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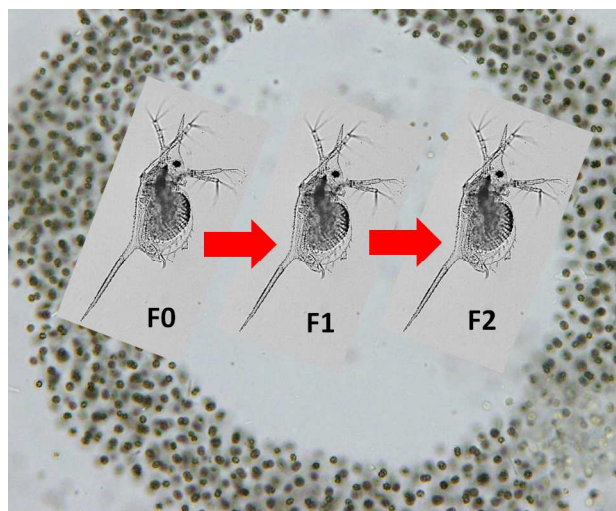
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Continuous exposure to microcystins

Environmentally relevant concentration

Life history traits:

- Survival
- Maturation
- Fecundity
- Body length

Weak negative effects in F0

Severe impacts on life traits in consecutive exposures

F1 generation showed no tolerance

F2 generation developed slight tolerance

1 Transgenerational effects of cyanobacterial toxins on a tropical micro-crustacean
2 *Daphnia lumholtzi* across three generations

3

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22

23 **Abstract**

24 Climate change and human activities induce an increased frequency and intensity of
25 cyanobacterial blooms which could release toxins to aquatic ecosystems.
26 Zooplankton communities belong to the first affected organisms, but in tropical
27 freshwater ecosystems, this issue has yet been poorly investigated. We tested two
28 questions (i) if the tropical *Daphnia lumholtzi* is capable to develop tolerance to an
29 ecologically relevant concentration of purified microcystin-LR and microcystins from
30 cyanobacterial extract transferable to F1 and F2 generations? And (ii) would F1 and
31 F2 generations recover if reared in toxin-free medium? To answer these questions,
32 we conducted two full factorial mutigenerational experiments, in which *D. lumholtzi*
33 was exposed to MC-LR and cyanobacterial extract at the concentration of $1 \mu\text{g L}^{-1}$
34 microcystin continuously for three generations. After each generation, each treatment
35 was split into two: one reared in the control (toxin free) while the other continued in
36 the respective exposure. Fitness-related traits including survival, maturity age, body
37 length, and fecundity of each *D. lumholtzi* generation were quantified. Though there
38 were only some weak negative effects of the toxins on the first generation (F0), we
39 found strong direct, accumulated and carried-over impacts of the toxins on life history
40 traits of *D. lumholtzi* on the F1 and F2, including reductions of survival, and
41 reproduction. The maturity age and body length showed some inconsistent patterns
42 between generations and need further investigations. The survival, maturity age (for
43 extract), and body length (for MC-LR) were only recovered when offspring from toxin
44 exposed mothers were raised in clean medium for two generations. Chronic
45 exposure to long lasting blooms, even at low density, evidently reduces survival of *D.*
46 *lumholtzi* in tropical lakes and reservoirs with ecological consequences.

47 **Key words:** microcystins, zooplankton, life history traits, tolerance, adaptation

48

49 **Capsule**

50 Exposure to environmentally relevant concentrations of cyanobacterial toxins for 3
51 generations tropical *Daphnia lumholtzi* developed no or marginal tolerance

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52 **1. Introduction**

53 Eutrophication and global climate change cause an increase of frequency and
54 intensity of cyanobacterial blooms with the occurrence of their toxic metabolites
55 (microcystins, MCs, amongst others; Harke et al., 2016). Besides being a public
56 health risk, cyanobacteria and their toxins can strongly alter the phytoplanktivorous
57 zooplankton communities, which are connecting the photosynthetic energy
58 acquaintance to consumption in food webs of aquatic ecosystems (Ger et al., 2016).
59 In standing or slow flowing tropical waters, the favorable temperature and nutrients
60 for cyanobacterial bloom are typically met all year round and MCs and other bioactive
61 cyanobacterial metabolites are commonly present (Chorus and Bartram, 1999; Mowe
62 et al., 2015). While MC-LR is one of the most potent MCs congeners to vertebrates,
63 other metabolites, e.g. microviridins, cyanopeptolines and others, could even
64 stronger impair daphnids than MCs (Ger et al., 2016). There is ample evidence
65 showing that exposures to cyanobacterial toxins and their metabolites can impair
66 behaviors, life history traits and biochemical responses of daphnids (e.g. Nizan et al.,
67 1986; DeMott et al., 1991; Ferrão-Filho et al., 2000; Rohrlack et al., 2001, 2004;
68 Wiegand et al., 2002; Lüring and Van der Grinten, 2003; Ghadouani et al., 2004;
69 Dao et al., 2013), but in most studies the exposure duration focused on one
70 generation and on species in northern temperate regions (reviewed in Ger et al.,
71 2016). Much less is known about how tropical daphnids deal with the cyanobacterial
72 toxins if exposure duration lasts for several generations. This question is particularly
73 relevant to the tropical freshwater ecosystems where cyanobacterial blooms are
74 predictable and last for months (Mowe et al., 2015) while the generation time of
75 tropical daphnia species such as *Daphnia lumholtzi* typically takes less than a week.

76 In *Daphnia*, maternal effects play an important role in their response to algal
77 toxins or contaminants across generations. Maternal effects may either increase or
78 decrease the offspring fitness. Many studies have showed that after exposure to
79 cyanobacterial toxins (Gustafsson and Hansson, 2004) or other contaminants
80 (Massarin et al., 2010; Krause et al., 2017) in the first generation, the next
81 generations showed an increase in tolerance. Typically in the the case when the
82 stressful conditions in which the mothers are living are predictable, they would invest
83 more in offspring fitness (Burgess and Marshall, 2014). This is supported by a
84 number of studies investigating the effects of cyanobacteria and their toxins beyond
85 one generation of the temperate species (e.g. *D. magna* Gustafsson and Hansson,
86 2004; Gustafsson et al., 2005; Ortiz-Rodriguez et al., 2012; von Elert et al., 2012) or
87 sub-tropical species (e.g. *D. carinata*; Jiang et al., 2013b). These studies consistently
88 revealed that the second generation of temperate daphnid species showed an
89 increased tolerance to cyanobacterial toxins that was associated with their elevated
90 base levels of detoxification enzymes e.g. glutathione S-transferase (Ortiz-Rodriguez
91 et al., 2012) or changing of digestive isoenzymes (von Elert et al., 2012). On the
92 other hand, several studies demonstrtd that parental exposure to harmful
93 compounds would have negative effects on offspring through adverse effects on
94 nutrition provisioning. This prediction was supported by a study from Beyer and
95 Hambright (2017) showing that the rotifer *Brachionus calyciflorus* exposed to
96 cyanobacteria produced offspring more vulnerable to algal toxins. Additionally, the
97 toxin tolerance of *D. magna* is clone specific (Gustafsson and Hansson, 2004;
98 Schwarzenberger et al., 2014). However, it is yet unknown if *Daphnia* species from
99 tropical regions may have different responses and toxin tolerance development
100 compared to their relatives from temperate and sub-tropical regions.

101 Most previous studies investigated the multigenerational effects on daphnids
102 using living cells of *Microcystis* rather than their extract or purified toxins (but see Dao
103 et al., 2010; Ortiz-Rodriguez et al., 2012). In nature, cyanobacteria typically form
104 large colonies (e.g. *Microcystis*) or long and big bunch filaments (e.g. *Anabaena*,
105 *Aphanizomenon*, *Planktothrix*) upon their mass development, which are not suitable
106 for consumption by micro-crustaceans such as *Daphnia* due to their size ($> 70 \mu\text{m}$;
107 Ebert, 2005) and unfavorable mucilage production (Rohrlack et al., 1999). Dissolved
108 cyanobacterial toxins, however, commonly occur and could last for days, weeks and
109 up to months, depending on the cyanobacterial lysis and the conditions in the water
110 (e.g. MCs; Chorus and Bartram, 1999; Giramida et al., 2013). Several studies
111 showed that living cells of cyanobacteria would induce stronger impacts than
112 dissolved cyanobacterial toxins on life history traits of daphnids (Nandini et al., 2017,
113 Lüring and Van der Grinten, 2003) due to nutritional insufficiency and feeding
114 inhibition.

115 One of the major gaps of knowledge that was highlighted in a review by Ger et
116 al. (2016) is whether tropical daphnid species may develop an increased tolerance to
117 cyanobacterial toxins after several generations have been exposed to the toxins as it
118 has been showed to many related temperate species (e.g. *D. magna*, Gustafsson
119 and Hansson, 2004). Tropical species or populations typically have faster life history
120 and shorter generation time and are more vulnerable to contaminants such as metals
121 than the temperate ones (Dinh Van et al., 2014). This is the result of the prioritizing
122 energy allocation to growth and development which in turn may trade off the energy
123 investing in elevating mechanisms to detoxify or excrete the toxins or contaminants
124 (Sibly and Calow, 1989; Congdon et al., 2001; Ortiz-Rodriguez et al., 2012). Hence it
125 could be that the offspring generation suffers more from the toxins as the

126 consequence of adverse effects on nutrition provisioning, or exposure during
127 embryonic development but this remains to be tested.

128 To address these issues, we conducted two full factorial mutigenerational
129 experiments in which the tropical micro-crustacean *D. lumholtzi* was exposed to the
130 cyanobacterial toxins both in purified form, MC-LR, and cyanobacterial extract for
131 three consecutive generations at $1 \mu\text{g L}^{-1}$ MCs. After each generation, each
132 treatment was split into two: one reared in the control (toxin free) and another one
133 continuously reared in the respective cyanobacterial toxins. This experimental design
134 resulted in 2 treatments in the F0 generation (control vs MC-LR or cyanobacterial
135 extract treatment), 4 and 8 treatments in generations F1 and F2, respectively. With
136 this approach, we tested two hypotheses: (1) the tropical *D. lumholtzi* develops an
137 increased tolerance to an ecologically relevant concentration of MCs in the next
138 generations (F1 and/or F2); and (2) F1 and F2 generations recover if reared in toxin-
139 free medium. Fitness-related traits such as survival, time to maturation, body length,
140 and fecundity of *D. lumholtzi* of each generation were quantified to examine these
141 hypotheses.

142

143 **2. Materials and methods**

144 **2.1 Chemicals and organisms for the tests**

145 Microcystin-LR (Enzo Life Science Inc) was dissolved in MeOH at a concentration of
146 1 mg mL^{-1} . The MCs-containing extract was prepared with reverse osmosis water
147 from a bloom of *Microcystis* in Dau Tieng Reservoir, Vietnam (Dao et al., 2014). The
148 extract was prepared from the collected bloom material by filtering, repeated
149 freeze/thaw cycles of the filters in distilled water to break the cells and centrifugation
150 to obtain the dissolved compounds; it was stored at -70°C after determining the MC-

151 LR ($20.3 \mu\text{g g}^{-1}$ dried weight, DW), MC-RR ($635 \mu\text{g g}^{-1}$ DW), and MC-YR ($31.7 \mu\text{g g}^{-1}$
152 DW) concentrations (Dao et al., 2014).

153 The tropical micro-crustacean *D. lumholtzi* (Bui et al., 2016) was used as test
154 organism. The culture of *D. lumholtzi* was initiated with more than 500 mothers
155 collected in a fish pond in northern Vietnam where the bloom of cyanobacteria had
156 not been previously observed since at least one year before. The culture has been
157 kept in the laboratory of Hochiminh City University of Technology for over 4 years
158 with the density of ca. 50 individuals L^{-1} . A previous study has showed that a
159 population of from 500 individuals is required to avoid gene diversity decrease (Colin
160 and Dam, 2004). The green alga *Chlorella* sp. and YTC (a mixture of yeast, cerrophyll
161 and trout chow digestion; US. EPA, 2002) were used as food for the daphnids. Both
162 *D. lumholtzi* and *Chlorella* sp. were in continuous culture in COMBO medium (Kilham
163 et al., 1998). The *D. lumholtzi* was fed *ad libitum* every second day with *Chlorella* and
164 YTC before the experiment.

165 **2.2 Experimental set up**

166 The experiments were conducted under laboratory conditions of 25 ± 1 °C, a
167 photoperiod of 12h light: 12h dark and the light intensity of around 1000 Lux (APHA,
168 2005) appropriate for the tropical *D. lumholtzi*. Tests were started with neonates of *D.*
169 *lumholtzi* (age ≤ 24 h, from 2nd – 3rd brood) obtained from a cohort of 50 mother
170 daphnids. Two experiments on the chronic effects of MC-LR or MCs-containing
171 cyanobacterial extract (E) at a concentration of $1 \mu\text{g L}^{-1}$ of either MC-LR or total MCs
172 from extract on *D. lumholtzi* were implemented according to Dao et al. (2010) with
173 minor modifications. Briefly, in the first experiment, neonates (called F0 *Daphnia*)
174 were randomly selected and individually incubated for each treatment in 50 mL glass
175 beakers containing 20 mL of exposure solutions (toxin free medium, C; and medium

176 containing MC-LR, M). Each treatment had 10 replicates ($n = 10$). The daphnids were
177 fed with 140,000 cells of *Chlorella* per mL (approximately 1 mg C L^{-1}) and 20 μL of
178 YTC. The test media and food were renewed every second day during the 14 days of
179 exposure. The offspring from the F0 control (F1 *Daphnia*) were split in 2 groups: a)
180 one was raised in control medium (CC) and b) one was raised in MC-LR containing
181 medium (CM). Similarly, the offspring from the MC-LR exposure were also split in 2
182 groups: a) raised in control medium (MC) and b) raised in medium containing MC-LR
183 (MM). The offspring from the second generation (called F2 *Daphnia*) were sampled
184 the same way and split and incubated in either control medium or MC-LR containing
185 medium, resulting in CCC, CCM, CMC, CMM, MCC, MCM, MMC, and MMM (Fig.
186 1a). The MeOH concentration in control was around $1 \mu\text{L L}^{-1}$, and this MeOH
187 concentration would not have side effects on *D. lumholtzi* because at the
188 concentration of $25 \mu\text{L L}^{-1}$ it showed no effects on life traits of *D. lumholtzi* during 21
189 days of incubation (our unpublished data).

190 Similarly, in the second experiment, the purified MC-LR was replaced by MCs-
191 containing cyanobacterial extract (E) at the concentration of $1 \mu\text{g MCs L}^{-1}$ (Fig 1b)
192 and *D. lumholtzi* were tested the same way as it was in the first experiment. The toxin
193 concentration of $1 \mu\text{g L}^{-1}$ was chosen because of three reasons: (i) the common
194 range of recorded dissolved MCs concentrations in natural water bodies ($0.1 - 10 \mu\text{g}$
195 L^{-1} , Chorus and Bartram, 1999), (ii) the WHO safety guideline value of MCs ($1 \mu\text{g L}^{-1}$
196 1) for drinking water supply (WHO, 1996), (iii) the range of cell bound MCs ($0.73 -$
197 $1.37 \mu\text{g L}^{-1}$) used in a three generational exposure to *D. magna* by Gustafsson et al.
198 (2005).

199 **2.3 Life history traits**

200 Life history traits of the *Daphnia* including mortality, maturity age, and reproduction
201 were scored daily. Maturity age was defined as the day on which the first egg
202 appeared in the brood chamber of the *Daphnia*. Numbers of neonates per clutch of
203 each mother daphnid were checked daily, collected and counted for clutch size to
204 evaluate the fecundity. Fecundity was calculated as total accumulated offspring
205 produced by a mother daphnid. When the tests terminated, living mother daphnids
206 were immediately fixed with Lugol solution (Sournia, 1978) and body length was
207 measured from the eye to the base of tail spine of the mothers, using a microscope
208 (Olympus BX 51) coupled with a digital camera (DP 71).

209 **2.4 Data analyses**

210 Survivorship rate of *D. lumholtzi* was calculated as percentage of which a gap of 20%
211 or more between two treatments was considered as significant difference (APHA,
212 2005). For other response variables, we ran general linear models for generations
213 F0, F1, and F2, respectively. In these models, direct exposure (F0) and the main
214 effects and interactions of direct exposure (F1 or F2) and previous exposures (F0
215 and/or F1) on *D. lumholtzi* was included as fixed factor(s) for generations F0, F1 and
216 F2, respectively. For body length of the third generation, because the body length
217 could not be measured for one treatment, ECC, we could only run the main effects of
218 E-F0, E-F1, E-F2 and the interactions of E-F1 and E-F2). The normal distribution of
219 data was tested by Shapiro-Wilk and the homogeneity of variances was tested by
220 Levene's tests. When there was a main effects or interactions of present and
221 previous exposures on a response variable, we performed a Bonferroni correction to
222 correct for multiple testing ($n = 1, 4$ and 8 Duncan's posthoc tests for the F0, F1 and
223 F2 generations, respectively; see appendix S1). All analyses were performed with
224 STATISTICA 12 (StatSoft Inc., Tulsa, OK, United States).

225

226 3. Results

227 3.1 Effects of microcystins on generation F0

228 In F0 generation, survival, maturity age and fecundity did not differ between the
229 control and MC-LR or cyanobacterial extract treatment (maturity age: MC-LR, $F_{1, 18} =$
230 0.24, $P = 0.63$ and cyanobacterial extract, $F_{1, 18} = 0.001$, $P = 0.99$; the fecundity: MC-
231 LR, $F_{1, 18} = 0.74$, $P = 0.40$ and cyanobacterial extract, $F_{1, 18} = 0.27$, $P = 0.61$, Fig. 2A-
232 E, G-H). For body length, there were no statistical differences between the control
233 and MC-LR treatment ($F_{1, 17} = 1.93$, $P = 0.18$), but *D. lumholtzi* exposed to
234 cyanobacterial extract grew slightly larger 0.1 mm (equivalent to 5%) in exposed
235 females compared to the control ($F_{1, 17} = 8.16$, $P = 0.011$, Fig. 2F).

236 3.2 Effects of microcystins on generation F1

237 In this exposure, most of the F1 that had been first time exposed to either MC-LR or
238 cyanobacterial extract (CM resp CE) confirmed the results of the F0 generation (M resp
239 E) concerning survival, maturity age, as well as body length for CM, and fecundity for
240 CE. However, some inconsistent responses occurred that body length decreased in
241 the CE treatment and fecundity in CM, both did not occur in the F0. Overall,
242 exposures to MC-LR and cyanobacterial extract for two continuous generations (MM,
243 EE, respectively) reduced survival, shortened body length, and lowered fecundity (all
244 P-corrected values < 0.05 , Fig. 3A-B, E-H). Exposure to MC-LR did not impact on
245 maturity age (Fig 3C) while cyanobacterial extract increased it by 2 days, equivalent
246 to 43 % of the development time (Fig 3D). The body length of MC-LR exposed
247 individuals was only significantly shortened after two generations, but for those
248 exposed to cyanobacterial extract, their body length was shorter with each

249 generation (11 % shorter in CE and 26 % in EE treatments; Fig. 3E, F) despite this
250 was not observed for the extract exposure in F0.

251 There were no signals of recovery when F1 individuals from exposed mothers
252 were reared in toxin-free medium (Fig. 4). Specifically, parental exposure to MC-LR
253 (MC) or cyanobacterial extract (EC) reduced survival, body length, and fecundity in
254 non exposed F1 to the same extent to what were observed in *D. lumholtzi* exposed to
255 MC-LR or cyanobacterial extract for two consecutive generations (MM and EE,
256 respectively, Fig 3; main effects of MC-LR-F0 and E-F0, Table S1 in Supplementary
257 1, Fig 4A-B, E-H). For example, when F1 individuals from exposed mothers were
258 reared in toxin free medium (MC and EC), the fecundities were still five times lower
259 than those whose mothers were cultured in clean medium (CC); this pattern was
260 comparable to what was observed in MM and EE to the control CC. MC-LR-exposed
261 mothers had no effect on maturity age in their offspring (Fig. 4C), but cyanobacterial
262 extract exposure of mothers caused a delayed maturity in their offspring (Fig. 4D).

263 **3.3 Effects of microcystins on generation F2**

264 In the third exposed generation (F2) occurred a tendency of a better survival in the
265 MC-LR or cyanobacterial extract exposed individuals, in comparison to the two
266 consecutive exposed generations before (F0 and F1) (Fig. 5A,B), indicating an
267 increase in tolerance. The third consecutive generation of exposed *D. lumholtzi*
268 survived better than the F2 but still not as good as after first exposure, and lower
269 than the controls (CCC).

270 Exposures to MC-LR for two (CMM) and three (MMM) consecutive
271 generations resulted in delayed maturation in F2 compared to those reared in control
272 (CCC) or exposed to MC-LR only in F2 (CCM) (Fig. 5C), but differed from F1 that
273 showed no difference in maturity age between CC and MM. *D. lumholtzi* exposed to

274 cyanobacterial extract, displayed the opposite of the inconsistent result between F1
275 and F2: EE in F1 was delayed in maturity, whereas CEE did not show a different
276 maturity age compared to CCC in F2. Delayed maturation only occurred in *D.*
277 *lumholtzi* exposed to cyanobacterial extract after three generations (Fig. 5D). Similar
278 to the maturity age, the inconsistent result of body length was also observed for the
279 F0 and F2 generations (Figs. 2F, 5F). Body length of *D. lumholtzi* was similar among
280 the exposure to MC-LR for one (CCM) two (CMM) or three (MMM) generations (Fig.
281 5E). For cyanobacterial extract, body length of F2 individuals was shortened after
282 exposure for two (CEE) or three (EEE) generations (Fig. 5F).

283 Fecundity dropped significantly when F2 individuals were exposed to MC-LR
284 for the first time (CCM, Fig 5G), and resulted in three times lower fecundity. No
285 further fecundity reduction occurred in F2 whose mother (CMM) or grand-mother
286 (MMM) were also exposed to MC-LR (Fig. 5G). For cyanobacterial extract, the
287 fecundity decreased after animals being exposed for two (CEE) and three
288 generations. Statistically, the fecundity of CEE and EEE was significantly lower than
289 that of CCC. However, significant difference was not observed between fecundity of
290 CCC and CCE, and CEE and EEE (Fig. 5H).

291 Hence, in extract exposure the better survival of the 3rd generation in
292 comparison to the 2nd was connected to a slower growth, which resulted in delayed
293 maturity, and consequently a lower fecundity. It is important to note that the fecundity
294 remained low or was even further decreased.

295 Similar to the comparison between the F0 and F1, most of the results focusing
296 on the third generation confirmed the previous observations, with some exceptions:
297 in the CMM treatment, maturity age was delayed, while it was neither significantly
298 different in F0 nor in F1. In contrast, the F2 CEE treated group did no longer suffer

299 from delayed maturity age, as did the EE treatment. Surprisingly, body length was no
300 longer significantly reduced in the CMM, as it was in the MM treatment of F1; and
301 similar for CCE versus CE treatment.

302 Some recovery occurred concerning survival (Fig. 6A, B) in the F2 generation
303 offspring from F0/F1 exposed mothers to both MC-LR and cyanobacterial extracts,
304 after one or two generations in toxin free environment (control). However, delayed
305 maturity age of MC-LR exposed-F0 offspring was not recovered after one and two
306 generations reared in toxin free environment (Fig 6C), nor did it completely disappear
307 in offspring of cyanobacterial extract-exposed F0 (Fig 6D). The reduced fecundity
308 was not recovered when offspring from F0-exposed animals were reared in toxin free
309 environment for one (MMC and MME) or two consecutive generations (MCC and
310 ECC, Fig. 6G-H). With few exceptions (survival and maturity age after extract
311 exposure), the recovery did not increase after 2 generations in toxin free medium.
312 Despite the observed recovery for some life traits, fecundity remained low.

313

314 4. Discussion

315 4.1 Effects of microcystins on generation F0

316 An ecologically relevant concentration of cyanobacterial toxins, either in form of the
317 pure MC-LR or as cyanobacterial extract, resulted in mild effects on fitness-related
318 traits including survival, and the accumulated number of neonates produced per
319 female *D. lumholtzi* in our study. The survival of *D. lumholtzi* in our study is in
320 agreement with previous studies in which *D. magna* exposed to similar
321 cyanobacterial toxin concentrations (e.g. 3.5 – 5 µg MC-LR L⁻¹; Lüring and Van der
322 Grinten, 2003; Dao et al., 2010). Exposure to higher densities of toxic *Microcystis*
323 may result in strong mortality of many *Daphnia* species such as *D. carinata*, *D.*

324 *magna*, *D. pulex*, *D. galeata*, *D. hyalina*, *D. pulicaria* (e.g. Rohrlack et al., 2001; Jiang
325 et al., 2013a). Also *D. lumholtzi* suffered more than 60% mortality when fed with
326 mixtures of *Scenedesmus* and *Microcystis* for 10 days at a higher density or
327 concentration (1 mg DW L⁻¹ of *Microcystis* equivalent to 280 µg MC L⁻¹) than
328 equivalent to our study (Semyalo et al., 2009). Higher concentrations, such as 5 and
329 50 µg L⁻¹ prolonged the developmental time and increased body length in *D. magna*
330 in a previous study (Dao et al., 2010). A longer body (0.1 mm, equivalent to 5%) was
331 surprisingly observed in *D. lumholtzi* after exposure to cyanobacterial extract. Despite
332 the significance of this result, the difference was in fact quite small and did not impact
333 on related life traits such as maturity age and fecundity (Fig. 2D, H).

334

335 **4.2 Effects of microcystins on generation F1**

336 Exposure to cyanobacterial toxins of the F1 generation whose mothers were reared
337 in toxin free medium confirmed most of the patterns of survival, maturity age, body
338 length (for MC-LR) and fecundity (for E) we found when exposing the F0 generation.
339 Some differences, however, occurred in body length of extract-exposed (CE) and the
340 fecundity of MC-LR exposed animals (CM). For the discrepancy concerning body
341 length, we cannot provide a sound explanation, however, the decrease of body
342 length in F1 was in line between the treatments (CC, CE, EE), which could hint to a
343 biological implication. The fecundity was declining with each MC-LR exposed
344 generation in the F1, evidencing the augmentation of the toxic impact. This could be
345 a consequence of a decreasing body length with the second continuously exposed
346 generation and is moreover connected to a reduction of the survival. Again, we could
347 not observe this in the treatment of the F0 for which we cannot provide a plausible
348 explanation at this point. Though MCs are very potently toxic to aquatic animals

349 (Stoner et al., 1989; Oberemm et al., 1999) other cyanobacterial metabolites from
350 extract might have generated the observed effects, but we didn't have the possibility
351 to determine in the current study.

352 Continuous exposure to both MC-LR and cyanobacterial extract resulted in
353 aggravated effects on fitness-related traits of F1 generation. This was expected, as
354 during exposure to low concentrations of cyanobacterial toxins, while not lethal,
355 *Daphnia* would have to spend more energy on amending the damages. In our study
356 we used MC-LR and MCs from extract at the concentration within the range that had
357 been tested with *D. magna* (0.07 – 6 $\mu\text{g L}^{-1}$), but much lower than used with *D.*
358 *carinata* (4.8 – 9.6 $\mu\text{g L}^{-1}$). Previous investigations showed that MCs deregulate
359 many processes in cells via protein phosphatases inhibition (MacKintosh et al.,
360 1990), enhance oxidative stress (Wiegand and Pflugmacher, 2005), and reduce the
361 ATP synthesis activity (Mikhailov et al., 2003), all of which to the expenditure of
362 energy to compensate. Exposed to MCs, *Daphnia* would spend energy for
363 physiological adjustments such as antioxidant and biotransformation enzyme
364 activities, toxin excretion and mechanisms of repairing damages that result in trade
365 offs concerning the energy for reproduction (Ortiz-Rodriguez et al., 2012).
366 Consequently, while F0 mothers *D. lumholtzi* could secure their survival, it can be
367 assumed that the energy allocated to cope with toxic stress in F0 mothers
368 diminished energetic resources and therewith the fitness of the F1 generation. This
369 can be interpreted as transmissive maternal effects (Marshall and Uller, 2007; Beyer
370 and Hambright, 2017).

371 Another important finding was that there was no signal of recovery when
372 offspring from F0-exposed *D. lumholtzi* were reared in toxin free medium. These
373 results are in agreement with previous studies (e.g., Gustafsson and Hansson, 2004;

374 Gustafsson et al., 2005). Dao et al. (2010) found a severe damage of embryos and
375 neonates inside brood chambers of mother *D. magna* exposed to MCs such as
376 decomposition, malformation and mortality. Probably, the neonate *D. lumholtzi* in the
377 current study were already negatively affected before released from their mothers'
378 brood chambers. Presumably these offspring did not develop sufficient physiological
379 ability to detoxify the harmful compounds. *D. lumholtzi* showed less tolerance
380 development than *D. magna* in a previous study, in which seven days of preexposure
381 of the parental generation induced detoxification and energy allocation enzymes
382 enabling the offspring to better withstand MC-LR (Ortiz-Rodriguez et al., 2012). In
383 that study, however, exposure of the mothers was clearly separated from exposure of
384 the offspring (Ortiz-Rodriguez et al., 2012), while in the current study, a continuous
385 exposure was chosen to mimic a more environmental relevant situation. Certain
386 temperate and sub-tropical daphnids such as *D. magna* and *D. carinata* however,
387 developed tolerance to toxins already in the next generation in similar experiments
388 after exposure to living cells of *Microcystis aeruginosa* containing around 5 – 7.5 µg
389 MCs L⁻¹ (Gustafsson and Hansson, 2004; Jiang et al., 2013b; Lyu et al., 2016).
390 These species specificities may be closely linked to the shift of zooplankton during
391 cyanobacterial blooms with the decrease of cladoceran abundance in temperate
392 water bodies (Hansson et al., 2007). Further *in situ* investigations on dynamics of
393 cyanobacterial biomass, toxins and cladoceran density in tropical freshwaters are
394 suggested.

395

396 **4.3 Direct and transgenerational effects of microcystins on generation F2**

397 In order to truly evaluate the transgenerational effects of contaminants or toxins on
398 species like *Daphnia* it is important to expose them to these stressors for at least

399 three generations (reviewed in Brander et al. 2017). So far, Gustafsson et al. (2005)
400 was the only study investigating impacts of toxic *Microcystis* on maturity ages, and
401 fecundity of the temperate species *D. magna* for 3 consecutive generations. They
402 evidenced increased fitness of *D. magna* already starting in the second generation
403 and no difference between the second and third generation (Gustafsson et al. 2005).
404 The authors used a *D. magna* clone isolated from a pond without cyanobacterial
405 blooms and preadapted for five months prior to their experiment. Tolerance of *D.*
406 *magna* to toxic *Microcystis* is clone specific (Gustafsson and Hansson, 2004). The *D.*
407 *lumholtzi* specimen used in our study originated as well from a pond without
408 cyanobacterial bloom but were cultivated in the laboratory for four years. While we do
409 not rule out a possibility for a genetic drift, the local adaptation to toxins from
410 cyanobacteria would be minor and indeed they showed a high sensitivity to both MC-
411 LR and extract at low concentration ($1 \mu\text{g L}^{-1}$). In our study, the second continuously
412 exposed *D. lumholtzi* generation (F1) was more vulnerable to MC-LR and MCs, while
413 there was visible increase of survival in the third continuously exposed generation
414 (F2). However, all other fitness-related traits were still below the control levels, hence
415 a complete tolerance development was not achieved. The better survival is, however,
416 to the expense of a later maturity in both treatments, which in turn is connected to a
417 decreased body length in the extract exposure and consequently to decreased
418 fecundity in both exposure scenarios.

419 It has been explained that the increased survival in offspring generations
420 derives from multiple factors: genetic selection, transgenerational or developmental
421 plasticity or maternal effects via epigenetics or provisioning (Brander et al., 2017). In
422 our study, the mortality was low therefore the decreased survival in F1 was unlikely a
423 result of genetic selection or stimulation as proposed by Gustafsson et al. (2005). It

424 could rather be a result of less energy allocated to the offspring or the adverse
425 effects of MCs during brood development (Dao et al. 2010) or both mechanisms.
426 Similarly, *Microcystis aeruginosa* decreased survival and fecundity of the rotifer
427 *Brachionus calyciflorus* probably due to constraints on the ability to up-regulate
428 detoxifying enzymes or to compensate for the nutritional inadequacy, or both (Beyer
429 and Hambright, 2017). Hence, toxic cyanobacterial biomass correlates negatively via
430 nutritional and toxin effects with cladoceran density (Ferrão-Filho et al., 2002;
431 Hansson et al., 2007). Bigger cladocerans were apparently more affected because
432 they unselectively ingested toxic cyanobacteria while smaller cladocerans seemed to
433 indirectly benefit, being more selective feeding groups. Consequently, toxic
434 cyanobacteria induce a shift in zooplankton size and community composition in
435 temperate inland waters (Hansson et al., 2007).

436 The reduced fecundity as total offspring was probably a result of delayed
437 maturity age, despite it did not occur in all the generations and with some
438 discrepancies between the generations. Start of reproduction is, however, a major
439 determinant of the reproductive output in copepod species, *Temora longicornis* (e.g.
440 Sichelau and Kiørboe, 2011), and cladoceran species, *D. magna* (Gustafsson et al.,
441 2005; Dao et al., 2010). Contrasting to the F1 generation, the body length was due to
442 the Bonferroni correction no longer significantly reduced in F2.

443 Importantly, the high mortality in F1 and the tendency of increased survival in
444 F2 suggest that maternal effects together with transgenerational, or developmental
445 plasticity may play a role in the slightly increased tolerance of *D. lumholtzi* to MCs
446 and cyanobacterial extracts. Whatever mechanisms, the consistent, slight increased
447 survival of *D. lumholtzi* to toxin in both forms: pure toxin and cyanobacterial extract is
448 especially important to explain the co-existence of *D. lumholtzi* with cyanobacteria

449 and MCs in tropical lakes. Even though F2 *D. lumholtzi* developed higher tolerance,
450 the still lower fecundity, however, possibly limits population survival in tropic lakes
451 with continuous cyanobacterial blooms if the following generations don't evolve a
452 better tolerance.

453 Our study revealed severe impairment of dissolved MCs at already $1 \mu\text{g L}^{-1}$ on
454 *D. lumholtzi* that may provide a mechanistic understanding to explain the low density
455 of *D. lumholtzi* in tropical lakes and reservoirs. It is also important to note that the
456 MC concentration of $1 \mu\text{g L}^{-1}$ is considered to be safe for drinking water for human
457 beings (WHO, 1996) while it has impairments on *D. lumholtzi* until at least the third
458 generation of this tropical *Daphnia* species. Further experiments are needed to
459 reveal differences between clones, and between populations of different exposure
460 and acclimation history.

461

462 **4.4 Conclusions**

463 Dissolved MCs at low concentration ($1 \mu\text{g L}^{-1}$) did not impact on life history traits of
464 F0 *D. lumholtzi*. Instead, continuously toxin exposure impaired the survivorship,
465 delayed maturation, and reproduction of the daphnids in F1 and F2 generations. The
466 trend of slightly recovery survival in F2 generation only partly support our first
467 hypothesis of an increased tolerance to ecologically relevant concentrations of MCs
468 within two generations. Our finding is controversial to previous investigations with
469 temperate and sub-tropical *Daphnia* species and suggests that adaptive maternal
470 effects are not applicable to all species of this genus. *D. lumholtzi* needed at least 2
471 consecutively exposed generations before signs of tolerance development appeared.
472 Only survival was moderately improved but not completely recovered when the
473 neonates from toxin experienced mother daphnids were raised in clean medium for

474 two generations. These results partly proved our second hypothesis of recovery
475 capacity of *D. lumholtzi* after three generations. Longer exposure duration is
476 therefore highly recommended to explicitly find out how many generations a tropical
477 daphnid like *D. lumholtzi* needs to adapt to low concentrations of cyanobacterial
478 toxins. Together with the study by Beyer and Hambright (2017), our study suggests
479 that mechanisms of adaptation to stress depend on the nature of the stressor, the
480 species and clone/population and most important the exposure history (including
481 their ancestors) of the specimen that are investigated. This challenges the
482 ecotoxicologists to identify which contaminants and zooplankton species would be
483 expected to rapidly increase in tolerance (e.g. Krause et al., 2017). Identifying this
484 requires comprehensive studies with different groups of zooplankton, different
485 classes of toxins and contaminants with multiple generations exposure durations, but
486 it would benefit conservation plans by identifying which are the most vulnerable
487 species in the tropical lakes and resevoirs. Furthermore, investigations on the
488 biochemical responses of *D. lumholtzi* exposed to MCs are suggested to unravel
489 underlying physiological mechanisms. Field monitoring on relation between
490 cladoceran community and MCs or cyanobacteria in tropical standing waters is
491 essential too.

492

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497

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652 **Figure legends**

653

654 **Fig. 1.** The experimental set up. C, control treatment; M, exposure solutions
655 containing $1 \mu\text{g L}^{-1}$ of MC-LR; E, exposure solutions containing $1 \mu\text{g L}^{-1}$ of MCs from
656 cyanobacterial extract. F0, F1 and F2 are the first, second and third generation of the
657 *D. lumholtzi*, respectively.

658

659 **Fig. 2.** Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and
660 fecundity (G, H) of *Daphnia lumholtzi* F0 generation in response to the MC-LR (M)
661 and cyanobacterial extract (E). Letters (a, b) on the bars indicate significant
662 difference among the exposures by Duncan's posthoc tests ($p < 0.05$).

663

664 **Fig. 3.** Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and
665 fecundity (G, H) of *Daphnia lumholtzi* F1 generation in response to exposures to the
666 MC-LR and cyanobacterial extract for one (CM or CE) and two (MM or EE)
667 consecutive generations. Letters (a, b, c) indicate significant difference among the
668 exposures by Duncan's posthoc tests ($p < 0.05$). Abbreviation as in Fig. 1.

669

670 **Fig. 4.** Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and
671 fecundity (G, H) of non-exposed F1 *Daphnia lumholtzi* after exposure of the F0 to
672 MC-LR and cyanobacterial extract. Letters (a, b) on the bars indicate significant
673 difference between the recovery of the F1 from non-exposed F0 and F1 from
674 exposed F0 by Duncan's posthoc tests ($p < 0.05$). Abbreviation as in Fig. 1.

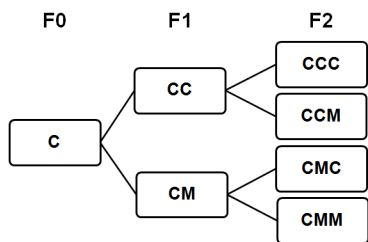
675

676 **Fig. 5.** Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and
677 fecundity (G, H) of *Daphnia lumholtzi* after one, two and three consecutive
678 generational exposure to MC-LR or MCs from cyanobacterial extract. Letters (a, b, c)
679 on the bars indicate significant difference among the exposures by Duncan's posthoc
680 tests ($p < 0.05$). Abbreviation as in Fig. 1.

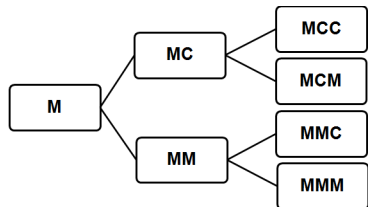
681

682 **Fig. 6.** Recovery capacity of *Daphnia lumholtzi* in generation F2 after one or two
683 generations reared in toxin free medium. Letters (a, b) on the bars indicate significant
684 difference among the treatments by Duncan's posthoc tests ($p < 0.05$). The body
685 length of the group ECC could not be measured. Abbreviation as in Fig. 1.

686

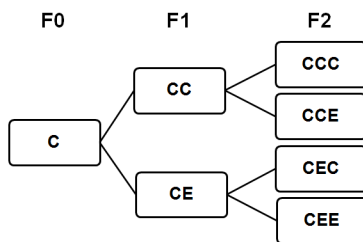


(a)

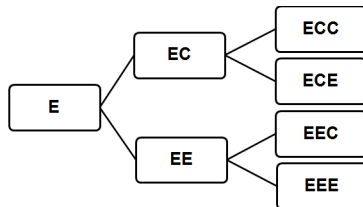


n = 10 replicates for each treatment

First experiment

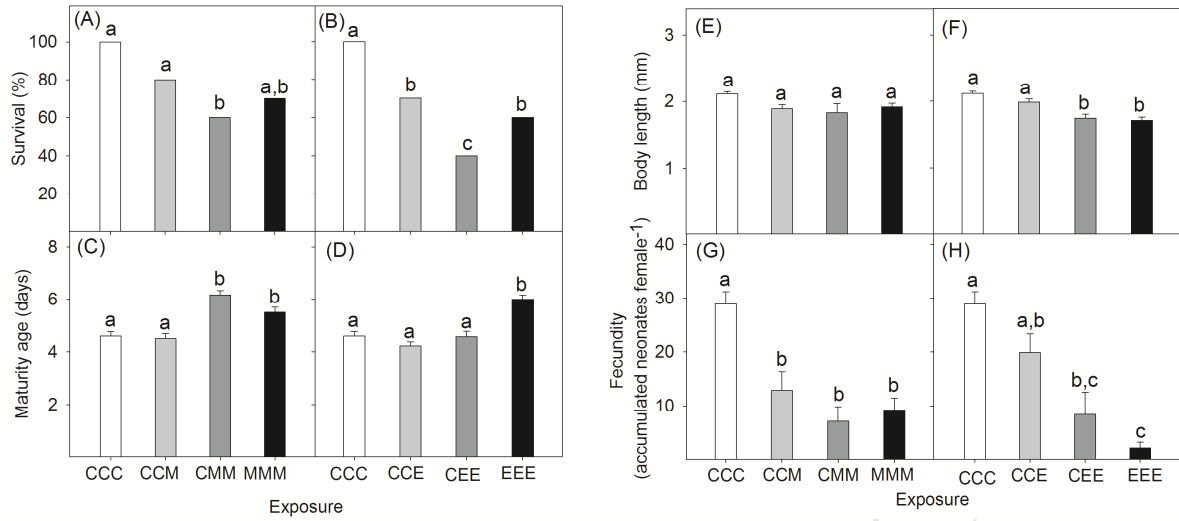


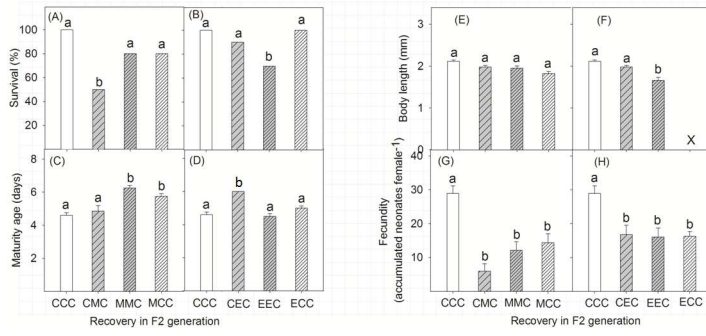
(b)

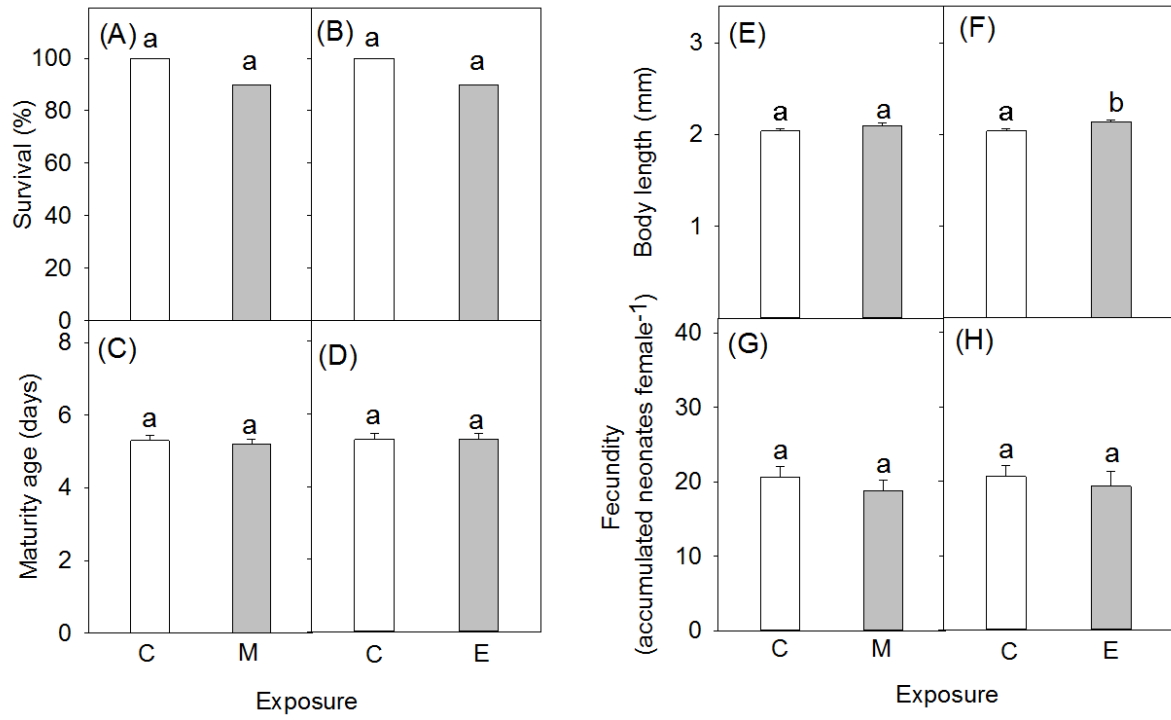


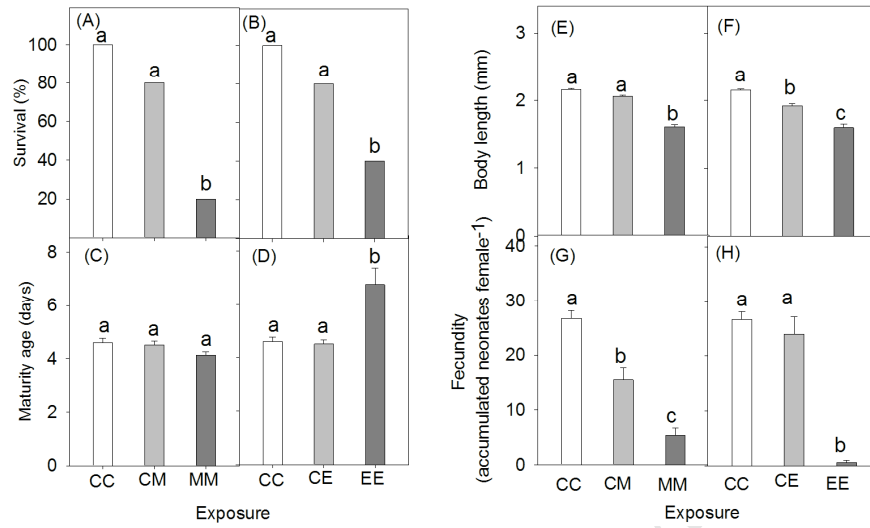
n = 10 replicates for each treatment

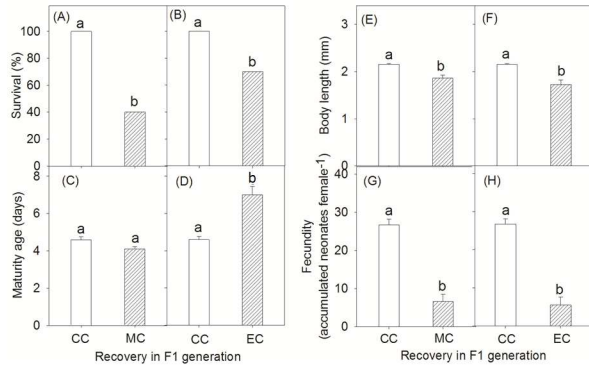
Second experiment











Highlights

- Tropical *Daphnia lumholtzi* exposed for 3 generations to cyanobacterial toxins
- Continuous toxin exposure impaired *D. lumholtzi* life traits in F1 and F2 generations
- *D. lumholtzi* started to develop tolerance after 2 consecutively exposed generations
- Maternal or transgenerational effects are lower in *D. lumholtzi* compared to *D. magna*