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2 **Cell free *Microcystis aeruginosa* spent medium affects** 3 ***Daphnia magna* survival and stress response**

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12

13 **Abstract:** Primary consumers in freshwater ecosystems, such as the zooplankton organism
14 *Daphnia magna*, are highly affected by cyanobacteria, both as they may use it as a food
15 source but also by cyanobacterial metabolites present in the water. Here, we investigate the
16 impacts of cyanobacterial metabolites focussing on the environmental realistic scenario of
17 the naturally released mixture without crushing cyanobacterial cells or their uptake as food.
18 Therefore, *D. magna* were exposed to two concentrations of cell free cyanobacterial spent
19 medium from *Microcystis aeruginosa* PCC 7806 to represent higher and lower ecologically-
20 relevant concentrations of cyanobacterial metabolites. Including microcystin-LR, 11
21 metabolites have been detected of which 5 were quantified. Hypothesising concentration and
22 time dependent negative impact, survival, gene expression marking digestion and
23 metabolism, oxidative stress response, cell cycle and molting as well as activities of
24 detoxification and antioxidant enzymes were followed for 7 days. *D. magna* suffered from
25 oxidative stress as both catalase and glutathione S-transferase enzyme activities significantly
26 decreased, suggesting enzyme exhaustibility after 3 and 7 days. Moreover, gene-expressions
27 of the 4 stress markers (glutathione S-transferase, glutathione peroxidase, catalase and
28 thioredoxin) were merely downregulated after 7 days of exposure. Energy allocation
29 (expression of Glyceraldehyde-3-phosphate dehydrogenase) was increased after 3 days but
30 decreased as well after 7 days exposure. Cell cycle was impacted time dependently but
31 differently by the two concentrations, along with an increasing downregulation of myosin
32 heavy chain responsible for cell arrangement and muscular movements. Deregulation of
33 nuclear hormone receptor genes indicate that *D. magna* hormonal steering including molting
34 seemed impaired despite no detection of microviridin J in the extracts. As a consequence of
35 all those responses and presumably of more than investigated molecular and physiological
36 changes, *D. magna* survival was impaired over time, in a concentration dependent manner.
37 Our results confirm that besides microcystin-LR, other secondary metabolites contribute to
38 negative impact on *D. magna* survival and stress response.

39

40

41 **Keywords:** zooplankton; cyanobacteria; secondary metabolites; PCC7806; oxidative stress;
42 transcriptomics

43 **Abbreviations**

44 MC-LR: microcystin-LR
45 des-MC-LR: des-microcystin-LR
46 CP: cyanopeptolin
47 AC: aerucyclamide
48 CP-A: cyanopeptolin A
49 AC-A: aerucyclamide A
50 AC-D: aerucyclamide D
51 LC: cyanobacterial spent medium diluted with the BG11 in the ratio 1:400
52 HC: cyanobacterial spent medium diluted with the BG11 in the ratio 1:40

53

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55

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75

76 **Declaration of interests**

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78 The authors declare that they have no known competing financial interests or personal
79 relationships that could have appeared to influence the work reported in this paper.

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1. INTRODUCTION

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Due to eutrophication in freshwater ecosystems, cyanobacterial proliferation frequently results in blooms that potentially produce bioactive or toxic metabolites that harm the environment and humans (Heisler *et al.*, 2008). *Microcystis* is one of the most widespread cyanobacterial genus in freshwaters, with *Microcystis aeruginosa* being one of the most commonly detected and investigated (Svirčev *et al.*, 2019). Like other cyanobacteria, *Microcystis* produces a diverse range of secondary metabolites that have been shown to impact life traits and physiology of aquatic organisms including zooplankton such as *Daphnia* (Lürling and van der Grinten, 2003; Merwe and Sebbag, 2012). One of the most detected toxins produced by cyanobacteria is microcystin (MC), with more than 250 variants described so far (Mowe *et al.*, 2015; Svirčev *et al.*, 2019). Microcystins change the phosphorylation state of proteins by inhibiting 1 and 2A protein phosphatases, thus disrupting pathways involving phosphorylation (MacKintosh *et al.*, 1990; Trinkle-Mulcahy and Lamond, 2006; Zurawell *et al.*, 2005). Furthermore, microcystins trigger oxidative stress in aquatic organisms (Amado and Monserrat, 2010).

Besides microcystins, *Microcystis sp.* produces a wide range of intracellular and extracellular secondary metabolites such as aeruginosins, cyanopeptolins, cyclamides, microginins and microviridins (Welker and Von Döhren, 2006), that have various negative effects on *Daphnia* physiology (Rohrlack *et al.* 2001, Bister *et al.* 2004, Ishida *et al.* 2007, von Elert *et al.* 2012). Similar to microcystins, cyanopeptolins (CP) are synthesized through non-ribosomal metabolic pathways, by non-ribosomal peptide synthetases (NRPSs) and polyketide synthase (PKS) (Welker and Von Döhren, 2006). CP are widely distributed and diverse compounds with more than 82 variants described so far (Gademann *et al.* 2010). Aeruginosins are synthesized non-ribosomally and they can inhibit trypsin-type serine proteases (Ishida *et al.* 2000, 2007). CPs and aeruginosins are potent inhibitors of the serine proteases trypsin and chymotrypsin, that are among the main digestive enzymes in *Daphnia* (von Elert *et al.* 2005; Gademann and Portmann 2008; Elkobi-Peer *et al.* 2013). Microviridins are one of the largest oligopeptides produced in cyanobacteria, with 13 to 14 amino acids (1600-1900 Da), contrasting to the other groups, they are ribosomally synthesized tricyclic depsipeptides. They are produced by different cyanobacterial genera, including *Microcystis sp.*, however, the natural

114 diversity of microviridins is still unknown (Gatte-Picchi *et al.*, 2014; Ziemert *et al.*, 2010).
115 Microviridin J has been identified as causing fatal molting disruption in *Daphnia pulicaria*
116 via inhibition of *Daphnia* proteases, thus leading to the death of the animals (Kaebernick *et*
117 *al.*, 2001; Rohrlack *et al.*, 2004). Cyclamides are cyclic hexapeptides with cytotoxic
118 properties to crustacea (Ishida *et al.*, 2000; Portmann *et al.*, 2008). Cyanopeptides can occur
119 in high frequency comparable to MC concentrations in aquatic environments and thus may
120 pose problems for drinking water purification from surface water reservoirs (Beverdors *et*
121 *al.*, 2018, 2017; Janssen, 2019; Natumi and Janssen, 2020). Concentrations of
122 cyanopeptolines ($< 7 \text{ ug/L}^{-1}$), anabaenopeptins ($< 1 \text{ ug/L}^{-1}$) and microginins ($< 1 \text{ ug/L}^{-1}$) have
123 been reported in surface waters (Beverdors *et al.*, 2017), however, more research is needed
124 regarding their diversity and concentration in aquatic environments. Furthermore, their effect
125 on organisms as a single compound or in a mixture are not investigated in detail.

126 Nevertheless, it has been suggested that in natural conditions some oligopeptides may be
127 even more harmful than MCs and other cyanotoxins to certain zooplankton species, in
128 particular as digestion inhibitors are active at much lower concentrations than the classical
129 cyanotoxins (Von Elert *et al.*, 2004). Digestive enzyme inhibition would cause starvation,
130 impair growth and reproduction, eventually leading to slow death (Von Elert *et al.*, 2004).

131 Among the freshwater zooplankton species, *Daphnia* graze on phytoplankton including
132 cyanobacteria, thereby connecting the primary production to the consumers within the
133 aquatic food web (Ger *et al.*, 2016). Dominance of nutritionally inadequate phytoplankton,
134 such as cyanobacteria, which lack important sterols and fatty acids necessary for *Daphnia*
135 growth and development, can represent an obstacle in efficient carbon transfer to higher
136 trophic levels (Martin-Creuzburg *et al.*, 2008). During their lifetime, however, *Daphnia* are
137 able to develop tolerance to cyanobacterial metabolites, as a physiological response to
138 bioactive compounds (Gustafsson and Hansson, 2004; Ortiz-Rodríguez *et al.*, 2012; Sarnelle
139 and Wilson, 2005). Several mechanisms have been described so far, such as *Daphnia*'s ability
140 to remodel their digestive enzymes (Schwarzenberger *et al.* 2012, von Elert *et al.* 2012), or
141 increase of antioxidant and biotransformation enzyme activity (Dao *et al.*, 2013; Ortiz-
142 Rodríguez and Wiegand, 2010; Sadler and von Elert, 2014a). When exposed to
143 cyanobacterial metabolites that are chymotrypsin and trypsin inhibitors, *Daphnia* switch to

144 chymotrypsins and trypsin isoenzymes with smaller molecular mass, which are less inhibited
145 (Schwarzenberger *et al.* 2012, von Elert *et al.* 2012). The antioxidative enzymatic defenses
146 include catalase (CAT), superoxide dismutase (SOD), glutathione-peroxidase (GPx), while
147 glutathione S-transferase (GST) is the detoxification mechanism (Ighodaro and Akinloye,
148 2018; Pflugmacher *et al.*, 1998). *Daphnia* can reduce oxidative stress caused by
149 cyanobacterial metabolites by increasing CAT activity (Ortiz-Rodríguez and Wiegand, 2010;
150 Wojtal-Frankiewicz *et al.* 2014). Similar increased activity of antioxidant SOD and CAT
151 enzyme providing cellular protection against ROS is found in a study where *Daphnia magna*
152 was fed with *Microcystis* diet (Lyu *et al.*, 2016b). *Daphnia* can reduce the toxicity of
153 microcystin by increasing the activity of GST catalysing the biotransformation via
154 conjugation to glutathione (Dao *et al.* 2010; Ortiz-Rodríguez *et al.*, 2012; Miles *et al.* 2016).

155 Furthermore, studies in *Daphnia* have shown that cyanobacterial metabolites can affect
156 expression of genes involved in digestive system (Asselman *et al.*, 2014; Drugă *et al.*, 2016;
157 Schwarzenberger *et al.*, 2012), cell cycle (De Coninck *et al.*, 2014b; Giraudo *et al.*, 2017;
158 Tong *et al.*, 2017), and oxidative stress (De Coninck *et al.*, 2014b; Lyu *et al.*, 2016a; Rhiannon
159 *et al.*, 2011). However, characterizing transcriptional responses of *Daphnia* to cyanobacterial
160 toxins so far focussed merely on effect of microcystin or cyanobacterial media without
161 considering other bioactive molecules that might be present apart from microcystin
162 (Asselman *et al.*, 2012; De Coninck *et al.*, 2014a; Drugă *et al.*, 2016; Lyu *et al.*, 2016b).

163 Besides being affected by grazing on cyanobacteria, zooplankton can also be affected by
164 naturally produced cyanobacterial metabolites released in water (Barrios *et al.*, 2015; Ferrão-
165 Filho *et al.*, 2014; Smutná *et al.*, 2014). Nevertheless, most of the studies investigate impact
166 of cyanobacteria on *Daphnia* grazing on them, or exposure to extracts obtained by crushing
167 cyanobacterial cells (from a culture or from a field sample), or to purified compounds (Dao
168 *et al.*, 2013; Esterhuizen-Londt *et al.*, 2016; Peng *et al.*, 2018). Hence, it is of interest to
169 investigate if *Daphnia* are impaired by the presence of cyanobacterial metabolites naturally
170 released during cyanobacterial normal growth, without necessarily feeding on them, or
171 crushing cells. Furthermore, knowing the composition and concentration of metabolites
172 present in the medium, naturally released as a product of cyanobacterial metabolic activity,
173 would provide further understanding of *Daphnia*'s response to cyanobacterial metabolites.

174 Therefore, the aim and novelty of this study is *Daphnia* exposure to cell free cyanobacterial
175 spent medium obtained from a two weeks culture of exponentially growing *M. aeruginosa*,
176 containing metabolites naturally released during that time. In addition, we detected and
177 quantified cyanobacterial metabolites present in the medium, beside commonly investigated
178 microcystin. We monitored markers of stress response in order to predict *Daphnia*
179 physiological phenotypes as a response to cyanobacterial spent medium. We analyzed the
180 effects of cell free cyanobacterial spent medium that contained a quantified mixture of
181 compounds on i) *Daphnia* survival, ii) activity of enzymes involved in oxidative stress and
182 detoxification and iii) expression of candidate genes involved in digestion, growth and
183 development, detoxification and oxidative stress response. We hypothesize a negative impact
184 on *Daphnia* survival, a trigger of CAT and GST activities, as well as expression of genes
185 involved in *Daphnia* growth and development, digestion, detoxification and oxidative stress.
186
187

2. MATERIALS AND METHODS

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2.1. Culture conditions

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2.1.1. *Microcystis aeruginosa* PCC 7806

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193

194 An axenic microcystin (MC) producing *M. aeruginosa* strain PCC 7806 was obtained
195 from the Pasteur Culture collection of Cyanobacteria in Paris, France
196 (<https://research.pasteur.fr/en/team/collection-of-cyanobacteria/>). The strain was grown in
197 100% cyanobacterial BG11 medium (SIGMA), under a 14h:10h light:dark regime using
198 daylight white fluorescent tubes (Toshiba, 15 W, FL15D) with 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$
199 illumination at a constant temperature of $20\pm 1^\circ\text{C}$ (Sanyo incubator). The culture was
200 maintained in exponential growth phase, while the axenicity was regularly evaluated as
201 described in (Briand et al., 2012).

202

2.1.2. *Daphnia magna*

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204

205 The *D. magna* clone was obtained from the PEARL INRA 1036 U3E. As the exposure
206 to cyanobacterial metabolites was realized via *M. aeruginosa* PCC 7806 spent medium, the
207 *D. magna* clone was acclimated to BG11 medium (SIGMA). Before performing experiments
208 we compared osmolarity of BG 11 medium with the osmolarity of commonly used Artificial
209 *Daphnia* medium, Elendt M4 and Elendt M7. Osmolarity of BG 11 medium was similar to
210 that of these *Daphnia* media, thus not affecting *Daphnia* survival. During the first three weeks
211 *D. magna* were slowly acclimated and adjusted to the cyanobacterial BG 11 medium that was
212 used in all the experiments, by gradually increasing the % of BG11 medium until it reached
213 100% in the end of the acclimation period. After the acclimation period *D. magna* were grown
214 in 100% of BG11 medium for a month, before neonates (< 36h old) were used in the
215 experiment. During the acclimation and cultivation period no irregularities in *Daphnia*
216 survival, eating, movement and growth were noticed, suggesting that *D. magna* were
217 successfully acclimated to the medium (Bojadzija Savic et al., 2020). *D. magna* were grown
218 and cultivated in the aquarium at a constant temperature of 20°C , light intensity of 15 μmol
219 $\text{photons m}^{-2} \text{ s}^{-1}$ having a day/night cycle of 14h:10h (Sanyo MIR 154) and daily renewal of

220 20% of the medium. *Daphnia* were fed daily with a fresh sub-culture of the green algae
221 *Scenedesmus communis* originating from lake Grand Lieu, France, that was isolated in our
222 laboratory (University of Rennes 1).

223 Sufficient food that 100 *Daphnia* (According to OECD guidelines for testing of
224 chemicals 202) that should be available daily in the medium is equivalent to $2.8 - 5.6 \times 10^8$
225 cells/mL of *S. communis*. *Daphnia* are usually maintained in medium that is not appropriate
226 for algal growth (Elendt M4, Elendt M7- OECD guidelines for testing of chemicals),
227 therefore when *S. communis* are introduced to *Daphnia* medium, they are consumed before
228 growing in high densities. However, in our cultures *Daphnia* were adjusted to BG11 medium
229 that was also used for *S. communis* culture. Hence, it allowed *S. communis* to grow and reach
230 high densities (10x higher than the initial one, within one week) while being consumed by
231 *Daphnia*. As high density of *S. communis* disrupts normal *Daphnia* functioning; its
232 concentration had to be adjusted to $2.8 - 5.6 \times 10^7$ cells/mL to maintain equilibrium between
233 growth and consumption. In order to keep the *S. communis* culture in more accessible form
234 for consumption, in particular by neonates (i.e. unicellular /in pairs instead of four cells
235 having long spikes), *Scenedesmus* culture was diluted every few days before feeding.

236

237 **2.2. Experimental design**

238

239 All experiments were performed in 5 replicates in 2 L aquariums and lasted for 3 and 7
240 days. All *Daphnia* were fed daily with *S. communis*, whose density was monitored and
241 adjusted daily. For the control, 150 *D. magna* neonates (< 36h old) per aquarium were raised
242 in BG11.

243 For the treatment, the cyanobacterial spent media was prepared in the following way: from
244 an exponentially growing *M. aeruginosa* PCC 7806 culture, centrifuged cells were
245 transferred in fresh sterile BG 11 medium to remove extracellular metabolites. From the
246 pellet, a cyanobacterial culture of 2×10^5 cells/mL initial density was grown for two weeks,
247 reaching a cell density of 1×10^6 cell/mL. From that culture, cell free cyanobacterial spent
248 medium was collected by filtering through 0.2 μm sterile cellulose nitrate filter. *D. magna*
249 were exposed to two concentrations of cyanobacterial spent medium: a) diluted with the
250 BG11 in the ratio 1:40 (thereafter called HC for high concentration) and b) 1:400 (thereafter

251 called LC for low concentration). These 2 dilutions provided environmentally relevant
252 concentrations of MC-LR in our experiments (final concentration of MC-LR: LC: 0.5 µg/L,
253 HC: 5 µg/L), as dissolved MC ranges in the environment between 0.2 and 11 µg/L (Lahti *et*
254 *al.*, 1997; Pawlik-Skowrońska *et al.*, 2008; Rastogi *et al.*, 2015; Su *et al.*, 2015).

255 *D. magna* neonates (150 per treatment) were exposed to 2 L of these two concentrations.
256 At the end of the exposure (3 days and respectively 7 days), *D. magna* samples
257 (biotransformation, oxidative stress, energetic profiles) were taken by collecting them over a
258 mesh, briefly rinsed with fresh BG11 media, and after removal of the excess media, snap
259 frozen in liquid nitrogen and stored at -80°C until analysis.

260

261 **2.3. Cyanobacterial secondary metabolites analysis**

262

263 Cyanobacterial secondary metabolites previously known to be produced by this
264 particular strain PCC7806 (Briand *et al.*, 2016; Rohrlack *et al.*, 2004; Sadler and von Elert,
265 2014b) were monitored after 2 weeks growth phase from initially 2×10^5 cells/mL to 1×10^6
266 cells/mL. Cyanobacterial cells were separated from the supernatant by centrifugation. Cell
267 free spent medium was filtered through 0.2 µm filter and 1 mL was lyophilized. Extraction
268 of lyophilized material was done in 0.5 ml 50% methanol and processed as described in
269 Bojadzija Savic *et al.*, 2019. Waters Acquity Ultra-High Performance Liquid
270 Chromatography coupled to a Xevo quadrupole time of flight mass spectrometer was used
271 for the metabolites analysis. Cyanobacterial peptides were detected using extracted ion
272 chromatograms for the respective specific masses of the different compounds (Bojadzija
273 Savic *et al.*, 2019). Microcystin-LR (MC-LR), Microcystin-des-LR (des-MC-LR),
274 cyanopeptolin A (CP-A), and aerucyclamide A (AC-A) and D (AC-D) were quantified using
275 linear relationship between peak area (MC-LR and des-MC-LR at 238 nm, CP-A at 220 nm,
276 and AC-A at 237 nm and AC-D at 240 nm) and known concentrations of the toxin standards.
277 The microcystin-LR standard was purified as previously described (Edwards *et al.*, 1996).
278 CP-A standard and AC-A and AC-D standard were purified using preparative HPLC (Biotage
279 Parallelex Flex, Cardiff, UK) and Flex V3 software for instrument control and data acquisition
280 as described in (Bojadzija Savic *et al.*, 2020). Detection and quantification of the
281 cyanobacterial peptides was done by MassLynx v4.1 software.

282

283 **2.3. *D. magna* survival**

284

285 Survival of *D. magna* was monitored daily, by counting and removing dead *Daphnia*
286 (identified as not moving and decaying *Daphnia* on the bottom of the aquarium) with results
287 expressed in percentages of total individuals at the start of the exposure.

288

289 **2.4. Enzyme extraction and measurement**

290

291 Fifty *Daphnia* were resuspended and homogenized in 1 mL of ice cold extraction buffer
292 (0.1 M phosphate buffer pH 6.5, glycerol, 1 mmol EDTA, and 1.4 mmol dithioerythritol)
293 using Lysing Beads-Matrix E (MPbio) in the Vibro-mill MM200, (RETSCH) for 3 min at
294 the frequency of 25 Hz to break the cells, followed by centrifugation (10,000 g, 10 min, 4°C,
295 Sigma 3K18C). The supernatant was used for enzyme measurements using a
296 spectrofluorometer (SAFAS Monaco Xenius XC, Monaco). Catalase (CAT) activity was
297 assayed by measuring the rate of disappearance of H₂O₂ at 240 nm (Chang and Kao, 1997).
298 GST was assayed at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. SOD
299 activity was determined using a photochemical assay based on the reduction of nitro blue
300 tetrazolium (NBT) according to total Superoxide Dismutase (T-SOD) assay kit
301 (Hydroxylamine method, SIGMA KIT), however SOD activity in our experiments remained
302 below the level of detection. All enzyme activities were related to the protein content in the
303 extract, measured according to Bradford (1976).

304

305 **2.5. Candidate function gene expression**

306

307 **2.5.1. RNA extraction and reverse transcription**

308

309 RNA extraction and reverse transcription was done according to (Colinet *et al.* 2010)
310 from the unexposed control and from *Daphnia* exposed to LC and HC after 3 and 7 days. For
311 each condition and sampling time point, five RNA samples (i.e. biological replicates), each
312 consisting of a pool of 50 *Daphnia*, were used. *Daphnia* were ground to fine powder in 1.5
313 mL tubes placed in liquid nitrogen. Samples were mixed with lysis buffer (containing 1% β-
314 mercaptoethanol) from RNA extraction kits (Qiagen) and crushed for 10 min to complete
315 homogenization. RNA extraction and purification was performed following the
316 manufacturer's instructions (Qiagen). Total RNA was eluted in 40 μL of DEPC-treated water.
317 RNA was quantified and quality-checked with a Nanodrop 1000 (Thermo Scientific,
318 Waltham, MA). Three hundred nanograms of total RNA was used in the reverse transcription
319 to cDNA, using the SuperScript ® III First-Strand Synthesis System for RT-PCR

320 (Invitrogen), according to the manufacturer's instructions. The undiluted cDNA was stored
 321 at 20°C until use.

322

323 2.5.2. Real-time PCR

324

325 We used primers of candidate genes involved in *Daphnia* energy (glyceraldehyde-3-
 326 phosphate dehydrogenase: *gapdh*), digestive system (protease: *ct383*), oxidative stress
 327 (glutathione S-transferase: *gst*, glutathione peroxidase: *gpx*, catalase: *cat*, thioredoxin: *trx*),
 328 cell cycle (histone: *h2a*, myosin heavy chain: *mhcr1*) and molting (nuclear hormone receptor:
 329 *hr3*, nuclear hormone receptor: *ftz-f1*) that potentially could be affected by cyanobacterial
 330 metabolites. We used β -actin as the reference gene. Oligonucleotide primers were obtained
 331 from the previously published references (see **Table 1.** for details), having efficiency between
 332 92% and 105% (Girauda *et al.*, 2017; Houde *et al.*, 2013; Lyu *et al.*, 2014; Rhiannon *et al.*,
 333 2011; Schwarzenberger *et al.*, 2010; Tong *et al.*, 2017; Wang *et al.*, 2016). Oligonucleotide
 334 primers were made by Integrated DNA Technologies, BVBA.

335

Table 1. Oligonucleotide primers used in the experiments.

Gene name	Symbol	Primer sequence 5'-3'	Amplicon size	Reference
Glyceraldehyd e-3-phosphate dehydrogenase	<i>gapdh</i>	F- TGCTGATGCCCAATGTTTGTGTTGT R-GCAGTTATGGCGTGGACGGTTGT	132	(Girauda <i>et al.</i> , 2017)
Protease CT383	<i>ct383</i>	F- TTGGCACCTTCCACCGAAT R- TCATCAGGACTGGAGAAACGC	183	(Schwarzenberge r <i>et al.</i> , 2010)
Glutathione S- transferase (NCBI: No. EFX81634.1)	<i>gst</i>	F- GGGAGTCTTTTACCACCGTTTC R- TCGCCAGCAGCATACTTGTT	150	(Wang <i>et al.</i> , 2016)
Glutathione peroxidase	<i>gpx</i>	F- AACGTTACGATGCCAGTTCC R- TCTTTCGAGCGGTTGAGATT	212	(Rhiannon <i>et al.</i> , 2011)
Catalase	<i>cat</i>	F- AGGTGCCTTTGGATACTTTGA R- TTGCGTATTCCCTGGTCAGTC	495	(Lyu <i>et al.</i> , 2014)
Thioredoxin	<i>trx</i>	F- GTATCCACGCCAGTCCTTGTT R- TCCTTCCACTTTTCCTCCCTTA	129	(Liu <i>et al.</i> , 2019)
Histone 2A	<i>h2A</i>	F- CTGGTGCCCTGTCTACCTA R- TAGGGAGGAGAACAGCCTGA	219	(Girauda <i>et al.</i> , 2017)
Myosin heavy chain	<i>mhcr1</i>	F - GATGCCGTTTCCGAGATGAG R - CTCGGCGGTCATGTGGTC	132	(Tong <i>et al.</i> , 2017)

Nuclear hormone receptor HR3	<i>hr3</i>	F- AAGGTCGAGGATGAAGTGCG R- AAAGACGCTACTATCGGGCG	81	(Girauda <i>et al.</i> , 2017)
Nuclear hormone receptor FTZ-F1	<i>ftz-f1</i>	F- TCTTACCGGACATTCACGCC R- ACAGCCGTTGAGATGCTTGA	71	(Girauda <i>et al.</i> , 2017)
Beta-actin	<i>β-actin</i>	F- GCCCTCTTCCAGCCCTCATTCT R- TGGGGCAAGGGCGGTGATT	189	(Houde <i>et al.</i> , 2013)

336

337 Real-time PCRs were performed on the LightCycler 480 system. Reactions were performed
338 in 384-well LightCycler plates, using LightCycler 480 High Resolution Melting Master Mix
339 and the crossing point (Cp), equivalent to the cycle threshold (Ct), estimates were obtained
340 using the absolute quantification module in the software package. The PCR reactions were
341 performed in four replicates, containing 4 μ L of cDNA sample, 2 μ m each primer, and 6 μ L
342 of the High Resolution Melting Master Mix. After 10 min at 95°C, the cycling conditions
343 were as follows: 60 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. To validate the
344 specificity of amplification, a post amplification melt curve analysis was performed.
345 Amplicons were first denatured at 95°C for 1 min, and then cooled to 65 °C, and the
346 temperature was then gradually raised to 95°C). Fluorescence data were recorded
347 continuously during this period, and subsequently analyzed using the Tm calling module in
348 the LightCycler 480 software.

349

$$R = \frac{(E_{target})^{\Delta CP_{target}(control-treated\ sample)}}{(E_{reference})^{\Delta CP_{target}(control-treated\ reference)}}$$

350 Relative expression ratios (R) (i.e. fold change) were calculated using the efficiency
351 calibrated model of (Colinet *et al.*, 2010; Pfaffl, 2001). In the Pfaffl model, CP is the crossing
352 point (i.e. Ct) and E the efficiency of PCRs. qPCR CT values of all candidate genes have
353 been provided in **Supplementary 1**. The ratio of the target gene is expressed in treated
354 samples versus matched controls (calibrators), and normalized using the housekeeping
355 reference gene.

356

357 **2.7. Statistical analyses**

358

359 R Core Team (2013) was used to access statistical analysis of the obtained data. All data
360 are presented as mean \pm standard deviation. Significant differences were determined at

361 $p < 0.05$. We performed t-tests to determine the difference between CAT, GST activities, as
362 well as gene expression between the control and treatment. Repeated-measures analysis of
363 variance was done to determine the difference in *Daphnia* survival between Control/LC,
364 Control/HC and LC/HC). Repeated-measures analysis of variance is a mixed linear model
365 with day, treatment (Control vs Treatment) and interaction between day and treatment
366 considering the repeated measures on replicates (random effect). Normality of residuals was
367 tested via Shapiro test (residuals normally distributed when $p > 0.05$). Anova was performed
368 to test the effects of the model. Pairwise comparison with correction for multiple comparison
369 was performed to check significant differences between control and the two treatments,
370 differences between LC and HC and if there was time dependency effect on control and
371 treatment.

372

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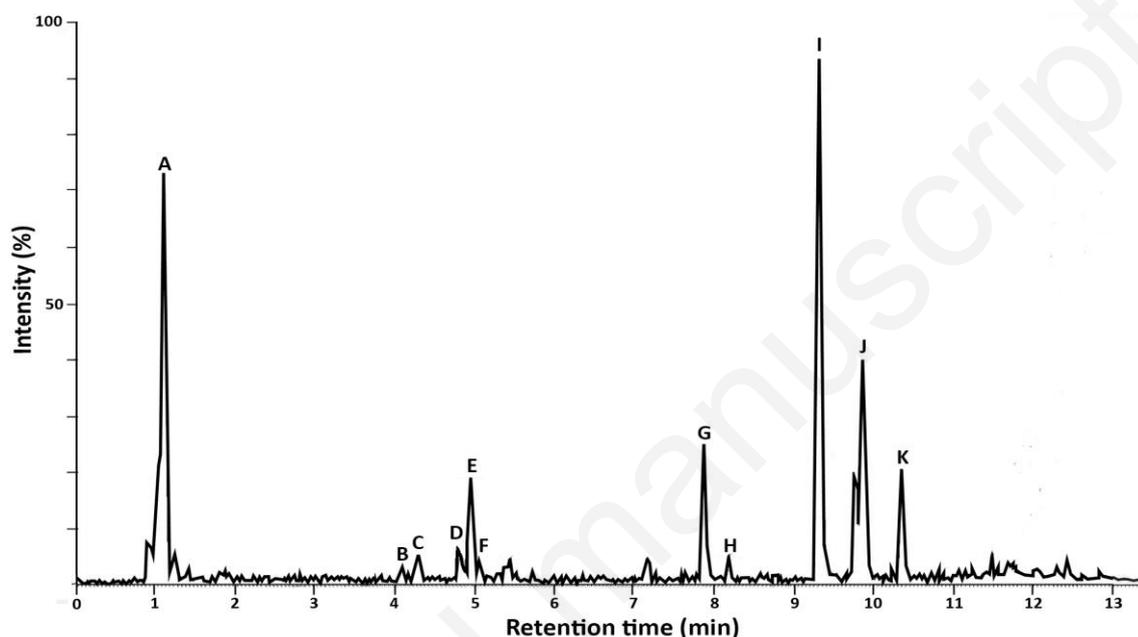
374 **3. RESULTS**

375

376 **3.1. Composition of the two weeks old *M. aeruginosa* PCC7806 medium**

377

378 In the media produced by exponentially growing *M. aeruginosa* PCC7806, 11
 379 metabolites were detected after 2 weeks of cultivation. *M. aeruginosa* PCC 7806 produced
 380 MC-LR and des-MC-LR, cyanopeptolins, CP (963A, A and B), aerucyclamides, AC (A,B,C
 381 and D) and aeruginosins (684 and 602) (**Figure 1**).



382

383 **Figure 1.** Secondary metabolites detected in *M. aeruginosa* PCC 7806. (A) aeruginosin 684, (B)
 384 cyanopeptolin B, (C) aeruginosin 602, (D) des-MCLR, (E) MC-LR, (F) CP A, (G) aerucyclamide D, (H)
 385 cyanopeptolin 963A, (I) aerucyclamide A, (J) aerucyclamide C, (K) aerucyclamide B.

386

387 The concentrations of five extracellular metabolites (MC-LR, des-MC-LR, CP-A, AC-
 388 D, AC-A) in *M. aeruginosa* PCC 7806 after 2 weeks growth phase from 2×10^5 cells/mL
 389 initially to 1×10^6 cells/mL, and in dilutions HC and LC are shown in **Table 2**.

390

Table 2. Concentrations of extracellular metabolites in original medium of the 2 weeks *M. aeruginosa* PCC 7806 and its HC and LC dilutions

Extracellular metabolite	Original spent <i>M. aeruginosa</i> PCC 7806 medium ($\mu\text{g/L}$)	HC ($\mu\text{g/L}$)	LC ($\mu\text{g/L}$)
MC-LR	218±21	5.45±0.53	0.55±0.05
Des-MC-LR	61±1	1.53±0.03	0.15±0.01
CP-A	135±11	3.38±0.28	0.34±0.03
AC-D	93±10	2.33±0.25	0.23±0.02

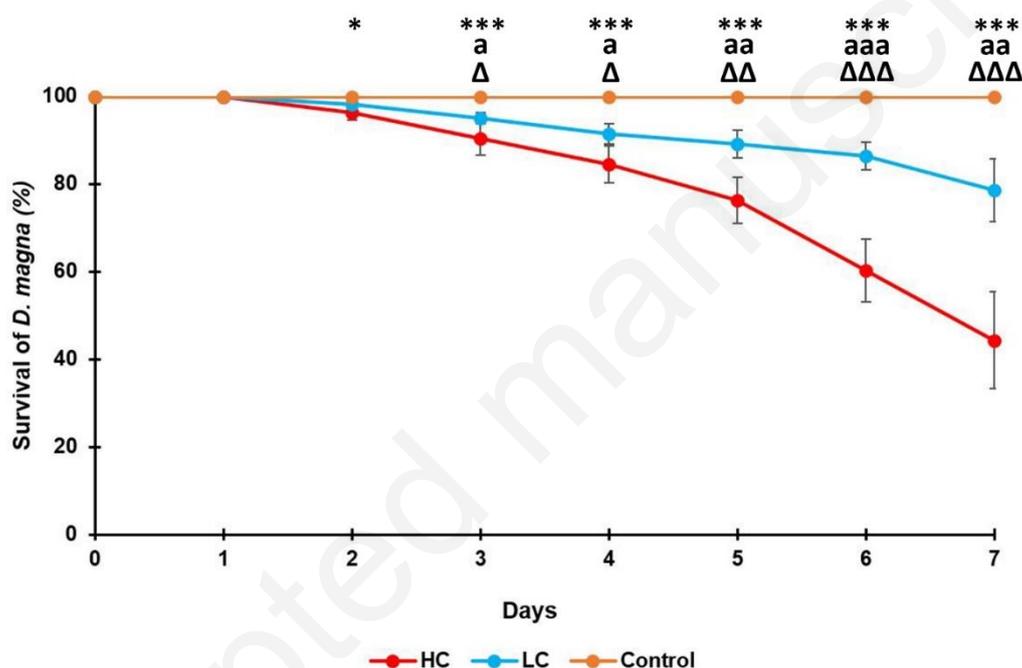
391

392 **3.1. *Daphnia* survival**

393

394 The high concentration of the cyanobacterial medium (HC) caused a significant decrease
 395 in survival from day 2 onwards, compared with the control, while exposure to the low
 396 concentration (LC) caused significant decrease after day 3. Both concentration levels
 397 significantly decreased *Daphnia* survival over the course of the experiment. *Daphnia* survival
 398 was significantly higher in LC exposure from day 3 onwards (80% survival after 7 days)
 399 compared with the HC exposure (40% survival after 7 days) (**Figure 2.**)

400



401

402 **Figure 2.** *D.magna* survival when exposed to HC and LC. Control vs HC: *; Control vs LC: a ; HC vs LC: Δ.
 403 *, a, Δ (p < 0.05), **, aa, ΔΔ (p < 0.01), ***, aaa, ΔΔΔ (p < 0.01); repeated-measures analysis of variance

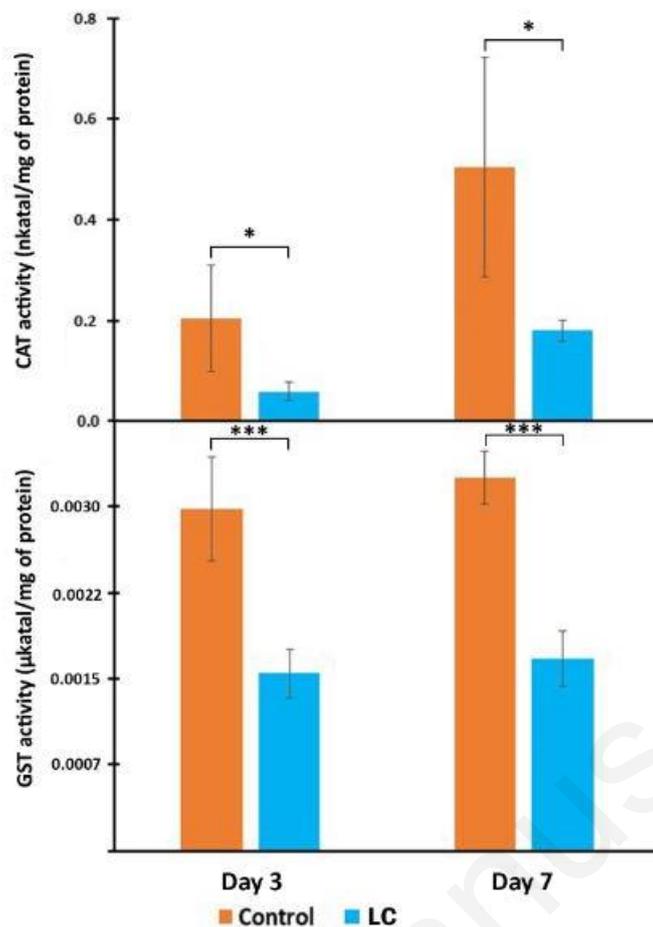
404

405 **3.2. Antioxidant and detoxification enzymes**

406

407 CAT activity was significantly lower on day 3 and day 7 in the LC exposure, compared
 408 to the control. Similarly GST activity was significantly lower on day 3 and 7 in the treatment
 409 exposed to LC, compared to the control. Due to the high mortality in the HC exposure, the
 410 biomass of the remaining *Daphnia* was insufficient for enzyme analysis on both days (**Figure**
 411 **3.**)

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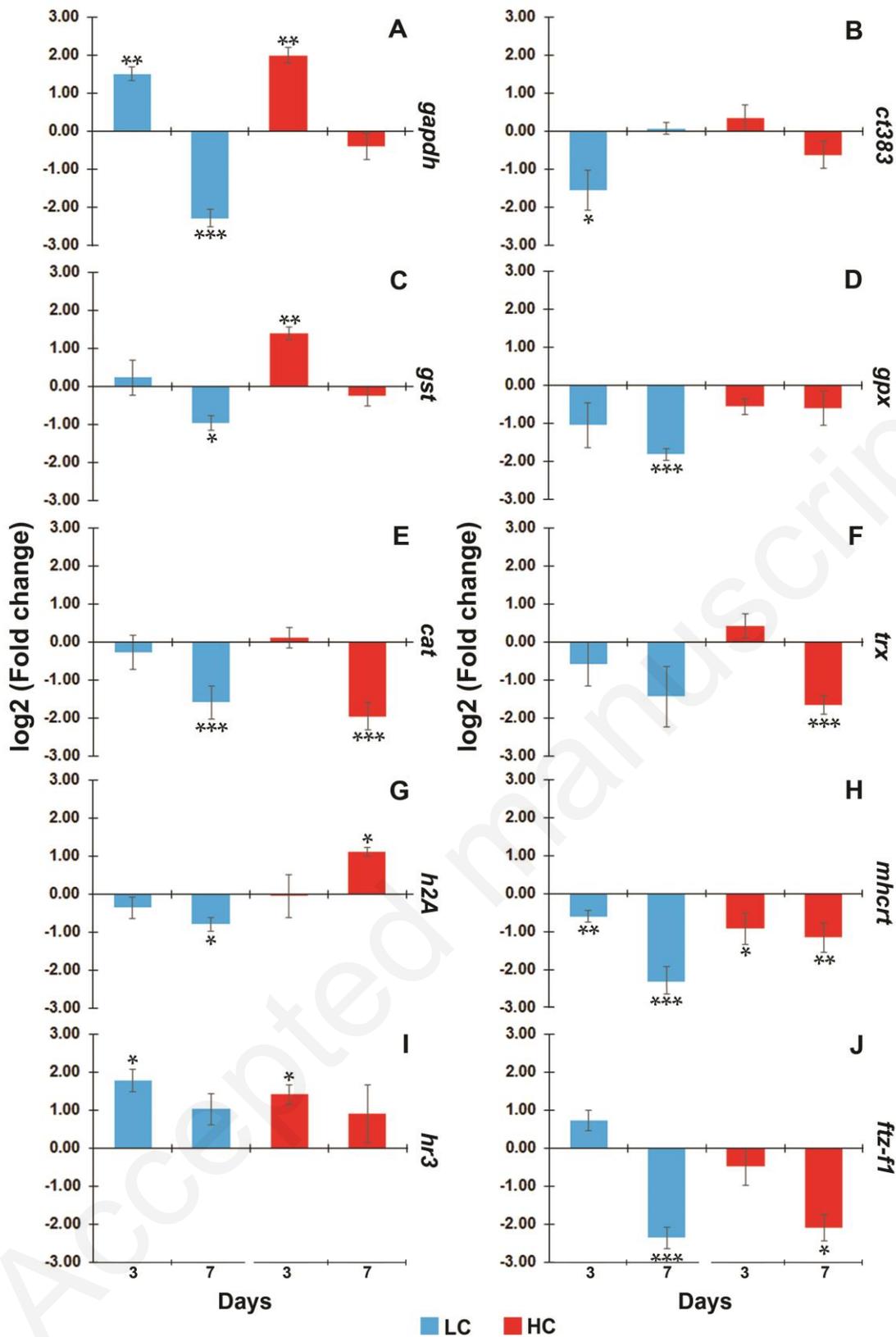
Figure 3. CAT and GST activity in *D.magna* exposed to LC * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.01$), t-test

416 **3.5. Selected genes expression**

417

418 Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was significantly overexpressed on
419 day 3 when *Daphnia* were exposed to both HC and LC, but downregulated after 7 days,
420 significant for LC (**Figure 4. A**). The gene coding for digestive enzyme Protease CT383
421 (*ct383*) was significantly downregulated after 3 days exposure to LC (day 3) of *Microcystis*
422 spent medium, but not in the other treatment (**Figure 4. B**). Concerning the oxidative stress
423 and detoxification related genes, merely downregulation was observed. The only exception
424 was the gene encoding for the detoxification enzyme Glutathione S-transferase (*gst*) which
425 was significantly upregulated on day 3 when exposed to HC, while on day 7, *gst* was
426 downregulated at LC treatment (**Figure 4. C**). Furthermore, Glutathione peroxidase (*gpx*)
427 expression was significantly downregulated on day 7 after *Daphnia* exposure to LC (**Figure**
428 **4. D**). Genes responsible for oxidative stress enzymes Catalase (*cat*) (**Figure 4. E**) and
429 Thioredoxin (*trx*) (**Figure 4. F**) were downregulated on day 3 (LC) and day 7 (HC and LC).
430 The response of the histone 2A (*h2A*) gene was the opposite of this, when exposed to HC and

431 LC: *h2A* showed significant overexpression respectively downregulated on day 7 when
432 exposed to HC or LC (**Figure 4. G**). HC and LC also had significant negative effect on gene
433 expression of Myosin heavy chain (*mhcr1*) that was significantly underregulated on day 3 and
434 7 (**Figure 4. H**). Cyanobacterial secondary metabolites affected two genes involved in the
435 molting cycle: the Nuclear hormone receptor HR3 (*hr3*) and FTZ-F1 (*ftz-f1*). While the *hr3*
436 was significantly upregulated on day 3 when exposed to both HC and LC (**Figure 4. I**) *ftz-*
437 was significantly downregulated on day 7 when exposed to both HC and LC (**Figure 4. J**).
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Figure 4. Effect of cyanobacterial media (LC and HC) on the relative gene expressions expressed as log₂(fold change). Relative expressions in *D. magna* for: A) Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), B) Protease CT383 (*ct383*), C) Glutathione S-transferase (*gst*), D) Glutathione peroxidase (*gpx*) E) Catalase (*cat*), F) Thioredoxin (*trx*), G) histone 2A (*h2A*), H) Myosin heavy chain (*mhcrt*), I) Nuclear hormone receptor HR3 (*hr3*), J) Nuclear hormone receptor FTZ-F1 (*ftz-f1*). Gene expressions were normalized to the control condition for each sampling time. Replicates of culture are represented as a mean and error bars represent the standard deviations (n = 5). * (p < 0.05), ** (p < 0.01), *** (p < 0.01), t-test

4. DISCUSSION

448
449

450 *Daphnia* are unselective phytoplankton filter feeders, and as such can be exposed to
451 cyanobacterial metabolites by direct grazing on cyanobacteria as well as by uptake of
452 metabolites released in the water. While previous studies have demonstrated the negative
453 impacts of cyanobacterial medium (Lanaras and Cook 1994, Neumann *et al.* 2000, Dao *et al.*
454 2010, 2013, Esterhuizen-Londt *et al.* 2016, Peng *et al.* 2018) on *Daphnia*, the novelty of this
455 study was to connect responses from selected markers on the gene-level to enzymatic
456 activities and to consequences for the survival of *D. magna* exposed to cyanobacterial cell-
457 free spent medium. From the mixture of cyanobacterial metabolites in the medium we
458 identified 11 and quantified 5.

459 As *M. aeruginosa* was grown in BG11, *D. magna* had to be acclimatised to this medium
460 during several weeks before the experiment. Survival of the control group throughout the
461 experiment was always high (>98%). These results are in line with the literature where non-
462 treated *D. magna* survival was always high (close to 100%) in the first 8 days of experiments,
463 although specific *D. magna* cultivation medium was used (Dao *et al.* 2010, Ortiz-Rodríguez
464 *et al.* 2012), suggesting that BG 11 medium used in our study did not have impact on the *D.*
465 *magna* survival. Apart from increased CAT activities no other changes were observed in the
466 control group over the exposure time, we nevertheless suggest for further studies to
467 compromise between both media by adding necessary minerals, e.g. calcium, using diluted
468 BG 11, while keeping the osmolarity as we ensured in our exposures.

469 Cyanobacterial spent medium had a dose and time dependent negative impact on *D.*
470 *magna* survival, verifying our hypothesis. The concentrations used (Table 2.) were
471 detrimental thus preventing *D. magna* from acclimatisation during the exposure period. These
472 detrimental effects within 7 days were not expected as these concentrations were chosen
473 according to previous studies showing that survival of *D. magna* rapidly decreased during 3
474 weeks exposure to crude extract containing 50 µg/L of dissolved microcystin, while exposure
475 to 5 µg/L did not impact *Daphnia* survival much (Dao *et al.*, 2010). Similar results were
476 observed by Lürding and van der Grinten (2003) where exposure to 3.5 µg/L of dissolved
477 microcystin showed no significant decrease in survival over 7 days. When exposed to crude
478 extract or artificial mixtures of pure microcystins containing 60 µg/L of total MC (MC-LR

479 21.1 ± 1.9 µg/L; MC-RR 37.2 ± 4.8 µg/L and MC-YR 6.1 ± 0.8 µg/L) it did not appear to
480 have an impact on *D. pulex* survival for 6 days (Esterhuizen-Londt *et al.*, 2016). Our results
481 are, however, in line with studies exposing *D. magna* to *M. aeruginosa* containing 1.4–9 µg/L
482 of MC, where feeding was inhibited, followed by increase of mortality in the first week of
483 the treatment (DeMott, 1999; Demott *et al.*, 1991; Ghadouani *et al.*, 2004; Rohrlack *et al.*,
484 2001). *D. laevis* (two clones) and *D. similis* exposed to five *Microcystis* extracts in which
485 microcystins were detected (434 - 538 mg/g of lyophilized sample) suffered decreased
486 survival, reproduction and disturbance in egg production after 48 h (Herrera *et al.* 2015).
487 Crude extract obtained from *Microcystis* spp. had lethal effect on *D. magna* neonates, with
488 LC50 (48 h) ranging from 168.3–442.7 mg microcystin (total) DW L⁻¹ (Pham, 2018).
489 Susceptibility to microcystin is, however, not only species-specific, but *Daphnia* species can
490 also show different responses within their clones (DeMott, 1999; Hairston *et al.*, 1999;
491 Rohrlack *et al.*, 2001).

492 As both concentrations in our experiment caused mortality (20% for LC and 60% for
493 HC) within the 7 days exposure, we suggest a high *D. magna* sensitivity to the other
494 cyanobacterial metabolites present in the spent medium, as besides MC-LR and desmethyl
495 MC-LR, cyanopeptolin-A, aerucyclamides A and D were quantified, and aeruginosins (602
496 and 684), cyanopeptolins (B and 963) and aerucyclamides (B and C) detected. HC and LC of
497 *Microcystis* spent medium downregulated a gene encoding the digestive enzyme protease,
498 *ct383*, thus potentially interfered with *D. magna* digestion and as a consequence could
499 contribute to the increasing mortality in a concentration and time dependent manner.
500 Similarly, when *D. magna* were fed with MC-producing strain of PCC 7806 wild type, *ct383*
501 was downregulated after 6 days, leading to reduced total chymotrypsin activity
502 (Schwarzenberger *et al.*, 2010).

503 When fed with *Microcystis* strain UWOC MRC (a non-microcystin producing strain)
504 a lethal molting disruption in *Daphnia* spp. has been observed, suggesting that cyanobacterial
505 proteases, other than microcystin, could interfere with the molting cycle (Kaebernick *et al.*,
506 2001). Despite the absence of microviridins J in our medium, known to impair the molting
507 cycle in *Daphnia* via protease inhibition (Rohrlack *et al.*, 2004), upregulation of a gene
508 coding for nuclear hormone receptor (*hr3*) followed by significant downregulation of *ftz-fl*

509 transcripts involved in crustacean molting and development (Street *et al.*, 2019) has been
510 observed. Our results suggest that other metabolites have a role in impairment of these
511 pathways which may affect *Daphnia* molting.

512 Activity of anti-oxidative stress enzymes is a crucial defense mechanism against the
513 induction of oxidative stress by microcystins (Amado and Monserrat, 2010). Through
514 increased oxidative stress enzyme activities and detoxication, *D. magna* are able to enhance
515 acclimation to cyanobacterial metabolites (Ortiz-Rodríguez *et al.*, 2012). CAT is a highly
516 efficient antioxidant enzyme, responsible for reduction of H₂O₂ concentrations in the cells
517 (Fridovich, 1998). Elevated CAT activity can prevent oxidative damage caused by
518 cyanotoxins thus provides oxidative protection for *Daphnia* (Wojtal-Frankiewicz *et al.*,
519 2013). Increased CAT activity was observed in adults and neonates after 24 h when exposed
520 to 100 µg/L pure MC-LR, while exposure to 5 µg/L MC-LR or less, CAT activity was similar
521 to control (Ortiz-Rodríguez *et al.*, 2012). In our experiments, genes of antioxidant enzymes,
522 *cat* and *trx*, were downregulated, confirming the results of the enzyme activity. The
523 significant decrease in CAT activity on days 3 and 7, suggests that these enzymes were
524 exhausted due to the presence of the mixture of cyanobacterial compounds in the
525 concentrations applied. Similarly, CAT decreased in *D. magna* after being exposed to
526 cyanobacterial crude extract (containing 60 µg/L total MC) during the whole exposure period
527 of 72 h while the pure toxin MC-LR increased its activity (Esterhuizen-Londt *et al.* 2016).
528 Even when *D. magna* were exposed to extracts from non-microcystin and non-
529 cylindrospermopsin medium, their CAT decreased after day 1 and 7 (Dao *et al.*, 2013). In
530 combination with the observed lethality, our results suggest that *D. magna* oxidative defence
531 response was repressed in such way that oxidative damages may have occurred resulting in
532 lethal effects.

533 In *D. magna* and *D. longispina*, increased GST activity can detoxify microcystin up to a
534 certain concentration or exposure duration, which can also be transferred to the next
535 generation (Ortiz-Rodrigues *et al.*, 2012; Wojtal-Frankiewicz *et al.* 2013, 2014). Despite the
536 upregulation of *gst* within the first 3 days, our exposures, however, decreased GST activity,
537 compared with the control on day 3 and day 7, where expression of *gst* gene was
538 downregulated as well. Our GST results concern one or at maximum two out of on average

539 7 GST isoenzymes present in an organism, hence genes regulating other GST isoenzymes
540 may have had a different response. Asselman *et al.* (2012) showed that sigma-class *gst* genes
541 in *D. pulex* were upregulated when fed on MC-producing *M. aeruginosa* after 16 days.
542 However, our results are in line with previous studies where short term exposure (48 h) to
543 microcystin-producing *M. aeruginosa* PCC7806 increased *delta-gst* transcript levels, while
544 long term exposure (15 days) downregulated them (Lyu *et al.*, 2016a). Similar decrease in
545 GST activities were observed in *D. magna* exposed to cyanobacterial medium (containing 60
546 µg/L total MC) after 24 h and 72 h (Esterhuizen-Londt *et al.*, 2016). Furthermore, GST
547 activity in *D. magna* decreased with increased MC-LR concentrations from 10 to 2000 µg/L
548 (Chen *et al.*, 2005), suggesting enzymes exhaustibility as also seen in (Dao *et al.*, 2013).

549 Our results suggest that even lower concentrations of MC in the cyanobacterial medium,
550 along with the other cyanobacterial metabolites can affect enzyme activity in a similar way
551 like cyanobacterial medium containing higher microcystin concentrations. Besides
552 cyanobacterial metabolites that were detected, cyanobacterial medium could contain
553 undetected compounds that could potentially interfere with overall ecotoxicity (Smutná *et al.*,
554 2014) or inhibit enzymes activity, such as microcin SF608 that was shown to have
555 inhibitory impact on GST (Wiegand *et al.*, 2002).

556 When *Daphnia* are in toxic environments, mobilization of detoxification and antioxidant
557 defense mechanisms, as well as growth and development, comes with energetic cost (Calow,
558 1991; McKee and Knowles, 1986; Pane *et al.*, 2004). For instance, *D. magna* exposed to
559 nickel (Pane *et al.*, 2004) or the fungicide tebuconazole (McKee and Knowles, 1986) showed
560 increased energy consumption, as glycogen and lipids levels were decreased. In our
561 experiments energetic resources were instantly used, visible by the induction of *gapdh*
562 (involved in glycolysis) at day 3. Upregulation of *gapdh* has been observed in *D. magna* in
563 response to diet containing microcystins after 4 days of the experiment, suggesting that
564 ingestion of microcystin induces glycolysis and protein catabolism (Schwarzenberger *et al.*,
565 2009). Our results further suggest that severe depletion of energy affected also the muscular
566 activity, as the myosin heavy chain was immediately and significantly downregulated in both
567 experimental exposures (HC and LC). Depletion of energy in combination with
568 downregulation of the myosin heavy chain could provide a mechanistic explanation of the

569 locomotory inhibition (feeding and swimming) observed in previous studies (e.g. Rohrlack
570 *et al.*, 2001; Ghadouani *et al.*, 2004).

571 The response of the histone 2A gene (*h2A*) (involved in normal cell cycle progression)
572 may indicate two different cellular pathways, depending on the exposure concentration. The
573 downregulation at LC hints on apoptotic processes, while the upregulation at HC exposure
574 may imply cellular dysfunction occurring during necrotic processes, indicating a stronger
575 negative effect of HC medium on *Daphnia*, that is in line with the higher mortality in this
576 exposure. Activation of apoptotic pathways are positively correlated with the decline in *mhcr1*
577 (Tong *et al.*, 2017) that was also observed in our study, suggesting apoptotic processes in *D.*
578 *magna*, caused by cyanobacterial metabolites. Used concentrations of cyanobacterial
579 metabolites in our exposures may have been too high for *Daphnia* to adapt, therefore use of
580 lower concentration in future studies would provide deeper understanding of *Daphnia*'s
581 molecular response to the cyanobacterial metabolites.

582

583 **5. CONCLUSION**

584

585 In conclusion, *D. magna* survival was strongly affected by cyanobacterial spent medium
586 at both LC and HC, due to the combined effect of MC and the other secondary metabolites
587 present. *D. magna* were not able to acclimate during the short exposure period. Medium with
588 the highest tested concentration of cyanobacterial metabolites was the most detrimental as
589 expected. Exposure to cyanobacterial compounds in the *M. aeruginosa* spent medium
590 affected *D. magna* genes involved in i) digestion, thus mobilization of the limited internal
591 energetic resources, ii) oxidative stress and detoxification, iii) muscular activity and iv) cell
592 regulation including the molting process. We also observed consequences of cyanobacterial
593 medium on detoxification and antioxidant capacities which were dose and in particular time-
594 dependent indicating an exhaustion of the enzymes with high concentration or long exposure
595 duration. Despite this, the concentrations were chosen based on available data of pure MCs
596 or MCs in crude extracts causing low lethality, for following sublethal effects an even lower
597 concentration is recommended.

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