Cross talk: two way allelopathic interactions between toxic Microcystis and Daphnia.

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

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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+**   *M. aeruginosa* 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 cyanobacteial 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*.

2. Materials and methods

2.1 Culture conditions

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 μmol photons $m^{-2} s^{-1}$ illumination at a constant temperature of $20 \pm 1°C$

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).

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 preforming

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

algae *Scenedesmus communis* (reaching max $\approx 2\times10^4$ 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).
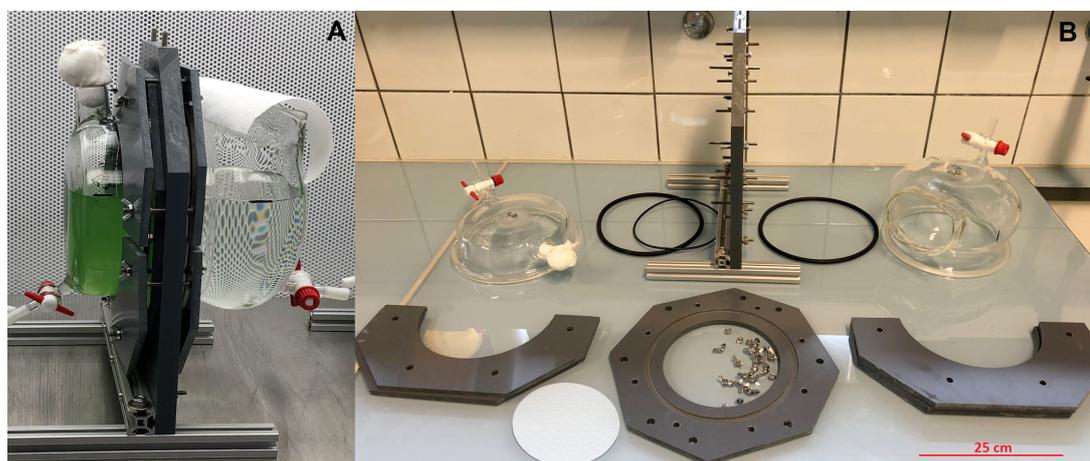
Figure 1. A The co-culture chamber during the exposure with the cyanobacterial culture at the left and the *D.*

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

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 as

175   example for a relatively large (995,2 g/mol) seconday 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 transfered to sterile 50%

185   BG11 medium until cell density reached $5x10^5$ 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.

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}).

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 µmol photons m^{-2} s^{-1}

208   ^{1}), while light intensity remained at 20 µmol photons m^{-2} 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   H_2O_2. 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   (ETRmax), an indicator of the algal physiological state, and precursor of growth (Briand et

221   al., 2012; Pannard et al., 2016). ETRmax 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). H$_2$DCFDA (2 ', 7'-dichlorodihydrofluorescein diacetate) is a non-fluorescent reagent

239   that is cleaved by intracellular esterases forming H$_2$DCF (dichlorodihydro-fluorescein).

240   H$_2$DCF reacts with intracellular ROS to form a fluorescent compound, DCF

241   (dichlorofluorescein). 5×10^6 cells were centrifuged and the pellets resuspended in 996 µL

242   NaPO$_4$ buffer 0.1M pH 6.5 and 4 µL of the H$_2$DCFDA 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 Parallex Flex, Cardiff, UK) and Flex V3 software for instrument control and data

264   acquisition. The separation was performed on Atlantis Prep C18 column (5 um particle size,

265   19 mm ID × 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 transfered 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 H_2O_2 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 H_2O_2 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 H_2O_2. Concentration of H_2O_2 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   secondes 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 normaly

324 distributed when $p>0.05$). Anova was preformed to test the effects of the model. Pairwise

325 comparison with correction for multiple comparison was preformed 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

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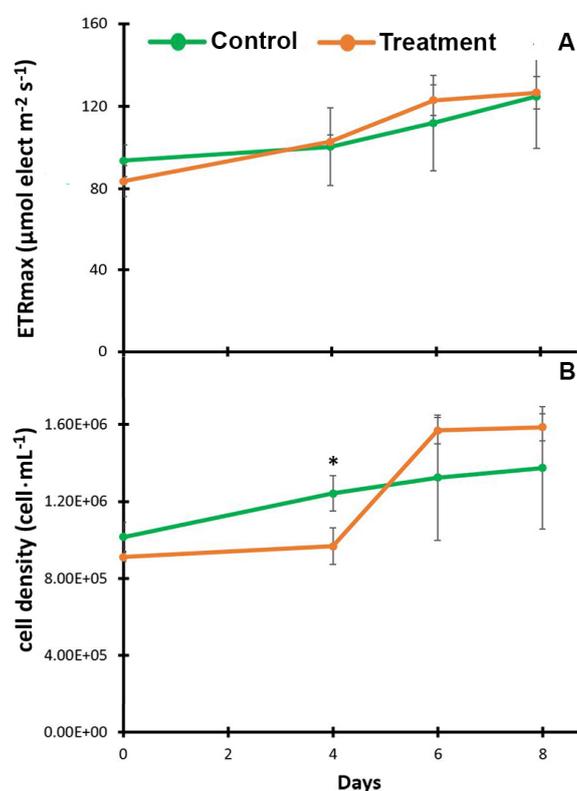
Figure 2. Photosynthetic activity and cyanobacterial biomass in control and in the presence of *D. magna*: **A** photosynthetic activity **B** cyanobacterial biomass. (p > 0.05) repeated-measures analysis of variance.

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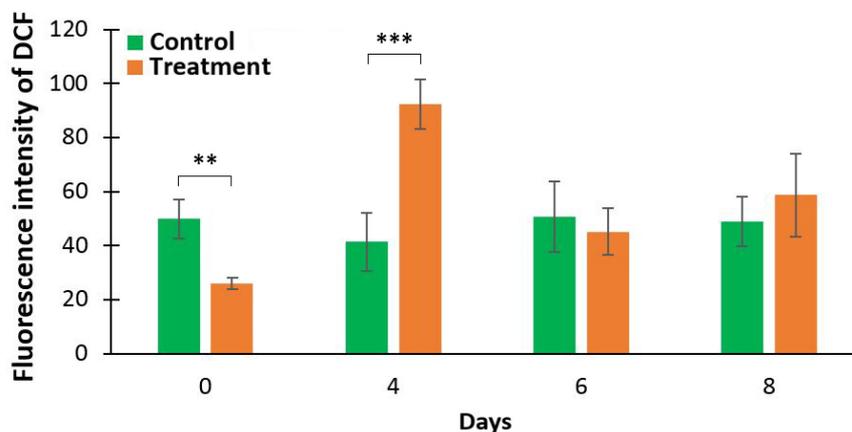
Figure 3. ROS content in MC+ in control and in the presence of *D. magna* ** ($p < 0.01$), *** ($p < 0.001$) repeated-measures analysis of variance.

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

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
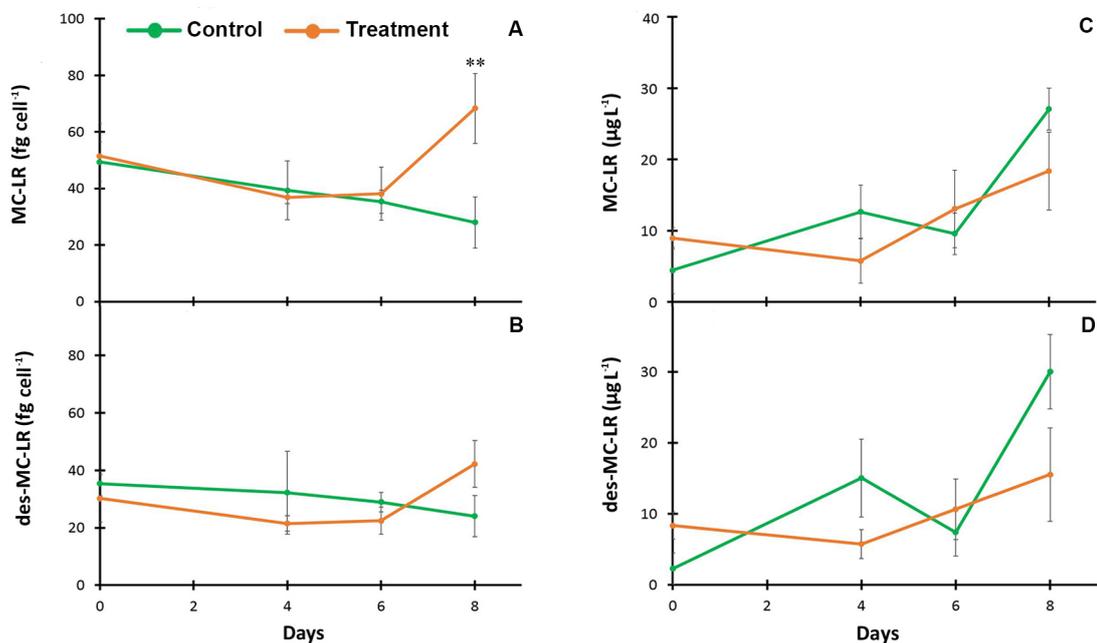
Figure 4. Dynamics of intracellular and extracellular metabolites in control and in the presence of *D. magna* in the cyanobacterial unit: **A** intracellular MC-LR, **B** intracellular des-MC-LR, **C** extracellular MC-LR, **D** extracellular des-MC-LR. * ($p < 0.05$), **($p < 0.01$), *** ($p < 0.001$) repeated-measures analysis of variance.
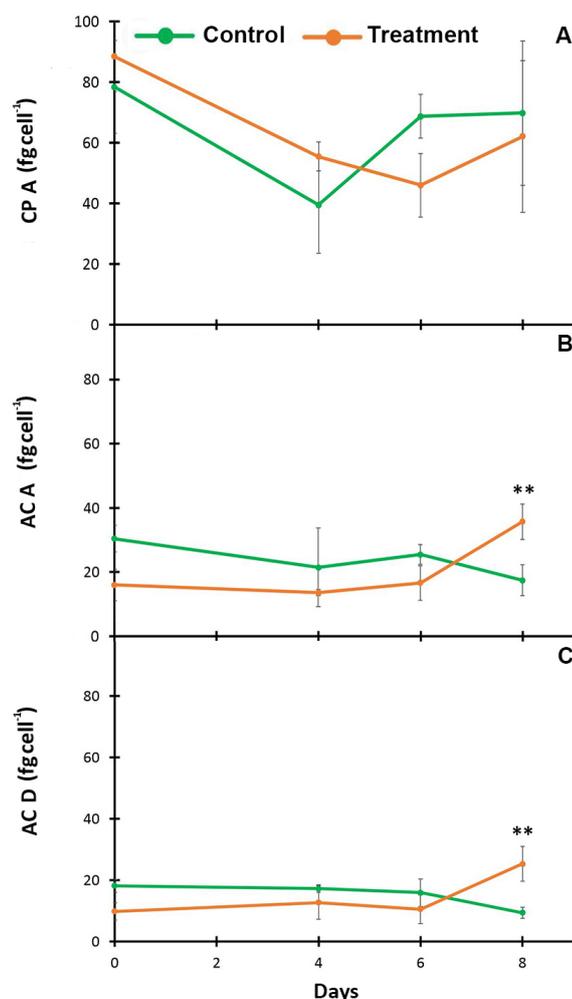
Figure 5. Dynamics of intracellular metabolites in control and in the presence of *D. magna* in cyanobacterial unit: **A** intracellular CP A, **B** intracellular AC A, **C** intracellular AC D. * ($p < 0.05$), *** ($p < 0.001$) repeated-measures analysis of variance.

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

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.**).
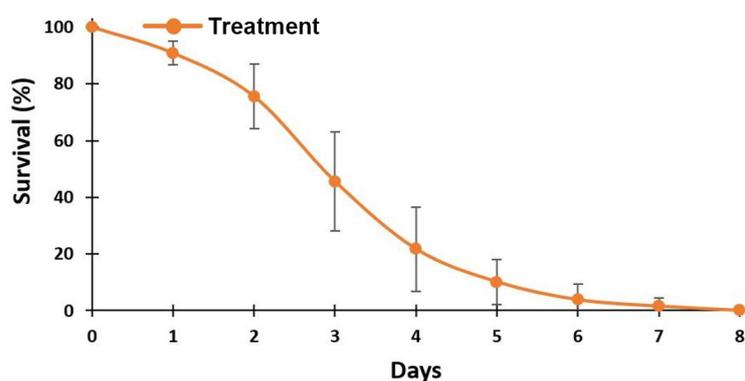
428

429 **Figure 6.** Neonates survival

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).
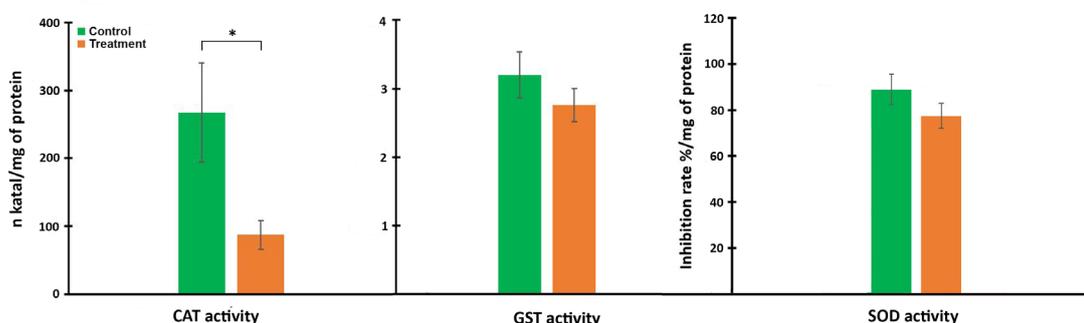
437

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

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

Reported studies on physiological or metabolic interactions between cyanobacteria and *Daphnia* using *Daphnia* individuals rather than medium are rare and involve very short exposures. For example, cell densities of *M. aeruginosa* clone LE-3 (initial cell density 4x10^6 cells mL^{-1}) were not significantly decreased when exposed to 120 *D. magna* individuals L^{-1} and 200 *D. pulex* individuals L^{-1} for 24h in a similar co-culture chamber (Harke et al., 2017). Exposure to spent medium from *D. magna* and *D. pulex* (300 *Daphnia* individuals cultivated for 24h) had no impact on growth rate of *M. aeruginosa* (5.8x10^6 cells mL^{-1}) (Yang and Li, 2007). Similar results were observed in 4 MC-producing strains (three strains of *M. aeruginosa* and one strain of *P. agardhii,*) exposed in their exponential growth phase to 10%, 25%, and 50% v/v of *D. magna* medium (from 200 non egg-bearing adults) which showed 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 ETRmax 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 corelation 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 ($0.5 - 4.5 \times 10^6$ 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 futher 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* delt 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 initialy 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 eventualy 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*.

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 $\mu g\ L^{-1}$) are high but environmentally relevant as concentrations of dissolved MC between 0.2

587 and 8.18 $\mu g\ L^{-1}$ 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 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),

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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1 **Supplementary info**
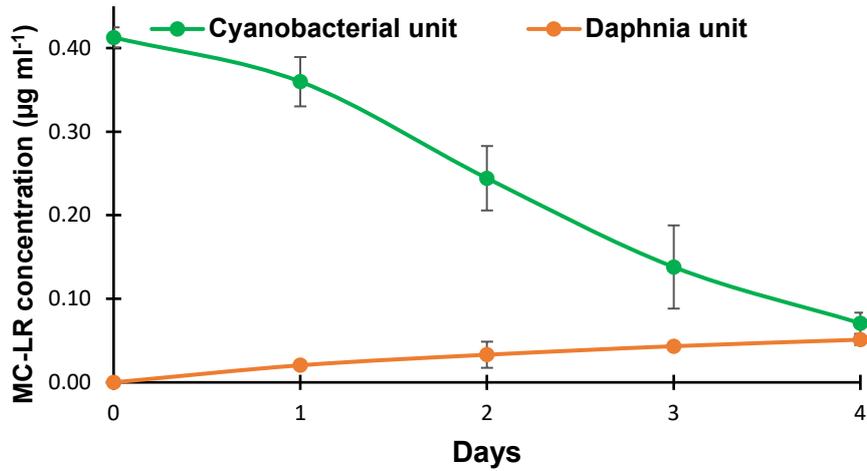
2

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