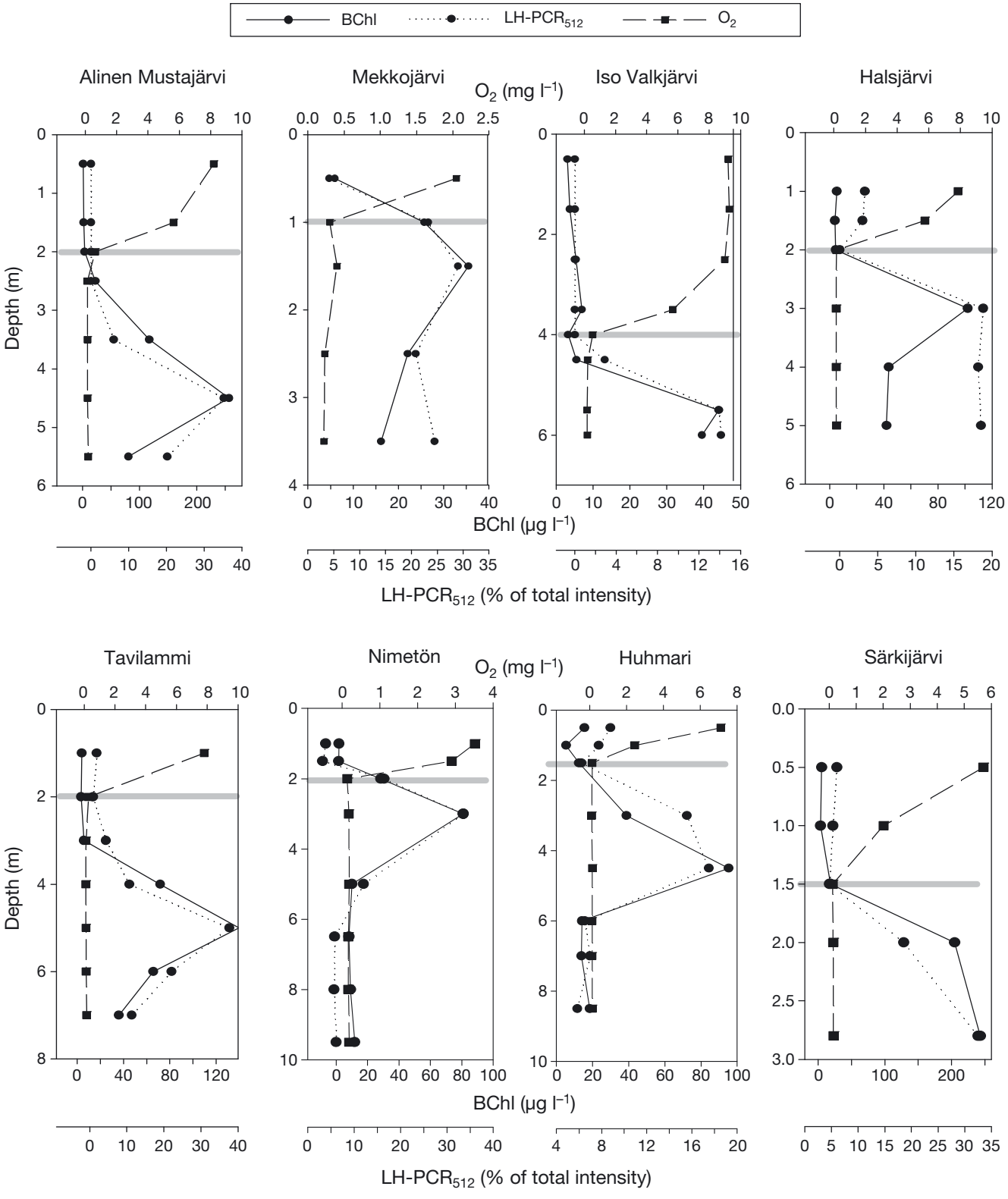

Fig. 1. Vertical summer profiles in study lakes of the green sulphur bacteria (GSB) based on the relative proportion of the LH-PCR$_{512}$ biomarker (in length heterogeneity–polymerase chain reaction analysis) with respect to the total PCR-amplified bacterial 16S rRNA genes, oxygen content and bacteriochlorophyll (BChl) concentrations in the GSB-positive lakes. The oxic–anoxic boundary layer is indicated with a grey line

results, *Chlorobium* sp. was also detected in Burton Lake (Antarctica), where light does not penetrate the water for 3 mo of the year (Burke & Burton 1988). The dominance of *Chlorobium* sp. in Lake Burton was explained by their efficient maintenance metabolism in winter and by their great efficiency in utilizing low-intensity light (Burke & Burton 1988).

Based on clone sequences recovered in libraries constructed from anoxic water, 13 bacterial phyla were identified, the main phyla being *Proteobacteria*, *Chlorobi* and *Bacteroidetes* (Fig. S4 in the supplement at www.int-res.com/articles/suppl/a068p267_supp.pdf). From the total of 192 16S rRNA gene clones, 25% of clones were identified as *Chlorobium* sp., and no other known BChls containing bacterial groups were detected, except 5 clones of *Chloroflexi* that were found in Halsjärvi. The sequencing analysis validated the LH-PCR$_{512}$ biomarker (512 ± 1 bp) for the analysis of GSB abundance in the study lakes, as no other bacterial groups had the same fragment size in the V1 to V3 16S rRNA region, and LH-PCR and sequencing results were consistent among the samples. The clone libraries revealed close phylogenetic relationships among the GSB communities of different lakes (Fig. 2). The clustering analysis in OTU$_{0.97}$ level (sequence length ≈ 880 bp) divided the clones into 4 clusters, of which 1 to 3 clusters were found in each lake. OTU1 comprised 36 reads; OTU2, 5 reads; and OTUs 3 and 4, 3 reads. OTU1 was the most homogenous of all OTUs, but it had the largest distance to any type strain. Still, OTU1 had the closest match to *Chlorobium clathratiforme*, which was also clustered with OTU2. OTU3 clustered with *Chlorobium ferroxidans*; and OTU4, with *Chlorobium phaeobacteroides* (Fig. 2).

Chlorobium clathratiforme is known to belong to the green subset of the GSB, and its pigment composition has been reported to be BChl *c*, *d*, or both (Gich et al. 2001). In Lago di Cadagno, *C. clathratiforme* covered 95% of the anoxygenic phototrophic community (Habicht et al. 2009). All the closest type strains assigned to our clones have been classified as freshwater species (Pfennig & Overmann 2001). In a previous study (Taipale et al. 2009), the GSB population of Mekkojärvi was assigned to *Chlorobium phaeobacteroides*. The difference in the phylogenetic affiliation was due to the length of the sequences, which was longer in the present study. The 16S rRNA structures of *Chlorobium* species are very similar (Imhoff 2003), which makes exact differentiation to species level challenging when using short sequences.

The measured BChl concentrations and the high proportion of the characteristic LH-PCR biomarker clearly proved that GSB were abundant in more than half of the study lakes. In those lakes where GSB were present, their biomarker accounted for 13 to 42% of the PCR-amplified bacterial 16S rRNA genes in the anoxic hypolimnia in July. However, the substantial increase of BChls suggested that the abundance of this group could be even higher by the end of the summer. Taipale et al. (2011) previously noted that during summer stratification, on

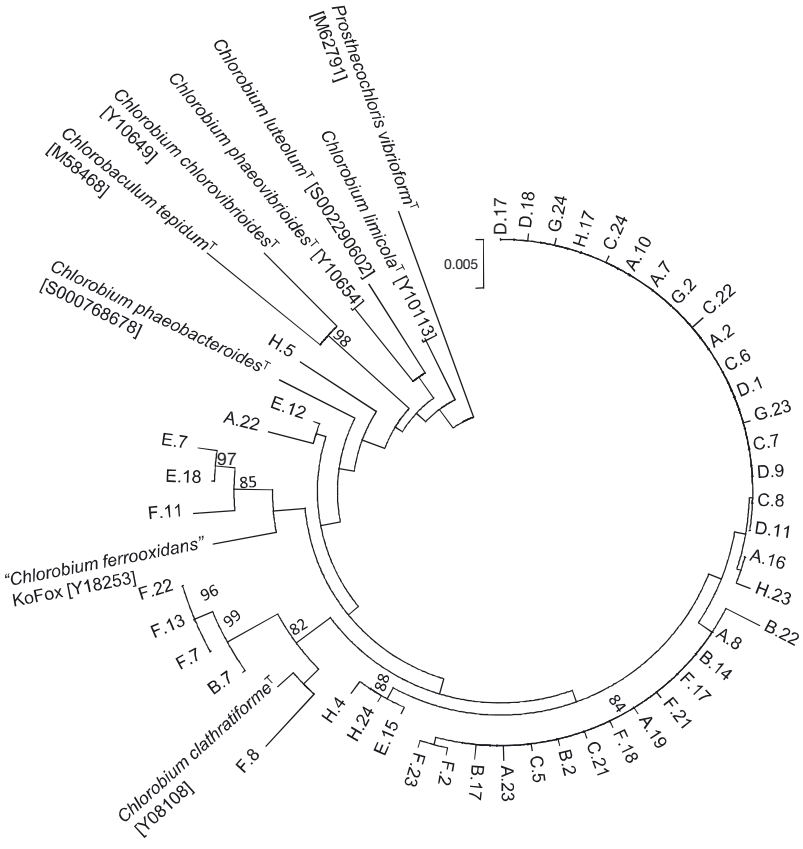

Fig. 2. Neighbour-joining tree of green sulphur bacteria in humic lakes and reference type strains of the genus *Chlorobium* based on partial 16S rRNA gene sequences. The tree was made of 798 aligned nucleotide positions. Bootstrap values >70% and clustering of the study sequences to 4 coherent OTU$_{0.97}$ (operational taxonomic unit) groups are shown. A: Alinen Mustajärvi; B: Halsjärvi; C: Mekkojärvi; D: Nimetön; E: Tavilammi; F: Särkijärvi; G: Iso-Valkjärvi; H: Huhmari

average, 47% of the PCR-amplified bacterial rRNA genes in Mekkojärvi were related to the GSB, which is slightly more than in our study. According to the results of Arvola et al. (1992), hypolimnetic GSB comprised 61 to 78% of the autochthonous carbon in Mekkojärvi in summer 1986. In the GSB-positive lakes, BChl made up roughly 52 to 98% of the total amount of chlorophyll (Fig. S3). Although the actual concentrations of chl *a* and BChl do not provide the whole picture for the number or biomass of GSB relative to phytoplankton, they can give some estimate of the relative photosynthetic potential of the GSB.

In conclusion, the results corroborate that the occurrence of GSB in small boreal lakes is widespread in spite of the poor PAR conditions. More attention should be paid to the actual photosynthetic activity and primary production rates of *Chlorobium* sp. cells before their ecological significance in the energy mobilization of boreal lakes can be determined.

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