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Differential susceptibility of cladoceran species to a saxitoxin-producer strain of *Cylindrospermopsis raciborskii* (cyanobacteria)

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Abstract

Toxic cyanobacteria can affect several organisms, including herbivorous zooplankton, as cladocerans. In this study we tested the hypothesis that cladoceran species of different body size, origin and degree of exposure to toxic cyanobacteria would respond differently to a saxitoxin(STX)-producing strain of the cyanobacterium *Cylindrospermopsis raciborskii* (CYRF-01). Newborns were exposed to increasing concentrations of the strain CYRF-01 mixed to a fixed proportion of green algae for 14 days and survivorship, age at first reproduction, clutch size, total offspring and the intrinsic rate of population increase (*r*), as a measure of fitness, were estimated. Different responses to the strain CYRF-01 were observed, with *Daphnia similis* (temperate, large size) being more sensitive, followed by *Moina micrura* (tropical, small size), both presenting symptoms of decreased fitness and paralysis. While *Ceriodaphnia richardi* (tropical, small size) *D. gessneri* (tropical, medium size) and *Diaphanosoma spinulosum* (tropical, small size) showed neither of these symptoms, the later species showed even increased fitness in the cyanobacterial treatments relative to control with green algae, suggesting not only a greater resistance to this strain, but also that it can serve as a nutritional supplement for this cladoceran. In conclusion, the results showed that while some cladocerans species may be negatively affected in their fitness, others may be not affected at all or even utilize STX-producer cyanobacteria as a complementary resource. This suggests that responses of cladocerans to toxic cyanobacteria in nature may vary with species and that the presence of toxic cyanobacteria may shape zooplankton communities to the dominance of more tolerant herbivorous species.

Keywords: cladocerans, cyanobacteria, fitness, saxitoxins, zooplankton

INTRODUCTION

Cyanobacterial blooms are becoming more frequent and long lasting and this has been considered a consequence of both eutrophication and climate change (Paerl & Huisman, 2008). Along with changes in species diversity, the presence of toxin producers may have a profound impact on aquatic communities, especially on primary consumers such as zooplankton. Bioaccumulation of cyanotoxins and toxic effects of cyanobacteria have been reported in all taxonomic levels of the aquatic biota (Ferrão-Filho & Kozlowsky-Suzuki, 2011), thus food chain structure can be altered by toxic cyanobacterial blooms.

Toxin production in cyanobacteria has been hypothesized as a chemical defense against grazing by zooplankton (Lampert, 1981; Kirk & Gilbert, 1992; Wilson & Hay, 2007). Although toxic cyanobacteria can exert a variety of negative effects on zooplankton, including feeding inhibition and decreased survival, growth, and reproduction (Haney *et al*., 1995; Lampert, 1981; Ferrão-Filho *et al*., 2000; Panosso & Lürling, 2010), these responses vary significantly among zooplankton species, and even among clones of the same

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species (DeMott *et al*., 1991; Ferrão-Filho *et al*., 2000; Alva-Martínez *et al*., 2007, Wilson & Hay, 2007; Ferrão-Filho *et al*., 2008). Also, there is not unequivocal evidence that microcystins, the most studied among cyanotoxins, is in fact the toxic agent in zooplankton-cyanobacteria studies (Ferrão-Filho & Kozlowsky-Suzuki, 2011; Wilson *et al*., 2006). Additionally, cyanobacteria may produce several unidentified compounds with toxic or inhibitory effects, potentially contributing the observed negative effects on zooplankton (Ferrão-Filho & Kozlowsky-Suzuki, 2011).

In fact, zooplanktonic species may be able to co-exist with toxic cyanobacteria through behavioral and/or physiological adaptations that increase their resistance to toxins, such as capacity to select food based on toxicity or morphological characteristics (DeMott & Moxter, 1991; DeMott *et al*., 1991), selection of resistant clones (Sarnelle & Wilson, 2005) or increased fitness, expressed as an increase in survivorship and fecundity, transferred from mother to offspring and leading to enhanced tolerance over generations (Ortiz-Rodríguez *et al*., 2012). Nevertheless, there is evidence that cyanobacterial blooms can have an effect on zooplankton population structure, leading to a decrease in biomass and changes in zooplankton species composition (Hansson *et al.*, 2007; Leonard & Paerl, 2005). Some studies also suggested that the high resistance of *C. raciborskii* to grazing by meso-zooplankton is determinant for the zooplankton community structure (Bouvy *et al*., 2001; Leonard & Paerl, 2005). These effects, however, have been ambiguously attributed to either mechanical interference of filaments or to toxin production.

The cyanobacterium *C. raciborskii* is originally described as a tropical-subtropical genus, but has been spreading fast throughout northern Europe and North America (Bonilla *et al*., 2012). It has become also an important component among toxin producing cyanobacteria in Brazilian water bodies (Bouvy *et al*. 2001; Soares *et al*., 2009a). In several countries, *C. raciborskii* has been described as a cylindrospermopsin (CYN)-producing, a potent cytotoxic alkaloid, while strains isolated in Brazil up to date have been described as saxitoxin (STX)-producing (Lagos *et al*., 1999; Molica *et al*., 2002). Saxitoxin (STX) and their analogs acts by blockage of sodium channels, preventing the influx of sodium ions and nerve impulse, which leads to paralysis and death by respiration arrest in mammals (Sivonen & Jones, 1999).

Notwithstanding the fact that there is still little information in literature about the bioaccumulation of STXs and effects of STX-producing cyanobacteria on zooplankton and on higher trophic levels of the aquatic food web (Ferrão-Filho & Kozlowsky-Suzuki, 2011), there is some evidence pointed out to the role of saxitoxins as strong, inhibitory compounds to cladocerans (Haney *et al*, 1995; Nogueira *et al*., 2004; Ferrão-Filho *et al*., 2008; 2010). Therefore, the aim of the present study was to test the hypothesis that a STX-producing strain of the cyanobacterium *Cylindrospermopsis raciborskii* (CYRF-01), previously known to cause acute effects (i.e. paralysis) in cladocerans (Ferrão-Filho *et al*., 2010), can affect differently the fitness of five cladoceran species differing in body size, origin and degree of exposure to toxic cyanobacteria.

MATERIAL AND METHODS

Origin and culture of organisms

The strain CYRF-01 of the species *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju was isolated from Funil Reservoir, in the State of Rio de Janeiro, Brazil and has been reported as a STX-producing (Ferrão-Filho *et al*., 2009). This strain was maintained in 500 mL batch cultures in ASM-1 medium (Gorham at al., 1964) pH 8.0, 23.5±1ºC, under 40-50 μE m^{-2} s^{-1} light intensity and 12/12 h light:dark cycle. The cell counting was performed on a Fuchs-Rosenthal chamber, and to establish the average size of each cell and each filament, measurements of at least 40 filaments (randomly chosen) had been made. Posteriorly, the number of cells could be determined by measuring all filaments in each field of the Fuchs-Rosenthal chamber and dividing the sum of filaments length by the previously established average cells size.

Cladocerans food consisted of the green algae *Ankistrodesmus falcatus* (Braun), grown in 1000 mL MBL medium (Stemberger, 1981) at pH 7.0, and the other culture conditions being the same as described for the cyanobacterial strains. Algal cell concentrates for feeding the animals were obtained every other day by centrifuging 400 mL of algal culture at 1000 g during 10 minutes. The same amount of MBL medium was replaced to keep the culture in exponential growth phase. The cell density was determined in a Fuchs-Rosenthal chamber and the carbon cell quota of 30 pg C $cell^{-1}$ (DeMott and Tessier, 2002) was used for the calculation of the food concentration. A total food concentration of 0.50 mg C L^{-1} was supplied every other day.

Four cladoceran clones were isolated from reservoirs in the State of Rio de Janeiro, Brazil: *Moina micrura* Kurz (adult size: ~0.9 mm) and *Daphnia gessneri* Herbst (~1.6 mm) were isolated in 2002 from Lajes Reservoir, an oligo-mesotrophic reservoir with no cyanobacterial blooms (Ferrão-Filho *et al*., 2009), and *Ceriodaphnia richardi* Sars (~0.7 mm) and *Diaphanosoma spinulosum* Herbst (~1.0 mm) were isolated in 2007 from the eutrophic Funil Reservoir, the same reservoir where the strain CYRF-01 was isolated (Ferrão-Filho *et al*., 2009). A clone of *Daphnia similis* Claus (~2.5 mm) was obtained from cultures of the Labtox-Biorio at the Federal University of Rio de Janeiro, Brazil. The history (lake origin) of the *D. similis* clone is unknown, but it has been reported as a widely distributed species occurring in Europe as well as in North and South America, mainly in temporary, shallow, turbid water ponds (Adamowicz *et al*., 2004). Also, *D. similis* is considered as a standard species, commonly used in ecotoxicological tests in Brazil. This clone has been maintained in laboratory cultures for >30 years in different Brazilian institutions. Cladocerans cultures were kept at 23.5±1ºC, 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% of lake water from a preserved area (State Park of Pedra Branca, City of Rio de

Janeiro, RJ, Brazil), filtered onto glass-fiber filter (Sartorius® 13400, Goettingen, Germany). The number of organisms never exceeded 20-30 animals per liter.

Assays with CYRF-01 strain

Two life-table experiments were performed to test the effect of CYRF-01 strain on fitness parameters of the cladoderan species, such as age at first reproduction (days), mean clutch size, total offspring per female and intrinsic rate of population increase (*r*). Samples for cell determination and biomass (dry weight, DW) were taken twice, one at the beginning and another in the middle of both experiments. Biomass was calculated by gravimetry, filtering 50 mL of the CYRF-01 culture onto a glass-fiber filter (Sartorius® 13400, Goettingen, Germany). Previous acute bioassays (Ferrão-Filho *et al*., 2008) showed that cladoceran species differed greatly in the range of sensitivity to strain CYRF-01, thus the experiments were performed at different cell density and biomasses ranges for each species (Table 1). Controls consisted of animals fed only 0.5 mg C L^{-1} of *A. falcatus* and this same amount of food was also added to treatments with cyanobacteria. Thus, assuming an algal carbon content of 50%, the estimated proportion of cyanobacteria in the food mixture varied from less than 1% for *D. similis*, from 2–8% for *C. richardi*, *D. spinulosum* and *M. micrura*, and from 8–30% for *D. gessneri* (Table 1). Fifteen newborns (<24h old) were placed individually in 30 mL flat-bottom glass tubes for each experimental condition. The animals were transferred daily to new medium with only food (control) or food+cyanobacteria. Experiments were performed at the same physical-chemical conditions described for the maintenance of cultures and lasted 14 days for all species, which is enough to reach at least three reproduction events (clutches). The survival and fecundity data were used to calculate the intrinsic rate of populate increase (*r*) using bootstrap technique with the use of *Rm* 2.0 program (Taberner *et al*., 1993), which produced 500 bootstrapped estimates of *r* and its 95% confidence interval by interaction of the stable age equation:

$$1 = \Sigma l_x m_x e^{-rx}$$

where l_x is age specific survival, m_x is age specific fecundity and *x* is age in days.

Saxitoxins analysis

Saxitoxins produced by CYRF-01 strain were analyzed by High Performance Liquid Chromatography (HPLC) according to the post-column derivatization method (Oshima, 1995). Two samples of 50 mL from CYRF-01 cultures were taken, one at the beginning and other in the middle of the experiments (coinciding with the exponential phase of growth), filtered onto glass-fiber filter (Sartorius® 13400) and frozen until the analysis. These samples were extracted with 2 mL 0.1N acetic acid and analyzed by HPLC following the protocol described in Ferrão-Filho *et al*. (2009). Standards solutions of saxitoxin (STX), decarbomoil-SXT (dc-STX), neosaxitoxin (NEO) and goniautoxins (GTX 1-4) were obtained from National Research Council (NRC), Institute of Marine Biosciences (Canada), and were used to quantify toxins in the samples analyzed. All solvent and reagents used were of analytical or HPLC grade. The detection limits for saxitoxins variants were: STX=0.89 ng L^{-1}, NeoSTX=2.33 ng L^{-1}, GTX 1=1.03 ng L^{-1}, GTX 2=0.72 ng L^{-1}, GTX 3=0.21 ng L^{-1}, GTX 4 = 0.24 ng L^{-1}. Saxitoxins were expressed as concentration of STX-equivalents.

Data analysis

The statistical analysis of the population parameters for each species was performed by one-way ANOVA and differences between treatments tested by Tukey multiple comparison test ($P < 0.05$) using SYSTAT V.9.0, 1998 (SPSS Inc., Chicago, IL, USA). For comparing statistical differences in the intrinsic rate of population increase (*r*) between controls and treatments with cyanobacteria we used the Student *t*-test.

RESULTS

Filaments measurements and toxin analysis

Average filament length varied between experiments, going from 308.3 (±202.7) mm in the experiment 1 to 96.8 (±40.4) mm in the experiment 2. The HPLC analysis showed that strain CYRF-01 presented only dc-SXT, NEO and GTX 2 and 3 variants. The STX-equivalent concentrations used in the experiments are presented in Table 1. Since experiment with *D. similis* and *D. gessneri* were run in a different date from the other species and with different CYRF-01 cultures, toxin concentrations and the relative toxin cell quota changed slightly between experiments (Table 1).

Assays with CYRF-01 strain

Cladoceran species differed greatly in their response to the strain CYRF-01. While *D. similis* (Fig. 1) and *M. micrura* (Fig. 2) showed to be sensitive to increasing concentrations of cyanobacteria, *C. richardi* (Fig. 3), *D. gessneri* (Fig. 4) and *D. spinulosum* (Fig. 5) showed to be little or not affected at all by the presence of toxic cells, despite the higher exposure concentrations used for those species.

Survivorship of *D. similis* and *M. micrura* was greatly affected with increasing concentrations of cyanobacteria (Fig. 1A and 2A). Overall, all population parameters such as age at first reproduction, clutch size and total offspring per female revealed significant differences between control and the treatments with cyanobacteria (Fig. 1B and 2B). However, a significant delay in the age at first reproduction ($F_{3,56} = 2.82$, $P < 0.03$) was observed only for *M. micrura* and only in the highest concentration (Fig. 2B). A significant reduction in the average clutch size ($F_{3,56} = 7.04–14.88$, $P < 0.001$) and in the total production of offspring ($F_{3,56} = 9.75–13.95$, $P < 0.001$) was also observed for both species (Fig. 1B and 2B), which

Table 1 Range of concentrations of cell density, biomass and toxins during the experiments. Percentage of cyanobacterial biomass in the total food offered is given in parenthesis. Saxitoxins are given as cell quota (pg×10^3 $cell^{-1}$) and as toxin concentration in the medium (ng L^{-1}) from two measurements during the experiments.

Experiment/ Date	Species	Cell density (cells mL^{-1})	Biomass (µg DW L^{-1})	Toxin cell quota STX-eq. (pg×10^3 $cell^{-1}$)	Toxin concentration STX-eq. (ng L^{-1})
1 27-Nov-09	*C. richardi*	250–1000	20–80 (2–8%)	1.9 – 4.6	1.2 – 4.6
	D. spinulosum	250–1000	20–80 (2–8%)	1.9 – 4.6	1.2 – 4.6
	M. micrura	250–1000	20–80 (2–8%)	1.9 – 4.6	1.2 – 4.6
2 19-May-10	*D. similis*	250–1000	12.5–50 (0.8–3%)	2.0 – 4.4	1.1 – 4.4
	D. gessneri	2500–10000	125–500 (8–30%)	2.0 – 4.4	11.0 – 44.0

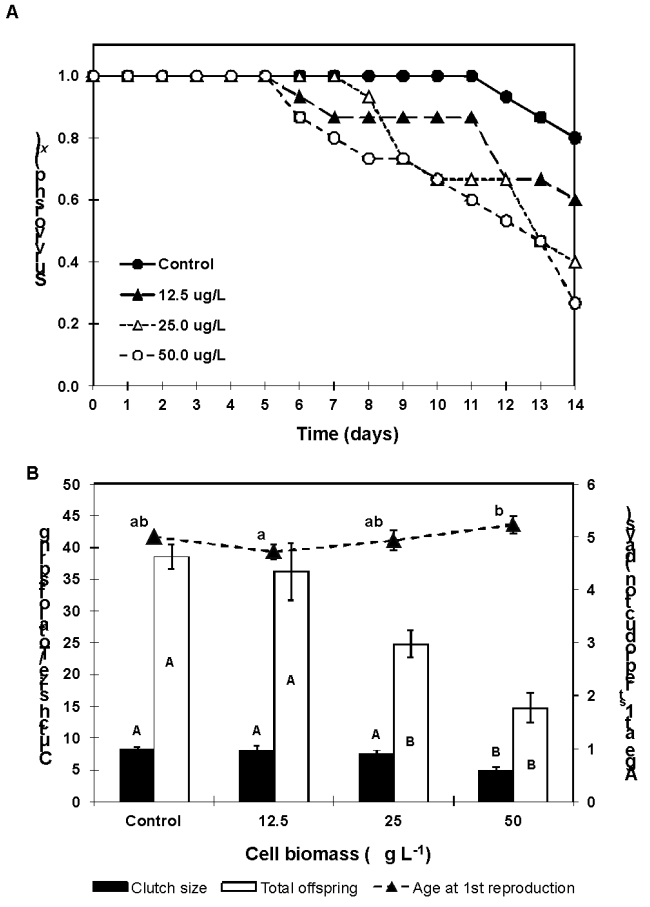

Fig. 1 Population parameters of *Daphnia similis* exposed to different concentrations (in µg DW L^{-1}) of strain CYRF-01 (*Cylindrospermopsis raciborskii*) mixed with 0.5 mg C L^{-1} of the green algae *A. falcatus* as food: (A) survivorship; (B) age at first reproduction (days), clutch size, and total offspring per female. Cell concentrations ranged between 250–1000 cells mL^{-1} and cyanobacterial biomass represented 0.8–3.0% of total food. Standard error bars are given. Different letters indicate significant differences (*post-hoc* Tukey test).

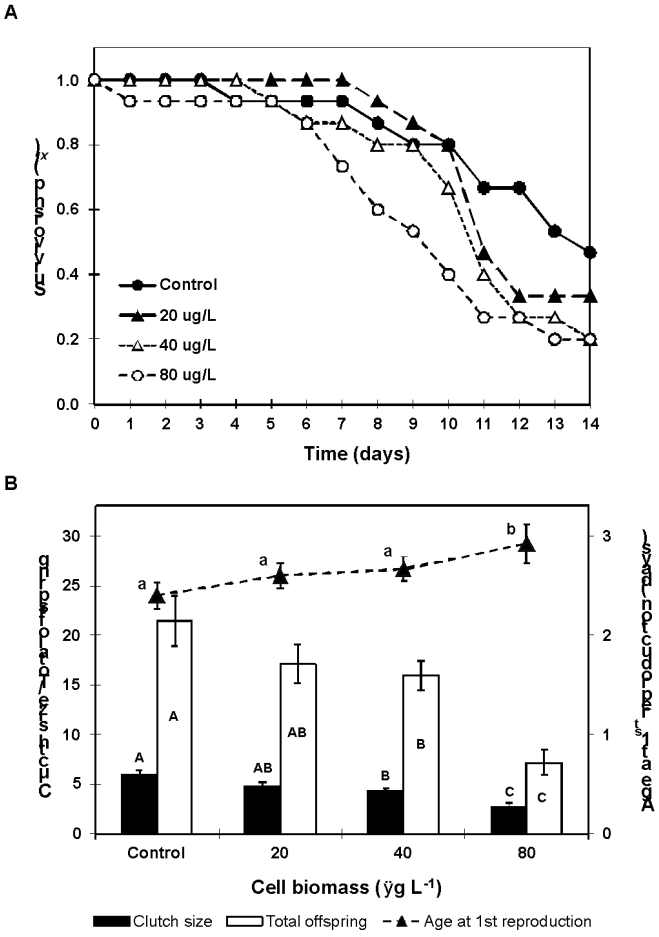

Fig. 2 Population parameters of *Moina micrura* exposed to different concentrations (in µg DW L^{-1}) of strain CYRF-01 (*Cylindrospermopsis raciborskii*) mixed with 0.5 mg C L^{-1} of the green algae *A. falcatus* as food: (A) survivorship; (B) age at first reproduction (days), clutch size, and total offspring per female. Cell concentrations ranged between 250–1000 cells mL^{-1} and cyanobacterial biomass represented 2–8% of total food. Standard error bars are given. Different letters indicate significant differences (*post-hoc* Tukey test).

resulted in a significant decrease (~20-40%) in the intrinsic rate of population increase (*r*-value) (*t*-test, $P < 0.05$; Table 2).

Except for *D. gessneri* in the highest concentration, the other cladocerans presented lower sensitivity to strain CYRF-01, showing little mortality rates (Figs. 3A– 5A). Population parameters of *C. richardi* (Fig. 3B) and *D. gessneri* (Fig. 4B) were not significantly affected, such as the age at first reproduction ($F_{3,56}$ = 0.66–3.95, $P > 0.05$), the average clutch

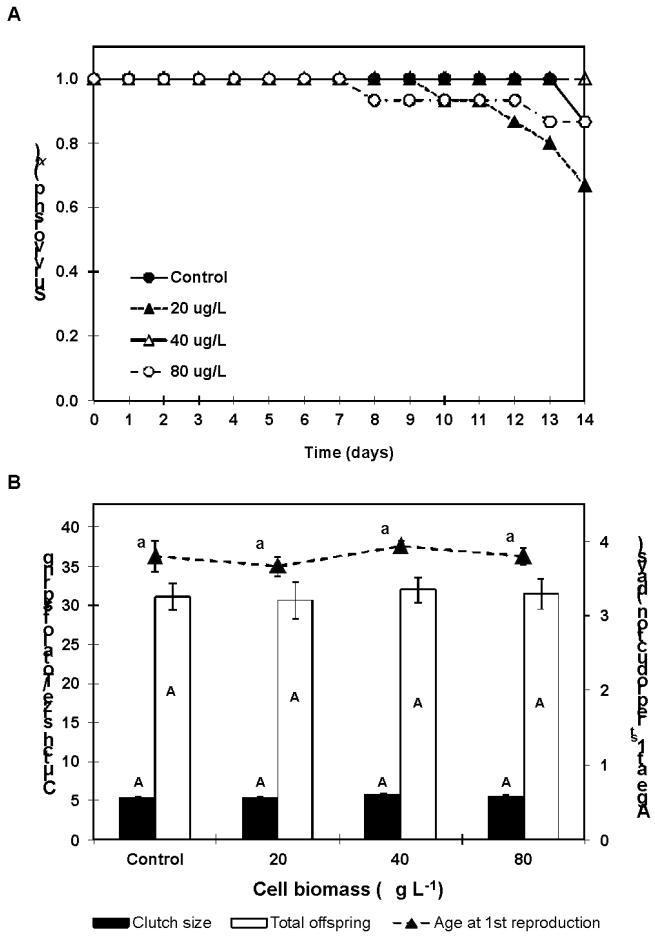

Fig. 3 Population parameters of *Ceriodaphnia richardi* exposed to different concentrations (in µg DW L^{-1}) of strain CYRF-01 (*Cylindrospermopsis raciborskii*) mixed with 0.5 mg C L^{-1} of the green algae *A. falcatus* as food: (A) survivorship; (B) age at first reproduction (days), clutch size, and total offspring per female. Cell concentrations ranged between 250–1000 cells mL^{-1} and cyanobacterial biomass represented 2–8% of total food. Standard error bars are given. Different letters indicate significant differences (*post-hoc* Tukey test).

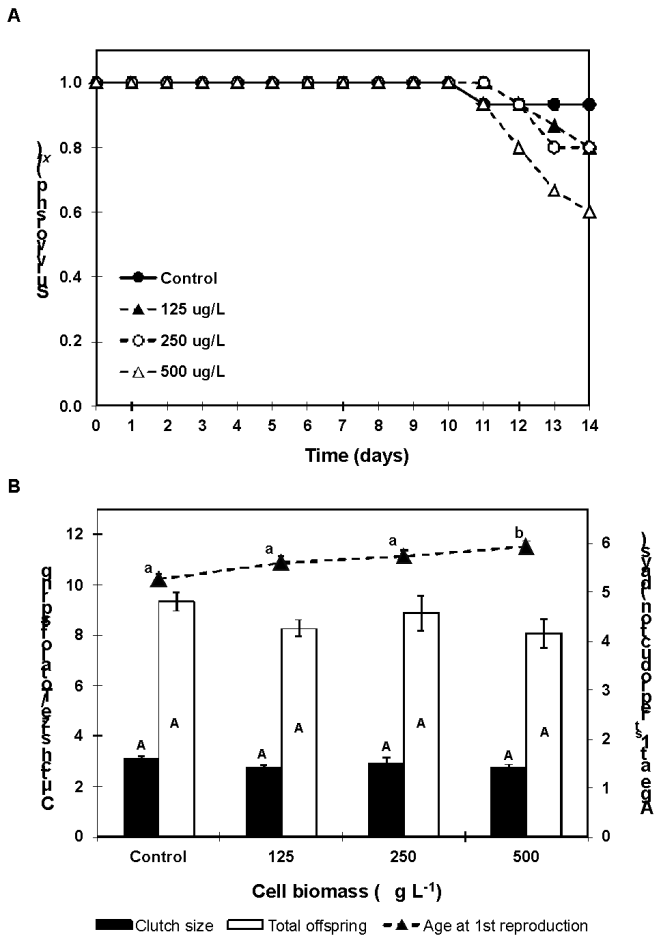

Fig. 4 Population parameters of *Daphnia gessneri* exposed to different concentrations (in µg DW L^{-1}) of strain CYRF-01 (*Cylindrospermopsis raciborskii*) mixed with 0.5 mg C L^{-1} of the green algae *A. falcatus* as food: (A) survivorship; (B) age at first reproduction (days), clutch size, and total offspring per female. Cell concentrations ranged between 2500–10000 cells mL^{-1} and cyanobacterial biomass represented 8–30% of total food. Standard error bars are given. Different letters indicate significant differences (*post-hoc* Tukey test).

size ($F_{3,56}$ = 0.58–0.83, $P > 0.05$) and the total production of offspring ($F_{3,56}$ = 0.09–1.11, $P > 0.05$). The *C. richardi*'s *r*-value presented a slightly, but not significant increase whereas *D. gessneri*'s *r*-value presented a slightly, but not significant decrease in the cyanobacterial treatments (*t*-test, $p > 0.05$; Table 2).

The cladoceran *D. spinulosum*, on the other hand, showed to be positively affected by the strain CYRF-01 (Fig. 5B), showing a significant increase in the average clutch size ($F_{3,56}$ = 6.805, $P = 0.001$) and in the total production of offspring ($F_{3,56}$ = 5.66, $P = 0.002$). The *r*-value increased significantly (up to 22%) with increasing concentrations of cyanobacteria (*t*-test, $P < 0.05$; Table 2).

Along with effects on population parameters, *D. similis* showed to be especially sensitive to CYRF-01 strain, presenting an inhibition of the swimming movements (data not shown) even in the lower concentration used (12.5 mg L^{-1}~1.1 ng STXeq. L^{-1}), which lasted until the end of the experiment but that not impeded this cladoceran to grow and reproduce. Initially, *M. micrura* also presented this inhibition of the swimming movements when exposed to strain CYRF-01. However, animals recovered swimming during the experiments. The other three cladoceran species (*C. richardi*, *D. gessneri* and *D. spinulosum*) did not show any sign of inhibition in the swimming movements.

DISCUSSION

Although culture conditions were the same in both experiments, there was an unexpected variation in filament length and toxin concentration. Filament length varied almost three times and saxitoxin cell quota varied about twice as much during the experiments 1 and 2 (Table 1). However, toxin cell quota was about in the same range between experiments. Some studies showed that filament length (Soares *et al*., 2013) and saxitoxins concentration in *C. raciborskii* may change

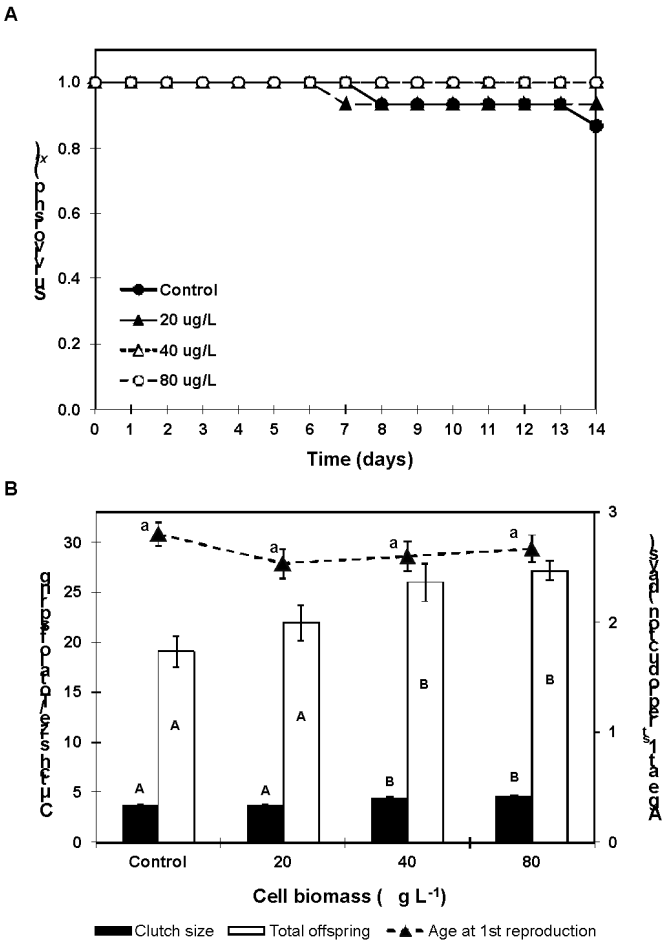

Fig. 5 Population parameters of *Diaphanosoma spinulosum* exposed to different concentrations (in µg DW L^{-1}) of strain CYRF-01 (*Cylindrospermopsis raciborskii*) mixed with 0.5 mg C L^{-1} of the green algae *A. falcatus* as food: (A) survivorship; (B) age at first reproduction (days), clutch size, and total offspring per female. Cell concentrations ranged between 250–1000 cells mL^{-1} and cyanobacterial biomass represented 2–8% of total food. Standard error bars are given. Different letters indicate significant differences (*post-hoc* Tukey test).

under different culture conditions (e.g. light and temprerature) and even show a daily rhythm of toxin production in the same culture (Carneiro *et al*., 2009). Thus, it is possible that slight variations in cultures conditions (i.e. temperature, light) or time of cell harvesting may lead to these variations.

Notwithstanding this fact, cladoceran species showed contrasting responses to the presence of toxic filaments of the cyanobacterium *C. raciborskii*. Despite the slight differences in the exposure concentrations of saxitoxins, *D. similis* and *M. micrura* were sensitive to the STX-producing strain CYRF-01, showing increased mortality and a reduction in fitness, expressed as a decline in both clutch size and in the intrinsic rate of population increase (*r*-value), while the other three species showed no sign of hazard in the cyanobacterial treatments. It is worth noting that the cladoceran *M. micrura* was sensitive in a higher range of concentrations than *D. similis*, while *D. gessneri*'s fitness was not affected at even higher concentrations of cyanobacteria in the diet.

Table 2 Intrinsic rate of population increase (*r*) and confidence interval (CI; 95%) for cladocerans exposed to strain CYRF-01. *P*-values refer to the comparisons between control and strain CYRF-01 concentrations (*t*-test).

Cladoceran species	Concentration (µg L^{-1})	Mean *r*-value	CI (95%)	*r*-value as % of control	*P*
D. similis	Control	0.418	0.406–0.431	---	---
	12.5	0.426	0.393–0.458	101.9	> 0.05
	25.0	0.413	0.391–0.436	98.8	> 0.05
	50.0	0.342	0.300–0.384	81.8	< 0.05
M. micrura	Control	0.592	0.545–0.638	---	---
	20.0	0.491	0.433–0.548	82.9	< 0.05
	40.0	0.509	0.451–0.567	86.0	< 0.05
	80.0	0.343	0.284–0.401	57.9	< 0.05
C. richardi	Control	0.386	0.366–0.405	---	---
	20.0	0.407	0.391–0.423	105.4	> 0.05
	40.0	0.391	0.380–0.402	101.3	> 0.05
	80.0	0.408	0.387–0.429	105.7	> 0.05
D. spinulosum	Control	0.372	0.343–0.402	---	---
	20.0	0.420	0.392–0.448	112.9	< 0.05
	40.0	0.445	0.406–0.484	119.6	< 0.05
	80.0	0.452	0.422–0.481	121.5	< 0.05
D gessneri .	Control	0.256	0.240–0.272	---	---
	125	0.238	0.228–0.248	93.0	> 0.05
	250	0.239	0.222–0.255	93.4	> 0.05
	500	0.241	0.216–0.265	94.1	> 0.05

Although not quantified, a paralysis effect was noticed in *M. micrura* and *D. similis*, partially or during the whole experiments, respectively. In a previous study, Ferrão-Filho *et al*. (2008) also showed that *M. micrura* were sensitive to another STX-producing *C. raciborskii* strain (T3), showing an inhibition of the swimming movements in concentrations as low as 100–1000 cells mL^{-1} (0.09–0.94 ng STXeq.L^{-1}), while *D. gessneri* showed no sign of inhibition in concentrations up to 10000 cells mL^{-1} (9.4 ng STXeq.L^{-1}). In another study

with the strain CYRF-01, Ferrão-Filho *et al*. (2010) showed the same symptom of paralysis of the swimming movements starting from biomass concentrations ranging 50–200 mg L^{-1} (0.26–1.10 ng STXeq.L^{-1}) for *D. pulex* and *M. micrura*, respectively. These studies show that both strains (T3 and CYRF-01) have comparable effects on cladocerans, knocking down their swimming capacity. In the present study, we aimed to test if similar concentrations (of cells or biomass) used in the two previous studies would cause effects on the fitness of the same cladoceran species.

However, since exposure to toxins depends also on ingestion rates, our results would benefit if we had measured clearance rates of animals in the different diets. Assuming that animals were feeding at non-limiting conditions and that there is no feeding inhibition or selective feeding (DeMott, 1982), the amount of cyanobacteria inside the gut would be a function of the amount ingested over time. Haney *et al.* (1995), however, verified that a filtrate of *Aphanizomenon flos-aquae* containing STXs lead to inhibition of the thoracic appendages beating rate (i.e. feeding rate) and increased post-abdominal rejection rate of *D. carinata*, which could ultimately lead to lower food ingestion and increased energy costs for those animals. Thus, as we do not know the ingestion rates of each cladoceran species on the different diets, our results are not readily comparable to each other. However, the difference observed between experiments, may be a result of how faster the animals can ingest and absorb the toxins in the gut. Therefore, considering that larger cladocerans such as *D. similis* and *D. gessneri* may have higher filtration rates than the smaller ones, once daphniids had their guts filled, it is likely that they would have been exposed to even higher concentrations of toxins than the other cladocerans.

Alternatively, the negative effects on the fitness of *D. similis* and *M. micrura* could have been explained by other hypothesis such as nutritional inadequacy, clogging of the filtering chamber, as well as increasing post-abdominal rejection movements, leading to high energy costs related to handling of cyanobacterial filaments (Porter & Orcutt, 1980; DeMott & Moxter, 1991; Leonard & Paerl, 2005). However, it is worth saying that there was no food limitation in our experiments since we added a sufficient amount of green algae with high nutritional value in all treatments. Also, in our study, negative effects in fitness were detected at very low proportions in the diet (<1–8%), which may suggest a truly toxic effect rather than food limitation. However, we cannot rule out that both mechanisms (i.e. feeding inhibition and paralysis) might be operating to cause the observed decrease in the fitness of *M. micrura* and *D. similis*, and could be a result of a jointing effect of both, starvation and toxicity.

The strain CYRF-01 presented a considerably large filament length (average length ~100–300 mm), which could cause constraints to ingestion by cladocerans. However, filamentous cyanobacteria such as *C. raciborskii* and *Planktothrix* spp. can be easily broken into small, edible sizes by zooplankton, including small cladocerans such as *C. cornuta* and *M. micrura* (Bouvy *et al*., 2001; Oberhaus *et al*., 2007; Kâ *et al*., 2012). Analysis of histogram frequency data (not shown) showed that during the experiments the filament length ranged mostly (>50%) between 70 and 300 mm in experiment 1 and between 25 and 100 mm in experiment 2. Thus, it is likely that a large proportion of the filaments were in the edible size range for cladocerans. Also, Panosso & Lürling (2010) showed that filament length of CYRF-01 (average range 61–137 mm) had little influence on the clearance rates of *D. magna* of 2.0–3.1 mm size, which is in a similar size range to *D. similis* adults. They concluded that "filament length of *C. raciborskii* should not be regarded *a priori* as an overriding factor affecting daphnids grazing. Alternatively, the degree of toxicity should be considered". Further, a meta-analysis of laboratory experiments conducted with a large dataset (n=597) to test the hypothesis that cyanobacteria cause detrimental effects to zooplankton came to the conclusion that both grazer groups (rotifers and cladocerans) were more inhibited by single celled cyanobacteria than by filamentous cyanobacteria, and that filamentous forms were better foods than single celled cyanobacteria (Wilson *et al*., 2006).

Other studies also found effects of *C. raciborskii* strains on the fitness of similar cladoceran species. Soares *et al*. (2009b) found effects of the strain CYRF-01 in survivorship and population growth rate in *D. magna* along with an inhibition of the feeding rate (i.e., clearance rate) but only in high proportions of cyanobacteria in the diet (75–100%), which is not the case in our study since we used lower proportions of CYRF-01 in the diet (up to 30%). No effect on mobility was reported in their study. The authors concluded that the effect of *C. raciborskii* seems to the more related to energy limitation than toxicity, but the food concentrations used seemed to be too high (~1.2–5.0 mg C L^{-1}) to cause food limitation. It is more likely that feeding inhibition by the toxins of the strain CYRF-01 have led to a starvation effect in animals. The study of Costa *et al*. (2013) tested the effects of a STX-producing (T3) and a non-STX-producing (NPLP-1) strains on the fitness of three cladoceran species and, similarly to our study, they showed that there was contrasting response between cladocerans, corroborated by a significant species *vs*. strain interaction. These results emphasize the diversity of responses that may be found in nature when toxic cyanobacteria dominate phytoplankton community.

In spite of its small size (~1.0 mm), the good performance of the *D. spinulosum* in the treatments with CYRF-01 suggests that it is able to utilize this strain as a food resource, providing biochemical compounds (i.e. proteins, lipids and carbohydrates) that may be not present in the green algae added as food. This hypothesis is contrary to some studies that suggest the low nutritional value of cyanobacteria, mainly due to the absence of essential polyunsaturated fatty acids (PUFA) and sterols (DeMott & Müller-Navarra, 1997). Some studies showed, however, that non-toxic cyanobacteria can be a complementary food source, providing a surplus of phosphorus and energy to cladocerans (DeMott & Müller-Navarra, 1997; DeMott & Tessier, 2002).

The lack of response of *C. richardi* and *D. gessneri* to the strain CYRF-01 might indicate that these cladocerans were

not using this resource. In spite from the fact that cladocerans are in general considered non-selective filter-feeders (DeMott, 1982), feeding experiments with *Daphnia* revealed that these animals may exhibit some selective feeding, discriminating between toxic *Microcystis* and non-toxic food (Ghadouani *et al*., 2004; Tillmanns *et al*., 2011). However, whether *C. richardi* and *D. gessneri* have a behavioral resistance (e.g. avoidance/rejection) or physiological tolerance to the STX-producing strain remains unclear and deserves more detailed studies. Nevertheless, in other experiments with *D. gessneri* submitted to a diet of only *C. raciborskii* (i.e. CYRF-01; non-published data), observation under the microscope showed that the gut was filled, suggesting ingestion of this cyanobacterium. Given its small size (~0.7 mm), it is likely that *C. richardi* would not be able to ingest most of the filaments due to carapace gap limitation (Gliwicz & Siedlar, 1980).

Alternatively, the absence of effect of strain CYRF-01 on *C. richardi* and *D. gessneri* suggests a possible resistance of these species to STXs. It is been hypothesized that tropical cladocerans might be more resistant than their temperate counterparts, since those species are exposed to heavier and long lasting blooms (Ferrão-Filho *et al*., 2000; Han *et al*., 2012). The clones of the species *C. richardi* and *D. spinulosum* came from the eutrophic Funil Reservoir, where cyanobacterial blooms are frequent and the strain CYRF-01 was isolated, whereas the clone of *D. gessneri* came from the oligo-mesotrophic Lajes Reservoir, where blooms of cyanobacteria have never been reported (Ferrão-Filho *et al*., 2009). This suggests that previous exposure to toxic cyanobacteria is not necessarily a pre-requirement to resistance (Wilson & Hay, 2007). Also, it must be emphasized that this species was exposed to a much higher range of STXs than the other species, which further suggests its higher resistance to strain CYRF-01's toxins.

In conclusion, our study shows that cladoceran species presented different responses to a STX-producing *C. raciborskii* strain. While the *D. similis* and the *M. micrura* showed decreased fitness in lower proportions of the strain CYRF-01 (<1–8%), the other cladocerans were either positively or not affected at all by the presence of cyanobacteria in the diet, even at higher proportions (up to 30%). This suggests that different susceptibilities to saxitoxins showed by cladoderans can lead to differences in competitive ability in environments dominated by STX-producing cyanobacteria, being thus a determinant factor in shaping zooplankton communities. Resistant species/clones that are able to co-exist and even utilize this cyanobacterium will gain an advantage over other, more sensitive species/clones.

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