Doi: 10.31276/VJSTE.61(4).70-75

# Toxicity of non-microcystin producing *Microcystis wesenbergii* isolated from the Tri An reservoir

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Received 5 August 2019; accepted 18 November 2019

***Abstract:***

**Harmful cyanobacterial blooms have become a global threat to human health and aquatic biota around the world. While the ecotoxicity of cyanobacterial toxins such as microcystins (MCs) has been studied extensively, the toxicity of non-toxin producing cyanobacteria has not been evaluated to the same extent. In this study, five strains of *Microcystis wesenbergii* were isolated from the Tri An reservoir and cultured under laboratory conditions. Microscopic observation was used for morphological identification. The MCs concentration was measured by high-performance liquid chromatography (HPLC). The microcrustacean (*Daphnia magna*) was exposed to different concentrations of crude extracts in a series of acute (48 h) and sub-chronic (15 day) toxicity experiments. The acute assay showed that crude extract from all isolated strains of *M. wesenbergii* generated toxic effects on *D. magna*, but no variant of MCs was detected in the cultures of *M. wesenbergii*. The 48 h $EC_{50}$ values of crude extracts of *M. wesenbergii* on *D. magna* ranged from 307.2-491.5 mg dry weight (dw)/l. Sub-chronic exposure of *D. magna* to the crude extract of *M. wesenbergii* at concentrations of 1, 10, 40, and 120 mg dw/l resulted in a decline of survival rates with dose dependence. Both maturation and reproduction of parent *D. magna* were inhibited with increasing concentrations of crude extract. This finding indicated that crude extracts from non-MC-producing *M. wesenbergii* isolated from the Tri An reservoir had significant acute and chronic toxic effects on *D. magna*.**

***Keywords:*** **acute, cyanobacteria, *Daphnia*, non-microcystins producing, Sub-chronic.**

***Classification number:*** **5.1**

## Introduction

Cyanobacterial blooms in eutrophic freshwater ecosystems have become an environmental concern worldwide [1]. *Microcystis* is one of the most common planktonic freshwater cyanobacterium, which frequently causes bloom-forming and toxin-producing genus in continental aquatic ecosystems. *Microcystis* is known to produce various toxins such as microcystins (MC), other bioactive peptides, alkaloid groups, and lipopolysaccharides (LPS) [2], all of which may generate toxic effects on aquatic organisms as well as human beings. In its natural environment, *Microcystis* blooms may contain either MC-producing or non-MC-producing strains [3]. However, previous studies have primarily focused on isolated MC or cyanobacterial extracts containing MC, but the roles of other toxic compounds present within a complex cyanobacterial extract have not been studied to the same extent [4]. There has been some evidence that various other cyanobacterial metabolites, including LPS, alkaloid groups or unknown secondary metabolites, and non-specific factors also contribute significantly to the adverse effects associated with blooms [4, 5].

In their natural environment, aquatic animals may be directly exposed to toxic cyanobacteria via feeding on toxic cyanobacterial cells or be indirectly exposed via ingestion of water contaminated with dissolved cyanotoxins [6]. Microcrustaceans play critical roles in aquatic ecosystems, serving as both feeders and consumers. As a filter-feeder, microcrustacean *Daphnia* spp. are potential consumers of planktonic cyanobacteria. These filter feeders are therefore seriously affected by the presence of toxic second metabolites released into the water column during cyanobacterial blooms or after the collapse of toxic cells at the end of the blooms [7]. Because of relatively

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high sensitivity to toxicants, rapid reproduction, and short lifetime, *D. magna* has been used extensively for ecotoxiclogical studies. Several studies [4, 7, 8] have examined the toxic effects of cyanobacterial bloom and MCs on *D. magna* in laboratory conditions. Acute exposure of *Daphnia* to cyanotoxins resulted in inhibition of filtration rate, decrease in swimming movements, and even death [4, 8]. Among chronic effects, literature reports decreased fecundity and population growth rate [7, 9]. Despite not producing MCs, some non-toxic cyanobacteria caused a significant increase of biotransformation enzyme activities from the exposed *D. magna* after a longer incubation [10]. However, the adverse effects of non-microcystin producing on microcrustaceans species in natural environments, where they may be exposed to a complex cyanobacterial biomass, remain somewhat unclear.

The non-microcystin producing *M. wesenbergii* are proliferate in many lakes, rivers and reservoirs in Vietnam. Little is known about their toxicity in the aquatic environment. In this study, we isolated five strains of the *M. wesenbergii* from the Tri An reservoir and maintained them in laboratory conditions. Microscopic observation was used for morphological identification. The MCs concentration was measured by HPLC. In addition, the toxic effects of a crude extract of *M. wesenbergii* on the freshwater *D. magna* were investigated.

## Materials and methods

### *Blooms collection and isolation of cyanobacteria*

Bloom samples from the Tri An reservoir were collected on surface water during July of 2017 (Fig. 1). Samples were then brought to the laboratory. Observation under microscope indicated several cyanobacteria dominant in the samples including *Microcystis*, *Oscillatoria*, and *Anabaena*. The colonies of the *M. wesenbergii* was identified 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 (2005) [11]. For isolation, single colonies of *M. wesenbergii* were picked out by micro-pipetting. After several times washing in milli-Q water, the colonies were transferred into tubes with Z8 medium and grew at a temperature of 28$^0$C under a 12:12 h light:dark cycle at an intensity of 50 µmol photons/m$^2$/s. The biomass of *M. wesenbergii* was collected onto GF/C fiberglass filters at stationary phase. After drying completely at 45$^0$C, the samples were kept at -20$^0$C prior to the experiment.

**Fig. 1. Collection of water bloom samples in the Tri An reservoir.**

### *Crude extract preparation and analysis*

The crude extracts of *M. wesenbergii* were prepared according to the method of Pietsch, et al. (2001) [12]. Briefly, a 1.0 g dry weight (dw) biomass of *M. wesenbergii* was dissolved into 100 ml milli-Q water and frozen at -70$^0$C, then thawed at room temperature. Then, the samples were sonicated for 3 min. This freeze-thaw-sonicate cycle was repeated five times. After centrifugation at 4000 rpm for 10 min, the supernatant was collected and kept at -20$^0$C. Subsamples of the crude extract were used for the measurement of MC by HPLC according to the methods reported previously by Pham, et al. (2015) [13]. Briefly, 100 µl of the supernatants was centrifuged at 4000 rpm for 15 min. The supernatant was collected into new glass tubes and dried completely. The MC's content in the samples were collected by re-constitute in 500 µl of 100% MeOH. MC concentrations were analyzed by an HPLC system with UV-visible photodiode array (PDA) detector (Shimadzu 10A series, Kyoto, Japan). Three commercial MCs included MC-RR, -LR, and -YR from Wako company (Osaka, Japan) were used as internal standards.

### *Acute and sub-chronic bioassays*

*D. magna* Straus purchased from the MicroBioTests Inc, Belgium has been permanently maintained for more than 3 years under controlled conditions: temperature 25 ± 1$^0$C and 14:10 h light:dark cycle in the ISO medium. The animals were fed by a mixture of viable green algae *Chlorella* sp. and *Scenedesmus* sp. Neonates less than 24 h were isolated for toxicity experiments.

Acute toxicity bioassays were performed according to the Protocol 202 of the Organization for the Economical Cooperation and Development (OECD) [14]. Briefly, *D. magna* neonates (<24 h old) were maintained in an ISO

medium with crude extracts of *M. wesenbergii*. At least six different concentrations with a dilution factor of 0.5 were tested in triplicate with 10 neonates per replicate. Test containers were placed at a controlled temperature of $25 \pm 1^0$C and a 14:10 h photoperiod during 48 h. The 48 h immobility of cladocerans was used to determine the half maximal effective concentration ($EC_{50}$) values with the 95% confidence interval by using the SPSS software.

Sub-chronic tests were performed with the crude extract of *M. wesenbergii* at four sublethal concentrations of supernatant (equal to 1, 10, 40, and 120 mg dw/l) and a control. Sub-chronic tests were done by using 50 ml beaker cups with 20 ml of ISO medium or exposure solutions. Neonates of *D. magna* less than 24 h in age were used. Each treatment contained 15 replicates (n=15). Test solutions were renewed every two days. A mixture of *Scenedesmus* sp. and *Chlorella* (approximately $1x10^6$ cells/ml) was provided as food for *Daphnia* in a two-day period. The mortality, maturation and production of live offspring were observed. The parent daphnid was checked daily for numbers of neonates per clutch. Reproduction was calculated by using the average of the number of neonate per female. The sub-chronic test was examined for 15 days. One-way ANOVA and Kruskal-Wallis test (Sigma Plot, version 12) was applied for calculations of the significant difference of the maturation and reproduction of *D. magna* between control and treatments.

## Results and discussion

### *Isolation and morphological characteristics*

Microscopic observation of the cyanobacterial bloom samples revealed the dominance of *Microcystis* spp. (mainly *M. aeruginosa*, *M. wesenbergii*, and *M. botrys*) and the less frequent occurrence of other genera (*Dolichospermum*, *Arthrospira*, *Planktothrix*, *Pseudanabaena*, and *Cylindrospermopsis*). Results of the present study are in accordance with previous observations that *Microcystis* was dominant group and the most common bloom-forming species in Vietnamese waters [7, 13, 15].

From bloom samples, five strains of *M. wesenbergii* were isolated and cultured in Z8 medium. Morphology examination from both field and isolated samples indicated that *M. wesenbergii* species forms elongate, often lobate, and sometimes spherical colonies that are commonly composed of sub-colonies with a distinct refractive mucilage edge. In nature, a sub-colony of this species is 15-100 µm in diameter and contains from a few (2-3) to many (>150) cells, depending on the age of the colony. Cells are more or less evenly spread throughout the colony, are 5.5-8.5 µm in diameter, and have many spherical aerotopes. Cells are slightly dark under a high magnification microscope. Colonies of this species forms an agar-like substrate that is soft and often breaks up in culture (Fig. 2).

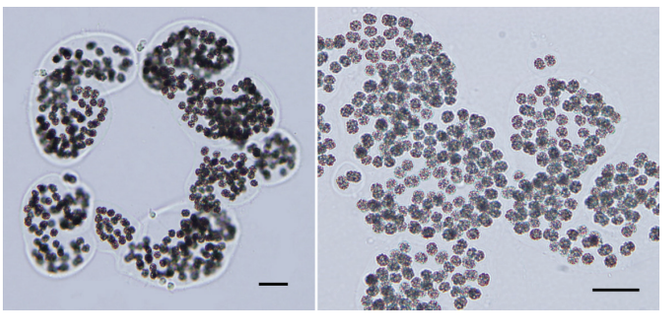

**Fig. 2. Morphology of *M. wesenbergii*** (scale bar: 20 µm).

*M. wesenbergii* is a common species of phytoplankton and sometimes forms surface blooms in many lakes and rivers worldwide. In Vietnam, the water bloom of *M. wesenbergii* has been reported from the Dau Tieng and Tri An reservoirs [7, 13], Hoan Kiem, and Nui Coc lake [15].

### *Measurement microcystins concentration from cultures*

Results of HPLC analysis indicated that the water bloom samples contained two variants of MCs including (MC-RR and MC-LR) with the highest concentration ranged from 778.2±12.6 µg/g dw (Fig. 3B and Table 1). But none of the isolated strains of *M. wesenbergii* produced microcystins (Fig. 3A). Three variants of MC, including MC-RR, MC-LR, and MC-RR from water blooms and isolated *Microcystis* species with the maximum concentration of 2130 µg/g dw have been reported from the Dau Tieng reservoir. Many strains of *M. aeruginosa* isolated from the Dau Tieng reservoir were reported to produce MC [13] but none of the strains of *M. wesenbergii* were MC-producing. It is possible that *M. aeruginosa* was the main toxin producer in the Dau Tieng reservoir. From the Tri An reservoir, Dao, et al. (2010) [7] reported four variants of MC, including MC-LR, MC-RR, MC-LA, MC-LY, and one unknown variant in the scum samples but none were found in the cultures. This may be a small number of strains of *Microcystis* were included in the examination. In this study several MCs variants were detected from the water bloom which indicated that the cyanobacterial community from the Tri An reservoir contained MC-producing and non-MC-producing cyanobacteria. Probably, the *M. wesenbergii* species is a non-toxic species. Further study is needed to determine the MC producers from the Tri An reservoir.

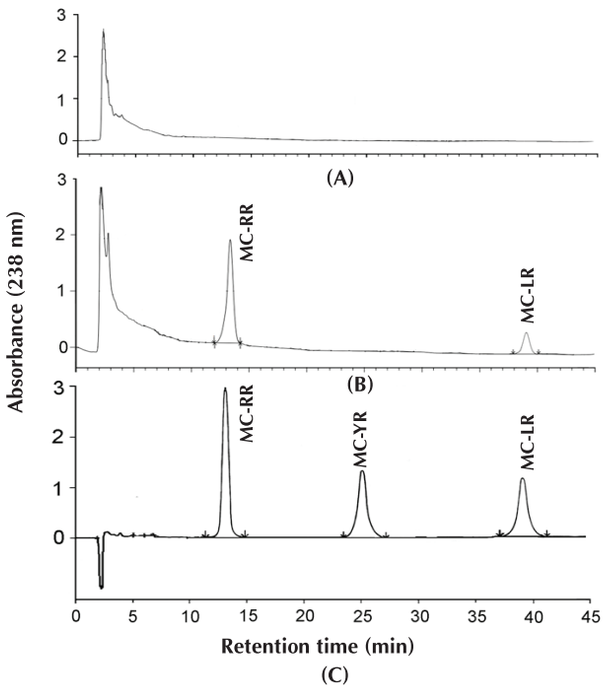


**Fig. 3. HPLC-chromatograms of (A) *M. wesenbergii*, (B) water bloom samples, and (C) microcystin standards.**

### *Acute bioassays with D. magna*

During the acute test, the survival of *D. magna* in the control was higher than 90%, and the highest concentration of crude extract (1.5 g/l) caused 100% mortality of *Daphnia* daphnids after 48 h. Therefore, the test met the requirement of the OECD (2004) [14] guideline for the acute test. The calculated 48 h $EC_{50}$ for the crude extracts of *M. wesenbergii* and water bloom samples are shown in Table 1. Although MCs were not detected in crude extracts of *M. wesenbergii*, all samples caused acute toxicity on *D. magna*. The $EC_{50}$ values of crude extracts of *M. wesenbergii* on *D. magna* after 48 h ranged from 307.2-491.5 mg dw/l (Table 1). The water bloom samples containing high concentrations of MCs also caused the highest toxicities to cladocerans. The calculated 48 h $EC_{50}$ value is 279.4 mg dw/l at 95% confidence interval (Table 1).

**Table 1. List of samples used for acute test with microcystin concentration and 48 h $EC_{50}$ values.**

| Strain name | Samples name | MC (µg/g dw) | 48 h $EC_{50}$ (mg dw biomass/l) |
|---|---|---|---|
| MW1 | *M. wesenbergii* | ND | 491.5 |
| MW2 | | ND | 307.2 |
| MW3 | | ND | 386.3 |
| MW4 | | ND | 383.1 |
| MW5 | | ND | 311.6 |
| BL-TA | Water bloom samples | 778.2±12.6 | 279.4 |

ND: no detectable microcystins.

Previous studies [16-18] have demonstrated various toxic effects on feeding behaviour and reproduction of daphnids after exposure to cyanobacterial cells or their purified toxins. However, the toxicity of the complex extract from non-MC-producing cyanobacteria is not examine in the same extent. Herrera, et al. (2014) [19] reported that the $EC_{50}$ values 48 h of a cyanobacteria bloom contained MC-LR (538 µg/g dw) collected from a reservoir in Colombia on *Daphnia* sp. were from 175-336 mg dw/l. The results of this study indicated that the toxic effects of non-MC-producing *M. wesenbergii* are somewhat lower than the water blooms samples. Probably, these water blooms samples contained other toxic compounds that contribute toxic effects other than MCs. The present results confirmed the toxic effect of non-MC-producing strains of *M. wesenbergii* on *D. magna*. In a recent study, Pawlik-Skowrońska, et al. (2019) [20] compared the toxic effects of purified MC and the extracts from *Microcystis*, *Planktothrix* and *Dolichospermum* on *Daphnia pulex*, and found that the toxicity of the crude extracts to *D. pulex* was higher than that from pure cyanotoxins. Authors reported that other toxic compounds present in the cyanobacterial extracts such as non-ribosomal oligopeptides and LPS may contribute to the toxic effects on cladocerans. The findings of this research are consistent with previous studies that natural extract from cyanobacteria contains various toxic compounds that may even be more toxic than cyanotoxins [4, 5, 9]. Further study is needed to understand the toxic effects of these compounds in cyanobacteria from the Tri An reservoir.

### *Sub-chronic toxicity and reproduction bioassay*

Sub-chronic toxic effects of crude extracts of *M. wesenbergii* (strain MW2) on *D. magna* over a period of 15 days revealed that the crude extracts of non-MC-producing *M. wesenbergii* had dose-dependent toxic effects on the survival of *D. magna* (Fig. 4).

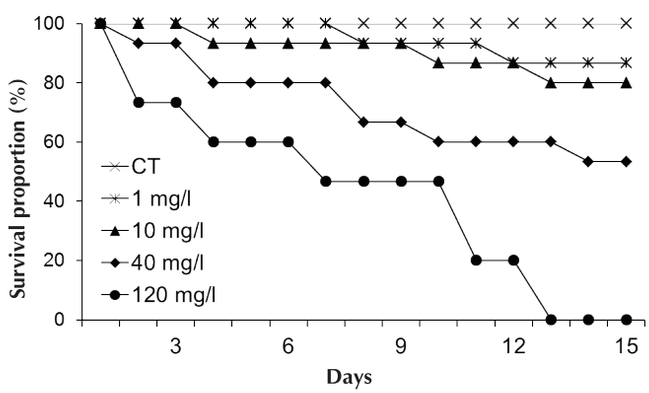


**Fig. 4. Effects of crude extracts of *M. wesenbergii* on survival of *D. magna*.**

No deaths of daphnids was recorded in the control treatment. But a mortality rate of 13% of the exposed daphnids was recorded in the treatment with 1 mg/l. The survival decreased to 80% in the 10 mg/l treatment by the end of the experiment. Only about 50% of daphnids survived in the 40 mg/l treatment. At the highest crude extract treatment (120 mg/l), mortality occurred quickly starting from day 2 and all the daphnids died after 13 days of exposure (Fig. 4).

Results of the maturation age and average number of offspring per female of *D. magna* exposed to different concentration of crude extracts of *M. wesenbergii* are shown in the results indicate that crude extracts of non-MC-producing *M. wesenbergii* at a concentration of 10 mg/l or higher inhibited the maturation and reproduction of parent daphnids. In the control and 1 mg/l treatments, there was no significant difference of maturation age between the two groups, where the maturation age of the daphnid was 5.4±0.3 days and 5.2±0.5 days, respectively. But the maturation ages of the exposures with 10 mg/l, 40 mg/l and 120 mg/l are significantly longer than the CT (Fig. 5A).

*wesenbergii* significantly delayed maturity age and caused a decline in the number of offspring of the parent daphnids. Smutná, et al. (2014) [4] exposed *D. magna* to both MC-containing and non-MC-containing cyanobacterial water bloom samples in a series of acute (48 h) and chronic (21 day) toxicity experiments. Results showed that high acute toxicity was observed for 6 of the 8 crude biomass samples. The chronic exposure assays indicated the complex biomass, the crude aqueous extract, and the microcystin-free extract all elicited similar and significant lethal effects on *D. magna*. The authors confirmed that cyanobacterial water blooms are highly toxic to zooplankton (both acutely and chronically) at environmentally relevant concentrations. Dao, et al. (2010) [7] reported malformation of neonates and cessation of the eggs/embryos of *D. magna* caused by cyanobacterial toxins from crude extract. In addition, the production of nonviable eggs and reduced fertility in *D. magna* were observed after exposure to toxic cyanobacteria [21]. The present study supports the previous findings that both toxic and non-toxic cyanobacteria exert significantly toxic effects on cladocerans.

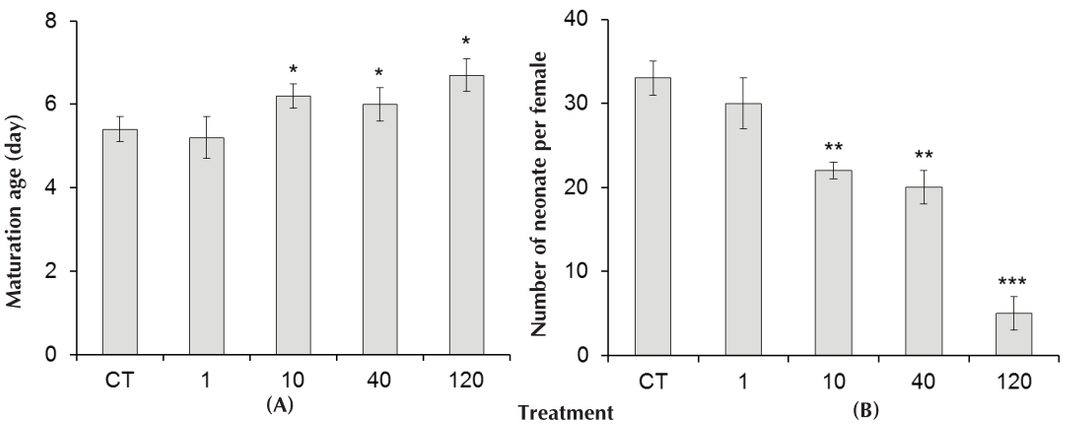


**Fig. 5. Maturation age (A) and number of offspring per female (B) of *D. magna* exposed to different concentration of crude extracts of *M. wesenbergii*.** Asterisks indicate significant difference between control (CT) and exposures.
*: p<0.05; **: p<0.01; ***: p<0.001.

During 15 days of the experiment, one parent *D. magna* in the CT treatment produced about 33±2 offspring, which was not significantly different than the 1 mg/l treatment. However, the treatment with 10, 40, and 120 mg/l resulted in significant decreases of the number of offspring per female (Fig. 5B). The reproduction results indicated that there is a concentration-response pattern in the parent daphnids exposed to crude extracts of non-MC-producing *M. wesenbergii.* Previous studies have confirmed the adverse effects of toxic cyanobacteria on *D. magna*. The toxicity includes inhibition of filtration rate, decrease in swimming movements, and fecundity or reduction population growth rate [7-9]. However, little is known about the chronic effects of non-MC-producing on cladocerans. The findings of this study revealed that crude extracts of non-MC-producing *M.*

## Conclusions

This study demonstrated that the bloom samples from the Tri An reservoir contained MCs, but no MC variant was detected from the cultures of five isolated strains of *M. wesenbergii*. The crude extracts from non-MC-producing *M. wesenbergii* isolated from the Tri An reservoir had significant acute and chronic toxic effects on *D. magna*. The present findings indicate that metabolites other than MC are likely to be responsible for the observed toxic effects, and that toxins produced from cyanobacteria may play only a minor role in the overall ecotoxicity of cyanobacterial blooms. MC producers and the toxicity mechanism of these unknown metabolites remain to be explored and need further investigation.

## ACKNOWLEDGEMENTS

This research was founded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.04-2018.314.

The author declares that there are no conflicts of interest regarding the publication of this article.

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