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1 **Responses of a tropical micro-crustacean, *Daphnia lumholtzi*, upon exposures to dissolved**
2 **toxins and living cells of cyanobacteria**

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15

16

17 **Abstract**

18 The mass development and expansion of cyanobacteria release cyanotoxins in the aquatic
19 environment and cause serious problems for grazers such as micro-zooplankton. In contrast to
20 aquatic ecosystems in temperate regions, impacts of cyanobacteria and their toxins on tropical
21 micro-crustaceans are relatively understudied. In this study, acute and chronic effects of pure
22 microcystin-LR (MC-LR), a crude extract of water bloom sample with a dominance of *Microcystis*
23 *aeruginosa* containing microcystins (MCE) and living cells of *Cylindrospermopsis curvispora* were
24 tested on a tropical micro-crustacean, *Daphnia lumholtzi*. The 24h- and 48h-LC₅₀ values for MC-
25 LR ranged from 247 – 299, and 331 – 409 µg MCE L⁻¹, respectively. Exposures to 1 – 25 µg MCE
26 L⁻¹ decreased survival, fecundity, and reproduction of *D. lumholtzi*. The impacts of *C. curvispora*
27 cells on life-history traits of *D. lumholtzi* were density-dependent and more severe than the impacts
28 of dissolved microcystins. It could be that the adverse effects of *C. curvispora* on *D. lumholtzi* are
29 linked to a combination of potential toxic metabolites, mal-nutrients, feeding and swimming
30 interference. *Daphnia lumholtzi* used in this study is more sensitive to microcystins compared to
31 *Daphnia* species from temperate regions. Therefore, we highly suggest using *D. lumholtzi* as a
32 model species for toxicity testing and monitoring on water quality, particularly in tropical countries.

33 **Keywords:** acute lethal concentration, clutch size, *Cylindrospermopsis curvispora*, microcystins,
34 reproduction, survival proportion

35

36 1. Introduction

37 Climate change and anthropogenic activities promote the global expansion and mass development
38 of harmful cyanobacteria in the aquatic environment (Huisman et al., 2018). In freshwater
39 ecosystems, bloom forming of cyanobacteria, especially *Microcystis* spp., causes serious problems
40 to organisms and wildlife due to oxygen depletion during their decay and the synthesis of bioactive
41 secondary metabolites (e.g. microcystins; Chorus and Bartram, 1999; Paerl and Fulton III, 2006, Li
42 et al., 2010). During blooms, cyanobacteria can remain in high densities in the aquatic environment
43 for several weeks; thus they can severely impact aquatic animals especially phytoplanktivorous
44 zooplankton, an intermediated position in the aquatic food chain (Lampert, 2006). Cyanobacteria
45 colonies or filaments are difficult to ingest, toxic and of low nutritional values for zooplankton as
46 they have low content of polyunsaturated fatty acids (PUFA) and lack sterols necessary for *Daphnia*
47 growth, development and reproduction (Martin-Creuzburg et al., 2008). As a consequence, the
48 abundance of cyanobacteria and their toxins correlates negatively with zooplankton biomass and
49 also induces a shift in zooplankton size and community composition (Hansson et al., 2007; Ger et
50 al., 2016).

51 Acute and chronic toxicity of cyanobacteria and their toxins and their detrimental impacts
52 on behaviors, biochemical responses, and life-history traits of zooplankton (e.g., *Daphnia*) are well
53 known (e.g., Ghadouani et al., 2004; Dao et al., 2010, 2013; Ger et al., 2016). Tests for median
54 lethal concentration (LC₅₀) of dissolved cyanotoxins to micro-crustaceans have been mainly
55 conducted with species of temperate regions. DeMott et al. (1991) treated temperate micro-
56 crustaceans with pure MC-LR and reported the 48h-LC₅₀ values ranging from 450 (*Diaptomus*
57 *birgei*) to 21,400 µg MC-LR L⁻¹ (*Daphnia pulex*). Other studies revealed the 48h-LC₅₀ values
58 from 84 – 2,550 µg MCE L⁻¹ for different clones of *Daphnia magna*, or from 34 – 1,906 µg MCE g
59 ¹ dry weight (DW) for *Daphnia similis* (Jungmann and Benndorf, 1994; Sotero-Santos et al., 2006;
60 Okumura et al., 2007; Smutna et al., 2014). Similar studies with tropical micro-crustaceans are rare,

61 with the 48h-LC₅₀ values of 80 – 460 µg MCE L⁻¹ for *Moina micrura*, and 120 – 180 µg MCE L⁻¹
62 for *Daphnia laevis* (Herrera et al., 2015). Sprague et al. (1971) suggested using 48h-LC₅₀ to
63 calculate safe concentrations (SC) of pollutants, which would not diminish the life-sustaining of
64 aquatic organisms such as fish. The authors suggested the SC value based on the 48h-LC₅₀
65 multiplied with an application factor of 0.01 – 0.04. Recently, Shen et al. (2019) calculated SC for
66 *D. magna* exposed to the dibutyl phthalate basing on the 24h- and 48-LC₅₀ values and the
67 application factor of 0.3. We will calculate SC for the investigated toxins and evaluate it in terms of
68 life trait consequences.

69 Likewise, investigations of chronic toxicity including life traits of cyanobacteria and their
70 toxins to *Daphnia* species have focused rather on temperate *Daphnia* (e.g., *D. magna*, *D. pulex*, *D.*
71 *similis*) than tropical *Daphnia* (e.g., *D. lumholtzi*) species (e.g., Hietala et al., 1997; Rohrlack et al.,
72 2001; Lürling and Van der Grinten, 2003; Dao et al., 2010; Smutna et al., 2014; Herrera et al.,
73 2015). Survival, body size, maturation, fecundity and reproduction of *D. magna* and *D. pulex* were
74 significantly reduced when they were fed with cyanobacterial isolates such as *Microcystis*
75 *aeruginosa* or *Cylindrospermopsis raciborskii* (Laurén-Määttä et al., 1997; Hietala et al., 1997;
76 Gustafsson and Hansson, 2004; Nogueira et al., 2004; Trubetskova and Haney, 2006).

77 The responses of tropical *Daphnia* chronically exposed to cyanobacterial toxins have only
78 been investigated recently. Living cells of *M. aeruginosa* impaired growth and survival of the
79 tropical micro-crustaceans *D. lumholtzi* during 10 days of incubation (Semyalo et al., 2009).
80 Moreover, *D. lumholtzi* suffered increasing impairment of life history traits over 3 generations
81 exposed to cyanobacterial toxins and very low recovery (Dao et al., 2018). Two other tropical
82 cladocerans (e.g. *D. laevis*, *M. micrura*) significantly reduced reproduction and enzymatic activity
83 (catalase, glutathione S-transferase) upon exposures to toxic cyanobacterial species, *M. aeruginosa*
84 and *C. raciborskii* (Ferrao-Filho et al., 2017).

85 In toxicity tests, widely distributed species *M. aeruginosa*, *Planktothrix agardhii* and *C.*
86 *raciborskii* are often used as living cyanobacterial cultures to test the toxicity on micro-crustaceans
87 via ingestions. Also, in tropical regions including Asia *Microcystis* sp. and *Cylindrospermopsis* sp.
88 are dominant, and alternating between wet and dry seasons (Move et al., 2015). The
89 cyanobacterium *Cylindrospermopsis curvispora* is a tropical and sub-tropical species, found in
90 Zambia, Botswana, Sri Lanka, Japan, Vietnam, and Southern Africa, and can produce the toxic
91 metabolite anatoxin-a (Cronberg and Komarek, 2004; Dao et al., 2016). However, the toxicity of
92 the cyanobacterium *C. curvispora*, to tropical cladocerans has not been reported. Investigating
93 effects of common cyanobacteria or cyanotoxins of tropical cyanobacteria (e.g. *C. curvispora*) on
94 the tropical cladocerans such as *D. lumholtzi* would enable us to have a more comprehensive
95 understanding and a better ecological risk assessment of cyanobacteria in tropical ecosystems.
96 Therefore, this study investigates the survivorship, fecundity, and reproduction of *D. lumholtzi* upon
97 both acute and chronic exposures to purified MC-LR, a crude extract of a water bloom sample with
98 dominance of *M. aeruginosa*, and living cells of the cyanobacterium *C. curvispora*.

99

100 **2. Materials and methods**

101 **2.1 Chemicals and organisms for the tests**

102 The cyanobacterium *Cylindrospermopsis curvispora* (Fig. 1a, b) was isolated from a pond in
103 District 7, Hochiminh City during its mass development and cultured in Z8 medium (Kotai, 1972).
104 The isolated strain is coiled with a diameter of between 20 – 25 µm, which is ingestible for *D.*
105 *lumholtzi*, and was a non-microcystin-producing strain (Dao et al., 2016). Microcystin-LR was
106 purchased from Enzo Life Science Inc. (USA). It was dissolved in reversed osmosis water prior to
107 the acute test and in methanol (MeOH) prior to the chronic test. The crude extract from
108 cyanobacterial bloom from Dau Tieng Reservoir, Vietnam, consisting of mainly *Microcystis*
109 *aeruginosa* producing microcystins, was used for toxicity tests. The extract was prepared from 5 g

110 dried weight (DW) of bloom material suspended into 100 mL reverse osmosis water according to
111 Pietsch et al. (2001). The extract (MCE) contained microcystin-LR, -RR, and -YR at the
112 concentrations of 20.3, 635.0 and 31.7 $\mu\text{g g}^{-1}$ DW, respectively (Dao et al., 2014) and was stored at
113 -70°C prior to experiment.

114 *Daphnia lumholtzi* (Fig. 1c) was used as a test organism, originating from a fishpond in
115 Northern Vietnam, that did not suffer from cyanobacterial blooms, and kept in culture for 7 years at
116 temperature ($25 \pm 1^{\circ}\text{C}$) and a light: dark cycle of 12h: 12h, with a light intensity of around 600 Lux
117 as described in Bui et al. (2016). *Daphnia lumholtzi* was fed with *Chlorella* sp. *at libitum*. Both *D.*
118 *lumholtzi* and *Chlorella* sp. were raised in COMBO medium (Kilham et al., 1998).

119

120 **2.2 Experimental setup**

121 The acute tests were conducted according to US EPA (2002) with minor modifications of the
122 temperature ($25 \pm 1^{\circ}\text{C}$) and light: dark cycle (12h: 12h), with a light intensity of around 600 Lux,
123 corresponding to the living conditions of the tropical *D. lumholtzi*. Neonates of individually raised
124 *D. lumholtzi* (age ≤ 24 h, from 2nd – 3rd clutch) were used for testing. Each treatment had four
125 replicates and each replicate contained 10 neonates in 40 mL of exposure solution in a 50-mL glass
126 vial. For acute tests seven concentrations of MC-LR (0, 100, 200, 300, 400, 500, 600 $\mu\text{g L}^{-1}$) or
127 microcystins-containing cyanobacterial extract (MCE) containing total microcystins (sum of MC-
128 LR, -YR, and -RR in the extract) of the same concentrations were prepared. The neonates were fed
129 before exposure but starved during the tests (US EPA, 2002). We checked every 24h and 48h
130 intervals for dead organisms, confirmed by the stop of the heartbeat, and removed them from the
131 vials. At the start and the end of the acute test, the pH (Metrohm 744) and dissolved oxygen (WTW
132 Oxil97i multi-detector) were measured.

133 Chronic tests were performed with the exposures of *D. lumholtzi* to MC-LR, MCE and
134 living cells of *C. curvispora* at the same laboratory conditions as the acute tests. The concentrations

135 of dissolved MC-LR or total microcystins from the extract were 0 (control), 1, 5 and 25 $\mu\text{g L}^{-1}$
136 (termed MC-LR1, MC-LR5, MC-LR25 and MCE1, MCE5, MCE25, respectively). Because MC-
137 LR used for the chronic test was dissolved in methanol (MeOH), another control was run with
138 MeOH at the concentration of 25 $\mu\text{L MeOH L}^{-1}$ (termed MeOH), the highest amount used to add
139 the toxin into the exposures. In the experiment with living *C. curvispora*, the *D. lumholtzi* were fed
140 with 3 regimes of food mixture (i) 90% *Chlorella* sp. + 10% *C. curvispora* (abbreviated as Cc10),
141 (ii) 50% *Chlorella* sp. + 50% *C. curvispora* (abbreviated as Cc50), and (iii) 100% *C. curvispora*
142 (abbreviated as Cc100). The total food (algae) in the test solution was 1 mg C L^{-1} , approximately
143 140,000 cells mL^{-1} of *Chlorella* sp., or 240,000 cells mL^{-1} of *C. curvispora* at 100% respectively
144 (Gustafsson and Hansson, 2004; Dao et al., 2017). Chronic tests were performed according to Dao
145 et al. (2010) with minor modifications. Briefly, 10 neonates of *D. lumholtzi* aged less than 24h were
146 individually incubated for each treatment in 50 mL glass vials containing 20 mL of exposure
147 solutions (n = 10). The test media were renewed every second day during the 21 days of exposure.
148 In each treatment, survival of mother *D. lumholtzi* was checked daily and the offspring was counted
149 and removed. In total, there were eleven treatments from the testing *D. lumholtzi* with dissolved
150 microcystins and living cells of *C. curvispora* (Table 1).

151

152 **2.3 Data analyses**

153 Median lethal concentrations with 95% confidence intervals (95% Cis) were calculated by Toxcalc
154 Program (Tidepool Scientific LLC. USA). The safe concentration (SC) of dissolved microcystins to
155 *D. lumholtzi* was calculated based on the 24h-LC₅₀ and 48h-LC₅₀ values according to Shen et al
156 (2019), $\text{SC} = (24\text{h-LC}_{50} \times 0.3) / (24\text{h-LC}_{50} / 48\text{h-LC}_{50})^3$.

157 Kruskal-Wallis test (Sigma Plot, version 12) was applied for testing the significances of
158 *Daphnia*' fecundity between control and toxin exposures. Survival proportion of *D. lumholtzi* in the

159 chronic test was calculated as the percentage of which a gap of 20% or more between two
160 treatments was considered as significant difference (APHA, 2012).

161

162 **3. Results and discussion**

163 ***3.1 Acute effects of dissolved MCs on D. lumholtzi***

164 In the acute test, pH and dissolved oxygen of the test solutions ranged from 6.6 – 7.6, and 6.5 – 7.7
165 mg L⁻¹, respectively, which is within the suitable range for *Daphnia* (APHA, 2012). Any negative
166 influences of the test solution characteristics on the survival of *D. lumholtzi* were unlikely. The 24h-
167 and 48h-LC₅₀ values for MC-LR exposures were 299 and 247 µg L⁻¹ and for MCE were 409 and
168 331 µg L⁻¹, respectively (Table 2). The cyanobacterial extract was presumably less toxic to *D.*
169 *lumholtzi* than purified MC-LR due to the high proportion of the less toxic congener, MC-RR
170 accounting for 92.4% of the total MCE in the cyanobacterial extract (Dao et al., 2014). Presumably,
171 the concentrations of other harmful cyanobacterial metabolites for *Daphnia* such as microviridins or
172 micropeptins that could be present in the extract were not sufficiently high to add to the overall
173 toxicity of the crude extract.

174 Interestingly, the 24h-LC₅₀ values for *D. lumholtzi* attained in our study (299 µg MC-LR L⁻¹
175 and 409 µg MCE L⁻¹) reveal this species more sensitive compared to other tropical species (Table
176 2). For example, *D. laevis* and *M. micrura* did not show mortality after 24h exposing to 1000 mg L⁻¹
177 of cyanobacterial dried mass (containing 538 µg MC-LR L⁻¹) (Herrera et al., 2015). In our study,
178 the 24h-LC₅₀ value for *D. lumholtzi* was equivalent to approximately 1020 mg L⁻¹ of cyanobacterial
179 dried mass of bloom material (containing 700 µg MCE L⁻¹). Similarly, 48h-LC₅₀ of *D. lumholtzi* to
180 MC-LR was 247 µg L⁻¹, suggesting that this species is more sensitive to MC-LR than temperate
181 micro-crustaceans such as *D. pulicaria*, *D. hyalina*, *D. pulex* and *Diaptomus birgei* as 48h-LC₅₀
182 values of these species range from 450 – 21,400 µg MC-LR L⁻¹ (DeMott et al., 1991; Table 2).
183 Correspondingly, *D. lumholtzi* seems to be more sensitive to MCE compared to *D. magna*, as 4 out

184 of 6 acute tests evidenced 48h-EC₅₀ values for immobilization for *D. magna* of at least 700 µg MCE
185 L⁻¹ (Smutna et al., 2014), which is more than two times higher than the 48h-LC₅₀ value for *D.*
186 *lumholtzi* obtained from our study (331 µg MCE L⁻¹; Table 2). In two other acute tests, the 48h-
187 EC₅₀ values for *D. magna* were > 84 and > 160 µg MCE L⁻¹ (Smutna et al., 2014) which were lower
188 than 48h-LC₅₀ value for *D. lumholtzi*. However, the difference of metabolites other than MCE
189 present in the bloom materials harvested at different times of the year might contribute to the
190 difference in the sensitivity of *D. magna* to the cyanobacterial extracts. Contrarily, the 48h-LC₅₀
191 values for tropical micro-crustaceans (*D. laevis* and *M. micrura*) varied between 80 – 460 µg MCE
192 L⁻¹ (Herrera et al., 2015), which covered the 48h-LC₅₀ values for *D. lumholtzi* in our study (Table
193 2). Therefore, there seems to be a general higher sensitivity of tropical micro-crustaceans than
194 temperate ones to MCE.

195 The calculated safe concentration (SC) values of MC-LR and MCE for *D. lumholtzi* were
196 35.5 and 38.5 µg L⁻¹, respectively, which is around 7 – 8.5 times lower than the 48h-LC₅₀ values
197 for *D. lumholtzi*. These SC concentrations are, however, higher than MC-LR and MCE
198 concentrations in the current chronic test which showed reductions in survival and reproduction of
199 *D. lumholtzi* from 1 µg L⁻¹ onwards (more details are presented below). Hence, this SC has limited
200 validity for the safety of the whole life span of the *Daphnia*. Sprague (1971) noted that the
201 application factor of 0.3 of the LC₅₀ value was suggested to attain a “limit of harmfulness or no
202 mortality” by pollutants (e.g. metals, pesticides) to aquatic animals (e.g. fish) in the field. The factor
203 value (0.3) was strongly supported by laboratory bioassay result with tubificids. Probably, different
204 species (fish, zooplankton) and their sizes would have different sensitiveness to one pollutant and
205 toxicity of one pollutant could be strongly regulated by some environmental conditions which are
206 not similar in the laboratory and *in situ*. The calculated SC values for neonate and adult *D. magna*
207 (0.56 and 0.98 mg dibutyl phthalate L⁻¹, respectively) were provided Shen et al. (2019) after testing
208 for max 94h, however, the authors did not discuss on their SC results. While this calculation seems

209 useful for acute toxicity testing, it has its limits for whole life cycle, hence population relevant
210 outcomes. Further investigations to determine the SC of cyanobacterial compounds to micro-
211 crustaceans would need to include measures for a whole life cycle to ensure survival at individual
212 and population levels.

213

214 **3.2 Chronic effects of dissolved MCs and live cells of *C. curvispora* on *D. lumholtzi***

215 **3.2.1 Effects on survivorship of *D. lumholtzi***

216 In our chronic tests, we found clear effects on MCE and live cells of *C. curvispora* on the mortality
217 of *D. lumholtzi* in a dose-response manner. Indeed, there was no mortality in control and MeOH
218 incubations (Fig. 2a), validating the control and solvent control treatment. The mortality of *D.*
219 *lumholtzi* increased with the levels of the MC-LR: MC-LR1 and MC-LR5 reduced the survival
220 proportion of *D. lumholtzi* down to 60% and 20%, respectively, by the end of the test and MC-LR25
221 caused 0% survival on day 12 of the exposure period. Similar to the acute test, the toxicity of the
222 MCE was lower than MC-RL: the survival rate of the animals in MCE1 and MCE5 were 70% and
223 30%, respectively, when the test was terminated, and 0% on the 14th day of the MCE25 treatment
224 (Fig. 2b). Survival proportion of *D. lumholtzi* exposed to living cyanobacterium *C. curvispora*
225 decreased over time in our study. In Cc10, more than 80% of *D. lumholtzi* survived during the first
226 12 days of incubation (Fig. 1c). However, the survival sharply declined thereafter to 20% by day 21
227 of the experiment. *Daphnia lumholtzi* in both Cc50 and Cc100 treatments already declined
228 substantially after 5 days of incubation. Only 10% of *D. lumholtzi* in Cc50 survived after 21 days of
229 exposure. In the Cc100, the last animal died on day 9 of the experiment (Fig. 2c).

230 In the exposure to pure MC-LR, in addition to its toxicity we can assume *D. lumholtzi*
231 suffered chronic effects via feeding inhibition or increasing costs for detoxification and mending
232 damages, as showed by Ghadouani et al. (2004) and Ortiz-Rodrigues et al. (2012). For the exposure
233 to the cyanobacterial extract, it can be assumed that other impacts, e.g. via impairing digestion (as

234 evidenced by e.g. Schwarzenberger et al., 2010) may add to the overall toxicity even though the
235 impact was lower in our study. In the exposures to *C. curvispora*, *D. lumholtzi* survival and
236 reproduction were already impaired from 0.1 mg C L⁻¹ of the cyanobacterium. Like other
237 cyanobacteria, *C. curvispora* is not a healthy food nor a rich nutrient (e.g. lacking PUFA) for micro-
238 crustaceans. On the other hand, it may contain toxic secondary metabolites (e.g. endotoxin
239 lipopolysaccharides as a gram negative bacterium, beside others). Moreover, the coil form of *C.*
240 *curvispora* may diminish the amount of *Chlorella* sp. filtered by the *D. lumholtzi*, and interfere the
241 normal swimming behavior of the animals.

242 Mortality rate of *D. magna* was not observed or very low if they are exposed to 3.5 µg MC-
243 LR L⁻¹ (Lüring and Van der Grinten, 2003), 50 µg MC-LR L⁻¹ (Dao et al., 2010) or even to 200 µg
244 MCE L⁻¹ (Smutna et al., 2014) during 21 days of incubation. Therefore, the cyanobacterial toxins
245 caused higher mortality to *D. lumholtzi* than to micro-crustaceans from temperate regions (e.g. *D.*
246 *magna*). The pure MC-LR is more toxic to *D. lumholtzi* than the MCE used in our study,
247 presumably due to its high content of MC-RR (92.4%). Our findings are consistent with the result
248 of Semyalo et al. (2009) also testing *D. lumholtzi* but using both, the toxic strain *M. aeruginosa*
249 PCC 7806 and its non-MCs producing mutant, evidencing a little higher effect of the MCE
250 producing one. The strain of *C. curvispora* used in our study did not contain microcystins (Dao et
251 al., 2016), but another strain of this species in Japan is known to produce anatoxin-a (Cronberg and
252 Komarek, 2004). We did not have a chance to analyze the anatoxin-a in our strain of *C. curvispora*.

253 We found a strong reduction of *D. lumholtzi* survival in the Cc10 (Fig. 1c) but the mortality
254 of *D. lumholtzi* by *C. curvispora* (Cc50) in our study seems to be lower than that by both
255 microcystins-producing and non-microcystins-producing *M. aeruginosa* (also 50% of food;
256 Semyalo et al., 2009). Ferrao-Filho et al. (2017) fed two micro-crustaceans species: *D. laevis* and
257 *M. micrura* with either 100% *M. aeruginosa* (producing microcystins) or 100% *C. raciborskii*
258 (producing saxitoxin) for 4 days. They found that *C. raciborskii* strongly reduced survival and

259 reproduction of the micro-crustaceans. *Moina micrura* was the most sensitive one among three
260 tested species. Hence, the influences of living cells of cyanobacteria on tropical micro-crustaceans
261 depend on the toxic metabolites and are species-specific.

262 In our previous study, Dao et al. (2016) incubated *D. magna* over 21 days with a mixture of
263 *C. curvispora* and *Scenedesmus* sp. at three similar ratios corresponding to the current study, 10%,
264 50% and 100% *C. curvispora*. Exposed *D. magna* showed high mortality rates of 33%, 60% and
265 67% in the 10%, 50% and 100% *C. curvispora*, respectively (Dao et al., 2016). Compared to the
266 current result, at the same ratio, the impact of *C. curvispora* on the survival of *D. lumholtzi* (Fig. 2c)
267 was even stronger than on *D. magna*. Keeping in mind that both species were tested at their optimal
268 temperatures (22 ± 1 and 25 ± 1 °C for *D. magna* and *D. lumholtzi*, respectively), this difference
269 could modify the toxicity but will ensure their best performance for other life traits. Hence the
270 tropical species *D. lumholtzi* seems to be more vulnerable to cyanobacterial living cells than the
271 temperate species, *D. magna* in their respective environment. The adverse effects of *C. curvispora*
272 seen in the current study could be closely linked to both, its poor nutritional characteristics and its
273 toxin-containing cells. It could be that toxicity of secondary metabolites (e.g. anatoxin-a,
274 cylindrospermopsin or PSP-toxins) in the cells of *C. curvispora* contributed to the higher mortality
275 of *D. lumholtzi*.

276

277 **3.2.2 Effects on fecundity and reproduction of *D. lumholtzi***

278 The fecundity of *D. lumholtzi* was alike in control and MeOH with 8 ± 2.7 and 9 ± 2.5 neonates per
279 clutch, respectively ($p = 0.105$, Kruskal-Wallis test). However, it decreased in the toxin treatments
280 (3 ± 1.7 in MC-LR1, $p < 0.001$; 5 ± 2.2 in MC-LR5, $p < 0.01$; and 2 ± 1.1 in MC-LR25, $p < 0.001$,
281 all compared to either control or MeOH), and, to a lesser extend also in the treatments with extract
282 (8 ± 4.5 in MCE1, $p = 0.685$; 6 ± 0.7 in MCE5, $p = 0.131$; and 2 ± 1.4 in MCE25, $p < 0.05$). In the

283 *C. curvispora* exposure, fecundity was reduced to 2.25 ± 0.9 in Cc10, ($p < 0.001$) and even
284 completely ceased to 0 in the higher Cc treatments (Fig. 3a).

285 During 21 days of incubation, the total neonates produced were 407 by control *D. lumholtzi*
286 and 442 by those in MeOH. The difference of cumulative offspring between control and MeOH was
287 around 8%, suggesting that MeOH did only slightly increase the reproduction of *D. lumholtzi*. The
288 total neonates of *D. lumholtzi* in MC-LR1, MC-LR5 and MC-LR25 decreased to 71, 34, and 8,
289 respectively. If we use the MeOH as the control for MC-LR exposures, the offspring proportion to
290 the solvent control (MeOH) of MC-LR1, MC-LR5, and MC-LR25 was 16%, 7.7% and 1.8%,
291 respectively. By the end of the chronic test, the *D. lumholtzi* in MCE1, MCE5 and MCE25
292 produced only 271, 11 and 4 neonates, respectively. Together with the LC_{50} values (Table 2) and
293 fecundity (Fig. 3a) it could be seen that the crude extract was less toxic than the pure MC-LR to *D.*
294 *lumholtzi*, again due to the less toxic microcystin variant MC-RR, being the main component in the
295 MCE. The cumulative offspring in MCE1 (271) are higher than in MC-LR1 (71). However, the
296 cumulative offspring in MC-LR5 (34) and MC-LR25 (8) were higher than those in MCE5 (11) and
297 MCE25 (4), respectively, which is surprising, and probably due to other compounds in the
298 cyanobacterial extract. It is the only parameter that shows a stronger effect of the crude extract and
299 indicates that other cyanobacterial metabolites add to the overall cyanobacterial toxicity. The
300 mother *D. lumholtzi* in Cc10 produced 54 neonates (Fig. 3b), whereas there was no offspring from
301 the Cc50 and Cc100 although not all mother *D. lumholtzi* died in Cc50 and Cc100 over 21 and 8
302 days, respectively (Fig. 2c).

303 Phytoplankton extract containing MC-LR similarly reduced fecundity and reproduction rates
304 in *D. laevis* (Herrera et al., 2015). In this study, the MC-LR concentrations of $1 - 25 \mu\text{g L}^{-1}$ caused a
305 reduction in reproduction of *D. lumholtzi*. In *D. magna*, no effect of MC-LR on the reproduction
306 was observed until the concentration of $3.5 \mu\text{g L}^{-1}$ (Lürding and Van der Grinten, 2003). The
307 negative effect of MC-LR on reproduction of *D. magna* was observed at the concentrations between

308 50 – 200 µg MC-LR L⁻¹ (Dao et al., 2010; Smutna et al., 2014) which is 3.5 – 200 times higher than
309 for *D. lumholtzi*, illustrating that the reproduction of *D. lumholtzi* is much more sensitive to MCE
310 exposures in than that of *D. magna*. The sensitivity of *D. lumholtzi* to other environmental stressors
311 such as trace metals was also higher than in temperate species such as *D. magna* (Dao et al., 2017).

312 Ferrao-Filho et al. (2017) reported a reduced clutch size and total offspring in *M. micrura*
313 exposed to around 3.2 ng saxitoxin L⁻¹, a very low toxin concentration of *C. raciborskii*. In our
314 study, we do not know whether *C. curvispora* can produce toxins (and it was negative for
315 microcystins), but fecundity and reproduction of Cc exposed *D. lumholtzi* were strongly reduced.
316 These results are consistent with above mentioned investigation of Ferrao-Filho et al. (2017).
317 *Daphnia magna* incubated with food ratios of 50%, and 100% *C. curvispora* produced 37 and 34
318 offsprings, respectively (Dao et al., 2016) whereas there was no accumulative offspring at all in the
319 experiment of *D. lumholtzi* treated with the same food ratios in the current study. This, again,
320 confirmed the lower tolerance of *D. lumholtzi* than *D. magna* to *C. curvispora*.

321 During chronic exposure, cyanobacteria can impair *Daphnia* not only via their toxicity, but
322 also by other mechanisms. Cyanobacterial compounds including microcystins can impair food
323 uptake of temperate and tropical cladocerans (Ghadouani et al., 2004; Ferrao-Filho et al., 2017).
324 Moreover, cyanobacterial compounds such as micropeptins directly inhibit digestive enzymes and
325 thus decrease the amount of energy that can be resorbed (Schwarzenberger et al., 2013), before an
326 acclimation takes place (Schwarzenberger et al., 2010). To some extent, microcystins can be
327 biotransformed and excreted in *Daphnia* as in other organisms and cause the oxidative stress and
328 other damages mended (Wiegand and Pflugmacher, 2005). The detoxification, however, increases
329 the energetic costs for maintenance, thus, in addition to the lower uptake and resorption, lowers
330 energy available for growth and reproduction (e.g. already described by Demott et al., 1999, Aleya
331 et al., 2006; Ortiz et al., 2012; Dao et al., 2013). Consequently, fecundity decreases in micro-

332 crustaceans as seen in this study and many others and will cause adverse outcomes on the
333 population level (Dao et al., 2010, 2018; Ferrao-Filho et al., 2017, 2019).

334 DEBtox theory and matrix population models are helpful tools to extrapolate the results
335 from the individual to the population level and can be used in understanding the interaction between
336 toxic cyanobacteria and zooplankton. Using DETBtox models, Billoir et al. (2009) correlated
337 impact on the tropical micro-crustaceans *M. micrura* with increasing food content of a *Microcystis*
338 sp. bloom. They documented that *M. micrura* decreased survival and reproduction at the
339 *Microcystis* sp. density of 0.25 mg C L⁻¹ or higher, regardless if the model took into consideration a
340 decrease in nutrient assimilation, or an increase in the costs for growth, maintenance or egg
341 production, or mortality during oogenesis.

342 In tropical lakes, zooplanktonic communities are characterized by low abundance, high
343 species richness and dominated by smaller and more selective grazers, and omnivorous organisms
344 like copepods (Ger et al., 2016; Rizo et al., 2017). Zooplankton communities are strongly controlled
345 by planktivorous fish, and the proportion between small (rotifers) and large (cladocerans)
346 zooplankton is regulated by food quality and quantity among other environmental factors (Wetzel,
347 2001; Rizo et al., 2017). Though high species richness of the genus *Daphnia* is a typical
348 characteristic of cladoceran assemblages, *D. lumholtzi* rarely occurs in temperate reservoirs and its
349 abundance is much lower than that of other *Daphnia* species (Havel et al., 1995; Pinto-Coelho et al.,
350 2005). Also, in tropical oligo- and eutrophic water bodies from Brazil and the Philippines *D.*
351 *lumholtzi* density was very low (Pinto-Coelho et al., 2005; Pascual et al., 2014). In the waterbodies
352 of Mexico, Figueroa-Sanchez et al. (2014) found many zooplankton species including cladocerans
353 such as *Bosmina longirostris*, *Ceriodaphnia lacustris*, *Daphnia laevis*, *Chydorus* cf. *sphaericus* and
354 *Alona* sp., but the density of *Daphnia* was low and only appeared for a short duration from January
355 to March. The authors also reported the dominance of cyanobacteria (*Microcystis*, *Anabaena*,
356 *Aphanizomenon*) among the phytoplankton associated with microcystins concentrations of

357 approximately $0.7 \mu\text{g L}^{-1}$, close to the lowest tested concentration in our study ($1 \mu\text{g L}^{-1}$).
358 Worldwide, more than 50% of lakes and reservoirs suffer eutrophication (Chorus and Bartram,
359 1999), out of which over 80% of freshwater ecosystems have been dominated by cyanobacterial
360 species of *Microcystis* and *Cylindrospermopsis* at least once over the last 30 years (Xiao et al.,
361 2020). The current laboratory study showed negative effects of dissolved microcystins and living
362 cyanobacteria on the survival, fecundity, and reproduction of *D. lumholtzi* that are three important
363 parameters for the population growth. Therefore, our results may be useful to explain that the low
364 density of *D. lumholtzi* in tropical lakes and reservoirs where cyanobacteria and potentially also
365 their toxins occur all year round.

366

367 **4. Conclusions and perspectives**

368 In conclusion, upon exposure to dissolved microcystins, the tropical *D. lumholtzi* seems more
369 sensitive than many other *Daphnia* species from temperate regions, in terms of survival, fecundity,
370 and reproduction. Because survival of *D. lumholtzi* was strongly impaired at low microcystins
371 concentrations, this species is recommended as a model species in studying the impacts of
372 cyanobacteria on tropical lakes. Living cells of *C. curvispora*, a non-microcystins-producing strain
373 from Vietnam, potentially caused mortality and inhibited reproduction of *D. lumholtzi*, evidencing that
374 this strain may contain toxic metabolites other than microcystins which need chemical
375 characterization to clarify. Besides, *C. curvispora* might have impaired the life traits of *D. lumholtzi*
376 by diminishing of feeding and swimming activity due to the filamentous form of the
377 cyanobacterium. Further studies on impacts of cyanobacterial toxins on behavior (e.g. food filtering
378 rate, swimming performance) and biochemical responses of *D. lumholtzi* are implied. The
379 abundance of *D. lumholtzi* and their interaction with cyanobacterial density and toxins in tropical
380 water bodies are suggested to investigate further. The potential invasion of *C. curvispora* into

381 temperate waterbodies and toxicity of this cyanobacterium to temperate zooplankton are
382 perspectives for *in situ* and empirical studies, respectively.

383

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388

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532

Accepted manuscript

533 **Table and figure legends**

534

535 Table 1. Summary of the chronic exposures of *Daphnia lumholtzi* to dissolved microcystins or live
536 cells of *Cylindrospermopsis curvispora*.

537

538 Table 2. Lethal concentration 48h-LC₅₀ (95% confidence-interval) of MC-LR and/or MCs-
539 containing cyanobacterial extract to zooplankton from this study compared to data from literature.
540 (*), 48h-EC₅₀ (median effective concentration 48h).

541

542 Fig. 1. The test organisms isolated from Vietnam; a and b, *Cylindrospermopsis curvispora* (scale
543 bars = 20 µm); and c, an adult *Daphnia lumholtzi* at its maturity age (scale bar = 500 µm).

544

545 Fig. 2. Survival proportion of *Daphnia lumholtzi* under chronic exposure to purified MC-LR (a),
546 cyanobacterial crude extract containing microcystins (b), and live cells of *Cylindrospermopsis*
547 *curvispora* (c). Abbreviations stand for treatments in Table 1.

548

549 Fig. 3. Fecundity (a) and reproduction (b) of *Daphnia lumholtzi* under chronic exposure to dissolved
550 microcystins and live cells of *Cylindrospermopsis curvispora*. The asterisks indicate the significant
551 difference by Kruskal-Wallis test (*, P < 0.05; **, P < 0.01; ***, P < 0.001) between control and
552 exposures. Abbreviations stand for treatments in Table 1.

553

554 Table 1. Summary of the chronic exposures of *Daphnia lumholtzi* to dissolved microcystins or live
 555 cells of *Cylindrospermopsis curvispora*

No.	Abbreviations	Exposures of <i>D. lumholtzi</i> to	Microcystins concentrations ($\mu\text{g L}^{-1}$)	Algae ratios (total food: 1 mg C L^{-1})
1	Control	No toxin, no methanol	0	100% <i>Chlorella</i> sp.
2	MeOH	25 $\mu\text{L MeOH L}^{-1}$	0	100% <i>Chlorella</i> sp.
3	MC-LR1	MC-LR	1	100% <i>Chlorella</i> sp.
4	MC-LR5	MC-LR	5	100% <i>Chlorella</i> sp.
5	MC-LR25	MC-LR	25	100% <i>Chlorella</i> sp.
6	MCE1	Microcystins containing crude extract	1	100% <i>Chlorella</i> sp.
7	MCE5	Microcystins containing crude extract	5	100% <i>Chlorella</i> sp.
8	MCE25	Microcystins containing crude extract	25	100% <i>Chlorella</i> sp.
9	Cc10	<i>Cylindrospermopsis curvispora</i>	0	90% <i>Chlorella</i> sp. + 10% <i>C. curvispora</i>
10	Cc50	<i>Cylindrospermopsis curvispora</i>	0	50% <i>Chlorella</i> sp. + 50% <i>C. curvispora</i>
11	Cc100	<i>Cylindrospermopsis curvispora</i>	0	100% <i>C. curvispora</i>

556

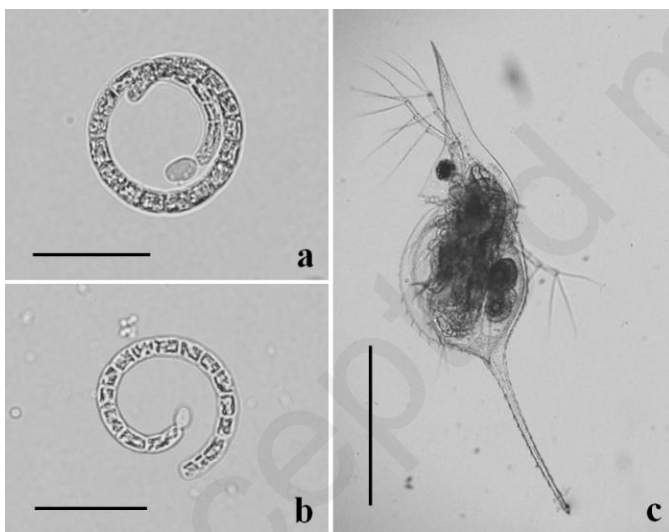
557

558 Table 2. Lethal concentration 48h-LC₅₀ (95% confidence-interval) of MC-LR and/or microcystins-
 559 containing cyanobacterial extract to zooplankton from this study compared to data from literature.
 560 (*), 48h-EC₅₀ (median effective concentration 48h).

Species	Toxins/crude extract	LC ₅₀ values	References
<i>Daphnia lumholtzi</i>	MC-LR	299 µg L ⁻¹ (265 – 338)	This study (24h-LC ₅₀)
<i>Daphnia lumholtzi</i>	MC-LR	247 µg L ⁻¹ (201 – 302)	This study (48h-LC ₅₀)
<i>Daphnia lumholtzi</i>	Microcystins- containing extract	409 µg L ⁻¹ (379 – 441)	This study (24h-LC ₅₀)
<i>Daphnia lumholtzi</i>	Microcystins- containing extract	331 µg L ⁻¹ (288 – 380)	This study (48h-LC ₅₀)
<i>Diaptomus birgei</i>	MC-LR	450 – 1,000 µg L ⁻¹	DeMott et al., 1991
<i>Daphnia pulicaria</i>	MC-LR	21,400 µg L ⁻¹	DeMott et al., 1991
<i>Daphnia hyalina</i>	MC-LR	11,600 µg L ⁻¹	DeMott et al., 1991
<i>Daphnia pulex</i>	MC-LR	9,600 µg L ⁻¹	DeMott et al., 1991
<i>Daphnia pulicaria</i>	crude extract of microcystins-	36,000 – 162,450 µg DW L ⁻¹	Jungmann and Benndorf, 1994;
<i>Daphnia similis</i>	producing <i>Microcystis</i>	34 – 1906 µg g ⁻¹ DW	Sotero-Santos et al., 2006; Okumura et al., 2007 ; Herrera et al., 2015
<i>Daphnia magna</i> *	crude extract of microcystins- containing cyanobacterial blooms	84 – > 2,550 µg L ⁻¹	Smutna et al., 2014
<i>Daphnia laevis</i>	phytoplankton extracts	120 – 180 µg MCs L ⁻¹	Herrera et al., 2015

	containing		
	microcystins		
<i>Moina micrura</i>	phytoplankton extracts	80 – 460 µg MCs L ⁻¹	Herrera et al., 2015
	containing		
	microcystins		
<i>Ceriodaphnia</i>	crude extract of	35.8 – 1,440 µg g ⁻¹ DW	Okumura et al., 2007
<i>silvestrii</i>	microcystins-		
	producing <i>Microcystis</i>		
<i>Ceriodaphnia</i>	crude extract of	32.6 – 1470 µg g ⁻¹ DW	Okumura et al., 2007
<i>dubia</i>	microcystins-		
	producing <i>Microcystis</i>		

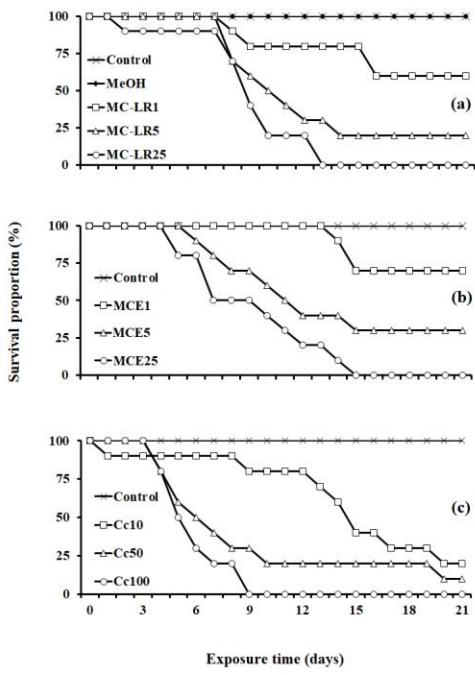
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563 Fig.1

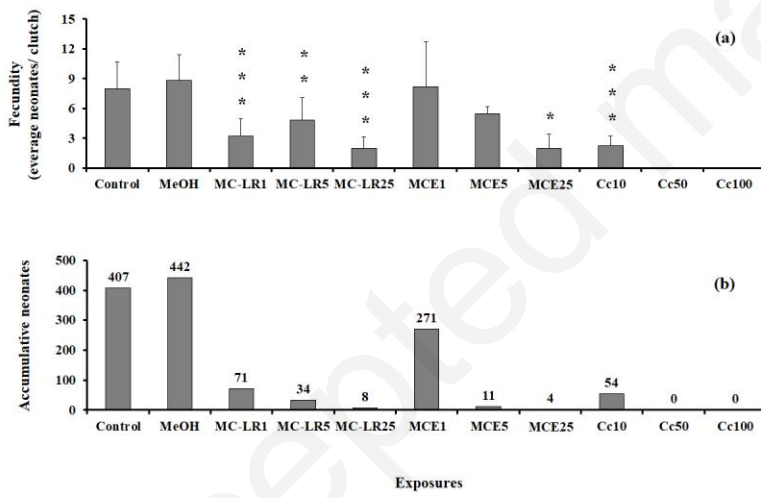
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566 Fig. 2

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569 Fig. 3