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Microcystin producing cyanobacterial communities in Amvrakikos Gulf (Mediterranean Sea, NW Greece) and toxin accumulation in mussels (*Mytilus galloprovincialis*)

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ABSTRACT

Various cyanobacterial species have the capacity to produce different types of toxins. Microcystins, the most prominent cyanotoxins are considered health hazards because of their potential hepatotoxic effects. They are well known to contaminate freshwater ecosystems but their presence in marine ecosystems has been reported only occasionally. We investigated seasonal changes of microcystin concentrations both in water and in the edible species of mussels *Mytilus galloprovincialis* collected from Amvrakikos Gulf (salinity ranging from 30‰ to 34‰), the biggest semi-enclosed basin in Greece. The microcystin concentrations in the water ranging from 0.003 to 19.8 ng l^{-1}, were below the World Health Organization (WHO) upper limit for recreational activities. In contrast, we found that microcystin concentrations in *M. galloprovincialis* mussels (ranging from 45 ± 2 to 141.5 ± 13.5 ng g^{-1} ww) exceeded the upper limit of the tolerable daily intake (TDI) of microcystin as determined by WHO.

Genotype composition of the total cyanobacterial community of the Gulf was analyzed by using denaturing gradient gel electrophoresis (DGGE) profiling of the rRNA internal transcribed spacer region (rRNA-ITS). The cyanobacterial community was found to be dominated almost exclusively by the cosmopolitan species *Synechococcus* – *Synechocystis*. In order to determine genes involved in the production of microcystins, a range of both specific and degenerate molecular primers against microcystin synthetase gene cluster (*mcyS*) was used.

To our knowledge this is the first report of the presence of the hepatotoxic microcystins in the Mediterranean Sea, the first study on the accumulation of these toxins in mussels from a Mediterranean marine ecosystem and one of the few published works suggesting a potential association of microcystins with *Synechococcus* and/or *Synechocystis* cyanobacteria.

The importance of our study is strengthened by the fact that Amvrakikos Gulf is among the most productive Greek "seafood" areas and a Mediterranean wetland of international significance according to Ramsar Convention.

1. Introduction

Cyanobacterial blooms are increasingly a major problem in freshwater ecosystems. There have been increasing public health concerns, since 60% (on average) of these cyanobacterial blooms are toxic (Watanabe and Oishi, 1982; Hawkins et al., 1985; Repavich et al., 1990; Carmichael and Falconer, 1993; Gobler et al., 2007; Vareli et al., 2009a,b). The most studied cyanobacterial toxins belong to a family of cyclic heptapeptide hepatotoxins called microcystins (Carmichael, 1994; van Apeldoorn et al., 2007). More than 60 micocystins 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 (Dawson, 1998; McElhiney and Lawton, 2005).

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Microcystins are synthesized non-ribosomally by the thiotemplate functions of large multifunctional enzyme complexes containing both non-ribosomal peptide synthase (PS) and polyketide synthase (PKS) (Pearson et al., 2008). The microcystin biosynthesis gene cluster (*mcyS*), has been sequenced and partially characterized in several cyanobacterial species (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). In *Microcystis aeruginosa*, the microcystin biosynthesis gene cluster (*mcyS*) spans 55 kb, comprises 10 genes arranged in two divergently transcribed operons (*mcyA–C* and *mcyD–J*) and includes genes encoding for peptide synthetases (*mcyA*, *mcyB* and *mcyC*), polyketide synthetases (*mcyD*), hybrid PS–PKS enzymes (*mcyE*, *mcyG*), and enzymes putatively involved in the tailoring (*mcyJ*, *mcyF*, and *mcyI*) and transporting (*mcyH*) of the toxin (Tillett et al., 2000).

Microcystin production in toxic cyanobacteria is thought to be influenced by a number of different physical and environmental parameters such as, nitrogen, phosphorus, trace metals, growth temperature, light, and pH (van derWesthuizen and Eloff, 1985; Sivonen, 1990; Lukac and Aegerter, 1993; Song et al., 1998). Interestingly, Long et al. (2001) suggested that the observed toxin fluctuations under different environmental conditions were probably due to the indirect effects on cell-growth rate. A closer examination of microcystin regulation at the molecular level revealed that high light intensities and red light were correlated with increased transcription, while blue light led to reduced transcript levels (Kaebernick et al., 2000).

The production of toxins by cyanobacteria, poses a serious problem to human health in relation to the consumption of contaminated drinking water or food. The cyanotoxins are collectively responsible for continued widespread poisoning of wild and domestic animals and human fatalities (Nishiwaki et al., 1994; Jochimsen et al., 1998; Carmichael et al., 2001; Briand et al., 2003; Jacquet et al., 2004; Falconer and Humpage, 2005; Wang et al., 2005; Xie et al., 2005; Soares et al., 2006; Yuan et al., 2006). Avian mortalities from cyanotoxins have been reported since the early 1900s (Schwimmer and Schwimmer, 1968). More recent reports of microcystin induced avian mortalities are from great blue herons (Driscoll et al., 2002) and flamingos (Ballot et al., 2002).

Although intoxication of aquatic organisms involving cyanobacterial toxins are documented worldwide in freshwater ecosystems (Codd et al., 2005), such intoxications of marine organisms have only occasionally been reported (Chen et al., 1993; Miller et al., 2010). As described for cyanobacteria blooms in freshwater and brackish waters, the increase in cyanobacteria bloom formation reported in coastal areas has been attributed to factors such as high irradiation, high temperatures and increased nutrient loading, as a consequence of human population growth near these locations (Camargo and Alonso, 2006; Ahern et al., 2007; Plinski et al., 2007). In the Baltic Sea, blooms of *Nodularia spumigena* are a common issue during the summer months and the production of the hepatotoxin Nodularin is common (Sivonen et al., 1989; Repka et al., 2004). Recently it was found that microcystin-LR levels are also high in the Baltic Sea (Karlsson et al., 2005a) and the most probable candidate organism for microcystin-LR production is thought to be *Anabaena flos-aquae* rather than *Anabaena lemmermannii* (Kankaanpaa et al., 2009).

Martins et al., 2007 showed that crude extracts of marine cyanobacterial species such as *Synechocystis* and *Synechococcus* had a negative effect on the survival and embryogenesis in a number of marine invertebrates. In a recent study (Carmichael and Li, 2006) it was found that in the inland hyper saline lake, the Salton Sea (California, USA), the genera producing measurable levels of microcystin included mainly *Synechococcus* and *Oscillatoria*. The production of microcystins by a *Synechococcus* strain closely related to marine *Synechococcus* indicates that microcystins may be more common in saline environments than previously thought.

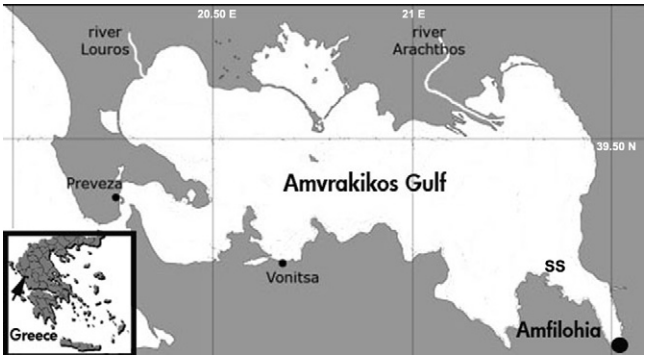

Fig. 1. Amvrakikos Gulf location in Northwestern Greece and a map of the Gulf. (SS: sample station.)

Cyanotoxins are also suggested to have a negative impact on selected aquaculture organisms (Carmichael and Li, 2006). The most well known is the loss of Atlantic net-pen reared salmon from microcystins produced by as yet unknown organisms (Andersen et al., 1993). These findings add concerns for potential negative impacts of cyanotoxins in the emerging business sector of fresh and marine aquaculture.

Mediterranean estuaries and enclosed basins, such as Amvrakikos Gulf, are areas of great ecological and economic importance. The Amvrakikos Gulf (Fig. 1) with an area of about 400 km^2 (salinity ranging from 24‰ to 36‰) is the biggest enclosed basin in Greece, among the most productive Greek "seafood" areas (Panayotidis et al., 1994; Economou et al., 2007), as well as a key wetland of international importance under the Ramsar Convention (www.ramsar.org). However, the area is at risk due to pollutants carried by the Louros and Arachthos Rivers and the wastewaters from the processing of agricultural products by small industries established in the area (Vassilopoulou et al., 2002). Anthropogenic inputs from agriculture, industry, and municipal wastes coupled with heavy nutrient loads from aquaculture industries that are stimulating factors for phytoplankton blooms and also putative toxigenic cyanobacterial blooms (Ignatiades and Gotsis-Skretas, 2010). Data on the trophic state of Amvrakikos Gulf are limited, usually concerning environmental parameters (e.g. nutrient loads) or sporadic biological parameters (e.g. chlorophyll measurements and fauna distribution) (Panayotidis et al., 1994). We studied microcystin concentrations for a one-year period in water (dissolved and particulate) and also in mussels collected from the SE less eutrophied region of the Gulf (Panayotidis et al., 1994). We chose to study mussels not only as bioindicators for the environmental quality assessment (Langston and Spence, 1995), but also due to their economic importance as edible products of the Gulf. We also mapped cyanobacteria species to identify potential toxic species in Amvrakikos Gulf. To our knowledge this is the first study to characterize cyanobacterial species in a Mediterranean semi-enclosed embayment by using molecular techniques and also the only study which has recorded microcystin concentrations in a Mediterranean marine ecosystem.

2. Materials and methods

2.1. Study area and sampling

Amvrakikos Gulf is a shallow (maximum depth: 60 m) semi-enclosed embayment in the Ionian Sea. It is connected with the Ionian Sea through a narrow channel (width 800 m, depth 12 m). At the northern part of the Gulf is situated the extensive deltas of Louros (mean annual discharge 19 $m^3 s^{-1}$) and Arachthos Rivers (mean annual discharge 70 $m^3 s^{-1}$) (Panayotidis et al., 1994).

Table 1
Seasonal fluctuations of nutrients, Chl *a* and microcystin loads in Amvrakikos Gulf sample station (N 38.912551, E 21.113870). Microcystin in both water and mussel tissues were measured by ELISA (Abraxis) (bdl: below detection limit, TDI: tolerable daily intake, na: not analyzed).

Date	T (°C)	pH	Salinity	NH_4 ($mg\,l^{-1}$)	NO_3 ($mg\,l^{-1}$)	NO_2 ($mg\,l^{-1}$)	Total phosphorus ($mg\,l^{-1}$)	Chl *a* ($\mu g\,l^{-1}$)	Microcystin-LR equiv. in seston (cell bound) ($\mu g\,g^{-1}$ dw)	Volumetric microcystin-LR equiv. ($ng\,l^{-1}$)	Microcystin-LR equiv. in *Mytilus galloprovincialis* tissues ($ng\,g^{-1}$ ww)	Critical amount for TDI (g tissue)
7/2006	28	8	34‰	0.16	bdl	0.012	0.01	2.7	0.9	0.95	56 ± 5	35.71
8/2006	27	8.2	34‰	bdl	bdl	bdl	0.02	1.39	1.6	0.95	73 ± 4	27.39
9/2006	25	7.9	32‰	bdl	bdl	bdl	0.03	1.35	12.3	10.2	77.5 ± 5.5	25.80
10/2006	20	7.5	32‰	bdl	0.1	bdl	0.01	0.92	66	19.8	101 ± 11	19.8
11/2006	16	7.9	32‰	0.65	3.6	0.01	0.04	1.67	3.4	3.9	110 ± 12	18.18
12/2006	13	7.9	32‰	0.13	3	bdl	0.2	1.89	0.09	0.076	132.5 ± 1.5	15.09
1/2007	12	8.1	31‰	0.17	19.1	bdl	0.9	5.7	0.004	0.938	117.5 ± 8.5	17.02
2/2007	12	7.7	31‰	0.12	22	0.006	0.1	6.3	0.015	0.003	135 ± 17	14.81
3/2007	16	7.8	32‰	0.37	24	0.02	0.3	13.3	0.011	0.008	141.5 ± 13.5	14.13
4/2007	18	8	30‰	0.15	18	0.002	0.1	12.7	0.085	0.3	50 ± 5	40
5/2007	20	8.2	31‰	0.18	11.7	0.004	0.09	7.5	0.073	0.004	47.5 ± 6.5	42.10
6/2007	21	8.3	na	0.45	4.9	bdl	0.02	6.4	0.005	0.015	45 ± 2	44.44

Samples were collected immediately below the surface next to Amfilohia (N 38.912551, E 21.113870) (Fig. 1). The station is of particular importance because most of the aquaculture activities related to mussel cultivation restricted to this area. According to previous studies the South East area is thought to be less eutrophied and with constant salinity (30–34‰) throughout the year while it is away from any freshwater inputs (Panayotidis et al., 1994) (see also Table 1).

During each sampling, general physical parameters (temperature, pH, conductivity) were measured via an YSI 556 MPS probe. Replicate 1 l sterile bottles were filled with water from 50 cm below the surface. Wild mussels (*Mytilus galloprovincialis*) of similar shell length (46.5 ± 0.8 mm) were collected monthly from Amvrakikos Gulf at the rocky shore 50 cm below the surface of water. Water and mussel samples were preserved in a portable refrigerator and then transported to the laboratory. Samples were kept cool until they were processed not longer than 1 h after collection.

2.2. Chlorophyll a measurement and chemical analysis

Samples for Chlorophyll *a* (Chl *a*) analysis were gently filtered onto glass fiber filters (Whatman GF/F), and stored frozen. Chl *a* was extracted and determined spectrophotometrically according to Standard Methods for the Examination of Water and Wastewater (APHA, AWWA and WPCF, 2005).

Nutrient samples were prefiltered onto glass fiber filters (GFF; for dissolved nutrients) and stored frozen. Nutrient samples were analyzed spectrophotometrically for nitrite, nitrate, phosphate, ammonia. Duplicate samples were measured also by the General State Chemical Laboratory (Division of Ioannina, Greece). In all cases measurements were made according to Standard Methods for the Examination of Water and Wastewater (APHA, AWWA and WPCF, 2005).

2.3. Microcystin extraction, ELISA measurements and LC/MS detection

Phytoplankton from water samples was collected by filtration (Millipore 0.45 μm) followed by resuspension and centrifugation (8500 × *g* for 10 min). Whole soft mussel tissue (ten specimens from each sampling round) was dissected for microcystin extraction. Phytoplankton pellets and mussel tissues were freeze-dried (Freeze drier vacuum concentrator Christ ALPHA1-2 LD). All the samples were weighed before and after drying.

Microcystin extraction from phytoplankton samples was performed as previously described (Karlsson et al., 2005b; Vareli et al., 2009a) with minor modifications as follows: 2–15 mg of lyophilized phytoplankton pellets were added to 2 ml of 75% methanol in water and 0.5 g zirconium beads (0.1 mm diameter). The samples were homogenized for 5 min by vigorous shaking (5000 rpm) on a Mini Bead-beater (Biospec products, Bartlesville, OK, USA) with intermittent cooling on ice. After homogenization, 8 ml of 75% methanol in water was added to the samples (final volume 10 ml). Subsequently, samples were sonicated for 15 min in a sonication water bath, and then incubated at room temperature for 20 min with vigorous shaking. The above step was repeated three times. At the end of the final incubation, the samples were centrifuged at 8500 rpm for 10 min. Supernatants were transferred to new tubes and stored at 4 °C. Pellets were resuspended in 10 ml of 75% methanol and incubated overnight (O/N) by vigorous shaking. The entire process was repeated twice the following day and all supernatants were pooled. Following extraction, the methanolic extract was stored at −20 °C until analysis.

For microcystin extraction from mussel tissues, whole mussel tissue (0.8–1.5 g ww) was lyophilized and stainless steel beads (Qiagen 5 mm diameter) were added before bead beating homogenization.

To assess the effectiveness of the extraction procedure, spiked recovery tests were carried out on both phytoplankton and mussel tissue. The homogenized samples were spiked (Garcia et al., 2010) with 0.75 $\mu g\,l^{-1}$ of microcystin-LR (Abraxis). Samples were then extracted and analyzed using the ELISA kit (Abraxis). Of the microcystin-LR spiked into phytoplankton samples 95% was recovered (0.71 $\mu g\,l^{-1} \pm 0.03$). Extraction efficiency in whole mussel tissue was found to be 87% (0.65 $\mu g\,l^{-1} \pm 0.04$).

The concentration for microcystins in the above samples was measured by a highly sensitive ELISA test (0.04 μg microcystin equiv. l^{-1}) for both microcystins and nodularins (Abraxis, Warminster, PA, USA) with a detection range of 0.05–5.0 μg microcystin l^{-1}. For the ELISA measurements, appropriate volumes of each sample were evaporated to dryness in a vacuum concentrator (SAVANT SpeedVac concentrator, SPD2010) at low temperatures and the residues were then dissolved in 50 μl of milliQ water.

In a seston sample (sample collected at 10/2006) containing high amounts of toxin (based on ELISA measurement) an HPLC–tandem MS procedure was used to confirm the presence and also the type of toxin while the Abraxis ELISA kit can not discriminate between microcystins and nodularins. HPLC–tandem MS was performed as previously described by Spoof et al. (2003). The HPLC system was a Waters Alliance 2795. The column we used was a Phenomenex Aqua C18 (250 mm × 2.0 mm, 5 μm particle size) protected by a Phenomenex SecurityGuard C18 4 mm × 2.0 mm cartridge. The flow rate was set to 0.2 ml/min, with mobile phase A being 0.5% formic acid in water and B consisting of 0.5% formic acid

in acetonitrile. The separation gradient was linear from 25% B at 0 min to 75% B at 50 min. Following that, the column was washed with 90% B (two column volumes) and 25% B (at least 5 column volumes). The mass spectrometer used was a Quattro Micro. Conditions were set according to Spoof et al. (2003). The chromatogram was monitored in the MRM mode for 11 transitions, the final fragment being always at 135.1, while the parent fragments were: 505.8 (didemethyl-RR), 512.8 (demethyl-RR), 519.8 (RR), 825.5 (Nodularin), 910.5 (LA), 967.5 (didemethyl-LR), 981.5 (demethyl-LR), 995.6 (LR), 1017.5 (didemethyl-YR), 1031.5 (demethyl-YR), 1045.5 (YR).

2.4. DNA extraction and PCR amplification

In our effort to molecularly characterize the cyanobacterial community of Amvrakikos Gulf and to identify potential microcystin producing genera we used a number of PCR approaches followed by either DGGE analysis or construction of clone libraries.

Initially, phytoplankton from water samples was collected by filtration (Millipore, 0.45 μm) followed by resuspension and centrifugation at 8500 × *g* for 10 min. For genomic DNA extraction we used the protocol previously described by Vareli et al. (2009a). Subsequently, the DNA was dissolved in MilliQ water and purified on a Wizard column (Promega, Madison, WI, USA) according to the manufacturer's recommendations.

PCR amplifications were performed in a Biorad iCycler in a 50 μl reaction volume containing approximately 100 ng of DNA.

For intergenic transcribed spacer sequences (ITS) amplification we used a combination of two cyanobacteria-specific primers: 16S GC-CSIF primer and 23S ULR primer. These amplicons, spanning the entire rRNA-ITS and referred to ITSc amplicons. Primer sequences and PCR conditions were as described earlier by Janse et al. (2003).

Potential *mcyS* genes were targeted with the use of four primer sets. Set1: primers mcy-R4 and mcyE-F2 against microcystin synthetase gene E (*mcyE*) of all potential microcystin producing freshwater genera (Rantala et al., 2006; Vareli et al., 2009a), Set2: degenerate primers DKF and DKR against PKSs (Schembri et al., 2001), Set3: primers mcyA-Cd 1F and mcyA-Cd 1R against to the condensation (C) domain of the *mcyA* synthetase gene (Hisbergues et al., 2003), Set4: primers MSF and MSR against N-methyl transferase (NMT) domain of the *mcyA* synthetase gene (Tillett et al., 2001). Initially, PCR conditions were according to the literature cited. Where no product was detected, PCR reactions were tested for contaminant inhibition by seeding with 10–100 ng of positive control DNA (plasmid DNA carrying either *Microcystis* sp., *Anabaena* sp. or *Planktothrix* sp. *mcyE* gene fragments previously cloned from Lake Ziros-Greece, Vareli et al., 2009a,b) and subsequently annealing temperatures were modified in order to obtain a PCR product (modified annealing temperatures for Set1: 50 °C, Set3: 53 °C, Set4: 50 °C).

The PCR reaction against the *mcyE* gene of all potential freshwater genera (primer set1) were also targeted with the same general forward primer (mcyE-F2) and one of the following genus-specific reverse primers mcyE-12R, mcyE-R8 and mcyE-plaR3 for *Anabaena*, *Microcystis* and *Planktothrix* spp. respectively (Rantala et al., 2006). For the semi-nested PCR procedure, 1–2 ml of the first PCR reaction was used as template for the second PCR reaction. PCRs were also performed as previously described (Rantala et al., 2006).

In all cases a proofreading DNA polymerase was used (Expand High Fidelity DNA polymerase, Roche).

2.5. Development of clone libraries of putative microcystin synthetase gene fragments

PCR products that had been amplified by using the previously mentioned primer sets (primer sets 1–4) were gel purified using a Macherey-Nagel DNA clean-up kit (Nucleospin Extract), and subsequently they were cloned using a TOPO TA cloning Kit (Invitrogen) according to the manufacturer's instructions. Twenty-five clones from each clone library were picked randomly. Plasmid DNA was extracted by using Nucleospin plasmid kit (Macherey-Nagel). Inserts were reamplified by using the original primer sets. Clones of the same size were screened further by HaeIII digestion in order to identify Restriction Fragment Length Polymorphisms (RFLPs) among them. Clones with different sizes and RFLP profiles were sequenced. Sequencing was performed by Macrogen Inc. Seoul, Korea.

2.6. Denaturing gradient gel electrophoresis (DGGE) cloning and sequencing

In order to study genotype composition of the total cyanobacterial community in the Gulf, PCR amplified products of the intergenic transcribed spacer region (ITSc amplicons) of cyanobacteria was analyzed by DGGE.

DGGE was performed essentially as described earlier by Muyzer et al. (1993) with minor modifications as described by Janse et al. (2003). 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 DDGE gel to confirm its identity. The new PCR products were purified using a Macherey-Nagel DNA clean-up kit (Nucleospin 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.

2.7. Phylogenetic analysis

All cloned ITS 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 version 4.1 (Tamura et al., 2007) and then automatically aligned using the integrated aligner tool. Phylogenetic analyses were also performed with MEGA 4.1. Trees were constructed using the Neighbour-Joining method with Jukes–Cantor distance correction. The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications.

2.8. Nucleotide sequences and accession numbers

The ITS sequences were deposited at GenBank and were assigned accession numbers HM244684–HM244704. The gene fragment isolated from the *mcyE* clone library was assigned accession number HM448449. Clones from the *mcyA*-Cd library were assigned accession numbers JF729340–JF729343. Clones isolated from the *mcyA*-NMT library were assigned accession numbers JF729336–JF729339 and JF729344. Clones amplified by using PKS degenerate primers were assigned accession numbers JF729325–JF729334.

3. Results

3.1. Environmental data and microcystin measurements

During this study, Gulf temperatures in Amfilohia sample station (Fig. 1) ranged between 12 °C during winter (January–February) and 28 °C during summer (July) (Table 1). Salinity ranged between 30‰ and 34‰. Higher Chl *a* concentrations were measured during spring (March–May). Lower Chl *a* concentrations were measured during late summer to mid winter. The lowest Chl *a*

concentration was measured in a sample collected at 10/2006 (Table 1). pH values ranged between 7.5 and 8.3. Inorganic nitrogen and phosphorus levels were high during most of the sampled dates with phosphorus and nitrate levels being higher during wet months (12/2006–4/2007).

Microscopic examination of the samples revealed that during periods of higher Chl *a* concentrations, diatoms are abundant. In contrast, during periods of lower Chl *a* levels coccoid cyanobacteria species (approximately 2.5 μm) prevailed (data not shown).

Microcystin (and/or nodularin) concentrations in water samples were measured by ELISA and were expressed as microcystin-LR equivalents. As shown in Table 1 microcystin (and/or nodularin) concentrations ranged from 66 μg g^{-1} dw (10/2006) to 0.004 μg g^{-1} dw (1/2007) in seston and from 19.8 ng l^{-1} (10/2006) to 0.004 ng l^{-1} (5/2007) in water (volumetric microcystin concentration: soluble and cell bound l^{-1}).

Microcystin concentration was also measured (by ELISA) in whole mussel tissue of wild mussels collected monthly from Amvrakikos Gulf. The highest microcystin (and/or nodularin) concentration was measured in mussel samples collected in 3/2007 and was 141.5 ± 13.5 ng g^{-1} tissue. The lowest microcystin (and/or nodularin) concentration was measured in samples collected three months later and it was 45 ± 2 ng g^{-1} tissue. Nevertheless, it must be highlighted that mussel tissue (throughout our study) from Amvrakikos Gulf contains microcystins (and/or nodularins) above the upper limit set by the WHO (0.04 μg kg^{-1} body weight, or 2 μg for an adult weighing 50 kg) (WHO, 1998) (Table 1).

A seston sample (collected at 10/2006), with the highest amount of microcystin measured during this study, was analyzed further by HPLC–tandem MS. HPLC–tandem MS analysis revealed that the toxin which is present in Amvrakikos Gulf is microcystin. Moreover, the main variants that found to be present were microcystin-LR and microcystin-YR (Fig. 2).

3.2. Clone of potential microcystin synthetase (mcyS) gene fragments

In order to identify the toxin producing cyanobacterium/a, we tried to study the composition of potential microcystin producing genera following a PCR approach based on the use of general primers against microcystin synthetase gene E (*mcyE*) of all known freshwater microcystin producing genera (Rantala et al., 2006). By using this PCR approach, in previous studies (Vareli et al., 2009a,b), our detection limit was set at 10 μg microcystin g^{-1} dw of seston, or 0.2 μg l^{-1} Chl *a* or 10 ng l^{-1} microcystin LR. The sample we used was the sample with the highest microcystin content −66 μg g^{-1} dw, 0.92 μg l^{-1} Chl *a*, 19.8 ng l^{-1} microcystin LR well above our detection limit. Unfortunately, we failed to amplify any product following standard PCR conditions (Vareli et al., 2009a; Rantala et al., 2006). Seeding of positive control DNA extracts into the samples resulted in positive amplification, indicating that contaminant inhibition of the PCR did not occur (Glas et al., 2010). In order to further expand our detection limit a semi nested PCR approach was used. By using this approach we managed to amplify *mcyE* gene fragments even in cases with undetectable microcystin amounts in seston samples as described earlier (Vareli et al., 2009b). For the semi nested PCR procedure, the initial PCR "product" was targeted with the same general forward primer and one genus specific reverse primer either for *Anabaena* sp., *Microcystis* sp., or *Planktothrix* sp. Once more we failed to amplify any PCR product.

Alternatively, we used also two other primer sets. A primer set (mcyA-Cd 1F and mcyA-Cd 1R) against to the condensation (C) domain of the *mcyA* synthetase gene (Hisbergues et al., 2003), and a primer set (MSF and MSR) against N-methyl transferase (NMT) domain of the *mcyA* synthetase gene (Tillett et al., 2001). No product following standard PCR conditions was detected.

In contrast, we managed to amplify PCR products by using all the above-mentioned primer sets (primers for *mcyE*, *mcyA-Cd* and *mcyA NMT*) under lower annealing temperature conditions. These products were cloned in order to develop clone libraries. Another clone library was constructed also containing putative PKSs gene fragments. Twenty-five randomly selected clones from each library (total 100 clones) were reamplified by using the original primer sets. Clones of the same size were screened further by HaeIII digestion in order to identify potential duplicate clones. Clones with different sizes and RFLP profiles were sequenced.

Unfortunately, only one clone upon BLASTn search found to have a significant homology to a known *mcyS* gene. This clone (clone 4, JF729328) was isolated from the PKS clone library and upon BLAST searching found to be 66%, 67%, 71% identical to *mcyG* gene from *Anabaena* sp. 90, *Planktothrix rubescens* NIVA CYA 98 and *M. aeruginosa* respectively (query coverage 59–73%). Upon BLASTx search this fragment was found to code for a putative PKS 62% identical to McyG from both *M. aeruginosa* (PCC7806, PCC7820, NIES-843) and *P. rubescens* NIVA-CYA 98 (99% query coverage).

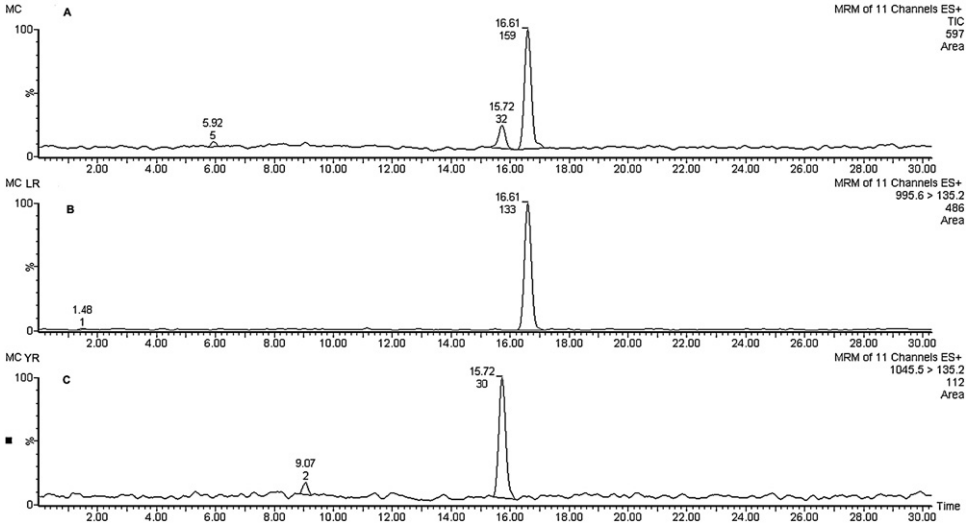

Fig. 2. LC–tandem MS. HPLC–Tandem MS analysis of a seston sample collected (10/2006) from Amvrakikos Gulf. (A) Total ion counts for all transitions. (B) Transition specific for microcystin LR. (C) Transition specific for microcystin YR.

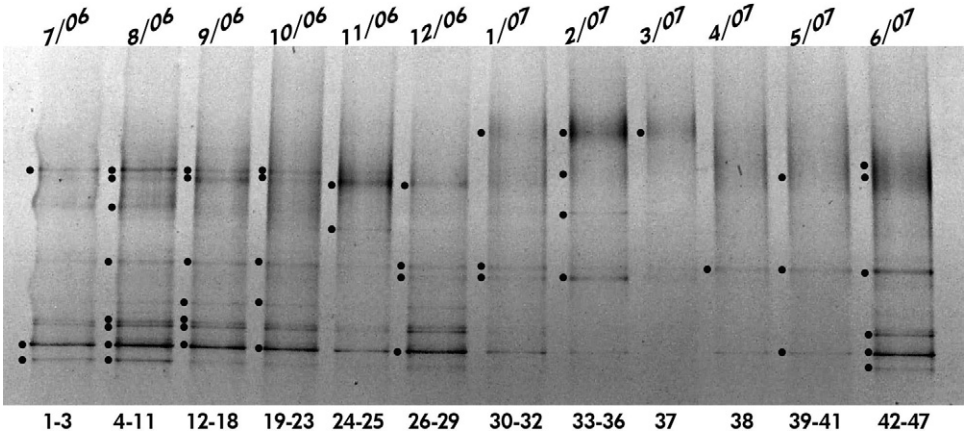

Fig. 3. Cyanobacterial species composition in Amvrakikos Gulf as revealed by ITSc DGGE profiles. Bands indicated by numbers 1–47 were excised, reamplified and sequenced.

3.3. Molecular characterization of the cyanobacterial community

To achieve high-resolution analysis for accurate identification of potential toxic cyanobacteria, we used the denaturing gradient gel electrophoresis profile of amplified ITSc fragments, spanning the entire rRNA-ITS. We used ITSc primer amplicons because they contain more sequence information than ITSa and ITSb amplicons. Moreover, for most tested genera, they yield sharp bands on DGGE gels (Janse et al., 2003). The cyanobacterial community profiles during the entire study period are shown in Fig. 3. Both prominent and faint bands were excised, re-amplified, cloned and sequenced.

BLAST searches revealed that most of the sequences had a highly homologous counterpart in the GenBank/EMBL/DDBJ databases. Thirty seven out of forty seven bands matched nearly completely (92–99%) with database entries, based on a comparison of the major part of rRNA-ITS (Table 2). These sequences were classified as *Synechococcus* sp. sequences. Phylogenetic relationships among *Synechococcus* sequences from Amvrakikos Gulf and those already deposited to GenBank are shown in the constructed phylogenetic tree (Fig. 4). Most of *Synechococcus* sequences from Amvrakikos Gulf are clustered into subcluster 5.3 (Choi and Noh, 2009). The remaining *Synechococcus* sequences are clustered into three previously identified clades (WPC1, I, and VI), with the exception of Amv.6 which along with the recently isolated from Pacific Ocean clone ES-0712-39 formed a clade closely related to WPC2 (Fig. 4) (Ahlgren and Rocap, 2006; Choi and Noh, 2009).

Sequences with lower similarities (84–86%), restricted only to two thirds of the total ITSs submitted so far, were classified as *Synechocystis* sp. sequences (Table 2). There are also five sequences corresponding to yet unknown marine cyanobacteria (bands numbered 15, 22, 39, 42, and 43).

4. Discussion

Intoxication of aquatic organisms involving cyanobacterial toxins is well documented in freshwater ecosystems worldwide (Codd et al., 2005), while intoxications of marine organisms have only occasionally been reported (Chen et al., 1993; Miller et al., 2010).

We decided to study cyanotoxins (microcystin and/or nodularin) in Amvrakikos Gulf because this semi-enclosed embayment in NW Greece is profiled as a major marine candidate for phytoplankton blooms and putative toxic cyanobacterial blooms due to its eutrophied character (Panayotidis et al., 1994; Vassilopoulou et al., 2002).

Table 2
Sequence analysis of excised denaturing gradient gel electrophoresis (DGGE) bands.

DGGE band. number	Base pairs sequenced	Identical to excised DGGE band	Closest matching organism	Base pairs compared	Similarity
Amv.1	561	4	*Synechocystis* sp. PAK 12 EF555570.1	414/487	85%
Amv.2	773	10,18,23,29	*Synechococcus* sp. KORDI-15 FJ497779	764/772	98%
Amv.3	773	11	*Synechococcus* sp. RCC307 CT978603.1	771/773	99%
Amv.5	925	13,20,24,26	*Synechococcus* sp. KORDI-62 FJ497768	918/924	99%
Amv.6	945		Uncultured *Synechococcus* sp. clone ES-0712-39	936/945	99%
Amv.7	773	14,21,27,31	*Synechococcus* sp. RCC307 CT978603.1	766/772	99%
Amv.8	773	16	*Synechococcus* sp. RCC307 CT978603.1	766/773	99%
Amv.9	773	17	*Synechococcus* sp. RCC307 CT978603.1	768/773	99%
Amv.12	558	19	*Synechocystis* sp. PAK 12 EF555570.1	411/484	84%
Amv.15	428	22	Uncultured Antarctic cyanobacterium clone N184-4 EU032391.1	121/125	96%
Amv.25	954		*Synechococcus* sp. KORDI-50 FJ497763	949/953	99%
Amv.28	773	32,36	*Synechococcus* sp. RCC307 CT978603.1	768/772	99%
Amv.30	937	33,37	*Synechococcus* sp. UW76 DQ351295.1	899/937	96%
Amv.34	772		*Synechococcus* sp. KORDI-15 FJ497779	738/746	98%
Amv.35	964		*Synechococcus* sp. KORDI-49 FJ497773.1	946/963	98%
Amv.38	773	40,44	*Synechococcus* sp. RCC307 CT978603.1	763/772	98%
Amv.39	475	43	Uncultured cyanobacterium EU23392.1	471/474	99%
Amv.41	773	46	*Synechococcus* sp. KORDI-15 FJ497779	768/772	99%
Amv.42	623		Unicellular cyanobacterium LLi67 DQ786165.1	528/706	74%
Amv.45	773		*Synechococcus* sp. RCC307 CT978603.1	767/772	99%
Amv.47	773		*Synechococcus* sp. RCC307 CT978603.1	768/772	99%

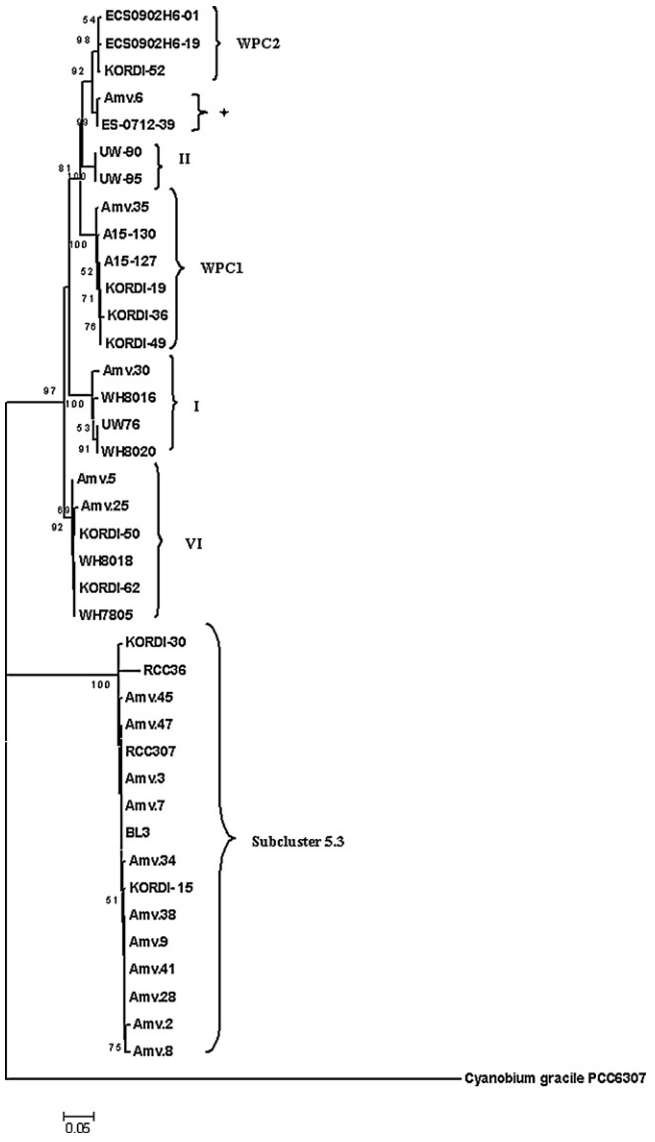

Fig. 4. Distance tree based on the alignment of *Synechococcus* sp. rRNA ITS sequences from Amvrakikos Gulf and a number of sequences with the highest similarities retrieved from GenBank/EMBL/DDBJ databases. Potential new clade or sub-clade is indicated by an asterisk. Bootstrap values higher than 50% are indicated. *Cyanobium gracile* PCC6307 was used as an outgroup according to Choi and Noh (2009). Scale bar, 0.05 nucleotide substitution per site.

During our study, inorganic nitrogen and phosphorus levels were high in most of the sampled dates with phosphorus (ranging between 0.01 and 0.9 mg l^{-1}) and nitrate (ranging between below detection limit and 24 mg l^{-1}) levels being higher during the wet months. The higher Chl *a* concentration was measured during spring (13.3 μg l^{-1}) and the lower during late summer to mid winter (0.92 μg l^{-1}). These data are in accordance with previously published studies confirming the eutrophic status of the Gulf (Panayotidis et al., 1994; Vassilopoulou et al., 2002). Volumetric cyanotoxin concentrations in water samples (cell bound l^{-1}) were measured by using an Abraxis ELISA kit and found to be low but detectable. Based on ELISA measurements we could not confirm that the toxin measured was either microcystin or nodularin or both, as the antibody used in the Abraxis ELISA test recognizes an epitope (the amino acid Adda: [2S,3S,8S,9S]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), which is identical in both toxins. However, by using HPLC–tandem MS we identified microcystin as the toxin present in Amvrakikos Gulf. Moreover, we found that microcystin-YR and microcystin-LR are the prevailing variants (Fig. 2).

The presence of tumor promoters of the microcystin family in oceanic and coastal waters was identified initially in mussels collected from the Northeastern Pacific, Eastern Canadian and European coasts (Chen et al., 1993). Recently, mussel samples collected from Amvrakikos Gulf were found to be Diarrheic Shellfish Poisoning (DSP) positive (Economou et al., 2007). Mice injected with extracts of the Amvrakikos samples, in addition to the typical DSP intoxication symptoms, exhibited a marked hypersensitivity 2 h before death, convulsions and shorter survival time. The authors suggested that these symptoms were indicative of the simultaneous presence of an unknown toxin along with okadaic acid and DTXs (Economou et al., 2007).

In our study we found that whole tissue samples of the edible mussel *M. galloprovincialis* collected from the Gulf are severely contaminated by microcystins. Microcystin concentrations in tissues, expressed as microcystin-LR equivalents in ng g^{-1} wet weight, exceed the upper limit of tolerable daily intake (TDI) of microcystin (0.04 μg kg^{-1} body weight, or 2 μg for an adult weighing 50 kg) determined by WHO (WHO, 1998) (Table 1). This is the first report suggesting that humans are at risk from microcystin poisoning when consuming shellfish harvested from a Mediterranean marine ecosystem. It is interesting to note that we measured high concentrations in the mussel tissues even in those samples collected during low microcystin concentrations in seston. A laboratory experiment (Amorim and Vasconcelos, 1999) showed that microcystin depuration in *M. galloprovincialis* mussels is not a very rapid process and contamination by feces containing microcystins is likely to occur and increase the persistence of these toxins in the mussels after the bloom disappearance. It has also been shown (Madenwald, 1985; Novaczek et al., 1992) that in the case of dinoflagellate toxins, depuration depends on temperature, salinity, size of the molluscs and season. Wild and farmed marine bivalves consumed by sea otters and humans exhibit high microcystin uptake and slow depuration under conditions that mimic natural exposure (Miller et al., 2010). These studies suggest that depuration of toxins proceeds less or more rapidly depending on environmental conditions. Moreover, Dahl et al. (1996) reported that *Mytilus edulis* mussels contaminated with okadaic acid from *Dinophysis* sp. retained the toxin throughout the winter until spring, even though they had been accumulated during autumn, an observation that is in agreement with our results.

Ocean discharge of freshwater microcystins was confirmed for three nutrient-impaired rivers flowing into the Monterey Bay National Marine Sanctuary, and microcystin concentrations up to 2900 ppm (2.9 million ppb) were detected in a freshwater lake and downstream tributaries within 1 km of the ocean (Miller et al., 2010). In the Northern part of Amvrakikos Gulf lie the extensive deltas of the Louros and Arachthos Rivers (Fig. 1). Microcystin concentrations in these rivers were found to be undetectable as expected, since neither the Louros River nor the Arachthos drained from lakes or reservoirs supporting cyanobacterial blooms (Cyanobacterial species composition in lakes of W. Greece, manuscript in preparation).

Attempting further to exclude the possibility of freshwater origins of the toxin detected both in water and in mussel tissue, we tried to amplify gene fragments corresponding to microcystin synthetase gene (*mcyE*) by using a combination of primer sets against all known toxic freshwater cyanobacterial species (Rantala et al., 2006; Vareli et al., 2009a,b).

Because it is known that PCR would fail to detect *mcyS* genes present in low abundance (Richardson et al., 2007; Glas et al., 2010) we used as a template DNA extracted from the sample with the highest microcystin concentration (sample collected at 10/

2006). This sample contains microcystin high enough to reach our detection limit as set by previously published studies (Vareli et al., 2009a,b). Moreover, the semi nested PCR procedure, enabled us to amplify *mcyE* gene fragments even in seston samples with undetectable amounts of microcystins (Vareli et al., 2009a,b). In the case of Amvrakikos Gulf, we failed to amplify any gene fragment by using either conventional or semi nested PCR. In order to exclude the possibility of the presence of potential inhibitors, we applied seeding of positive control DNA extract into the sample which resulted in positive amplification (Glas et al., 2010). Moreover, DNA of the same sample was successfully amplified by using PCR primers against cyanobacterial ITS. Thus, although we cannot fully exclude the possibility that the lack of PCR products is probably due to other reasons such as an extremely low abundance of the microcystin producing organism, we propose that microcystin presence in the Gulf is more likely to have a marine origin.

Recently, common marine cyanobacteria strains of *Oscillatoria*, *Synechocystis* and *Synechococcus* genera isolated from Portuguese coastal waters were found to cause toxic effects on mice. The histopathological changes observed in the liver of treated mice were indicative of hepatotoxicity. Seventeen strains screened for microcystins by ELISA and seven of them (four *Synechococcus* strains, one *Synechocystis* strain, one *Oscillatoria* strain and one *Cyanobacterium stanieri* strain) were found to produce small quantities of microcystins (Martins et al., 2005).

In another study (Carmichael and Li, 2006), microcystins were detected by ELISA in the majority of liver and intestine samples from grebes collected due to morbidity or mortality from the Salton Sea, the largest inland body of water in California with salinities varying from freshwater/brackish water at the major river outlets to hypersaline conditions in the sea proper. Isolation, culture and ELISA testing for microcystin of 50 strain isolates showed that microcystins were produced by all strains—albeit at low levels ($<1\ \mu g\ g^{-1}$ dw). The genera producing measurable levels of microcystin included mainly *Synechococcus* and *Oscillatoria*. The most notable result of the study was that a microcystin—producing *Synechococcus* strain (SS-1) was found by 16S rRNA analyses to be closer to marine strains of *Synechococcus* than to freshwater strains of this genus. Moreover, *Synechococcus* isolates from the Salton Sea were shown to produce both microcystin LR and microcystin YR by LC/MS (Carmichael and Li, 2006).

Unfortunately, in both studies no attempt was made in order to characterize potential *mcyS* genes from the cultivated marine microcystin producing cyanobacterial strains.

This was however done by Richardson et al. (2007) with two black band disease (BBD) related marine cyanobacteria isolates (*Geitlerinema* sp. and *Leptolyngbya* sp.) which were found to be microcystin producers. In this study by using two primer sets against *mcyA* synthetase gene the authors managed to amplify a putative *mcyA* gene fragment from a *Geitlerinema* strain and another from a *Leptolyngya* strain. The *Geitlerinema*'s *mcyA* gene fragment was found to be 100% homologous only in a small part of the total sequence (3% query coverage) of the *M. aeruginosa mcyA* gene. The same was found for *Leptolyngbya*'s *mcyA* gene fragment (100% homologous, 8% query coverage of *Planctothrix* NIVA-CYA34 *mcyA* gene).

More recently, Frazao et al. (2010) by using a number of PCR primer sets against *mcyS* gene cluster managed to amplify *mcyE* gene fragments from *Leptolyngbya* and *Oscillatoria* strains initially isolated from Portuguese coastal waters. These fragments showed 99% similarity with the *mcyE* gene of a *Microcystis* sp. strain. However, the above mentioned strains were not found to be microcystin producers. While the authors did not manage to amplify other genes of the *mcyS* gene cluster, they hypothesized that deletion events may have occurred resulting in the loss of these genes (Frazao et al., 2010).

In the case of Amvrakikos Gulf we tried to amplify potential *mcyS* gene fragments by using primer sets against *mcyE*, *mcyA*-Cd, *mcy*-NMT and PKS. With the exception of the PKS primer set, all the other primer sets failed to amplify any gene product with a significant BLASTn hit. Among putative PKS gene fragments, clone 4 (JF729328) is the most promising while BLASTn searching revealed 66%, 67%, 71%, identity to *mcyG* gene from *Anabaena* sp., *P. rubescens* NIVA-CYA 98 and *M. aeruginosa*, respectively (query coverage 59–73%). Upon BLASTx search this fragment was found to code for a putative PKS 62% identical to McyG from both *M. aeruginosa* (PCC7806, PCC7820, NIES-843) and *P. rubescens* NIVA-CYA 98 (99% query coverage).

In conclusion, our marine putative *mcyS* gene fragment was found to be more closely related to known freshwater *mcyS* genes than the previously described *Geitlerinema*'s and *Leptolyngya*'s putative *mcyA* genes (Richardson et al., 2007). In contrast, *mcyE* gene fragments from *Leptolyngbya* and *Oscillatoria* strains initially isolated from Portuguese coastal waters were found to be more closely related to known freshwater *mcyS* genes than ours (Frazao et al., 2010). It is important to note that BBD *Geitlerinema* and *Leptolyngbya* are microcystin producers while Portuguese *Leptolyngya* and *Oscillatoria* are not. It seems likely that there are differences between *mcyS* genes from certain marine microcystin producing cyanobacteria and their freshwater counterparts. This is not improbable, although the similarity of *mcyS* gene clusters between distantly related genera is astonishingly high (Kurmayer and Christiansen, 2009). For instance, comparison of *mcyE* gene from *Anabaena* strain 90 with those *mcyE* genes sequenced from other freshwater cyanobacteria revealed 75% identity to *M. aeruginosa* and 78% identity to *Planktothrix agardii* (Rouhiainen et al., 2004). The homologies are even lower for *mcyA* gene, 69% identity to *M. aeruginosa mcyA* gene and 67% identity to *Planktothrix agardii mcyA* gene (Rouhiainen et al., 2004). Based on these sequence differences, Rantala et al. (2006) developed genus specific primer sets in order to identify putative toxic cyanobacterial genera in mixed toxic freshwater cyanobacterial populations.

In order to identify potential marine toxic species in the Gulf, we decided to characterize the cyanobacterial community by using culture independent molecular methods. We found that the cyanobacterial community was dominated by the cosmopolitan genus *Synechococcus.* Thirty seven out of forty seven DGGE bands that have been cloned and sequenced upon BLAST search were found to be *Synechococcus* sequences. Based on ITS sequence phylogenies, *Synechococcus* genotypes from Amvrakikos Gulf were clustered into five (subcluster 5.3, clade I, VI, WPC1 and WPC2) out of 18 previously identified clades (Ahlgren and Rocap, 2006; Choi and Noh, 2009) (Fig. 4). It is noteworthy that subcluster 5.3 (exclade X) dominated in the Gulf (Fig. 4). While some of the clades possess distinguished physiological characters that render them ecologically distinct (Ahlgren and Rocap, 2006), a lot of work needs to be done in order to explain the dominance of the above-mentioned clade in the Amvrakikos Gulf. The low levels of environmental detection of subcluster 5.3 by using oligonucleotide probes thus far preclude generalization about the ecological distribution of the clade (Scanlan et al., 2009). Other *Synechococcus* genotypes are clustered as members of clade I (Amv.30) and VI (Amv.5 and 25). Members of the clade I are frequent in coastal and/or temperate mesotrophic open ocean waters largely above 30°N and below 30°S, while members of the clade VI are relatively widely distributed in low abundance but have been seen to dominate mesotrophic upwelling regions (Scanlan et al., 2009).

Two other sequences (Amv.35 and Amv.6) formed clades with the clone sequences KORDI-49 and Uncultured *Synechococcus* sp. clone ES-0712-39, respectively (Fig. 4). KORDI-49 was previously characterized as a member of a distinct novel *Synechococcus* clade

named WPC-1 (Choi and Noh, 2009). Clone ES-0712-39 which has been isolated recently from north-western Pacific Ocean, along with Amv.6 formed a clade closely related to WPC2. WPC-1 and WPC-2 clades initially seem to be restricted to the East China Sea. Based on our results it seems likely that WPC-1 and WPC-2 clades are more widespread than previously considered.

From the remaining ten genotypes, four genotypes were found to be homologous to *Synechocystis* sequences and the other six genotypes to unknown cyanobacterial species. It should be noted that *Synechocystis* genotypes are present only during periods of high microcystin contents in seston. Moreover, the similarity of ITS sequences among *Synechocystis* sequences from Amvrakikos Gulf and the closest relative in GenBank was very low (c. 85% to *Synechocystis* sp. PAK12, EF555570.1) (Korelusova et al., 2009). Thus, *Synechocystis* species from Amvrakikos Gulf deserve further investigation due to their phylogenetic distinction from other populations worldwide.

In summary, the present study is the first report on the presence of the hepatotoxin microcystin in the Mediterranean Sea, the first one to examine accumulation of the toxin in mussels from a Mediterranean marine ecosystem and one of the few studies that correlate the presence of the toxin with the cosmopolitan cyanobacterial species *Synechococcus* and *Synechocystis*. Moreover, while Amvrakikos Gulf is one of the most productive Greek sea food areas, our results indicate that there is also risk of Hepatotoxic shellfish poisoning (HSP) along with the previously reported DSP risk (Economou et al., 2007).

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