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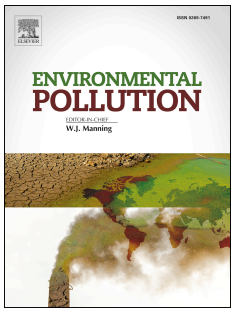

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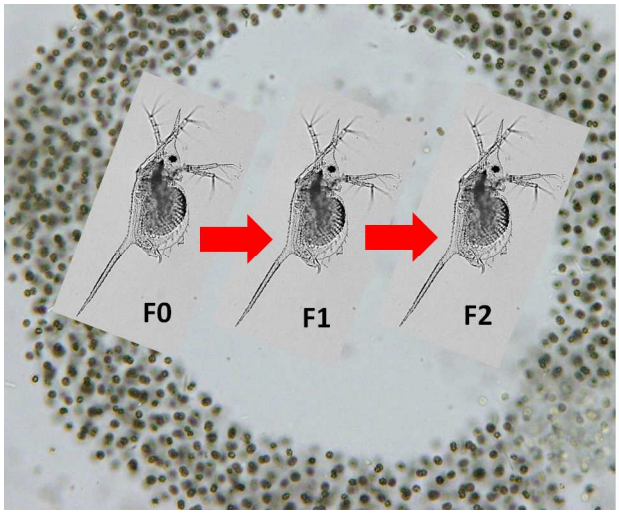

Continuous exposure to microcystins

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

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

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Abstract

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

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

Capsule

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1 Chemicals and organisms for the tests

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

LR (20.3 µg g^{-1} dried weight, DW), MC-RR (635 µg g^{-1} DW), and MC-YR (31.7 µg g^{-1} DW) concentrations (Dao et al., 2014).

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

2.2 Experimental set up

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

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

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

2.3 Life history traits

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

2.4 Data analyses

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

3. Results

3.1 Effects of microcystins on generation F0

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

3.2 Effects of microcystins on generation F1

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

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

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

3.3 Effects of microcystins on generation F2

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

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

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

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

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

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

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

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

4. Discussion

4.1 Effects of microcystins on generation F0

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

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

4.2 Effects of microcystins on generation F1

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

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

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

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

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

4.3 Direct and transgenerational effects of microcystins on generation F2

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

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

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

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

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

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

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

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

4.4 Conclusions

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

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

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Figure legends

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

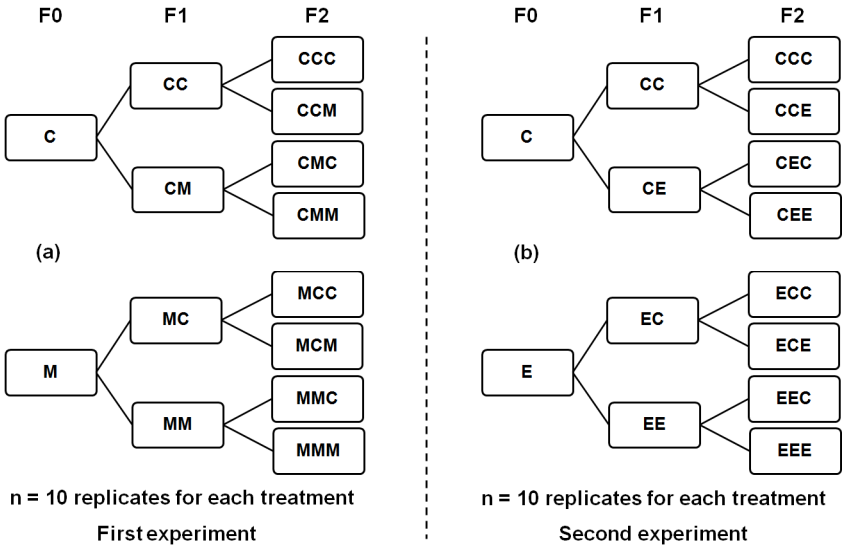
Fig. 2. Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and fecundity (G, H) of *Daphnia lumholtzi* F0 generation in response to the MC-LR (M) and cyanobacterial extract (E). Letters (a, b) on the bars indicate significant difference among the exposures by Duncan's posthoc tests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

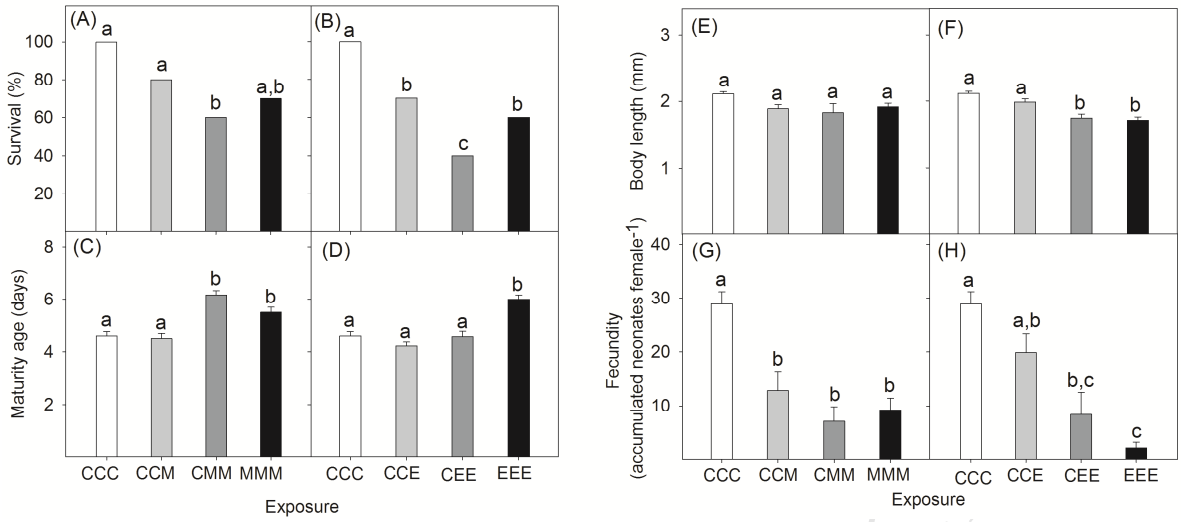
Fig. 3. Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and fecundity (G, H) of *Daphnia lumholtzi* F1 generation in response to exposures to the MC-LR and cyanobacterial extract for one (CM or CE) and two (MM or EE) consecutive generations. Letters (a, b, c) indicate significant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

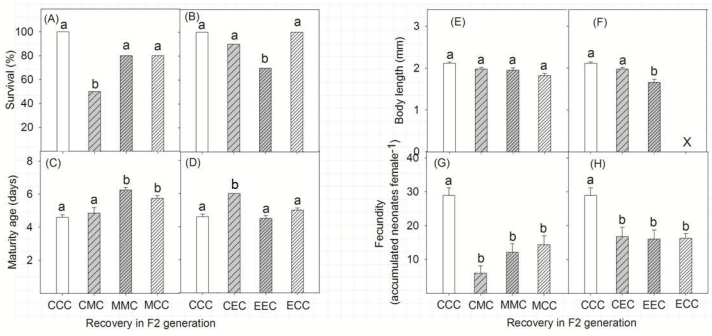
Fig. 4. Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and fecundity (G, H) of non-exposed F1 *Daphnia lumholtzi* after exposure of the F0 to MC-LR and cyanobacterial extract. Letters (a, b) on the bars indicate significant difference between the recovery of the F1 from non-exposed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

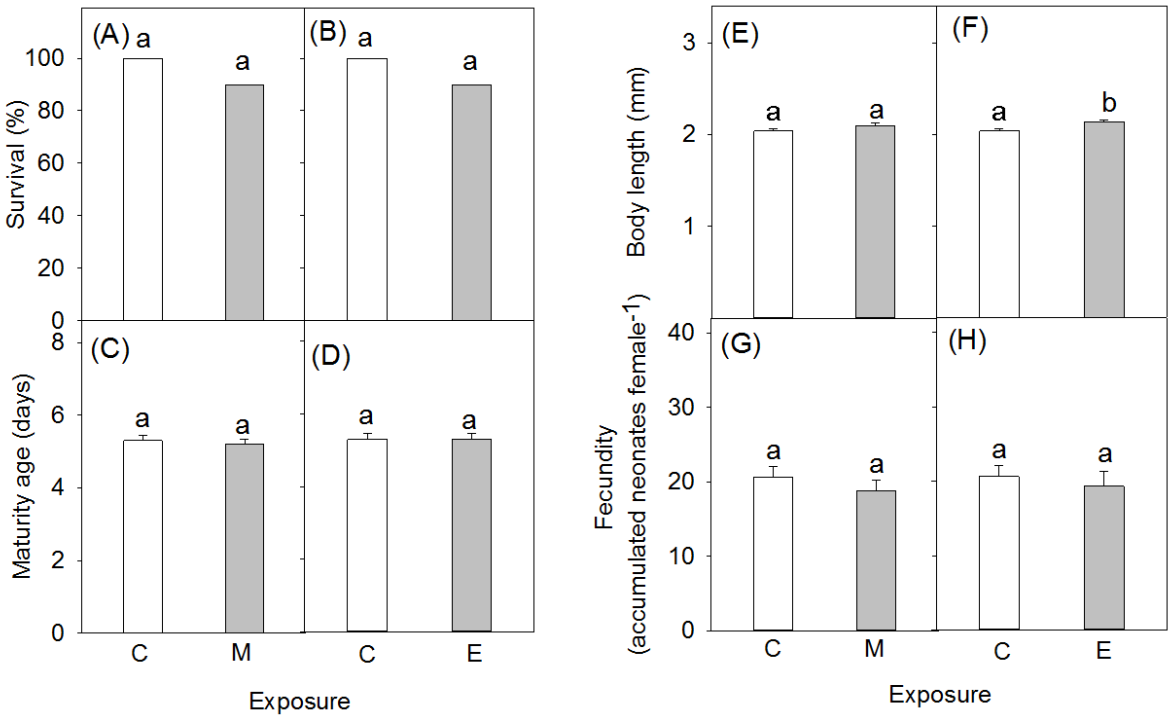
Fig. 5. Means + 1 SE of survival (A, B), maturity age (C, D), body length (E, F), and fecundity (G, H) of *Daphnia lumholtzi* after one, two and three consecutive generational exposure to MC-LR or MCs from cyanobacterial extract. Letters (a, b, c) on the bars indicate significant difference among the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

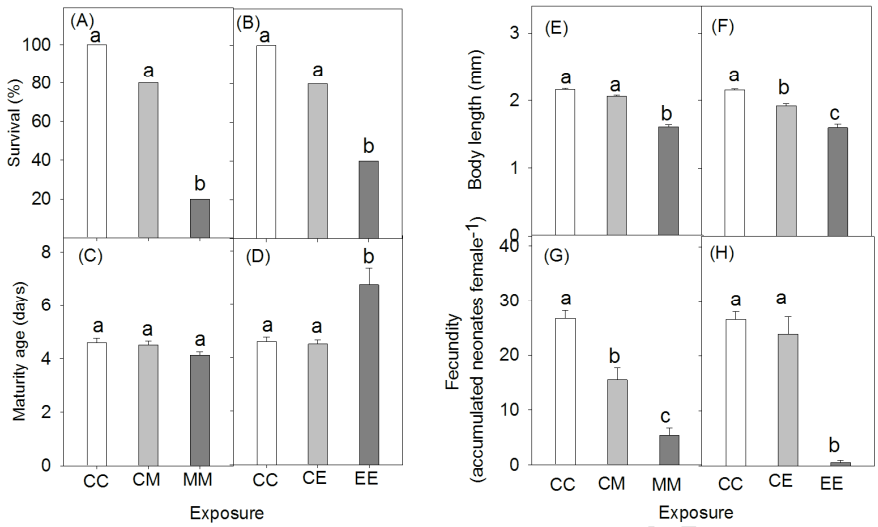
Fig. 6. Recovery capacity of *Daphnia lumholtzi* in generation F2 after one or two generations reared in toxin free medium. Letters (a, b) on the bars indicate significant difference among the treatments by Duncan's posthoc tests ($p < 0.05$). The body length of the group ECC could not be measured. Abbreviation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

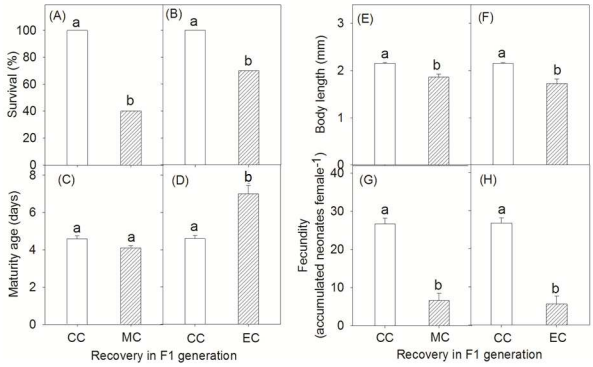












Highlights

- 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*