

# Quantitative PCR Enumeration of Total/Toxic *Planktothrix rubescens* and Total Cyanobacteria in Preserved DNA Isolated from Lake Sediments<sup>∇†</sup>

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**The variability of spatial distribution and the determinism of cyanobacterial blooms, as well as their impact at the lake scale, are still not understood, partly due to the lack of long-term climatic and environmental monitoring data. The paucity of these data can be alleviated by the use of proxy data from high-resolution sampling of sediments. Coupling paleolimnological and molecular tools and using biomarkers such as preserved DNA are promising approaches, although they have not been performed often enough so far. In our study, a quantitative PCR (qPCR) technique was applied to enumerate total cyanobacterial and total and toxic *Planktothrix* communities in preserved DNA derived from sediments of three lakes located in the French Alps (Lake Geneva, Lake Bourget, and Lake Annecy), containing a wide range of cyanobacterial species. Preserved DNA from lake sediments was analyzed to assess its quality, quantity, and integrity, with further application for qPCR. We applied the qPCR assay to enumerate the total cyanobacterial community, and multiplex qPCR assays were applied to quantify total and microcystin-producing *Planktothrix* populations in a single reaction tube. These methods were optimized, calibrated, and applied to sediment samples, and the specificity and reproducibility of qPCR enumeration were tested. Accurate estimation of potential inhibition within sediment samples was performed to assess the sensitivity of such enumeration by qPCR. Some precautions needed for interpreting qPCR results in the context of paleolimnological approaches are discussed. We concluded that the qPCR assay can be used successfully for the analysis of lake sediments when DNA is well preserved in order to assess the presence and dominance of cyanobacterial and *Planktothrix* communities.**

Eutrophication of freshwater ecosystems is accompanied by changes in diversity and abundance of the phytoplankton, leading to a decrease in planktonic diversity and, usually, a dominance of cyanobacteria (14, 15). Proliferation of toxic cyanobacteria in aquatic ecosystems has been associated with major ecological disturbances, such as a decrease in aquatic biodiversity, with potential consequences for the entire food web. The toxic potential of several genera of planktonic freshwater cyanobacteria producing microcystins (MCs), the most prevalent cyanobacterial hepatotoxins in freshwaters, may also represent a considerable health risk for both wild and domestic animals as well as for human beings (7). Toxic cyanobacteria can even occur in less eutrophic systems, such as deep mesotrophic lakes in the Alps (7, 29). In the latter case, *Planktothrix rubescens* is currently the dominant species reported for the following European subalpine lakes: Zurich (Switzerland) (55, 56), Garda

(Italy) (48), Mondsee (Austria) (27), Nantua (France) (17), and Bourget (France) (23, 24). *Planktothrix* is one of the most important MC-producing genera in temperate lakes (27). Various chemical, physical, and biological parameters are involved in the development of cyanobacteria (12), and the variability of the spatial distribution (biogeography), the determinism of cyanobacterial blooms, and their impact at the lake scale are not understood. Several studies have highlighted the importance of long-term data sets in attempting to elucidate these ecological questions (2, 3, 20, 24, 57). However, existing long-term biomonitoring programs are usually incomplete and started, at best, in the middle of the 1950s, when the majority of the lakes (in temperate regions) were already impacted by cyanobacterial blooms. The paucity of long-term climatic and environmental monitoring data on the water body can be compensated by the use of proxy data from high-resolution sedimentary records (22). Fossilized organic components provide an archive of ancient aquatic microbial communities and hence can be used to reconstruct variations in climate and their impact on biodiversity (9). Preserved DNA can provide information at the species level by phylogenetic comparison, as well as data on quantitative proportions of species, and DNA has been shown to be preserved in the sediments of stratified lakes with anoxic and cold bottom waters (5, 9, 11). A strong DNA

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adsorption to mineral and organic matrices in sediments is also thought to be responsible for enhanced preservation due to protection against degradation by nucleases (4, 10, 44, 47, 49). Consequently, the cells of different microorganisms may often be preserved in deep lake sediments with sufficient fidelity to allow taxonomic identification or ecological inference, thus producing an archive of limnological history.

To date, most preserved DNA analyses have been performed on DNA from permafrost zones, halite crystals, amber, and marine sediments (4, 9, 10, 13, 41). Sediment records have been applied to a range of global environmental issues, including lake acidification, eutrophication, climate change (22), and the effect of human activity on the environment (34). In this sense, molecular biology-based techniques, such as PCR and quantitative PCR (qPCR), represent powerful tools for providing valuable information about *in situ* microbial dominance and diversity. Recently, *Taq* nuclease assays (TNAs) were designed and applied for quantification of total and toxic *Planktothrix* populations in water samples (6, 40, 50). However, to our knowledge, no comparable approach has been reported from the paleolimnological point of view, particularly regarding the quantification of *Planktothrix* and cyanobacteria in the preserved DNA derived from lake sediments. However, the comparability of such studies between environments, or even between different depths in the same sediment core, depends on various factors, e.g., sedimentary processes (ultimately explaining the efficiency of the preservation of organic matter) or methodological issues such as nucleic acid yield and amplification efficiency for each particular sediment type (33). Additionally, studies on preserved DNA are complicated by the extreme sensitivity of analytical techniques to DNA contamination and degradation, requiring adequate test procedures for both experimental and authentication methodology. The sensitivity of qPCR to even a small amount of coextracted inhibitors, such as organic matter in humus-rich sediments, might cause erroneously low estimates of the target. Therefore, these facts necessitate proper validation of the method before applying it to real environmental sediment samples.

The primary goal of this study was to demonstrate that qPCR can be applied successfully to enumerate total cyanobacterial and total/toxic *Planktothrix rubescens* and *Planktothrix agardhii* populations in preserved DNA derived from the sediments of three lakes located in the French Alps. This methodological validation represents an essential step for developing the analytical approach for further application to large-scale analyses of whole deep-sediment cores in order to understand the historical presence of cyanobacteria and their impact on lake ecology.

## MATERIALS AND METHODS

**Culture sample collection.** A total of 21 *P. rubescens*, 17 *P. agardhii*, and 26 other cyanobacterial and algal clonal but nonaxenic strains were used for the testing of primers and probes (see Table S1 in the supplemental material). Strains were either obtained from the Thonon Culture Collection (TCC) (INRA, France) or supplied by international culture collections. All strains were grown under sterile conditions in BG11 medium (46) under continuous light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at  $20 \pm 2^\circ\text{C}$ . Cells were harvested during the early stationary phase and subjected to counting by microscopy or DNA extraction as detailed below.

**Lake sediment samples.** Our approach was based on analyses of sediment cores from three lakes located in the French Alps (Lake Geneva [Leman], Lake Bourget, and Lake Annecy) (see Table S2 in the supplemental material). Lake

Bourget ( $45^\circ44'\text{N}$ ,  $5^\circ52'\text{E}$ ; 18 km long by 2.8 km wide; maximum depth, 145 m; mesotrophic) is a hard-water lake at the northwestern edge of the French Alps (19). Five sediment cores were obtained from both the southern basin and the northern basin of Lake Bourget (cores B1 to B5) (see details in Table S2). One sediment core from Lake Annecy (A1) was retrieved at the central point, at a 65-m depth, in 2010. Lake Annecy ( $45^\circ51'\text{N}$ ,  $6^\circ10'\text{E}$ ; 14.6 km long by 3.2 km wide; maximum depth, 82 m; oligotrophic) is known as "Europe's cleanest lake." Sediment core L1 from Lake Geneva ( $46^\circ26'\text{N}$ ,  $6^\circ33'\text{E}$ ; 73 km long by 14 km wide; maximum depth, 310 m; mesotrophic), the largest lake on the border between France and Switzerland, at the northern end of the French Alps, was taken at the deepest point of the lake. All cores were retrieved by means of a UWITEC gravity corer (Mondsee, Austria) and dated by radionuclides ( $^{210}\text{Pb}$ ,  $^{137}\text{Cs}$ , and  $^{241}\text{Am}$ ) and lamination counting as previously described (19, 39).

Four different slices were subsampled along the deep cores taken from Lake Bourget (B5-1, B5-4, B5-10, and B5-24), Lake Annecy (A1-1, A1-4, A1-10, and A1-24), and Lake Geneva (L1-1, L1-4, L1-10, and L1-24); these slices (1-cm height) were taken at 1-, 4-, 10-, and 24-cm core depths, respectively, to compare distinct regions of the cores which may correspond to the different trophic periods in the lakes (oligotrophic to eutrophic) (19, 39). The dating chronology of the sediment is presented in Table S2 in the supplemental material. For the sediment cores B1 to B4 from Lake Bourget, samples were taken only at a 4-cm depth in each core (samples B1-4, B2-4, B3-4, and B4-4) in order to obtain repeated measures, at the same depth, from different cores taken in the same lake. For each sediment sample, an aliquot of approximately 10 g of homogenized wet sediment was dried at  $50^\circ\text{C}$  and weighed. The rest of the sample was subjected to molecular analysis.

Due to the risk of contamination of the sediment samples by modern DNA, special laboratory precautions against contamination were applied as described previously (separated stations for sampling, DNA extractions, and PCR, sterile disposable labware, and negative controls for PCR) (9, 34).

**Total cell counts from culture samples by microscopic analysis.** In order to test the accuracy of qPCR, *P. rubescens* strain TCC 29 cell numbers determined by microscopy were related to those estimated by qPCR with DNA extracts. *P. rubescens* TCC 29 cell densities were determined from freshly grown cultures preserved with Lugol's solution by use of an optical microscope (Olympus BH-2) equipped with a hemocytometer (Malassez cells). Samples were diluted up to 100-fold (depending on the population density), *P. rubescens* TCC 29 filament lengths were measured at a magnification of  $\times 20$ , and the results were averaged for at least duplicate whole-chamber counting. The number of TCC 29 cells making up the filament was estimated by measuring the total filament length and mean cell length ( $5 \mu\text{m}$  as measured at a magnification of  $\times 400$ ). All image combining, processing, and analysis were performed with the standard software package provided by Olympus (BH-2).

**DNA extraction from culture and sediment samples.** Genomic DNAs (gDNAs) from a wide range of cyanobacterial and other algal cultures were extracted by the DNA extraction protocol with GenElute LPA (linear polyacrylamide) modified by Jardillier et al. (26). Modification included the addition of  $1 \mu\text{l}$  of GenElute LPA (Sigma-Aldrich, Inc.) to aid in DNA recovery.

Genomic DNAs preserved in sediment samples were extracted from 2 or 3 subsamples of 1 g of wet sediment each to avoid any heterogeneity, and resulting DNA extracts were pooled together. DNA was extracted using an UltraClean soil DNA isolation kit (Mobio, Carlsbad, CA) according to the manufacturer's instructions. The DNA extraction was evaluated on the basis of recovery efficiency, reproducibility, resulting DNA integrity, and performance in qPCR analyses. The total DNA concentration was measured using a Quant-iT PicoGreen kit (Invitrogen) on a Rotor Gene 3000 qPCR cyclor with software version 6.0 (Corbett Research, Mortlake, Australia). Quantification with lambda DNA standards (Invitrogen) was performed in triplicate. The DNA was stored at  $-20^\circ\text{C}$  until further analysis.

**Preserved DNA quality check by amplification of the 16S rRNA-ITS region.** Each preserved DNA extract was analyzed for the presence of the cyanobacterial 16S rRNA-internal transcribed spacer (ITS) gene region by PCR (25) to determine the quality and fragment integrity of the DNA throughout the core and to exclude the possibility of PCR inhibition causing negative qPCR results. Thus, the presence of 16S rRNA-ITS gene amplification products (around 1,500 bp) was used as a reference to standardize the PCR. PCR amplification was performed in a volume of  $50 \mu\text{l}$  containing up to 50 ng of a template DNA,  $5 \mu\text{l}$  of  $10\times$  PCR buffer (Bioline), 500 nM (each) forward (CYA371F [5'-CCTACGG GAGGCAGCAGTGGGGAATTTC-3']) and reverse (373R [5'-CTAACCA CCTGAGCTAAT-3']) primers (Biomers), 1.2 mM  $\text{MgCl}_2$  (Bioline), 1 U of *Taq* DNA polymerase (Bioline), deoxynucleoside triphosphates (a 200  $\mu\text{M}$  concentration of each; Bioline), and 1  $\text{mg ml}^{-1}$  bovine serum albumin (BSA; Sigma). All PCRs were performed using a TProfessional Basic gradient thermocycler

(Biometra) and included an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, an annealing temperature of 60°C for 1 min, and elongation at 72°C for 1 min. The amplification products of the 16S rRNA-ITS gene region from preserved DNA were sequenced (Beckman Coulter Genomics) as described previously (31). Obtained sequences were checked for chimera formation and compared with similar sequences available in GenBank by a BLAST search (1).

**qPCR protocols.** Two types of qPCR chemistries were applied in our study: a nonselective fluorescent dye, SYBR green (SG), was explored to quantify total cyanobacteria, while TNAs were applied to enumerate (i) the total population of *Planktothrix* by amplification of two alternatives (the intergenic spacer region of the phycocyanin operon [PC-IGS] region [two alternative primer-probe sets] and the 16S rRNA gene [TNA-16S rRNA]) and (ii) the population of MC-producing *Planktothrix* species via amplification of the *mcyA* region, which is indicative of microcystin biosynthesis (see Table S3 in the supplemental material) (21). Multiplex qPCR assays were then applied to quantify total and toxic *Planktothrix* spp. in a single reaction tube for all sediment samples.

All amplifications were performed in triplicate, using a Rotor Gene 3000 instrument with software version 6.0 (Corbett Research, Mortlake, Australia). Each multiplex reaction mix (25  $\mu$ l) contained 12.5  $\mu$ l of 2 $\times$  Rotor Gene multiplex PCR master mix (Qiagen), a 300 nM concentration of each primer, a 200 nM concentration of each TaqMan probe, and 2  $\mu$ l of template DNA. No-temperature controls and respective positive controls were included in all runs. The TaqMan probes had a fluorescent reporter dye (6-carboxyfluorescein [FAM] or 6-carboxyl-X-rhodamine [ROX]) attached covalently to the 5' end and black hole quencher 1 (BHQ-1) or black hole quencher 2 (BHQ-2) dye (see Table S3 in the supplemental material). The following three qPCR master mixes were evaluated based on their performance during amplification: QuantiTect Probe PCR master mix, with and without ROX passive reference dye (Qiagen), and Rotor Gene multiplex PCR master mix (Qiagen). The singleplex TNA reaction mix (25  $\mu$ l) consisted of 12.5  $\mu$ l of one of the 2 $\times$  PCR master mixes mentioned above, 300 nM (each) forward and reverse primers (Biomers), 200 nM TaqMan probe (Biomers), and 1 to 5  $\mu$ l of template DNA. TNA reactions were initiated by either a 5-min (for Rotor Gene multiplex PCR master mix) or 15-min (for QuantiTect Probe PCR master mixes) hold at 95°C to activate the hot start polymerase, followed by 40 cycles of a two-step PCR consisting of a denaturation step at 95°C for 15 s and subsequent annealing and elongation steps at 60°C for either 15 s (for Rotor Gene multiplex PCR master mix) or 60 s (for QuantiTect Probe PCR master mixes).

For the SYBR green assay, two alternative primer sets were checked to enumerate total cyanobacteria (25): (i) CYA371F-CYA783R, targeting the 16S rRNA gene, and (ii) CSIF-373R, targeting the 16S rRNA-ITS gene region. qPCRs were performed in a 25- $\mu$ l volume containing 12.5  $\mu$ l of 2 $\times$  QuantiTect SYBR green PCR master mix (Qiagen), 500 nM (each) forward and reverse primers (Biomers), and 2  $\mu$ l of template DNA. The qPCR program consisted of an initial polymerase activation step (95°C for 15 min) followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. The qPCR program was followed by a melting curve determination step (from 70°C to 95°C at a transition rate of 1°C every 5 s) to determine the specificity of the amplification. As an additional post-PCR analysis (51), all qPCR products from the SYBR green-rRNA-ITS assays were analyzed for unspecific PCR amplification by running in a 2% agarose gel for 99 min at 50 V.

**Assessment of specificity of qPCR assays.** The specificities of primers and TaqMan probes as well as the optimal annealing temperatures for all qPCR assays were evaluated both *in silico* by Primer-BLAST (1) and experimentally by amplification tests with a wide range of target and nontarget algal species (see Table S1 in the supplemental material). The specificity and robustness of each TNA were tested by adding different amounts of DNA originating from other organisms as a background (40). DNAs extracted from *Microcystis* strain HUB 524, *Lyngbya* strain TCC 3a, *Synechococcus* strain TCC 32, and *Anabaena* strain TCC 79 were added to the DNA of *P. rubescens* strain TCC 29 (at 1.25 ng of DNA per template) and mixed at ratios of 1:100 and 1:1, and the resulting mixtures were analyzed by TNAs amplifying the PC-IGS region and the 16S rRNA gene. The TNA targeting the *mcyA* region was considered highly specific (6), and only DNA from *Synechococcus* strain TCC 32 was added to the DNA of *P. rubescens* strain TCC 29 (at 0.5, 5, and 50 ng of DNA per template) and mixed at ratios of 1:100, 1:10, and 1:1. In order to verify the specificity of the qPCR assays performed with sediment samples, the resulting qPCR amplification products (for enumeration of total cyanobacteria and toxic *Planktothrix* spp.) from preserved DNA were sequenced as described above.

**Standardization and amplification efficiency.** In our study, the minimum information for publication of quantitative PCR experiments (MIQE) guidelines (8) were followed in order to standardize the experimental practice and termi-

nology and to promote consistency between different results (the quantification cycle [ $C_q$ ] was used instead of the threshold cycle [ $C_T$ ]).

Tenfold dilution standards were prepared from gDNA for each qPCR assay, ranging from 2.5 to  $2.5 \times 10^{-5}$  ng gDNA  $\mu$ l $^{-1}$ . For each standard, the concentration (number of copies) of diluted DNA was plotted against the mean  $C_q$  value (determined in triplicate), and the slopes of the standard curves were calculated by performing a linear regression analysis with Rotor Gene software (version 6.0; Corbett Research, Mortlake, Australia). The *mcyA*, 16S rRNA, and PC-IGS gene copy numbers of the DNAs of the standard strains of *Planktothrix agardhii* strain CYA126/8 genome has only one PC-IGS and one *mcyA* gene and four copies of the 16S rRNA gene (R. Kurmayer, unpublished data). Cell numbers for total cyanobacterial counts were estimated using an average 16S rRNA gene copy number of 3 (18, 41). The approximate genome size of *Planktothrix* spp. was determined to be 5.0 Mb, and the average size of cyanobacterial genomes (4.2 Mb) was used to estimate their total counts (54; <http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1>). It was calculated that 25 ng of each gDNA standard corresponded to averages of  $4.63 \times 10^6$  and  $5.48 \times 10^6$  cells for *Planktothrix* spp. and total cyanobacteria, respectively, according to the following equation: number of copies per microliter = DNA concentration ( $\mu$ g/ $\mu$ l)  $\times 10^6$  (pg/ $\mu$ g)  $\times$  (1 pmol/660 pg  $\times$  genome size [bp])  $\times 6.022 \times 10^{23}$  (copies/mol)  $\times 10^{-12}$  (mol/pmol).

$C_q$  values of the sediment samples were determined for each TNA and SYBR green assay by importing the respective standard curve from each qPCR assay and adjusting it to the  $C_q$  value of the  $10^{-4}$  or  $10^{-5}$  gDNA standard, used as a respective positive control in each run. The accuracy of the qPCR assay was confirmed by relating the cell numbers determined by microscopy and estimated by qPCR with those determined by TNA-16S rRNA with DNA extracts, and the regression equation defining this relationship was established by standard Excel software.

**Assessment of inhibition in sediment samples.** TNA-16S rRNA and TNA-PICP (50) were performed with a series of increasing amounts of genomic DNA of *P. rubescens* strain TCC 29 (0.125, 1.25, 12.5, and 125 ng per reaction mix) in the presence of increasing amounts of sediment DNAs (extracted from the Lake Bourget core at different depths [1, 10, and 24 cm]), mixed at ratios of 1:1, 1:10, 1:100, and 1:1,000. Efficiencies of qPCR performed with the resulting mixtures and also with DNA extracts of *P. rubescens* strain TCC 29 in the absence of sediment DNAs were compared based on the obtained  $C_q$  values. To test the effect of sediment addition (potential inhibition) on the qPCR results, the data were subjected to one-way analysis of variance (ANOVA) (the equal variance of groups and the normal distribution of residuals were tested). XLstats software was used to perform this statistical analysis.

**Nucleotide sequence accession numbers.** The sequences of the amplification products of the 16S rRNA-ITS gene region from preserved DNAs were deposited in the GenBank database under accession numbers JN098337 to JN098376. The sequences of qPCR amplification products from preserved DNAs were deposited in the GenBank database under accession numbers JN703798 to JN703887.

## RESULTS AND DISCUSSION

**Specificity of qPCR assays.** For all TNAs, the assessment of primer and probe specificities was performed previously with respective controls (6, 40, 50); however, in order to confirm their specificities with other isolates, additional *in silico* and experimental amplification tests were performed in our study. The results revealed that *Planktothrix*-specific primers and TaqMan probes were specific for *P. rubescens* and *P. agardhii* but not for other species of cyanobacteria. The PC-IGS and 16S rRNA gene fragments were amplified from all *P. agardhii* and *P. rubescens* strains tested, whereas the *mcyA* gene fragment was amplified only from MC-producing *Planktothrix* strains (see Table S1 in the supplemental material). All nontarget DNAs could be amplified by targeting the cyanobacterium-specific 16S rRNA-ITS gene region but gave no products when those DNAs were amplified with *Planktothrix*-specific primer-probe sets. The presence of as much as 0.0125 to 1.25 ng of *Microcystis*, *Lyngbya*, *Synechococcus*, and *Anabaena*

TABLE 1. qPCR assay specificity check for *Planktothrix rubescens* based on  $C_q$  values obtained for mixtures of target and nontarget species DNAs

Organism(s) <sup>a</sup>	Strain(s)	Ratio of mixture	$C_q$ value (mean ± SD)				
			TNA-PIPC	TNA-16S rRNA	TNA-MAP with dilution factor of:		
					1	0.1	0.01
<i>P. rubescens</i>	TCC 29		21.52 ± 0.04	19.57 ± 0.05	16.82 ± 0.07	20.58 ± 0.11	24.83 ± 0.08
<i>P. rubescens</i> and <i>Microcystis</i>	TCC 29/HUB 524	1:1	21.74 ± 0.07	19.61 ± 0.03			
	TCC 29/HUB 524	1:100	21.94 ± 0.03	19.89 ± 0.19			
<i>Microcystis</i>	HUB 524		33.11 ± 0.04	33.82 ± 0.07			
<i>P. rubescens</i> and <i>Lyngbya</i>	TCC 29/TCC 3a	1:1	21.95 ± 0.13	20.15 ± 0.04			
	TCC 29/TCC 3a	1:100	21.81 ± 0.03	19.98 ± 0.08			
<i>Lyngbya</i>	TCC 3a		34.12 ± 0.07	33.91 ± 0.03			
<i>P. rubescens</i> and <i>Synechococcus</i>	TCC 29/TCC 32	1:1	21.78 ± 0.02	19.58 ± 0.08	16.68 ± 0.06	20.12 ± 0.04	24.89 ± 0.02
	TCC 29/TCC 32	1:10			16.97 ± 0.05	20.59 ± 0.08	24.53 ± 0.23
	TCC 29/TCC 32	1:100	22.03 ± 0.02	19.95 ± 0.09	16.74 ± 0.05	20.22 ± 0.05	24.78 ± 0.03
<i>Synechococcus</i>	TCC 32		32.17 ± 0.15	32.27 ± 0.07	31.72 ± 0.22	— <sup>b</sup>	— <sup>b</sup>
<i>P. rubescens</i> and <i>Anabaena</i>	TCC 29/TCC 79	1:1	22.32 ± 0.12	20.14 ± 0.09			
	TCC 29/TCC 79	1:100	21.84 ± 0.06	19.96 ± 0.12			
<i>Anabaena</i>	TCC 79		32.59 ± 0.04	32.56 ± 0.05			

<sup>a</sup> Mixes of *P. rubescens* strain TCC 29 and background DNAs originating from other organisms (*Microcystis* strain HUB 524, *Lyngbya* strain TCC 3a, *Synechococcus* strain TCC 32, and *Anabaena* strain TCC 79).  
<sup>b</sup> —, below the detection limit.

DNAs in the qPCR mixtures did not show a significant effect on  $C_q$  values of the samples which had only *P. rubescens* DNA (Table 1). For *mcyA*, the majority of nonspecific templates did not show any amplification, but in some cases, late amplification signals ( $C_q > 31$ ) were observed for nontarget species, which could be abundant in the lake sediments (e.g., *Synechococcus*, *Microcystis*, and *Anabaena*). Nevertheless, the sequencing results for the *mcyA* amplicons from the qPCR runs with sediment samples from 1-, 4-, and 24-cm core depths (16 sequences for each depth) confirmed the specificity of TNA-MAP (6), demonstrating the specific amplification of the target *mcyA* gene of *P. rubescens* and *P. agardhii* only from sediment samples.

The application of SYBR green with optimal primer sets can be as sensitive as qPCR using TaqMan probes (35), but it is much cheaper to operate. In our study, SYBR green assay with the CYA371F-CYA783R primer set did not show sufficient specificity over the wide range of annealing temperatures examined (52 to 60°C). The alternative primer set, CSIF-373R, appeared to be highly specific for cyanobacteria, and a single amplification product with a characteristic melting temperature was observed when both genomic DNAs from a variety of algal cultures and preserved DNAs from sediment samples were analyzed. Importantly, the characteristic melting temperatures of different cyanobacterial standard strains, including *P. rubescens* strains TCC 29 and TCC 38 (86 ± 0.1°C), *P. agardhii* strains SAG 5.81 and CCAP 1459/15 (86.1 ± 0.1°C), *Anabaena* strain TCC 79 (85.8 ± 0.2°C), *Microcystis* strains HUB 5.3 and HUB 524 (85.9 ± 0.1°C), and *Synechococcus* strains TCC 32 (86.2 ± 0.3°C) and TCC 173 and 175 (87.3 ± 0.6°C), corresponded to those for DNAs amplified from sediment samples

(86.1 ± 0.2°C). This result confirms that specific amplification occurred with preserved DNAs from sediment samples. The sizes of the PCR products varied among the different cyanobacterial genera (275 bp to 350 bp) (25), which resulted in small differences (<1.5°C) in the characteristic melting temperatures. When a DNA mix containing known amounts of specific and nonspecific templates was analyzed by qPCR, only specific amplification was observed, based on the presence of a single melting peak. Post-PCR analyses by inspection of an agarose gel containing each sediment sample showed no unspecific products, which confirmed the results of melting curve analyses. Primer dimers were not formed during qPCR experiments, as shown by the negative-control samples and postamplification melting curve analyses for every run. Moreover, since the application of SYBR green with relatively large (~300-bp) qPCR products might be questionable for environmental samples, the resulting rRNA-ITS gene amplification products of qPCR runs were sequenced to obtain the ultimate proof of the specificity of this assay for sediment samples. The sequencing results (44 sequences) obtained for depths of 1, 4, 10, and 24 cm revealed a wide range of cyanobacterial diversity, and no unspecific sequences were observed. The majority of clones were affiliated with *Synechococcus* spp., *Planktothrix*, *Anabaena*, and *Microcystis* spp. Thus, qPCR with SYBR green chemistry was found to be specific even with sediment samples, was reliable and robust enough to prevent false-positive results, and therefore could be applied successfully to enumerate total cyanobacteria in sediment samples.

**Sensitivity and amplification efficiency of qPCR assays performed with culture and preserved DNA samples.** Standard curves based on serial dilutions of genomic DNA were con-

structured for all four qPCR assays, and they were not extrapolated beyond the ranges of dilution, which were defined as  $23.2 \times 10^5$  to 23 and  $27.4 \times 10^5$  to 27 copies per template for each of the target regions detected by TNAs and SYBR green reactions, respectively. The amplification efficiencies ( $E$ ) for all qPCR assays were in the range of 92 to 103% (Table 2). Since quantification errors typically occur toward both ends of a calibration curve, cell quantification for sediment samples was achieved by direction toward the central region of the standard curves, which is found to be the most resistant against background effects (28). A significant relationship between the *Planktothrix* cell numbers estimated by microscopic counting and those estimated by TNA-16S rRNA was found (Fig. 1C). The cell number per milliliter estimated by qPCR with DNA extracts was  $1.11 \times 10^7 \pm 9.81 \times 10^5$ , compared to  $2.08 \times 10^7 \pm 0.56 \times 10^6$  for enumeration by direct microscopic counting. The regression equation was as follows:  $y = 4.38x - 1,184$  ( $R^2 = 0.97$ ;  $P < 10^{-4}$ ;  $n = 18$ ), where  $y$  and  $x$  are the log cell numbers determined by microscopic and TNA-16S rRNA methods, respectively.

Controls for evaluating the performances of the multiple assays, amplifying each target individually and comparing the results with those for the multiplex assays, were performed for both cultures and sediment samples. Resulting  $C_q$  values for all assays were highly comparable, independent of the sample type and target gene (TCC 29 had  $C_q$  values of  $16.66 \pm 0.45$  [singleplex] and  $16.39 \pm 0.18$  [multiplex], and B5-1 had values of  $27.14 \pm 0.40$  [singleplex] and  $26.52 \pm 0.09$  [multiplex], as determined by TNA-PIPC). The different qPCR mixes tested did not reveal significant differences in performance, demonstrating similar  $C_q$  values for both culture and sediment samples as determined by either TNA-PIPC or TNA-16S rRNA (data not shown). However, Rotor Gene multiplex PCR master mix, optimized specifically for Rotor Gene cyclers, had a shorter analysis time and was therefore used for all further qPCRs.

When two alternative TNAs for enumeration of total *Planktothrix* populations via the PC-IGS region of *Planktothrix*, i.e., TNA-PC and TNA-PIPC, were compared, the latter demonstrated a higher sensitivity of detection, showing lower  $C_q$  values ( $12.80 \pm 0.35$  for TCC 29 and  $10.86 \pm 0.24$  for TCC 83-1 with TNA-PIPC versus  $15.38 \pm 0.37$  for TCC 29 and  $13.52 \pm 0.07$  for TCC 83-1 with TNA-PC). Thus, all qPCR assays with sediment samples were performed by TNA-PIPC. Total *Planktothrix* enumeration by either 16S rRNA or PC-IGS gene-targeting primer-probe sets revealed comparable results for both sediment and culture samples, showing the high reproducibility and reliability of these genetic markers. Generally,  $C_q$  values obtained by TNA-16S rRNA were the lowest among the other TNAs for all preserved DNA samples (Table 2). This was likely due to the multiple copy number of the 16S rRNA gene (four copies of the 16S rRNA gene are found in *P. agardhii* CYA126/8). It was feasible to detect small numbers of *Planktothrix*, down to 3 copies per template, by TNA-16S rRNA assay, while such detection was not reliable by TNA-PIPC. Thus, qPCR targeting the 16S rRNA gene of *Planktothrix* spp. has certain advantages over the application of PC-IGS-based qPCR, since it has increased sensitivity and enables the detection of rare organisms in a sample (34, 35, 50).

TABLE 2. Parameters for standard curves of qPCR amplifications of *Planktothrix rubescens* obtained from culture (TCC 29 and TCC 83-1) and sediment (B5-1 and B5-24) samples

Parameter <sup>a</sup>	Value														
	TNA-16S rRNA			TNA-PIPC			TNA-MAP			SG-rRNA ITS					
	TCC 29	TCC 83-1	B5-1	TCC 29	TCC 83-1	B5-1	TCC 29	TCC 83-1	B5-1	TCC 29	TCC 83-1	B5-1	TCC 29	TCC 83-1	B5-1
Slope	-3.37	-3.34	-3.27	-3.35	-3.31	-3.2	-3.25	-3.15	-3.39	-3.51	-3.40	-3.52	-3.51	-3.40	-3.35
$R^2$	0.998	0.998	0.998	0.998	0.999	0.996	0.997	0.996	0.994	0.998	0.998	0.998	0.998	0.998	0.999
$E$ (%)	98.6	99.3	102.1	98.6	99.9	102.3	103.2	101	97.0	93.6	96.8	92.0	93.6	96.8	99.0
$y$ intercept	35.67	36.61	32.89	38.14	36.26	34.57	38.75	38.32	39.39	36.02	32.54	36.73	36.02	32.54	36.48
Dilution factor	$1-10^{-5}$	$1-10^{-5}$	$1-10^{-2}$	$1-10^{-4}$	$1-10^{-4}$	$1-10^{-2}$	$1-10^{-4}$	$1-10^{-4}$	$1-10^{-2}$	$1-10^{-5}$	$1-10^{-5}$	$1-10^{-2}$	$1-10^{-5}$	$1-10^{-5}$	$1, 0, 1$

<sup>a</sup>  $R^2$ , correlation coefficient;  $E$ , amplification efficiency.

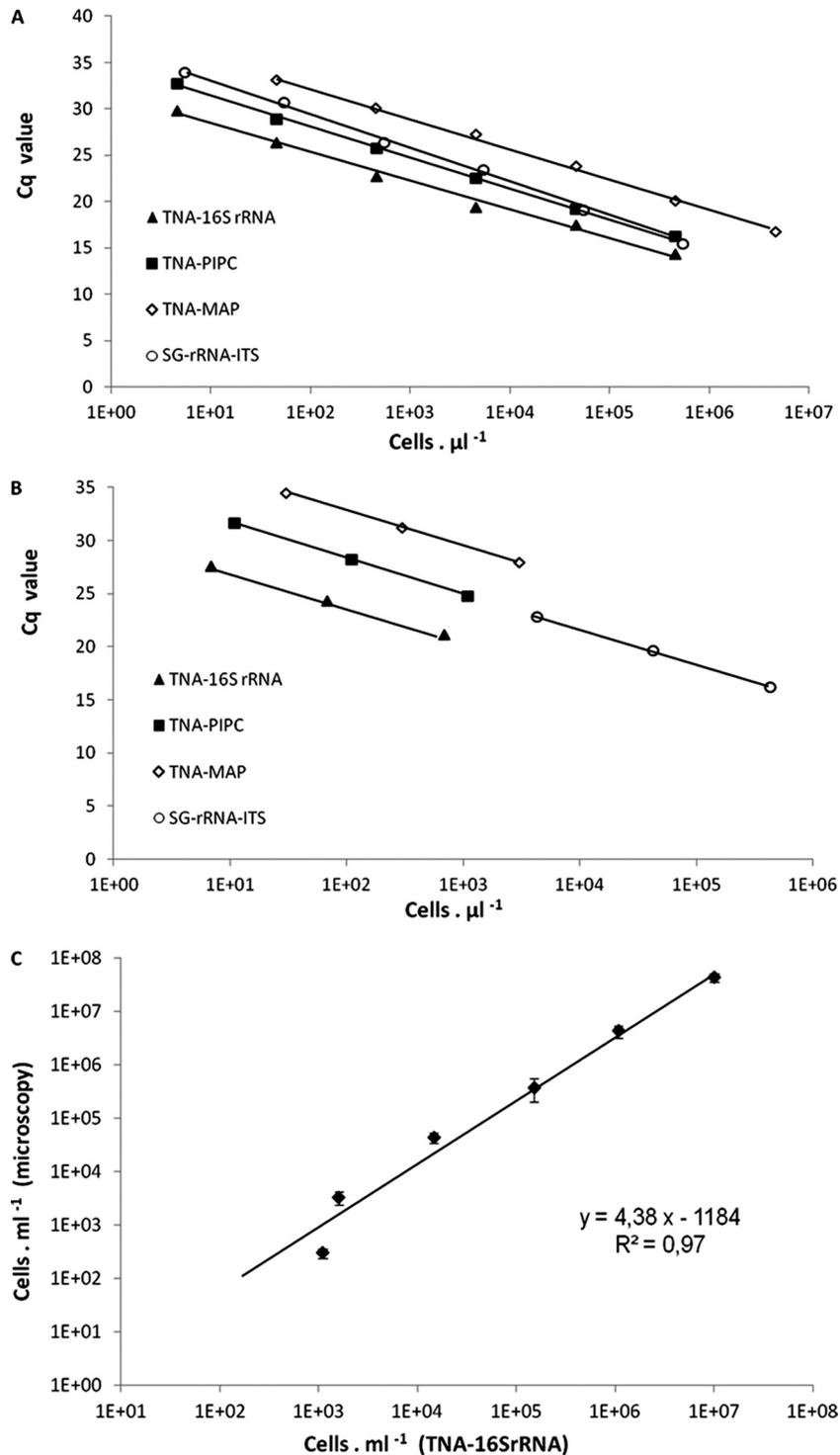


FIG. 1. Examples of qPCR standard curves obtained for *Taq* nuclease assays (TNA-16S rRNA, TNA-PIPC, and TNA-MAP) and the SYBR green assay (SG-rRNA-ITS), generated after amplification of predetermined concentrations (log scale) of MC-producing *P. rubescens* strain TCC 29 (A) and Lake Bourget sediment sample B5-1 (B). For the description of all curves, see Table 2. (C) Correlation between *P. rubescens* strain TCC 29 cell densities estimated by microscopic counting and those estimated by TNA-16S rRNA. Error bars, which are hidden by the symbols in almost all cases, give the standard deviations for three independent amplifications.

**Assessment of inhibition due to coextracted materials from sediment samples.** Due to the potentially high humic acid content in sediment samples, an absence of inhibition should be demonstrated when performing qPCR with such samples.

The approach applied in this case was based on the assumption that inhibitors are diluted out when a log-linear relationship is achieved between  $C_q$  and the dilution factor (33). No inhibition effect was noticed when preserved DNAs from sediment sam-

ples from different depths along the sediment core were mixed with *P. rubescens* strain TCC 29 at any ratio, as assessed by either TNA-16S rRNA or TNA-PIPC. Further ANOVA showed no significant effect of sediment DNA addition on  $C_q$  values at any dilution rate ( $P = 0.935$  and  $P = 0.584$  for TNA-16S rRNA and TNA-PIPC, respectively).  $C_q$  values of all mixes were  $15.62 \pm 0.14$  and  $11.06 \pm 0.33$  by TNA-PIPC and TNA-16S rRNA, respectively, while  $C_q$  values of strain TCC 29 in the absence of sediment samples were  $15.46 \pm 0.27$  and  $10.86 \pm 0.51$ , respectively. When both culture and preserved DNAs were diluted 10-, 100-, and 1,000-fold and mixed at a ratio of 1:1,  $C_q$  values of those mixes were not changed compared with those of TCC 29 alone at the respective dilution rate. Additionally, a  $C_q$  value of  $11.19 \pm 0.73$  was achieved for all nondiluted culture and sediment samples mixed at a ratio of 1:1, as determined by TNA-16S rRNA, which was close to the  $C_q$  value for TCC 29 alone ( $10.86 \pm 0.51$ ). Importantly, the amplification efficiencies of all qPCR assays performed with preserved DNAs from sediment samples (0.87 to 1.16) (Table 2; Fig. 1B) were as high as those with the respective standards (0.91 to 1.05) (Table 2; Fig. 1A). Similar qPCR amplification efficiencies ensured that no PCR-inhibiting contaminants were present in the sediment samples. Based on the obtained results, we concluded that qPCRs were not inhibited, which is a prerequisite for reliable enumeration of both toxic and total *Planktothrix* spp. and total cyanobacteria in preserved DNA.

**Quantification of total/toxic *Planktothrix* spp. and total cyanobacteria in preserved DNAs from lake sediments.** Sediment sampling sites were selected based on the fact that Lake Geneva, Lake Annecy, and Lake Bourget are deep large lakes (with anoxic bottom waters over at least the last few decades) from the same region but show different histories in terms of their trophic status and, consequently, different diversity and quantities of cyanobacteria (16, 24). Thus, if DNA is preserved in the laminated sediments of these lakes, DNA-based analyses of sediment samples should reveal these differences regarding planktonic communities, and more particularly, qPCR analyses are likely to reflect the abundance and dominance of a wide range of cyanobacterial species in water columns.

Lake sediments contained 0.24 (L1) to 5.95 (B5-10)  $\mu\text{g}$  DNA per gram of dry sediment. Lake Annecy sediments had smaller amounts of genomic DNA extracted than those of Lake Bourget and Lake Geneva, probably due to low productivity. The DNAs from lake sediments resulted in amplification of 1,500-bp PCR products from the 16S rRNA-ITS gene region; thus, as reported before by Coolen et al. (9) for other ecosystems, fragmentation of this region of DNA during burial in the sediments did not occur, or at least was rare enough to favor the preservation of a sufficient quantity and quality of DNAs from several cyanobacterial groups. Moreover, the amplification products of the 16S rRNA-ITS gene region were sequenced, and around 100 sequences per core depth were obtained (O. Savichtcheva et al., unpublished data). To date, sequencing analyses with preserved DNAs from sediments have not been performed often enough, indicating the necessity and novelty of our approach. In this study, we focused mainly on demonstration of the quality and integrity of a preserved DNA fragment, rather than discussing the diversity of various cyanobacterial groups, by sequencing the 16S rRNA-ITS gene region. The sequencing results obtained for depths of

1, 4, 10, and 24 cm revealed a wide range of cyanobacterial diversity, and no chimeric sequences were observed. The majority of clones were affiliated with *Synechococcus* spp. but also included *Cyanobium*, *Anabaena*, *Nostoc*, *Microcystis*, and *Planktothrix* spp. Thus, these results demonstrate sufficient quality and fragment integrity of the preserved DNA.

We determined the amounts of DNA derived from *Planktothrix* and cyanobacteria in sediment cores by qPCR. Overall, qPCR assays performed with preserved DNAs from different lake sediments revealed clear differences in the quantitative compositions of total and toxic *Planktothrix* and total cyanobacteria (Fig. 2). Thus, qPCR enumeration showed high cyanobacterial counts in Lake Bourget sediments and very low ones in Lake Annecy sediments, which is in good correspondence with available data on lake water monitoring (16, 24, 43). Lake Bourget and Lake Geneva were indeed affected by eutrophication in the second half of the 20th century, and these systems are now recovering toward an oligotrophic status, while Lake Annecy has not been impacted by such strong eutrophication, and its oligotrophy has been preserved over the past decade (42). Lake Geneva underwent strong eutrophication in the early 1960s, but following the implementation of phosphorus reduction measures in the 1970s, the phosphorus concentration decreased from  $\sim 90 \mu\text{g liter}^{-1}$  in the mid-1970s to  $\sim 30 \mu\text{g liter}^{-1}$  in 2005 in Lake Geneva (53) and from 120 to  $26 \mu\text{g liter}^{-1}$  between 1980 and 2001 in Lake Bourget (24).

Among all the lakes, Lake Bourget had the highest counts for total (as determined by both 16S rRNA and PC-IGS gene-based qPCR) and toxic (sample B5-1) *Planktothrix* populations as well as for total cyanobacteria (sample B5-1). Total *Planktothrix* quantifications based on the 16S rRNA and PC-IGS genes showed comparable results and demonstrated similar trends for all sediment cores (Fig. 2A). Generally, *mcvA*-based counts exceeded those of 16S rRNA or PC-IGS gene-based assays (Fig. 2A). The fact that sequencing results for the *mcvA* amplicons confirmed the amplification of only *Planktothrix* spp. rejects the idea that the primers targeting this conserved condensation domain (21) might be able to coamplify other MC-producing taxa. At present, the highest *mcvA* proportions ( $>100\%$ ) in sediment samples cannot be explained. From these preliminary results, it was concluded that the proportion of the *mcvA* gene has remained high in Lake Bourget since the 1950s and in Lake Geneva since the 1980s. In the future, alternative qPCR assays to enumerate toxic *Planktothrix* spp. should be applied to validate those results.

For Lake Bourget, the proliferation of MC-producing *P. rubescens* has been reported previously (7, 24); the dominance of this taxon in Lake Bourget has been known at least since 1996 and is associated mainly with the process of restoring this ecosystem. In Lake Annecy sediments, no *Planktothrix* spp. were detected by qPCR, and *Planktothrix* has never been reported for planktonic assemblages in this lake. The presence of *P. rubescens* has been detected in the pelagic compartment of Lake Geneva, but generally in small amounts (compared to those in Lake Bourget). *Planktothrix* spp. have been detected in Lake Geneva, especially during the years 1963 to 1985 (16). Importantly, we observed the same trend by performing qPCR assays with lake sediments, which confirmed the lower *Planktothrix* counts in Lake Geneva than in Lake Bourget.

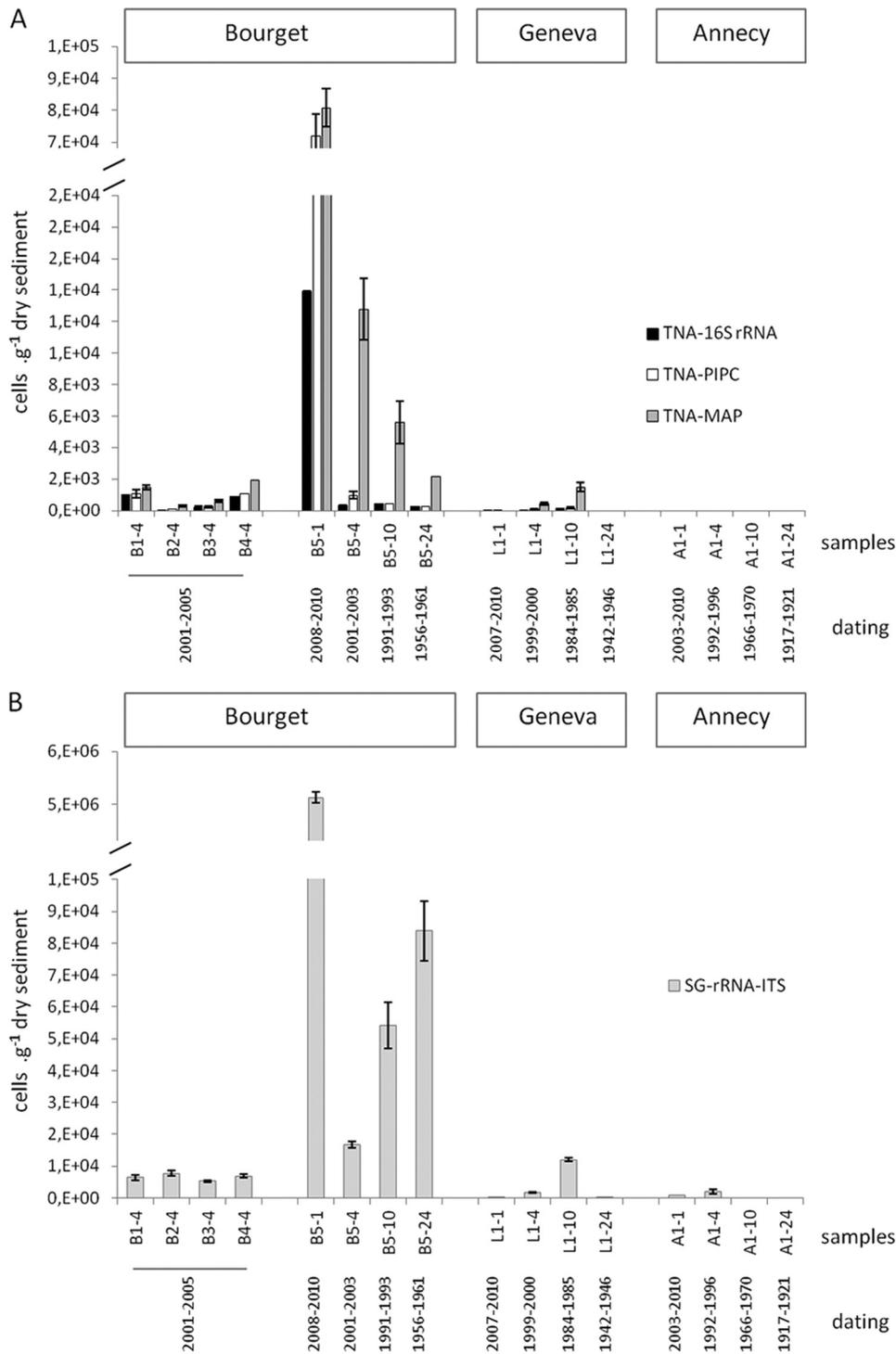


FIG. 2. Abundances of total (TNA-16S rRNA and TNA-PIPC) and MC-producing (TNA-MAP) *Planktothrix* (A) and total cyanobacteria (SG-rRNA-ITS) (B), estimated by qPCR, in the sediment samples from three lakes located in the French Alps (Lake Geneva, Lake Bourget, and Lake Annecy). Error bars, which are hidden by the symbols in some cases, give the standard deviations for three independent amplifications.

Within the Lake Geneva sediment core, total and toxic *Planktothrix* spp. were detected at 1-, 4-, and 10-cm depths (for dating, see Table S2 in the supplemental material) but not at the 24-cm core depth, while other cyanobacteria were detected down to the 24-cm layer. Interestingly, total cyanobacterial

qPCR counts increased gradually from 1 to 10 cm, consistent with the period of strong eutrophication observed in the late 1980s (53), and decreased again at the 24-cm core depth (consistent with a period of pre-eutrophication according to data from the long-term monitoring of deep perialpine lakes).

Within the Lake Bourget sediment core, particularly high *Planktothrix* and cyanobacterial counts were observed at a 1-cm core depth (Fig. 2), and they decreased with the depth but remained at high levels at 4-, 10-, and 24-cm core depths. This suggests that the presence of *P. rubescens* is ancient in this lake (the 24-cm depth in sediment corresponds to the period from 1956 to 1961 [see Table S2 in the supplemental material]). Some older data on planktonic counts (performed occasionally) confirm the presence of this taxon in Lake Bourget in 1913 (32).

In the Lake Annecy sediment core, cyanobacteria were detected by qPCR at 1- and 4-cm depths but not at 10- and 24-cm core depths (Fig. 2). The values were generally low, with the highest quantification recorded for the 4-cm layer (corresponding to the period from 1992 to 1996).

The data obtained in our study are consistent with the past dynamics of cyanobacteria and likely reflect the temporal variations in *Planktothrix* and cyanobacterial species in these deep lakes. Thus, combining paleolimnology and molecular tools, the amounts of cyanobacterial and *Planktothrix* compositional turnover in sediments from different lakes could be analyzed, and distinct regions of the sediment core, corresponding to different trophic periods in the lake (oligotrophic to eutrophic), could be identified, inferring the recent history of the lake ecosystems.

**Limitations of the use of qPCR for paleolimnology, with future perspectives.** One of the major limitations in the use of the qPCR approach for paleolimnology is the efficiency of DNA preservation, which could vary from 1 year to another, especially depending on the length or stability of anoxia. Even though it is difficult to prove that no shifts in degradation processes occurred from 1 year to the other during the past century in the three studied lakes, it could be expected that rather good preservation conditions have occurred during the last century in these three cold, anoxic aquatic sediments (19). Paleolimnological studies performed recently on Lake Bourget and Lake Annecy (19, 39, 42) provided converging information about the efficient storage of organic material in these lakes during the last century. Additionally, we showed that high-quality DNA could be extracted from these sediments, and since a short DNA region is targeted in performing qPCR, the qPCR method should be considered even with partial degradation of preserved DNA. Consequently, we assume that full analyses of these sediment cores by using the qPCR approach and sequencing in order to target cyanobacteria (and potentially other phytoplanktonic taxa, such as diatoms) are of special interest to reconstruct the comparative history of these lakes (19). Moreover, to avoid the obstacles related to the different DNA preservation levels within sediment layers, it would be useful to apply the relative counts (e.g., the ratios of *Planktothrix* counts to total cyanobacterial counts) instead of their absolute numbers.

On the other hand, not all cellular material is transported equally well to sediments, and there are species- or cell-specific variations in the level of (post)depositional degradation of intracellular DNA (10). We assume that obtained DNAs represent preserved DNAs from *Planktothrix* and other cyanobacteria residing in the water column rather than DNAs derived from physiologically active microorganisms residing in the sediments. *P. rubescens* is characterized by its location in the

metalimnic layer of the water column, and internal waves appear to have a major impact on its spatial distribution and proliferation, influencing the growth of this species by a direct impact on light availability (12). This species is particularly well adapted for growing at low light intensities and also at low temperatures (56). In contrast, other cyanobacterial colonies (especially *Microcystis* spp.) have a benthic life cycle, where they form blooms during the summer, sink and reach sediments in autumn, remain in the sediments as vegetative cells during winter, and provide an inoculum for the water column in spring (30). For *Planktothrix*, such a benthic life cycle has not been reported. All of these factors could introduce a certain level of discrepancy and error to the qPCR-based approach performed with preserved DNA, especially where comparisons between different phytoplanktonic groups (e.g., diatoms, cyanobacteria, and chlorophyceae) are concerned. We should thus be cautious in using DNA as a quantitative indicator of past phytoplankton community structure. The combined application of DNA and traditional biomarkers, such as lipids or pigments, would also provide converging information about the importance of phytoplankton groups stored in sediments.

Further studies on the application of qPCR to sediment samples, as well as development of the data set by sequencing of the 16S rRNA-ITS gene region, should be conducted. Only a few characteristics about cell viability of cyanobacteria in sediments have been studied yet; thus, the identification of the active microbial population within sediment samples can be addressed by targeting rRNA content directly rather than targeting DNA. Moreover, the preservation (during sedimentation processes and after deposition on sediments), origin, and size of DNA in sediments should not be neglected. Even though it is expected that similar preservation conditions might occur in various anoxic aquatic sediments, further work examining whether the DNAs from different species are equally well preserved should be performed.

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