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Diversity of cyanobacterial phylotypes in a Mediterranean drinking water reservoir (Marathonas, Greece)

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Abstract The structure of the cyanobacterial community in a large drinking water reservoir (Marathonas, Greece) was investigated in October 2007 and September 2008. Cyanobacteria-specific primers were used for the PCR amplification of cyanobacterial 16S rDNAs from three water column sites and the water collection tank. In total, 199 clones were sequenced representing 52 unique cyanobacterial, including chloroplast-related, and 11 non-cyanobacterial phylotypes. All cyanobacterial phylotypes belonged to the order Chroococcales. Cluster analysis showed that the cyanobacterial communities in 2007 in the three water column sites showed high similarity between the stations and low diversity ($H = 1.17 - 1.44$), due to the occurring common phylotypes, while all sites in 2008 had very low similarities between them and higher diversity ($H = 1.56 - 2.40$). Some of the most abundant phylotypes were closely related (>98%) to members of the genus *Gloeocapsa* and a potentially toxin-producing strain of *Microcystis aeruginosa*. The non-cyanobacterial phylotypes were either unaffiliated or belonged to the Verrucomicrobia, and were related with sequences originating from lake water habitats.

Keywords *Microcystis aeruginosa* · 16S rRNA · Drinking water quality · Verrucomicrobia

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Introduction

Cyanobacterial dominance in phytoplankton has become a common feature of eutrophic freshwaters worldwide. Phytoplankton studies in Greek freshwaters have shown that prolonged cyanobacterial dominance resulting in water blooms is a frequent phenomenon (Moustaka-Gouni 1993; Moustaka-Gouni et al. 2007). Cyanobacterial toxin-producing species in Greece were found in all freshwaters studied (Vardaka et al. 2005) whereas hepatotoxic cyanobacterial blooms have been reported in several Greek freshwaters (Cook

et al. 2004). The presence of toxin-producing cyanobacterial species and cyanotoxins in drinking water reservoirs is known to pose a potential hazard to humans and wildlife (Falconer 1999). However, in Greece freshwater ecosystems are yet under no management regarding cyanotoxins (Cook et al. 2004; Vardaka et al. 2005).

Cyanobacteria, traditionally, have been identified on the basis of their morphology according to the botanical classification system (Komárek and Anagnostidis 1989) whereas some genera have been given validly published names under the Bacteriological Code (Wilmotte and Herdman 2001). Polyphasic evaluation of culture strains of some cyanobacterial genera is becoming more frequently applied (Gkelis et al. 2005a, b; Li et al. 2008). Morphological differences in several cyanobacterial species and genera are reflected in different clusters in the phylogenetic trees of 16S rDNA (Ballot et al. 2008). Nevertheless, high morphological diversity without corroboration between morphology and phylogeny of other taxa, such as *Microcystis* species complex, is known (Otsuka et al. 1998). Recently, natural populations of the species dominating cyanobacterial blooms in Greek freshwaters such as *Cylindrospermopsis raciborskii* have been characterized both morphologically and phylogenetically (Moustaka-Gouni et al. 2009), depicting the need for accurate identification of Cyanobacteria, especially when potentially toxic species are included in lake water blooms.

The most common approach for studying the composition of microbial communities in aquatic environments involves the construction of 16S rDNA libraries, since aquatic prokaryotes are difficult to obtain in pure culture (Kemp and Aller 2004a, b). This approach has revealed a previously unsuspected prokaryotic diversity and has led to the accumulation of a huge number of sequences in global databases, affiliated to phylogenetic groups that were either unknown or thought to be absent from the aquatic ecosystems (Kemp and Aller 2004b). Furthermore, recent studies have started to reveal groups of ubiquitously distributed bacterial phylotypes in freshwater lakes and reservoirs (Zwart et al. 2002; Newton et al. 2006).

To the best of our knowledge this study constitutes the first report on cyanobacterial phylotypes of natural populations from the Marathonas drinking water reservoir. Using cyanobacterial-specific 16S rDNA clone libraries, we examined the diversity of Cyanobacteria in two consecutive years, in order to reveal phylotypes of toxin-producing species in the water column of four sampling sites.

Materials and methods

Marathonas Reservoir is located in Attiki, Greece, about 35 km north-eastern from the city of Athens. It has a surface area of 2.5 km^2 and a mean depth of 15 m. Its drainage basin is 118 km^2 with a total water circulation of 14,400,000 m^3 per year, when the yearly mean rainfall value is 580 mm. Marathonas Reservoir was the main water resource for the supply of Athens from 1931, when its operation began, until 1959. Nowadays, Marathonas Reservoir's water content is adequate only for a few days for the water supply of the city of Athens and its use is occasionally supplementary. Water retention time is about 187–200 days (EYDAP SA, unpublished). During sampling, air temperature in the area ranged between 13 and 22°C and 21–32°C on 30 October 2007 and 08 September 2008, respectively (Hellenic National Meteorological Service, unpublished).

Water samples were collected from four different sites of the reservoir (Fig. 1): site A (38° 10′32.59″ N–23°53′1.33″ E), site B (38°10′45.41″ N–23°54′18.77″ E), site K (38°10′9.91″ N–23°53′ 58.91″ E), and site VE (water collection tower, 38°9′55.77″ N–23°54′0.68″ E). Previous investigations (Katsiapi et al., submitted), showed that *Microcystis*-like morphotypes were the most frequently occurring Cyanobacteria in this reservoir. Since *Microcystis* appears late (i.e., late summer/autumn) in species succession and it is known that all *Microcystis* that have been found in Greece are toxic (Gkelis et al. 2005a, b), the most likely season for *Microcystis* occurrence was autumn. We conducted our sampling in that time of the autumn (30 October 2007 and 08 September 2008) when we observed microscopically such Cyanobacteria.

Samples were collected from approximately 0.5 m below surface in sterile bottles. The sam-

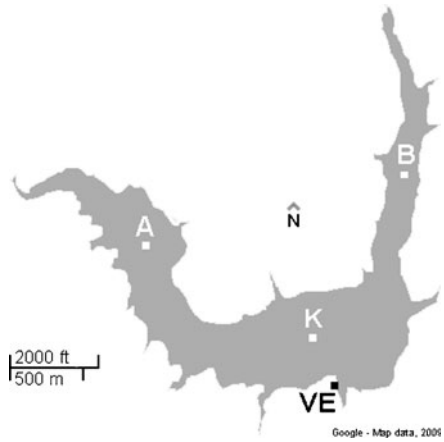

Fig. 1 Sampling sites in the Marathonas drinking water reservoir, Greece. Site VE is the water collection tower

ples were kept cool and in darkness until they were processed (<4 h after collection). Water samples for microscopical analysis were preserved in formaldehyde. Water samples of 10 to 14 L from each site were filtered through 0.22 μm isopore polycarbonate filters (Millipore, France) and filters were stored at −20°C until further processing.

Preserved samples were examined under a light microscope (Nikon Eclipse TE2000-S) and cyanobacteria species identified according to the Botanical classification system (Komárek and Anagnostidis 1989).

DNA was extracted using the Ultra Clean Mega Soil DNA Isolation Kit (MoBio Laboratories Inc., USA) following the manufacturer's protocol and dissolved in 1 ml of PCR water. The DNA was diluted 1:10 with PCR water before PCR amplification, to overcome persistent PCR inhibition problems. For the cyanobacterial 16S rDNA amplification, nested PCR had to be applied. The first PCR was performed using primers BAC-8F (5'-AGAGTTTGATCCTGGCTCAG-3') (Lane 1991) and BAC-1390R (5'-TGTACACACCGCCCGTC-3') (Zheng et al. 1996). For the first PCR run, an initial denaturation step at 94°C for 1 min was followed by 30 PCR cycles (94°C denaturation for 45 s; primer annealing at 52.5°C for 45 s; and primer extension at 72°C for 2 min), and a final 7 min elongation step at 72°C. These PCR products were re-amplified in a second PCR run using the cyanobacterial primers CYA-359F and CYA-781R (Nübel et al. 1997). This PCR consisted of an initial denaturation step at 94°C for 1 min followed by 26–30 PCR cycles (94°C denaturation for 1 min; primer annealing at 59°C for 1 min; and primer extension at 72°C for 2 min), and a final 7 min elongation step at 72°C. PCR products were visualized on a 1.2% agarose gel under UV light, bands were excised, and PCR products were extracted with the Nucleospin Extract II PCR Clean-up kit (Macherey-Nagel GmbH and Co. KG, Germany) following the manufacturer's protocol.

The PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen Inc, USA) and chemical transformation, according to the manufacturer's specifications. For each clone library a maximum of 40 clones containing an insert of ca. 400 bp were grown in liquid LB medium with kanamycin and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel GmbH and Co. KG, Germany) for DNA sequencing. Sequence data were obtained by capillary electrophoresis (Macrogen Inc., Korea) using the BigDye Terminator kit (Applied Biosystems Inc., USA) with the primer M13F (5'-GTAAAACGACGGCCAG-3'). Each sequence read was approximately 850 bp and each sequence was checked for chimeras using the CHIMERA-CHECK function of the Ribosomal Database Project II (Maidak et al. 2001). For the detection of closest relatives, all sequences were compared with the BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence data were compiled using the MEGA4 software (Tamura et al. 2007) and aligned with sequences obtained from the GenBank (www.ncbi.nlm.nih.gov) databases, using the ClustalX aligning utility. Phylogenetic analyses were performed using minimum evolution and parsimony methods implemented in MEGA4 (Tamura et al. 2007). Heuristic searches under minimum evolution criteria used 1,000 random-addition replicates per data set, each followed by tree bisection–reconnection topological rearrangements. The topology of the tree was based on neighbor-joining according to Jukes-

Cantor. Bootstrapping under parsimony criteria was performed with 1,000 replicates. Sequences of unique phylotypes found in this study have GenBank accession numbers FJ774008–FJ774070. Library clone coverage was calculated by the formula $[1-(n_1/N)]$ (Good 1953), where n_1 is the number of phylotypes represented by only one clone and N is the total number of the clones examined in each library.

The Shannon–Wiener index H' was used as a diversity index and was calculated as follows: $H' = -\Sigma(p_i)(\log\ p_i)$, where the summation is over all phylotypes i, and p_i is the proportion of phylotypes relative to the sum of all phylotypes.

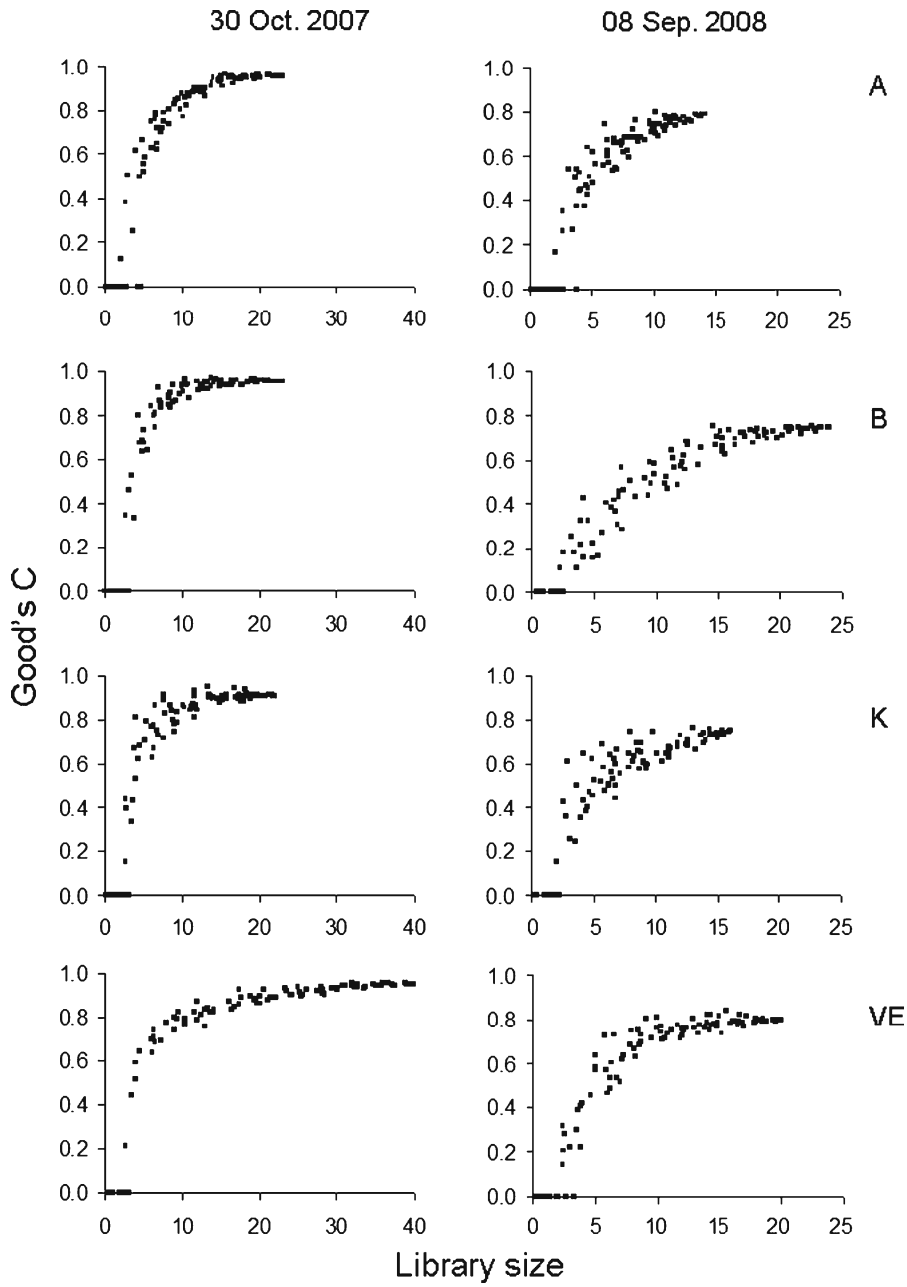

Fig. 2 Cyanobacterial clone library coverage based on Good's C estimator from three water column stations (*A*, *B*, *K*) and the water collection tower (*VE*) in Marathonas Reservoir, Greece

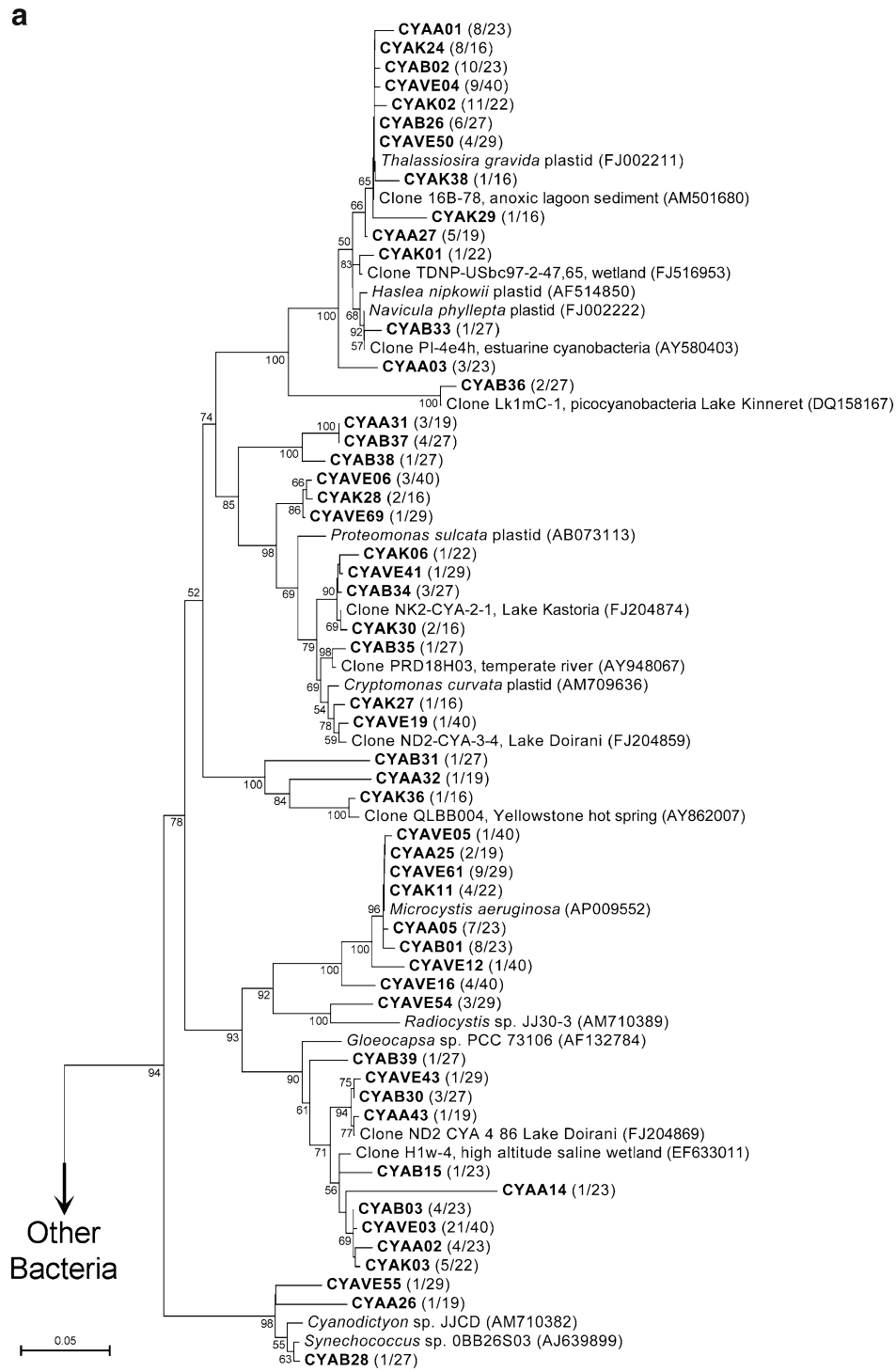

Fig. 3 **a**, **b** Phylogenetic tree of the cyanobacterial 16S rDNA (ca. 420 bp) from Marathonas Reservoir, based on the neighbor-joining method as determined by distance Jukes-Cantor analysis. One thousand bootstrap analyses (distance) were conducted, and percentages greater than 50% are indicated at the nodes. The tree was rooted with *Pantoea agglomerans*. Clones (*bold letters*) that have ≥98% similar nucleic acid sequences within each sampling site are represented by a single sequence, with the number of clones out of the total in *parentheses*. The *numbers in brackets* are GenBank accession numbers. *Scale bar* represents 5% estimated distance

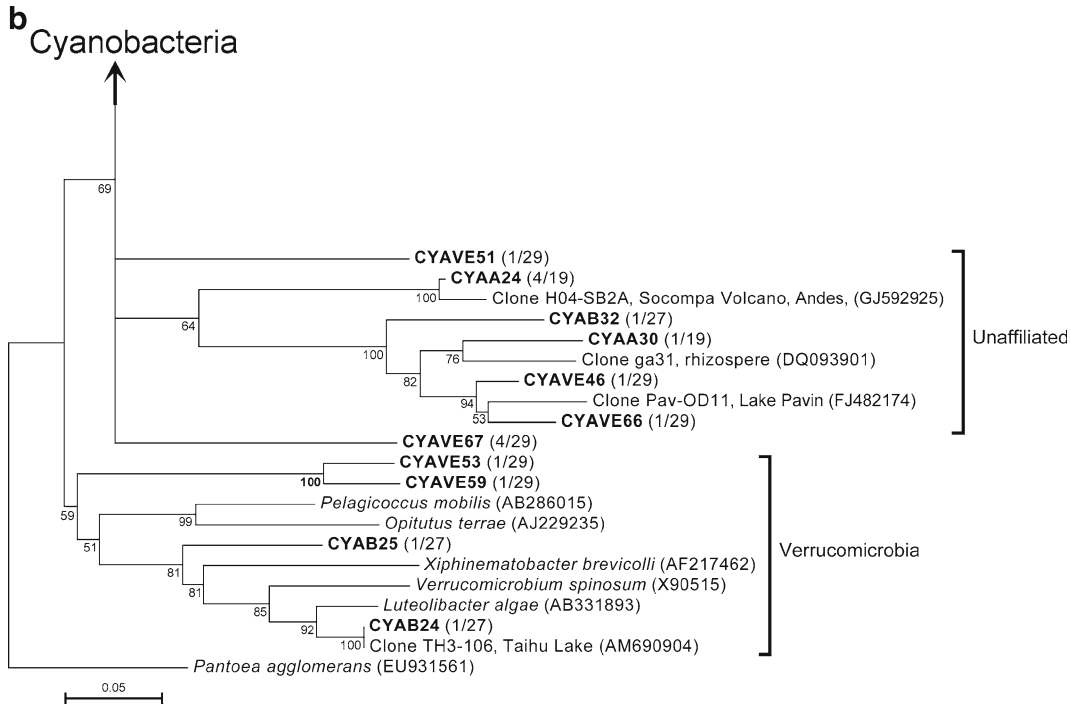

Fig. 3 (continued)

The Pielou evenness index J was calculated as $J = H'/\ln S$, where S is the number of phylotypes (Shannon and Weaver 1949; Pielou 1969).

Results

Three cyanobacterial morphospecies were identified in the water samples of Marathon Reservoir during the study. *Microcystis aeruginosa* and *Chroococcus dispersus* were abundant whereas small colonies of pico-sized chroococcal cyanobacteria were rare.

According to the Good's C estimator for the libraries clone coverage (Kemp and Aller 2004b), curves from all sampling sites in 2007 reached a plateau above 0.90, while in 2008 all curves were curvilinear above 0.75 (Fig. 2).

A total of 108 and 91 clones were sequenced corresponding to 21 and 42 unique phylotypes in 2007 and 2008 samples, respectively (Table S1, Fig. 3a, b). In both samplings, all the found cyanophylotypes belonged to the order Chroococcales, with a total of 13 unique phylotypes related to algal plastids. Eight phylotypes were closely related to *M. aeruginosa*, *Gloeocapsa* sp., and *Radiocystis* sp. One phylotype was closely related to *Synechococcus* sp. and two phylotypes were distantly related to the same group.

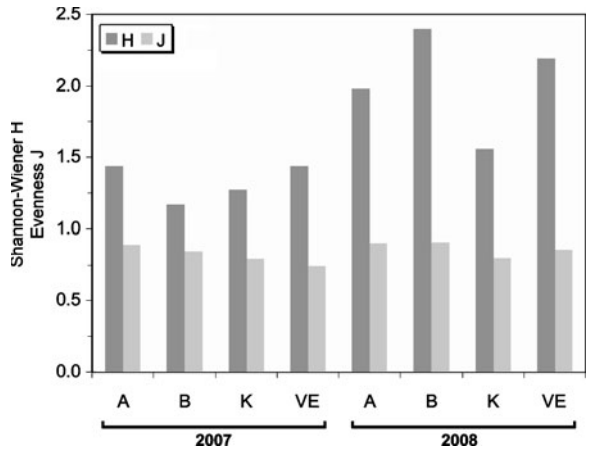

Fig. 4 Comparisons between the Shannon–Wiener richness (H) and the Pielou evenness (J) index of the cyanobacterial phylotypes from three water column stations (A, B, K) and the water collection tower (VE) in Marathonas Reservoir, Greece

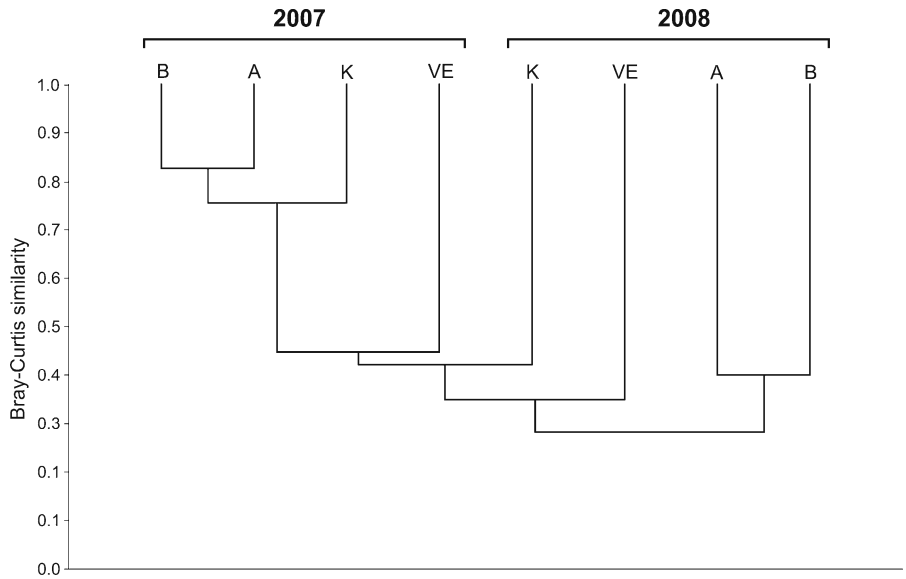

Fig. 5 Cluster analysis of the cyanobacterial phylotypes from three water column stations (*A*, *B*, *K*) and the water collection tower (*VE*) in Marathonas Reservoir, Greece

Only one phylotype (CYAA01) was found to occur in all stations and both years (Table S1) and was related to algal plastids. This phylotype was the most dominant one in stations A, B, K, and the second most abundant in station VE in both samplings. Two phylotypes (CYAA02 and CYAA05) was found in all stations only in 2007 and was among the most abundant clones, with the exception of station VE in 2007 where it was the dominant one. Phylotype CYAA02 was related to *Gloeocapsa* sp. while CYAA05 was practically identical to *M. aeruginosa* (Fig. 3a). The rest of the phylotypes showed very low abundance, with the majority being singletons, and had sporadic occurrence in the four stations and two samplings.

Eleven phylotypes, with very low or singleton abundance in the clone libraries, were not affiliated to the Cyanobacteria (Fig. 3b). Four of them belonged to the Verrucomicrobia, with one of them being practically identical to a phylotype from the Lake Taihu (AM690904), while the rest were distantly related to any cultivated or uncultivated members of the phylum. The last seven phylotypes, although they could not be affiliated to any of the known bacterial phyla, had closest relatives from other lake or soil habitats (Fig. 3b).

In the 2007 sampling, cyanobacterial diversity index *H* (Fig. 4) ranged between 1.17 (Site B) and 1.44 (Site VE) while in the 2008 sampling it ranged from 1.56 (Site K) to 2.40 (Site B). The increase of *H* was attributed to the increased contribution of singleton—i.e., those which appear only once in the clone library—phylotypes as was shown by the regression of the *H* index vs. the percentage of singletons ($R^2 = 0.698$, $p < 0.02$). The distribution of phylotypes in each site's populations was satisfactory even ($J = 0.74 - 0.91$).

Cluster analysis based on phylotype composition (Fig. 5) showed that in 2007 stations A, B, and K were highly similar (>75%) but VE was not similar to these three stations (ca. 45%). In 2008, the stations did not show any similarity. The two samplings in 2007 and 2008 were ca. 40% similar to each other.

Discussion

In this study, the molecular diversity of Cyanobacteria was studied in three water column sites and the water collection tank of the Marathonas drinking water Reservoir, during two periods with 1-year interval, in order to detect phylotypes that are attributed to potentially toxic species. Preliminary morphological analysis of water samples revealed three chroococcal species with the most abundant *M. aeruginosa*, a toxin-producing species known to occur in all Greek freshwaters with water blooms (Gkelis et al. 2005a, b).

The satisfactory coverage of our clone libraries, based on the Good's C estimator (Kemp and Aller 2004b), along with the high number of singleton phylotypes we found, suggested that the major part of the existing cyanobacterial diversity in the studied samples has been revealed. This renders the use of diversity indices feasible and the Shannon–Wiener diversity has been suggested as the most appropriate for estimating the diversity of prokaryotic communities (Hill et al. 2003).

All of the cyanobacterial phylotypes found in the Marathonas Reservoir belonged to the order Chroococcales, agreeing with the morphological analysis, which was based on the Botanical classification system (Komárek and Anagnostidis 1989), or were attributed as algal plastids. Chroococcales is the most diverse in species number among the other cyanobacterial orders in other Greek lakes and in the drinking water Reservoir Polyphytos (Chrisostomou et al. 2009). The Chroococcales do not form a phylogenetically coherent clade. In addition, apart from the genera *Microcystis* and *Cyanobium*, the vast majority of the existing sequences pointed towards a polyphyletic group (Castenholtz 2001). Algal plastids are considered to be of cyanobacterial origin (Sagan 1967) and thus, it is possible that they represent true Cyanobacteria. For example, *Cryptomonas curvata* rhodoplast, which is closely related to some of the Marathonas Reservoir phylotypes (Fig. 3a), is closely affiliated to free living species of *Prochlorococcus* and *Synechococcus* (Marin et al. 2007). However, *Cryptomonas* species have been identified in the reservoir's phytoplankton community (Katsiapi et al., submitted). Also, the only phylotype (CYAA01) that was found in all stations in both samplings and was dominant in terms of relative abundance in the clone libraries, was an algal plastid of Thalassiosiraceae. Small centric diatoms of the family of Thalassiosiraceae were observed in the reservoir's water samples as revealed by light microscopy. The above suggest that the occurrence of algal-related 16S rRNA sequences in monitoring projects should always be coupled with morphological observations (i.e., microscopy) in order to clarify their true ecophysiological significance.

The absence of filamentous Cyanobacteria—but possibly present under the detection limit of the methodology we used in this work—implies that several of the potentially toxic species of the families Oscillatoriales and Nostocales do not pose a threat for the Marathonas Reservoir during the sampling periods of the present study. Most Nostocales are usually abundant in summer and early autumn whereas Oscillatoriales are abundant later in autumn in natural Greek lakes (Moustaka-Gouni 1993). Hydraulic flushing of a Greek lake was very effective in breaking the biomass increase of oscillatorialean and nostocalean species, which are considered sensitive to flushing events (Reynolds et al. 2002), but the same did not apply for the chroococcalean *M. aeruginosa*, a species which appears late in the phytoplankton succession (Moustaka-Gouni et al. 2007). Similarly, the absence of filamentous Cyanobacteria in the Marathonas Reservoir could be possibly due to withdrawal of the reservoir's water for drinking purposes resulting in increasing flushing rate (up to 0.7% of the reservoir volume per day in summer).

Although no blooms of *M. aeruginosa* have been reported for the Marathonas Reservoir, we found a clade of eight phylotypes closely related to *M. aeruginosa*. Members of this species, which appear late in the phytoplankton succession, are less exposed to high flushing (Moustaka-Gouni et al. 2007) and thus could impose an environmental threat for the Marathonas Reservoir. In addition, the found phylotype related to *Microcystis*, was practically identical to a *M. aeruginosa* strain whose genome bears the whole *mcy* operon of microcystin synthesis (Kaneko et al. 2007) which renders it a potential a toxin producer and, thus, a threat for the Marathonas Reservoir. However, this needs further confirmation with sequencing of the full length of the phylotype's 16S rRNA gene and the detection of microcystin-producing genes, such as the *mcy*. The relationship between toxicity and phylogeny within the genus *Microcystis* is still unresolved (Mikalsen et al. 2003); however, the genetic diversity of the *mcy* genes is attributed to both intragenic and intergenic recombination events that might occur (Tanabe et al. 2004). The occurrence of a potentially toxic cyanobacterium in the Marathonas Reservoir, should be carefully

taken into account for the reservoir's future management after the extensive fires that happened in the surrounding drainage area in August 2009. The expected increased nutrient loading in the forthcoming years could trigger water blooms, that of *Microcystis* included.

The *Gleocapsa* group phylotype, on the basis of morphological analysis possibly corresponds to *C. dispersus*. Although cell and colony morphology are the main characteristics used in the botanical classification system to distinguish between these genera, overlapping makes distinction problematic. In the past, strains of *Chroococcus* assigned to *Gloecapsa* group were reassigned to *Chroococcus* genus on the basis of 16S rRNA gene sequences (Herdman et al. 2001).

In the second sampling, the cyanobacterial community was more diverse and also the sites were more dissimilar to each other compared to the first sampling. This increase in diversity was due to the increase of singletons abundance. The fact that in 2007, stations A, B, and K were clustered together was due to sharing the same phylotype. Site VE was not clustered with the water column sites as it had a different dominant phylotype and also includes two bacterial phylotypes strictly restricted in this habitat, probably adapted to less light intensity. Site VE is the water collection tower through which the reservoir water heads to the water refineries, whenever it is needed. Thus, darkness and water retention time in the tower (2 to 4 weeks) determine vigorously its cyanobacterial content (Lindström et al. 2006). The 2008's clustering was very distant for all sampling sites not only between them, but even more with the 2007's clustering. However, this is not surprising since all sites shared only one common phylotype, the one that was the most abundant of 2007, and all other phylotypes appeared in two or three out of the four sampling sites, in different relative abundances.

Although the primers used in this study are considered cyanobacterial-specific (Nübel et al. 1997), seven phylotypes that could not be affiliated to any known phyla and four phylotypes which belonged to the Verrucomicrobia were also retrieved, possibly due to primer mismatches. However, all but one of these phylotypes were singletons and were related to sequences retrieved from lake water column (Fig. 3b). In particular, the Verrucomicrobia are considered to be "globally distributed" in lake ecosystems (Lemke et al. 2009; Eiler and Bertilsson 2004).

In conclusion, the application of cyanobacterial-specific PCR coupled with morphological observations in the water column of the Marathonas Reservoir, Greece, revealed the clear dominance of chroococcalean Cyanobacteria. Only one phylotype could impose a threat for the system as it was practically identical to a toxin-producing *Microcystis* strain. The diversity of the cyanobacterial community increased from the first to the second year due to changes in the dominant cyanobacterial phylotypes and the relative abundances of the common phylotypes occurring in the water column of the sampling sites.

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