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Effects of an Anatoxin-a(s)-Producing Strain of *Anabaena spiroides* (Cyanobacteria) on the Survivorship and Somatic Growth of Two *Daphnia similis* Clones

Fábio Q. de Abreu, Aloysio da S. Ferrão-Filho

Laboratório de Avaliação e Promoção da Saúde Ambiental, Instituto Oswaldo Cruz-FIOCRUZ, Rio de Janeiro, Brasil.
Email: aloysio@ioc.fiocruz.br

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ABSTRACT

The toxicity of an anatoxin-a(s) producer strain of *Anabaena spiroides* (ITEP-024) was estimated through sub-chronic bioassays with two clones of *Daphnia similis* (Labtox and Itajubá), both with intact cells and aqueous extracts of lyophilized material. Animals were grown as clonal cultures in the lab with mineral water plus 20% lake water. The concentrations used in the bioassays were 0.125, 0.25, 0.375, 0.50 and 1.00 mg·L^{-1} for intact cell cultures and 10, 25, 50 and 100 mg·L^{-1} for aqueous extracts. Controls with nutritive food were used. The bioassays lasted 72 hours for the aqueous extracts and 96 hours for the intact cell cultures, with measurements of survivorship every day and body length at the beginning and at the end of the bioassays. Both kinds of samples affected survivorship and growth rate of both *Daphnia* clones, with the intact cell samples being more effective than aqueous extracts. Regarding survivorship, the clone Itajubá was more sensitive to aqueous extracts than the clone Labtox (LC_{50} = 54.4 and 83.1 mg·L^{-1}, respectively). No significant difference was found between clones in the intact cell bioassays. Regarding growth rates, a significant difference was found between clones in both samples. However, growth rate was significantly decreased in much lower concentrations of intact cells than in aqueous extracts of cyanobacteria. A stimulus of growth was found in the lower concentration (10 mg·L^{-1}) of aqueous extracts, which is consistent with a hormetic response. In spite of its known high neurotoxicity to mice, ITEP-024 strain caused any effect on mobility of both clones. The effects on survivorship and growth of *Daphnia* caused by ITEP-024 strain in much higher concentrations of aqueous extracts suggests that uptake of toxins from the water are not so effective as the uptake trough the gut when intact cells are ingested.

Keywords: Daphnia; Cyanobacteria; Anatoxina-a(s); *Anabaena spriroides*; Fitness; Growth Rate; Zooplankton

1. Introduction

Cyanobacteria proliferate in eutrophic aquatic ecosystems and impose risks to aquatic fauna and human health [1,2]. However, although mass mortality of aquatic vertebrates have been frequently associated with cyanobacterial blooms [3], there is little evidence of mass mortality of invertebrates (e.g. *Daphnia*) related to massive development of cyanobacterial blooms [4].

Most of the studies up-to-date have focused on the hepatotoxins microcystins (MCs) and on MCs-producer cyanobacteria effects on aquatic biota while a few have focused on neurotoxins, mainly from the group of saxitoxins (STXs) and anatoxins (ANTX-a and ANTX-a(s)) [2,5].

ANTX-a(s) is produced by species of the genus *Anabaena*, such as *A. flos-aquae*, *A. iemmermannii* and *A. spiroides* [6,7]. It is a natural organophosphate which irreversibly inhibits acetylcholinesterase (AChE), similar to organophosphorous and carbamate insecticides [8]. When AChE is inhibited, the neurotransmitter acetylcholine is no longer hydrolyzed in the synapse, the postsynaptic membrane cannot be repolarized, and nerve influx is blocked. Anatoxin-a(s) is highly toxic for mammals, causing symptoms such as hypersalivation and convulsions and also death by respiration arrest [1,8]. This toxin acts quickly, killing mice in a few minutes (2 - 30 min) after intraperitonial injection [9]. According to Devic *et al.* [10], anatoxin-a(s) is among the most neurotoxic compounds since the inhibition rates of AChE are higher than those obtained for most organophosphorous insecticides.

The mechanism of action of ANTX-a(s) in invertebrates in not well studied, yet effects of living cells of ANTX-a(s)-producers have been tested in copepods and cladocerans [11,12]. Here, we report the effects of an ANTX-a(s)-producer strain of *Anabaena spiroides* (ITEP-024) on two *Daphnia similis* clones using both intact cell cultures and aqueous extracts from lyophilized cultures.

2. Materials and Methods

2.1. Cultures of Algae and Cyanobacteria

The chlorophyceans of the species *Ankistrodesmus falcatus* (Braun) e *Pseudokirchneriella subcaptata* (Korshikov) Hindak (ex-*Selenastrum capricornutum*) were cultured in 1 L of MBL medium adjusted to pH 7.0, with aeration, at 23.5˚C ± 1˚C, 40-50 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity and 12/12 h light:dark cycle. A strain of *Anabaena spiroides* (ITEP-024), isolated from Tapacurá reservoir (Pernambuco, Brazil), was kindly provided by Dr. R. Molica and maintained in ASM-1 medium adjusted to pH 8.0, without aeration, and other conditions being the same as the chlorophytes. This strain was described as forming solitary coiled trichomes, with spirals of 32 - 62 μm wide and a distance of 10 - 50 μm between the coils [7]. However, in our cultures the ITEP-024 strain presented as short, straight trichomes of variable length, formed by single cells to a few cells.

2.2. Cultures of *Daphnia similis*

Two clones of *Daphnia similis* Claus (~2.5 mm adult size) were obtained from different sources: 1) cultures of the Labtox-Biorio at the Federal University of Rio de Janeiro, RJ State, Brazil; 2) cultures from the Dr. A.L. Fonseca, at Federal University of Itajubá, Minas Gerais State, Brazil. For practical purposes, the clones were designated herein as "Labtox" and "Itajubá". The origin of these clones is uncertain, however, it has been reported as a widely distributed species occurring in Europe as well as in North and South America [13], mainly in temporary, shallow, turbid water ponds [14]. *D. similis* is considered as a standard species, commonly used in ecotoxicological tests in Brazil. These clones have been maintained in lab as clonal cultures for a long time (>20 years) in different Brazilian institutions.

Cladocerans cultures were kept at 23.5˚C ± 1˚C, under dim light and 12/12 h light:dark cycle, in 500 or 1000 mL beakers with commercial mineral water as the culture medium combined with 20% - 30% of filtered lake water from a preserved area in the State Park of Pedra Branca, Rio de Janeiro (RJ State, Brazil). The number of organisms never exceeded 25 animals per liter and were fed a mixture of the green algae at a total food concentration of 1.5 mg $DW \cdot L^{-1}$, supplied in the ratio of 2:1 of *A. falcatus*/*P. subcaptata*.

2.3. Sub-Chronic Bioassays

Two bioassays were performed, one with intact cells from concentrated stock cultures (centrifuged at 1000 g for 10 min.), and another one with aqueous extracts from lyophilized cultures. For the bioassays with intact cells, appropriate dilutions were made based on cell concentrates diluted in *Daphnia* medium. Biomass (dry weight = DW) of the cell concentrates were based on gravimetric analysis of glass-fiber filters samples. For the bioassays with aqueous extracts, an appropriate amount of lyophilized cultures was weighed and diluted in deionized water, being sonicated for 10 minutes for breaking the cells and centrifuged to remove cell debris. After that, dilutions of both samples were made with *Daphnia* medium (only mineral water) to the concentrations used in the bioassays: 0.125, 0.25, 0.375, 0.50 and 1.00 mg $DW \cdot L^{-1}$ for intact cell cultures, and 10, 25, 50 and 100 mg $DW \cdot L^{-1}$ for aqueous extracts. Ten neonates (<24 h) were placed in flat-bottom test tubes, with three replicates per treatment. Controls (3 replicates) with only mineral water and chlorophytes were run in parallel. The bioassays with intact cells lasted 96 hours while the bioassays with aqueous extracts lasted 72 hours (due to scarcity of material), with renewal of the test medium every day. At the beginning and at the end of bioassays, animals were measured for the body length from the top of the head to the base of caudal spine. Body growth rate was calculated with the formulae: $g = \ln(l_f - l_i)/\text{days of the experiment}$, where l_i is the body length of neonates at the beginning and l_f is the length of surviving animals at the end of the bioassays.

2.4. Statistical Analysis

For the sake of comparison, the median lethal concentration in 72 hours (LC_{50}) was calculated with the use of two statistical approaches: Probit (SPSS Statistical Package, SPSS Inc., Chicago, IL, USA) and Trimmed Spearman-Karber analysis [15]. The survivorship and growth rate at the end of each test were analyzed by one-way ANOVA and treatments were compared by Tukey tests (Systat version 9; SPSS, Chicago, Illinois, USA). Two-way ANOVA was used to compare clones regarding growth rates.

3. Results

Regarding the bioassays with intact cells, there was a significant effect in the survivorship of both *Daphnia* clones (**Figures 1(A)** and **2(A)**). For the clone Labtox it was possible to estimate LC_{50}(72 h) values by the two statistical methods and the values found were similar, with an overlap in the 95% confidence intervals (**Table 1**).

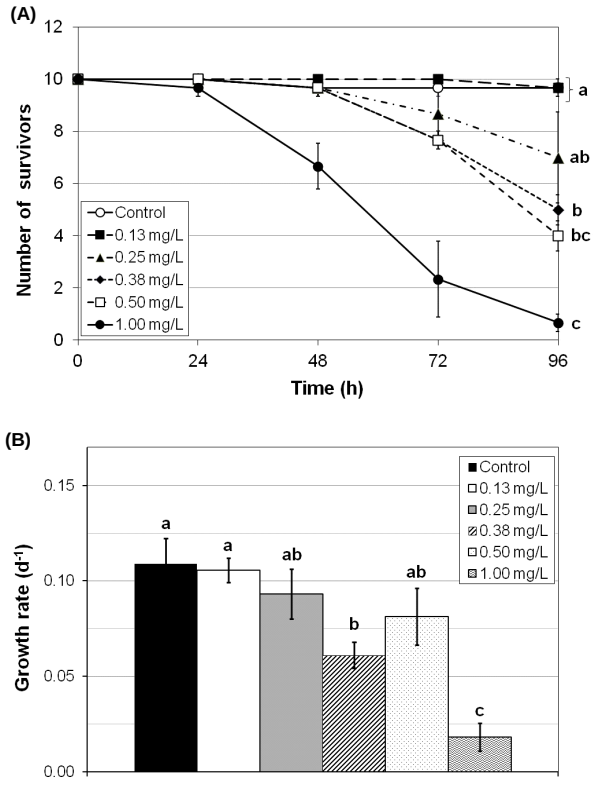

Figure 1. Survivorship (A) and growth rate (B) for the clone Labtox in the bioassays with intact cells of the strain ITEP-024. Concentrations are given in mg DW·L^{-1}. Significant differences are indicated by different letters (Tukey test; P < 0.05).

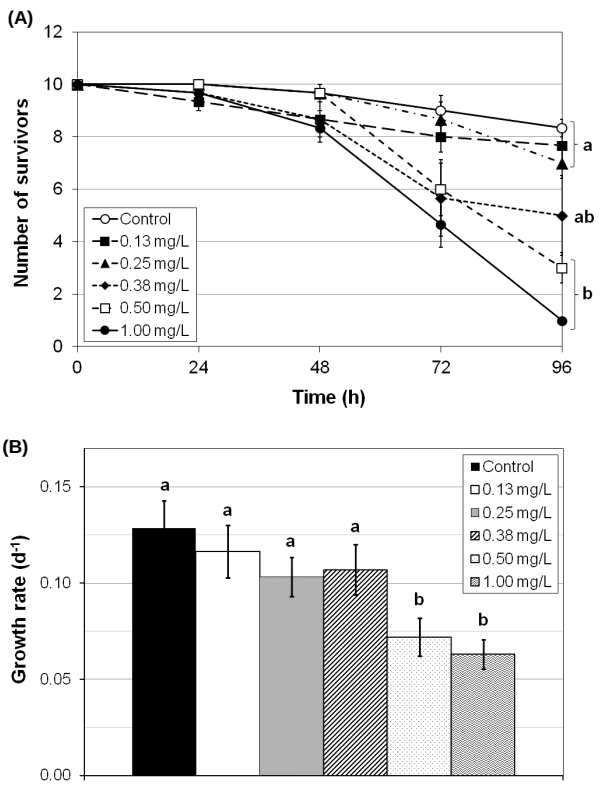

Figure 2. Survivorship (A) and growth rate (B) for the clone Itajubá in the bioassays with intact cells of the strain ITEP-024. Concentrations are given in mg DW·L^{-1}. Significant differences are indicated by different letters (Tukey test; P < 0.05).

Differently, for the clone Itajubá LC_{50}(72 h) values were only possible to estimate by the Probit analysis, and its 95% confidence interval overlapped completely with that estimated for the clone Labtox, showing that there was no significant differences between clones in the sensitivity to intact cells (**Table 1**).

Intact cells significantly affected body growth of both *Daphnia* clones. There was a decrease in growth rate of both clones with increasing concentrations of intact cells, being significant only in the higher concentrations (**Figures 1(B)** and **2(B)**). Differently from survivorship, there was a significant difference between clones regarding growth rate (two-way ANOVA, $F_{1,97} = 5.86$, $P = 0.017$). The clone Itajubá had higher growth rates than the clone Labtox.

Regarding bioassays with aqueous extracts, both clones had significant increased mortality only in the highest concentration, equivalent to 100 mg·L^{-1} of lyophilized material (**Figures 3(A)** and **4(A)**). Again, for the clone Labtox it was possible to estimate LC_{50}(72 h) values by the two statistical methods and the values found were similar, with an overlap in the 95% confidence intervals (**Table 2**). For the clone Itajubá, LC_{50}(72 h) values were only possible to estimate by Trimmed Spearman-Karber method, and the LC_{50}(72 h) value found was lower than that found for the clone Labtox, with a lower range also in the 95% confidence interval (**Table 2**), showing that there was a significant difference between clones in the sensitivity to aqueous extracts.

Aqueous extracts also significantly affected body growth of both *Daphnia* clones. However, differently from intact cells, aqueous extracts seemed to stimulate growth in the lower concentration (equivalent to 10 mg DW·L^{-1} of lyophilized material), being significantly higher than the control only for the clone Labtox (**Figures 3(B)** and **4(B)**). The other concentrations did not affect or significantly inhibited growth relative to control. However, all treatments showed positive growth (above 0.1 d^{-1}). There was also a significant difference between clones regarding growth rate (two-way ANOVA, $F_{1,84} = 23.6$, $P < 0.001$) with the clone Itajubá having higher growth rates than the clone Labtox.

4. Discussion

In this study, both clones of *D. similis* responded in a similar fashion to intact cells and aqueous extracts to

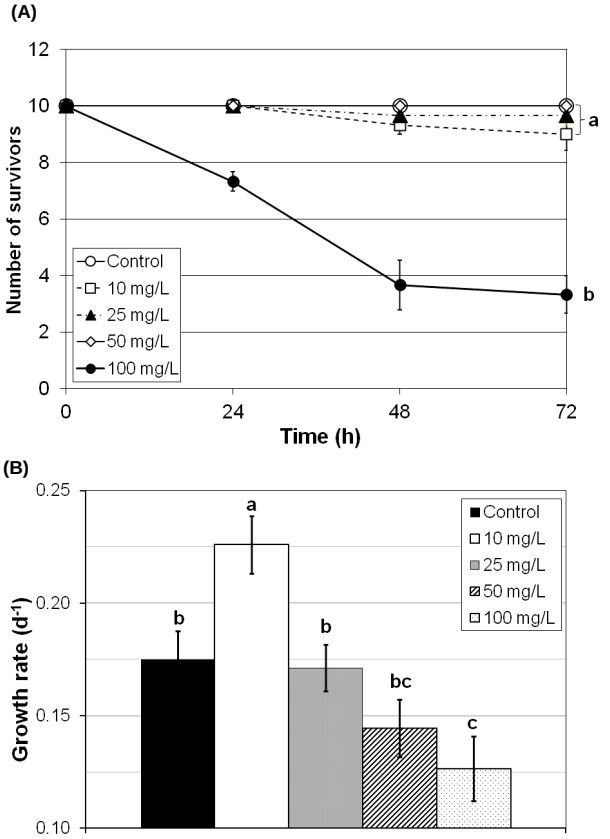

Figure 3. Survivorship (A) and growth rate (B) for the clone Labtox in the bioassays with aqueous extracts of the strain ITEP-024. Concentrations are given in mg DW·L^{-1}. Significant differences are indicated by different letters (Tukey test; P < 0.05).

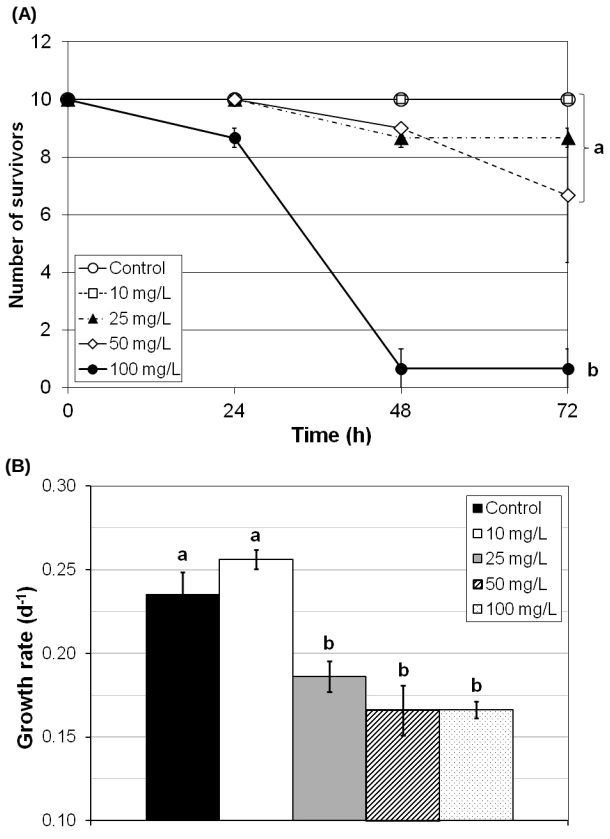

Figure 4. Survivorship (A) and growth rate (B) for the clone Itajubá in the bioassays with aqueous extracts of the strain ITEP-024. Concentrations are given in mg DW·L^{-1}. Significant differences are indicated by different letters (Tukey test; P < 0.05).

anatoxin-a(s)-producer *A. spiroides*, with decreases in survivorship and growth rate. However, a difference in the sensitivity between clones, regarding survivorship, was found in the bioassay with aqueous extracts, with the clone Itajubá being more sensitive than the clone Labtox. According to Costa [12], the sensitivity among cladocerans can vary as a function of different factors, such as physiological resistance of the species, toxin uptake capacity relative to the administered form (*i.e.* intact cells, cell extracts or purified), cyanobacterial morphology (*i.e.* single cells, colonies or filaments), and strain toxicity.

Differences in the sensitivity between several *Daphnia* clones and species exposed to aqueous extracts or purified toxins and toxic and non-toxic cells of cyanobacteria were found in other studies [11,16-19]. However, few studies tested the effects of ANTX-a(s) producer strains of cyanobacteria on *Daphnia*. DeMott *et al.* [11] tested the effects of *Anabaena flos-aquae* strain NRC 525-17 and found that the cladoceran *D. pulicaria* and the copepod *Diaptomus birgei* exhibited rapid mortality at concentrations of 10^4 and 10^5 cells·mL^{-1} after 48 hours (>50%), similarly to our study.

Costa [12] found a significant effect of strain ITEP-024 aqueous extracts on the survivorship of *Moina micrura*, with a LC_{50}(96 h) of 41.1 (35.1 - 50.0) mg DW·L^{-1}, but not in two *Daphnia* species (*i.e.*, *D. gessneri* and *D. pulex*). However, intact cells of this strain did not affected the survivorship of any of the cladocerans tested, including *M. micrura* and the two *Daphnia* species. Probably, the concentrations of extract used (5.0 - 50 mg DW·L^{-1}) were too low to cause any toxic effect on *Daphnia*. Further, as stated by the author, the morphology of the strain in those experiments were different, with strain ITEP-024 presenting coiled trichomes with more than 100 μm in length, which could difficult ingestion of toxic cells by cladocerans. In our experiments, however, ITEP-024 strain presented as single cells or short thricomes of <50 μm in length, being thus possible to be ingested by *Daphnia*.

Effects of other neurotoxic strains of *Anabaena* have been tested on zooplankton. Gilbert [20] tested the effects of intact cells of *A. flos-aquae* (anatoxin-a producer

Table 1. Values of LC_{50}(72 h)(mg DW·L^{-1}) and its 95% confidence intervals (CI) for the two *Daphnia* clones in the tests with intact cells of the strain ITEP-024, calculated by two statistical methods, Trimmed Spearman-Karber (TKS) and Probit.

Clone	LC_{50} (95% CI)	
	TKS	Probit
Labtox	0.715 (0.622 - 0.821)	0.724 (0.627 - 0.862)
Itajubá	-	0.815 (0.629 - 1.232)

Table 2. Values of LC_{50}(72 h)(mg DW·L^{-1}) and its 95% confidence intervals (CI) for the two *Daphnia* clones in the tests with aqueous extracts of the strain ITEP-024, calculated by two statistical methods, Trimmed Spearman-Karber (TKS) and Probit.

Clone	LC_{50} (95% CI)	
	TKS	Probit
Labtox	83.06 (72.05 - 95.74)	90.99 (70.61 - 135.16)
Itajubá	54.36 (45.06 - 65.60)	-

strain IC-1) on rotifer species and found effects in concentrations as low as 0.5 - 4.0 mg DW·L^{-1}. These values are in the range of LC_{50}(72 h) found in this study (0.7 - 0.8 mg DW·L^{-1}), even considering that anatoxin-a and anatoxina-a(s) have different mechanism of action in mammals [1,8]. These values can be considered very low, compared to values found in other studies for other toxins [2] and points out to the high toxicity of this strain to cladocerans.

Comparing the results of bioassays with intact cell with the ones with aqueous extracts it is clearly observable that the dose necessary to kill the same amount of organisms (LC_{50}) is much higher in the case of aqueous extracts, being about 100 times higher than that of intact cells. These findings are in accordance to that of DeMott *et al.* [11], who found that the toxin (MC-LR) concentration needed to produce a lethal effect was about five orders of magnitude (equivalent to 10 mg DW·mL^{-1}) greater than the concentration of intact cell (~0.2 μg DW·mL^{-1}) to produce the same response. This author argued that the mode of delivery (*i.e.* intact cells, extracts, or purified toxins) can affect the results, and there is no reason to expect that comparable concentrations of dissolved and ingested toxins would have comparable effects. Rohrlack *et al.* [21] suggested that the mechanism of intoxication by MCs in *Daphnia* is primarily a mechanism of intestinal uptake, after the ingestion and digestion of the cells accumulated in the midgut and transport throughout the gut epithelium to the blood. Also, the uptake of dissolved toxins by gills of aquatic organisms seems to be low [22-24].

Other neurotoxic strains are reported to cause effects on the motor activity of cladoceran species. Ferrão-Filho *et al.* [25,26] showed that two saxitoxin-producer strains of *Cylindrospermopsis raciborskii* can inhibit the swimming movements of *D. pulex* and *M. micrura*. Saxitoxins, however, have a very different mode of action, blocking sodium ion channels in neurons and leading to paralysis of muscles [9]. In spite of the high neurotoxicity of ITEP-024 strain to mice, causing convulsions and respiratory arrest in minutes [7], this strain did not cause any effect on the mobility of both *Daphnia* clones in our study. This elicits two hypotheses: first, anatoxin-a(s) have a different mechanism of action on cladocerans; and second, other, unknown toxins produced by ITEP-024 strain are causing the lethal effect. The first hypothesis is unlikely, since vertebrate AChE is inhibited in a similar fashion to invertebrate's [10]. It is more likely that other, unknown toxic compound(s) produced by this *Anabaena* strain is the cause of the toxic effect observed.

In spite of the negative effect in *Daphnia* fitness in higher concentrations of ITEP-024 strain in the diet, all treatments showed positive growth and there was, indeed, a stimulus in growth in the lower concentration (10 mg·L^{-1}) of aqueous extracts, which is consistent with a hormetic response [27]. Although toxic, cyanobacteria can have some nutritional constituents that may favor zooplankton growth when in low proportions in the diet [28]. It is uncertain, however, if the hormetic response is relative to ITEP-024 strain toxins or to other biochemical compounds (*i.e.* proteins, lipids or carbohydrates) contained in the aqueous extracts.

Although some studies showed effects of other filamentous, neurotoxic cyanobacteria on the fitness of cladocerans, this is the first report of the effect of an ANTX-a(s)-producer strain of cyanobacteria on the fitness of *Daphnia*. Nogueira *et al.* [29] showed that both somatic and population growth rate (*r*) of this *D. magna* were negatively affected by a saxitoxin-producer strain of *Aphanizomenon issatschenkoi*. Soares *et al.* [30] showed that *D. magna* had its body growth and *r* depressed in high proportions (75% - 100%) of *C. raciborskii* (CYRF-01) in the diet, and concluded that energy limitation, not toxicity, might be the dominant factor affecting growth of large-bodied cladocerans. Costa *et al.* [31] showed that while the intrinsic rate of population increase (*r*) of *D. pulex* and *M. micrura* was negatively affected by a STXs-producer strain of *C. raciborskii* (T3), it was stimulated by a non STXs-producer strain (NPLP-1) of the same species. In contrast, *D. gessneri* was stimulated by strain T3 and depressed by the strain NPLP-1, suggesting resistance to STXs produced by the strain T3 and sensitivity to some bioactive compound(s) produced by the

strain NPLP-1. These results corroborate ours, showing that sensitivity to toxic cyanobacteria seems to be species or clone-specific. Additionally, this emphasizes that the effects (positive or negative) of cyanobacteria on the fitness of cladoceran species can be a strong selective factor in the shaping of zooplankton communities.

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