Introduction

Cyanobacteria are photosynthetic, prokaryotic organisms which occur primarily in freshwater and saline environments, but also in terrestrial ecosystems. Their presence in lakes with high nutrient levels can lead to a mass increase in cyanobacterial cell numbers, with the formation of blooms, which results in a depreciation of water quality [1]. Cyanobacterial harmful algal blooms (or CyanoHABs) represent one of the most conspicuous waterborne microbial hazards to human and agricultural water supplies, fishery production, and freshwater and marine ecosystems [2]. This hazard results from the production of cyanotoxins, harmful secondary metabolites, which can have deleterious effects within reservoirs and in downstream receiving water systems during releases [3].

In Greece, common bloom-forming cyanobacteria mainly belong to the genera Microcystis and Anabaena, followed by Cylindrospermopsis and Aphanothece [1, 4, 5]. In addition to the bloom-forming cyanobacteria, a wide range of less abundant and lesser-known cyanobacteria, such as, filamentous (e.g. Pseudanabaena) or colonial (e.g. Aphanocapsa, Chroococcus, Cyanodictyon) nanoplancktonic (2-20 μm) species [4] and Synechococcus-type picocyanobacteria (<2 μm) [6] are present in blooms that rarely become dominant, but can represent an important part of the total cyanobacterial biomass. Occasionally, benthic and/or periphytic cyanobacteria can be observed in phytoplankton.

The characterization of the bloom communities' structure remains problematic because the cyanobacterial taxonomy of certain genera has not yet been resolved [7]. Today, cyanobacterial diversity is examined using a polyphasic approach by assessing morphological and molecular data (e.g. 8, 9), often combined with toxicological characters [10, 11]. The traditional cyanobacterial classification [12-15] and the bacteriological classification [16] are based on morphological and genotypic (partial 16S rRNA gene sequences) data [9]. The comparison of morphological and genetic data is sometimes hindered by the lack of cultures of several cyanobacterial species.

Abstract: Cyanobacterial harmful algal blooms (or CyanoHABs) represent one of the most conspicuous waterborne microbial hazards. The characterization of the bloom communities remains problematic because the cyanobacterial taxonomy of certain genera has not yet been resolved. In this study, 29 planktic and benthic cyanobacterial strains were isolated from freshwaters located in Greece. The strains were assigned to the genera Chroococcus, Microcystis, Synechococcus, Jaaginema, Limnothrix, Pseudanabaena, Anabaena, and Calothrix and screened for the production of the cyanotoxins microcystins (MCs), cylindrospermopsins (CYNs), and saxitoxins (STXs) using molecular (PCR amplification of seven genes implicated in cyanotoxin biosynthesis) and immunological (ELISA) methods. This study presents, for the first time, a cyanobacteria culture collection from Greece, thus providing missing study material for the understanding of bloom formation and cyanotoxin production in the Mediterranean and for the polyphasic characterization of important components of the phytoplankton. The combined use of molecular and immunochemical methods allowed the identification of MC producing strains, but further data are needed for CYN- and STX-producing cyanobacteria. The high percentage of MC-producing Microcystis strains in the urban Lakes Kastoria and Pamvotis, frequently used for agriculture irrigation, fishing and recreation, highlights the potential risk for human health.

Keywords: Microcystis, Anabaena, Limnothrix, Calothrix, cyanotoxins, molecular detection, lakes, ELISA
morpheospes and inadequate morphological data of sequenced strains [8]. Furthermore, in order to evaluate the phenotypic plasticity within defined taxa, the variability observed in cultures has to be compared to the range in natural variation [17].

The taxonomy of some of the potentially toxic cyanobacteria remains challenging [18], especially due to the co-occurrence of several different morphotypes [7]. In Greece, cyanobacteria diversity and toxicity is mainly known by field (e.g. [4, 5, 19]) and culture-independent 16S rRNA gene studies (e.g. [7]); only one publication [18] refers to Limnothrix cyanobacteria isolates. The objective of this paper is to isolate and characterize cyanobacteria from freshwaters of Greece, with respect to their ability to produce cyanotoxins.

2 Experimental Procedures

2.1 Growth media and growth conditions

Solid growth medium: agar plates 53 and 90 mm in diameter containing BG-11 media [20] with or without (for the nitrogen-fixing strains) nitrogen, 1.2% w/v [21] agar (Sigma-Aldrich, Germany). Liquid growth medium: BG-11 with or without nitrogen in 100, 250 and 500 mL culture flasks.

Cultures were grown as liquid batch cultures at 20±2°C or 25±1°C (for Microcystis) at a photosynthetic photon flux density of 20 μmol m⁻² s⁻¹ provided by cool white light fluorescent lamps (Sylvania Standard F36W/154-T8, SLI) in a 16:8 h light:dark cycle.

2.2 Sampling Sites and Strain isolation

Strains were isolated from surface water samples collected from freshwaters of Greece between 1999 and 2010 (Table 1); for a description of the Kerkini Reservoir and Lakes Amvrakia, Doirani, Kastoria, Mikri Prespa, Pamvotis, Paralimni, Volvi, see [1 and 4]. Lake Pikrolimni is located in the basin of Kilkis plain, near Thessaloniki (23 km), in northern Greece. It is a small, shallow lake which usually dries out during summer. It has an average depth of about 0.5–0.7 m and covers an area about 4.5 km² when it is flooded [22]. Strains were isolated on solid growth media using classical microbiological techniques and grown as batch clonal unialgal cultures. The strains were purified unialgal by repeated transfer of single colonies or trichomes of cyanobacteria on BG-11 medium agar plates; all strains were derived from a single colony or trichome. The isolates were deposited in Aristotle University of Thessaloniki (AUTH) microalgae collection (Department of Botany, School of Biology) and can be accessed in http://cyanobacteria.myspecies.info/.

2.3 Light microscopy

A Zeiss Axio imager z2 (Carl Zeiss, Germany) microscope using bright field and differential interference contrast (EC Plan-Neofluar 5x/0.16, EC Plan-Neofluar 10x/0.3, Plan-Apochromat 20x/0.8, Plan-Neofluor 40x/0.75 DIC, Plan-Neofluor 63x/1.25 Oil DIC, Plan-Neofluor 100x/1.30 Oil DIC) was used. Microphotographs were taken with an Axio Cam MRc5 digital camera (Carl Zeiss, Germany).

2.4 Identification

The strains were identified to the species or genus level according to Anagnostidis & Komárek [12, 13], Komárek & Anagnostidis (14, 15, 23, 24), Castenholz [16], taking into consideration current taxonomic status [17].

2.5 DNA extraction and PCR analyses

In order to identify toxic strains, different primer pairs, previously described in the literature, were used to detect different gene targets known to be involved in the biosynthesis of either MC, CYN or STX. DNA was extracted using the protocol described in Atashpaz et al. [25] for Gram negative bacteria. PCR was carried out on the DNA extracts using the primer pairs shown in Table 2 and PCR conditions described in detail by Gkelis & Zaoutsos [5]. Thermal cycling was carried out using an Eppendorf MasterCycler Pro (Eppendorf). PCR products were separated by 1.5% (w/v) agarose gel in 1X TAE buffer. The gels were stained with ethidium bromide and photographed under UV transillumination.

DNA extracted from Microcystis aeruginosa M6 strain was used as positive control for the amplification of mcyA, mcyB and mcyE gene targets; DNA from Cylindrospermopsis raciborskii Aq5 strain was used as positive control for the amplification of the ps (peptide syntethase) and pks (polyketide synthase) genetic determinants; DNA from Aphanizomenon gracile A040 strain was used as positive control for the detection of sxtI target gene (see [26]). All positive controls we used produced an amplification product under the tested conditions.

2.6 Cyanotoxin analyses

The Abraxis Microcystin (520011), Saxitoxin (52255B), and Cylindrospermopsin (522011) Microtiter Plate Kits were
used to determine the presence of Microcystins (MCs), Saxitoxins (STXs), and Cylindrospermopsins (CYNs), respectively. Eight-to-ten mL from each culture were centrifuged and the pellet was freeze-dried.

MC, STX and CYN from each strain were extracted by placing up to 1200 mg of freeze-dried material in eight mL of water in glass tubes, immersed in ice and sonicated for 10 min. After sonication, the mixture was stirred for 30 min at room temperature, centrifuged for 10 min at 13,000 g and the supernatant was collected. The pellet was resuspended in eight mL of water and re-extracted. The resulting solutions were then applied to the above mentioned ELISA kits following the manufacturer’s instructions.

The microtiter plates were read at 450 and 630 nm, for MCs and at 450 nm for CYNs and STXs, and $B_0$ values (%) were calculated. Samples with a coefficient of variation percentage of >15% were not accepted. Strains were considered positive for a cyanotoxin when concentration was higher of the lowest concentration of the standards provided for each cyanotoxin.

### 3 Results

Twenty-nine strains were isolated from surface water samples collected from nine lakes (Amvrakia, Cheimaditis, Doirani, Kastoria, Mikri Prespa, Pamvotis, Paralimni, Pikrolimni and Volvi) and one reservoir (Kerkini) located in Greece (Table 1). Isolation procedures resulted in 25 planktic, and four benthic isolates (Table 1) representing eight genera (Figure 1). The isolated cyanobacterial strains were assigned to the following genera: strain 0599, *Chroococcus*; strains 0410, 0610, 0710, 1410, 1510, 1610, 1710, 1810, 2010, 2110, 2310, 2410, *Synechococcus* sp. 0499, 3010, *Limnothrix* redekei 0310, *Jaaginema* sp. 0110, 2210, *Pseudanabaena* sp. 0104, 0199, 0299, 0799, 0899, 2510, 2610, 2710, *Calothrix* sp. 0399.
Isolation and characterization of cyanobacteria strains


A PCR product of about 300 bp was obtained using the mcyA-Cd 1F/mcyA-Cd 1R primer pair, indicating the presence of mcyA gene, in eight out of the 12 *Microcystis* isolates; six of those strains gave positive ELISA results for MCs (Table 3). We also obtained fragments targeting the mcyB, mcyE, and mcyE/ndaF genes in 10, 10 and 9 *Microcystis* isolates, respectively (Table 3). All three primer pairs were co-amplified, except for one strain (*Microcystis* sp. AUTH 0710) where mcyE/ndaF gene fragment was not amplified (Table 3). Only the mcyB gene fragment was amplified in strains *Synechococcus* sp. AUTH 0499 and *Anabaena* sp. AUTH 0299; only mcyE/ndaF gene fragment was amplified in strains *Pseudanabaena* sp. AUTH 0104 and *Anabaena* sp. AUTH 0799; only mcyE gene fragment was amplified in strain *Anabaena* sp. AUTH 2710; none of the strains where only one MC gene fragment was amplified gave positive ELISA results for MCs (Table 3).

None of the assayed isolates gave positive PCR results using the psM13/PSM14 primer pair, thus suggesting the absence of the ps gene in the strains. However, the 422 bp fragment of the pks gene was amplified using the primer

**Figure 1**: Microphotographs of strains representing eight genera of cyanobacteria isolated from freshwaters of Greece. [a] *Chroococcus minutus* AUTH 0599; [b] *Microcystis flos-aquae* AUTH 1410; [c] *Synechococcus* sp. AUTH 3010; [d] *Limnothrix redekei* AUTH 0310; [e] *Jaaginema* sp. AUTH 0110; [f] *Pseudanabaena* sp. AUTH 0104; [g] *Anabaena* sp. AUTH 0899; [h] *Calothrix* sp. AUTH 0399. Bars, 20 μm.
management of toxic cyanobacteria, awareness of benthic strains is important because toxic benthic cyanobacteria have caused animal deaths in Scotland and in Switzerland [28].

Microcystins were found in 90% of the Microcystis strains examined in this study thus providing further evidence that in Greece cyanobacteria blooms, often dominated by Microcystis spp., are highly likely to contain microcystins [5, 19]. ELISA detection of microcystins was in accordance with PCR amplification of the mcy regions, especially the mcyA gene region. This is in accordance with Vasconcelos et al. [26] that detected MCs using ELISA only in samples where the mcyA gene region was amplified. However, Gkelis & Zaoutsos [5] recently reported that in environmental samples the mcyA region was amplified only where Microcystis formed blooms and where MC concentrations were >40 μg L⁻¹, indicating that mcyA gene region is not always amplified or is amplified when Microcystis reaches high biovolumes. Thus, we suggest that mcyA gene is a suitable molecular marker for MC production in the presence of bloom-forming Microcystis populations.

In some Synechococcus, Pseuadanabaena, and Anabaena strains of this study mcyB, mcyE and mcyE/ndaF regions were amplified, but no MCs were found using ELISA. The mcyB2959F/mcyB3278R primary targets mcyB gene [29], which encodes a peptide synthetase containing two modules each possessing adenylation, thiolation, and condensation domains. The mcyB may be involved with the activation of “variable L-amino acids” [30]. The PKEF1/PKER1 primer pair targets the pair pksM4/PKSK18 in strains Microcystis sp. AUTH 2310 and Anabaena sp. AUTH 0899; both strains were found positive for CYN in ELISA (Table 3).

None of the isolates gave positive PCR results for the presence of the sxtI gene indicating the absence of STX producing strains (Table 3). However, nine strains (belonging to Microcystis, Synechococcus, Jaaginema, and Anabaena) were found positive for STX using ELISA (Table 3).

### 4 Discussion

The strains isolated in this study were assigned to species and genera known to occur and/or form blooms in Greek lakes. Chroococcus minutus, Microcystis flos-aquae, M. aeruginosa, and M. viridis have been previously recorded in the phytoplankton of Lakes Mikri Prespa [27], Pamvotis, and Kastoria [4] where they were isolated from. This is the first report of Limnothrix redekei occurring in Lake Doirani. At the collection dates, water blooms formed mainly by Microcystis spp., Anabaena spp., Aphanizomenon issatschenkoi, and Cylindrospermopsis raciborskii were observed in Kerkini Reservoir and Lakes Cheimaditis, Doirani, Mikri Prespa, Kastoria, and Pamvotis [1, 5, 19]. All of these genera, with the exception of the benthic Calothrix, are known to occur in Greek lakes [4, 6]. To the best of our knowledge, this is the first report of Calothrix occurring in Greek freshwaters. It is known that, occasionally, benthic cyanobacteria can be observed in phytoplankton [28], especially when surface samples are collected from littoral stations, like in this study. For monitoring and management of toxic cyanobacteria, awareness of benthic strains is important because toxic benthic cyanobacteria have caused animal deaths in Scotland and in Switzerland [28].

Microcystins were found in 90% of the Microcystis strains examined in this study thus providing further evidence that in Greece cyanobacteria blooms, often dominated by Microcystis spp., are highly likely to contain microcystins [5, 19]. ELISA detection of microcystins was in accordance with PCR amplification of the mcy regions, especially the mcyA gene region. This is in accordance with Vasconcelos et al. [26] that detected MCs using ELISA only in samples where the mcyA gene region was amplified. However, Gkelis & Zaoutsos [5] recently reported that in environmental samples the mcyA gene region was amplified only where Microcystis formed blooms and where MC concentrations were >40 μg L⁻¹, indicating that mcyA gene region is not always amplified or is amplified when Microcystis reaches high biovolumes. Thus, we suggest that mcyA gene is a suitable molecular marker for MC production in the presence of bloom-forming Microcystis populations.
Isolation and characterization of cyanobacteria strains

The mcyE gene in all the MC-producing strains [31]. The mcyE gene codes for the glutamate-1-semialdehyde aminotransferase (GSA-AMT) domain, whose role in MC biosynthesis is to supply the glutamate group to Adda [32]. In addition, the HEPF/HEPR primer pair targets the AMT domain of either mcyE or ndaF, involved in the production of MC or nodularin, respectively [33]. Species of the genus *Anabaena* are well known for microcystin production [34] and are very often one of the dominant bloom-forming species in Greece [4, 5, 19]. However, the planktic and benthic strains we isolated from Lakes Doirani, Kerkini, and Paralimni seem to be incapable of production of MC or nodularin, respectively [33].

### Table 3: Cyanobacteria strains evaluated for cyanotoxin production by ELISA and genetic properties. Bold letter mark strains where toxicity was confirmed by both PCR and ELISA for a cyanotoxin; *indicates strains where toxicity was confirmed only by ELISA; MC: microcystin; CYN: cylindrospermopsin; STX: saxitoxin; n.a.: not analysed. Cyanotoxin genes are explained in Table 2.

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</table>
producing MCs, with the exception of strain Anabaena sp. AUTH 0899 which needs further investigation to clarify the discrepancy of the ELISA positive results and the absence of amplification of mcy regions. No Synechococcus strain has been reported to produce MCs. Nonetheless, recent findings [35, 36] associate marine Synechococcus with MCs. Pseudanabaena spp. are also not considered microcystin-producing; however, a new Pseudanabaena species of low microcystin toxicity was recently found [11].

Two strains (Microcystis sp. AUTH 2310 and Anabaena sp. AUTH 0899) were found positive for CYN, but only one region (ps) targeting CYN-implicated genes was amplified. The ps (aoaB/cyrB) gene spans 8.7 kb and catalyzes a step in the CYN synthesis. It encodes a PKS/NRPS hybrid [37]. The pks (aoaC/cyrC) gene spans 5.0 kb and encodes a PKS [36]. Other studies have shown the presence of parts of aoaC/cyrC, aoaB/cyrB (and also aoaA/cyrA) genes associated with the biosynthesis of CYN in non-CYN-producing cyanobacterial strains [38, 39]. Up to now, no Microcystis species has been found to produce CYN or contain any gene found in the cluster responsible for CYN biosynthesis [40]. However, cylindrospermopsin genes have been reported from Oscillatoria sp. PCC6506 [41]. Genetic analyses have shown that the CYN genes may have been horizontally transferred [40]. CYN was also detected in three Microcystis-dominated blooms in Greece (in two of them, fragments targeting the pks (aoaC/cyrC) gene were also obtained) [5]. In contrast, CYN production has been reported, although rarely, for Anabaena strains [42, 43]. In view of the absence of cyrb genes, further studies are needed to assess whether strains Microcystis sp. AUTH 2310 and Anabaena sp. AUTH 0899 are capable of producing CYN.

In all of the strains found positive for STX in this study the sxtl gene region was not amplified. The sxtl gene product is a putative carbamoyltransferase of the STX biosynthetic cluster that catalyzes the transfer of a carbamoyl group in STX biosynthesis [44]. The sxtl gene is exclusively present in PSP toxin producing strains of Anabaena, Aphanizomenon, Cylindrospermopsis, and Lyngbya [44, 45]. Up to now, no Microcystis, Synechococcus or Jaaginema strains has been reported to produce STX; furthermore, no cyanobacterium has been reported to produce STX in the absence of sxtl gene. Thus, the possibility of ELISA false-positive results has to be considered and further studies are needed to investigate the strains found positive for STX, although no false-positive results have been reported for STX [10] when analyzed with both chromatographic methods and ELISA.

5 Conclusions

This study presents, for the first time, a cyanobacteria culture collection from Greece, thus providing missing study material for the understanding of bloom formation and cyanotoxin production in the Mediterranean and for the polyphasic characterization of important components of the phytoplankton. The data conclude that Microcystis are the main MC producing strains, but further data are needed to assess CYN and STX producing cyanobacteria strains. The high percentage of MC-producing Microcystis strains in the urban Lakes Kastoria and Pamvotis, frequently used for agriculture irrigation, fishing and recreation, highlights the potential risk for human health.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

References


