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1 **Cross talk: two way allelopathic interactions between toxic** 2 ***Microcystis* and *Daphnia***

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10

11 **Abstract**

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

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

36 **Keywords:** zooplankton, cyanobacteria, secondary metabolites, PCC7806, toxic, oxidative
37 stress

38 **Abbreviations**

39	MC+	<i>M. aeruginosa</i> PCC7806
40	MC-LR	microcystin LR
41	des-MC-LR	des microcystin LR
42	CP	cyanopeptolin
43	AC	aerucyclamide
44	CP A	cyanopeptolin A
45	AC A	aerucyclamide A
46	AC D	aerucyclamide D
47	CAT	catalase

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

52 **1. Introduction**

53 Many aquatic ecosystems experience cyanobacterial blooms as a consequence of
54 eutrophication, and global warming (O’Neil et al., 2012; Paerl and Otten, 2013). High
55 abundance of cyanobacteria leads to elevated concentrations of diverse cyanobacterial
56 secondary metabolites some of which pose health hazards to humans, but also to aquatic
57 organisms, such as zooplankton (Li et al., 2010; Holland and Kinnear, 2013). As filter feeders
58 some zooplankton species, including *Daphnia* sp. graze on phytoplankton, including
59 cyanobacteria, reducing cyanobacterial population in aquatic environments up to certain
60 densities (Sarnelle, 2007; Chislock et al., 2013; Ekvall et al., 2014). In addition to producing
61 harmful secondary metabolites, cyanobacteria are a nutritionally inadequate food source, as
62 they lack sterols and essential fatty acids necessary for *Daphnia* growth, development and
63 reproduction (Lynch et al., 1986; Müller-Navarra et al., 2000; Martin-Creuzburg et al., 2008).
64 The cyanobacterium *Microcystis aeruginosa* is commonly distributed worldwide, and known
65 to produce microcystin, one of the most investigated cyanobacterial toxins (O’Neil et al.,
66 2012; Svirčev et al., 2019). Microcystin toxicity is mediated through its ability to inhibit
67 protein phosphatases 1 and 2A (MacKintosh et al., 1990), causing cytoskeletal derangements
68 as well as promotion of oxidative stress (Amado and Monserrat, 2010), which together may
69 eventually lead to cell death (Carmichael, 1992; Zaccaroni and Scaravelli, 2008). Besides
70 microcystins, that can have negative effect on *Daphnia* life traits, *M. aeruginosa* is known to
71 produce other bioactive metabolites, such as cyanopeptolins, aerucyclamides and
72 aeruginosins, known as protease inhibitors, affecting the digestion in *Daphnia* (Czarnecki et

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

75 Unraveling the natural role of microcystin and its dynamics for the cyanobacteria has
76 been a topic of many studies with the emphasis on the influence of abiotic factors (such as
77 light, nutrients, or temperature) (Gobler et al., 2007; Jähnichen et al., 2007; Schatz et al.,
78 2007; Alexova et al., 2016). More recent studies focused on changes in the production of
79 cyanobacterial metabolites mediated by biotic factors, such as the presence of grazers (Jang
80 et al., 2003, 2004; van Gremberghe et al., 2009; Sadler and von Elert, 2014a; Harke et al.,
81 2017; Bojadzija Savic et al., 2019). These studies suggest that the presence of *Daphnia* can
82 trigger the production of cyanobacterial secondary metabolites via the substances they release
83 in the water (infochemicals), which could be an antigrazing response. Considering that the
84 cyanobacteria and their genes responsible for toxin production are much older than complex
85 organisms, such as their grazers, it has been suggested that an antigrazing role could have
86 been obtained and kept over time due to grazing pressure (Wilken et al., 2010; Chislock et
87 al., 2013). Increase of microcystin concentration in the presence of *Daphnia* infochemicals
88 has been recorded in several studies (Jang et al., 2003, 2007; Izydorczyk et al., 2008; Kaplan
89 et al., 2012; Pérez-Morales et al., 2015), as well as the higher export of microcyclamide
90 7806A (Sadler and von Elert, 2014a), and cyanopeptolin A (Bojadzija Savic et al., 2019)
91 supporting this hypothesis. Cyanobacterial response seems to be strain dependant, as 4 out of
92 8 strains increased the production of microcystin in the presence of *Daphnia* medium (van
93 Gremberghe et al., 2009). On the other hand, the presence of *Daphnia* medium can also
94 decrease microcystin concentration (Becker et al., 2010; Bojadzija Savic et al., 2019), as well
95 as cyanopeptolin and aerucyclamide (Bojadzija Savic et al., 2019), leaving this role arguable.

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

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

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

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

122 We hypothesize that:

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

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

127 **2. Materials and methods**

128 **2.1 Culture conditions**

129 **2.1.1 *Microcystis aeruginosa* PCC 7806**

130 An axenic strain of microcystin (MC) producing *M. aeruginosa* PCC 7806 (MC+) was
131 obtained from the Pasteur Culture collection of Cyanobacteria in Paris
132 (<https://research.pasteur.fr/en/team/collection-of-cyanobacteria/>). The strain was grown in
133 50% cyanobacterial BG11 medium (SIGMA), diluted with sterile 50% reverse osmosis
134 water, under a 14h:10h light:dark regime using daylight white fluorescent tubes (Toshiba, 15
135 W, FL15D) with 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ illumination at a constant temperature of $20 \pm 1^\circ\text{C}$
136 (Sanyo incubator). The culture was maintained in exponential growth phase, while the
137 axenicity was regularly evaluated as described in Briand et al. (2012).

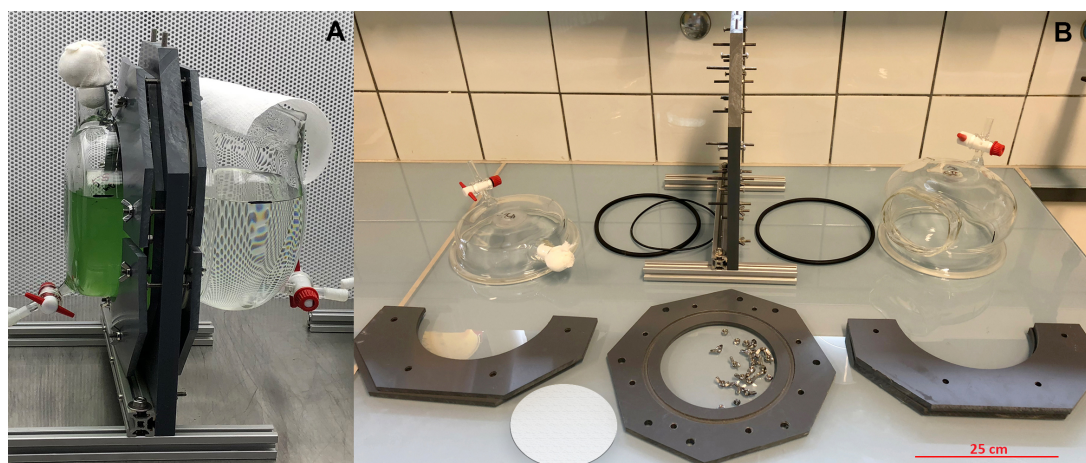
138 **2.1.2 *Daphnia magna***

139 The *D. magna* clone (originating from INERIS) was obtained from the PEARL INRA
140 1036 U3E (Rennes, France). Before the start of the experiments, *D. magna* were
141 progressively acclimatized during three weeks to cyanobacterial BG11 medium (SIGMA)
142 diluted to 50% with reverse osmosis water. Furthermore, to make sure that osmolarity would
143 not affect *Daphnia* life traits, we compared osmolarity of BG11 medium with the osmolarity
144 of commonly used Artificial *Daphnia* medium, Elendt M4 and Elendt M7 before performing
145 the experiments. Osmolarity of BG medium was similar to osmolarity of commonly used
146 *Daphnia* media, thus did not affect *Daphnia* survival. *D. magna* were fed daily with the green

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

151 2.2 Exposure set up

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



155
156 **Figure 1. A** The co-culture chamber during the exposure with the cyanobacterial culture at the left and the *D.*
157 *magna* on the right side; **B** The disassembled device with all of the parts required for its setup.

158 The disassembled co-culture chamber (**Figure 1. B**) was comprised of two glass
159 compartments separated by a 0.2 μm cellulose nitrate membrane filter (142 mm diameter,
160 Whatman, Buckinghamshire, UK). The glass material was preferred over plastic for easier
161 autoclaving and to avoid the release of plastic compounds. The cyanobacterial co-culture unit
162 (0.6 L volume **Fig 1. A** left side, hereafter named cyanobacterial unit) had a 20 mm opening
163 at the top, while the *Daphnia* co-culture unit (2L volume **Fig 1. A**) right side, hereafter named
164 *Daphnia* unit) had an opening 10 x10 cm. The openings allowed oxygen exchange, as well

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

172 **2.3 Diffusion pre-test**

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

182 **2.4 Experimental design of the monoculture controls**

183 **2.4.1 *M. aeruginosa* PCC7806**

184 Exponentially growing MC+ was centrifuged and cells were transferred to sterile 50%
185 BG11 medium until cell density reached 5×10^5 cells mL⁻¹. Inoculating in fresh medium
186 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 9.5×10^5 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 4×10^7 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* (4×10^7 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* (4×10^7 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 4×10^7 cells L⁻¹. During the experiment the *Daphnia* unit
207 was covered with paper cloth that reduced light intensity (from 20 to 15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$
208 ¹), while light intensity remained at 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the cyanobacterial unit.

209 Cyanobacterial photosynthetic activity, cell density, reactive oxidative species (ROS)
210 and metabolite dynamics were monitored at days 0, 4, 6 and 8. *Daphnia* were sampled on the
211 last day of the experiments for analysis of the enzymes (Superoxide dismutase (SOD),
212 Glutathione S-transferase (GST), Catalase (CAT), glycogen, lipids and hydrogen peroxide,
213 H₂O₂. Neonates that were born during the exposure were collected on the last day of the
214 experiment and kept in the medium obtained from the *Daphnia* unit, where their survival was
215 observed over 7 days. After removing of excess water, adult *Daphnia* samples were snap
216 frozen in liquid nitrogen and stored at -80°C. All experiments were performed in four
217 replicates with a duration of 8 days.

218 **2.6 Photosynthetic activity**

219 Photosynthetic activity is often measured through maximum electron transport rate
220 (ETR_{max}), an indicator of the algal physiological state, and precursor of growth (Briand et
221 al., 2012; Pannard et al., 2016). ETR_{max} values reaching 100 and above for the strain PCC
222 7806 are an indicator of good physiological state, while values below 30 are associated with
223 physiological stress from which cyanobacteria cannot recover (Briand et al., 2012, 2016).
224 The electron transport rate (ETR) was measured on days 0, 4, 6 and 8 with a pulse-amplitude-
225 modulated fluorescence monitoring system (PhytoPAM, Walz, Germany) (Schreiber et al.,
226 1998; Pannard et al., 2016). PhytoPam settings used in our experiments were as described in
227 Bojadzija Savic et al. (2019).

228 **2.7 Cyanobacterial cell density**

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

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

236 **2.8 ROS production in cyanobacteria**

237 Cyanobacterial oxidative stress was measured via total ROS production (Rajneesh *et al.*
238 2017). H₂DCFDA (2', 7'-dichlorodihydrofluorescein diacetate) is a non-fluorescent reagent
239 that is cleaved by intracellular esterases forming H₂DCF (dichlorodihydro-fluorescein).
240 H₂DCF reacts with intracellular ROS to form a fluorescent compound, DCF
241 (dichlorofluorescein). 5x10⁶ cells were centrifuged and the pellets resuspended in 996 µL
242 NaPO₄ buffer 0.1M pH 6.5 and 4 µL of the H₂DCFDA diluted with ethanol (1:8). Samples
243 were incubated for one hour in the dark and fluorescence of DCF was measured
244 spectrophotometrically at 485 nm.

245 **2.9 Cyanobacterial secondary metabolites**

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

256 MC-LR, des-MC-LR, cyanopeptolin A, and aerucyclamide A and D were quantified in
257 this experiment using linear relationship between peak area (MC-LR and des-MC-LR at 238
258 nm, cyanopeptolin A (CP A) at 220 nm, and aerucyclamide A (AC A) at 237 nm and D (AC
259 D) at 240 nm) and known concentrations of the toxin standards. MassLynx v4.1 was used for
260 both detection and quantification of the cyanobacterial peptides. The microcystin-LR
261 standard was purified as previously described (Edwards et al., 1996). CP A and AC A and D
262 standard were purified using preparative HPLC (high performance liquid chromatography)
263 (Biotage Parallelex Flex, Cardiff, UK) and Flex V3 software for instrument control and data
264 acquisition. The separation was performed on Atlantis Prep C18 column (5 μ m particle size,
265 19 mm ID \times 300 mm long; Waters, Elstree, UK) using a 30-min linear gradient from 60% to
266 100% methanol in MilliQ water. The flow rate was 20 mL/min and 4 mL fractions were
267 collected (Bojadzija Savic et al., 2019).

268 **2.10 *D. magna* survival**

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

277 **2.11 Enzyme extraction and measurement**

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

282 dithioerythritol) using Lysing Beads-Matrix E (MPbio) in the Vibro-mill MM200,
283 (RETSCH) for 3 minutes at the frequency of 25 Hz to break the cells. The homogenates were
284 then centrifuged at 10,000 g for 10 minutes at 4°C (Sigma 3K18C). Supernatant was
285 separated from pellets and used in enzyme measurements. Catalase (CAT) activity was
286 assayed by measuring the rate of disappearance of H₂O₂ at 240 nm (Chang and Kao, 1997).
287 GST was assayed at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Habig
288 et al., 1974). Superoxide dismutase (SOD) activity was determined using a photochemical
289 assay based on the reduction of nitro blue tetrazolium (NBT) according to Total Superoxide
290 Dismutase (T-SOD) assay kit (Hydroxylamine method, SIGMA KIT). All enzyme activities
291 were related to protein content in the extract, measured according to Bradford (1976).

292 **2.12 H₂O₂ measurement**

293 In order to measure hydrogen peroxide concentration in *Daphnia*, they were resuspended
294 and homogenized in 600 µL of 0.1 M phosphate buffer pH 6.5 using Lysing Beads-Matrix
295 E, MPbio. Samples were homogenized for 3 minutes at the frequency of 25 Hz (bead-beater
296 Vibro-mill MM200, RETSCH) followed by centrifugation at 15,000 g for 20 minutes at 4°C
297 (Sigma 3K18C). The supernatant was transferred to a microtube and stored in ice for protein
298 determination (Bradford, 1976) and quantification of H₂O₂. Concentration of H₂O₂ was
299 measured according to Sasadhar and Monojit (1981) at 410 nm.

300 **2.13 Energetic resources**

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

308 water. Phases were separated by centrifugation at 4°C at 180xg for 15 minutes and kept at -
309 20°C over night. The following day, 300 µl of the chloroform phase was transferred in a new
310 tube and used for triglyceride measurements. The pellet between the two phases was used for
311 measuring glycogen content according to Foray et al. (2012). Triglycerides were measured
312 after evaporation of the chloroform using a triglycerides colorimetric assay (Triglycerides kit
313 reference CC02200, LTA srl, Italy), however, triglycerides in our samples were below the
314 level of detection.

315 **2.14 Statistical analyses**

316 R Core Team (2013) was used to access statistical analysis of the obtained data. All data
317 are presented as mean ± standard deviation. Significant differences were determined at
318 $p < 0.05$. We performed repeated-measures analysis of variance to determine the difference
319 between cell density, photosynthetic activity, ROS and concentration of intracellular and
320 extracellular metabolites between the control and treatment for MC+. Repeated-measures
321 analysis of variance is a mixed linear model with day, treatment (Control vs Treatment) and
322 interaction between day and treatment considering the repeated measures on replicates
323 (random effect). Normality of residuals was tested via Shapiro test (residuals normally
324 distributed when $p > 0.05$). Anova was performed to test the effects of the model. Pairwise
325 comparison with correction for multiple comparison was performed to check significant
326 differences between control and the treatment and if there was time dependency effect on
327 control and treatment (* ($p < 0.05$), **($p < 0.01$), *** ($p < 0.001$)).

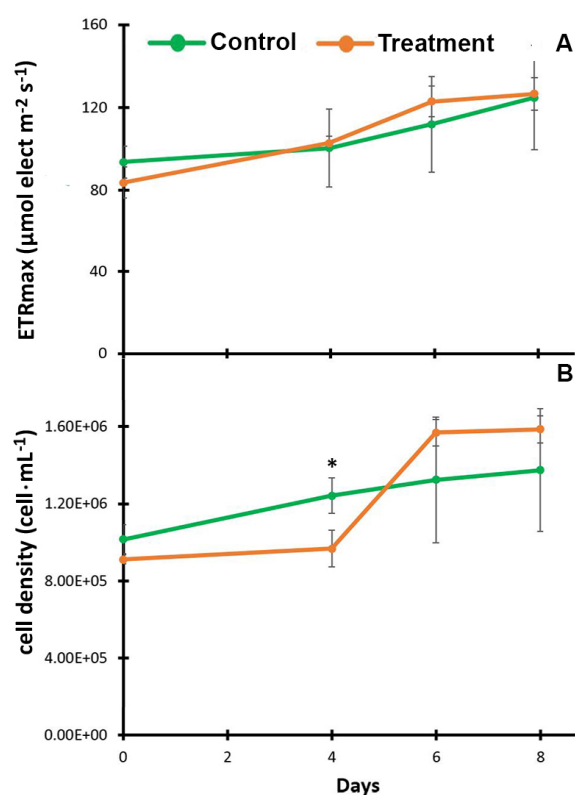
328 T-test was used to compare *Daphnia* enzyme activity and energetic resources between
329 control and treatment.

330 **3. Results**

331 **3.1 Cyanobacterial growth and photosynthetic activity**

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

334 treatment ($p > 0.05$) (**Figure 2. A**). Whereas it didn't change over time in the control ($p >$
 335 0.05), a time effect was observed for the treatment where photosynthetic activities were
 336 superior on day 6 ($p < 0.05$) and day 8 ($p < 0.01$) to the photosynthetic activity on day 0.
 337 Cyanobacterial cell density was not significantly different between control and treatment (p
 338 > 0.05). However, an effect of time was observed in the control, where cyanobacterial cell
 339 density on day 8 was higher compared with day 0 ($p < 0.05$). Furthermore, a time effect was
 340 observed in the treatment, where cyanobacterial biomass was significantly higher on day 6
 341 and day 8 compared with biomass on day 0 ($p < 0.001$), and day 4 ($p < 0.01$) (**Figure 2. B**).



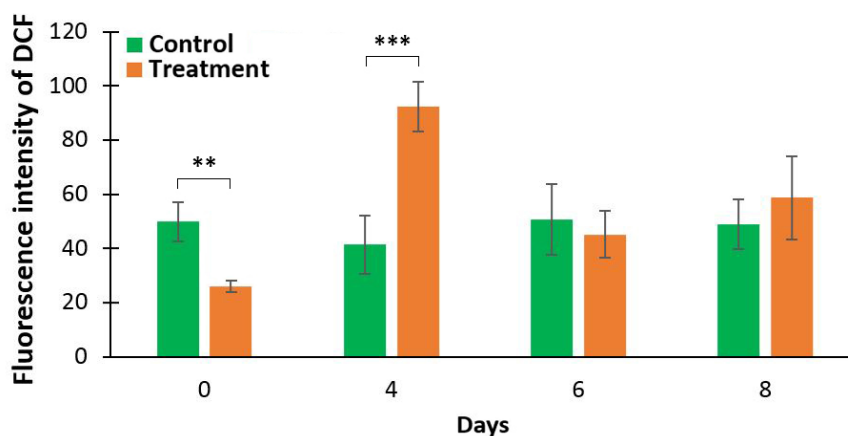
342

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

346 3.1 ROS in cyanobacteria

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

351 throughout the experiment ($p > 0.05$), while in the treatment, ROS content was
 352 significantly higher on day 4, day 6 and day 8 than ROS content observed on day 0 ($p <$
 353 0.001). Furthermore, ROS content in the treatment was significantly lower on day 6 and day
 354 8, compared with day 4 ($p < 0.001$).



355

356 **Figure 3.** ROS content in MC+ in control and in the presence of *D. magna* ** ($p < 0.01$), *** ($p <$
 357 0.001) repeated-measures analysis of variance.

358 3.3 Cyanobacterial metabolites

359 3.3.1 Detected cyanobacterial metabolites in control and treatment

360 In this study, eleven metabolites were detected: MC-LR, des-MC-LR, cyanopeptoline
 361 (953A, A, B, C), aerucyclamide (A, B, C, D), aeruginosin 684 and 602 (**Table S1.**). In the
 362 cyanobacterial co-culture unit, all these metabolites were present both intracellularly and
 363 extracellularly throughout the experiment. In the *Daphnia* co-culture unit, all cyanobacterial
 364 metabolites remained below detection limit until day 4 (**Table S1.**). Nine metabolites were
 365 detected from day 4 onwards: MC-LR, des-MC-LR, cyanopeptoline (CP 953A, A, C),
 366 aerucyclamide (AC A, B, C), aeruginosin 684 and 602. CP B was not detected until the day
 367 8, while AC D remained below the detection limit in the *Daphnia* co-culture unit during the
 368 whole experiment. The same metabolites were observed in the control and the treatment at
 369 the corresponding time points (**Table S1.**).

370 3.3.2 Quantified cyanobacterial metabolites

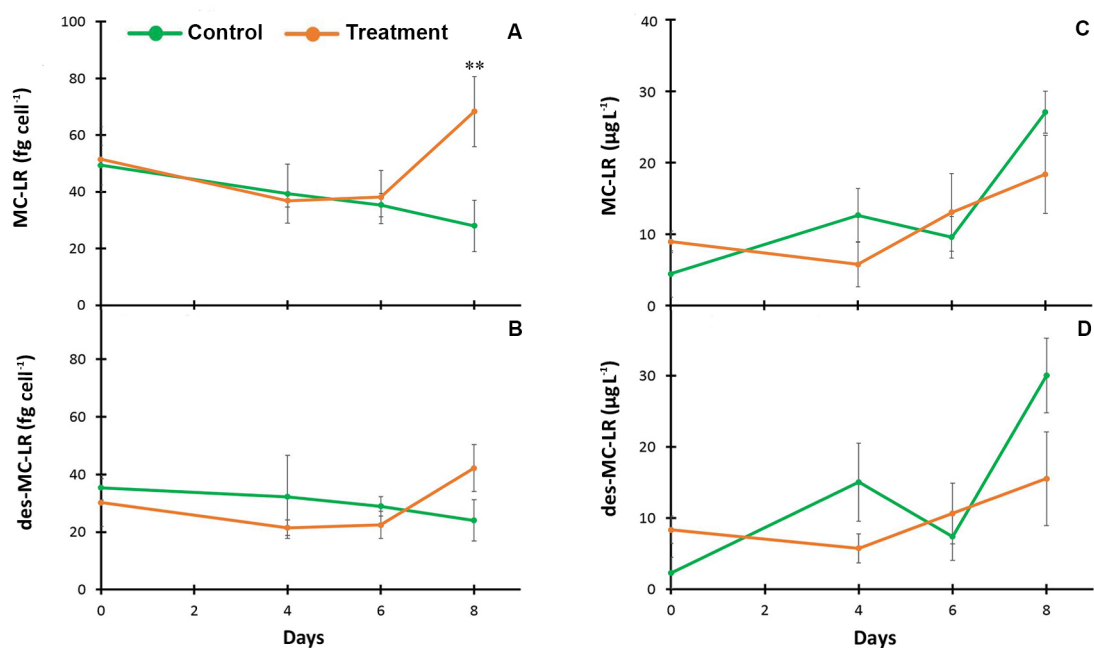
371 3.3.2.1 Cyanobacterial unit

372 Intracellular concentration of MC-LR and des-MC-LR (fg cell^{-1}) in MC+ remained
373 constant throughout the experiment in the control (**Figure 4A**). In the presence of *D. magna*,
374 the intracellular concentration of both was not significantly different from the control during
375 the first 6 days of the experiment but increased significantly on day 8 ($p < 0.001$, respectively
376 $p < 0.05$), also in comparison with day 4 and day 6 ($p < 0.001$, respectively $p < 0.01$) (**Figure**
377 **4A**). A similar trend was observed for AC A ($p < 0.001$) and AC D ($p < 0.001$), where
378 intracellular concentration in the presence of *D. magna* increased only on the last day of the
379 experiment in MC+ (**Figure 5**). Concentration of intracellular AC A started significantly
380 higher in the control compared with the treatment ($p < 0.05$) (**Figure 5B**). Furthermore, in
381 the control, intracellular concentration of AC A and AC D were significantly lower on day 8,
382 compared with day 0, whereas they significantly increased in the treatment on day 8,
383 compared with day 0, day 4 and day 6 ($p < 0.001$). Intracellular CP A was the only metabolite
384 showing no significant difference between control and treatment, ($p > 0.05$) (**Figure 5A**). In
385 both, the control and treatment, it even decreased, significant for the control on day 4 ($p <$
386 0.01), compared with day 0, before it increased again.

387 Extracellular MC-LR was significantly higher in the control compared to the treatment
388 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
389 8 ($p < 0.001$) (**Figure 4C, D**). Extracellular concentration of MC-LR and des-MC-LR
390 significantly changed over time in the control: MC-LR: significant decrease on day 6,
391 compared with day 4 ($p < 0.01$); des-MC-LR: significant increase on day 6 and day 4,
392 compared with day 0 ($p < 0.001$) and significant increase on day 8, compared with day 4 (p
393 < 0.001).

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

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



399

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

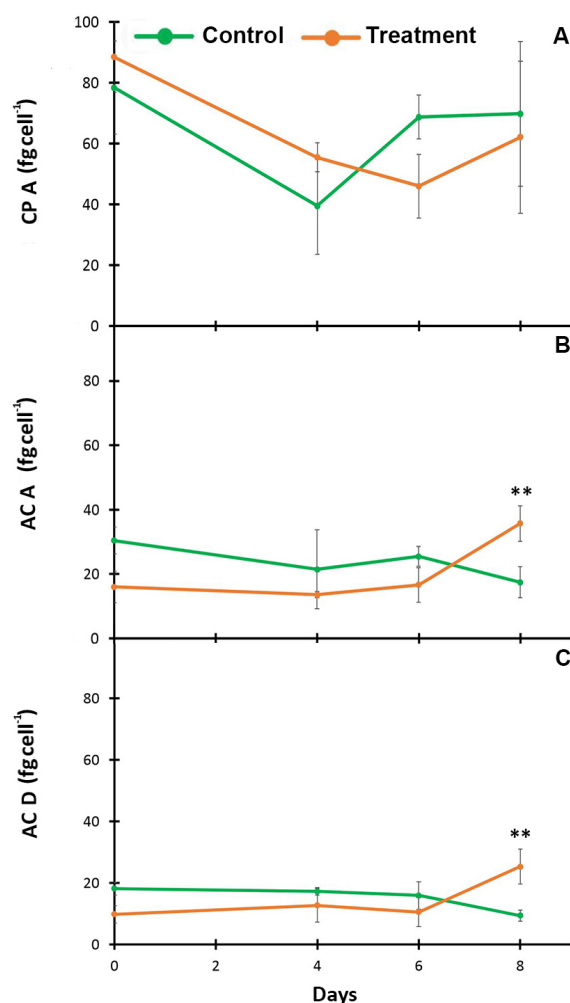


Figure 5. Dynamics of intracellular metabolites in control and in the presence of *D. magna* in cyanobacterial unit: **A** intracellular CP A, **B** intracellular AC A, **C** intracellular AC D. * ($p < 0.05$), *** ($p < 0.001$) repeated-measures analysis of variance.

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405
406
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3.3.2.2 *Daphnia* unit

410 MC-LR and des-MC-LR remained below the level of detection in the medium of the *D.*
411 *magna* until day 4 (**Table 1**). Both peaked at day 6 in the control but increased with time in
412 the presence of *D. magna*. MC-LR was significantly higher at days 4 ($p < 0.05$) and 8 ($p <$
413 0.001) in the presence of *D. magna*, while des-MC-LR was significantly higher on the day 8
414 in treatment compared with the control in the *Daphnia* co-culture unit ($p < 0.001$) (**Table 1**).
415 Over time the extracellular concentration of MC-LR was higher on day 6 in the control,
416 compared with day 4 ($p < 0.01$) and extracellular concentration of des-MC-LR was
417 significantly lower on day 8, compared with day 6 ($p < 0.001$). Furthermore, in the treatment,
418 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.

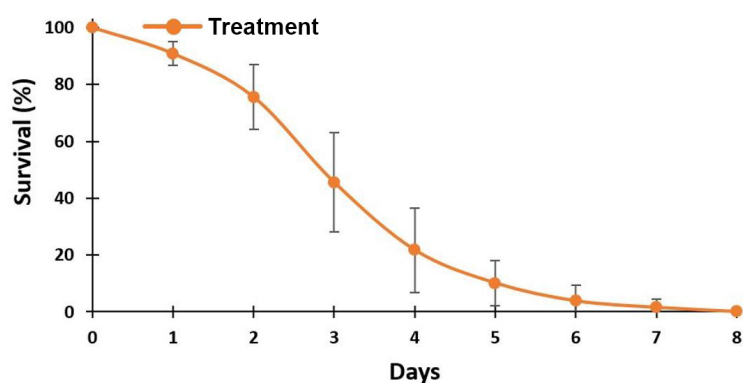
Dynamics of extracellular MC-LR and des-MC-LR in the *Daphnia* unit in the control and the treatment. * ($p < 0.05$), *** ($p < 0.001$) repeated-measures analysis of variance.

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



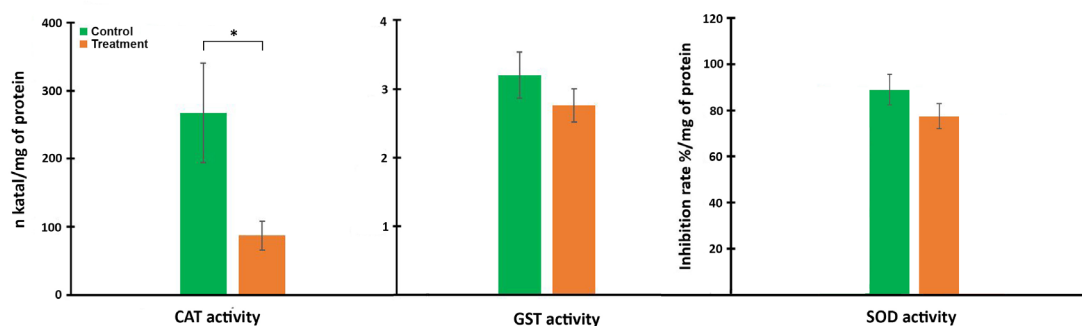
428

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 H₂O₂ 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 ETR_{max} 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 4x10⁶
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* (5.8x10⁶ 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).

480 In contrast with these results, van Gremberghe et al. (2009) observed strain specific
481 response when exposed to *Daphnia* spent medium, as biomass decreased in 4 out of 8
482 *Microcystis* strains isolated from two lakes. Our results are in line with these results, as
483 growth on day 4 showed decreasing tendencies, together with a significant increase in ROS
484 production demonstrating that the cyanobacterial strain suffered from stress, possibly caused
485 by *Daphnia* infochemicals. Excess production of ROS is one of the first signs that
486 phytoplankton is undergoing stress (Hirata et al., 2004; Diaz and Plummer, 2018). In their
487 natural habitat cyanobacteria are exposed to constantly changing abiotic and biotic factors,
488 therefore their ability to initiate rapid antioxidant defences in order to overcome ROS is
489 crucial for their survival (Latifi et al., 2009). Allelochemicals produced by submerged
490 macrophytes can cause oxidative damage in *M. aeruginosa* (Shao et al., 2009; Wang et al.,
491 2011). This study adds to the knowledge that also *Daphnia* infochemicals cause oxidative
492 stress in *M. aeruginosa* via ROS production. The decline tendencies in growth on day 4,
493 might be due to the energy allocation either towards anti-oxidant stress enzymes, or towards
494 other defense mechanisms against *Daphnia*. As the photosynthetic activity ETR_{max} was not
495 affected by the presence of *Daphnia* and as the cyanobacterial strain recovered by day 6, we
496 suggest that the cost may be transient as the cyanobacterial strain managed to deal with the
497 stress and acclimate to *Daphnia* exposure.

498 **4.2 MC production in response to presence of *D. magna* (as medium or individuals)**

499 Concentration of intracellular MC-LR significantly increased after day 6 when exposed
500 to *D. magna* infochemicals, suggesting that the presence of *Daphnia* even though not in direct
501 contact, induced the production of MC-LR as a response of *Microcystis*. Similarly, in a Polish
502 Reservoir, Izydorczyk et al. (2008) found a significantly positive correlation between increase
503 of intracellular MC concentrations and density of *D. pulex* and *D. cucullata*. Additionally,
504 increasing densities of *D. pulex* (100–500 individuals L⁻¹) provoked increasing
505 concentrations of MC in *Microcystis* spp at different densities (0.5 – 4.5 × 10⁶ cells mL⁻¹),

506 suggesting an anti-grazer role (Pérez-Morales et al., 2015). Compared with juveniles and
507 neonates, adult zooplankton the quantity or quality of infochemicals resulted in a greater
508 increase in both MC production and release (Jang et al., 2007). Extracellular MC was
509 significantly higher in the treatment in the *Daphnia* unit compared with the control,
510 suggesting overall higher export of MC-LR in the presence of *Daphnia* infochemicals,
511 gradually diffusing through the membrane. Our results are in line with these studies,
512 suggesting a stress response role for MC, when *M. aeruginosa* is suffering from stress caused
513 by *Daphnia* infochemicals or as grazing defense. Further research is needed to confirm this
514 role and to generalize in terms of both cyanobacterial and *Daphnia* strains and species and
515 the respective densities. The way *Daphnia* infochemicals are introduced in the cyanobacterial
516 medium plays a role in the reaction of the cyanobacteria. When *M. aeruginosa* is exposed to
517 *Daphnia* infochemicals by sudden introduction, it causes an intense stress: in a
518 precedent experiment we observed that MC+ exposed to *D. magna* spent medium (200 all
519 age individuals of *D. magna* L⁻¹ cultivated for 24 h) significantly decreased intracellular MC-
520 LR concentration, and general physiological state (Bojadzija Savic et al., 2019). Similarly,
521 *Microcystis* stopped producing MC, and its growth was impaired when it was exposed to
522 *Daphnia* medium (165 *D. magna* individuals L⁻¹ grown in 24 h WC medium) (Becker et al.,
523 2010). Apparently, *Daphnia* spent medium contains metabolites harmful to the
524 cyanobacterial cells but a gradual introduction would allow them to acclimate. A general
525 stress reaction observed during the gradual introduction was the formation of ROS, occurring
526 on day 4 but reduced thereafter. This reduction might not be possible during a sudden
527 introduction as Schuurmans *et al.* (2018) provided further clarification indicating that MC
528 can inhibit antioxidant stress enzymes activity by binding to the same target proteins
529 (Schuurmans et al., 2018).

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

532 (Utkilen and Gjølme, 1992; Wiedner et al., 2003), as well as anti-grazing (Rohrlack et al.,
533 1999; Jang et al., 2004, 2007; Sadler and von Elert, 2014a). Our study supports an anti-stress
534 role of this cyanobacterial metabolite, however it could have more biological roles, as known
535 so far and their clarification still need further investigations.

536 **4.3 Cyanobacterial production of secondary metabolites other than toxins in the** 537 **presence of *Daphnia***

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

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

565 **4.4 Cyanobacterial response to *Scenedesmus communis* used as food source for** 566 ***Daphnia***

567 Through allelopathic interactions, *Scenedesmus* can negatively impact growth and
568 photosynthetic activity of *M. aeruginosa* that eventually lead to cell lysis (Jia et al., 2008;
569 Kaplan et al., 2012; Bittencourt-Oliveira et al., 2014). However, a co-culture experimental
570 setup, where *M. aeruginosa* (SAG 14.85) was cultivated with *S. obliquus* (SAG 276-3a)
571 demonstrated minor effects of interspecific interference between both species, but no
572 inhibition of *M. aeruginosa* (Dunker et al., 2013). In our experimental setup, *Scenedesmus*
573 was used as a food for *Daphnia*, therefore, its metabolites could have been present in the
574 *Daphnia* co-culture unit and eventually diffuse to the cyanobacterial co-culture unit.
575 However, our results show that *Scenedesmus* metabolites did not have a negative effect on
576 *M. aeruginosa* PCC7806, as they showed in the control (without *D. magna* but with
577 *Scenedesmus*) constantly good photosynthetic activity, increasing biomass over time and no
578 change in ROS production, which would have indicated stress. Therefore, it is highly unlikely
579 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 $\mu\text{g L}^{-1}$) are high but environmentally relevant as concentrations of dissolved MC between 0.2
587 and 8.18 $\mu\text{g L}^{-1}$ 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 $\mu\text{g MC-LR}$ that mildly affected its survival
592 (Lürding and van der Grinten, 2003) and 5 $\mu\text{g MC-LR}$ 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 impacts 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 dealt 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 $\mu\text{g L}^{-1}$)
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),

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

677 **5. Conclusions**

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

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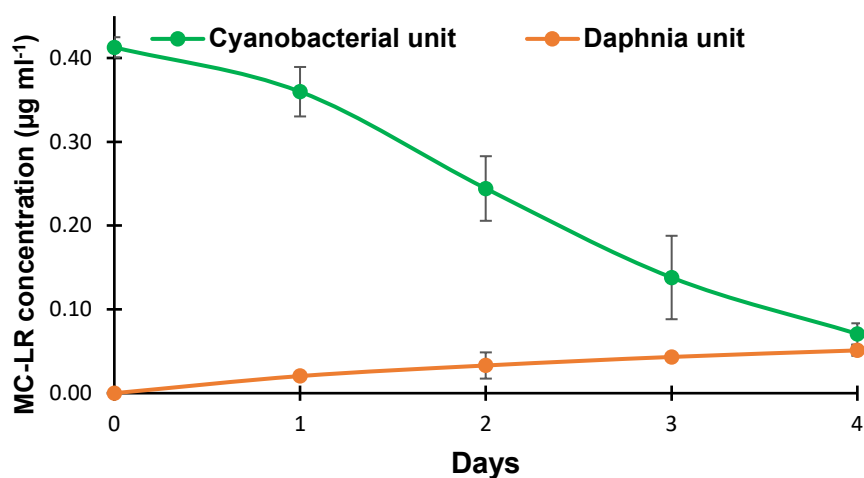
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959

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	+	+	+

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