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DETECTION OF POTENTIALLY TOXIGENIC *MICROCYSTIS* STRAINS FROM DAU TIENG RESERVOIR

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SUMMARY

Microcystis is a bloom-forming, common cyanobacterium in Dau Tieng reservoir used for public water supply. To assess the presence of potentially microcystin-producing *Microcystis*, molecular techniques were conducted and acute toxicity bioassays were performed with the microcrustacean *Daphnia magna* exposed to cyanobacterial crude extracts. Potentially toxigenic of isolated strains was characterized by amplifying *mcyD* genes and identification of *Microcystis* was confirmed by 16S rRNA amplification. Microcystins (MCs) concentration in bloom samples and cultured strains were quantified by High Performance Liquid Chromatography (HPLC). Results showed that there were 9/15 strains showed positive with the *mcyD* marker indicating that they are toxic strains. Three MCs variants including MC-RR, -LR and -YR were found in all extracts of toxic strains with the highest concentration of 1,218 µg/g dry weight (DW). The acute toxicity bioassays revealed that both toxic and non-toxic crude extracts elicited significant lethal effects on the tested animal with LC50 values ranged from 189-411 mg DW/L. The toxic effects of isolated strains were independent from the MCs concentration in some strains suggesting the presence of other metabolites contributed to the biological effects. In conclusion, microcystin-producing *Microcystis* from the Dau Tieng reservoir warn about possible toxic effects for aquatic biota and human health.

Keywords: *Microcystin producing, Microcystis, mcy gene, PCR detection, Dau Tieng reservoir*

INTRODUCTION

Blooms of cyanobacteria (blue-green algae) have been occurred in eutrophication freshwater bodies all over the world, including Vietnam. Bloom forming of cyanobacteria has created a significant water quality problem, as some species are capable of producing cyanotoxins. Among cyanotoxins, microcystins (MCs) are the most prominent cyanobacterial hepatotoxins in freshwater (Chorus, Bartram, 1999). The MCs are cyclic heptapeptide hepatotoxins synthesized non-ribosomally by a multifunctional enzyme complex that includes peptide synthetase (NRPS) and polyketide synthase (PKS) modules, both of which are encoded by the microcystin synthetase gene (*mcy*) cluster, which contains 55 kb of DNA and has been characterized in many cyanobacterial genera (Nishizawa *et al.,* 1999; Tillett *et al.*, 2000; Rouhiainen *et al.*, 2004). More than 80 MCs structural variants have so far been reported worldwide (Dittmann, Wiegand, 2006). MCs are powerful inhibitors of the proteins phosphatases (PP) 1 and (PP) 2A, causing strong hepatic hemorrhage.

Many species belonging to the genera such as *Dolichospermum, Microcystis*, *Oscillatoria*, *Nostoc*, *Aphanizomenon* and *Pseudanabaena* can produced MCs (Chorus, Bartram, 1999; Ballot *et al*., 2004). Morphological methods could be used to identify difference genera. However, these methods could not be used to recognize toxic and non-toxic cyanobacterial species because many strains of cyanobacteria appear to be identical under the microscope. Blooms of cyanobacteria usually consist of toxic and nontoxic strains (Janse *et al*., 2004). Several techniques to identify toxigenic strains have been developed for cyanobacteria. Among them, the presence or absence of the *mcy* gene cluster has been widely used as a means for distinguishing the two genotypes and has been recently used to reveal the presence of MC-producing cyanobacteria in both

environmental samples and axenic cultures (Nishizawa *et al*., 2000; Rantala *et al*., 2006; Pedro *et al*., 2011).

Cladocerans are the most used group in ecotoxicological studies, especially the daphnia species *Daphnia magna* (Sarma, Nandini, 2006). However, previous studies often used purified toxins for toxicity test. The toxicity assessment of purified cyanotoxins on *Daphnia* may not reflect at all events occurring in the environments, as it has been reported that the toxicity of *Microcystis* is susceptible to be modified when they are associated to other molecules (Burýšková *et al*., 2006). Hence, it is better to use crude extracts for evaluating the toxic effects as they have occurred in natural environments after blooms decay.

Long term blooms of cyanobacteria are common in Dau Tieng reservoir (Pham *et al*., 2015). This problem poses a risk not only for the aquatic organisms but also for human and biota of the neighbouring areas. Monitoring quality of water destined to public supply includes identification of potentially toxic cyanobacteria and their toxicity. This information is extremely useful to prevent against the possible risk of intoxication when human populations or natural biota are exposed to water from sites where *Microcystis* blooms are occurring. Considering the increasingly frequent toxic blooms in tropical aquatic ecosystems and the scarcity of reports on potentially toxin-producing cyanobacteria populations and their toxicity in Vietnamese waters, this study aimed to detect *Microcystis* strains with the genetic potential to produce MCs independent of their taxonomic category and their toxicity at relevant environmental conditions.

MATERIALS AND METHODS

Sample collection

Bloom samples from the Dau Tieng reservoir were collected by skimming across the water surface using bolting silk plankton net of 25 micron mesh size. These samples were kept cool (25°C) and brought to the laboratory. Samplings were performed only during July of 2016, when the selected sites contained predominantly blooms of *Microcystis* sp. The samples were concentrated by placing the material in glass cylinders and the buoyant cyanobacterial scum collected from the surface. The natural biomass samples were dried at 45°C overnight and kept at -20°C until further processes.

Isolation and cultivation of cyanobacteria

Cyanobacteria were isolated by micropipetting and washing. A single cyanobacterial colony of *Microcystis* was isolated by micropipetting, washed, and transferred into cyanobacterial growth medium (Belcher, Swale, 1988). Cyanobacteria were grown in Z8 medium (Kotai, 1972). All cultures were grown on a 12h:12h light:dark cycle at temperature of 28°C under light conditions provided by 40-W fluorescent lamps, which provided an approximate luminic intensity of 20 µmol photons/m^2/s. Biomasses of cyanobacterial cultures were harvested onto GF/C fiberglass filters (Whatman, Kent, England), dry at 45°C overnight and kept at -20°C until further processes.

Identification of cyanobacteria

Cyanobacteria were observed at 400 × magnification under a microscope Olympus CK40-F200 equipped with a digital camera (Olympus, Tokyo, Japan). Taxonomic classification was based on the system of Komárek and Anagnostidis (1989, 1999, 2005). Descriptions of cyanobacteria were based on observations of both preserved and cultured samples.

Preparation of the crude extract

Cyanobacterial crude extracts (CCE) were prepared according to Pietsch *et al.,* (2001) with some modifications. Briefly, 2 g dry weight (DW) of the bloom material or isolated culture was dissolved into distilled water, frozen at -70°C then thawed at room temperature. After the materials thawed completely, they were sonicated for three min. This freeze–thaw–sonicate cycle was repeated five times. The samples were then centrifuged at 2000 × g for 10 min to remove cell debris. The supernatant was collected and kept at -20°C until use for the toxicity experiments.

Sub samples of the CCE supernatant were used for MC analysis as previously reported by Pham *et al.,* (2015). Briefly, 100 µL of the supernatants was centrifuged at 4000×g for 15 min. The supernatant was collected, dried completely, and re-dissolved in 500 µL of 100% MeOH. The samples were analyzed by HPLC system with UV-visible photodiode array (PDA) detector (Shimadzu 10A series, Kyoto, Japan). Commercial MCs from Wako Company (Osaka, Japan) were used as standards.

Acute toxicity bioassays

D. magna Straus purchased from the MicroBioTests Inc, Belgium was used for the test. The animal were raised in ISO medium and fed by a mixture of viable green algae *Chlorella* sp. and *Scenedesmus* sp., which were cultivated in COMBO medium (Kilham *et al*., 1998) with continuous aeration. Both *Daphnia* and algae were maintained in the laboratory conditions at 25 ± 1°C, with a 14h:10h light:dark cycle.

Acute toxicity bioassays were performed, according to the Protocol 202 of the Organization for the Economical Cooperation and Development (OECD, 2004), compatible with the procedure proposed by the U.S. EPA (2002). Briefly, *D. magna* neonates (< 24 h-old) were maintained in ISO medium containing CCE. For each crude extracts, at least five different concentrations with a dilution factor of 0.5 were tested in triplicate by exposing 10 neonates per replicate. Test containers were placed in an environmental beaker at a controlled temperature of 25°C and a 14:10 h photoperiod during 48 h. The assessed response was immobility or death of cladocerans. The criterion for test acceptance was a survival higher or equal to 90% in the control group. Finally, mortality data recorded at the end of the toxicity tests (48 h) were used to determine the Median Lethal Concentration (LC50) through Probit analyses by using the SPSS software according to the method of Stephan (1977).

DNA extraction

Total genomic DNA was extracted from cyanobacterial retained on filters following the methods described previously in Hisbergues *et al.,* (2003) with minor modifications. Briefly, the filters contained cyanobacterial cells were suspended in TE buffer (50 mM Tris/HCl, 40 mM EDTA, pH 8.0). An aliquot of 30 µL of 10% SDS (sodium dodecyl sulfate) and proteinase K (final concentration: 100 µg/mL in 0.5% SDS) was then added and incubated for 60 min at 37°C. Then 5 M NaCl (100 µL) and CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) (80 µL) were added, and the samples were incubated for 10 min at 65°C. DNA was then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v). After centrifugation for 5 min at 6000 × *g* at 4°C, the supernatant was collected and transferred to a fresh tube. The DNA was then rinsed with 1 mL of 70% ethanol and dried under vacuum. The final DNA sample was rehydrated in 20 µL of 1 × TE buffer (10 mM Tris and 1 mM EDTA pH 8.0).

PCR amplification

The polyketide synthase fragment (*mcyD*, 297 bp) was amplified using primer pair mcyD-F2/mcyD-R2 (Kaebernick *et al*., 2000). To detect the presence of cyanobacterial DNA, the CYA primer pair (Urbach *et al*., 1992) was used to amplify a 1200 bp fragment of the 16S rRNA gene common to all cyanobacteria. For each sample, two separate PCRs were conducted. All PCR reactions were prepared in a volume of 20 µL containing 2 µL of 10 × Ex-Taq Buffer, 200 µM of each dNTP, 0.5 µL of each primer (10 µM), 0.5 U of Ex-Taq polymerase (Takara Bio Inc., Shiga, Japan), and 20-25 ng of template DNA. Amplification was performed in a Thermal Cycler (Applied Biosystems, Foster City, California, USA) follow the condition: initial denaturation at 95°C for 10 min, 35 cycles (94°C/1 min, 54°C/1 min, 72°C/1.5 min) and a final extension step at 72°C for 10 min. PCR products were examined on 1.5% (w/v) agarose gels stained with ethidium bromide and photographed under UV transillumination.

HPLC quantification of microcystins

Microcystins concentration was quantified by HPLC system following the methods described previously in Pham *et al.,* (2015). Briefly, a reverse-phase HPLC system with UV-visible photodiode array (PDA) detector (Shimadzu 10A series, Kyoto, Japan) was equipped with a silica-based, reverse-phase C_{18} column (Waters SunFire^{TM} 5 µm, 3 × 250 mm, Milford, Massachusetts, USA) and maintained at 40°C. The MCs content in samples were separated with a mobile phase consisting of methanol: 0.05 M phosphate buffer (pH 2.5; 50:50 v/v) at a flow rate of 0.58 mL/min. Microcystin congeners were detected by UV detection at 238 nm and identified on the basis of both their retention time and characteristic UV spectra. Microcystins purchased from Wako Pure Chemical Industries, Ltd. (Chuoku, Osaka, Japan), were used as standards.

RESULTS

Isolation and morphological characteristics of cyanobacteria

Microscopic examination of the cyanobacterial bloom samples revealed the dominance of *Microcystis* (mainly *M. aeruginosa*) and the less frequent occurrence of other genera (*Dolichospermum*, *Arthrospira*, *Planktothrix*, *Pseudanabaena*, and

Cylindrospermopsis). Identification of individual *Microcystis* colonies revealed the occurrence of four species: *M. aeruginosa*, *M. botrys*, *M. wesenbergii* and *M. panniformis* (Fig. 1). A total of 15 *Microcystis* strains were isolated from the cyanobacterial and maintained them in cultures.

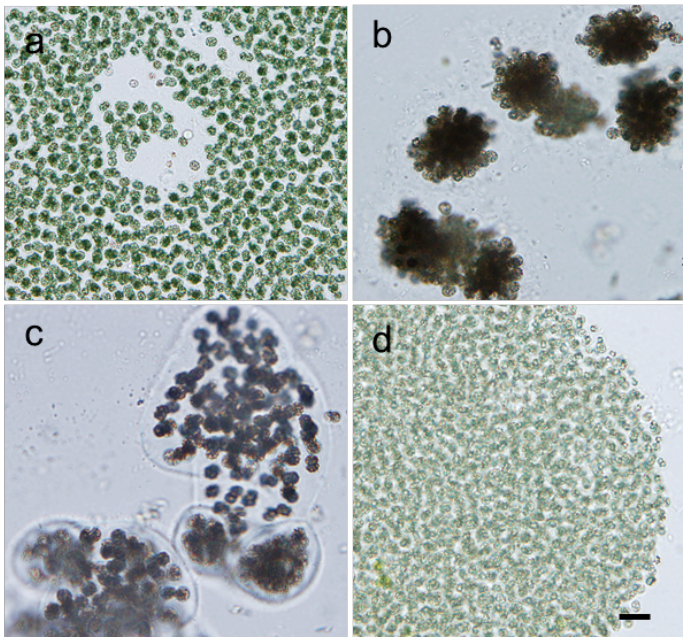

Figure 1. (a) *M. aeruginosa*, (b) *M. botrys*, (c) *M. wesenbergii*, (d) *M. panniformis*. Scale bar: 10 µm.

Molecular characterization of the *Microcystis* isolates

Isolated strains were examined by the 16S rRNA and the *mcyD* fragments. The 16S rRNA fragments presented in all strains confirmed that all strains examined were cyanobacteria. The use of the mcyD-F2/mcyD-R2 primers in the PCR yielded 300 bp amplicons of the different studied strains, indicating the presence of *mcyD* genes in these strains. In total, the *mcyD* region was amplified for 10 of the 15 strains (Fig. 2). This amplicon was obtained from *Microcystis* isolates, which correspond to potential toxigenic strains, since they presented the *mcy* genes. The strains DT-bo10, DT-bo12, DT-bo13 corresponding to the cyanobacterium *M. botrys* and the strains DT-we14, DT-we15 corresponding to the cyanobacterium *M. wesenbergii* could not be amplified despite the fact that the procedure was repeated several times.

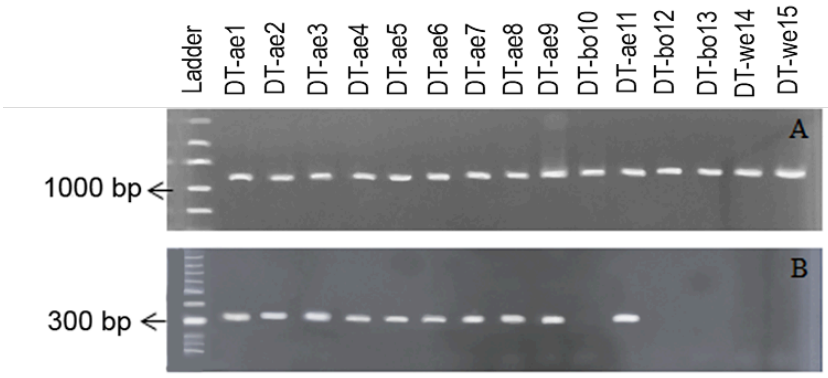

Figure 2. Ethidium bromide stained agarose electrophoresis gels showing PCR amplification products from selected strains. A: 16S rRNA amplification products. B: *mcyD* amplification products. Ladder: PHY ladder; other lanes represent the different strains in the study (See list in Table 1).

Quantification of microcystins with HPLC

Results of HPLC analysis indicated that 10/15 cultured strains contained MCs. All strains positive with the *mcyD* produced MCs. MCs producing strains were classified into the *M. aeruginosa* (DT-ae1, DT-ae2, DT-ae3, DT-ae4, DT-ae5, DT-ae6, DT-ae7, DT-ae8, DT-ae9 and DT-ae11), while non MC producing strains were belonging to *M. botrys* and *M. wesenbergii* (DT-bo10, DT-bo12, DT-bo13, DT-we14 and DT-we15). The total concentration of MCs from toxic isolated strains ranged from 89.1 to 1,218 µg/g DW (Table 1). The MCs content of these strains was quite variable. The minimum content of MCs was found in the strain DT-ae4 (89.1 µg/g DW) and the maximum in DT-ae11 (1,218 µg/g DW).

Table 1. List of isolated strains and bloom samples showing taxonomic assignment, amplification of the *mcyD* region, total microcystin content, and LC50 values

Source	Strain	Taxonomic assignment	mcyD	MC (µg/g DW)	LC50 (mg DW biomass/L)
Dau Tieng reservoir	DT-ae1	*M. aeruginosa*	+	113.5	214
	DT-ae2	*M. aeruginosa*	+	237.9	320
	DT-ae3	*M. aeruginosa*	+	146.8	275
	DT-ae4	*M. aeruginosa*	+	89.1	256
	DT-ae5	*M. aeruginosa*	+	548.4	189
	DT-ae6	*M. aeruginosa*	+	1013.4	196
	DT-ae7	*M. aeruginosa*	+	134.7	264
	DT-ae8	*M. aeruginosa*	+	297.8	198
	DT-ae9	*M. aeruginosa*	+	687.4	192
	DT-bo10	*M. botrys*	–	–	328
	DT-ae11	*M. aeruginosa*	+	1,218	255
	DT-bo12	*M. botrys*	–	–	272
	DT-bo13	*M. botrys*	–	–	411
	DT-we14	*M. wesenbergii*	–	–	380
	DT-we15	*M. wesenbergii*	–	–	365
	Bloom 1			568.3	169
	Bloom 2	Collected natural biomass		465.8	183

Acute toxicity bioassays with *D. magna*

No mortality was observed at 48 h in the control. On the other hand, exposure to some of the crude extracts induced mortality in *D. magna* neonates, which allowed for the calculation of the 48 h LC50 for those cases in which 50% mortality was within the interval of the tested concentrations. The means of the 48 h LC50 were shown in table 1. Strains DT-bo10, DT-bo12, DT-bo13 (*M. botrys*) and DT-we14, Dt-we15 (*M. wesenbergii*) and DT-ae2 (*M. aeruginosa*) had the highest LC50 values (272 to 411 mg/L dry biomass) and DT-ae5, DT-ae6, DT-ae8, DT-ae9 were the most toxic strains to *D. magna*. As can be seen in Table 1, although MCs were not detected in all of the strains, all of the strains induced acute toxicity, the highest LC50 corresponding to the strain DT-bo11 (*M. botrys*) which did not produce MCs. In addition, comparison of the mortality results of collected natural biomass and isolated strains in Tables 1 revealed that the amount of biomass required to produce the lethal effect was, in general, lower when using collected material than when using the biomass from the culture of toxigenic strains isolated from the same sites. This indicated that the biomass of cyanobacteria from natural bloom caused more toxic effects than the cultured biomass did (Table 1).

DISCUSSION

In Vietnam, there are many artificial lakes used for water supplies and recreational activities in

which cyanobacterial bloom formation associated with MCs production is frequently increased due to the high degree of eutrophication (Hummert *et al*., 2001; Duong 2014; Dao *et al*., 2016; Pham *et al*., 2017). MCs concentration exceed the WHO guideline value of 1.0 µg/L have also been reported in the Tri An and Dau Tieng reservoirs (Pham *et al*., 2015; Dao *et al*., 2016). This deserves special attention given the potential risk to human health and animal sanitation posed by blooms, since toxicity has been document to 75% of blooms cases (Chorus, Batram, 1999). Among the toxic species, *M. aeruginosa* is one of the most common and widespread bloom-forming cyanobacteria in freshwater environments. This species was also reported the bloom-forming in many Vietnamese water bodies (Hummert *et al*., 2001; Duong 2014; Dao *et al*., 2016; Pham *et al*., 2017). In this study we found that *M. aeruginosa* was the bloom-forming and the main toxin produces in the Dau Tieng reservoir. We strongly recommend further investigations to elucidate the cause and mitigate of these blooms.

PCR-based detection of genes involved in the synthesis of MCs is a reliable technique and has been successfully applied for determination of toxic and non-toxic cyanobacteria worldwide (Hisbergues *et al*., 2003; Bittencourt-Oliveira *et al*., 2010; Martins *et al*., 2011). Previous study have showed that the *mcyD* which encodes for parts of both the β-ketoacyl synthase and the acyltransferase domains (Rantala *et al*., 2004; Pham *et al*., 2015) is one of the best molecular markers for determination of potential toxicity of cyanobacteria. In this study, the amplification of the *mcyD* showed again reliable results in the distinguish toxic and non-toxic *Microcystis*. Therefore, we recommend using this fragment for the determination of toxic genetic of *Microcystis* in other Vietnamese waters.

The acute toxicity bioassays with CCE of bloom biomass and cultured *Microcystis* can affect cladocerans adversely, obtaining similar results to those found by Arzate-Cárdenas (2010). The LC50 calculated for the assays performed with bloom biomass from the sampling sites was lower in all cases than that of the isolated *Microcystis* strains from the same sites. Probably due to the fact that the biomass of the blooms is constituted by a mixture of MCs and other cyanotoxins such as anatoxins, cylindropermopsin, so that could contribute to the toxic effect on *Daphnia*.

It was found that both toxic and non-toxic strains caused death of *D. magna* neonates. These results were well in agreement to findings by other researcher (FerrÃo-Filho *et al.,* 2000; Dao *et al.,* 2013). And *Microcystis* strains with different MCs contents resulted in different LD50 on *D. magna* neonates. This could be explained by the fact that different strains produced different chemical structure of MCs, since the structure affects its toxic properties (Prieto *et al.,* 2006). *Microcystis* strains are able to produce more than one MC variant (Mowe *et al.,* 2014; Pham *et al.,* 2015), which could be related to the genetic structure of the *mcy* genes cluster (Mikalsen *et al.,* 2003). In addition, Dao *et al.,* (2013) found that not all crude extracts exert the same effects on tested organisms and not all organisms react in the same way with the harmful substances. Burýšková *et al.,* (2006) points out that MCs are not the only or major toxic compound in the complex cyanobacterial samples and it is necessary to study in more detail the possible interactions of other toxic compound in the cyanobacterial biomass. This issue must be further investigated.

CONCLUSIONS

Bloom of cyanobacteria and *Microcystis* strains with a large toxigenic potential were found in the Dau Tieng reservoir, which could pose risk on the aquatic communities and human health. The results revealed that toxic effects on *D. magna* were not only related with the MCs concentration, but other substances and metabolites present in the crude extracts also exerted acute toxic effect. The analytical methods in this study (PCR and HPLC) were useful and reliable for determining MC production and its potential contribution to the acute toxic effects.

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PHÂN TÍCH KHẢ NĂNG SINH ĐỘC TỐ CỦA MỘT SỐ CHỦNG VI KHUẨN LAM *MICROCYSTIS* Ở HỒ DẦU TIẾNG

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TÓM TẮT

Vi khuẩn lam *Microcystis* thường gây nở hoa ở hồ Dầu Tiếng dùng cho cấp nước sinh hoạt. Trong nghiên cứu này, phương pháp sinh học phân tử và phương pháp thử nghiệm cấp tính với vi giáp xác *Daphnia magna* được sử dụng để phân tích khả năng sinh độc tố và xác định tính độc của các chủng *Microcystis*. Đoạn gen 16S rRNA được sử dụng để nhận dạng vi khuẩn lam và phương pháp kiểm tra đoạn gen *mcyD* được sử dụng để kiểm tra khả năng sinh độc tố của các chủng *Microcystis*. Hàm lượng độc tố microcystins (MCs) được phân tích bằng phương pháp sắc ký lỏng hiệu năng cao (HPLC). Kết quả cho thấy có 9/15 chủng *Microcystis* dương tính với đoạn gen *mcyD* từ đó giúp kết luận các chủng này là các chủng có khả năng sinh độc. Độc tố MCs (MC-RR, MC-LR và MC-YR) được tìm thấy trong sinh khối của tất cả các chủng sinh độc với hàm lượng cao nhất là 1218 µg/g trọng lượng khô (DW). Thí nghiệm cấp tính với *Daphnia* cho thấy các chủng sinh độc và không sinh độc đều gây chết động vật thí nghiệm với liều gây chết 50% (LD50) dao động từ 189 đến 411 mg DW/L. Tính độc lên *Daphnia* ở các chủng phân lập được không phụ thuộc vào hàm lượng độc tố do chúng sinh ra. Điều này cho thấy có sự hiện diện của các hợp chất gây độc khác ngoài MCs trong sinh khối của các chủng *Microcystis*. Kết quả của nghiên cứu cho thấy vi khuẩn lam ở hồ Dầu Tiếng gây ảnh hưởng xấu đến quần xã thủy sinh vật và sức khỏe con người.

Từ khóa: *Sinh độc tố microcystin, Microcystis, mcy gen, phương pháp PCR, Hồ Dầu Tiếng*