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Experimental Exposure of the Mediterranean Mussels *Mytilus galloprovincialis* to Potentially Toxic Cyanobacteria (*Synechocystis* sp.) and Detection of Microcystins

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Abstract

The effect of microcystins (MCs) was assessed on Mediterranean mussels *Mytilus galloprovincialis* during experimental exposure to marine cyanobacteria belonging to the genus *Synechocystis*. The strain was isolated for the first time in Greece, from the Thermaikos gulf (Central Macedonia, Northern Greece). All mussels used for the experiment were collected from farms located in the mentioned area. No toxic algae or phytoplankton populations were observed during the experimental period. The mussels were divided in three different groups; two of the three groups were fed daily with 10,000 cells/mL and 100,000 cells/mL of *Synechocystis* sp., respectively, whereas the third group was not fed at all. MCs were detected using a specific, validated direct competitive enzyme-linked immunosorbent assay. The experimental exposure was conducted in triplicates. The results were confirmed by ultra-high-performance liquid chromatography-tandem mass spectrometry. It was demonstrated that the Mediterranean mussels *M. galloprovincialis* could accumulate MCs up to 6.85±0.220 μg/kg after 72 hours of exposure at a density of 100,000 cells/mL to the marine strain *Synechocystis* sp.

Keywords: Cyanobacteria, microcystins, *Mytilus galloprovincialis*, *Synechocystis* sp.

Introduction

Cyanobacteria are prokaryotic, photosynthetic, gram-negative microorganisms, which can be unicellular or filamentous and are distributed worldwide in fresh, brackish, and marine waters, even in extreme environments (Ward et al., 2012). Toxic cyanobacteria and their secondary metabolites, the cyanotoxins, are of great concern because they induce toxic effects in humans, domestic animals, and wildlife and can cause damage to the ecosystems (Preece et al., 2017; Vareli et al., 2012). The most common toxic cyanobacteria reported belong to species of the genera *Microcystis*, *Anabaena*, *Dolichospermum*, *Planktothrix*, *Aphanizomenon*, *Cuspidothrix*, *Nodularia*, *Cylindrospermopsis*, *Lyngbya*, and *Oscillatoria*, as well as to the picoplanktonic species of *Synechocystis*, *Synechococcus*, and *Prochlorococcus* (Deniz and Taş, 2009; Jakubowska and Szeląg-Wasielewska, 2015; Turner et al., 2018; Vareli et al., 2012). One of the most important groups of cyanotoxins is microcystins (MCs). They induce hepatotoxicity, and their analog MC-LR has been classified as a potential carcinogen, group 2B, by the International Agency for Research on Cancer (IARC) (IARC Monographs, 2010; Svirčev et al., 2010). Moreover, MCs affect aquatic animals, inducing damages in the liver, kidneys, gills, intestine, and heart. They promote changes in the immunological indices, enzyme activities,

and blood cells and lead to chronic toxicity in fish, inducing osmoregulatory imbalance (Pavagadhi and Balasubramanian, 2013; Pham and Utsumi, 2018).

Although MC production from freshwater cyanobacteria species has been documented, there is a lack of data regarding MC production from marine picoplanktonic ones. The potential production of MCs from *Synechocystis* sp. strains has been described in fresh, brackish, and marine waters (Barboza et al., 2017; Jakubowska and Szeląg-Wasielewska, 2015). For example, Lincoln and Carmichael (1981) have described the MC from picoplanktonic *Synechococcus* sp. and *Synechocystis* sp. as potentially toxic. Moreover, MC-producing strains of *Synechocystis* sp. have been reported by Domingos et al. (1999) in a brackish lagoon reservoir in Rio de Janeiro and during algal blooms in Sepetiba Bay in Brazil (Magalhães et al., 2003). Similar studies have been conducted in wastewater treatment plant for agricultural reuse in both Morocco (Oudra et al., 2002) and Portuguese coasts (Martins et al., 2005). Nascimento and de Oliveira e Azevedo (1999) have reported the production of MCs by the strain *Synechocystis aquatilis* f. *salina* that were isolated from saline water in Barra lagoon at Rio de Janeiro, and fish mortality was recorded.

Ignatiades and Gotsis-Skretas (2010) have reported an incident of blooming owing to *Synechocystis sallensis* in Evoikos gulf (Central Greece). In 2012 during an algal bloom in Amvrakikos gulf in Greece, both *Synechocystis* sp. and *Synechococcus* sp. were detected (Vareli et al., 2012). It was demonstrated that the accumulation of MCs in the mussels *Mytilus galloprovincialis* ranged from 45±2 ng/g to 141.5±13.5 ng/g wet weight, values that exceed the upper limit of the tolerable daily intake for human consumption (TDI, 0.04 µg/kg/day bodyweight) (World Health Organization [WHO], 2003).

This study aimed to investigate the potential production of MCs from the marine strain of *Synechocystis* sp., its accumulation in the mussels' tissues, and its relationship with the mentioned cyanobacterium population density.

Materials and Methods

Sampling area
All mussels used for the experiment were collected from mussel farms located in the sea area of Chalastra near the Delta of Axios, Loudias, and Aliakmonas rivers in Thermaikos gulf, Central Macedonia Greece (40°32′20.12′′N and 22°44′56.63′′E), during July 2015 (Figure 1). Thermaikos is a semi-closed, shallow gulf (maximum depth 90 meters), with a surface area of 5,100 km², located in the north-west Aegean Sea. According to the laboratory unit for harmful marine microalgae of Aristotle University of Thessaloniki, which is authorized for the Toxic Phytoplankton Surveillance Program, neither toxic phytoplankton nor algal blooms were recorded during the sampling period.

Figure 1. Sampling areas of Chalastra and Aggelochori in Thermaikos gulf, Central Macedonia, Greece

In total, 700 mussels *M. galloprovincialis*, which were 13-month-old, were collected and sent within 2 hours to the Laboratory of Ichthyology, School of Veterinary Medicine, Aristotle University of Thessaloniki, using proper ice boxes. Only alive mussels were used and measured. The mean length was found to be 7±1.0 cm (n=10), and the mean bodyweight was 6.4±0.30 g (n=10).

Chemicals and reagents
Brain heart infusion broth, glycerol, and sheep blood agar were purchased from Oxoid (Basingstoke, United Kingdom), MacConkey agar from Merck (Darmstadt, Germany), and antibiotic supplement from BioRad (Munchen, Germany). Inorganic solvents and biotin were purchased from Sigma Aldrich Co. (St. Luis, USA) and organic solvents from Thermo Fisher Scientific (Waltham, MA, USA). HLB OASIS cartridges (Hydrophilic-Lipophilic Balanced) used for solid-phase extraction were purchased from Waters (Milford, MA, USA). Marine salt minerals were obtained from TROPIC MARIN (Wartenberg, Germany); the certified reference material (CRM) of MC-LR, MC-RR, and MC-YR were obtained from CIFGA (Lugo, Spain); and uncontaminated mussels tissue (CRM-Zero-Mus) were obtained from the National Research Council (Newland and Labrador, Canada). The enzyme-linked immunosorbent assay (ELISA) kit was purchased from CD Creative Diagnostics (New York, USA). The Acclaim Polar Advantage II kit including a C18 reverse-phase column Acclaim Polar Advantage II 3 µm 3×150 mm, guard column 2/pk, and Holder-Coupler used for ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Isolation and inoculation of *Synechocystis* sp.
In 2013, an algal bloom occurred in the Thermaikos gulf near the sea area of Aggelochori, Thessaloniki region, (40° 29′30.05′′N and 22° 49′11.79′′E) (Figure 1), and potential toxic cyanobacteria were detected (Kalaitzidou et al., 2015). The strain *Synechocystis* sp. used in this study was isolated from the above area for the first time in Greece and classified according to Anag-

nostidis and Komárek (1985). The axenic cultures obtained by Kalaitzidou et al. (2015) were identified with polymerase chain reaction analysis according to Webb and Maas (2002) protocol. The isolate was stored at −70°C in 15% v/v glycerol/brain heart infusion broth.

For the preparation of the batch culture, the frozen cells were thawed for 30 minutes at room temperature (22°C) and then for 30 minutes at 30°C. Before preparation of the enrichment medium, the isolated cells were cultured on the sheep blood agar and MacConkey agar and were checked for possible contamination. After 2 days of incubation and microscopic examination, 100 μL was passed in MA selective liquid medium for cyanobacteria. For the MA preparation, ultrapure water was used, inorganic substances were added according to the manufacturer's instructions (NIES collection, Microbial Culture Collection, Ibaraki, Japan), and the pH was adjusted at 8.00. Imipenem (50 mg/L), kanamycin (50 mg/L), nystatin (50 mg/L), and biotin (0.1 μg/L) were added after sterilization and cooling of the medium.

The incubation was performed for 7 days at 25°C in a refrigerated incubator (VOW/ShelLab 1535, Sheldon Manufacturing, USA). The batch culture was kept under ventilation using an air pump at a rate of 2±0.5 mL/s under cool white fluorescent light (700 lx), with a 14/10 light/dark cycle.

Cell counting procedure and experimental design
Cell counting was monitored on the 5th, 6th, and 7th day of incubation. Cell samples were collected with a sterile pipette and then placed in Neubauer improved hemocytometer chamber (0.1-mm depth) and allowed to set for 10 minutes (Ichor et al., 2016; Ji and Yildiz, 2018). After cell counting, decimal dilutions were performed to achieve 10^4 cells/mL and 10^5 cells/mL of *Synechocystis* sp.

The experiment was conducted in triplicates in tanks filled with 20 L of artificial sea water. In total, 40 mussels were placed in each tank. All tanks were cleaned and disinfected using suitable for food contact materials (FD 18 detergent and Combimat BAC-100disinfectant, Greece) to avoid residues that could affect mussels and have matrix effects during ELISA analysis. Min-

erals (Tropic Marin PRO-REEF) were added into ultrapure water at a salinity of 32±1.0 parts per trillion. The pH was adjusted at 8±0.5, and water temperature was maintained at 15°C±2.0°C. In addition, the water was continually aerated (2±0.5 mL/s). The mussels were allowed to acclimate for 24 hours in continually flowing, filtered artificial sea water. Temperature, salinity, pH, and dissolved oxygen were monitored using a handheld multiparameter instrument (YSI 556, YSI Incorporated, Ohio, USA) (Table 1).

A total of three tanks were used, marked as Tank A, B, and C. The mussels in Tank A were daily fed with population density of *Synechocystis* sp. at 10^4 cells/mL and in Tank B were daily fed with population density of *Synechocystis* sp. at 10^5 cells/mL; in Tank C, the mussels were kept as control. From each tank, a sample of 10 mussels was removed 24, 48, and 72 hours after toxin challenge for analysis. All samples were replaced by equal number of marked mussels to maintain the same population density throughout the experimental period.

Mussels' soft tissues were removed gently, placed in a sieve to drain, and homogenized in a blender, and 5±0.1 g of mussels were transferred in a 50 mL polypropylene centrifuge tube and stored at −20°C. No formation of pseudofeces was observed during the experiment.

Extraction and clean-up procedure
The extraction procedure was performed with an aqueous solution of 75% methanol (75:25 v/v methanol:ultrapure water) because it is considered a proper solvent for best recovery and repeatability (Fastner et al., 1998). The frozen homogenized samples were allowed to thaw overnight at 4°C. In addition, 10 mL of methanol was added, and the samples were homogenized using Ultra Turrax™ (IKA, Germany). Sonication was performed for 15 minutes using an ultrasonic bath (Bestultrasonic Co, UK), and the samples were centrifuged at 3,000 g for 10 minutes at 4°C. The supernatant in each tube was transferred in another 50 mL polypropylene centrifuge tube. The extraction procedure was repeated twice, and all the supernatants were pooled. The solvent of the extractions was evaporated by rotary evaporator (Stuart RE300 rotary evaporator, Keison Products, United Kingdom) at 40°C and was re-diluted according to De Pace et al. (2014), and 1 mL of ultrapure water was added to 0.5 mL of the extraction.

Before ELISA analysis, a clean-up procedure by solid-phase extraction using C18 columns was performed with the use of vacuum manifold (Alltech® Vacuum Manifold, 2051 Waukegan Rd, Bannockburn, USA). The cartridges were first conditioned with 5 mL of 100% methanol followed by 5 mL of Milli-Q water. The samples were loaded to HLB OASIS cartridges (200 mg/6 cc) and were washed with 10 mL of Milli-Q water and 2% aqueous solution of methanol to remove the interferences of the

Table 1. Physicochemical parameters during the experiment

| Day | Parameters | | |
	Temperature (°C)	pH	Salinity (ppt)
	Mean±SD	Mean±SD	Mean±SD
1	15.0±0.16	8.20±0.280	32.10±0.260
2	15.0±0.22	8.10±0.120	32.22±0.180
3	15.0±0.13	8.00±0.260	32.18±0.210

ppt: part per trillion; SD: standard deviation.

matrix. The fraction, which was possibly containing toxins, was collected by elution with 5 mL of methanol in glass tubes. The extracts were evaporated under nitrogen stream and reconstituted with 1 mL of Milli-Q water.

Detection of MCs by ELISA and UHPLC-MS
The qualitative and quantitative detection of MCs was performed in triplicates using direct competitive MCs ([(2S,3S,4E,6E,8S,9S)-3-amino-9-methoxy-2,6,8-   trimethyl-10-phenyldeca-4,6-dienoic acid, ADDA)-direct monoclonal (DM) ELISA according to the manufacturer's instructions. Commercial high-sensitivity ELISA kits are considered suitable for screening MCs because they detect many analogs (Gurbuz et al., 2012). Moreover, Preece et al. (2015) proved that DM ELISA is more reliable for the detection of MCs in seafood such as mussels than using other commercial ELISA kits such as anti-ADDA ELISA. According to the manufacturer, no cross-reactivity with other non-related toxins or compounds is expected, and the limit of detection (LOD) on the basis of MC-LR was set at 0.10 parts per billion (ppb) of MCs and nodularins. The working range was 0.15 ppb to 5 ppb (standards solutions: 0.15, 0.40, 1, 2, and 5 ppb). A control at 0.75±0.185 ppb and a negative calibrator (standard 0) were measured in every batch. All standards and controls were measured in duplicate. A spiked sample of uncontaminated mussel (Zero Mus) with MC-LR at 2 µg/g was also measured 10 times before the assay to determine the reproducibility and recovery of the method. The absorbance was measured at 450 nm with a spectrophotometer (DAS model A3, Italy). The coefficients of variation were less than 10% for the standards and less than 15% for the samples; the average recovery rate for MC-LR was 89%. The values were calculated by the standard curve, and the results were expressed as MC-LR equivalents and nodularins.

The results obtained by ELISA were confirmed by the analytical method of UHPLC-MS. Several methods have been described

for the quantitative and/or qualitative detection of MCs, such as protein phosphatase inhibition assay, mouse bioassay, high-performance capillary electrophoresis, gas chromatography, and high-performance liquid chromatography with ultraviolet. UHPLC-MS is considered the best because it has high sensitivity, selectivity, and robustness toward all the analogs of MCs and their derivatives (Massey et al., 2020; Turner et al., 2017). The instrumentation used for the method development was a high-resolution mass spectrometer Orbitrap and Q Exactive Focus coupled to an Ultimate 3000 ultra-high-performance liquid chromatography system (Thermo Scientific, USA). The separation of the analytes was performed using a C18 reverse-phase column Acclaim Polar Advantage II 3 µm 3×150 mm in conjunction with a guard column 2/pk and a Holder-Coupler (Thermo scientific, USA). The column was kept at 40°C, the injection volume was 20 µL, the flow rate was 0.3 mL/min, and the run time was 11 minutes. The mobile phases were water (mobile phase A) and acetonitrile (mobile phase B), both with 0.1% formic acid. Regarding the Orbitrap conditions, multiple reaction monitoring was selected at a range of 5,000,000 to 11,000,000 (m/z) using positive ion mode for each toxin; the capillary source temperature was kept at 320°C, the gas heater temperature was 413°C, and the spray voltage was 3.50 KV. Limit of quantification (LOQ), Limit of detection (LOD) and recovery were calculated according to the International Union of Pure and Applied Chemistry before the method implementation, and the linearity for the calibration curves was following the criterion of coefficients greater than 0.99 (r^2).

Statistical analysis
Statistical analysis was conducted using the Statistical Package for the Social Sciences software program, and the presence of MCs was considered as a potential risk at values higher than 1 ppb (µg/kg wet weight). Differences between the mean values of specific factors were evaluated by the Duncan's new multiple range test. Results were expressed as mean±standard deviation. Significance was declared at p≤0.05, unless otherwise noted.

Results

During the exposure of mussels to *Synechocystis* sp. culture, it was demonstrated that toxins accumulated in the whole-body tissue of the mussels between 48 and 72 hours of exposure (Table 2). During the first 24 hours, MCs accumulation reached 0.32±0.040 µg/kg and 2.20±0.110 µg/kg of wet weight in Tank A and Tank B, respectively. The toxins detected by UHPLC-MS at the first 24 hours were lower than the LOQ of the method. The levels were similar after 48 hours of contamination. The highest value of toxins (10.83±0.260 µg/kg wet weight according to ELISA and 6.85±0.220 µg/kg according to UHPLC-MS) was recorded after 72 hours in Tank B. It was found that the abundance of 10^4 cells/mL led to accumulation of toxins at the level of

Table 2. ELISA screening concentration of microcystins in the mussels *Mytilus galloprovincialis* during the exposure to *Synechocystis* sp.

	Concentration of microcystins	
	P1	**P2**
Time (hours)	**Mean±SD (µg/kg)**	**Mean±SD (µg/kg)**
24	0.31±3.510[A,a]	2.2±1.00[a,B]
48	0.51±3.510[B,b]	5.3±6.11[b,B]
72	0.86±6.650[c,A]	10.83±0.260[c,C]

[a]Values with different exponents with small letters indicate statistically significant difference (p≤0.05) in the same cell density.
Values with different exponents with capital letters indicate statistically significant difference (p≤0.05) between different cell densities on the same sampling time.
P1: Population of 104 cells/mL of *Synechocystis* sp.
P2; Population of 105 cells/mL of *Synechocystis* sp.
SD: standard deviation; ELISA: enzyme-linked immunosorbent assay.

Table 3. Performance criteria of the method

	MC-LR	MC-RR	MC-YR
LOQ[a]	1.15	1.01	1.00
LOD[a]	0.35	0.31	0.30
Recovery (%)	112	92	85

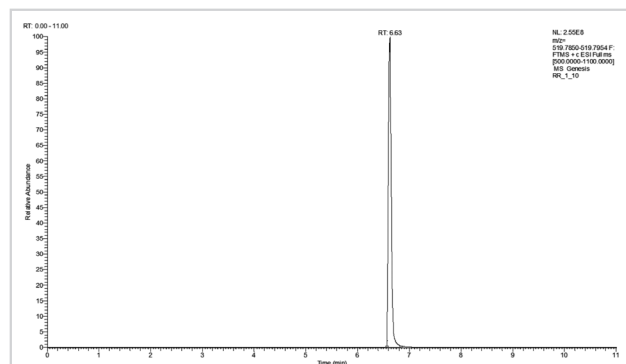[a]µg/kg wet weight. MC: microcystin; LOQ: limit of quantitation; LOD: limit of detection

Figure 2. Chromatogram of standard of microcystin-RR. The retention time is 6.63 min

0.86±0.070 µg/kg wet weight during the same time. Moreover, the toxins detected at that level by UHPLC-MS were lower than the LOQ of the method. After 48 hours of exposure at the 10^5 cells/mL, the toxin accumulation in the mussels' tissue was recorded at values as high as 10.83±0.260 µg/kg and 6.85±0.220 µg/kg wet weight by ELISA and UHPLC-MS, respectively. The experiment revealed that the toxin production is time and cyanobacterial population is density dependent. In contrast, low values of toxins were detected even after 72 hours of exposure at 10^4 cells/mL abundance.

The toxin that was detected by UHPLC-MS after 72 hours at the 10^5 cells/mL abundance was MC-RR. The retention time was 6.62 minutes and 6.63 minutes for the sample and the standard of the toxin, respectively, as it is shown in the chromatographs (base peak) (Figures 2 and 3). In addition, traces of MC-LR were detected. The mass spectrum of the MCs is shown in Figure 3. No MC-YR was detected. The performance criteria of the method are presented in Table 3.

Discussion

The presence of potential toxic cyanobacteria species in marine ecosystems has been described as an arising risk (Preece et al., 2017), and their secondary metabolites, the cyanotoxins, are
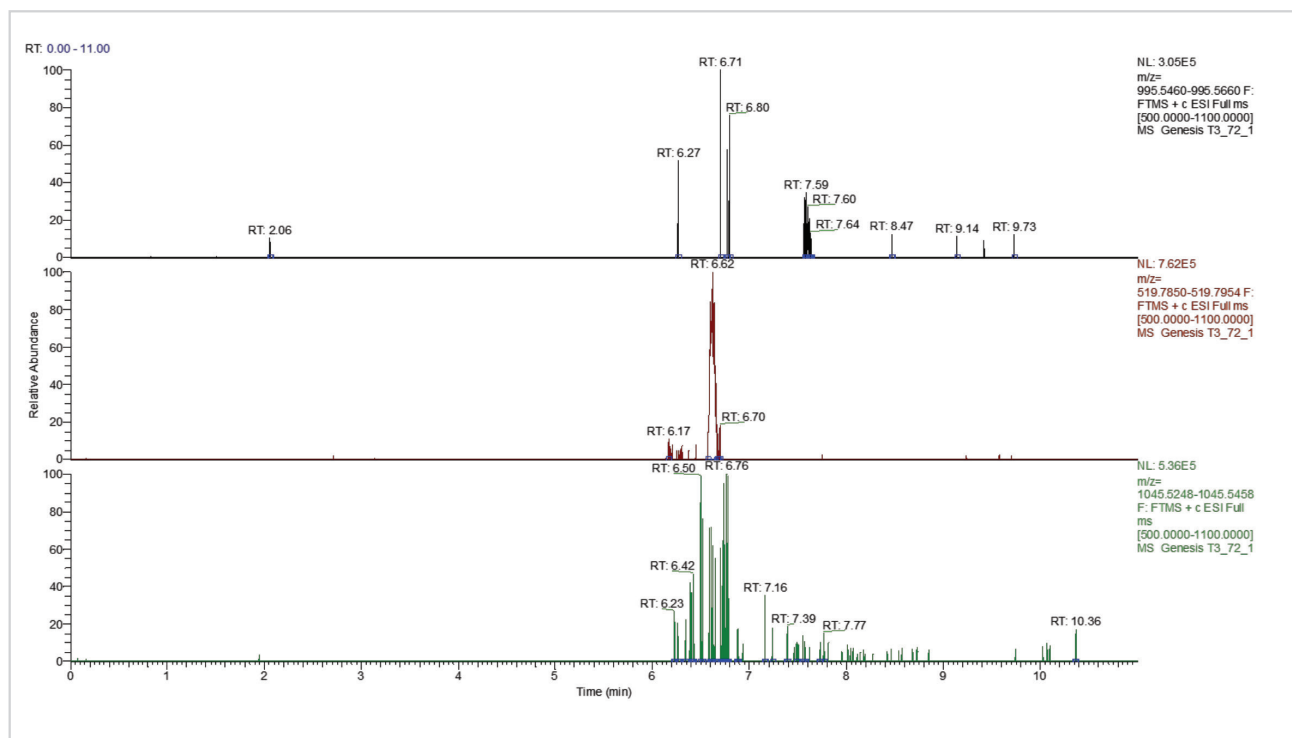

Figure 3. Chromatogram of mussels *Mytilus galloprovincialis* contaminated with *Synechocystis* sp. at the abundance of 105 cells/mL after 72 hours of contamination. The retention time of microcystin-RR is 6.62 min. The mass spectrum m/z is 519.7850 to 519.7954. Traces of microcystin-LR are detected (retention time is 7.59 and m/z is 995.5460 to 995.5660)

considered to be emerging toxins (Gerssen and Gago-Martínez, 2019). Moreover, De Pace et al. (2014) have proved that marine waters can be affected by toxic cyanobacterial blooms owing to the expansion from internal reservoirs, and mussels and fish can be contaminated with MCs. Our study focused on the detection of MCs in sea water with emphasis on *Synechocystis* sp. toxicity. *Synechocystis* sp. was isolated for the first time in Greece from shallow waters in Thermaikos gulf. The morphological characteristics of the gulf; the enrichments in the coastline by three big rivers, Axios, Loudias, and Aliakmonas; and the climate conditions indicate eutrophication problems and algal blooms in the coastal zones (Nikolaidis et al., 2006).

MC production from the mentioned strain is considered important because, according to the existing literature, MCs remain under-investigated in marine waters (Vareli et al., 2012) along with the abundance of *Synechocystis* sp. The WHO has set the concentration of 1 µg/L expressed as MC-LR as a provisional guideline value for potable water and 0.04 µg/kg/day bodyweight as a chronic TDI for human consumption (Chorus, 2012; Li et al., 2017). Moreover, cyanobacterial population density of 10^5 cells/mL is considered the limit of transition from low level 1 to alert level 2 for recreational water activities (Chorus, 2012; WHO, 2003).

Our findings indicate that a population density of 10^5 cells/mL can produce MCs, reaching levels higher than the TDI in 72 hours' time span. Similar studies have declared that concentrations at levels similar to those in our study could be hazardous for public health (Amorim and Vasconcelos, 1999; Vasconcelos, 1995). Moreover, research in bivalve mollusks related to the population of other potential toxic cyanobacteria have shown that 10^5 cells/mL could produce MCs at levels above the TDI. Vasconcelos (1995) studied the uptake of MCs in *M. galloprovincialis* soft tissues during their exposure at 10^5 cells/mL of toxic freshwater cyanobacterium *Microcystis aeruginosa* for 16 days. The highest value was observed on the 10th day and reached 10.52 mg/g dry weight. A rapid uptake of toxins was observed on the 2nd day, as in our research (Table 2). In another experiment, Amorim and Vasconcelos (1999) have reported that 10^5 cells/mL of the toxic *Microcystis aeruginosa* produced MCs at a concentration of 10.7 µg/g dry weight on day 4 of the experiment. Moreover, Morais et al. (2008) have studied the bioavailability of MCs after different cooking conditions. Mussels *M. galloprovincialis* were exposed at 10^5 cells/mL of the toxic freshwater cyanobacterium *Microcystis aeruginosa* for 4 days, reaching at MC concentration of 16.5 ng/g wet weight. It was demonstrated that common cooking, as boiling at 100°C, had no significant effect on MC concentration in the mussels.

In contrast, Amorim (1997) fed *M. galloprovincialis* with population density of 10, 10^2, and 10^3 *Microcystis aeruginosa* cells/mL.

After 8 days, MCs were not detectable. Menezes et al. (2017) have studied the relationship between the abundance of toxic cyanobacteria (including *Synechocystis* sp.) and the production of MCs. The results were obtained after a 14-year monitoring program in eight Portuguese recreational freshwater reservoirs. The program focused on MC production related to the abundance of potential toxic cyanobacteria. It was shown that at population density of up to 10^4 cells/mL, low levels of MCs were detected. In contrast, high levels of MCs were detected in 24% of samples at population density of 10^5 cells/mL. In our study, toxin production was detected (0.86±0.07 µg/kg wet weight from ELISA) when the mussels were fed with 10^4 cells/mL of *Synechocystis* sp., a value that is not considered hazardous (WHO, 2003) as mentioned earlier.

The toxin production of *Synechocystis salina* was studied by Baptista et al. (2015) on the mussel *M. galloprovincialis*. In their experiment, they have demonstrated that this strain at a population density of 10^5 cells/mL produced the neurotoxin beta-N-methylamino-L-alanine in 15 days of exposure. This toxin is connected to amyotrophic lateral sclerosis/parkinsonism-dementia complex (Brand et al., 2010; Lance et al., 2018). In addition, Baptista et al. (2015) have demonstrated decreased metabolic activity of the cells of the gills and hepatopancreas of the mussels fed with *Synechocystis salina* for longer periods of time. The toxicity of the *Synechocystis* sp. was also assessed by Martins et al. (2007) on marine invertebrates exposed to freeze-dried cyanobacterial material extracts at concentrations of 100 mg/mL of sea water. They described inhibition of embryogenesis of the sea urchin *Paracentrotus lividus,* with most of the embryos reaching only the morula stage. The authors have reported that toxic effects were observed on fertilized eggs of the mussel *M. galloprovincialis,* affecting the normal development of embryos that reached the D-larvae stage. In addition, they proved that after 24–48 hours of exposure to crude extracts of *Synechocystis* sp. on *Artemia salina* nauplii, acute toxicity caused 100% mortality of the nauplii.

During this experiment, no dead animals were recorded, which is in accordance with the findings of Vasconcelos (1995), Amorim and Vasconcelos (1999), and Morais et al. (2008). It seems that this toxicity cannot lead the mussels to death. The same authors have mentioned mortality as low as 1%. According to Pires et al. (2004), the bivalves are able to survive during their exposure to toxic species of cyanobacteria and show resistance to cyanotoxins owing to the metabolic mechanisms of detoxification. Fernandes et al. (2009) have reported that the resistance of mussels to cyanotoxins is due to the detoxification activity by glutathione-S-transferases. Similar findings have been reported by other researchers that also confirm the resistance of bivalves to toxic cyanobacteria and their tolerance to cyanotoxins (Gazuhla et al., 2012; Pham et al., 2015).

The comparison of the results from our study with ELISA and UHPLC-MS shows that the concentrations of MCs obtained using ELISA could be overestimated. This could be explained owing to the cross-reactivity of ELISA with other matrices. Bogialli et al. (2005) have reported that results of MCs obtained using ELISA in the liver of treated animals were 1,000 times higher than the ones by liquid chromatography (LC)/mass spectrometry (MS). This was attributed to the matrix effect of ELISA with other compounds. In addition, De Pace et al. (2014) during a monitoring study on the mussels *M. galloprovincialis*, contaminated after a cyanobacterial bloom, have reported that the levels of MCs using ELISA ranged from 1.73 ng/g to 256 ng/g. The presence of MCs was confirmed using MS method (UHPLC, ESI, Orbitrap and Q Exactive Focus coupled to anUltimate 3000 ultra-high-performance liquid chromatographysystem). It has been shown in some cases that some analogs could not be detected using ELISA, such as desMe-MC-RR. It has been demonstrated that ELISA is a particularly good screening method, and chromatography-tandem MS is selected for confirmation and quantitation of the results.

Our study showed that the marine cyanobacterium *Synechocystis* sp., isolated from the Thermaikos gulf can be toxic and can produce MCs. The mussels *M. galloprovincialis* accumulated these toxins when they were exposed at a population density of 10^5 cells/mL. This density meets the alert guideline values for recreational activities as defined by the WHO. The area of the Thermaikos gulf is particularly important for the local economy owing to the farming of *M. galloprovincialis*, fishing, recreational activities, and tourism development in terms of public health. It has also been demonstrated that ELISA could be a rapid screening method, but the results should be confirmed by chromatography-tandem MS because ELISA cannot distinguish the toxin analogs and may reveal overestimation. For these reasons, further research on the toxicity of marine cyanobacteria species is required to protect human and animal health, aquatic organisms, and ecosystems.

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