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First Report on Chronic Effects of Non-Microcystin Producing Cyanobacteria, *Cylindrospermopsis Curvispora* and *Planktothrix* sp., on *Daphnia Magna*

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Abstract

Cyanobacteria are an essential part of aquatic ecosystems. However, they can cause detrimental impacts on other organisms of higher tropic levels in water bodies because of their potent toxic metabolites (e.g. microcystin) and other bioactive compounds. In this study we tested the long-term and negative effects of two non-microcystin producing cyanobacteria *Cylindrospermopsis curvispora* and *Planktothrix* sp. from Vietnam on *Daphnia magna* under the laboratory conditions. The animal was fed with mixtures of green alga, *Scenedesmus* sp., and *C. curvispora* or *Planktothrix* sp. at different ratios (100 % *Scenedesmus,* 10 %

cyanobacteria + 90 % *Scenedesmus*, 50 % cyanobacteria + 50 % *Scenedesmus*, 100 % cyanobacteria) over a period of 21days. The results showed that the *D. magna* fed with from 10 to 100 % cyanobacteria reduced their survival with density dependence, delayed or postponed its maturation. Besides, the cyanobacteria also inhibited the reproduction of adult *D. magna* consequently strongly prevent the next population development of *D. magna*. The species *C. curvispora* had stronger effect on survival, but less impact on maturation and reproduction of *D. magna* than *Planktothrix* sp, negatively. To our knowledge, this is the first report on negative effects of *C. curvispora* and *Planktothrix* sp. from Vietnam on life history traits of *D. magna*. Additionally, our results revealed that even non-microcystin producing cyanobacteria at low density could also have negative impacts on zooplankton consequently ecological balance interference. In situ investigations on the effects of cyanobacteria on zooplankton are suggested for more understanding on the ecological interactions of the two trophic levels of aquatic ecosystem.

Keywords: Negative effects, *Daphnia magna, Cylindrospermopsis curvispora, Planktothrix* sp.

1. Introduction

A century of eutrophication has resulted in a worldwide increase of cyanobacterial abundance in lakes (Hallegraeff 1993). Many studies showed that cyanobacteria negatively affect zooplankton, even if they do not produce toxins. Cyanobacterial exposure can cause severe impairment on life history traits of daphnids (Porter 1980). Hietala *et al.* (1997) fed *Daphnia pulex* with toxic *Microcyst*is and observed the mortality intensification in the exposures. Investigation of Rorhlack *et al.* (2005) indicated that *Planktothrix* produced compound inhibiting the trypsin activity of *D. magna*. Besides, non-toxic *Cylindrospermopsis raciborskii* caused abortion of *Daphnia pulicaria* (Bednarska and Slusarczyk 2013). *Daphnia magna* fed with toxic *Microcystis* decreased its dry mass and the effect was density dependent (Trubetskova and Haney 2006). Mother *Daphnia* exposed to the cyanobacterial toxin, microcysin-LR, induced a decrease of dry mass of its offspring even though the offspring were raised in non-toxic medium (Ortiz-Rodriguez *et al.* 2012). Beside effects on survival or reproduction, DeMott (1999) also showed an inhibition on feeding rate when he conducted experiments of five *Daphnia* species exposed to mixtures of green alga, *Scenedesmus*, and cyanobacterium, *Microcystis*, for 7 days.

Guo and Xie (2006) hypothesized the tolerance development against toxic *M. aeruginosa* in *Daphnia carinata, Ceriodaphnia cornuta, Moina micriura* after exposing the 3 daphnid species to mixture of *Scenedesmus* and *Microcystis* trans-generationally for 4 weeks. The results clearly showed the development of tolerance in *C. cornuta* and *M. micriura* but *D. carinata*, to toxic *Microcystis*. Similarly, Gustafsson and Hansson (2004) and Gustafsson et al. (2005) reported that *Daphnia* pre-exposed of toxic *Microcystis* had lower mortality and growth faster than *Daphnia* in control indicating maternal effect to *Daphnia* under exposure to toxic *Microcystis*.

Nowadays, the nutrient value of non-toxic cyanobacterial to zooplankton has been a matter of controversy. While many studies has showed that non-toxic cyanobacteria are a poor quality

food for zooplankton (Porter 1980, Infante and Abella 1985, Hazanato and Yasuno 1987, Matveev and Balseiro 1990, Lundstedt and Brett 1991, Smith and Gilbert 1995), other studies have shown that some species of zooplankton exhibited good survival and growth when fed on cyanobacteria (Burns and Xu 1990, Gliwicz 1990, Fulton and Jones 1991). Over all, toxicity of cyanobacteria to *Daphnia* has been studied mainly with the toxic and non-toxic species of *M. aeruginosa, Planktothrix agardhii* and *C. raciborskii*. However, toxicity of the species *Cylindrospermopsis curvispora* to zooplankton has not been known and there have been few studies of the negative effects of non-microcystin producing filamentous cyanobacteria *Planktothrix* on micro-crustaceans. Therefore, in this study, we investigate adverse effects of two non-microcystin producing cyanobacteria *C. curvispora* and *Planktothrix* sp. isolated from Vietnam on *D. magna*.

2. Materials and Methods

2.1 The Test Organisms

Daphnia magna Straus was purchased from the MicroBioTests Inc, Belgium. The animal has been fed with green alga *Scenedesmus* sp. and maintained in the laboratory conditions of 22 ± 1 ℃, dim light and light dark cycle of 14h light: 10h dark. Two cyanobacterial strains, *Cylindrospermopsis curvispora* and *Planktothrix* sp., were used for exposure to *D. magna* (Fig. 1). The cyanobacterium *C. curvispora* was isolated from a small pond in District 7, Hochiminh City, during its mass development, while the other species, *Planktothrix* sp. was isolated from Dau Tieng Reservoir, Tay Ninh Province, Vietnam. Both cyanobacterial strains were cultivated in Z8 medium (Kotai 1972) with continuous aeration and under the laboratory conditions of 25 ± 1 ℃, light intensity of around 3000 Lux, and light dark cycle of 12h light: 12h dark.

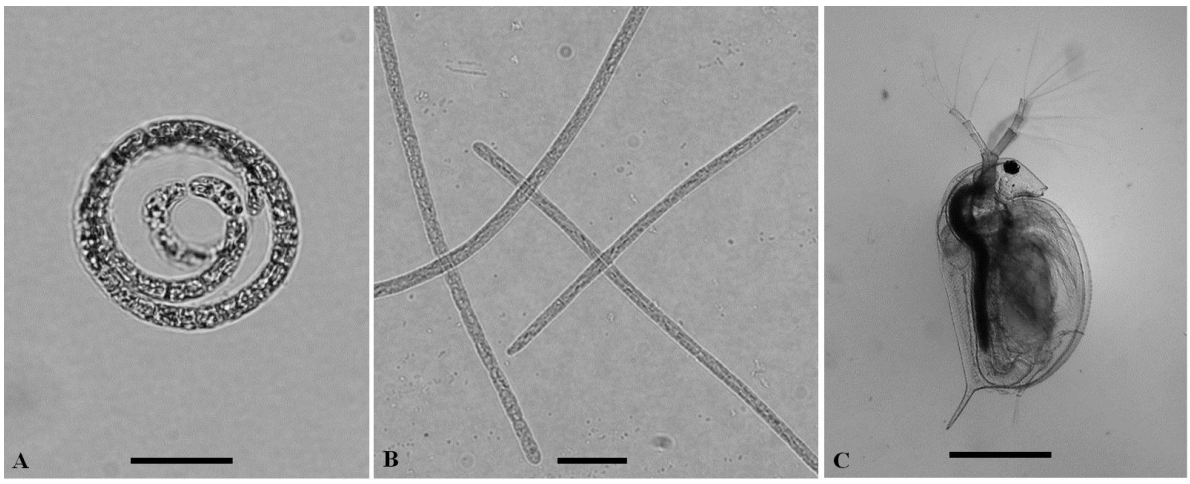

Figure 1. The organisms for the toxicity test. A, *Cylindrosmeropsis curvispora*; B, *Planktothrix* sp.; C: newly born *Daphnia magna*. Scale bars of A and B = 20 μm; scale bar of C = 300 μm

2.2 Toxin Analysis

Both cyanobacterial isolates were used for microcystins (MCs) characterization. Each

cyanobacterial culture was harvested during the exponential growth phase and filtered onto GF/A filters (Fiore, France), dried at 50 ℃ over night and stored at –70 ℃ prior to toxin determination. For MCs determination, the filters containing microbes were cut into small pieces with scissors. Extraction of MCs was conducted according to Pham *et al.* (2015). Briefly MCs were firstly extracted in 5 mL of 100 % (vol/vol) aqueous methanol by shaken for 60 min followed by 2 x 60 min of extraction in 3 mL of 75 % aqueous methanol. Each extraction step was followed by centrifugation (4 500 rpm, 30 min, 4 ℃). The supernatants of all extractions from each sample were pooled, dried at room temperature, re-dissolved in 0.5 mL MeOH (100%) and centrifuged at 8.000 rpm, 4 ℃ for 5 minutes. The supernatant was passed through a Minisart RC 4 filter membrane (0.2 µm pore size, Sartorius, Germany) prior to HPLC analysis. The HPLC (Shimadzu, Japan) equipped with a silica based reverse phase C_{18} column (Waters SunFire™, Ireland), maintained at 40 ℃. A 0.05 M phosphate buffer (pH 2.5) in MeOH (50/50, v/v) was used as mobile phase, at a flow rate of 0.58 mL/min. MCs congeners were detected by the UV detection at 238 nm with a photodiode UV-visible array detector. Microcystin-LR, -RR and -YR purchased from Wako chemicals company (Osaka, Japan) were used as standards. The HPLC system had a detection limit of 0.01 µg/L.

2.3 Toxicity Test

Fifteen neonates (< 24h old) were used for each chronic experiment (Adema 1978) and individually raised in 50 mL beakers containing 20 mL of medium (Dao *et al.* 2010). In the control experiment, the *Daphnia* was fed with 100 % of green alga *Scenedesmus* sp. In exposures, *Daphnia* was fed with a mixture of *Scenedesmus* and cyanobacteria (either *C. curvispora* or *Planktothrix* sp.) with total concentration of 1 mg C/L/day (Gustafsson *et al.* 2005) at three different regimes (1) 10 % *Scenedesmus* + 90 % cyanobacteria; (2) 50 % *Scenedesmus* + 50 % cyanobacteria; and (3) 100 % cyanobacteria (Table 1). In total, seven incubations including one control and six different exposures were conducted. All medium and food were renewed every two days. The life history traits of *Daphnia* such as survival, maturity age, reproduction were daily observed. The incubations lasted for 21 days.

Table 1. Summary of the treatments in the toxicity test.

No.	Treatments	Biomass proportion of food (green alga and cyanobacteria)		
		Scenedesmus sp.	*Cylindropsermopsis curvispora*	*Planktothrix* sp.
1	Control	100 %	0 %	0 %
2	10% Cc	90 %	10 %	0 %
3	50% Cc	50 %	50 %	0 %
4	100% Cc	0 %	100 %	0 %
5	10% Pl	90 %	0 %	10 %
6	50% Pl	50 %	0 %	50 %
7	100% Pl	0 %	0 %	100 %

2.4 Statistical Analysis

Sigmaplot version 12 was used for the data treatment. Kruskal-Wallis test was applied for calculation on statistically significant difference of the maturation of *D. magna*.

3. Results

3.1 Effects of Cyanobacteria on the Survivorship of Daphnia magna

The toxin analysis showed that both cyanobacterial species, *C. curvispora* and *Planktothrix* sp., did not produce MCs. Therefore, the used cyanobacterial strains for *Daphnia* experiments could be considered as non-MCs producing cyanobacterial strains.

In control, all *D. magna* were well alive by the end of experiment. On the other hand, *Daphnia* started to die after 2 weeks of incubation in 10 % Cc but it did within the first week in 50 % Cc and 100 % Cc. By the end of incubation, the *Daphnia* exposed to 10 %, 50 % and 100 % Cc reduced their survival by 33 %, 60 % and 67 %, respectively (Fig. 2a). Exposure to 10 % and 50 % Pl caused a reduction of 20 % of *Daphnia* population after 3 weeks of incubation. Besides, 47 % of population of *Daphnia* died after 3 weeks feeding on *Planktothrix* sorely (Fig. 2b). Mortality of *Daphnia* occurred after 4 – 5 days of exposure in all three *Planktothrix* treatments. The higher concentration of cyanobacteria the animals exposed the lower survival they had.

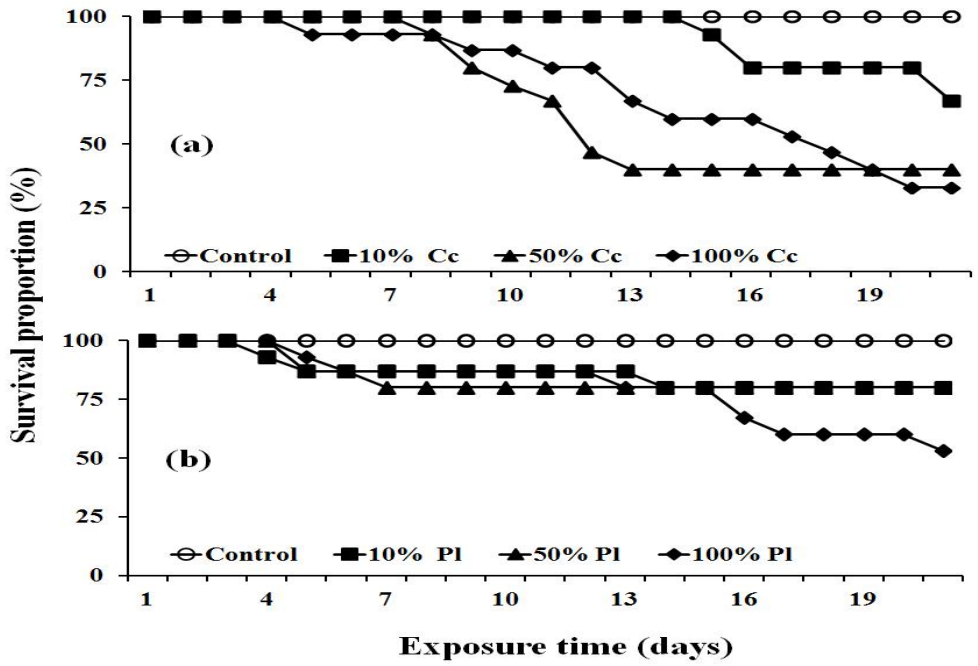

Figure 2. Survival of *Daphnia magna* from control and exposures during 3 weeks of incubation. Abbreviation as in Table 1

3.2 Effects of Cyanobacteria on the Maturation of Daphnia Magna

Daphnia raised in control reached its maturity at the age of around 6 days old. However, the animals exposed to cyanobacterial isolates delayed their maturation, from 7.5 – 9 days old (Fig. 3). Seriously, those in the treatment of 100% Pl were not able to reach their maturation (Fig. 3) although 53% of the population was alive till the last day of experiment (Fig. 2b).

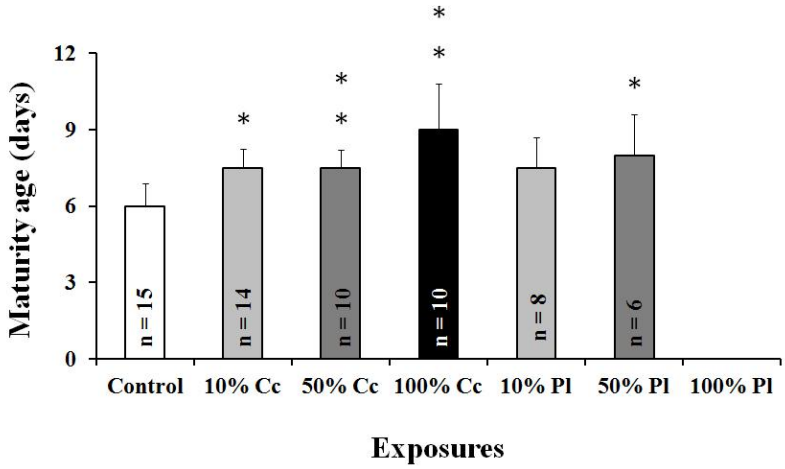

Figure 3. Maturation of *Daphnia magna* (mean value ± SD of n as indicated in the columns) from control and exposures during 3 weeks of incubation. Asterisks indicate significant difference by Kruskal-Wallis test (*, $p < 0.01$; **, $p < 0.001$). Abbreviations as in Table 1.

3.3 Effects of Cyanobacteria on the Reproduction of Daphnia magna

The first offspring of *Daphnia* were observed at the ninth day in the control and *Cylindrospermopsis* incubations (Fig. 4a, b). However, they were recorded earlier in 10% Pl treatment, after 7 days of treatment (Fig. 4c). The number of offspring in control was linear in increase during the experiment. Nevertheless, it was slowly increased in the cyanobacterial exposures (Fig. 4). The accumulative neonates in control was 479, much higher than those in the cyanobacterial exposures, 123 neonates from 10 % Cc, 37 from 50 % Cc, 34 from 100 % Cc, 3 from 10% Pl, and 19 from 50% Pl. No neonate was obtained from 100 % Pl treatment (Table 2).

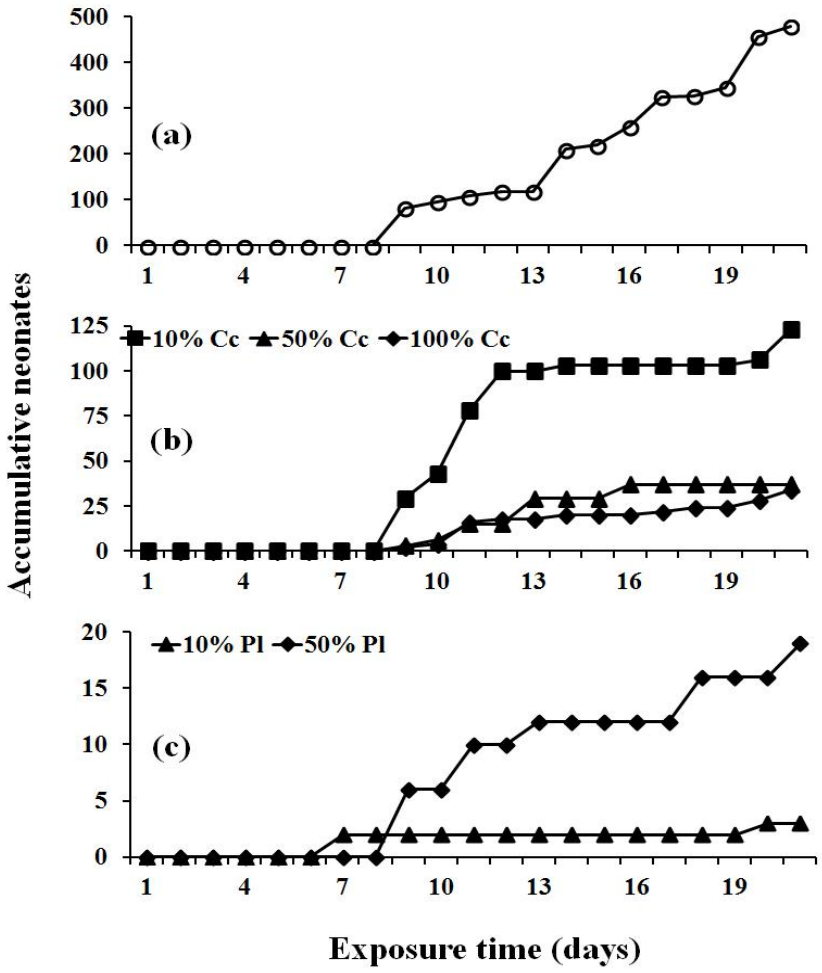

Figure 4. Reproduction of mother *D. magna* during control incubation (a), *Cylindrospermopsis* treatment (b) and *Planktothrix* exposure (c). Abbreviation as in Table 1.

Table 2: Accumulative neonates of *D. magna* after three weeks of incubation. Abbreviation as in Table 1.

	Control	10% Cc	50% Cc	100% Cc	10% Pl	50% Pl	100% Pl
Accumulative neonates	479	123	37	34	3	19	0

4. Discussion

As the live cells of cyanobacteria (*C. curvispora* and *Planktothrix* sp.) and green alga (*Scenedesmus*) were added into the medium as food, the physical characteristics of the test medium (e.g. pH, alkalinity, hardness) after food addition might be slightly changed and should result in similar physical characteristics, biologically. Besides, the secondary metabolites in cyanobacteria (e.g. microcystin, bioactive compounds) are cell-bound substances. Therefore, the observed effects on *D. magna* in the exposures should be induced by the cyanobacterial isolates used as food in our experiment.

The mortality of exposed *D. magna* in our study was in agreement with the investigation of Da Costa *et al.* (2013), in which non-toxic *C. raciborskii* reduced the survival of *D. pulex*, *M. micrura* during 12 – 15 experimental days. Oberhaus *et al.* (2007) reported that no negative effects on *Daphnia*'s survival feeding on toxic *P. rubescens* and *P. agardhii*. Hence the cyanobacterium *Planktothrix* sp. in the current study showed apparent toxicity to *Daphnia* which could be explained as different species or strains of cyanobacteria would have different toxic capacity and toxin production (Cronberg and Annadotter 2006). Generally, *C. curvispora* had stronger impact on survival of *Daphnia* than *Planktothrix* sp. (Fig. 2a, b). The hypothesis is suggested that both of cyanobacterial species may produce some toxic compounds other than MCs, but the concentration of toxic compounds in *Cylindrospermopsis* was higher than that in *Planktothrix* sp. This observation may be explained as (i) the used cyanobacterial species for our experiments could produce other toxic bio-active compounds to *Daphnia*, and (ii) cyanobacteria are low nutritional value (e.g. absence of essential polyunsaturated fatty acid and sterols) for *Daphnia* which was reported elsewhere (Brett et al. 1997, Von Elert 2002).

Cyanobacteria are not nutrient food for *Daphnia*'s growth while green alga *Scenedesmus* was used as a good food for the animal. So, the postponement of maturation should be closely related to toxic bio-active compounds from the cyanobacteria at least in the lower cyanobacterial proportion treatments (10 % and 50 % Cc, 10 % and 50 % Pl). We recognized that the body size of the animals exposed to cyanobacteria was smaller than animals in control treatment (data not showed). According to Green (1956) and Ebert (1991), smaller *Daphnia* would take more instars to mature than larger *Daphnia* (Green 1956, Ebert 1991). Therefore, the maturation of *Daphnia* in cyanobacterial treatments delayed compared to control.

Our record on reproduction of *D. magna* supported the results in a previous investigation (Lürling and Van der Grinten 2003). The same authors showed the negative effects of non-toxic *M. aeruginosa* on body length, reproduction (number of new born per female) and clearance rate of *Daphnia*. In the natural environment, the effects of cyanobacteria, even non-toxic, on zooplankton can have some implications. For example, it can reduce zooplankton's vitality and growth rate, lead their populations to decline. Therefore, the effect of cyanobacteria on zooplankton can interfere in some ways with decreasing the fitness of these species

5. Conclusions

Though non-MCs producers, both *C. curvispora* and *Planktothrix* sp. negatively affected life history traits of *D. magna*, including mortality increase, maturation postponement or inhibition and reproduction reduction. The adverse effects were concentration dependent. The toxicity of *C. curvispora* was more potent than *Planktothrix* sp. To the best of our knowledge, this is the first information on chronic effects of non-MCs producing cyanobacteria, *C. curvispora* and *Planktothrix* sp. on *D. magna*. This study revealed that these cyanobacterial species may product some toxic bio-active compounds other than MC which need further analysis with modern equipment, e.g. LC/MS, GC/MS. Besides, more attention to the

presence, distribution of cyanobacteria in nature should be paid. Negative impacts of *C. curvispora* and *Planktothrix* sp. on other aquatic organisms (e.g. aquatic plants, fish, micro-algae) should be investigated to get more understanding on the toxicity of these cyanobacteria on the aquatic ecosystem.

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