

Development of a real-time PCR method for the detection of fossil 16S rDNA fragments of phototrophic sulfur bacteria in the sediments of Lake Cadagno

D. F. RAVASI,¹ S. PEDUZZI,^{1,2} V. GUIDI,^{2,3} R. PEDUZZI,¹ S. B. WIRTH,⁴ A. GILLI⁴ AND M. TONOLLA^{1,2,3}

¹Piora Alpine Biology Centre Foundation, c/o Cantonal Institute of Microbiology, Bellinzona, Switzerland

²Cantonal Institute of Microbiology, Bellinzona, Switzerland

³Microbial Ecology Group, Microbiology Unit, Plant Biology Department, University of Geneva, Geneva, Switzerland

⁴Geological Institute, ETH Zurich, Zurich, Switzerland

ABSTRACT

Lake Cadagno is a crenogenic meromictic lake situated in the southern range of the Swiss Alps characterized by a compact chemocline that has been the object of many ecological studies. The population dynamics of phototrophic sulfur bacteria in the chemocline has been monitored since 1994 with molecular methods such as 16S rRNA gene clone library analysis. To reconstruct paleo-microbial community dynamics, we developed a quantitative real-time PCR methodology for specific detection of 16S rRNA gene sequences of purple and green sulfur bacteria populations from sediment samples. We detected fossil 16S rDNA of nine populations of phototrophic sulfur bacteria down to 9-m sediment depth, corresponding to about 9500 years of the lake's biogeological history. These results provide the first evidence for the presence of 16S rDNA of anoxygenic phototrophic bacteria in Holocene sediments of an alpine meromictic lake and indicate that the water column stratification and the bacterial plume were already present in Lake Cadagno thousands of years ago. The finding of *Chlorobium clathratiforme* remains in all the samples analyzed shows that this population, identified in the water column only in 2001, was already a part of the lake's biota in the past.

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Corresponding author: D. F. Ravasi. Tel.: +41 91 814 6037; fax: +41 91 814 6019; e-mail: damiana.ravasi@ti.ch

INTRODUCTION

To predict how organisms interact and adapt to environmental alterations, it is essential to understand the ecosystem responses to change over long timescales. The geological record offers an invaluable opportunity to investigate the variations in biological systems over long periods of time. Molecular approaches have recently been successfully employed to analyze ancient DNA (i.e. fossil DNA) preserved in sedimentary records, such as lake sediments, to reconstruct past ecosystems (Coolen & Overmann, 1998; Coolen *et al.*, 2004a,b, 2008; Bissett *et al.*, 2005; D'Andrea *et al.*, 2006; Coolen & Gibson, 2009; Epp *et al.*, 2010). Permanently stratified lakes especially provide excellent preservation conditions for ancient DNA because of their anoxic bottom waters and undisturbed laminated sediments (Coolen & Gibson, 2009). So far, intact DNA of anoxygenic phototrophic

bacteria could be extracted from up to 9100-year-old Holocene lake sediments (Mahoney Lake, Canada) and was analyzed by polymerase chain reaction (PCR) amplification and sequencing (Coolen & Overmann, 1998).

Lake Cadagno, in the southern Swiss Alps ($46^{\circ}33'N$, $8^{\circ}43'E$), has an estimated age of 10 000–12 000 years and is the result of glacial erosion during the last glacial period and the formation of a moraine acting as a small dam (Krig, 1918; Boucherle & Züllig, 1988; Stapfer, 1991; Del Don *et al.*, 2001). It represents a unique opportunity for the study of past ecosystems in permanently stratified (i.e. meromictic) lakes in the Alps, as only very few lakes presenting the characteristic stratification remain in this mountain range. The permanent stratification of Lake Cadagno is induced by the presence of underwater saline springs that create a density difference between surface (mixolimnion) and bottom (monimolimnion) waters (Hanselmann & Hutter, 1998; Peduzzi

et al., 1998; Tonolla *et al.*, 1999; Del Don *et al.*, 2001; Tonolla & Peduzzi, 2006). At the interface between the two water layers, a zone of steep gradients of oxygen, sulfide, and light, namely the chemocline, supports the development of a dense microbial community, mainly composed of anoxygenic phototrophic bacteria. The bacteria in the chemocline have been identified using molecular methods, such as 16S rRNA gene clone library analysis and subsequent *in situ* hybridization with specific fluorescent probes, and belong mainly to two families: the anoxygenic phototrophic purple (Chromatiaceae) and green (Chlorobiaceae) sulfur bacteria (Tonolla *et al.*, 1999, 2003, 2004; Peduzzi *et al.*, 2003a,b).

Yearly monitoring carried out between 1994 and 2003 on the chemocline's bacterial populations showed large temporal shifts in abundance between purple and green sulfur bacteria. Purple sulfur bacteria were the most abundant bacteria in the chemocline between 1994 and 2001, representing between 70% and 95% of the phototrophic sulfur bacteria community (Tonolla *et al.*, 2003). A shift in dominance from purple sulfur bacteria to green sulfur bacteria was observed between 2001 and 2003, with the species *Chlorobium clathratiforme* reaching about 95% of the phototrophic sulfur bacteria (Tonolla *et al.*, 2003, 2005; Decristophoris *et al.*, 2009; Gregersen *et al.*, 2009; Habicht *et al.*, 2011). It was suggested that a disruption to the chemocline in 2000 owing to natural causes (i.e. a violent storm) may have altered environmental niches and populations in subsequent years (Tonolla *et al.*, 2005; Decristophoris *et al.*, 2009).

In 1988, Boucherle and Züllig analyzed the sediment of Lake Cadagno. Their work focused on the concentration of the carotenoid okenone, a pigment produced by purple sulfur bacteria, and other carotenoids in the first 170 cm of the sediment. Although their investigation gave some clue about the impact of environmental changes on the bacterial populations in the upper part of the sediment, molecular analyses on the DNA remains of the bacterial populations through the entire lake's sediment record have not yet been conducted.

In September 2009, sediment cores were recovered from the depositional centre of the lake covering the entire Holocene. This continuous record of 11 000-year-old sediments offered a unique opportunity to study the population dynamics of purple and green sulfur bacteria during the lake's entire history based on the analysis of preserved molecular remains. The aims of the present study were (i) to develop a quantitative real-time PCR methodology for specific detection of 16S rRNA gene sequences of Chromatiaceae and Chlorobiaceae populations in the sediment layers of Lake Cadagno; (ii) to perform a quantitative relative comparison of microbial communities in order to reconstruct past population dynamics and environmental conditions, in particular the presence of meromixis; and (iii) to ascertain whether the predominance of *C. clathratiforme* was a previous condition of the lake or is a distinctive trait of the present state.

METHODS

Design of primers and TaqMan probes

Primers and oligonucleotide probes (Microsynth AG, Balgach, Switzerland) (Table 1) were designed using MEGA (v. 4.1) software (available online: <http://www.megasoftware.net/>) based on reference probes previously used for *in situ* hybridization (Tonolla *et al.*, 1999, 2003, 2005; Peduzzi *et al.*, 2011) and modified for real-time PCR. The primers targeted two groups of phototrophic sulfur bacteria that are presently found in the lake's chemocline: purple sulfur bacteria from the Chromatiaceae family (i.e. *Chromatium okenii*, *Lamprocystis purpurea*, *Lamprocystis roseopersicina*, *Lamprocystis* sp. population D, *Candidatus 'Thiodictyon syntrophicum'* population F, *Thiocystis chemoclinalis*, and *Thiocystis cadagnonensis*) and green sulfur bacteria from the Chlorobiaceae family (i.e. *C. clathratiforme* and *Chlorobium phaeobacteroides*). *Thiocystis chemoclinalis* and *T. cadagnonensis* have recently been recognized as species (Peduzzi *et al.*, 2011) as well as *Thiodictyon* sp. population F described as *Candidatus 'Thiodictyon syntrophicum'* sp. nov. (Peduzzi *et al.*, 2012). Partial (98-bp-long on average) bacterial 16S rRNA gene sequences were selectively amplified using population-specific forward primers (Table 1). TaqMan probes and reverse primers were group specific (Table 1). The internal oligonucleotide probes were labeled at the 5'-end with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) and at the 3'-end with the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) (Microsynth AG). Specificity of primers and probes was checked *in silico* by comparing the sequences with those in GenBank database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Real-time quantitative PCR

The real-time PCR amplification reactions were conducted using a 7500 Fast Real-time PCR System instrument (Applied Biosystems, Rotkreuz, Switzerland). Optimal conditions were 2 min at 50 °C, 10 min at 95 °C followed by 50 cycles including denaturing (15 s at 95 °C) and primer annealing (1 min at 60 °C). The 20-μL reactions contained 10 μL of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), 0.9 μM of forward and reverse primers, 0.2 μM of probe, and 2 μL of DNA template. The lack of inhibition was checked by adding a TaqMan® Exogenous Internal Positive Control – VIC™ Probe (Applied Biosystems) to each reaction tube. In addition, each amplification run comprised one reaction devoid of DNA template as a negative control. Finally, quantification of fossil bacterial DNA was performed in duplicates to test reproducibility of the results. The data were analyzed with the 7500 Fast System SDS Software v. 1.4.0 (Applied Biosystems) with the auto settings used for the baseline.

Table 1 Primers and probes used for the real-time PCR

Primers/probes	Target*	Oligonucleotide sequence (5' → 3')	Reference or source
ProbePSBbis(f)	Chromatiaceae, 16S rDNA, pos. 443–468	CACAAAAGAACCGGCTAACTCCG	This study (consensus)
S453Cbis(f)	<i>Chromatium okenii</i> (DSM 169), 16S rDNA, pos. 431–453	AACCTGGTGTAAATACCCATCG	Tonolla <i>et al.</i> (1999)
S453Abis4(f)	<i>Lamprocystis purpurea</i> (DSM 4197), 16S rDNA, pos. 438–463	GTCGTTGGATAATACCCCTGGCGA	Tonolla <i>et al.</i> (1999)
S453Ebis(f)	<i>Lamprocystis roseopersicina</i> (DSM 229), 16S rDNA, pos. 433–458	GCATTTGGTTAACCTGGGAATG	Tonolla <i>et al.</i> (1999)
S453Dbis3(f)	<i>Lamprocystis</i> sp. population D (clone 261), 16S rDNA, pos. 433–458	GCGGCTGGTTAACCTGGGCTG	Tonolla <i>et al.</i> (1999)
S453Fbis2(f)	<i>Candidatus 'Thiodictyon syntrophicum'</i> (clone 371), 16S rDNA, pos. 434–458	GCCTTGTYTAATACCCATGAGGG	Peduzzi <i>et al.</i> (2012)
S453Hbis2(f)	<i>Thiocystis chemoclinalis</i> (clone 222), 16S rDNA, pos. 436–456	GGGCGTTAACCGTCCCGTC	Peduzzi <i>et al.</i> (2011)
S448bis3(f)	<i>Thiocystis cadagnonensis</i> (clone 249), 16S rDNA, pos. 436–454	CGGGGGTCAATACCCCTGC	Peduzzi <i>et al.</i> (2011)
PSB(r)	Chromatiaceae, 16S rDNA, pos. 483–502	GCTTGACCCCTCCGTATTAC	This study (consensus)
ProbeGSBbis(f)	<i>Chlorobium clathratiforme</i> (DSM 5477), 16S rDNA, pos. 156–181	TCGCCAAAGGATGAACCTATATCCCA	This study
Pld190bis(f)	<i>C. clathratiforme</i> (DSM 5477), 16S rDNA, pos. 125–145	ACAATCGATGGTTCTGC	Tonolla <i>et al.</i> (2005)
GSBbis(r)	<i>C. clathratiforme</i> (DSM 5477), 16S rDNA, pos. 220–239	TCTCAGACCGACTACCCGT	This study
ProbeGSBbis2(f)	<i>Chlorobium phaeobacteroides</i> , (DSM 266), 16S rDNA, pos. 398–421	CGGGACTGACGGTACTCCCGAAT	Tonolla <i>et al.</i> (2003)
Chlp441bis(f)	<i>C. phaeobacteroides</i> , (DSM 266), 16S rDNA, pos. 373–395	GTGGAGGAAGAATATCCCGATT	Tonolla <i>et al.</i> (2003)
GSBbis3(r)	<i>C. phaeobacteroides</i> , (DSM 266), 16S rDNA, pos. 510–535	GGATTAAACCCCCGACTTATGAATCC	Tonolla <i>et al.</i> (2003)

f, forward; r, reverse.

*pos., position on the 16S rDNA according to the *Escherichia coli* numbering (Brosius *et al.*, 1981).

Standard curves and detection limits determination

Total DNA from pure culture cells of the nine targeted bacterial populations was extracted using the InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. The DNA extracted from each bacterial culture was then subjected to PCR by using the primers previously described (Table 1) to amplify the targeted 16S rDNA fragments. The amplicons were purified by gel filtration using Sephadex® G-100 (Sigma-Aldrich, St. Louis, MO, USA) and quantified with the fluorescent dye Pico-Green® (Molecular Probes, Eugene, OR, USA). Serial tenfold dilutions of purified amplicons ranging from 1×10^7 to 1×10^0 copies μL^{-1} were prepared in TE buffer solution. A quantitative standard curve was plotted with the C_t values (threshold cycle) as a function of the amplicons concentration. The threshold was set at 0.02. PCR efficacies were calculated with the formula $[(10^{-1/\text{slope}})-1] \times 100$ (Cordier *et al.*, 2007). The detection limit of the assay was defined as the lowest number of copies that could be detected.

Between 10^2 and 10^6 copies μL^{-1} (tenfold dilution series) of specific 16S rDNA amplicons were added to reaction mixtures and served as standards during real-time PCR to calibrate the copy numbers of template DNA in the sediment samples.

Verification of real-time PCR inclusivity and exclusivity

Real-time PCR inclusivity and exclusivity were verified using pure cultures of the nine bacterial populations included in this study. For each culture, we performed a real-time PCR using the population-specific primers-probe set, as well as the primers-probe sets specific to the other eight populations. Additional tests were performed on a control sediment sample free of targeted bacteria obtained from Lake Maggiore, Ticino.

The sediment was first dried at 60 °C overnight. Five-gram samples of the control sediment were inoculated with 10 μL of DNA extracted from the pure culture of all nine study populations. The samples were then submitted to DNA extraction with PowerMax™ Soil DNA Isolation Kit (Mobio, Carlsbad, CA, USA) following the recommendations of the manufacturer. The DNA extracted from the subsamples was then subjected to real-time PCR with the primers and probes for the study populations.

Verification of real-time PCR specificity

The European standard ISO 16140 defines qPCR specificity as target detection without interference of sample components. To verify qPCR specificity, we initially quantified the number of cells contained in 50 μL of a pure culture of *Candidatus 'Thiodictyon syntrophicum'*. Ten microliters of the culture were stained with 4'-6-Diamidino-2-phenylindole (DAPI), and the bacterial cells were counted under a fluorescence microscope. The number of cells was extrapolated to 2.93×10^7 cells mL^{-1} of culture. A dried control sediment sample obtained from Lake Maggiore, previously described, was used for the test. We performed three extractions from (i) 5 g sediment inoculated with 50 μL of solution containing 1.18×10^6 cells of *Candidatus 'Thiodictyon syntrophicum'* (corresponding to 2.36×10^5 cells g^{-1}); (ii) 50 μL of solution containing the same amount of cells of *Candidatus 'Thiodictyon syntrophicum'* without sediment; (iii) 5 g sediment inoculated with 10- μL amplicons (0.5×10^6 copies μL^{-1}) from *Candidatus 'Thiodictyon syntrophicum'*, corresponding to a final concentration of 1×10^6 copies g^{-1} of sediment. For the extraction tests (i) and (ii), a DNase treatment step was performed on the cell suspension before sediment inoculation to eliminate free DNA. Briefly, 5 μL

DNase buffer 10× (400 mmol⁻¹ Tris-HCl, pH 8.0, 30 mmol⁻¹ MgCl₂, 30 mmol⁻¹ MgSO₄) and 200 U mL⁻¹ DNase I (Roche Diagnostic AG, Rotkreuz, Switzerland) were added to cell culture, which was incubated at 25 °C for 30 min. The enzyme was heat-inactivated at 75 °C for 20 min. For all three tests, samples were subjected to DNA extraction as described previously. The DNA extracted was diluted in 70-µL TE buffer solution. The number of copies of 16S rDNA of *Candidatus 'Thiodictyon syntrophicum'* was quantified through real-time PCR.

Sediment core retrieval and age model

Two composite cores (inner diameter, 5.9 cm) were retrieved in September 2009 at the same location in the deepest part of the Cadagno lake basin (i.e. 21-m water depth) using a UWITEC percussion piston corer. The undisturbed water sediment interface was recovered with a gravity short corer. Core 1 was transferred to the ETH, Zürich, for detailed sedimentological analysis and radiocarbon dating from terrestrial organic remains. Core 2 was longitudinally split, opened, photographed, and sampled at the Alpine Biology Center in Piora, immediately after its retrieval. Core 2 was sampled in the uppermost meter in 5-cm sections and below into a total of 85 10-cm-long sample intervals. Precautions were taken to prevent cross-contamination as described by Coolen & Overmann (1998), and samples for DNA extraction were stored in 50-mL tubes at -20 °C. The robust age model of core 1 could be transferred to core 2 on the basis of over 68 tie points established by visual correlation. The radiocarbon ages were calibrated using the online calibration software OxCal 4.1 (<https://c14.arch.ox.ac.uk/oxcal/OxCal.html>) (Bronk Ramsey, 2009) and the Intcal09 calibration curve (Reimer *et al.*, 2009). All ages are reported as calibrated years before present (cal yr BP), where 1950 is by convention the origin of the BP scale. It is however important to note that the sedimentary column of Lake Cadagno consists more than half of mass-movement material and flood deposits, which contain no paleoenvironmental information. Therefore, sample intervals containing solely reworked material were excluded from the DNA extraction. Sixteen samples were then chosen for DNA extraction in this study.

Extraction of total DNA

Total DNA was extracted from 5 g of wet weight sample using the PowerMax™ Soil DNA Isolation Kit (Mobio). Precautions against contamination of the samples with foreign DNA were applied following Coolen & Overmann (1998). DNA was diluted with 50 µL TE buffer solution and the concentration of extracted DNA was quantified with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To standardize the results, dry

weight was calculated for each sample by allowing 1 g of wet sample to dry overnight at 60 °C. The percentage of humidity was calculated as (wet weight-dry weight)/wet weight × 100. The dry weight of the 5-g sediment sample was then extrapolated. For each of the sediment core samples, the number of copies of 16S rDNA of the nine targeted bacterial populations was quantified by real-time PCR. The results obtained by real-time PCR were normalized to copies µg⁻¹ of extracted DNA to compensate for possible variation in the extraction efficiencies between core samples.

Test of variance on extraction

A test of variance on extraction and real-time PCR was conducted using four subsamples of one sediment from Lake Cadagno known to contain fossil DNA of *C. okenii*. Total DNA was extracted from the four subsamples using the PowerMax™ Soil DNA Isolation Kit (Mobio) as described previously. The number of copies (g dry weight sample)⁻¹ of 16S rDNA of *C. okenii* was then quantified by using real-time PCR with the specific primers and probe. Mean number of copies was 6×10^3 copies (g dry weight sample)⁻¹. Variability among the samples was very low (Var = 0.0006). Therefore, one subsample was used for the DNA extraction from sediments.

RESULTS

Standard curves and detection limits

Standard curves were created for each primers/probe set (for each of the nine targeted bacterial populations) by plotting the *C_t* values (threshold cycle) as a function of the number of 16S rDNA copies. All standard curves showed a good confidence in correlating the quantity values to *C_t* values ($R^2 > 0.96$ in all cases). The amplification efficiency was on average 104% ($\pm SD$ 13.9). The detection limit (i.e. the sensitivity) of the TaqMan real-time PCR varied among the targeted bacterial populations. *Chromatium okenii*, *Candidatus 'Thiodictyon syntrophicum'*, and *C. phaeobacteroides* showed the highest sensitivity with down to 5 16S rDNA copies µL⁻¹ detected. Most of the populations (i.e. *L. purpurea*, *L. roseopersicina*, *T. chemoclinalis*, and *C. clathratiforme*) showed a limit of 50 16S rDNA copies µL⁻¹ detected. Two populations (i.e. *T. cadagnonensis* and *Lamprocystis* sp. population D) showed a sensitivity of only 500 copies of 16S rDNA µL⁻¹ detected.

Verification of real-time PCR inclusivity and exclusivity

To verify real-time PCR inclusivity and exclusivity, tests were performed on the pure cultures of the nine bacterial study populations. Each pure culture was subjected to real-time PCR using the primers-probe set specific to the population as

well as the primers–probe set specific to the other eight populations. In addition, nine control sediments were inoculated each time with 10 µL of DNA extracted from the pure culture of a bacterial population, for all nine study populations. In each case, the amplification of the targeted bacterial population by real-time-PCR-specific primers–probe sets was positive (data not shown), demonstrating the inclusivity of the real-time PCR. The real-time PCR performed with primers–probe sets for the non-targeted bacterial populations did not show amplification (data not shown), demonstrating the exclusivity of the real-time PCR.

Verification of real-time PCR specificity

The number of copies recovered by real-time PCR following the extraction from (i) a control sediment inoculated with 2.36×10^5 cells g⁻¹ of *Candidatus ‘Thiodictyon syntrophicum’* and (ii) a solution containing the same number of cells but without sediment were in the same order of magnitude (3.2×10^5 and 4.8×10^5 copies g⁻¹ dry weight sample, respectively). These results showed that the presence of sediment did not affect the efficiency of DNA extraction of the bacterial population and the qPCR specificity. The number of copies recovered by real-time PCR from (iii) the control sediment inoculated with 1×10^6 copies g⁻¹ of *Candidatus ‘Thiodictyon syntrophicum’*-specific amplicons, was 8.5×10^2 copies g⁻¹ dry weight sample, thus much lower than the number that was originally inoculated in the sediment. This could be due to the much smaller size of amplicons (approximately 100 bp) that we used for this test, compared to whole cell DNA. The small-sized amplicons are possibly more likely to be retained by the sediment during the extraction process. Consequently, only a part of the inoculated amplicons might have been extracted from the sediment and quantified through real-time PCR.

Total DNA extracted from the sediment core

After optimization of the extraction protocol, genomic DNA was isolated from the 16 sediment samples from core 2. Between 1 and 126 µg of DNA was extracted per g of dry weight sample (Fig. 1). Total DNA concentration varied among the different sediment depths. In the older part of the sediment core from 9500 to 1120 cal yr BP, total DNA concentration did not exceed 10 µg g⁻¹ dry weight sample (Fig. 1). The amount of DNA gradually increased in the upper sediment core. Between 1100 and 100 cal yr BP, total DNA concentration per g dry weight sample varied between 10 and 40 µg. The highest amount of DNA was recovered in the first 10 cm of the sediment, which corresponds to the most recent century of the lake’s history (Fig. 1).

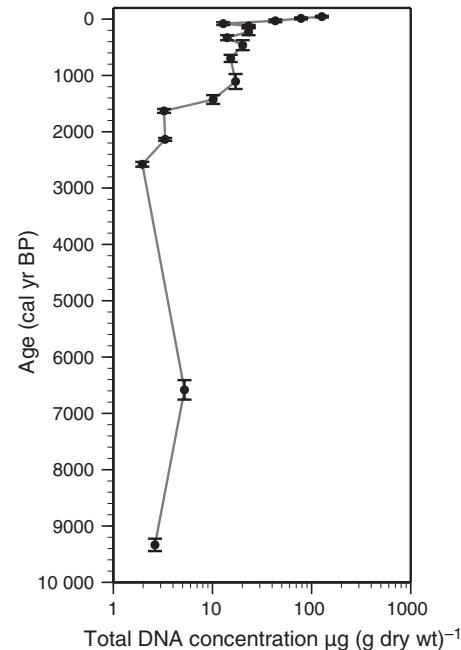


Fig. 1 Total DNA concentration in µg g⁻¹ dry sediment in sediment core samples from Lake Cadagno. One-sigma error of the total DNA concentrations is for all samples smaller than the size of the symbol and therefore not displayed. The vertical error bars represent the age range covered by the samples.

Specific amplification and quantification of 16S rDNA from purple and green sulfur bacteria

The results obtained by real-time PCR were normalized to copies µg⁻¹ of extracted DNA to compensate for possible variation in the extraction efficiencies between core samples (Fig. 2). The real-time PCR analysis revealed that there was always at least one of the studied species of bacteria present in each sediment sample analyzed, down to a depth of almost 9 m, corresponding to approximately 9450 cal yr BP (Fig. 2). A lower amount of 16S rDNA was recovered in older sediments compared to more recent ones, although variations were present between the populations. For *Candidatus ‘Thiodictyon syntrophicum’*, 16S rDNA was detected starting from 2535 to 2620 cal yr BP, while for three other populations, namely *L. purpurea*, *T. cadagnonensis*, and, to some extent, *L. roseopersicina*, the fossil 16S rDNA was not detected before 2110–2160 cal yr BP. In contrast, the 16S rDNA of *Lamprocystis* sp. population D was not found in sediments between 1595 and 2620 cal yr BP, but was present in older sediments as well as in the upper part of the core. For all nine populations of phototrophic bacteria analyzed, the highest values of 16S rDNA were detected in the most recent deposited sediment (Fig. 2). Higher amounts of 16S rDNA were found in the sediment samples younger than 140 cal yr BP compared to the older part of the sediment core. The fossil 16S rDNA of *C. okenii* and *C. phaeobacteroides* was found in all analyzed samples and showed less variability compared to the other

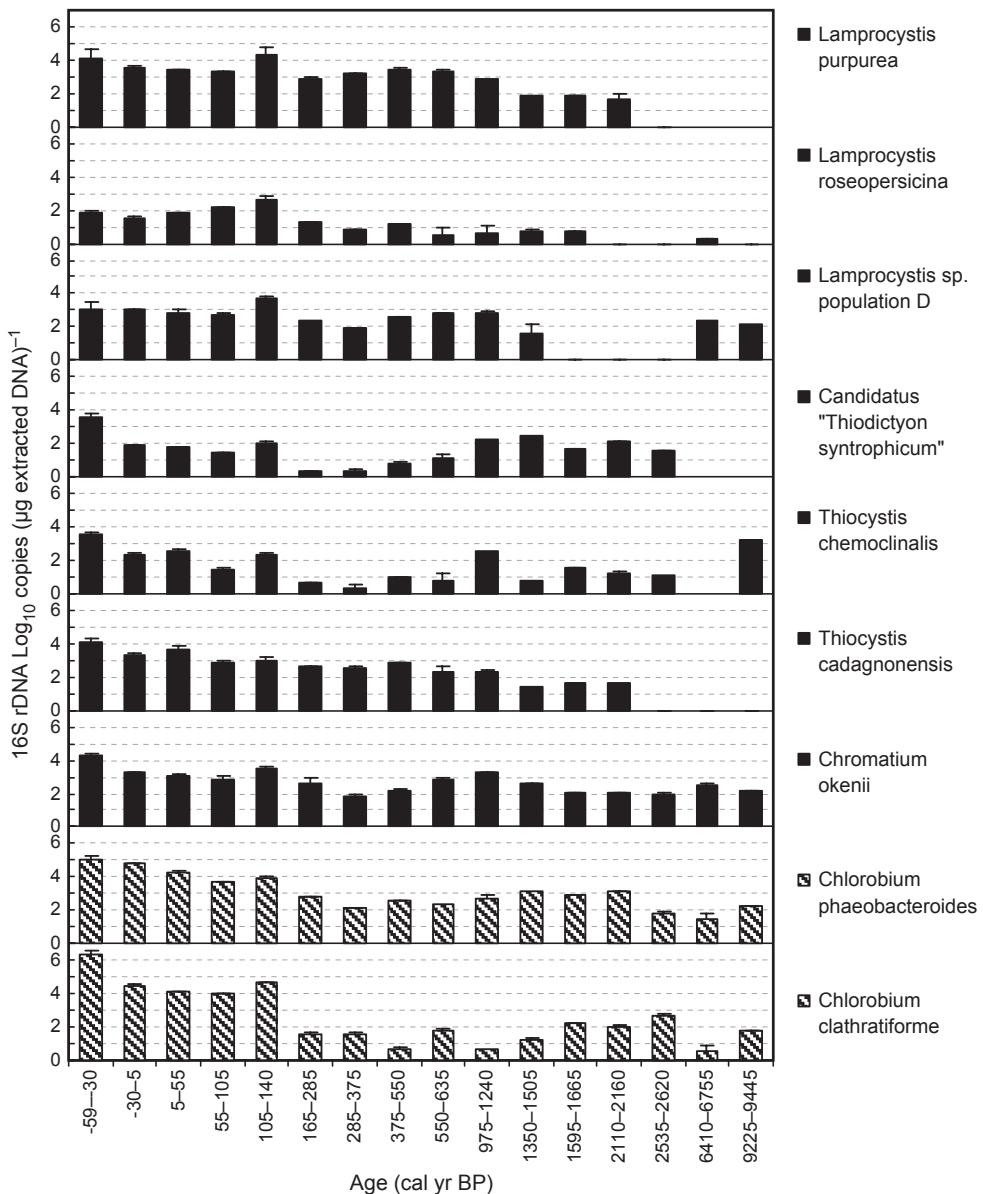


Fig. 2 Number of 16S rDNA copies μg^{-1} extracted DNA for each of the nine targeted bacterial populations as a function of the sediment age. Purple sulfur bacteria are represented by black columns; green sulfur bacteria are represented by a diagonal pattern. Ages are reported in cal yr BP rounded to the nearest 5. Negative years indicate depositional ages younger than 1950 due to the convention that the origin of the BP scale is 1950.

populations. *Chlorobium clathratiforme* was also recovered from all analyzed sediment samples and showed a clear increase starting from 105 to 140 cal yr BP upwards.

DISCUSSION

In this study, we were able to develop a highly specific real-time PCR method for the detection and quantification of nine phototrophic sulfur bacterial populations in sediment samples. This method is based on a TaqMan real-time PCR amplification of partial bacterial 16S rDNA gene sequences (98 bp

on average). The real-time PCR was sensitive, although the detection limit varied among the targeted populations. The method was consequently applied to the analysis of the fossil remains of the nine bacterial populations in a sediment core extracted from Lake Cadagno.

We were able to successfully extract fossil DNA from Lake Cadagno's sediment down to a depth of 9 m. Through amplification of fossil 16S rDNA sequences, we were also able to detect the presence of fossil DNA of phototrophic sulfur bacteria back to approximately 9500 years BP, providing the first evidence for the presence of DNA of anoxygenic

phototrophic bacteria in Holocene sediments of an alpine meromictic lake. It is thus reasonable to conclude that the water stratification (i.e. meromixis) and phototrophic sulfur bacteria have been present in Lake Cadagno since then. The importance of phototrophic sulfur bacteria for the sulfur, carbon, and nitrogen cycles in Lake Cadagno has already been underlined by various studies (i.e. Luthy *et al.*, 2000; Camacho *et al.*, 2001; Halm *et al.*, 2009; respectively). The efficient preservation of fossil DNA in Lake Cadagno's sediment can be attributed to low temperatures (Willerslev *et al.*, 1999), anoxic conditions, and a high concentration of organic material (Coolen & Gibson, 2009).

Our study shows a general decrease in the amount of fossil 16S rDNA quantified from sediments older than 200 years (corresponding to a sediment depth of approximately 24 cm, Fig. 1) in all studied populations, suggesting the possibility of DNA degradation after a given time after deposition. According to a study by Birch *et al.* (1996) on a short sediment core, the content of water and organic matter in the sediments showed a gradual decrease from the subsurface downwards to 50 cm sediment depth, indicative of a slightly increasing compaction and decomposition with depth, and a corresponding decrease in porosity. This is also confirmed by our age model showing a strong decrease in sedimentation rate in the top-most 50 cm. However, while remains of the populations *L. purpurea* and *T. cadagnonensis* were not found in sediment samples older than 2200 years (Fig. 2), vestiges of the other five populations were found in the oldest part of the sediment core, thus providing evidence in favor of the idea that in Lake Cadagno, 16S rRNA gene fragments remain preserved in the order of magnitude of thousands of years after its deposition. The extent to which fossil DNA is preserved and the factors that control the preservation of DNA remain largely unknown (Coolen & Gibson, 2009). A study by Coolen & Overmann (1998) in similar settings showed that the majority of the genomic DNA was degraded to shorter fragments (approximately 600 bp) within the first several thousand of years after deposition. The same study also showed that further decrease in fragment size did not occur between 4000 and 10 000 cal yr BP. From the results of the present study, it seems that degradation to smaller than 100-bp-long fragments did not occur. Thus, possible real-time PCR bias owing to degradation was very limited. When quantifying the amount of 16S rRNA gene fragments from sediments, it is however important to take into account other methodological aspects like lysis efficiency in DNA extraction, amplification efficiency, and detection limits of the PCR (Lenk, 2006).

A general higher quantity of fossil 16S rDNA in the core samples <200 years old could possibly be linked to the increased presence of human habitations and agriculture around the lake, followed by anthropogenic emissions in Lake Cadagno and consequent eutrophication (Boucherle & Züllig, 1988). The construction of the dam in 1948 could also have had an impact on the bacterial production and on

the deposition of organic matter, which could be reflected in the first few cm of the sediment. However, in contrast to the large increase in pigment concentration that was observed by Boucherle & Züllig (1988), no particular effects on the amount of total DNA in the time period since the construction of the dam were noticed (Fig. 1).

In addition to the successful extraction and amplification of fossil 16S rDNA of phototrophic sulfur bacteria, nine populations of the two groups of purple- and green sulfur bacteria could also be individually distinguished (Fig. 2). Although other studies on the sediments of Lake Cadagno showed the presence of Cladocera (Crustacea) remains, bacterial pigments (Boucherle & Züllig, 1988), and sulfate-reducing bacteria and methanogenic Archaea (Bottinelli, 2008) down to 170-cm sediment depth, this is the first study that proves that specific sulfur bacterial populations have been present since the early Holocene soon after the formation of the lake. Because of the variability in the quantity of population-specific 16S rDNA fragments amplified from various sediment depths, we can speculate that complex population dynamics occurred throughout the existence of Lake Cadagno. The comparison of 16S rDNA quantities between the nine studied bacterial populations showed that at least one population of purple sulfur bacteria was always present in the sedimentary record (Fig. 2). This corresponds to the constant recovery by Züllig (1985) of the pigment okenone, typically found in this group of bacteria, in the first 170 cm of sediment. There did not seem to be a striking dominance of one or more populations over the others (Fig. 2).

We were surprised to recover remains of the green sulfur bacterium *C. clathratiforme* from the sediments. According to the yearly analyses on the water column carried out since 1994, this population was not present in the lake before 2001 and was thought to be a new comer (Bossard *et al.*, 2000a,b; Tonolla *et al.*, 2005; Gregersen *et al.*, 2009). However, the present study demonstrated that the population was already part of the lake's biota in the past (Fig. 2). The identification of *C. clathratiforme* from the sediment does not confirm Gregersen *et al.*'s (2009) hypothesis that the strain might have been recently introduced into the lake through human intervention. Instead, it supports the hypothesis that *C. clathratiforme* was already present in the lake before 2000 as an undetectable population and that a change in environmental conditions allowed it to bloom (Tonolla *et al.*, 2005; Gregersen *et al.*, 2009).

CONCLUSIONS

This work contributes to the knowledge on the microbial ecology of Lake Cadagno by providing new information for the reconstruction of past population dynamics of phototrophic sulfur bacteria. It was also possible to determine that the green sulfur bacteria *C. clathratiforme* was already part of the lake's biota in the past and it is thus not a newcomer

characterizing the present lake state. Future studies could extend the identification range of fossil bacterial remains by sequencing and subsequent phylogenetic analysis to better understand the small-scale microbial dynamics in this specific lake setting. In addition, sediment cores from different depth zones of the lake could be used to confirm our results and constrain the variability in the lateral extent of the chemocline.

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