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



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## ABSTRACT

The mass development and expansion of cyanobacteria release cyanotoxins in the aquatic environment and cause serious problems for grazers such as micro-zooplankton. In contrast to aquatic ecosystems in temperate regions, impacts of cyanobacteria and their toxins on tropical micro-crustaceans are relatively understudied. In this study, acute and chronic effects of pure microcystin-LR (MC-LR), a crude extract of water bloom sample with a dominance of *Microcystis aeruginosa* containing microcystins (MCE) and living cells of *Cylindrospermopsis curvispora* were tested on a tropical micro-crustacean, *Daphnia lumholtzi*. The 24 h- and 48 h-LC<sub>50</sub> values for MC-LR ranged from 247–299, and 331–409  $\mu\text{g MCE L}^{-1}$ , respectively. Exposures to 1–25  $\mu\text{g MCE L}^{-1}$  decreased survival, fecundity, and reproduction of *D. lumholtzi*. The impacts of *C. curvispora* cells on life-history traits of *D. lumholtzi* were density-dependent and more severe than the impacts of dissolved microcystins. It could be that the adverse effects of *C. curvispora* on *D. lumholtzi* are linked to a combination of potential toxic metabolites, mal-nutrients, feeding and swimming interference. *Daphnia lumholtzi* used in this study is more sensitive to microcystins compared to *Daphnia* species from temperate regions. Therefore, we highly suggest using *D. lumholtzi* as a model species for toxicity testing and monitoring on water quality, particularly in tropical countries.

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## 1. Introduction

Climate change and anthropogenic activities promote the global expansion and mass development of harmful cyanobacteria in the aquatic environment (Huisman et al., 2018). In freshwater ecosystems, bloom forming of cyanobacteria, especially *Microcystis* spp., causes serious problems to organisms and wildlife due to oxygen depletion during their

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decay and the synthesis of bioactive secondary metabolites (e.g. microcystins; Chorus and Bartram, 1999; Paerl and Fulton III, 2006; Li et al., 2010). During blooms, cyanobacteria can remain in high densities in the aquatic environment for several weeks; thus they can severely impact aquatic animals especially phytoplanktivorous zooplankton, an intermediated position in the aquatic food chain (Lampert, 2006). Cyanobacteria colonies or filaments are difficult to ingest, toxic and of low nutritional values for zooplankton as they have low content of polyunsaturated fatty acids (PUFA) and lack sterols necessary for *Daphnia* growth, development and reproduction (Martin-Creuzburg et al., 2008). As a consequence, the abundance of cyanobacteria and their toxins correlates negatively with zooplankton biomass and also induces a shift in zooplankton size and community composition (Hansson et al., 2007; Ger et al., 2016).

Acute and chronic toxicity of cyanobacteria and their toxins and their detrimental impacts on behaviors, biochemical responses, and life-history traits of zooplankton (e.g., *Daphnia*) are well known (e.g., Ghadouani et al., 2004; Dao et al., 2010, 2013; Ger et al., 2016). Tests for median lethal concentration (LC<sub>50</sub>) of dissolved cyanotoxins to micro-crustaceans have been mainly conducted with species of temperate regions. DeMott et al. (1991) treated temperate micro-crustaceans with pure MC-LR and reported the 48h-LC<sub>50</sub> values ranging from 450 (*Diatomus birgei*) to 21,400 µg MC-LR L<sup>-1</sup> (*Daphnia pulicaria*). Other studies revealed the 48h-LC<sub>50</sub> values from 84–2550 µg MCE L<sup>-1</sup> for different clones of *Daphnia magna*, or from 34–1906 µg MCE g<sup>-1</sup> dry weight (DW) for *Daphnia similis* (Jungmann and Benndorf, 1994; Sotero-Santos et al., 2006; Okumura et al., 2007; Smutna et al., 2014). Similar studies with tropical micro-crustaceans are rare, with the 48h-LC<sub>50</sub> values of 80–460 µg MCE L<sup>-1</sup> for *Moina micrura*, and 120–180 µg MCE L<sup>-1</sup> for *Daphnia laevis* (Herrera et al., 2015). Sprague (1971) suggested using 48h-LC<sub>50</sub> to calculate safe concentrations (SC) of pollutants, which would not diminish the life-sustaining of aquatic organisms such as fish. The authors suggested the SC value based on the 48h-LC<sub>50</sub> multiplied with an application factor of 0.01–0.04. Recently, Shen et al. (2019) calculated SC for *D. magna* exposed to the dibutyl phthalate basing on the 24h- and 48-LC<sub>50</sub> values and the application factor of 0.3. We will calculate SC for the investigated toxins and evaluate it in terms of life trait consequences.

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

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

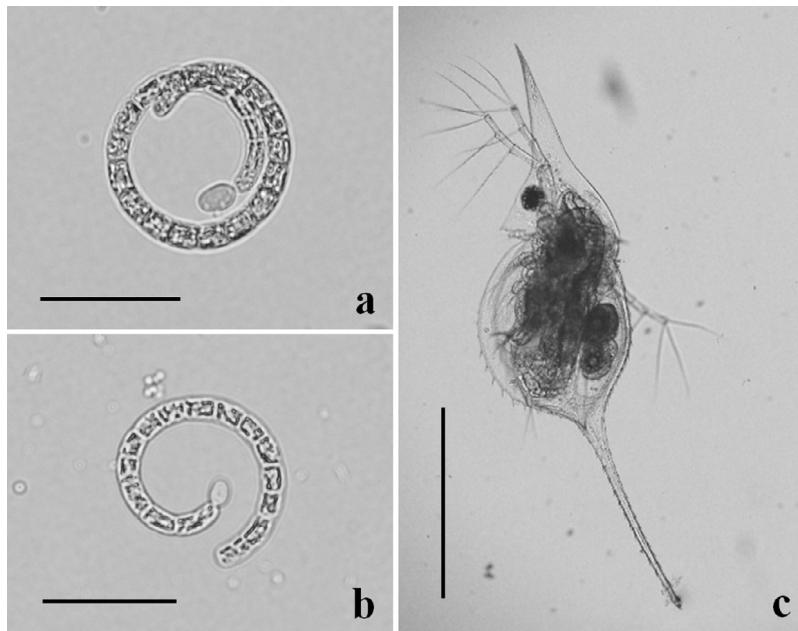
In toxicity tests, widely distributed species *M. aeruginosa*, *Planktothrix agardhii* and *C. raciborskii* are often used as living cyanobacterial cultures to test the toxicity on micro-crustaceans via ingestions. Also, in tropical regions including Asia *Microcystis* sp. and *Cylindrospermopsis* sp.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

## 2. Materials and methods

### 2.1. Chemicals and organisms for the tests

The cyanobacterium *Cylindrospermopsis curvispora* (Fig. 1a, b) was isolated from a pond in District 7, Hochiminh City during its mass development and cultured in Z8 medium (Kotai, 1972). The isolated strain is coiled with a diameter of between 20 – 25 µm, which is ingestible for *D. lumholtzi*, and was a non-microcystin-producing strain (Dao et al., 2016). Microcystin-LR was purchased from Enzo Life Science Inc. (USA). It was dissolved in reversed osmosis water prior to the acute test and in methanol (MeOH) prior to the chronic test. The crude extract from cyanobacterial bloom from Dau Tieng Reservoir, Vietnam, consisting of mainly *Microcystis aeruginosa* producing microcystins, was used for toxicity tests. The extract was prepared from 5 g dried weight (DW) of bloom material suspended into 100 mL reverse osmosis water according to Pietsch et al. (2001). The extract (MCE) contained microcystin-LR, -RR, and -YR at the concentrations of 20.3, 635.0 and 31.7 µg g<sup>-1</sup> DW, respectively (Dao et al., 2014) and was stored at -70 °C prior to experiment.

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



**Fig. 1.** The test organisms isolated from Vietnam; a and b, *Cylindrospermopsis curvispora* (scale bars = 20  $\mu\text{m}$ ); and c, an adult *Daphnia lumholtzi* at its maturity age (scale bar = 500  $\mu\text{m}$ ).

## 2.2. Experimental setup

The acute tests were conducted according to US EPA (2002) with minor modifications of the temperature ( $25 \pm 1^\circ\text{C}$ ) and light: dark cycle (12h: 12h), with a light intensity of around 600 Lux, corresponding to the living conditions of the tropical *D. lumholtzi*. Neonates of individually raised *D. lumholtzi* (age  $\leq 24$  h, from 2nd–3rd clutch) were used for testing. Each treatment had four replicates and each replicate contained 10 neonates in 40 mL of exposure solution in a 50-mL glass vial. For acute tests seven concentrations of MC-LR (0, 100, 200, 300, 400, 500, 600  $\mu\text{g L}^{-1}$ ) or microcystins-containing cyanobacterial extract (MCE) containing total microcystins (sum of MC-LR, -YR, and -RR in the extract) of the same concentrations were prepared. The neonates were fed before exposure but starved during the tests (US EPA, 2002). We checked every 24h and 48h intervals for dead organisms, confirmed by the stop of the heartbeat, and removed them from the vials. At the start and the end of the acute test, the pH (Metrohm 744) and dissolved oxygen (WTW Oxi197i multi-detector) were measured.

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

## 2.3. Data analyses

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

Kruskal–Wallis test (Sigma Plot, version 12) was applied for testing the significances of *Daphnia*' fecundity between control and toxin exposures. Survival proportion of *D. lumholtzi* in the chronic test was calculated as the percentage of which a gap of 20% or more between two treatments was considered as significant difference (APHA, 2012).

**Table 1**Summary of the chronic exposures of *Daphnia lumholtzi* to dissolved microcystins or live 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 <sup>-1</sup> )
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>

### 3. Results and discussion

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

In the acute test, pH and dissolved oxygen of the test solutions ranged from 6.6 – 7.6, and 6.5 – 7.7 mg L<sup>-1</sup>, respectively, which is within the suitable range for *Daphnia* (APHA, 2012). Any negative influences of the test solution characteristics on the survival of *D. lumholtzi* were unlikely. The 24h- and 48h-LC<sub>50</sub> values for MC-LR exposures were 299 and 247  $\mu\text{g L}^{-1}$  and for MCE were 409 and 331  $\mu\text{g L}^{-1}$ , respectively (Table 2). The cyanobacterial extract was presumably less toxic to *D. lumholtzi* than purified MC-LR due to the high proportion of the less toxic congener, MC-RR accounting for 92.4% of the total MCE in the cyanobacterial extract (Dao et al., 2014). Presumably, the concentrations of other harmful cyanobacterial metabolites for *Daphnia* such as microviridins or micropeptins that could be present in the extract were not sufficiently high to add to the overall toxicity of the crude extract.

Interestingly, the 24h-LC<sub>50</sub> values for *D. lumholtzi* attained in our study (299  $\mu\text{g MC-LR L}^{-1}$  and 409  $\mu\text{g MCE L}^{-1}$ ) reveal this species more sensitive compared to other tropical species (Table 2). For example, *D. laevis* and *M. micrura* did not show mortality after 24h exposing to 1000 mg L<sup>-1</sup> of cyanobacterial dried mass (containing 538  $\mu\text{g MC-LR L}^{-1}$ ) (Herrera et al., 2015). In our study, the 24h-LC<sub>50</sub> value for *D. lumholtzi* was equivalent to approximately 1020 mg L<sup>-1</sup> of cyanobacterial dried mass of bloom material (containing 700  $\mu\text{g MCE L}^{-1}$ ). Similarly, 48h-LC<sub>50</sub> of *D. lumholtzi* to MC-LR was 247  $\mu\text{g L}^{-1}$ , suggesting that this species is more sensitive to MC-LR than temperate micro-crustaceans such as *D. pulicaria*, *D. hyalina*, *D. pulex* and *Diaptomus birgei* as 48h-LC<sub>50</sub> values of these species range from 450–21,400  $\mu\text{g MC-LR L}^{-1}$  (DeMott et al., 1991; Table 2). Correspondingly, *D. lumholtzi* seems to be more sensitive to MCE compared to *D. magna*, as 4 out of 6 acute tests evidenced 48h-EC<sub>50</sub> values for immobilization for *D. magna* of at least 700  $\mu\text{g MCE L}^{-1}$  (Smutna et al., 2014), which is more than two times higher than the 48h-LC<sub>50</sub> value for *D. lumholtzi* obtained from our study (331  $\mu\text{g MCE L}^{-1}$ ; Table 2). In two other acute tests, the 48h-EC<sub>50</sub> values for *D. magna* were > 84 and > 160  $\mu\text{g MCE L}^{-1}$  (Smutna et al., 2014) which were lower than 48h-LC<sub>50</sub> value for *D. lumholtzi*. However, the difference of metabolites other than MCE present in the bloom materials harvested at different times of the year might contribute to the difference in the sensitivity of *D. magna* to the cyanobacterial extracts. Contrarily, the 48h-LC<sub>50</sub> values for tropical micro-crustaceans (*D. laevis* and *M. micrura*) varied between 80–460  $\mu\text{g MCE L}^{-1}$  (Herrera et al., 2015), which covered the 48h-LC<sub>50</sub> values for *D. lumholtzi* in our study (Table 2). Therefore, there seems to be a general higher sensitivity of tropical micro-crustaceans than temperate ones to MCE.

The calculated safe concentration (SC) values of MC-LR and MCE for *D. lumholtzi* were 35.5 and 38.5  $\mu\text{g L}^{-1}$ , respectively, which is around 7 – 8.5 times lower than the 48h-LC<sub>50</sub> values for *D. lumholtzi*. These SC concentrations are, however, higher than MC-LR and MCE concentrations in the current chronic test which showed reductions in survival and reproduction of *D. lumholtzi* from 1  $\mu\text{g L}^{-1}$  onwards (more details are presented below). Hence, this SC has limited validity for the safety of the whole life span of the *Daphnia*. Sprague (1971) noted that the application factor of 0.3 of the LC<sub>50</sub> value was suggested to attain a “limit of harmfulness or no mortality” by pollutants (e.g. metals, pesticides) to aquatic animals (e.g. fish) in the field. The factor value (0.3) was strongly supported by laboratory bioassay result with tubificids. Probably, different species (fish, zooplankton) and their sizes would have different sensitiveness to one pollutant and toxicity of one pollutant could be strongly regulated by some environmental conditions which are not similar in the laboratory and *in situ*. The calculated SC values for neonate and adult *D. magna* (0.56 and 0.98 mg dibutyl phthalate L<sup>-1</sup>, respectively) were provided Shen et al. (2019) after testing for max 94h, however, the authors did not discuss on their SC results. While this calculation seems useful for acute toxicity testing, it has its limits for whole life cycle, hence population relevant

**Table 2**

Lethal concentration 48h-LC<sub>50</sub> (95% confidence-interval) of MC-LR and/or microcystins-containing cyanobacterial extract to zooplankton from this study compared to data from literature. (\*), 48h-EC<sub>50</sub> (median effective concentration 48h).

Species	Toxins/crude extract	LC <sub>50</sub> values	References
<i>Daphnia lumholtzi</i>	MC-LR	299 $\mu\text{g L}^{-1}$ (265 – 338)	This study (24h-LC <sub>50</sub> )
<i>Daphnia lumholtzi</i>	MC-LR	247 $\mu\text{g L}^{-1}$ (201 – 302)	This study (48h-LC <sub>50</sub> )
<i>Daphnia lumholtzi</i>	Microcystins-containing extract	409 $\mu\text{g L}^{-1}$ (379 – 441)	This study (24h-LC <sub>50</sub> )
<i>Daphnia lumholtzi</i>	Microcystins-containing extract	331 $\mu\text{g L}^{-1}$ (288 – 380)	This study (48h-LC <sub>50</sub> )
<i>Diatomus birgei</i>	MC-LR	450 – 1,000 $\mu\text{g L}^{-1}$	DeMott et al. (1991)
<i>Daphnia pulicaria</i>	MC-LR	21,400 $\mu\text{g L}^{-1}$	DeMott et al. (1991)
<i>Daphnia hyalina</i>	MC-LR	11,600 $\mu\text{g L}^{-1}$	DeMott et al. (1991)
<i>Daphnia pulex</i>	MC-LR	9,600 $\mu\text{g L}^{-1}$	DeMott et al. (1991)
<i>Daphnia pulicaria</i>	crude extract of microcystins-producing <i>Microcystis</i>	36,000 – 162,450 $\mu\text{g DW L}^{-1}$	Jungmann and Benndorf (1994), Sotero-Santos et al. (2006), Okumura et al. (2007), Herrera et al. (2015)
<i>Daphnia similis</i>		34 – 1906 $\mu\text{g g}^{-1}\text{DW}$	
<i>Daphnia magna</i> *	crude extract of microcystins-containing cyanobacterial blooms	84 – > 2,550 $\mu\text{g L}^{-1}$	Smutna et al. (2014)
<i>Daphnia laevis</i>	phytoplankton extracts containing microcystins	120 – 180 $\mu\text{g MCs L}^{-1}$	Herrera et al. (2015)
<i>Moina micrura</i>	phytoplankton extracts containing microcystins	80 – 460 $\mu\text{g MCs L}^{-1}$	Herrera et al. (2015)
<i>Ceriodaphnia silvestrii</i>	crude extract of microcystins-producing <i>Microcystis</i>	35.8 – 1,440 $\mu\text{g g}^{-1}\text{DW}$	Okumura et al. (2007)
<i>Ceriodaphnia dubia</i>	crude extract of microcystins-producing <i>Microcystis</i>	32.6 – 1470 $\mu\text{g g}^{-1}\text{DW}$	Okumura et al. (2007)

outcomes Further investigations to determine the SC of cyanobacterial compounds to micro-crustaceans would need to include measures for a whole life cycle to ensure survival at individual and population levels.

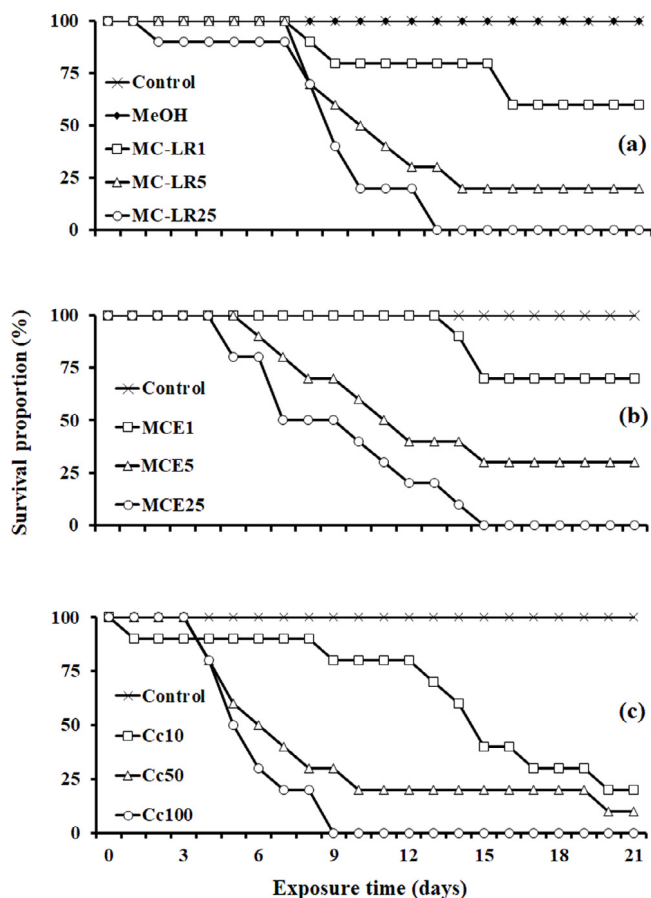
### 3.2. Chronic effects of dissolved MCs and live cells of *C. curvispora* on *D. lumholtzi*

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

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

In the exposure to pure MC-LR, in addition to its toxicity we can assume *D. lumholtzi* suffered chronic effects via feeding inhibition or increasing costs for detoxification and mending damages, as showed by Ghadouani et al. (2004) and Ortiz-Rodriguez et al. (2012). For the exposure to the cyanobacterial extract, it can be assumed that other impacts, e.g. via impairing digestion (as evidenced by e.g. Schwarzenberger et al., 2010) may add to the overall toxicity even though the impact was lower in our study. In the exposures to *C. curvispora*, *D. lumholtzi* survival and reproduction were already impaired from 0.1 mg C L<sup>-1</sup> of the cyanobacterium. Like other cyanobacteria, *C. curvispora* is not a healthy food nor a rich nutrient (e.g. lacking PUFA) for micro-crustaceans. On the other hand, it may contain toxic secondary metabolites (e.g. endotoxin lipopolysaccharides as a gram negative bacterium, beside others). Moreover, the coil form of *C. curvispora* may diminish the amount of *Chlorella* sp. filtered by the *D. lumholtzi*, and interfere the normal swimming behavior of the animals.

Mortality rate of *D. magna* was not observed or very low if they are exposed to 3.5  $\mu\text{g MC-LR L}^{-1}$  (Lürling and Van der Grinten, 2003), 50  $\mu\text{g MC-LR L}^{-1}$  (Dao et al., 2010) or even to 200  $\mu\text{g MCE L}^{-1}$  (Smutna et al., 2014) during 21 days

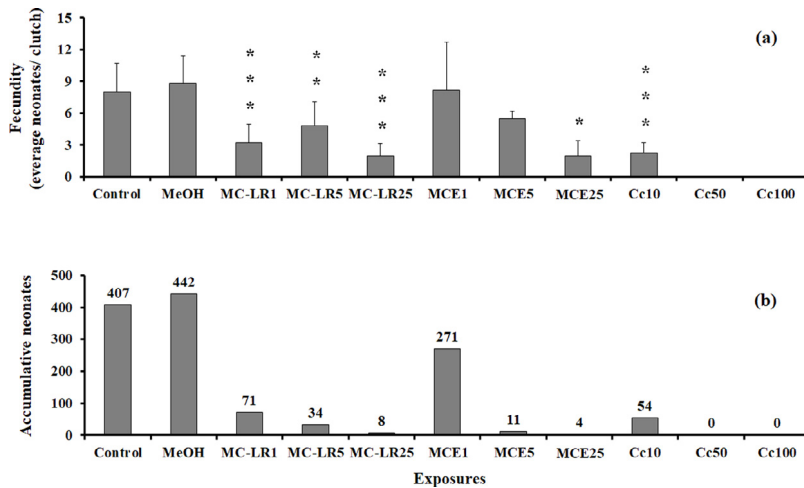


**Fig. 2.** Survival proportion of *Daphnia lumholtzi* under chronic exposure to purified MC-LR (a), cyanobacterial crude extract containing microcystins (b), and live cells of *Cyndrospermopsis curvispora* (c). Abbreviations stand for treatments in Table 1.

of incubation. Therefore, the cyanobacterial toxins caused higher mortality to *D. lumholtzi* than to micro-crustaceans from temperate regions (e.g. *D. magna*). The pure MC-LR is more toxic to *D. lumholtzi* than the MCE used in our study, presumably due to its high content of MC-RR (92.4%). Our findings are consistent with the result of Semyalo et al. (2009) also testing *D. lumholtzi* but using both, the toxic strain *M. aeruginosa* PCC 7806 and its non-MCs producing mutant, evidencing a little higher effect of the MCE producing one. The strain of *C. curvispora* used in our study did not contain microcystins (Dao et al., 2016), but another strain of this species in Japan is known to produce anatoxin-a (Cronberg and Komarek, 2004). We did not have a chance to analyze the anatoxin-a in our strain of *C. curvispora*.

We found a strong reduction of *D. lumholtzi* survival in the Cc10 (Fig. 1c) but the mortality of *D. lumholtzi* by *C. curvispora* (Cc50) in our study seems to be lower than that by both microcystins-producing and non-microcystins-producing *M. aeruginosa* (also 50% of food; Semyalo et al., 2009). Ferrao-Filho et al. (2017) fed two micro-crustaceans species: *D. laevis* and *M. micrura* with either 100% *M. aeruginosa* (producing microcystins) or 100% *C. raciborskii* (producing saxitoxin) for 4 days. They found that *C. raciborskii* strongly reduced survival and reproduction of the micro-crustaceans. *Moina micrura* was the most sensitive one among three tested species. Hence, the influences of living cells of cyanobacteria on tropical micro-crustaceans depend on the toxic metabolites and are species-specific.

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



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

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

The fecundity of *D. lumholtzi* was alike in control and MeOH with  $8 \pm 2.7$  and  $9 \pm 2.5$  neonates per clutch, respectively ( $p = 0.105$ , Kruskal–Wallis test). However, it decreased in the toxin treatments ( $3 \pm 1.7$  in MC-LR1,  $p < 0.001$ ;  $5 \pm 2.2$  in MC-LR5,  $p < 0.01$ ; and  $2 \pm 1.1$  in MC-LR25,  $p < 0.001$ , all compared to either control or MeOH), and, to a lesser extent also in the treatments with extract ( $8 \pm 4.5$  in MCE1,  $p = 0.685$ ;  $6 \pm 0.7$  in MCE5,  $p = 0.131$ ; and  $2 \pm 1.4$  in MCE25,  $p < 0.05$ ). In the *C. curvispora* exposure, fecundity was reduced to  $2.25 \pm 0.9$  in Cc10, ( $p < 0.001$ ) and even completely ceased to 0 in the higher Cc treatments (Fig. 3a).

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

Phytoplankton extract containing MC-LR similarly reduced fecundity and reproduction rates in *D. laevis* (Herrera et al., 2015). In this study, the MC-LR concentrations of  $1 - 25 \mu\text{g L}^{-1}$  caused a reduction in reproduction of *D. lumholtzi*. In *D. magna*, no effect of MC-LR on the reproduction was observed until the concentration of  $3.5 \mu\text{g L}^{-1}$  (Lürding and Van der Grinten, 2003). The negative effect of MC-LR on reproduction of *D. magna* was observed at the concentrations between  $50 - 200 \mu\text{g MC-LR L}^{-1}$  (Dao et al., 2010; Smutna et al., 2014) which is 3.5 – 200 times higher than for *D. lumholtzi*, illustrating that the reproduction of *D. lumholtzi* is much more sensitive to MCE exposures in than that of *D. magna*. The sensitivity of *D. lumholtzi* to other environmental stressors such as trace metals was also higher than in temperate species such as *D. magna* (Dao et al., 2017).

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

During chronic exposure, cyanobacteria can impair *Daphnia* not only via their toxicity, but also by other mechanisms. Cyanobacterial compounds including microcystins can impair food uptake of temperate and tropical cladocerans



(Ghadouani et al., 2004; Ferrao-Filho et al., 2017). Moreover, cyanobacterial compounds such as micropeptins directly inhibit digestive enzymes and thus decrease the amount of energy that can be resorbed (Schwarzenberger et al., 2013), before an acclimation takes place (Schwarzenberger et al., 2010). To some extent, microcystins can be biotransformed and excreted in *Daphnia* as in other organisms and cause the oxidative stress and other damages mended (Wiegand and Pflugmacher, 2005). The detoxification, however, increases the energetic costs for maintenance, thus, in addition to the lower uptake and resorption, lowers energy available for growth and reproduction (e.g. already described by DeMott, 1999; Aleya et al., 2006; Ortiz-Rodriguez et al., 2012; Dao et al., 2013). Consequently, fecundity decreases in micro-crustaceans as seen in this study and many others and will cause adverse outcomes on the population level (Dao et al., 2010, 2018; Ferrao-Filho et al., 2017, 2019).

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

In tropical lakes, zooplanktonic communities are characterized by low abundance, high species richness and dominated by smaller and more selective grazers, and omnivorous organisms like copepods (Ger et al., 2016; Rizo et al., 2017). Zooplankton communities are strongly controlled by planktivorous fish, and the proportion between small (rotifers) and large (cladocerans) zooplankton is regulated by food quality and quantity among other environmental factors (Wetzel, 2001; Rizo et al., 2017). Though high species richness of the genus *Daphnia* is a typical characteristic of cladoceran assemblages, *D. lumholtzi* rarely occurs in temperate reservoirs and its abundance is much lower than that of other *Daphnia* species (Havel et al., 1995; Pinto-Coelho et al., 2005). Also, in tropical oligo- and eutrophic water bodies from Brazil and the Philippines *D. lumholtzi* density was very low (Pinto-Coelho et al., 2005; Pascual et al., 2014). In the waterbodies of Mexico, Figueroa-Sanchez et al. (2014) found many zooplankton species including cladocerans such as *Bosmina longirostris*, *Ceriodaphnia lacustris*, *Daphnia laevis*, *Chydorus* cf. *sphaericus* and *Alona* sp., but the density of *Daphnia* was low and only appeared for a short duration from January to March. The authors also reported the dominance of cyanobacteria (*Microcystis*, *Anabaena*, *Aphanizomenon*) among the phytoplankton associated with microcystins concentrations of approximately  $0.7 \mu\text{g L}^{-1}$ , close to the lowest tested concentration in our study ( $1 \mu\text{g L}^{-1}$ ). Worldwide, more than 50% of lakes and reservoirs suffer eutrophication (Chorus and Bartram, 1999), out of which over 80% of freshwater ecosystems have been dominated by cyanobacterial species of *Microcystis* and *Cylindrospermopsis* at least once over the last 30 years (Xiao et al., 2020). The current laboratory study showed negative effects of dissolved microcystins and living cyanobacteria on the survival, fecundity, and reproduction of *D. lumholtzi* that are three important parameters for the population growth. Therefore, our results may be useful to explain that the low density of *D. lumholtzi* in tropical lakes and reservoirs where cyanobacteria and potentially also their toxins occur all year round.

#### 4. Conclusions and perspectives

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

#### CRedit authorship contribution statement

TSD raised the conceptualization and did the funding acquisition. TMCV and BTB did the investigation, methodology and data curation. TMCV wrote the original draft. All authors wrote the final manuscript. CW and VKD edited, structured the manuscript and polished the language.

#### Declaration of competing interest

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

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