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RESEARCH ARTICLE

Dominance of a clonal green sulfur bacterial population in a stratified lake

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Chlorobium; clonal population; bloom; green sulfur bacteria; Lake Cadagno.

Abstract

For many years, the chemocline of the meromictic Lake Cadagno, Switzerland, was dominated by purple sulfur bacteria. However, following a major community shift in recent years, green sulfur bacteria (GSB) have come to dominate. We investigated this community by performing microbial diversity surveys using FISH cell counting and population multilocus sequence typing [clone library sequence analysis of the small subunit (SSU) rRNA locus and two loci involved in photosynthesis in GSB: *fmoA* and *csmCA*]. All bacterial populations clearly stratified according to water column chemistry. The GSB population peaked in the chemocline (*c.* 8×10^6 GSB cells mL^{-1}) and constituted about 50% of all cells in the anoxic zones of the water column. At least 99.5% of these GSB cells had SSU rRNA, *fmoA*, and *csmCA* sequences essentially identical to that of the previously isolated and genome-sequenced GSB *Chlorobium clathratiforme* strain BU-1 (DSM 5477). This ribotype was not detected in Lake Cadagno before the bloom of GSB. These observations suggest that the *C. clathratiforme* population that has stabilized in Lake Cadagno is clonal. We speculate that such a clonal bloom could be caused by environmental disturbance, mutational adaptation, or invasion.

Introduction

Phototrophic sulfur bacteria often form massive blooms in aquatic environments where anoxic, sulfide-containing zones in the sediment or the water column receive light. In the water column of stratified lakes, this occurs around the chemocline where chemical gradients allow formation of a complex microbial community (van Gemerden & Mas, 1995; Overmann, 2008). These phototrophic sulfur bacteria couple anoxic oxidation of inorganic sulfur compounds to CO_2 fixation. Sulfide is usually the most readily available sulfur compound under natural conditions although other inorganic compounds (e.g. elemental sulfur, thiosulfate, H_2, and Fe^{2+}) also function as electron donors in some strains (Frigaard & Dahl, 2008). Two distinct groups of phototrophic sulfur bacteria are found: the green sulfur bacteria (GSB; family *Chlorobiaceae*) (Garrity & Holt, 2001) and the purple sulfur bacteria (PSB; families *Chromatiaceae* and *Ectothiorhodospiraceae*) (Imhoff, 2006). Although evolutionarily and physiologically distinct, these two groups share a similar ecological role as phototrophic, anoxic oxidizers of inorganic sulfur compounds in aquatic environments. The GSB have limited physiological capabilities and are thought to be obligate photoautotrophs. The PSB are capable of both photoautotrophy and photoheterotrophy and some strains can grow as chemotrophs. GSB are often found below PSB in stratified microbial communities, one reason being that GSB possess much more efficient light-harvesting antenna systems (chlorosomes) than PSB (van Gemerden & Mas, 1995; Overmann, 2008). The factors that lead to blooms of phototrophic sulfur bacteria are at present unclear.

Lake Cadagno in the Swiss Alps is a permanently stratified lake with a chemocline that harbors a large population of phototrophic sulfur bacteria (Tonolla & Peduzzi, 2006). Historically, the phototrophic community in Lake Cadagno was dominated by PSB, but since 2000, there has been a dramatic change, leading to a community dominated by GSB containing bacteriochlorophyll (BChl) *e* (Tonolla *et al.*,

2005; Decristophoris *et al.*, 2009). In order to investigate the structure of this new community, we characterized the microbial diversity at different depths in the water column using FISH and sequence information derived from small subunit (SSU) rRNA clone libraries. We also determined the content of photosynthetic pigments in the water column. To examine the genetic heterogeneity of the GSB population in more detail, we also analyzed clone libraries of two other loci conserved in all GSB: the *fmoA* gene encoding the light harvesting, BChl *a*-containing Fenna–Matthews–Olson protein, FmoA (Olson, 2004), and the *csmCA* operon encoding the two most important chlorosome proteins, CsmC and CsmA (Frigaard & Bryant, 2006).

Materials and methods

Study site

Lake Cadagno is located in the Swiss Alps (46°33′N, 8°43′E), 1923 m above sea level (Tonolla & Peduzzi, 2006). Its surface area is 0.26 km^2 and the lake has a maximum depth of 21 m. The lake is fed by subsurface aquatic springs whose chemical composition is derived from the surrounding dolomitic rock, causing a rather high conductivity (200–300 $\mu S\,cm^{-1}$) and a high sulfate concentration (1.5–2.0 mM) in the lake bottom water.

Water samples were obtained from a pontoon positioned at the deepest point on the lake. Samples used for cell enumeration and FISH analysis during the period 1994–2007 were retrieved from the turbidity maximum in the chemocline and stored at −70 °C until use. Samples for pigment and DNA analysis were obtained in the afternoons in the period September 25–28, 2006 using a double-conical sampler (Jørgensen *et al.*, 1979) connected to a battery-driven pump.

Physicochemical measurements

A submersible probe was used to measure temperature, conductivity, pH, redox potential, and turbidity (YSI 6000, YSI Hydrodata Ltd, UK). Light intensity was measured using a spherical LI-193 detector fitted to an LI-1000 data logger (LI-COR, Lincoln, NE). The sensor used is sensitive between 400 and 700 nm and thus covers the blue and green light that penetrates into the water column. Samples for sulfide measurement were fixed in $ZnCl_2$ (5 mL lake water and 2 mL of 1% $ZnCl_2$) and the sulfide concentration was determined using Cline's reagent (Cline, 1969).

Pigment analysis

Lake water was prefiltered on a Spectra/Mesh macroporous polypropylene filter with a mesh opening of 149 μm (Spectrum Laboratories, Rancho Dominguez, CA) to exclude large organisms and particles. Samples for pigment analysis (100–1000 mL of prefiltered lake water) were then collected on GF5 or GF75 glass fiber filters (47 mm diameter) using a vacuum pump. We confirmed that no measurable pigments were obtained after passing the filtrate from the glass fiber filters through a 0.22-μm filter. Filters were either extracted and analyzed immediately or stored at −20 °C until extraction.

For HPLC analysis, glass fiber filters were extracted with methanol and injected onto an octadecylsilyl-bonded silica column fitted with a diode-array detector as described previously (Frigaard *et al.*, 1997). The pigment content was quantified using the following absorption coefficients determined for a methanol solution at the specified wavelengths that correspond to a suitable absorption maximum: BChl *a*, 60 $L\,g^{-1}\,cm^{-1}$ at 770 nm (Permentier *et al.*, 2000); BChl *e*, 43 $L\,g^{-1}\,cm^{-1}$ at 656 nm (assuming a molecular weight of 835) (Borrego *et al.*, 1999); Chl *a*, 79 $L\,g^{-1}\,cm^{-1}$ at 664 nm (Lichtenthaler, 1987); and Chl *c*, 120 $L\,g^{-1}\,cm^{-1}$ at 450 nm (Jeffrey, 1963).

For absorption spectroscopy, glass fiber filters were extracted with 5 mL of acetone : methanol (7 : 2 v/v) in the dark for 20–30 min with intermittent agitation. After centrifugation, the pigment content was quantified using the following absorption coefficients at the Q_y absorption maximum: BChl *a*, 65 $L\,g^{-1}\,cm^{-1}$ at 772 nm (Permentier *et al.*, 2000); BChl *e*, 49 $L\,g^{-1}\,cm^{-1}$ at 652 nm (assuming a molecular weight of 835) (Borrego *et al.*, 1999); and Chl *a*, 85 $L\,g^{-1}\,cm^{-1}$ at 664 nm (Lichtenthaler, 1987). Absorption coefficients for BChl *a* and Chl *a* in acetone : methanol (7 : 2 v/v) are not available. Therefore, the values for ethanol were used because the absorption coefficient for BChl *e* in acetone : methanol is very similar to that in ethanol and intermediate between those in pure acetone and in pure methanol (Borrego *et al.*, 1999).

In some pigment extracts, BChl *a*, BChl *e*, and Chl *a* were simultaneously present in significant amounts. All these pigments contribute to absorption at 652 and 664 nm, whereas only BChl *a* absorbs at 772 nm. Chl *c* absorption above 500 nm was negligible in our samples due to the relatively small amounts present. Therefore, the absorption at 652 and 664 nm can be described as

$$A_{652} = A_{652}^{\text{BChl}\,e} + A_{652}^{\text{Chl}\,a} + A_{652}^{\text{BChl}\,a} \quad (1)$$

$$A_{664} = A_{664}^{\text{BChl}\,e} + A_{664}^{\text{Chl}\,a} + A_{664}^{\text{BChl}\,a} \quad (2)$$

Rearrangement of these equations allows the absorption at 652 and 664 nm specifically due to BChl *e* and Chl *a* to be calculated as follows:

$$A_{652}^{\text{BChl}\,e} = (A_{652} - \delta A_{664} - (\alpha - \beta\delta)A_{772})/(1 - \gamma\delta) \quad (3)$$

$$A_{664}^{\text{Chl}\,a} = (A_{664} - \gamma A_{652} - (\beta - \alpha\gamma)A_{772})/(1 - \gamma\delta) \quad (4)$$

where $\alpha = A_{652}/A_{772}$ (for pure BChl *a*) ≈ 0.10, $\beta = A_{664}/A_{772}$ (for pure BChl *a*) ≈ 0.11, $\gamma = A_{664}/A_{652}$ (for pure BChl *e*)

≈ 0.61, and $\delta = A_{652}/A_{664}$ (for pure Chl *a*) ≈ 0.72. The values of α and β were empirically determined from an absorption spectrum of pure BChl *a*. The value of γ was empirically determined from an absorption spectrum of a lake extract containing BChl *e* and BChl *a* and no other chlorophylls. The value of δ was empirically determined from an absorption spectrum of a lake extract containing Chl *a* and no other chlorophylls (other than Chl *c* in negligible amounts). If $A_{652}^{BChl\,e}$ or $A_{664}^{Chl\,a}$ are known, Eqs (3) and (4) simplify to

$$A_{652}^{BChl\,e} = A_{652} - \delta A_{664}^{Chl\,a} - \alpha A_{772} \quad (5)$$

$$A_{664}^{Chl\,a} = A_{664} - \gamma A_{652}^{BChl\,e} - \beta A_{772} \quad (6)$$

Enumeration of planktonic cells

Details are described in the Supporting Information. In brief, planktonic cells were collected (without prefiltration) on polycarbonate membrane filters (0.22 μm pore size) and preserved using paraformaldehyde. Total cell counts were performed using 4′-6-diamidino-2-phenylindole (DAPI) staining. Specific populations were counted using FISH with population-specific, rRNA-targeting oligonucleotide probes listed in the Supporting Information.

DNA preparation, sequence generation, and sequence analysis

Details are described in the Supporting Information. In brief, lake water was prefiltered on polypropylene filters (149 μm pore size). Planktonic cells were then collected on polycarbonate membrane filters (0.2 μm pore size) and preserved before DNA extraction. PCR was performed using degenerate primers specific for the SSU rRNA, *fmoA*, or the *csmCA* locus. The PCR products were inserted into a 3′-dA-overhang-dependent cloning vector and cloned into *Escherichia coli*. Clones were sequenced using primers specific for either the vector (*csmCA* gene fragments) or the insert (SSU rRNA and *fmoA* gene fragments).

The SSU rRNA sequences were analyzed by BLAST and by comparison with the publicly available SSU rRNA databases GenBank (http://www.ncbi.nlm.nih.gov) and Greengenes (DeSantis *et al.*, 2006). Rarefaction curves and diversity calculations were constructed using MEGA (Tamura *et al.*, 2007) and DOTUR (Schloss & Handelsman, 2005). Phylogenetic trees were constructed using MEGA. For details, see the Supporting Information.

Nucleotide sequence accession numbers

The SSU rRNA sequences reported in this work were deposited in GenBank as sequence sets (PopSet groups). GSB sequences that were $> 99\%$ identical to the sequence of *Chlorobium clathratiforme* DSM 5477 (accession number Y08108) were not deposited. Bacterial sequences from 10 m contain the label 'LC10' (accession numbers FJ546750–FJ546842), sequences from 11.5 m contain the label 'LC115' (accession numbers FJ546983–FJ547019), and sequences from 15 m contain the label 'LC15' (accession numbers FJ547020–FJ547058). One archaeal SSU rRNA sequence (LC15_L00B08) was deposited with the accession number FJ547059.

Results

Physicochemical parameters

The water column was divided into three zones: the oxic mixolimnion (from the surface to around 11.5 m), the anoxic chemocline (between 11.5 and 12 m), and the anoxic, sulfide-containing monimolimnion (from 12 m to the bottom at around 21 m) (Fig. 1). When the samples were taken, there was no overlap between measurable oxygen and sulfide at the chemocline. The cell density estimated from turbidity measurements peaked at about 12 m between the sulfide- and the non-sulfide-containing layers. This cell density peak coincided with the peak in BChl *e*-containing GSB as shown by the measured BChl *e* concentrations. Chl *a* and BChl *a* peaked somewhat higher in the water column at around 11.5 m, indicating that the algal and PSB populations peaked at this depth. The photon fluence rate at midday with a cloudless sky was 6 μmol photons $m^{-2}\,s^{-1}$ at the Chl *a* and BChl *a* maxima (11.5 m) and 0.5 μmol photons $m^{-2}\,s^{-1}$ at the BChl *e* maximum (12 m). The concentrations of ammonium and phosphate at the top of the turbidity maximum were below the limit of detection (< 0.5 μM; results not shown).

Three samples were retrieved for PCR clone library preparation (SSU rRNA and functional genes) and detailed pigment analysis by HPLC. They represented the oxic mixolimnion (depth, 10.0 m; oxygen, 4.0 mg L^{-1}; sulfide, not detectable), the anoxic chemocline (depth 11.5 m; oxygen, not detectable; sulfide, *c.* 1 μM), and the anoxic monimolimnion (depth 15.0 m; sulfide, 240 μM).

Enumeration of planktonic cells

The total cell numbers in the anoxic water column were determined by DAPI staining (Fig. 2). The cell density throughout the profile was $> 10^7$ cells mL^{-1}, with a maximum at the chemocline at 11.8 m (1.3×10^7 cells mL^{-1}).

The numerical abundance of GSB was determined by microscopy using FISH probes. The GSB was by far the most abundant cell type present in the anoxic water column, ranging from 8.3×10^6 cells mL^{-1} (66% of the DAPI counts) in the chemocline (11.8 m) to 3.6×10^6 cells mL^{-1} (36% of the DAPI counts) at the deepest sample location (17 m) (Fig. 2). The probe targeting the *C. clathratiforme* DSM 5477

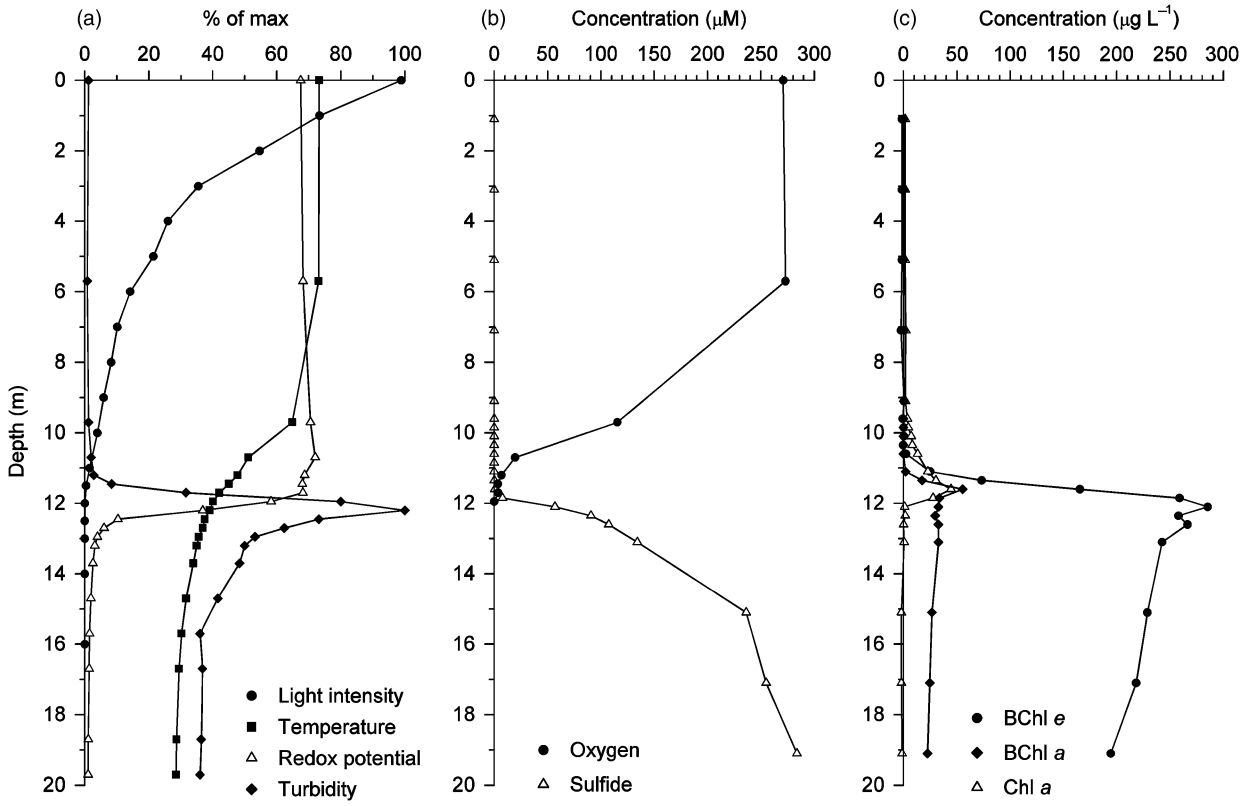

Fig. 1. Profiles of physicochemical parameters and microbial chlorophylls (Chl) and bacteriochlorophylls (BChl) in the water column of Lake Cadagno in September 2006. (a) ●, Light intensity (100% = 1300 μmol photons m^{-2} s^{-1}); ■, temperature (100% = 16 °C); △, redox potential (0% = −300 mV, 100% = 300 mV); ◆, turbidity (100% = 25 FTU). (b) •, Dissolved oxygen; △, sulfide. (c) ●, BChl *e*; ◆, BChl *a*; △, Chl *a*.

ribotype (Tonolla *et al.*, 2005) accounted for > 99.5% of the GSB counts at all depths.

The numerical abundance of PSB was determined by FISH using probes for *Lamprocystis* spp., *Thiocystis* spp., and *Chromatium okenii* (Tonolla *et al.*, 2005). PSB accounted for only 2–3% of the DAPI counts in the anoxic water column (Fig. 2). *Lamprocystis* spp. and *Thiocystis* spp. were present throughout the anoxic water column with $(2–3) \times 10^5$ cells mL^{-1} and $(1–2) \times 10^4$ cells mL^{-1}, respectively. *Chromatium okenii* peaked in the chemocline with $(1–5) \times 10^4$ cells mL^{-1} at 11–12 m, and was found in the monimolimnion with *c.* 10 times lower density.

Overall, the total count of GSB cells was *c.* 20–30 higher than the total count of PSB cells throughout the anoxic water. Taken together, the phototrophic sulfur bacteria (GSB and PSB) constituted 70% of the DAPI-stained cells in the chemocline, and 97% of these were labeled with a probe that targets *C. clathratiforme* DSM 5477.

Figure 3 shows changes in the bacterial populations in the chemocline since 1994. The total number of cells (determined by DAPI staining) was in the range $(1–6) \times 10^6$ cells mL^{-1} up until 2001. Since the rise of *C. clathratiforme* after 2002, this number has increased to $(1–3) \times 10^7$ cells mL^{-1}. *Chlorobium clathratiforme* was not detected by FISH before 2000. Since 2002, the *C. clathratiforme* population detected by FISH has been around 10^7 cells mL^{-1}, corresponding to about half of the DAPI counts. *Chlorobium phaeobacteroides* were present at around 10^5 cells mL^{-1} up to 2000, but have declined by a factor of 5–10 as *C. clathratiforme* has increased.

Photosynthetic pigments

The only chlorophyll pigments found in the water column by HPLC with diode array detection were Chl *a* and Chl *c* at 10 m; Chl *a*, Chl *c*, BChl *a*, and BChl *e* at 11.5 m; and BChl *a* and BChl *e* at 15 m (data not shown). Because Chl *b* was not found at any depth, we conclude that green algae were not present in significant amounts. The ratio of Chl *c* to Chl *a* was similar at 10 and 11.5 m, *c.* 0.25, which is typical of Chl *c*-containing algae growing at low light intensities (Fujiki & Taguchi, 2002).

Analysis of the overlapping spectra in the red region of Chl *a*, BChl *a*, and BChl *e* in the organic solvent allowed these to be determined by absorption spectroscopy (see Materials and methods). Chl *c* cannot be determined using this method because it is present in very small amounts and

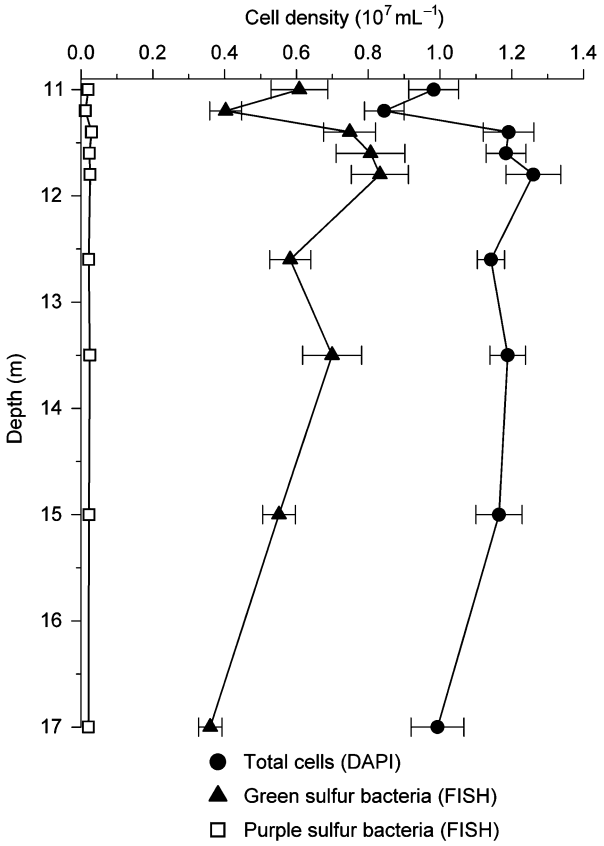

Fig. 2. Profiles of the density of all planktonic cells (●, determined by DAPI staining), GSB cells (▲, determined by FISH), and PSB cells (□, determined by FISH) in the anoxic water column of Lake Cadagno in September 2006.

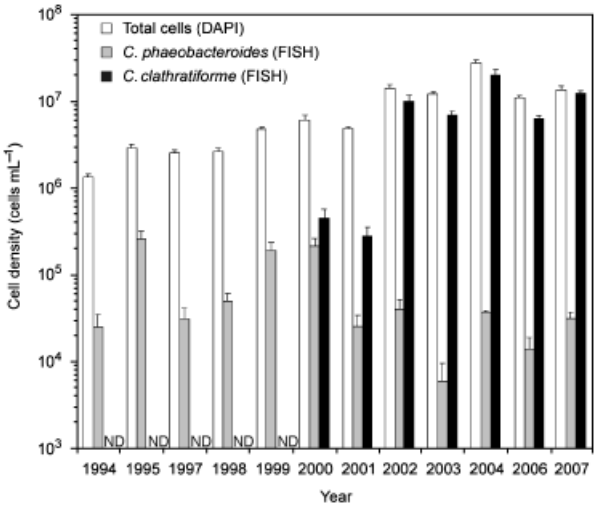

Fig. 3. Density of all planktonic cells (determined by DAPI staining, white bars), *Chlorobium phaeobacteroides* (determined by FISH using probe Chlp441, gray bars), and *Chlorobium clathratiforme* (determined by FISH using probe Chlc190; black bars) in the chemocline of Lake Cadagno in September or October from 1994 to 2007. ND, not detected (detection limit: $< 10^2$ cells mL^{-1}).

its specific absorption coefficient in the red region in organic extracts is much lower than that of other chlorophylls.

The chlorophyll pigment profiles are shown in Fig. 1c. The highest concentration of BChl *e* was 285 μg L^{-1} at 12.1 m and the integrated amount of BChl *e* through the water column was about 2.5 g m^{-2}, which is typical for lakes with blooms of GSB (van Gemerden & Mas, 1995). The highest BChl *a*-to-BChl *e* ratio (0.33) and the highest BChl *a* concentration (55 μg L^{-1}) were found in the chemocline at 11.6 m due to the presence of PSB. The BChl *a*-to-BChl *e* ratio was rather similar (*c.* 0.12) between the monimolimnion from 12 m and the deepest measurement at 19 m (Fig. 1c). Although GSB account for most of the cells in the chemocline, individual cells are much smaller than PSB: the biovolume of a GSB cell is 5–10 times smaller than that of a small-celled PSB (e.g. *Lamprocystis* spp.) and 50–100 times smaller than that of the large-celled PSB *C. okenii* (Tonolla *et al.*, 2003). In addition, BChl *a* is only a minor constituent of GSB because the main light-harvesting pigment in these organisms is BChl *e*. Thus, most of BChl *a* in the chemocline, and a significant proportion of the BChl *a* in the monimolimnion, apparently originates from PSB.

BChl *e* from GSB consists of a number of homologs with different side chains on the chlorin ring combined with different esterifying alcohols. The BChl *e* homolog distribution was found to be indistinguishable between 11.5 m (0.6 m above the BChl *e* maximum) and 15 m (2.9 m below the BChl *e* maximum) (Supporting Information, Fig. S1).

Genetic diversity of the GSB

Microscopical examination with FISH probes is only specific for a small part of the SSU rRNA molecule. More detailed information about the structure of the GSB population in Lake Cadagno was obtained by a sequencing approach involving construction and analysis of clone libraries for three different loci: SSU rRNA and two loci encoding three proteins involved in photosynthesis.

The SSU rRNA clone libraries were constructed using general bacterial primers for PCR. GSB dominated the SSU rRNA clone libraries obtained from the anoxic water column, making up 63% of all sequences from the chemocline and 74% of all sequences from the monimolimnion (Table S1). With one exception (LC15_L00C03), the GSB sequences obtained were identical to the SSU rRNA sequences on the genome of *C. clathratiforme* DSM 5477 (accession number CP001110) within the uncertainty due to sequencing errors (Fig. 4). Position 266 in the *C. clathratiforme* SSU rRNA sequences from the lake was

hypervariable: 70% was adenine and 30% was guanine (Fig. S2). The genome of *C. clathratiforme* DSM 5477 has three SSU rRNA genes that differ by one base at position 266: two of the genes have an adenine and the other a guanine. Thus, the experimentally obtained base frequency in position 266 of the *C. clathratiforme* SSU rRNA sequences from the lake (70% adenine, 30% guanine) closely matches the expected frequency (2/3 adenine, 1/3 guanine) from the SSU rRNA microheterogeneity in *C. clathratiforme* DSM 5477. Disregarding position 266, 82% of the *C. clathratiforme* SSU rRNA sequences from the lake had a perfect match to the sequence of strain DSM 5477, 14% differed by a single base, and the rest by no more than three bases, with all differences randomly distributed over >700 bases (Fig. S2). Speksnijder *et al.* (2001) investigated sequencing artifacts in a model system consisting of a mixture of clones with closely related sequences. They found at least 12 errors in 66 partial SSU rRNA gene sequences of 162 bp, corresponding to at least 0.11% per base. Our results for the *C. clathratiforme* SSU rRNA sequences from the lake exhibited *c.* 0.03% differences per base (disregarding position 266), which is below the level of experimental artifacts observed by Speksnijder *et al.* (2001).

Further information about the GSB population was obtained by generating clone libraries for the *fmoA* and *csmCA* loci (Figs S3 and S4). These loci exhibit considerable variation among different characterized strains of GSB due to changes in the third base of codons and the presence of an intergenic region in the *csmCA* loci (Fig. S5). Twenty-five of 26 *fmoA* and all 39 *csmCA* sequences from the lake were identical to the corresponding sequences from *C. clathratiforme* DSM 5477 within the uncertainty due to sequencing errors. One environmental *fmoA* sequence was identical to the corresponding sequence from *C. phaeobacteroides* DSM 266. The overall variation from the consensus sequences was *c.* 0.10% at random positions, which is within the range expected for a clonal population. We therefore conclude that the majority of the GSB in Lake Cadagno are closely related and are likely to represent a clonal population that is very similar, if not identical, to *C. clathratiforme* DSM 5477.

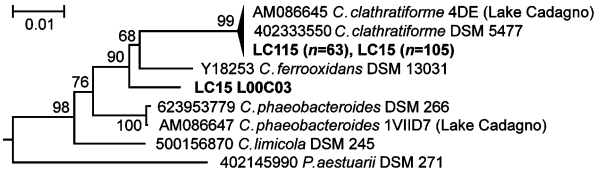

Fig. 4. Phylogenetic tree of the SSU rRNA sequences of GSB obtained from Lake Cadagno (outgroup: *Chloroflexus aurantiacus* J-10-fl, accession number CP000909; not shown). The phylogenetic analysis was performed using the neighbor-joining method; bootstrap values (in percent based on 1000 replicates) of at least 50% are shown. Names in bold represent sequences from the current work. Sequences labeled LC115 are from 11.5 m; sequences labeled LC15 are from 15 m. See the text for details.

Diversity parameters for the entire bacterial community

The SSU rRNA diversity was studied in samples at three depths representing the mixolimnion (10 m), chemocline (11.5 m), and monimolimnion (15 m). PCR amplification with general primers for bacterial SSU rRNA generated a total of 359 sequences from all depths. Of these, 337 were clearly bacterial SSU rRNA sequences, whereas 14 were from plastids from cryptomonad algae.

Table 1 shows estimations of diversity, evenness, richness, and coverage of the bacterial SSU rRNA sequences. The diversity ($H=3.35$) and evenness ($E=0.91$) in the mixolimnion was similar to what has been observed in the mixolimnion of other meromictic lakes (Lehours *et al.*, 2007; Dimitriu *et al.*, 2008). However, the diversity ($H=1.43$ and 1.69) and evenness ($E=0.41$ and 0.54) in the anoxic zones (chemocline and monimolimnion) were significantly lower than that in the oxic mixolimnion and the anoxic zones of other meromictic lakes. This is due to the high proportion of essentially identical GSB sequences that constitute a single operational taxonomic unit (OTU) in Lake Cadagno. Nevertheless, the richness was highest in the monimolimnion (Chao1 = 221) similar to what has been observed in the monimolimnion of other meromictic lakes (Lehours *et al.*, 2007; Dimitriu *et al.*, 2008). This indicates that the variety of bacteria found in the monimolimnion of Lake Cadagno is

Table 1. Diversity, evenness, richness, and coverage of the bacterial SSU rRNA sequences from Lake Cadagno

Sample	Number of sequences	Number of OTUs	Shannon diversity	Pielou evenness	Chao1 richness	Good coverage
Mixolimnion (10 m)	89	40	3.35 (3.14–3.55)	0.91 (0.85–0.96)	76 (53–143)	0.43
Chemocline (11.5 m)	99	23	1.69 (1.35–2.03)	0.54 (0.43–0.65)	41 (28–83)	0.35
Monimolimnion (15 m)	141	32	1.43 (1.10–1.75)	0.41 (0.32–0.51)	221 (92–624)	0.13

Calculated using DOTUR (Schloss & Handelsman, 2005). OTUs were defined at 97% sequence identity. The numbers in parenthesis under diversity, evenness, and richness correspond to the 95% confidence interval as calculated by DOTUR. Formulas employed: Shannon's diversity index: $H=-\Sigma(n_i/N)\ln(n_i/N)$; Pielou's evenness index: $E=H/\ln(S)$; Good's coverage index: $C=1-s'/S$, where N is the total number of sequences, n_i is the number of sequences in the i'th OTU, S is the total number of OTUs and s' is the number of OTUs with only one sequence.

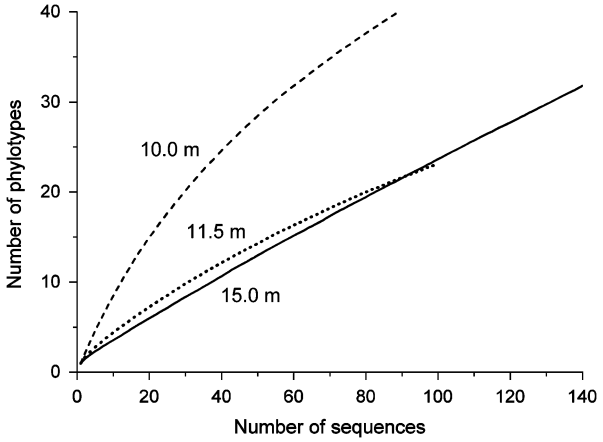

Fig. 5. Rarefaction curves for the three bacterial SSU rRNA clone libraries (10 m, dashed line; 11.5 m, dotted line; 15 m, solid line). The sequences were grouped into phylotypes based on the furthest neighbor model (complete linkage) using a 97% sequence identity cut-off to distinguish unique phylotypes.

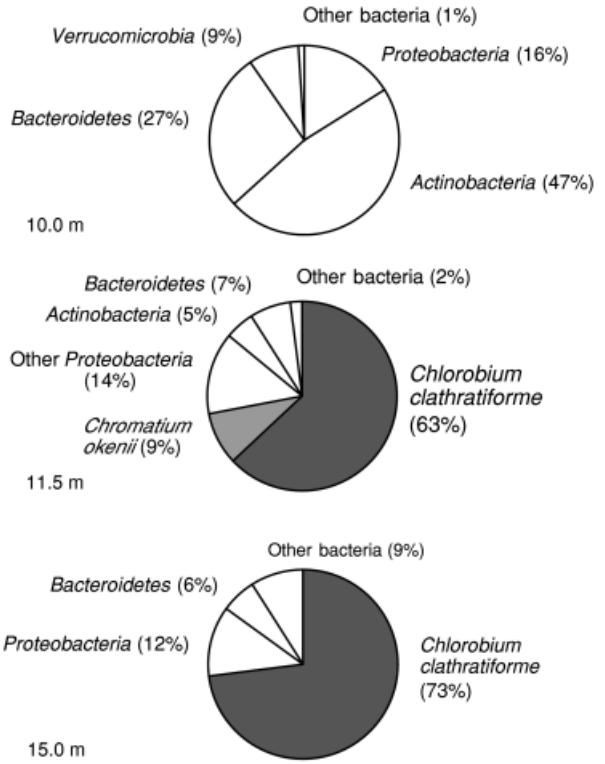

Fig. 6. Relative abundance of phylogenetic groups of the bacterial SSU rRNA sequences from depths of 10, 11.5, and 15 m. The total numbers of bacterial SSU rRNA sequences were 93 (10 m), 100 (11.5 m), and 144 (15 m). See Table S1 for absolute numbers.

higher than that in the upper layers although they are greatly outnumbered by GSB.

Rarefaction analysis (Fig. 5) supports the notion that the mixolimnion has a higher evenness than the anoxic zones because the initial slope of the mixolimnion's rarefaction curve is much greater. The observation that the initial slope of the mixolimnion's rarefaction curve is close to 1.0 is expected if all OTUs are approximately equally abundant because this would cause approximately even sampling among all OTUs. In contrast, initial sampling from the anoxic zones is very uneven among the OTUs because GSB sequences are encountered more often than any of the other OTUs. On the other hand, the monimolimnion appears to have the highest richness because the monimolimnion's rarefaction curve appears to have the highest asymptotic value. However, the monimolimnion's rarefaction curve also appears to be the furthest from reaching its asymptotic value, consistent with a poor coverage of the OTUs present. This means that a very large number of sequences would have to be samples to reveal the true diversity in the monimolimnion, even though the vast majority of sequences would originate from the same *C. clathratiforme* OTU.

Diversity of microbial groups apart from GSB

The overall distribution of the SSU rRNA sequences among the phylogenetic groups in samples taken from different depths is shown in Fig. 6 and Table S1. Phylogenetic trees of dominant phyla (*Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*) are shown in Figs S6–S8.

Sequences from the oxic mixolimnion were predominantly from *Actinobacteria* (47% of all sequences at 10 m). Most of our sequences showed a very high degree of similarity to sequences derived from other lakes (Fig. S6); 76% of our sequences belonged to the acI and acII groups, which are typical of mountain lakes (Warnecke *et al.*, 2004, 2005; Allgaier & Grossart, 2006). Actinobacterial sequences were almost absent from the anoxic zones (11.5 and 15 m). The second most abundant group of sequences in the oxic mixolimnion belonged to the phylum *Bacteroidetes* (27% of all sequences at 10 m). The majority of the remaining sequences from the mixolimnion were from the *Proteobacteria* and *Verrucomicrobia*.

Although Chl *a* is present, no sequences from cyanobacteria were obtained with the general primers used. Microscopical studies of chemocline samples also revealed no obvious cyanobacteria, but numerous eukaryotic algae containing Chl *a*.

The *Bacteroidetes* sequences from the entire water column were distributed into two distinct polyphyletic groups (Fig. S7): one group of clades (labeled 'oxic') containing sequences only from the oxic zone and the oxic/anoxic interface and another group of clades (labeled 'anoxic') containing sequences only from the anoxic zone and oxic/anoxic interface.

There are significant concentrations of methane in the anoxic water column in Lake Cadagno, but undetectable

concentrations in the oxic layer, suggesting the presence of methanotrophs in the chemocline. We obtained two clades of sequences from methylotrophic bacteria: one containing *Methylobacter* and *Crenothrix* spp. (*Gammaproteobacteria*) (Rahalkar *et al*., 2007) and another containing *Methylophilus* and *Methylotenera* spp. (*Betaproteobacteria*) (Kalyuzhnaya *et al*., 2006) (Fig. S8). Sequences from Lake Cadagno related to these bacteria were found at all three depths, but were concentrated in the chemocline, where they comprised *c*. 5% of all the sequences obtained.

Apart from the GSB, the most abundant group of sequences in the chemocline (9% at this depth) constituted a single phylotype of PSB with sequences that were $>$ 99% identical to *C. okenii* DSM 169. *Chromatium okenii* is a large-celled, motile PSB of a distinctive type (Imhoff, 2006) that was readily observed by microscopy. This phylotype was the only PSB sequence found in the chemocline and was not found in the deepest layer.

The high concentration of sulfide in the anoxic zone demonstrates the importance of sulfate-reducing bacteria. Sulfate-reducing *Deltaproteobacteria* constituted 3% of the sequences at 15 m. These included sequences nearly identical to *Desulfocapsa* sp. Cad626 isolated from Lake Cadagno (Peduzzi *et al*., 2003), as well as two sequences similar to those previously detected in Lake Cadagno (clones 22 and 166) (Tonolla *et al*., 2004).

A small number of epsilonproteobacterial sequences were obtained (2% of all sequences), all of which were similar to sequences from microorganisms or microbial communities that transform sulfur compounds. These sequences grouped into two clades: one group having 98% identity with *Sulfuricurvum kujiense* and another group having 94% identity with *Sulfurovum lithotrophicum*. These organisms represent a group of recently characterized bacteria that use inorganic sulfur compounds as electron donors for growth and O_2 or nitrate as an electron acceptor (Inagaki *et al*., 2004; Kodama & Watanabe, 2004). Thus, the epsilonproteobacterial sequences in Lake Cadagno may represent similar chemothioautotrophic organisms that live around the chemocline.

PCR amplification with general primers for archaeal SSU rRNA (see Supporting Information for details) was not successful; out of 47 obtained sequences, only one was archaeal, the rest being eukaryotic SSU rRNA due to nonspecific amplification. Archaea detectable with the general primers used were thus not abundant in the water column. The single archaeal sequence obtained at 15 m (LC15_L00B08) was closely related to a group of uncultivated methanogenic Euryarchaea. It is of interest that archaeal SSU rRNA sequences related to methanogens have been obtained from the sediment (Bottinelli, 2008). Methanogens and methane in millimolar concentrations have been detected previously in the sediment of Lake Cadagno (Wagener *et al*, 1990). Thus, methanogenesis is active in the sediment rather than in the water column. PCR amplification with general primers for eukaryotic SSU rRNA was successful and a detailed analysis of these data will be presented elsewhere (M.F. Jørgensen, L.H. Gregersen, N.-U. Frigaard, unpublished data).

Relationship between cell counts, clone counts, and biovolume

The cell volumes of the phototrophs in Lake Cadagno show considerable variation. In particular, the PSB *C. okenii* has a much greater cell volume than *C. clathratiforme*. This raises the possibility that the relative numbers of sequences generated in clone libraries are not a reliable indicator of the relative contribution of the populations to processes in the lake. Figure 7 shows a comparison of three quantitative measures of the phototrophs in the chemocline of Lake Cadagno. This shows that allowing for the large cell size of *C. okenii* provides a better correlation with clone library frequencies than simple cell counts. This could be explained by greater numbers of SSU rRNA genes in the larger cell as a result of multiple genome copies per cell or multiple SSU rRNA gene copies per genome or both. It is noteworthy that a much smaller number of *Lamprocystis* sequences were obtained than expected based on the number of cell counts. One possible explanation is that the aggregating nature of the *Lamprocystis* cells caused a reduced DNA extraction or the cells to be trapped on the prefilter.

Discussion

Our results show that the bacterial population in Lake Cadagno resembles other freshwater lakes, with the exception of an unusual result with the GSB. We have used a sequencing approach to demonstrate that the vast majority

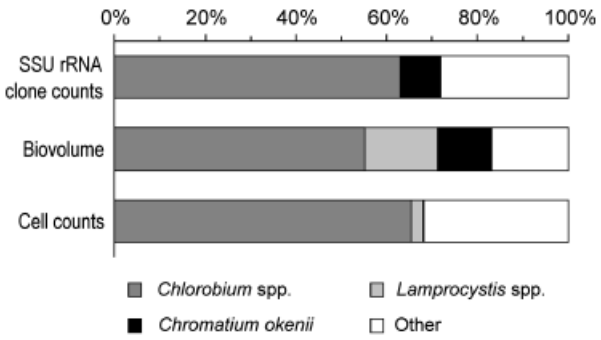

Fig. 7. Comparison of the relative abundance of various microbial groups in the chemocline at 11.5 m calculated using three different methods: occurrence in the SSU rRNA clone libraries (upper bars), estimated biovolume (middle bars), and direct cell counts using DAPI and FISH (lower bars). The biovolume values were derived from the direct cell counts using the following cell volumes: *Chlorobium* spp., 0.8 μm^3; *Lamprocystis* spp., 6 μm^3; *Chromatium okenii*, 60 μm^3; and other cells, 0.5 μm^3 (Tonolla *et al*., 2003).

of the GSB – themselves comprising more than half the SSU rRNA clones and microscopic cell counts obtained in the anoxic water column – have essentially identical sequences not only for the SSU rRNA but also for two other loci encoding proteins specific for the light-harvesting apparatus of the GSB. The small differences in the sequences obtained ($< 0.1\%$) showed no systematic pattern and we ascribe these to sequencing artifacts. We thus conclude that the GSB in the lake is essentially a clonal population.

The emergence of this new population is a relatively recent event. Before 2000 the anoxygenic phototrophs in the lake were mostly PSB and the minority of GSB belonged to another species (*C. phaeobacteroides*) than the currently dominant population (*C. clathratiforme*). In addition to the data from FISH counts (Fig. 3), this conclusion is supported by the results of Bosshard *et al.* (2000), who generated an SSU rRNA clone library from a chemocline sample collected in 1996; 13 out of 45 clones were PSB and the single GSB sequence was identical to *C. phaeobacteroides* DSM 266. As far as we are aware, mass occurrence of a clonal population of GSB has not been reported previously in a typical aquatic environment. Low SSU rRNA sequence diversity has been observed in extreme environments with GSB populations present in small abundance such as the chemocline of the Black Sea (Manske *et al.*, 2005) and sediment from the saline lake Bad Water in Death Valley (CA) (Alexander & Imhoff, 2006). Low SSU rRNA sequence diversity was also reported for one sediment sample from the Sippewisset salt marsh (MA), but a wider diversity was found in another sample from the same site (Alexander & Imhoff, 2006). Lake Kaiike in Japan is a meromictic lake with a bloom of GSB similar to that in Lake Cadagno, but diversity studies using SSU rRNA with group-specific FISH probes and denaturing gradient gel electrophoresis (DGGE) revealed three distinct populations of GSB (Koizumi *et al.*, 2004) even without the greater discrimination provided by multilocus sequence typing as used in the current work. A diversity of GSB populations was also observed in samples from the anoxic water column from Mariager Fjord, Denmark, after sequencing of bands excised from DGGE gels of SSU rRNA obtained with GSB-specific primers (T. Gregersen, J.S. Pedersen & R.P. Cox, unpublished data).

The strain of GSB that now dominates the microbiota of Lake Cadagno obviously has characteristics that differ from the strain that was dominant before 2000. On the basis of the three loci sequenced, the *C. clathratiforme* currently dominating in Lake Cadagno is identical to a GSB with a sequenced genome, *C. clathratiforme* DSM 5477 isolated from Lake Buchensee, a meromictic lake in southern Germany (Overmann & Pfennig, 1989) (accession number CP001110). The previously dominant GSB, represented by *C. phaeobacteroides* strain 1VIID7 previously isolated from Lake Cadagno (S. Peduzzi & M. Tonolla, unpublished data), has an SSU rRNA sequence with a single base difference from that of *C. phaeobacteroides* DSM 266 for which the genome sequence is also available (accession number CP000492). Comparison of the two genome sequences reveals three possibly significant differences: *C. clathratiforme* has gas vesicles, the ability to utilize thiosulfate, and the high-affinity cbb_3 oxidase – properties that *C. phaeobacteroides* lacks. Gas vesicles appear not to yield GSB positive buoyancy, but to decrease the rate of sedimentation in the water column, providing a competitive advantage by allowing the cells to remain for longer periods in the zone where light for photosynthesis is available (Overmann *et al.*, 1991; Garrity & Holt, 2001). Little is known about the utilization of thiosulfate in natural environments (Overmann, 2008), but thiosulfate is generated abiotically at the chemocline by the reaction of sulfide and oxygen (Chen & Morris, 1972) and so it is a potential reductant for CO_2 fixation at the top of the anoxic layer. A high-affinity oxidase could scavenge toxic oxygen and allow growth at the top of the zone containing sulfide or thiosulfate (H. Li, A.M. Jubelirer, A.M.G. Costas, N.-U. Frigaard & D.A. Bryant, unpublished data).

GSB are present at high cell counts from the chemocline to the sediment in Lake Cadagno. It is clear that at the pigment concentrations that are observed, the amount of light reaching the lower levels is so low that phototrophic growth with generation times sufficient to account for the observed population can be excluded. It is noteworthy that the same GSB SSU rRNA sequences were obtained at both 11.5 and 15 m and that the BChl *e* homolog distributions were the same, and so there was no indication of genotypic or phenotypic adaptation to light intensity. Either the GSB in the monimolimnion are metabolically inactive or, less likely, obtain energy from some type of dark metabolism previously not known to occur in GSB. In any case, dwelling in the monimolimnion largely excludes predation by aerobic protists and (if the GSB are metabolically inactive) bacteriophage.

All current approaches to the study of microbial diversity in complex environments suffer from methodological problems. Apart from the limitations caused by nonrepresentative DNA extraction and PCR bias, quantitative analysis of clone libraries is in principle a count of the number of SSU rRNA genes in the sample and thus not directly comparable to cell counting using FISH. The quantitative importance of one type of GSB in Lake Cadagno is supported by clone counts, FISH labeling, and also by direct microscopy of the morphologically distinct cells of *C. clathratiforme*. However, there are discrepancies in our estimates of some other bacterial types that can be ascribed to methodological problems. *Lamprocystis* spp. is under-represented in the SSU rRNA clones, possibly because aggregates were trapped on the prefiliter used. In contrast, the clone library contains more SSU rRNA sequences from *C. okenii* than would be expected from the FISH counts. There is a better match to

biovolume and it would be of interest to investigate whether these unusually large cells contain large numbers of SSU rRNA genes because of some combination of multiple genome copies per cell and multiple rRNA operons on the genome.

It is of interest to speculate about the cause of the relatively rapid population change in the GSB that has occurred since 2000. We refer to this as a bloom, although there is no sign of a reversion to the original situation over the time scale of years, and so the situation is rather different from the temporary population explosions that are commonly observed in aquatic environments. Some of these ephemeral blooms are clonal; for example, Pernthaler *et al.* (2004) reported a clonal bloom of a filamentous bacterium belonging to the *Bacteroidetes*.

At least three types of explanation can be envisaged for the changes in Lake Cadagno. One possibility is a change in the environmental conditions that allowed a previously undetectable ecotype to bloom. There is evidence of an extreme mixing event in 1998–1999 that could have increased the nutrient concentrations in the chemocline, leading to increased light absorbance in the oxic zone, creating light conditions favoring GSB (Tonolla *et al.*, 2005). A second possibility is a genetic change in a single cell that allowed its progeny to dominate the lake after a period of a few years. A third explanation is the introduction of a new strain into the lake, either by natural causes or through human intervention; Lake Cadagno is the site of extensive teaching and research activities and introduction of a new strain via sampling equipment is not inconceivable.

The predominance of a genetically homogenous bacterial population in Lake Cadagno makes this an ideal site for the study of GSB in natural environments, and more generally for the application of transcriptomics and proteomics to environmental samples.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. HPLC chromatogram at 656 nm of pigments extracted from planktonic cells.

Fig. S2. Alignment of 128 random environmental *C. clathratiforme* SSU rRNA sequences.

Fig. S3. Alignment of 23 random environmental *fmoA* sequences from Lake Cadagno.
Fig. S4. Alignment of 39 random environmental *csmCA* sequences from Lake Cadagno.
Fig. S5. Depiction of sequence divergence between *fmoA*, *csmCA*, and SSU rRNA..
Fig. S6. Phylogenetic tree of the SSU rRNA sequences of *Actinobacteria*.
Fig. S7. Phylogenetic tree of the SSU rRNA sequences of *Bacteroidetes*.
Fig. S8. Phylogenetic tree of the SSU rRNA sequences of *Proteobacteria*.
Table S1. Distribution of phylogenetic groups at different depths.

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