Cross talk: Two way allelopathic interactions between toxic Microcystis and Daphnia

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Abstract

Due to eutrophication, freshwater ecosystems frequently experience cyanobacterial blooms, many of which produce bioactive metabolites that can affect vertebrates and invertebrates life traits. Zooplankton are able to develop tolerance as a physiological response to cyanobacteria and their bioactive compounds, however, this comes with energetic cost that in turn influence *Daphnia* life traits and may impair populations. Vice versa, it has been suggested that *Daphnia* are able to reduce cyanobacterial dominance until a certain cyanobacterial density; it remains unclear whether *Daphnia* metabolites alone influence the physiological state and bioactive metabolites production of cyanobacteria. Hence, this study investigates mutual physiological reactions of toxic *Microcystis aeruginosa* PCC7806 and *Daphnia magna*. We hypothesize that a) the presence of *D. magna* will negatively affect growth, increase stress response and metabolites production in *M. aeruginosa* PCC7806 and b) the presence of *M. aeruginosa* PCC7806 will negatively affect physiological responses and life traits in *D. magna*. In order to test these hypotheses experiments were conducted in a specially designed co-culture chamber that allows exchange of the metabolites without

direct contact. A clear mutual impact was evidenced. Cyanobacterial metabolites reduced survival of *D.magna* and decreased oxidative stress enzyme activity. Simultaneously, presence of *D.magna* did not affect photosynthetic activity. However, ROS increase and tendencies in cell density decrease were observed on the same day, suggesting possible energy allocation towards anti-oxidative stress enzymes, or other protection mechanisms against *Daphnia* infochemicals, as the strain managed to recover. Elevated concentration of intracellular and overall extracellular microcystin MC-LR, as well as intracellular concentrations of aerucyclamide A and D in the presence of *Daphnia*, indicating a potential protective or anti-grazing function. However, more research is needed to confirm these findings.

Keywords: zooplankton, cyanobacteria, secondary metabolites, PCC7806, toxic, oxidative stress

Abbreviations

MC+	*M. aeruginosa* PCC7806
MC-LR	microcystin LR
des-MC-LR	des microcystin LR
CP	cyanopeptolin
AC	aerucyclamide
CP A	cyanopeptolin A
AC A	aerucyclamide A
AC D	aerucyclamide D
CAT	catalase

SOD	superoxide dismutase
GST	glutathione-s-transferase
ROS	reactive oxidative species
HPLC	high performance liquid chromatography

1. Introduction

Many aquatic ecosystems experience cyanobacterial blooms as a consequence of eutrophication, and global warming (O'Neil et al., 2012; Paerl and Otten, 2013). High abundance of cyanobacteria leads to elevated concentrations of diverse cyanobacterial secondary metabolites some of which pose health hazards to humans, but also to aquatic organisms, such as zooplankton (Li et al., 2010; Holland and Kinnear, 2013). As filter feeders some zooplankton species, including *Daphnia* sp. graze on phytoplankton, including cyanobacteria, reducing cyanobacterial population in aquatic environments up to certain densities (Sarnelle, 2007; Chislock et al., 2013; Ekvall et al., 2014). In addition to producing harmful secondary metabolites, cyanobacteria are a nutritionally inadequate food source, as they lack sterols and essential fatty acids necessary for *Daphnia* growth, development and reproduction (Lynch et al., 1986; Müller-Navarra et al., 2000; Martin-Creuzburg et al., 2008). The cyanobacterium *Microcystis aeruginosa* is commonly distributed worldwide, and known to produce microcystin, one of the most investigated cyanobacterial toxins (O'Neil et al., 2012; Svirčev et al., 2019). Microcystin toxicity is mediated through its ability to inhibit protein phosphatases 1 and 2A (MacKintosh et al., 1990), causing cytoskeletal derangements as well as promotion of oxidative stress (Amado and Monserrat, 2010), which together may eventually lead to cell death (Carmichael, 1992; Zaccaroni and Scaravelli, 2008). Besides microcystins, that can have negative effect on *Daphnia* life traits, *M. aeruginosa* is known to produce other bioactive metabolites, such as cyanopeptolins, aerucyclamides and aeruginosins, known as protease inhibitors, affecting the digestion in *Daphnia* (Czarnecki et

al., 2006). The function of those compounds within the cyanobacteial cell and the dynamics of their production is however not yet fully resolved.

Unraveling the natural role of microcystin and its dynamics for the cyanobacteria has been a topic of many studies with the emphasis on the influence of abiotic factors (such as light, nutrients, or temperature) (Gobler et al., 2007; Jähnichen et al., 2007; Schatz et al., 2007; Alexova et al., 2016). More recent studies focused on changes in the production of cyanobacterial metabolites mediated by biotic factors, such as the presence of grazers (Jang et al., 2003, 2004; van Gremberghe et al., 2009; Sadler and von Elert, 2014a; Harke et al., 2017; Bojadzija Savic et al., 2019). These studies suggest that the presence of *Daphnia* can trigger the production of cyanobacterial secondary metabolites via the substances they release in the water (infochemicals), which could be an antigrazing response. Considering that the cyanobacteria and their genes responsible for toxin production are much older than complex organisms, such as their grazers, it has been suggested that an antigrazing role could have been obtained and kept over time due to grazing pressure (Wilken et al., 2010; Chislock et al., 2013). Increase of microcystin concentration in the presence of *Daphnia* infochemicals has been recorded in several studies (Jang et al., 2003, 2007; Izydorczyk et al., 2008; Kaplan et al., 2012; Pérez-Morales et al., 2015), as well as the higher export of microcyclamide 7806A (Sadler and von Elert, 2014a), and cyanopeptolin A (Bojadzija Savic et al., 2019) supporting this hypothesis. Cyanobacterial response seems to be strain dependant, as 4 out of 8 strains increased the production of microcystin in the presence of *Daphnia* medium (van Gremberghe et al., 2009). On the other hand, the presence of *Daphnia* medium can also decrease microcystin concentration (Becker et al., 2010; Bojadzija Savic et al., 2019), as well as cyanopeptolin and aerucyclamide (Bojadzija Savic et al., 2019), leaving this role arguable.

Furthermore, increase of microcystin has been associated with the intracellular protective role against oxidative stress, providing toxic strains advantage in stress inducing environments (Briand et al., 2008, 2012; Zilliges et al., 2011; Paerl and Otten, 2013).

However, this function is not entirely proven, as a recent study suggests that microcystin binds to proteins involved with antioxidative stress enzymes (peroxiredoxin, thioredoxine) interfering with their activity, and increasing the sensitivity of the microcystin producing strain (Schuurmans et al., 2018).

During their lifetime, *Daphnia magna* are able to develop tolerance as a physiological response to cyanobacterial metabolites, but only few of the mechanisms involved have been revealed so far, such as increase in detoxification and oxidative stress enzyme activity, and remodeling their digestive enzymes (Ortiz-Rodríguez et al., 2012; von Elert et al., 2012; Meissner et al., 2013; Sadler and von Elert, 2014b). These mechanisms contribute to the development of tolerance, however, they come with energetic cost that in turn influences *Daphnia* life traits and may impair populations (Ortiz-Rodríguez et al., 2012, Wojtal-Frankiewicz et al. 2014). Nevertheless, zooplankton often coexists with toxic cyanobacterial blooms, through co-acclimation, in mutual two-way interactions that are yet to be disentangled. It remains unclear whether *Daphnia* infochemicals could have negative impact on cyanobacterial growth and photosynthetic activity, due to contradicting results in the literature (Jang et al., 2007; Yang and Li 2007; Bojadzija Savic et al., 2019). Furthermore, whether cyanobacteria suffer from oxidative stress when exposed to daphnids requires further clarification.

The focus of this study was to investigate the mutual physiological and metabolic reactions of microcystin producing *Microcystis aeruginosa* PCC7806 strain and its potential grazer *Daphnia magna* during interaction. Experiments were conducted in a purposely-designed co-culture chamber that allowed the exchange of metabolites produced by both organisms, in the presence of the other, without direct contact.

We hypothesize that:

a) The presence of *D. magna* will negatively affect growth, increase stress response and metabolite production in *M. aeruginosa* PCC7806.

b) The presence of *M. aeruginosa* PCC7806 will negatively affect physiological responses and life traits in *D. magna*.

2. Materials and methods

2.1 Culture conditions

2.1.1 *Microcystis aeruginosa* PCC 7806

An axenic strain of microcystin (MC) producing *M. aeruginosa* PCC 7806 (MC+) was obtained from the Pasteur Culture collection of Cyanobacteria in Paris (https://research.pasteur.fr/en/team/collection-of-cyanobacteria/). The strain was grown in 50% cyanobacterial BG11 medium (SIGMA), diluted with sterile 50% reverse osmosis water, under a 14h:10h light:dark regime using daylight white fluorescent tubes (Toshiba, 15 W, FL15D) with 20 µmol photons m^{-2} s^{-1} illumination at a constant temperature of 20 ± 1°C (Sanyo incubator). The culture was maintained in exponential growth phase, while the axenicity was regularly evaluated as described in Briand et al. (2012).

2.1.2 *Daphnia magna*

The *D. magna* clone (originating from INERIS) was obtained from the PEARL INRA 1036 U3E (Rennes, France). Before the start of the experiments, *D. magna* were progressively acclimatized during three weeks to cyanobacterial BG11 medium (SIGMA) diluted to 50% with reverse osmosis water. Furthermore, to make sure that osmolarity would not affect *Daphnia* life traits, we compared osmolarity of BG11 medium with the osmolarity of commonly used Artificial *Daphnia* medium, Elendt M4 and Elendt M7 before preforming the experiments. Osmolarity of BG medium was similar to osmolarity of commonly used *Daphnia* media, thus did not affect *Daphnia* survival. *D. magna* were fed daily with the green

algae *Scenedesmus communis* (reaching max ≈ 2×10^4 cells mL^{-1} in the aquarium at feeding time). *S. communis* originated from lake Grand Lieu (France) was isolated in our laboratory (University of Rennes 1, UR1). *D. magna* were cultivated at a constant temperature of 20°C, light intensity of 15 µmol photons m^{-2} s^{-1} and a day/night cycle of 14h:10h (Sanyo incubator).

2.2 Exposure set up

Experiments were performed in an innovative co-culture glass chamber that was purposely designed and built in house (**Figure 1. A**). The glass compartments were from the Glass Workshop of the Department of Physics (UR1).

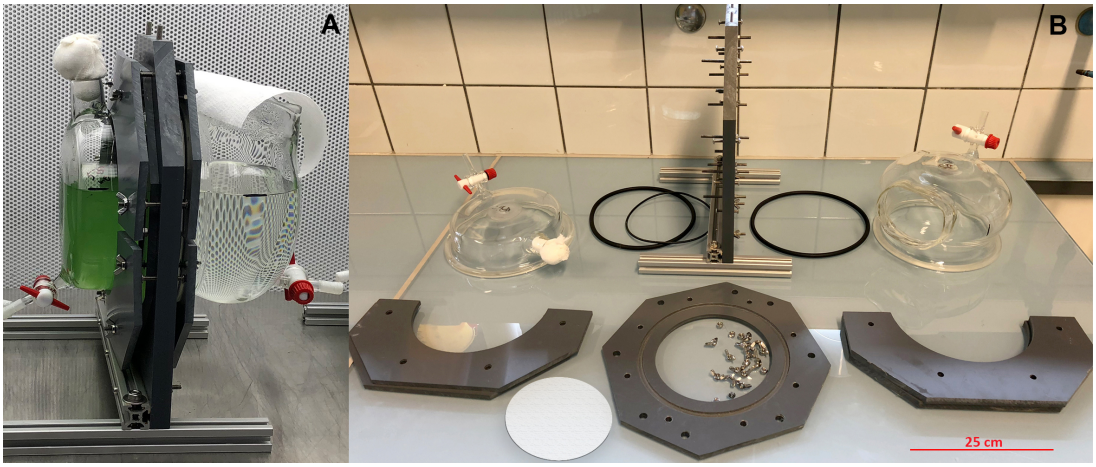


Figure 1. A The co-culture chamber during the exposure with the cyanobacterial culture at the left and the *D. magna* on the right side; **B** The disassembled device with all of the parts required for its setup.

The disassembled co-culture chamber (**Figure 1. B**) was comprised of two glass compartments separated by a 0.2 µm cellulose nitrate membrane filter (142 mm diameter, Whatman, Buckinghamshire, UK). The glass material was preferred over plastic for easier autoclaving and to avoid the release of plastic compounds. The cyanobacterial co-culture unit (0.6 L volume **Fig 1. A** left side, hereafter named cyanobacterial unit) had a 20 mm opening at the top, while the *Daphnia* co-culture unit (2L volume **Fig 1. A)** right side, hereafter named *Daphnia* unit) had an opening 10 x10 cm. The openings allowed oxygen exchange, as well

as filling the co-culture unit with the medium and food in the case of *D. magna*, and sampling during the experiment. Both co-culture units were sealed with screws and held together with the plastic support (**Figure 1. B**). This approach allowed exchange of fluids and dissolved metabolites, without direct contact between testing organisms (Briand et al., 2016). Both chamber units were cleaned and autoclaved before being used in the experiments. The cyanobacterial co-culture unit was closed with a sterile cotton plug. The 0.2 μm cellulose nitrate membrane filter also guaranteed that the *M. aeruginosa* remained axenic.

2.3 Diffusion pre-test

Before the experiments, the time required for microcystin-LR (MC-LR) to diffuse and reach equilibrium in the co-culture chamber was determined. MC-LR was chosen as as example for a relatively large (995,2 g/mol) seconday metabolite of *M. aeruginosa*. The co-culture chamber was filled with 50% BG11 medium on both sides, and 0.4 μg/ml of pure MC-LR was added to the cyanobacterial unit. Medium was gently mixed in both co-culture chamber units daily. Samples were taken every 24h from both co-culture chamber units, revealing that it took 4 days for MC-LR to reach equilibrium between the chambers (**Figure S1.**). Therefore the experiment length was determined to be 8 days, giving sufficient time for metabolites to reach equilibrium and organisms to respond.

2.4 Experimental design of the monoculture controls

2.4.1 *M. aeruginosa* PCC7806

Exponentially growing MC+ was centrifuged and cells were transfered to sterile 50% BG11 medium until cell density reached $5x10^5$ cells mL^{-1}. Inoculating in fresh medium allowed reduction in extracellular compounds released in the used medium at start of the

187 experiment. MC+ was grown for one week (reaching cell density $9.5x10^5$ cells mL^{-1}) and
188 then used in the experiment. The co-culture chamber was assembled and filled under the
189 sterile hood in order to maintain axenic conditions. Both chambers were filled simultaneously
190 to avoid a transfer of the media through the membrane: the cyanobacterial unit with the one
191 week old cyanobacterial culture, and the *Daphnia* unit with fresh 50% BG11 medium
192 (previously oxygenized for 2 days, but without *Daphnia*) and the addition of *S. communis* to
193 a density of $4x10^7$ cells L^{-1}, the green algae used in *Daphnia* cultivation. *S. communis* density
194 was chosen as it was equivalent to the daily feed of *D. magna* in the co-culture treatment.
195 Medium was gently mixed in both units every day.

196 **2.4.2 *D. magna***

197 Three hundred 2-4 days old, non-egg bearing *D. magna* individuals were grown in fresh
198 50% BG11 medium (previously oxygenized for 2 days) in 2 L aquariums and fed daily with
199 *S. communis* ($4x10^7$ cells L^{-1}).

200 **2.5 Experimental design of the co-culture treatment**

201 For the co-culture experiment, preparation of the cyanobacteria was performed as for the
202 control experiment, as well as the assemblage and filling of both chambers. This was
203 followed by adding three hundred 2-4 days old, non-egg bearing *D. magna* individuals in the
204 *Daphnia* unit as well as *S. communis* ($4x10^7$ cells L^{-1}). Experiment lasted 8 days, therefore it
205 required daily *Daphnia* feeding adjustment. Hence, the cell density of *Scenedesmus* was
206 measured daily and readjusted to $4x10^7$ cells L^{-1}. During the experiment the *Daphnia* unit
207 was covered with paper cloth that reduced light intensity (from 20 to 15 μmol photons m^{-2} s^{-1}),
208 while light intensity remained at 20 μmol photons m^{-2} s^{-1} in the cyanobacterial unit.

Cyanobacterial photosynthetic activity, cell density, reactive oxidative species (ROS) and metabolite dynamics were monitored at days 0, 4, 6 and 8. *Daphnia* were sampled on the last day of the experiments for analysis of the enzymes (Superoxide dismutase (SOD), Glutathione S-transferase (GST), Catalase (CAT), glycogen, lipids and hydrogen peroxide, H_2O_2. Neonates that were born during the exposure were collected on the last day of the experiment and kept in the medium obtained from the *Daphnia* unit, where their survival was observed over 7 days. After removing of excess water, adult *Daphnia* samples were snap frozen in liquid nitrogen and stored at -80°C. All experiments were performed in four replicates with a duration of 8 days.

2.6 Photosynthetic activity

Photosynthetic activity is often measured through maximum electron transport rate (ETRmax), an indicator of the algal physiological state, and precursor of growth (Briand et al., 2012; Pannard et al., 2016). ETRmax values reaching 100 and above for the strain PCC 7806 are an indicator of good physiological state, while values below 30 are associated with physiological stress from which cyanobacteria cannot recover (Briand et al., 2012, 2016). The electron transport rate (ETR) was measured on days 0, 4, 6 and 8 with a pulse-amplitude-modulated fluorescence monitoring system (PhytoPAM, Walz, Germany) (Schreiber et al., 1998; Pannard et al., 2016). PhytoPam settings used in our experiments were as described in Bojadzija Savic et al. (2019).

2.7 Cyanobacterial cell density

Cyanobacterial cell density was obtained at days 0, 4, 6 and 8 by measuring optical density with a spectrophotometer (UVIKONxs SECOMAN) at the absorbance of 750 nm

(Briand et al., 2012). 50% BG11 medium was used as reference (blank). In order to establish a relationship between optical density at 750 nm and cell density, a calibration of *Microcystis* cells number mL^{-1} versus absorbance was established for the strain PCC 7806 (Pannard et al., 2016). A nageotte cell (Marienfeld) was used for cyanobacterial cell counting and observed under a Olympus BX50 microscope (objective 40x) as in Pannard et al. (2016).

2.8 ROS production in cyanobacteria

Cyanobacterial oxidative stress was measured via total ROS production (Rajneesh *et al.* 2017). H_2DCFDA (2 ', 7'-dichlorodihydrofluorescein diacetate) is a non-fluorescent reagent that is cleaved by intracellular esterases forming H_2DCF (dichlorodihydro-fluorescein). H_2DCF reacts with intracellular ROS to form a fluorescent compound, DCF (dichlorofluorescein). $5x10^6$ cells were centrifuged and the pellets resuspended in 996 µL $NaPO_4$ buffer 0.1M pH 6.5 and 4 µL of the H_2DCFDA diluted with ethanol (1:8). Samples were incubated for one hour in the dark and fluorescence of DCF was measured spectrophotometrically at 485 nm.

2.9 Cyanobacterial secondary metabolites

Dynamics of intra and extracellular cyanobacterial secondary metabolites known to be produced by the MC+ strain (Rohrlack et al., 2004; Sadler and von Elert, 2014b; Briand et al., 2016) have been monitored in the cyanobacterial unit and the *Daphnia* unit. Metabolites were analysed with a Waters Acquity Ultra-High Performance Liquid Chromatography coupled to a Xevo quadrupole time of flight mass spectrometer. Samples (5 mL) were centrifuged, and cells (for intracellular metabolites), and supernatant (for extracellular metabolites) separated and lyophilized. Dried material was extracted in 0.5 ml 50% methanol and processed as described in Bojadzija Savic et al. (2019). Cyanobacterial peptides were detected using extracted ion chromatograms for the respective specific masses of the different compounds (Bojadzija Savic et al., 2019).

MC-LR, des-MC-LR, cyanopeptolin A, and aerucyclamide A and D were quantified in this experiment using linear relationship between peak area (MC-LR and des-MC-LR at 238 nm, cyanopeptolin A (CP A) at 220 nm, and aerucyclamide A (AC A) at 237 nm and D (AC D) at 240 nm) and known concentrations of the toxin standards. MassLynx v4.1 was used for both detection and quantification of the cyanobacterial peptides. The microcystin-LR standard was purified as previously described (Edwards et al., 1996). CP A and AC A and D standard were purified using preparative HPLC (high performance liquid chromatography) (Biotage Parallex Flex, Cardiff, UK) and Flex V3 software for instrument control and data acquisition. The separation was performed on Atlantis Prep C18 column (5 um particle size, 19 mm ID × 300 mm long; Waters, Elstree, UK) using a 30-min linear gradient from 60% to 100% methanol in MilliQ water. The flow rate was 20 mL/min and 4 mL fractions were collected (Bojadzija Savic et al., 2019).

2.10 *D. magna* survival

D. magna adults survival was measured by counting dead *Daphnia* every day in each unit and results were expressed in percentages of total individuals. In the last two days of the co-culture chamber experiment, neonates were produced. At termination of the experiment they were transfered into beakers filled along with the medium from the co-culture chambers. Neonates were fed every day and grown for 8 days, with the intention of comparing their life traits with the life traits of the parent generation. Survival of the neonates was measured by counting dead *Daphnia* every day in each unit and results were expressed in percentages of total individuals.

2.11 Enzyme extraction and measurement

Daphnia individuals were collected at the end of the experiment, briefly rinsed and blotted, then frozen in liquid nitrogen and stored at -80°C. Throughout the extraction the samples were kept on ice. *Daphnia* were resuspended and homogenized in 1 mL of extraction buffer (0.1 M phosphate buffer pH 6.5, glycerol, 1 mmol EDTA, and 1.4 mmol

dithioerythritol) using Lysing Beads-Matrix E (MPbio) in the Vibro-mill MM200, (RETSCH) for 3 minutes at the frequency of 25 Hz to break the cells. The homogenates were then centrifuged at 10,000 g for 10 minutes at 4°C (Sigma 3K18C). Supernatant was separated from pellets and used in enzyme measurements. Catalase (CAT) activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm (Chang and Kao, 1997). GST was assayed at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). Superoxide dismutase (SOD) activity was determined using a photochemical assay based on the reduction of nitro blue tetrazolium (NBT) according to Total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method, SIGMA KIT). All enzyme activities were related to protein content in the extract, measured according to Bradford (1976).

2.12 H_2O_2 measurement

In order to measure hydrogen peroxide concentration in *Daphnia*, they were resuspended and homogenized in 600 µL of 0.1 M phosphate buffer pH 6.5 using Lysing Beads-Matrix E, MPbio. Samples were homogenized for 3 minutes at the frequency of 25 Hz (bead-beater Vibro-mill MM200, RETSCH) followed by centrifugation at 15,000 g for 20 minutes at 4°C (Sigma 3K18C). The supernatant was transferred to a microtube and stored in ice for protein determination (Bradford, 1976) and quantification of H_2O_2. Concentration of H_2O_2 was measured according to Sasadhar and Monojit (1981) at 410 nm.

2.13 Energetic resources

Samples were lyophilised and weighed before the extraction (Balance XP2U Mettler Toledo, Columbus). Further steps were done on ice: 600 µl of phosphate buffer was added to each sample, homogenized with Lysing Beads-Matrix E (MPbio) for 1 minute and 30 secondes at 24 HZ (bead beater Vibro-mill MM200, RETSCH), centrifuged (500xg, 10 minutes, 4°C) and supernatant transferred to a new tube. 50 µl of the supernatant was used for protein measurements (Bradford, 1976). For triglyceride and glycogen determination, 300 µl of the supernatant was mixed with 900 µl chloroform:methanol (2:1) and 100 µl of MilliQ

water. Phases were separated by centrifugation at 4°C at 180xg for 15 minutes and kept at -20°C over night. The following day, 300 µl of the chloroform phase was transferred in a new tube and used for triglyceride measurements. The pellet between the two phases was used for measuring glycogen content according to Foray et al. (2012). Triglycerides were measured after evaporation of the chloroform using a triglycerides colorimetric assay (Triglycerides kit reference CC02200, LTA srl, Italy), however, triglycerides in our samples were below the level of detection.

2.14 Statistical analyses

R Core Team (2013) was used to access statistical analysis of the obtained data. All data are presented as mean ± standard deviation. Significant differences were determined at $p<0.05$. We performed repeated-measures analysis of variance to determine the difference between cell density, photosynthetic activity, ROS and concentration of intracellular and extracellular metabolites between the control and treatment for MC+. Repeated-measures analysis of variance is a mixed linear model with day, treatment (Control vs Treatment) and interaction between day and treatment considering the repeated measures on replicates (random effect). Normality of residuals was tested via Shapiro test (residuals normaly distributed when $p>0.05$). Anova was preformed to test the effects of the model. Pairwise comparison with correction for multiple comparison was preformed to check significant differences between control and the treatment and if there was time dependency effect on control and treatment (* ($p < 0.05$), **($p < 0.01$), *** ($p < 0.001$)).

T-test was used to compare *Daphnia* enzyme activity and energetic resources between control and treatment.

3. Results

3.1 Cyanobacterial growth and photosynthetic activity

Differences of photosynthetic activity, measured as maximum electron transport rate (ETR max) were not statistically significant during the entire experiment between control and

treatment ($p > 0.05$) (**Figure 2**. **A**). Whereas it didn't change over time in the control ($p > 0.05$), a time effect was observed for the treatment where photosynthetic activities were superior on day 6 ($p < 0.05$) and day 8 ($p < 0.01$) to the photosynthetic activity on day 0. Cyanobacterial cell density was not significantly different between control and treatment ($p > 0.05$). However, an effect of time was observed in the control, where cyanobacterial cell density on day 8 was higher compared with day 0 ($p < 0.05$). Furthermore, a time effect was observed in the treatment, where cyanobacterial biomass was significantly higher on day 6 and day 8 compared with biomass on day 0 ($p < 0.001$), and day 4 ($p < 0.01$) (**Figure 2**. **B**).

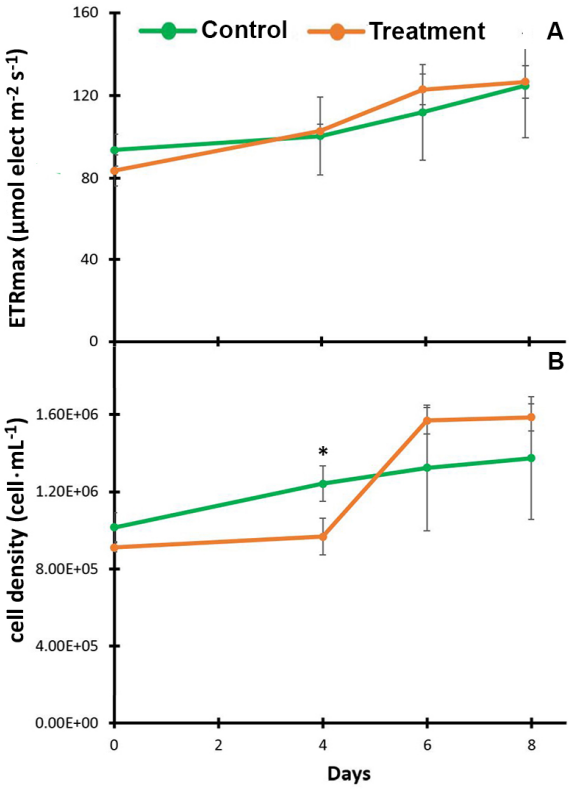


Figure 2. Photosynthetic activity and cyanobacterial biomass in control and in the presence of *D. magna*: **A** photosynthetic activity **B** cyanobacterial biomass. ($p > 0.05$) repeated-measures analysis of variance.

3.1 ROS in cyanobacteria

Total reactive oxygen species (ROS) were significantly lower on day 0 ($p < 0.01$) in the presence of *D. magna*, compared with the control, while on day 4 it was significantly higher ($p < 0.001$) (**Figure 3.**). On days 6 and 8 there were no significant differences in the ROS content between control and treatment ($p > 0.05$). ROS content was constant in the control

throughought the experiment ($p > 0.05$), while in the treatment, ROS content was significantly higher on day 4, day 6 and day 8 then ROS content observed on day 0 ($p < 0.001$). Furthermore, ROS content in the treatment was significantly lower on day 6 and day 8, compared with day 4 ($p < 0.001$).

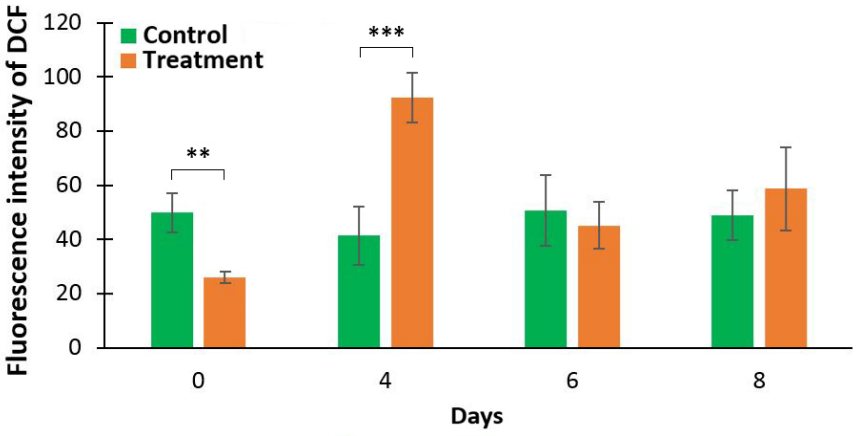


Figure 3. ROS content in MC+ in control and in the presence of *D. magna* ** ($p < 0.01$), *** ($p < 0.001$) repeated-measures analysis of variance.

3.3 Cyanobacterial metabolites

3.3.1 Detected cyanobacterial metabolites in control and treatment

In this study, eleven metabolites were detected: MC-LR, des-MC-LR, cyanopeptoline (953A, A, B, C), aerucyclamide (A, B, C, D), aeruginosin 684 and 602 (**Table S1.**). In the cyanobacterial co-culture unit, all these metabolites were present both intracellularly and extracellularly throughout the experiment. In the *Daphnia* co-culture unit, all cyanobacterial metabolites remained below detection limit until day 4 (**Table S1.**). Nine metabolites were detected from day 4 onwards: MC-LR, des-MC-LR, cyanopeptoline (CP 953A, A, C), aerucyclamide (AC A, B, C), aeruginosin 684 and 602. CP B was not detected until the day 8, while AC D remained below the detection limit in the *Daphnia* co-culture unit during the whole experiment. The same metabolites were observed in the control and the treatment at the corresponding time points (**Table S1.**).

3.3.2 Quantified cyanobacterial metabolites

3.3.2.1 Cyanobacterial unit

Intracellular concentration of MC-LR and des-MC-LR (fg $cell^{-1}$) in MC+ remained constant throughout the experiment in the control (**Figure 4A**). In the presence of *D. magna*, the intracellular concentration of both was not significantly different from the control during the first 6 days of the experiment but increased significantly on day 8 ($p < 0.001$, respectively $p < 0.05$), also in comparison with day 4 and day 6 ($p < 0.001$, respectively $p < 0.01$) (**Figure 4A**). A similar trend was observed for AC A ($p < 0.001$) and AC D ($p < 0.001$), where intracellular concentration in the presence of *D. magna* increased only on the last day of the experiment in MC+ (**Figure 5**). Concentration of intracellular AC A started significantly higher in the control compared with the treatment ($p < 0.05$) (**Figure 5B).** Furthermore, in the control, intracellular concentration of AC A and AC D were significantly lower on day 8, compared with day 0, whereas they significantly increased in the treatment on day 8, compared with day 0, day 4 and day 6 ($p < 0.001$). Intracellular CP A was the only metabolite showing no significant difference between control and treatment, ($p > 0.05$) (**Figure 5A**). In both, the control and treatment, it even decreased, significant for the control on day 4 ($p < 0.01$), compared with day 0, before it increased again.

Extracellular MC-LR was significantly higher in the control compared to the treatment on day 4 ($p < 0.05$), and day 8 ($p < 0.01$), as well as des-MC-LR on day 4 ($p < 0.05$), and day 8 ($p < 0.001$) (**Figure 4C, D**). Extracellular concentration of MC-LR and des-MC-LR significantly changed over time in the control: MC-LR: significant decrease on day 6, compared with day 4 ($p < 0.01$); des-MC-LR: significant increase on day 6 and day 4, compared with day 0 ($p < 0.001$) and significant increase on day 8, compared with day 4 ($p < 0.001$).

Extracellular MC-LR significantly increased over time on day 6 ($p < 0.05$) compared with day 4 and on day 8 compared with day 0 and day 4 ($p < 0.001$); similarly, des-MC-LR: significantly increased on day 8, compared with day 4 and day 6 ($p < 0.01$). (**Figure 4C, D**).

Extracellular CP A, AC A, and AC D were detected, remained however below the limit of quantification.

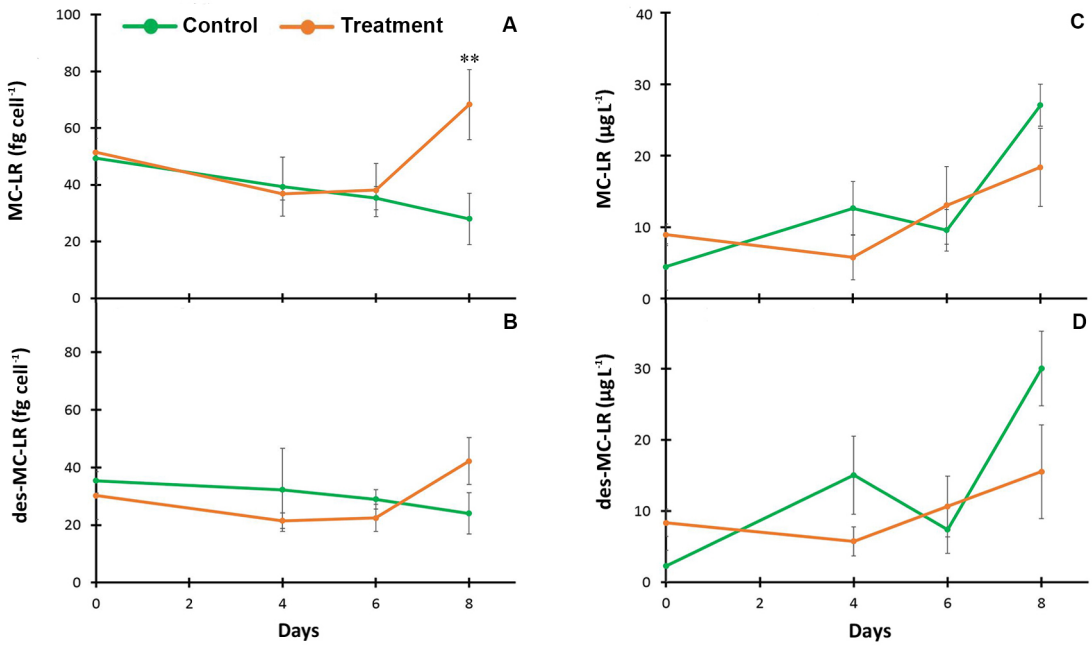


Figure 4. Dynamics of intracellular and extracellular metabolites in control and in the presence of *D. magna* in the cyanobacterial unit: **A** intracellular MC-LR, **B** intracellular des-MC-LR, **C** extracellular MC-LR, **D** extracellular des-MC-LR. * ($p < 0.05$), **($p < 0.01$), *** ($p < 0.001$) repeated-measures analysis of variance.

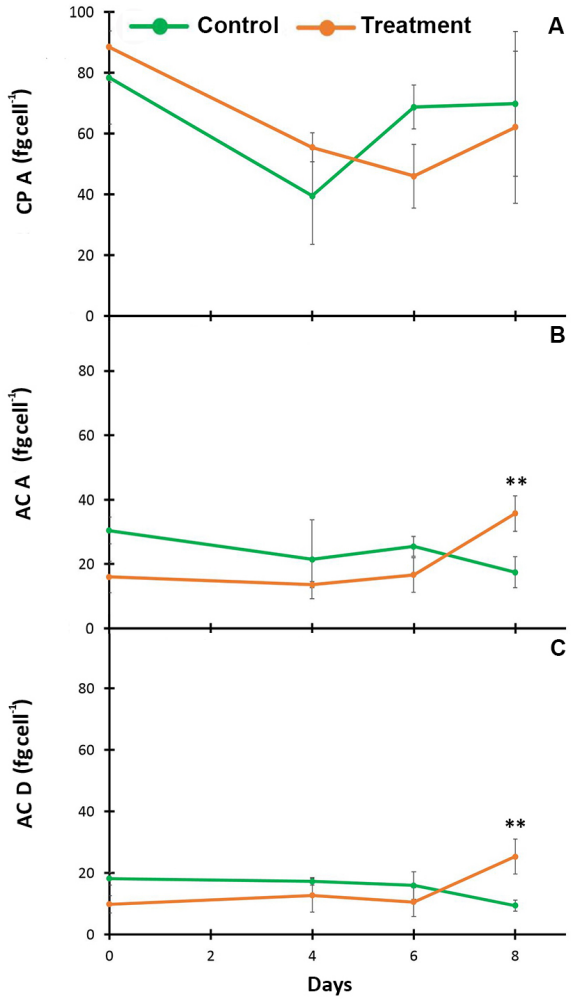


Figure 5. Dynamics of intracellular metabolites in control and in the presence of *D. magna* in cyanobacterial unit: **A** intracellular CP A, **B** intracellular AC A, **C** intracellular AC D. * ($p < 0.05$), *** ($p < 0.001$) repeated-measures analysis of variance.

3.3.2.2 *Daphnia* unit

MC-LR and des-MC-LR remained below the level of detection in the medium of the *D. magna* until day 4 (**Table 1.**). Both peaked at day 6 in the control but increased with time in the presence of *D. magna*. MC-LR was significantly higher at days 4 ($p < 0.05$) and 8 ($p < 0.001$) in the presence of *D. magna*, while des-MC-LR was significantly higher on the day 8 in treatment compared with the control in the *Daphnia* co-culture unit ($p < 0.001$) (**Table 1.**). Over time the extracellular concentration of MC-LR was higher on day 6 in the control, compared with day 4 ($p < 0.01$) and extracellular concentration of des-MC-LR was significantly lower on day 8, compared with day 6 ($p < 0.001$). Furthermore, in the treatment, extracellular concentration of MC-LR was significantly higher on day 8, compared with day

419 4 and day 6 ($p < 0.001$), while extracellular concentration of des-MC-LR was significantly
420 higher on day 6 and day 8, compared with day 4 ($p < 0.01$). CP A, AC A and AC D were
421 below the quantification limit in the *Daphnia* unit during the whole experiment.

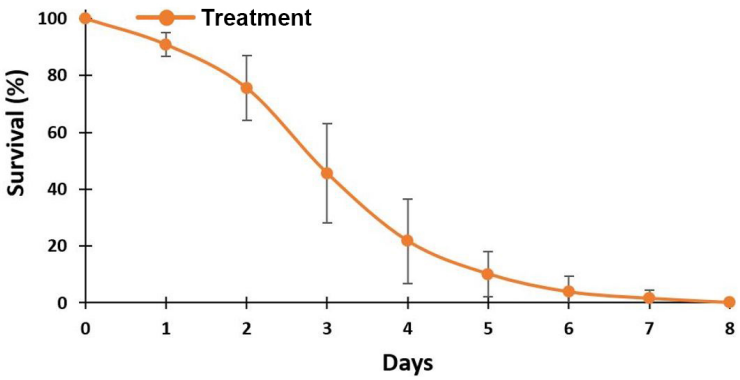
Table 1.
Dynamics of extracellular MC-LR and des-MC-LR in the *Daphnia* unit in the control and the treatment. * ($p < 0.05$), *** ($p < 0.001$) repeated-measures analysis of variance.

	Day	Control	Treatment
Extracellular MC-LR (µg L^{-1})	1	< LOD	< LOD
	4	0.95±0.13	3.21±0.81 *
	6	3.76±0.98	3.26±1.19
	8	2.80±1.01	6.30±1.79 ***
Extracellular des-MC-LR (µg L^{-1})	1	< LOD	< LOD
	4	< LOD	3.64±0.96
	6	4.48±1.01	5.56±0.72
	8	2.28±0.78	5.57±2.33***

422

423 **3.4 *Daphnia* survival**

424 On day 8 of the experiment, survival of adult *D. magna* in the co culture unit was 80 ±
425 3.65%. Survival of neonates that hatched in the co-culture chamber during exposure to
426 cyanobacterial metabolites and raised after termination of the experiment was drastically
427 impaired with mortality of 100% on day 8 (**Figure 6.**).



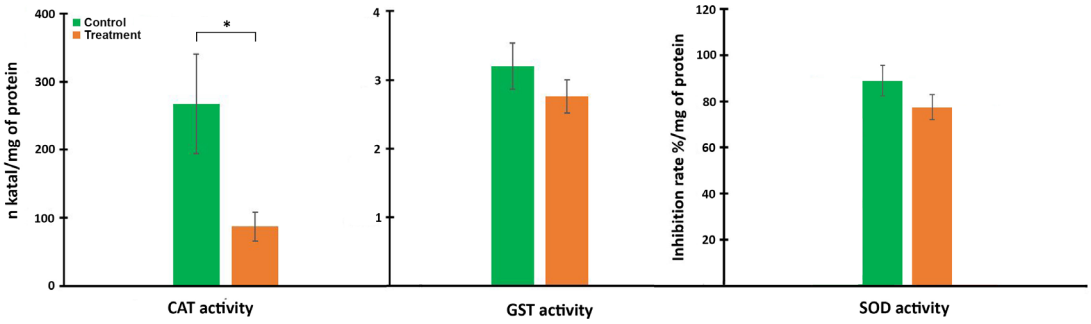
428

429 **Figure 6.** Neonates survival

430 **3.5 Oxidative stress**

431 GST and SOD activities in *D. magna* were not significantly different on day 8 between
432 control and treatment (**Figure 7.**). However, decreasing tendencies have been observed in
433 both of these enzyme activities in the presence of MC+. CAT activity was significantly lower

434 in the treatment, compared to the control on day 8 (**Figure 7.**). Concentration of H_2O_2 in
435 *Daphnia* cells was not statistically different between control and treatment on day 8 (data not
436 shown).



437

438 **Figure 7.** *D. magna* enzyme activities in control and in the presence of MC+ after 8 days of co-culture: GST
439 activity, CAT activity, SOD activity * ($p < 0.05$) t-test.

440 **3.6 Glycogen and protein contents**

441 A slight but not statistically significant decrease in glycogen and protein contents were
442 observed between control and treatment on day 8 (**Table 2.**).

Table 2.
Glycogen and protein content in *D.magna* in control and treatment ($p>0.05$) t-test.

	Days	Control	Treatment
Glycogen content (mg/mg DW)	8	0.136±0.042	0.098±0.032
Protein content (mg/ml)	8	0.451±0.041	0.402±0.093

443

444 **4. Discussion**

445 This is the first study reporting on the mutual metabolic interactions between a MC-
446 producing *M. aeruginosa* strain and *D. magna* mediated only via diffused metabolites, co-
447 culturing them without direct contact, over a relatively long exposure time of 8 days.

448 **4.1 Physiological changes in cyanobacteria**

449 Infochemicals from *Daphnia* are known to induce physiological changes in
450 cyanobacteria, but it is not known whether their production results from the presence of
451 cyanobacteria (possibly associated with the breaking down of cells while ingesting) or from
452 their natural metabolism. Indeed, most reported studies used *Daphnia* spent medium rather
453 than *Daphnia* individuals (Jang et al., 2007, 2008; van Gremberghe et al., 2009; Becker et

454 al., 2010; Sadler and von Elert, 2014a; Bojadzija Savic et al., 2019). In this study, toxic
455 cyanobacteria exposed to individuals of *D. magna*, did not suffer negative physiological
456 impact by *Daphnia* infochemicals, as the ETRmax values were above 80, reaching 100,
457 although the *Daphnia* density was high (starting with 150 L^{-1} 2-4 days old, non-egg bearing
458 *Daphnia* individuals). Whereas, comparing impact of *D. magna* densities and culturing time
459 we recently showed that spent medium obtained from the highest density (200 L^{-1}) cultivated
460 for the shortest time (24h) provoked the strongest negative effect on the photosynthetic
461 activity of the same strain *M. aeruginosa* PCC 7806 (Bojadzija Savic et al., 2019). Altogether,
462 these results suggest that *D. magna* medium effect on MC+ cells depends not only on
463 *Daphnia* density and time of the cultivation, but also on how *Daphnia* medium is introduced
464 to the cyanobacterial culture. Our results showed that MC+ acclimated better to *Daphnia*
465 medium when the infochemicals were gradually diffused via the membrane of the co-culture
466 chamber, despite their increase over time, in contrast to being directly introduced to the
467 cyanobacterial culture. Moreover, the present study was conducted with a higher density of
468 *Daphnia,* than the one impairing the cyanobacteria in the previous study.

469 Reported studies on physiological or metabolic interactions between cyanobacteria and
470 *Daphnia* using *Daphnia* individuals rather than medium are rare and involve very short
471 exposures. For example, cell densities of *M. aeruginosa* clone LE-3 (initial cell density $4x10^6$
472 cells mL^{-1}) were not significantly decreased when exposed to 120 *D. magna* individuals L^{-1}
473 and 200 *D. pulex* individuals L^{-1} for 24h in a similar co-culture chamber (Harke et al., 2017).
474 Exposure to spent medium from *D. magna* and *D. pulex* (300 *Daphnia* individuals cultivated
475 for 24h) had no impact on growth rate of *M. aeruginosa* ($5.8xl0^6$ cells mL^{-1}) (Yang and Li,
476 2007). Similar results were observed in 4 MC-producing strains (three strains of *M.*
477 *aeruginosa* and one strain of *P. agardhii,*) exposed in their exponential growth phase to 10%,
478 25%, and 50% v/v of *D. magna* medium (from 200 non egg-bearing adults) which showed
479 no significant change in biomass (Jang et al., 2007).

In contrast with these results, van Gremberghe et al. (2009) observed strain specific response when exposed to *Daphnia* spent medium, as biomass decreased in 4 out of 8 *Microcystis* strains isolated from two lakes. Our results are in line with these results, as growth on day 4 showed decreasing tendencies, together with a significant increase in ROS production demonstrating that the cyanobacterial strain suffered from stress, possibly caused by *Daphnia* infochemicals. Excess production of ROS is one of the first signs that phytoplankton is undergoing stress (Hirata et al., 2004; Diaz and Plummer, 2018). In their natural habitat cyanobacteria are exposed to constantly changing abiotic and biotic factors, therefore their ability to initiate rapid antioxidant defences in order to overcome ROS is crucial for their survival (Latifi et al., 2009). Allelochemicals produced by submerged macrophytes can cause oxidative damage in *M. aeruginosa* (Shao et al., 2009; Wang et al., 2011). This study adds to the knowledge that also *Daphnia* infochemicals cause oxidative stress in *M. aeruginosa* via ROS production. The decline tendencies in growth on day 4, might be due to the energy allocation either towards anti-oxidant stress enzymes, or towards other defense mechanisms against *Daphnia*. As the photosynthetic activity ETRmax was not affected by the presence of *Daphnia* and as the cyanobacterial strain recovered by day 6, we suggest that the cost may be transient as the cyanobacterial strain managed to deal with the stress and acclimate to *Daphnia* exposure.

4.2 MC production in response to presence of *D. magna* (as medium or individuals)

Concentration of intracellular MC-LR significantly increased after day 6 when exposed to *D. magna* infochemicals, suggesting that the presence of *Daphnia* even though not in direct contact, induced the production of MC-LR as a response of *Microcystis*. Similarly, in a Polish Reservoir, Izydorczyk et al. (2008) found a significantly positive corelation between increase of intracellular MC concentrations and density of *D. pulex and D. cucullata.* Additionally, increasing densities of *D. pulex* (100–500 individuals L^{-1}) provoked increasing concentrations of MC in *Microcystis* spp at different densities ($0.5 – 4.5 \times 10^6$ cells mL^{-1}),

suggesting an anti-grazer role (Pérez-Morales et al., 2015). Compared with juveniles and neonates, adult zooplankton the quantity or quality of infochemicals resulted in a greater increase in both MC production and release (Jang et al., 2007). Extracellular MC was significantly higher in the treatment in the *Daphnia* unit compared with the control, suggesting overall higher export of MC-LR in the presence of *Daphnia* infochemicals, gradually diffusing through the membrane. Our results are in line with these studies, suggesting a stress response role for MC, when *M. aeruginosa* is suffering from stress caused by *Daphnia* infochemicals or as grazing defense. Further research is needed to confirm this role and to generalize in terms of both cyanobacterial and *Daphnia* strains and species and the respective densities. The way *Daphnia* infochemicals are introduced in the cyanobacterial medium plays a role in the reaction of the cyanobacteria. When *M. aeruginosa* is exposed to *Daphnia* infochemicals by sudden introduction, it causes an intense stress: in a precedent experiment we observed that MC+ exposed to *D. magna* spent medium (200 all age individuals of *D. magna* L^{-1} cultivated for 24 h) significantly decreased intracellular MC-LR concentration, and general physiological state (Bojadzija Savic et al., 2019). Similarly, *Microcystis* stopped producing MC, and its growth was impaired when it was exposed to *Daphnia* medium (165 *D. magna* individuals L^{-1} grown in 24 h WC medium) (Becker et al., 2010). Apparently, *Daphnia* spent medium contains metabolites harmful to the cyanobacterial cells but a gradual introduction would allow them to acclimate. A general stress reaction observed during the gradual introduction was the formation of ROS, occurring on day 4 but reduced thereafter. This reduction might not be possible during a sudden introduction as Schuurmans *et al*. (2018) provided further clarification indicating that MC can inhibit antioxidant stress enzymes activity by binding to the same target proteins (Schuurmans et al., 2018).

Various roles of MCs have been suggested such as intracellular protection against oxidative stress (Dziallas and Grossart, 2011; Zilliges et al., 2011), role in photosynthesis

(Utkilen and Gjølme, 1992; Wiedner et al., 2003), as well as anti-grazing (Rohrlack et al., 1999; Jang et al., 2004, 2007; Sadler and von Elert, 2014a). Our study supports an anti-stress role of this cyanobacterial metabolite, however it could have more biological roles, as known so far and their clarification still need futher investigations.

4.3 Cyanobacterial production of secondary metabolites other than toxins in the presence of *Daphnia*

When exposed to *Daphnia* spent medium, elevated production of AC B and D, CP B, and microcyclamide 7806A have been observed in *M. aeruginosa,* while relative and total amounts of CP A and C were not affected (Sadler and von Elert, 2014a). Furthermore, active export into the surrounding medium of microcyclamide 7806A was elevated in the presence of *Daphnia* (Sadler and von Elert, 2014a). In contrast to a previous study, we evidenced elevated concentration of CP A in the presence of *D. magna* spent medium (Bojadzija Savic et al., 2019) supporting a potential defensive mechanism of this molecule. When indirectly exposed to *D. magna* and *D. pulex* for 24h, transcriptomic response of genes encoding CPs, microviridins and aeruginosins were not significantly different from the control in a co-culture chamber setup (Harke et al., 2017). In our study, intracellular concentrations of AC A and D significantly increased after 6 days, when *D. magna* infochemicals gradually diffused into the cyanobacterial side of the co-culture chamber. However, as the concentration of extracellular AC A, and AC D remained below the detection limit, we do not know if intracellular increase was followed by active export of these metabolites due to the *Daphnia* exposure. Bojadzija Savic et al. (2019) reported that the non-MC-producing mutant strain of *M. aeruginosa* delt better with stress caused by *D. magna* metabolites than the MC-producing wild type. Furthermore, the non-MC-producing mutant strain of *M. aeruginosa* was initialy producing almost twice the amount of the intracellular CP A and AC D (Bojadzija Savic et al., 2019). Also in monocultures higher concentrations of cyanopeptolins, aerucyclamides, and aeruginosins were produced by the non-MC-producing

mutant in comparison with the wild type (Briand et al., 2016). Initial higher amount of the secondary metabolites in non-toxic strains (Bojadzija Savic et al., 2019; Briand et al., 2016), might be involved in a yet unknown protective role of the cell, and increase of these metabolites in the MC-producing strain could be a stress response mechanism when exposed to stress induced by *D. magna* metabolites. However, unravelling the potential anti-grazing function of these compounds, as well as potential protective roles would require further research.

4.4 Cyanobacterial response to *Scenedesmus communis* used as food source for *Daphnia*

Through allelopathic interactions, *Scenedesmus* can negatively impact growth and photosynthetic activity of *M. aeruginosa* that eventualy lead to cell lysis (Jia et al., 2008; Kaplan et al., 2012; Bittencourt-Oliveira et al., 2014). However, a co-culture experimental setup, where *M. aeruginosa* (SAG 14.85) was cultivated with *S. obliquus* (SAG 276-3a) demonstrated minor effects of interspecific interference between both species, but no inhibition of *M. aeruginosa* (Dunker et al., 2013). In our experimental setup, *Scenedesmus* was used as a food for *Daphnia*, therefore, its metabolites could have been present in the *Daphnia* co-culture unit and eventually diffuse to the cyanobacterial co-culture unit. However, our results show that *Scenedesmus* metabolites did not have a negative effect on *M. aeruginosa* PCC7806, as they showed in the control (without *D. magna* but with *Scenedesmus*) constantly good photosynthetic activity, increasing biomass over time and no change in ROS production, which would have indicated stress. Therefore, it is highly unlikely that it had a significant impact on cyanobacteria physiology and metabolites production in

580 either the control or the treatment, as it was used as food source only and fastly consumed by
581 *Daphnia*.

582 **4.5 *Daphnia* response to toxic cyanobacteria**

583 In our study *D. magna* survival, enzyme activities and energetic profiles were affected
584 by *M.aeruginosa* PCC7806 metabolites gradually introduced through the membrane.
585 Concentration of MC-LR in the *Daphnia* unit between day for and the end of the study (3-6
586 μg L^{-1}) are high but environmentally relevant as concentrations of dissolved MC between 0.2
587 and 8.18 μg L^{-1} have been reported in aquatic environments (Lahti et al., 1997; Rastogi et al.,
588 2015; Su et al., 2015). Hence we assume that the concentration of the other metabolites would
589 be in the environmental relevant range too, as we could not quantify them. Survival of adult
590 *D. magna* (80%) in our study was in agreement with previous studies in which *D. magna* was
591 exposed to similar MC-LR concentrations 3.5–5 μg MC-LR that mildly affected its survival
592 (Lürling and van der Grinten, 2003) and 5 μg MC-LR decreasing 10% of adult *D. magna*
593 within their lifetime (Dao et al., 2010). Usually, non-treated adults show high survival (close
594 to 100%) in the first 8 days of experiments (Dao et al. 2010, Ortiz-Rodríguez et al. 2012,
595 Pérez-Fuentetaja and Goodberry, 2016), suggesting that the higher mortality in our study is
596 apparently due to the presence of other cyanobacterial metabolites. Although below the level
597 of quantification, we detected cyanopeptolines and aerucyclamides in the medium in
598 *Daphnia* co-culture unit that could together with MC-LR impact *Daphnia* life traits.
599 Cyanopeptolines are protease inhibitors of the serine proteases trypsin and chymotrypsin, the
600 main digestion enzymes (Gademann and Portmann, 2008), having detrimental effect on
601 *Daphnia* and other zooplankton species due to interference with their nutrition uptake

602 (Agrawal et al., 2005; Schwarzenberger et al., 2012; von Elert et al., 2012). Furthermore,
603 aerucyclamides are a class of cyanobacterial peptides having cytotoxic effect (Ishida et al.,
604 2000), detrimental for crustaceans (Portmann et al., 2008). The kinetics of these metabolites
605 and their impact alone and in mixture on zooplankton however, is still an area of research.
606 The combination of these impacts, however, was mortal for the juveniles during their first
607 week, where intensive nutrient uptake and cell division is needed for growth, besides the
608 toxic impacs of cyanobacterial metabolites. Usual survival rate of non treated neonates in the
609 first week is around 90 to 100% (Yang et al. 2012, Dao et al. 2014). However in our study,
610 neonates survival completely declined after 8 days (100%) when grown in the beakers filled
611 with the medium from co-culture chambers collected on the last day of the experiment. These
612 results suggest either a high sensitive to metabolites from the media, or that they suffered
613 already damages during their embryonic development as they are not sufficiently protected
614 under the mothers' carapax. Juveniles from exposed mothers are evidently more susceptible,
615 they suffered still higher mortality, even when raised in toxin-free medium (Dao et al., 2010).
616 Moreover, Akbar et al. (2017) showed the dependence of offspring survival on the mothers
617 treatment with different conditioned cyanobacteria: if cyanobacteria were pre-stimulated by
618 the presence of zooplankton, their harmful effect increased. This could also apply for the
619 cyanobacteria in the co-culture-chamber exposure of this study.
620 Cyanobacterial secondary metabolites can promote oxidative stress and reactive
621 oxidative species production in aquatic species including zooplankton (Zanchett and
622 Oliveira-Filho, 2013). If not detoxified, increased ROS can lead to protein, lipid and DNA
623 damage eventually leading to cell apoptosis (Amado and Monserrat, 2010). Elevated CAT
624 activity was recorded in neonates and in adult *Daphnia,* providing protection against

625 hydrogen peroxide, one of the ROS generated during oxidative stress (Ortiz-Rodríguez and
626 Wiegand, 2010). *Daphnia* spp. from a cyanobacterial dominated lake showed increased
627 levels of CAT activity compared to *Daphnia* spp. from a lake where cyanobacterial
628 occurrence was significantly lower (Wojtal-Frankiewicz et al., 2013). These results suggest
629 stronger antioxidant capabilities in *Daphnia* spp. populations with longer history of exposure
630 to cyanobacteria (Wojtal-Frankiewicz et al., 2013).

631 *Daphnia* are able to reduce toxic MC effect by biotransforming MC to more water-
632 soluble MC glutathione conjugate via enzymes of glutathione S-transferases (sGST)
633 (Pflugmacher et al., 1998; Meissner et al., 2013). The biotransformation enzyme sGST
634 increased in both *D. magna* adults and neonates when exposed to MC-LR (Ortiz-Rodríguez
635 and Wiegand, 2010). Furthermore, when the parental generation was exposed to MC, the next
636 generation of *D. magna* showed increased activity of GST, CAT and MDH, suggesting
637 maternal transfer of activation factors (Ortiz-Rodríguez et al., 2012). Therefore, through
638 increased detoxication and oxidative stress enzyme activities, *D. magna* are able to enhance
639 adaptation to cyanobacterial metabolites, increasing chances of survival of the next
640 generation (Ortiz-Rodríguez et al., 2012, Wojtal-Frankiewicz et al. 2013, 2014). However,
641 our results showed significant CAT activity decrease on day 8, as well as decreasing
642 tendencies of SOD and GST enzyme activities in the treatment. It is probable, that without
643 previous acclimation, these enzymes and other processes in the cells became exhausted by
644 the constant presence of microcystins and other cyanobacterial compounds. Similar
645 exhaustion of GST enzymes and CAT occurred after exposure of *D. magna* to extracts from
646 non-MC and non-CYN producing cyanobacteria for 3 days (Dao et al., 2013). Decreasing
647 CAT activities could be related to the *Daphnia* development, as a similar age-related pattern
648 have been observed in other studies, where juveniles delt better than adults with oxidative
649 stress concerning CAT activity (Barata et al., 2005; Ortiz-Rodríguez and Wiegand, 2010;

650 Alberto et al., 2011). A similar decrease related with the age of *Daphnia* was observed for
651 SOD (Alberto et al., 2011).

652 *Daphnia* as other organisms allocate energy reserved for growth, development and
653 reproduction to detoxification and repairing processes, when grown in stress promoting
654 conditions (McKee and Knowles, 1986; Calow, 1991; Pane et al., 2004). Due to this energy
655 allocation, *D. magna* dry mass was reduced after 7 days exposure to MC-LR (50 μg L^{-1})
656 (Ortiz-Rodríguez et al., 2012). Furthermore, when exposed to toxic environments containing
657 nickel (Pane et al., 2004) or the fungicide tebuconazole (McKee and Knowles, 1986), total
658 carbohydrates and glycogen content in *D. magna* decreased, suggesting energetic cost
659 allocation to handle the toxic stress. Our results showed, although not significant, decreasing
660 tendencies of the energetic budget in the treatment compared to the control. However, since
661 our results did not show increase in CAT, GST and SOD, energy allocation was possibly
662 towards other mechanisms involved in stress response and mending of cellular damages that
663 were not investigated in this study.

664 Allelopathic interactions between cyanobacteria and zooplankton in natural
665 environments are dynamic, due to the diversity of potential cyanobacterial defense traits and
666 the factors controlling them, along with zooplankton tolerance development and acclimation
667 to cyanobacterial metabolites (Ger et al. 2016). Furthermore, co-existence with other aquatic
668 organisms and microbial communities makes it challenging to analyze only interactions
669 between *Daphnia* and cyanobacteria. Our approach provides a step forward in disentangling
670 complex mutual interactions between these organisms, separated by a membrane in a co-
671 culture chamber. Our results suggest that *Daphnia* infochemicals alone could modulate the
672 dynamics of cyanobacterial metabolites, in the lab, so it may happen also in natural aquatic
673 environments. Indeed, an increase of MCs content in *Microcystis* in the presence of *Daphnia*
674 has been recorded for example in the Sulejow Reservoir (Poland) (Izydorczyk et al. 2008),

where in turn also the detoxification in *Daphnia* spp. correlated with longer exposure history to cyanobacteria (Wojtal-Frankiewicz et al. 2014).

5. Conclusions

This study demonstrated two-way physiological and metabolic responses of a toxic *Microcystis* strain and *D. magna* exposed to the presence of each other without direct contact. These results confirm a cross talk between the organisms only through diffusing metabolites. Daphnia life traits, oxidative stress and energy allocation were affected by the toxic cyanobacteria. Simultaneously, *Microcystis* showed an antioxidative stress response to *Daphnia* infochemicals and increased the production of several secondary metabolites suggesting either a protective or antigrazer response.

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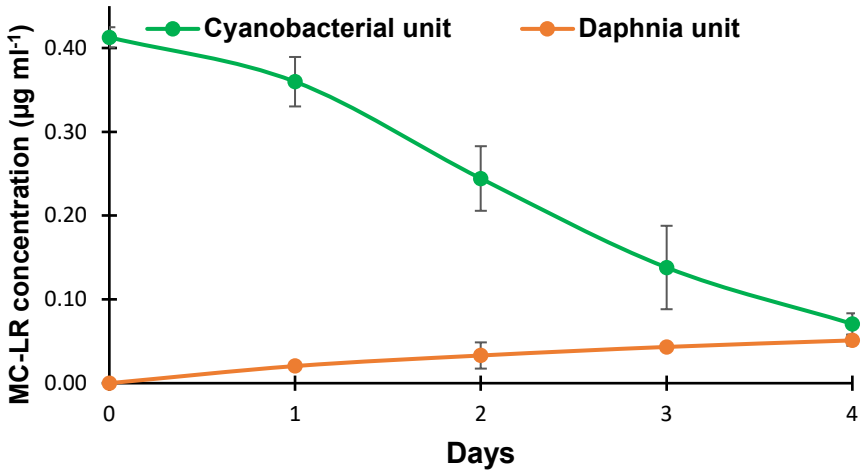
1 **Supplementary info**



2

3 **Figure S1.** Diffusion of MC-LR in the co-culture chamber

4

Table S1.
Detected secondary metabolites produced by *M.aeruginosa* PCC7806 in the co-culture chamber in the control and the treatment

	Cyanobacterial unit								***Daphnia* unit**			
	Intracellular				**Extracellular**				**Extracellular**			
Days	0	4	6	8	0	4	6	8	0	4	6	8
MC-LR	+	+	+	+	+	+	+	+	< LOD	+	+	+
Des-MC-LR	+	+	+	+	+	+	+	+	< LOD	+	+	+
CP 963A	+	+	+	+	+	+	+	+	< LOD	+	+	+
CP A	+	+	+	+	+	+	+	+	< LOD	+	+	+
CP B	+	+	+	+	+	+	+	+	< LOD	< LOD	< LOD	+
AC A	+	+	+	+	+	+	+	+	< LOD	+	+	+
AC B	+	+	+	+	+	+	+	+	< LOD	+	+	+
AC C	+	+	+	+	+	+	+	+	< LOD	+	+	+
AC D	+	+	+	+	+	+	+	+	< LOD	< LOD	< LOD	< LOD
Aeruginosin 684	+	+	+	+	+	+	+	+	< LOD	+	+	+
Aeruginosin 602	+	+	+	+	+	+	+	+	< LOD	+	+	+

5