Molecular characterization of cyanobacterial diversity and yearly fluctuations of microcystin loads in a suburban Mediterranean Lake (Lake Pamvotis, Greece)†

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Cyanobacterial blooms are a frequent phenomenon in eutrophic freshwaters worldwide, and are considered as potential hazards to ecosystems and human health, while it has been shown that on average 60% of these cyanobacterial blooms are toxic. Hepatotoxic blooms are more common than neurotoxic ones and microcystins have been found to be the most prevalent cyanobacterial hepatotoxins. Lake Pamvotis is an ancient (having been in continual existence throughout the Pliopleistocene period) suburban Mediterranean Lake used for recreation, fishing and irrigation purposes which has suffered eutrophication for the last three decades. We investigated cyanobacterial species composition and microcystin loads in this lake over a 16-month period. The highest microcystin concentrations were recorded in autumn, one to two months after the midsummer severe bloom. With the exception of the winter months, microcystin concentrations exceed the WHO upper limits for drinking water but not for recreational waters. Seasonal changes of microcystin bioaccumulation in edible species were also investigated. Microcystin concentrations never exceed the WHO upper limits in those species with the exception of bivalves. For a detailed characterization of the cyanobacterial species composition of the lake, we used polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) between 16S and 23S rRNA genes, in combination with denaturing gradient gel electrophoresis (DGGE). ITS sequences from Lake Pamvotis revealed that the cyanobacterial community of this lake is made of two major populations. A population well defined both microscopically and molecularly as Microcystis sp. dominated during autumn, and another population of filamentous cyanobacteria microscopically characterized as Anabaena sp./Anabaenopsis sp. dominated during midsummer blooms. Sequences of filamentous cyanobacteria from Lake Pamvotis revealed that this cyanobacterial population is homogeneous, although divergent from other populations worldwide. Finally, by using a combination of general and genus specific primer sets against the mcyE gene, we identified Microcystis as the only genus responsible for microcystin production in Lake Pamvotis.

Introduction

Planktonic cyanobacteria of the genera Microcystis, Anabaena, Anabaenopsis, Nostoc and Planktothrix (Oscillatoria) are commonly found in freshwater lakes and reservoirs worldwide. Under optimal mass-reproduction conditions they result in seasonal algal blooms. Although recently it has been suggested that some species of cyanobacteria could even be used as a substantial part of human food supplies, definite cyanobacterial strains have the potential to produce different types of toxins.1–3 The most studied cyanobacterial toxins belong to a family of cyclic heptapeptide hepatotoxins called microcystins.4,5 More than 60 microcystins have been identified to date, all of which have the amino acid ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) combined with six other amino acids.6,7 Being a first trophic level product, microcystins have a tendency to accumulate in the food chain. In this context it has been shown that carnivorous fish have higher microcystin concentrations in muscle than phytoplanktivorous fish.8 There is increasing evidence that microcystins can result in health hazards for exposed populations.9,10 Toxic effects of cyanobacteria to adult animals and developmental defects in animal embryos have been reported.11–13 Moreover, cyanobacterial toxins have been accused of contributing to the mass flamingo deaths in Africa.14,15 Microcystin LR is considered a hepatotoxin. The health hazards some microcystin species can pose to humans started receiving attention following a biological accident in Caruaru,
Lake Pamvotis has been in existence throughout the Plio-Pleistocene period. Therefore, it attracts research interests as a sedimentary archive on long term environmental and climate history and as a hot spot for European biodiversity. The latter is shown by the presence of several endemic mollusc taxa which are known to be 500 000 years old.28 In another study, Lake Pamvotis has been characterized as a Quaternary refugium, an ecological stable area critical not only for the long-term survival of species, but also for the emergence of new ones.32 Thus, the biological interest of this study is broadened by the ecological importance of Lake Pamvotis which is one of the few European lakes old enough to feature endemic faunas and floras and which is also considered one of the “hot spots” for European biodiversity.25,32

Materials and methods

Field sampling
Lake Pamvotis water was sampled from August 2004 to December 2005 at monthly or biweekly intervals. Water samples were collected immediately below the surface, in the middle of the lake (39.65828N, 20.88691E) in sterile bottles. Samples were kept cool until they were processed within 1 h from collection. Fauna samples were preserved in a portable refrigerator and then transported to the laboratory. The fish and mussels were dissected immediately and then freeze-dried for microcystin analysis. (See ESI for further details.)†

Microscopic analysis and biovolume measurements.
Cyanobacterial biovolumes were calculated from counted cells. For counting, water samples were preserved with Lugol’s Iodine directly after sampling and stored at 4 °C in the dark. For counting Microcystis sp. cell numbers, samples containing Microcystis sp. colonies were first disintegrated according to Janse et al., 2004.48 Cyanobacterial cell densities were determined by the inverted microscope technique.33,34 (See ESI for further details.)‡

Chlorophyl measurements, and chemical analysis.
Chlorophyl a was determined by fluorimetry following filtration and extraction with 90% (w/v) aceton.46 Chemical analysis was carried out 1 h after sampling according to Standard Methods for the Examination of Water and Waste-water (21st edition 2005, A.P.H.A., A.W.W.A. and W.P.C.F.). (See ESI for further details.)‡

Microcystin extraction and ELISA measurement.
Cyanobacterial pellets and tissues from different faunal species were lyophilized within 1 hr of collection. Microcystin extraction was performed according to Karlsson et al.,36 with minor modifications as previously described by Vareli et al.37 (see ESI).† We used commercially available ELISA kits (Abraxis, Warminster, PA, USA) to measure microcystins according to the manufacturer’s instructions. The Abraxis ELISA kit quantifies most types of microcystins (microcystin-LR, microcystin-RR, microcystin-YR, microcystin-LF, microcystin-LW, demethylated-microcystin-RR,

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DNA isolation and PCR amplification

The cyanobacterial cells in the lake samples were concentrated by centrifugation. For genomic DNA extraction we used a protocol previously described.37

PCR amplification was performed in a Biorad iCycler in a 50 μL reaction volume containing approximately 100 ng of DNA. For internal transcribed spacer sequence (ITS) amplification, we used a combination of two cyanobacteria-specific primers: 16S GC-CSIF primer and 23S ULR primer. The resulted amplicons, spanning the entire rRNA-ITS and are referred to as ITS control amplicons. Primer sequences and PCR conditions were as described earlier.41 For PCR amplification of mcyE gene fragment we used three primer sets as described earlier.42 The mcyE gene is often used to track microcystin synthesis in wild populations of cyanobacteria. The mcyE gene has commonly been used to detect microcystin production in lakes with toxic cyanobacterial blooms.43 All potential microcystin producing genera were targeted with the use of a general reverse primer (mcy-R4) and a general forward primer (mcy-E-F2). Microcystin producing Anabaena sp. or Microcystis sp. were targeted with the same general forward primer (mcy-E-F2) and one of the following genus specific reverse primers mcyE-12R and mcyE-R8 respectively.44 For the semi-nested PCR procedure, 1–2μL of the first PCR reaction was used as template for the second PCR reaction.45 PCR products were also performed as previously described.46,47 As positive controls, plasmids carrying either Microcystis sp. or Anabaena sp. mcyE gene fragments previously cloned from a neighbour lake (Lake Ziros) were used.48 In the case of negative controls, cyanobacterial DNA samples were processed like all the other samples except that DNA polymerase was not added. In all cases, a proofreading DNA polymerase was used (Expand High Fidelity DNA polymerase, Roche).

Denaturing gradient gel electrophoresis (DGGE), cloning and sequencing

DGGE was performed essentially as described earlier by Muyzer et al.,41 with minor modifications as described by Janse et al.42 A small piece of the gel from the middle of all bands detected after ethidium bromide staining was excised and incubated in 50 μL sterile MilliQ water O/N at 4 °C. The eluent was reamplified by using the original primer set and run on a DGGE gel to confirm its identity. The new PCR products were purified using a Macherey–Nagel DNA clean-up kit (Nuclceosin Extract), and subsequently they were cloned using a TOPO TA cloning Kit (Invitrogen) according to the manufacturer’s instructions. Inserts were fully determined by sequencing both strands. Sequencing was performed by Macrogen Inc. Seoul, Korea. (Details of quality assurance procedures and protocols are provided in the ESI.)†

Nucleotide sequences and accession numbers

The sequences were deposited at GenBank and were assigned accession numbers EF150948–EF151001.

Phylogenetic trees and statistical analysis

All cloned sequences were compared with GenBank entries using BLAST in order to select reference sequences and obtain a preliminary phylogenetic affiliation of the clones. All sequences were imported into MEGA 3.1 and then automatically aligned using the integrated aligner tool. Phylogenetic analyses were also performed with MEGA3.1. Trees were constructed using the Neighbour-Joining method with Jukes–Cantor distance correction. Statistical analysis was done with Instat V3.05 package (Graphpad Software Inc., San Diego, CA, USA).

Results and discussion

Mass occurrences of cyanobacteria in freshwater bodies are generally promoted by a combination of environmental factors such as nutrient enrichment, high water temperatures, long day length and high pH.42,43 Such environmental conditions were present in the case of the Mediterranean Lake Pamvotis (Table 1, ESI),† which has been suffering yearly from cyanobacterial blooms during the last two to three decades.35 During this study, lake temperatures rose from 5.3 °C in February 2005 to 26.2 °C during summer (late July) and dropped to 7.4 °C at 5/12/05 (Table 1, ESI).† Inorganic nitrogen and phosphorus levels were high during the study with phosphorus levels being higher during wet months.

It was already known that a number of dominant genera in Lake Pamvotis were potential microcystin producing genera. However this environmental hazard has been inadequately investigated. Microcystin concentrations have only been calculated in limited samples acquired during the hot seasons of 1999 and 2000 have been assessed to be approximately 1 mg g−1 scum dry weight.28,30

In our analysis, lake water samples collected at monthly or biweekly intervals during the studying period from August 2004 to December 2005. Microcystin concentrations in lake water samples were measured by using ELISA and were expressed as microcystin-LR equivalents. Table 1 (ESI)† shows microcystin concentrations which ranged from 0.01 μg L−1 to 19.5 μg L−1. The two highest microcystin concentrations were recorded in autumn: 19.5 μg L−1 in September 2004 and 18.2 μg L−1 in February 2005. Afterwards, in both years, microcystin levels declined but remained high until October. During late winter and spring, microcystin levels remained low but detectable and started rising in late summer (Table 1, ESI).† Chlorophyll α measurements revealed that high microcystin concentrations do not coincide with the highest chlorophyll α values. The highest chlorophyll α value (80.41 μg L−1) was recorded at 20/7/05. At the same date the microcystin concentration was found to be 4.5 μg L−1, four times less than microcystin levels measured at September of the same year. Thus, the highest microcystin levels were recorded not during the warmer period at the zenith of the main annual cyanobacterial bloom but a few months later.
Microscopic examination of the cyanobacterial samples collected throughout the study revealed that two cyanobacterial groups are prominent. One group well defined as *Microcystis* sp. and another group of filamentous cyanobacteria (*Anabaena* sp. and/or *Aphanizomenon* sp.) (Fig. 1, C and D). Filamentous cyanobacteria were found to be prominent during the midsummer bloom at 20/7/05 (Fig. 1C and Table 1, ESI†) where filamentous cyanobacteria cell counts were found to be 9 x 10^7 and *Microcystis* sp. cell counts, 3.2 x 10^7. In contrast, *Microcystis* sp. was the dominant species during autumnal blooms (at 28/9/04: *Microcystis* sp. cell counts: 1.7 x 10^8, filamentous cyanobacteria cell counts: 7.9 x 10^6; and *Microcystis* sp. cell counts: 1.4 x 10^8, filamentous cyanobacteria cell counts: 8 x 10^6 at 29/9/05). During autumnal blooms we also measured the highest microcystin concentrations: 19.5 μg L^-1 at 28/9/04 and 18.2 μg L^-1 at 29/9/05 (Table 1, ESI).† This observation led us to conclude that *Microcystis* sp. is probably the main toxin producing genus.

Moreover, a Pearson’s test demonstrated a highly significant correlation of microcystin concentrations with *Microcystis* cell loads (p < 0.0001) but not with filamentous cyanobacteria (p = 0.6).

To further investigate the above observation, we studied the composition of potential microcystin producing genera following a PCR approach based on the use of general and genus-specific primers against microcystin synthetase gene E (*mcyE*).39,37 The *mcyE* gene has commonly been used to detect microcystin production both in lakes and rivers with toxic cyanobacterial blooms.39,44

When using general *mcyE* primers, potential microcystin producers found to be present throughout the study period (Fig.2, A1). When using primers specific for *Microcystis* sp. *mcyE* gene, PCR products corresponding to the *Microcystis* sp. *mcyE* gene fragment were detected throughout the study (Fig. 2, A2). The samples were also analyzed with two other genus specific primers suitable for specific amplification of *Anabaena* sp.*mcyE* gene fragment. No product was amplified (data not shown). Even when we used the general amplification product as a template in a semi-nested PCR procedure as previously described,37 no product corresponding to *Anabaena* sp.*mcyE* gene was amplified (Fig. 2, B). This is not a common feature for eutrophic lakes examined so far, where more than one genus tends to produce microcystins.39

To achieve high resolution analysis of the cyanobacterial species and/or strains of Lake Pamvotis, we used the denaturing gradient gel electrophoresis (DGGE) profile of amplified ITSc fragments, spanning the entire rRNA-ITS.38,45 We used ITSc primer amplicons because these amplicons contain more sequence information than ITSa and ITSb amplicons.38 Moreover, for most tested genera, they yielded sharp bands on DGGE gels. Fig. 3 shows the cyanobacterial community profiles during the entire study period. Both prominent and faint bands were excised, reamplified, cloned and sequenced. BLAST searches revealed that most of the sequences do not have a completely homologous counterpart in the GenBank/EMBL/DDBJ databases (Table 2, ESI).† Bands numbered 1, 3, 5, 6, 9, 10, 14, 24, 29, and 45 matched completely (100%) with database entries, based on a comparison of the major part of rRNA-ITS. There are also many sequences with high similarity to database entries from 97% to 99%. All the above mentioned sequences upon BLAST searches found to be *Microcystis* sequences (Table 2, ESI).† The constructed distance tree revealed that *Microcystis* sequences from Lake Pamvotis are clustering with known *Microcystis* sequences from other lakes worldwide.
Moreover, based on a previously published work, these ITSc sequences were found to be homologous to ITSc sequences from already characterized toxic or non-toxic Microcystis strains. If it is possible to discriminate microcystin producing Microcystis strains from non producing strains based on ITSc sequences, as suggested by Janse et al., then potentially non-toxic genotypes in Lake Pamvotis were more abundant during our study.

Other sequences with lower similarities (94%–96%), restricted only to half of the total ITSs submitted so far, are classified as sequences belonging to filamentous cyanobacteria (Table 2, ESI). Based on these ITSc sequences we failed, as expected, to discriminate between Anabaena and Aphanizomenon genotypes, because they both have been shown to share a high sequence similarity of 16S rDNA. In contrast, it is noteworthy that filamentous cyanobacteria genotypes from Lake Pamvotis are quite distinct from those already characterized from other lakes worldwide (Table 2, ESI), with the exception of ITS sequences recently isolated from the neighbouring lake, Lake Ziros, an observation that is expanded by the constructed distance tree (Fig. 4). The long history of the lake and the geographical isolation present reasonable explanations to this finding. Moreover, databank sequences with the highest similarities to our sequences are the ITS sequences from Anabaena spiroides PMC9702, Aphanizomenon sp. W35 and Aphanizomenon sp. T51, strains that have been already characterized as non-toxin producers. For all the above mentioned reasons, filamentous cyanobacteria of Lake Pamvotis deserve investigation for their phylogenetic distinction from other populations worldwide.

Finally, we measured microcystin levels in a number of different faunal species of the lake, for the reason that several fish species of Lake Pamvotis are a food source for the local population. Freshwater mussels are not considered edible by local people. However, we chose to measure microcystin levels in Anodonta cygnea (Swan mussel), which is a characteristic bivalve...
of the lake, because bivalves tend to accumulate microcystins due to their filter-feeding function. For this reason, microcystin levels are expected to be high in bivalve tissues even at times when dissolved levels are marginally detectable. Microcystin levels were also measured in the tissues (liver and muscle) of a number of fish species collected from Lake Pamvotis. We chose liver because microcystins tend to accumulate in this tissue and muscle because this is the edible part of the above species. All species were collected during periods of high and low cyanobacterial toxic contents. In bivalve tissues, microcystin concentration ranged from 49.8 to 102.2 ng g⁻¹ w/w (Table 3, ESI).† At 28/9/2004 we measured the highest microcystin concentration for the year 2004. However, microcystin levels in Anodonta cygnea mussels measured at 26/10/2004 did not surpass the concentrations measured later when the cyanobacterial content was lower, (6/2005) or when they increased back in autumn 2005 (9/2005). No statistical correlation was found between microcystin levels in lake water and levels found in animal tissues (p = 0.18 for muscle concentrations and p = 0.26 for liver tissue) (Table 3, ESI).† This finding was common for all species studied and is in agreement with other reports.⁶⁻⁹ In muscle of different fish species the microcystin content ranged from 2.06 to 4.71 ng g⁻¹ w/w, while in liver it ranged from 14.2 to 56.2 ng g⁻¹ w/w. The highest muscle microcystin concentration was found in a Rutillus ylkiensis (an endemic roach of Greek freshwaters) specimen collected at 19/3/2005 and it was 4.71 ng g⁻¹/C0. The highest liver microcystins concentration was measured in a Barbus albanicus (Albanian barbel) specimen collected at 30/9/2005 and it was 56.2 ng g⁻¹ w/w (Table 3, ESI).† Thus, only Anodonta cygnea tissues were found to be severely contaminated by hepatotoxic microcystins. Although the feeding habits of people around Lake Pamvotis do not include Anodonta cygnea and the muscles from edible fish species contain microcystins low enough to reach the WHO tolerable daily intake, we suggest that microcystin content in fishery products of this lake should be monitored to prevent health hazards in the future.

Conclusions

A suburban Mediterranean ancient lake, Lake Pamvotis, which is considered a “hot spot” for European biodiversity, suffers yearly cyanobacterial blooms. We found that filamentous cyanobacteria of Lake Pamvotis are phylogenetically distinct from other populations worldwide, and microcystin production is attributed only to Microcystis sp., an observation that is not common for eutrophic lakes. Although faunal species from Lake Pamvotis were not found to be heavily contaminated with microcystins (except Anodonta cygnea species), the establishment of a cyanobacterial and cyanotoxin monitoring program is needed. Finally, Lake Pamvotis, a subject of intense restoration efforts, can be considered an ideal system for studying cyanobacterial community changes and evaluating undertaken restoration efforts in shallow eutrophic Mediterranean lakes.

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