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# Competitive exclusion of toxic cyanobacterial species by an allelopathic strain of *Phormidium*

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**Abstract** Blooms of freshwater toxic cyanobacteria are a growing environmental health problem, enhanced by anthropogenic eutrophication and climate change. A variety of techniques were tested for their remediation, from physical methods using artificial mixing or flocculation, to chemical methods employing synthetic and natural compounds, as well as constructed wetlands. In this work, we conducted an evaluation at microcosm scale of the usefulness of the allelochemicals produced by a strain of the filamentous cyanobacteria *Phormidium* sp. for the bioremediation of proliferations of four strains of toxic freshwater cyanobacteria (*Cylindrospermopsis raciborskii*, *Chrysosporum ovalisporum*, *Anabaena* sp. and *Nodularia* sp.). Allelochemicals produced by this strain of *Phormidium* sp. belong to the portoamides compounds family. Their effect was tested in bioassays using cell-free filtrate, the results showing that all the four strains were sensitive. In addition, we performed phosphate-limited long-term competition experiments in continuous cultures, in which *Phormidium* sp. was co-cultured with each of the toxic cyanobacterial strains. The purpose of these later experiments was to demonstrate that allelopathy and not resource competition was responsible for the ecological exclusion of the toxic cyanobacteria strains, and also to employ higher population abundances to test the effectiveness of the allelochemicals. Before that, we needed to estimate the competitive ability of each species to limit the resource that we employed (phosphate). *Phormidium* sp. were clearly a better competitor for phosphate than *Anabaena* sp., worse than *Nodularia* sp., and very similar to *C. raciborskii* and *C. ovalisporum*. Only in the case of *Nodularia* sp. could we demonstrate that the ecological exclusion of the toxic cyanobacteria was caused by allelopathy. However, the rapid exclusion shown in our experiments suggests that allelopathy was the main cause in all cases. An inter-specific competition model including only competition for phosphate and an allelopathic interaction was able to accurately describe the patterns of population dynamics observed in our experiments.

**Keywords** Cyanobacteria · Allelochemicals · Culture · Portoamides · *Phormidium* · Inter-specific competition · Phosphate

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## Introduction

Phytoplankton blooms are particularly dangerous when they are caused by cyanobacteria, since many species in this group produce toxins. Genera associated with common toxic cyanobacterial blooms in freshwaters include *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia* and *Planktothrix* (Buratti et al. 2017; Hudnell 2008; Huisman et al. 2018; Paerl and Otten 2013; Vasconcelos 1999). Damage to other aquatic organisms may be the result of either the production of potent toxins or hypoxia caused by high biomass conditions. These blooms also affect human health and economic activities, such as water consumption, aquaculture, fisheries, tourism and recreational uses of water (Chorus and Bartram 1999; Mur et al. 1999; Carmichael 2001; Kurmayer et al. 2004; Huisman et al. 2018).

Blooms could be enhanced by natural conditions (ideal levels of solar radiation, high temperature, elevated nutrient loads, low turbidity) but they are also favored by anthropogenic disturbances such as eutrophication (specially by increased phosphorus, Dokulil and Teubner 2000) and climate change (Paerl and Huisman 2008). Eutrophication is originated by nutrients from several natural and/or anthropogenic sources, such as domestic and industrial effluents, agricultural fertilizers and soil erosion. Climate change favors cyanobacterial development by increasing atmospheric temperatures and solar radiation (Carey et al. 2012; O'Neil et al. 2012; Paerl and Paul 2012).

Several methods have been tested to combat cyanobacterial blooms, such as the control of nutrients by dredging, sealing of sediments, flocculants (Sengco and Anderson 2004), mechanical methods: surface water filtration, artificial mixing (Visser et al. 1996), ultrasonication, chemical methods: nutrient chelators (Robb et al. 2003), detergents, pesticides, algicides (Peterson et al. 1997), reactive oxygen forms (Matthijs et al. 2012) and biological methods: viral, bacterial agents (Li et al. 2016) or periphyton (Wu et al. 2011), extracts or molecules from plants or algae (Yang et al. 2017) and constructed wetlands (Wang et al. 2018). The majority of these methods have shortcomings: limited effectiveness, high application costs, alterations to the ecosystem, having negative effects on many organisms (Cobo 2015; Matthijs et al. 2016). For this reason, an effective, economical, sustainable, and environmentally friendly solution to control blooms is needed.

Among the biological methods, the use of chemical products of biological origin was tested with relatively good success at laboratory scale (Yang et al. 2017). They have the advantage of being more environmentally friendly than other methods and could potentially be produced sustainably. Allelochemicals produced by microalgae or cyanobacteria are candidates to be employed as biological methods of bloom development control. Among phytoplanktonic organisms, cyanobacteria are the group for which most examples of allelopathy are known (Sliwinska-Wilczewska et al. 2021). This could be due to the fact that cyanobacteria are more frequently screened for allelopathy, but also because it is a larger and more diverse group than others. Examples of relevant genera of cyanobacteria in which allelopathy has been detected are *Anabaena*, *Aphanizomenon*, *Microcystis*, *Cylindrospermopsis*, *Oscillatoria*, *Planktothrix*, *Lyngbya*, *Nostoc*, *Synechococcus* (Antunes et al. 2012; Becher et al. 2005; Brilisauer et al. 2019; Ma et al. 2015; Puschner 2018; Suikkanen et al. 2004). The allelopathic compounds are not always known, but there are some allelopathic and/or cytotoxic compounds first described in some of the previous relevant genera of cyanobacteria: 7-deoxysedoheptulose (*Synechococcus*, Brilisauer et al. 2019), lyngbyatoxin A (*Lyngbya*, Berman et al. 1999), nostocyclamide (*Nostoc*, Todorova et al. 1995), homoanatoxin-a (*Oscillatoria*, Puschner 2018), microviridin (*Microcystis*, Puschner 2018).

The only allelochemicals produced by the freshwater cyanobacteria *Phormidium* sp. strain LEGE 05,292 (previous *Oscillatoria* sp.) were shown to be portoamides A-D (Leão et al. 2010) small cyclic peptides with selective effects on some green algae and bacteria (Leão et al. 2010; Barreiro and Vasconcelos 2014; Antunes et al. 2019). The mechanism of action of portoamides seems to be related to the respiratory chain and other basic mitochondrial functions (Ribeiro et al. 2017; Sousa et al. 2020).

Theoretical models suggested that allelopathy could be a mechanism of competition that reverses the outcome predicted by classic resource competition theory for a single limiting resource (Durret and Levin 1997; Roy 2009). The conditions for this are as follows: in a two-species system of inter-specific competition, if there is a trade-off between

allelopathic properties and the ability to exploit the limiting resource, the winner of the competition depends on the initial relative abundance of both species. In classic resource competition theory, however, the winner of the competition will always be the best competitor for the resource (Tilman 1977). To date, there is only one experimental demonstration of this principle for allelopathy (Barreiro Felpeto et al. 2018). Under these conditions (existence of the trade-off), it is possible, then, to distinguish between the effects of allelopathy and resource competition in long-term competition experiments (Roy 2009, Barreiro Felpeto et al. 2018). Following this principle, in this work we test whether allelochemical compounds from *Phormidium* sp. strain LEGE 05,292 can competitively exclude populations of several bloom-forming toxic cyanobacteria. Demonstrating that these allelochemicals are responsible for the exclusion of a large population of toxic cyanobacteria would show that they have potential as a biocontrol method of toxic cyanobacterial blooms. The first steps that to be performed to accomplish this aim are to test whether several toxic cyanobacteria are sensitive to *Phormidium* sp. allelopathy and estimate the competitive ability of all the species employed for a resource that limits their growth.

The specific aims of this work are (I) to test the allelochemical effect of *Phormidium sp.* strain LEGE 05,292 on four bloom-forming toxic cyanobacterial species; if these species are sensitive, then (II) to determine the competitive ability of the species for inorganic phosphorus, and (III) to test in long-term inter-specific competition experiments using phosphorus as the limiting resource, whether the allelopathic effect of *Phormidium sp.* is effective in outcompeting the populations of the toxic species. This later could be only demonstrated, as stated above, given the existence of a trade-off between the ability to exploit phosphorus and allelopathy. These experiments will also provide insights into whether allelopathy is effective with larger population abundances and longer exposure times than the bioassays. We selected phosphorus as a limiting resource for several reasons: portoamides are nitrogen rich molecules, so using nitrogen as a limiting resource might interfere with its production. Phosphate limitation is the macronutrient that most often limits primary production in freshwater environments (Elser et al. 2007). There are few literature reports on the parameters for phosphate uptake and growth for cyanobacteria, and phytoplankton in general. The scarcity of these data led to the development of phylogenetic methods for the estimates for these parameters, when needed (Bruggeman et al. 2009).

## Methods

### Cyanobacteria species

The species used in this work were: *Phormidium* sp. (strain LEGE 05,292), formerly classified as *Oscillatoria* sp. (Barreiro and Vasconcelos 2014; Barreiro Felpeto et al. 2018; Leão et al. 2009, 2010) *Chrysosporum ovalisporum* (strain LEGE X-001), *Anabaena* sp. (strain LEGE X-002), *Nodularia* sp. (strain LEGE 06,071) and *Cylindrospermopsis raciborskii* (strain LEGE 97,047). These species are all potentially bloom-forming and produce the toxins shown in Table 1. Cultures of these species were maintained in flasks of 250 mL, unmixed, with a growth medium whose composition is detailed in Barreiro and Vasconcelos (2014). This medium was specifically designed to provide a higher variety of macronutrients that could be suitable to maintain any freshwater phytoplankton species, providing their concentrations in excess and an N:P of 3200:200 µM. This is the same medium that will be used in all further experiments, altering only the N and P concentrations when necessary. The medium was always prepared with ultrapure water (0.056 µS cm at 23 °C) with a Lab Tower EDI 15 (Thermo Fisher Scientific®, Niederelbert, Germany) pro water purification system and sterilized in a Uniclave 88 (AJC Lda. Cacém, Portugal) autoclave system. Vitamins (filtered through 0.22 µm Millipore ®Express PES membrane filters) were added to the autoclaved medium after cooling. Cultures were kept in a culture room with 12:12 light conditions provided by cool-white fluorescent tubes model ARFX 25 W, brand ORNI-EX Lda. Portugal (intensity measured on top of the cultures surface of 60 µmol photons $m^{-2}$ $s^{-1}$, measured with a Digital Lux meter LU8500, TQC Sheen UK) and at 24 °C.

### Allelopathy bioassays

The bioassays carried out to test the effect of portoamides produced by *Phormidium* sp. were

**Table 1** Toxins produced by the species employed in our study (according to Chorus and Bartram 1999; Huisman et al. 2018)

| Toxins produced | Species | | | |
|---|---|---|---|---|
| | *Anabaena* sp. | *C. ovalisporum* | *C. raciborskii* | *Nodularia* sp. |
| *Hepatotoxic* | | | | |
| Cylindrospermopsin | | X | X | |
| Microcystin | X | | | |
| Nodularin | | | | X |
| Neurotoxic | | | | |
| Anatoxin-a | X | | | |
| Anatoxin-a(s) | X | | | |
| Saxitoxin | X | | X | |
| *Irritant* | | | | |
| Lipopolysaccharides (LPS) | X | X | X | X |

performed by testing the cell-free filtrate effect of *Phormidium* sp. on the four toxic strains.

In order to obtain cell-free filtrate for the assays, *Phormidium* sp. was grown in a continuous culture with the culture medium cited above, a N:P of 3200:200 μM, and maintained with a dilution rate that allowed permanent exponential growth (0.4 $day^{-1}$) and strong mixing, since portoamides are produced at higher rates during exponential growth (Barreiro et al. unpublished). The target cyanobacterial species were obtained from semi-continuous cultures (renewing 50% of the medium weekly) using the same culture medium.

The experimental set-up for the bioassays consisted of a treatment of different concentrations of the cell-free filtrate, in 8 mL glass vials containing 5 mL of cell suspensions of the target toxic strains. The concentrations of cell-free filtrate ranged from 0 (control) to 60% (Table 2). To avoid confusion between allelopathy and the effects of nutrient limitation, the same volume of a concentrate amount of highly enriched medium (same as above, Vasconcelos and Barreiro 2014) was added to every treatment level, producing final N:P concentrations of 640:40 μM with all micronutrients in excess, enough to guarantee the absence of nutrient limitation during the short duration of the experiment. The final volumes of the vials were equalized by adding the corresponding amounts of the same medium without the macronutrients and iron. The different treatment levels had different amounts of filtrate added, but this would simply increase the macronutrient concentrations. Because the basal N:P of 640:40 μM is already an excess of nutrients for the duration of this experiment, these alterations would not have an effect on growth. Five replicates were used for each filtrate concentration. The administration of the experimental treatments to the vials was done in one pool per cell-free filtrate concentration. Then, each replicate was pipetted from the same pool, minimizing the initial variation among replicates.

The initial abundances of the target strains are shown in Table 2. Equalization of the species in terms of biovolume was attempted. These abundances were selected in an attempt to make them as low as possible while still being able to obtain representative cell counts in a Neubauer hemocytometer. This obviously depends on the species with the lowest numbers (highest biovolume). Using excessively high abundances could compromise the detection of the allelopathic effect, because in these bioassays the allelopathic effect is weaker than when there is closer cell contact and continuous release of allelochemicals.

**Table 2** Cell abundances in the bioassays

| Toxic species | Initial abundance (cells $mL^{-1}$) | Tested filtrate concentrations (%) |
|---|---|---|
| *Anabaena* sp. | 63,611 ± 1944 | 0–25–50–60 |
| *C. ovalisporum* | 256,203 ± 8759 | 0–20–40–60 |
| *C. raciborskii* | 262,847 ± 30,436 | 0–20–40–60 |
| *Nodularia* sp. | 244,351 ± 13,056 | 0–25–50–60 |

The vials were incubated in a growth chamber with the growth conditions detailed in the previous section. Cell growth was estimated for all species after 48 h, except for *C. raciborskii* which needed 120 h due to its lower growth rate. Cell counts were performed using a Leica DM LB (Leica Microsystems, Wetzlar, Germany) microscope at 10 × or 40 × magnification, using a Neubauer chamber.

The growth rate was calculated as:

$$r = \frac{\ln\left(\frac{n_2}{n_1}\right)}{t_2 - t_1}$$

where $n_i$ is the abundance of the species in the cells $mL^{-1}$ at $t = i$, and $t_i$ the time step in days. The allelopathic effect for each species was estimated by linear regression between the dependent variable "growth rate" and the independent variable "% of cell-free culture filtrate" of *Phormidium* sp. A significant negative slope for this regression indicates the existence of an allelopathic effect. The linear regression and Mann–Whitney pair-wise comparisons were performed using the *lm* and *wilcox.test* functions available in the *stats* package of the *R* program (R Core Team, 2020).

## Phosphate uptake experiments

A 50 mL flask of batch culture from each species was precultured for 6 days with the same medium composition as in the first section, but with low phosphate concentration (1 µM). Then, all the 50 mL cultures were transferred to a 250 mL culture flask, adding up to 250 mL of the same medium without phosphate. These cultures were kept under these conditions for 24 h. After this time, a pulse of approximately 5 µM of phosphate was added to these cultures and phosphate concentrations were monitored for 18 h. Cell abundances were only estimated at time 0, assuming a negligible growth during 18 h (time steps of 0, 0.5, 1, 2, 4, 7, 11 and 18 h). Three replicates of 7 mL for phosphate analysis were sampled at each time step. They were filtered through 0.22 µm membrane filters (Millipore® Express PES) and kept in the freezer at − 20 °C until they were analyzed in a Skalar Sanplus nutrient autoanalyzer using the Skalar M461-318 (EPA 353.2) method. The uptake of phosphate was assumed to follow a Monod model, where $V_{max}$ is the maximum uptake rate and $H_{PO4}$ the half-saturation constant:

$$V = \frac{V_{\max} PO_4}{H_{PO_4} + PO_4}$$

For the estimate of the maximum uptake rate of each species, the slope of a linear regression performed between external phosphate concentration and time was calculated, considering only the first hours of the experiment, when the uptake is faster. Half-saturation constant estimates were obtained by fitting the experimental data to the above equation using a nonlinear least-squares regression method, with the *nls* function coded in the *stats* package from R (R Core Team 2020).

## Growth experiments

In order to estimate the growth parameters of the five species, experiments were performed on batch cultures with phosphate as the limiting nutrient. Growth conditions and medium composition were the same as in the above sections, but with an N:P ratio of 3200:20 µM (160:1) thereby making it strongly phosphate limited. Cell abundances and phosphate concentration were monitored every 24 h. For the phosphate analysis, two replicates of 7 mL were taken and processed in the same way as described above. Cell counts were also performed, and the growth rate was calculated, also as indicated in the previous sections. Monitoring was ended after 14 days, a few days after each species reached the stationary phase. It was assumed that population growth rate follows the Monod model, where $\mu_{max}$ is the maximum growth rate and $K_{PO4}$ the half-saturation constant for phosphate.

$$\mu = \frac{\mu_{\max} PO_4}{K_{PO_4} PO_4}$$

The maximum growth rate of each species was estimated as the slope of a linear regression performed with cell densities (log-transformed) against time, during the period when the cultures showed faster growth, and the estimates of the half-saturation constant were obtained by fitting our experimental data to the above Monod equation using a nonlinear least squares algorithm implemented in the *nls* function, from the *stats* package from *R* software (R Core Team 2020).

### Long-term competition experiments in continuous cultures

In these experiments, *Phormidium* sp. competed for inorganic phosphorus against each of the toxic cyanobacteria species separately. The continuous cultures were maintained under the same growth conditions as detailed above, but with mixing provided by aeration and daily scratching of walls with a magnet to avoid biofilm formation. The culture medium was the same, with an N:P of 3200:20 μM, one order of magnitude greater than the theoretical Redfield's optimal of 16:1, to guarantee phosphate limitation. Flow rate of the chemostat system was 0.3 $day^{-1}$. *Phormidium* sp. was always sonicated prior to the inoculation, to disentangle large filament balls and homogenize the culture. For those toxic cyanobacteria that resulted to be better competitors for phosphate than *Phormidium* sp., two kinds of experiments were performed: (A) Higher initial abundance of *Phormidium* sp. relative to the toxic species and (B) Higher initial abundance of the toxic species relative to *Phormidium* sp. The rationale behind these experiments is that if the toxic strain is a better competitor for the limiting resource, in agreement with the ecological theory of competition for resources (Tilman 1977) it will win the competition regardless of the relative initial abundances of both species. However, according to models and experiments involving allelopathy in interplay with resource competition (Barreiro Felpeto et al. 2018) the relative initial abundances would actually determine whether the winner of competition is the best competitor for the resource, or the allelopathic species. For the A experiments, the allelopathic (*Phormidium* sp.) is expected to win the competition and for the B experiments, the toxic strain, better competitor for phosphate, is expected to win the competition.

Cell abundances were estimated every day, and counts were made in Neubauer and Sedgewick-Rafter chambers of whether the population abundances were high or low, respectively.

### Competition model formulation

In order to represent, predict and analyze the results of the long-term competition experiments described above, we formulated, optimized and simulated a model consisting of coupled ordinary differential equations. This model represents the dynamics of the populations of the two competing species based on two mechanisms: competition for the limiting resource (phosphate) and the allelopathic effect of *Phormidium* sp. against the other species. The model formulation is as follows:

$$\frac{dP}{dt} = D(P_0 - P) - F_1(P)\frac{1}{\eta_1} - F_2(P)\frac{1}{\eta_2}$$

$$\frac{dS_1}{dt} = F_1(P) - \gamma S_2 S_1 - DS_1$$

$$\frac{dS_2}{dt} = F_2(P) - DS_2$$

where,

$$F_i(P)(i = 1, 2) = \frac{\mu_i S_i P}{K_i + P}$$

where $P$ is the actual phosphate concentration in the culture, $D$ is the dilution rate of the chemostat system, $P_0$ is the inflow phosphate concentration, $F_i(P)$ are the growth functions for species, where $i = 1$ is one of the toxic cyanobacteria and 2 is *Phormidium* sp., $\mu_i$ is the maximum growth rate of species $i$, and $K_i$ the half-saturation constant for phosphate of species $i$. Parameters $\eta_i$ are the yield coefficients of species $i$, as a ratio of the mass of cells to the mass of nutrient consumed.

$S_i$ are the population abundances of toxic cyanobacteria ($i = 1$) and *Phormidium* sp. ($i = 2$) $\gamma$ represents the allelopathic effect, that is, the rate of reduction in population growth of $S_1$ caused per cell of $S_2$.

### Model parameterization and optimization

The initial parameter estimates for $\mu_i$, and $K_i$ come from the growth experiments. Other parameters were set experimentally ($D$ = 0.4 $day^{-1}$, $P_0$ = 20 μM). The rest of the parameters were unknown, and their initial values were set tentatively for estimation.

Parameter optimization was performed in two steps, first by applying a global optimization method, simulated annealing, and then, with the estimates obtained from it, a local optimization method based on the Nelder-Mead algorithm (Nelder and Mead 1965). The simulated annealing was implemented with the *GenSA* function from the *GenSA R* package. The Nelder-Mead algorithm was implemented with the *optim* function from the stats R package. In both global

and local optimization, the objective functions were set according to a version of the Levenberg-Mardquart minimization criterion (Levenberg 1944):

$$E = \sum_{t=1}^{t=n} \left[ \left( \frac{\left| P_{1\text{pred}} - P_{1\text{obs}} \right|}{P_{1\text{obs}}} \times \frac{1}{CV_{P1}} \right) + \left( \frac{\left| P_{2\text{pred}} - P_{2\text{obs}} \right|}{P_{2\text{obs}}} \times \frac{1}{CV_{P2}} \right) \right]$$

The parameter set providing the minimum value of $E$ is considered the best estimate. In this formula, $P_{i\text{pred}}$ (where $i = 1$ for each toxic cyanobacteria, 2 for *Phormidium* sp.) correspond to the abundances of each species predicted by the model for times $t = 1$ to $t = n$, and $P_{i\text{obs}}$, the same abundances from the real data. $CV_{Pi}$ is the coefficient of variation of the observed population abundances. For the values of the observed population abundances, the real abundances were smoothed with a nonparametric regression method (Ellner et al., 2002).

### Model simulation

When a set of optimal parameter estimates was obtained for the experimental data of each pair toxic cyanobacteria-*Phormidium* sp., the model was extrapolated to test the outcome of inter-specific competition under a wider range of initial conditions (relative proportions of the competing species). The aim of this extrapolation is to check whether the predictions hold for a wider range of conditions, and it will be particularly interesting only if there is a trade-off between competition for the limiting resource and allelopathy, since, in that case, the winner of the competition will depend on the initial relative proportions of the species, as explained above.

The model simulations were performed on a grid with all possible combinations of initial abundances of the two competing species from 1.000 to 3.000.000 cells $mL^{-1}$, in steps of 1000. This makes a total of 9.000.000 simulations. The simulations were initially run for 100 time steps (days), to eliminate initial transient phases, and, taking the values at day 100 as the new initial values, they were run for an additional 200 days. The density of each species at day 200 was then assumed to be the steady-state equilibrium abundance for the winner of the competition when the abundance of the species excluded is 0.

## Results

### Sensitivity to Phormidium sp. allelopathy

Cell-free filtrate from *Phormidium* sp. culture showed a significant allelopathic effect against all of our target species in the bioassays (Figs. 1, 2). All the slopes of the linear regressions in Fig. 1 were statistically significant: *C. ovalisporum* slope = − 0.004 (t-student = − 2.603, $p = 0.018$); *Anabaena* sp. slope = − 0.004 (t-student = − 7.053, $p < 0.001$); *Nodularia* sp. slope = − 0.003 (t-student = − 5.819, $p < 0.001$) and *C. raciborskii* slope = − 0.003 (t-student = − 9.295, $p < 0.001$). We consider a demonstrated allelopathic effect when there is a significant reduction in the growth of the target species. This negative effect is supported by the statistical significance of the slope of a linear regression between the concentration of the filtrate and the growth rate. The slope of the regressions for the different species (Fig. 1) showed similar values, so the strength of the negative allelopathic effect found was of the same magnitude across the target species. Besides the linear regression analysis, the nonparametric Mann–Whitney tests performed to compare all pairs of filtrate concentrations (Fig. 2) always showed significant differences between the control and the highest concentration of filtrate. Other pairwise comparisons also showing significant differences were the following: between the control and 25% of filtrate for *Anabaena* sp. and *Nodularia* sp. ($p = 0.016$ and $p = 0.028$, respectively) and between the control and 40% filtrate for *C. raciborskii* ($p = 0.011$).

### Competitive ability for phosphate

The results of the parameterization of phosphate uptake are shown in Table 3. According to the proportion of the maximum uptake rate ($V_{\max}$) to the half-saturation constant ($H_{PO4}$), employed as an approximate estimation of affinity, the species with highest affinity for phosphate clearly appears to be *C. ovalisporum* and the one with the least affinity seems to be *C. raciborskii*. However, the differences between *C. raciborskii*, *Anabaena* sp., *Nodularia* sp. and *Phormidium* sp. are not very clear. The proportion of these parameters is very similar for all four species. In addition, it is difficult to obtain significant half-saturation constants because this parameter defines a

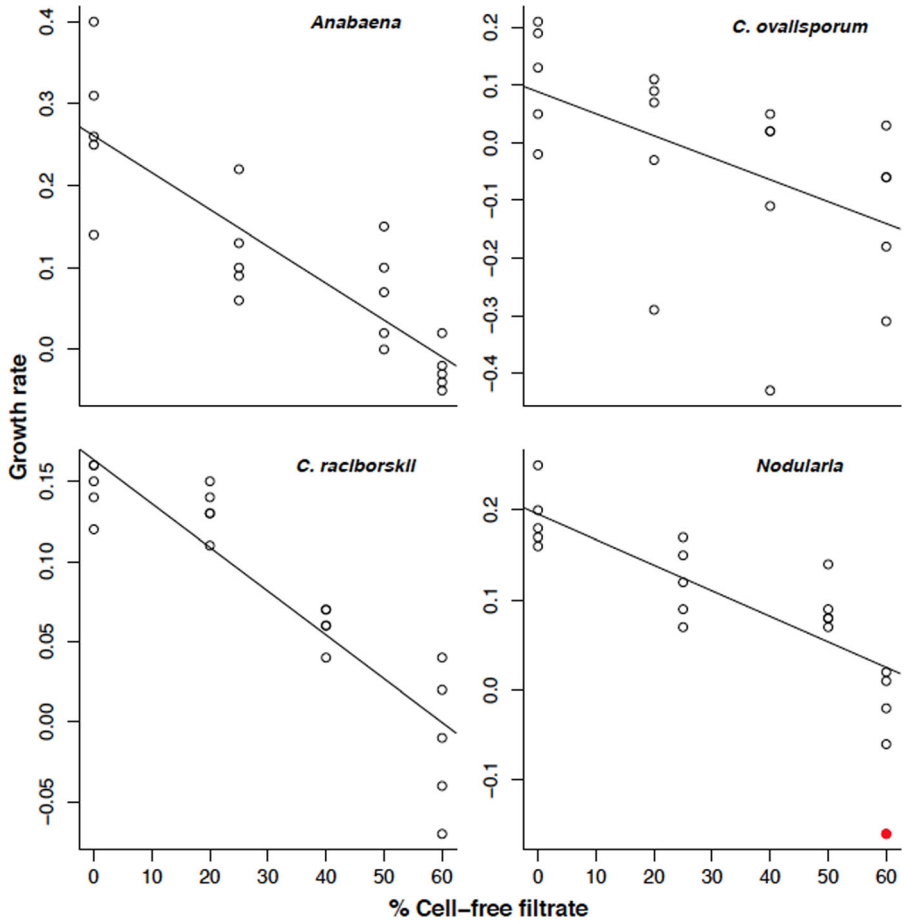


**Fig. 1** Results of bioassays showing the effect of cell-free filtrate on each of the target species. Individual points are the independent replicates, and plotted lines show the linear regressions. The red dot in the *Nodularia* panel is an outlier that was not included in the regression model

very sensitive region of the curve, with a high variation in the response variable ($V$) over a small range of the predictor ($PO_4$). Despite the relatively low number of data points, however, two of the five estimated half-saturation constants were statistically significant (for *Phormidium* sp. and *C. ovalisporum*) and for *Anabaena* sp. marginally significant (Table 3).

The results of the parameterization of growth are shown in Table 4. The ratio of the maximum growth rate to the half-saturation constant, employed as an approximate indicator of affinity, shows that *Nodularia* sp. is the best competitor and *Anabaena* the worst. Among the rest of the species, the values are very close and, in this situation, this ratio is not a reliable indicator, particularly due to the uncertainty in the estimation of the half-saturation constants. Two of the five half-saturation constants were found to be statistically significant (*Phormidium* sp. and *C. raciborskii*).

As an approximate indicator of the overall rank as competitors, we could consider the sum of the ratios $V_{max}/H$ and $\mu_{max}/K$, which give us the following ranking: *Nodularia* sp. = 0.149; *C. ovalisporum* = 0.034; *Phormidium* sp. = 0.027; *C. raciborskii* = 0.014; *Anabaena* sp. = 0.012.

As mentioned in the Introduction, there must be a trade-off between allelopathy and competitive ability for phosphate to demonstrate that allelopathy can exclude the best competitor in these long-term competition experiments. The existence of this trade-off implies that the winner of competition will be the one with higher initial relative abundance. Then, the effects of the competition for nutrients and allelopathy could be disentangled.

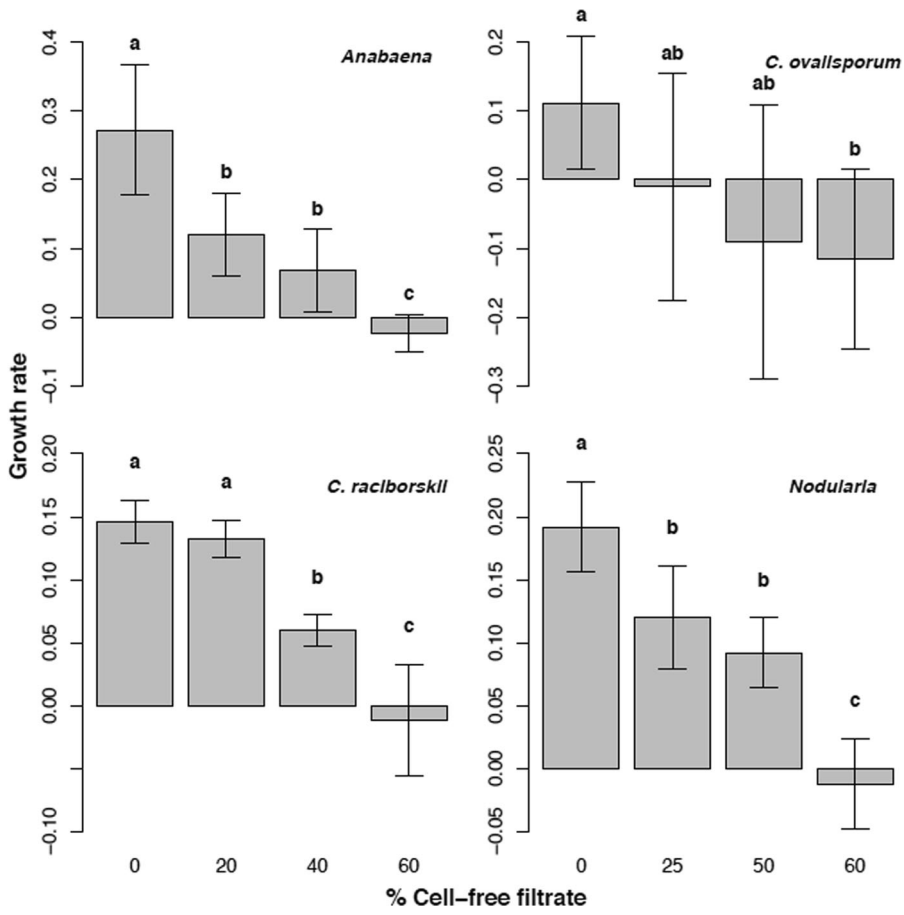


**Fig. 2** Barplots of mean ± SD of growth rate for each % of filtrate. The letters on top of each bar indicate significant differences according to Mann–Whitney tests

**Table 3** Best parameter estimates for the uptake Monod equation obtained from the uptake experiment, indicating the statistical significance for the estimated half-saturation constants (students-t value and associated *p* value)

| Species | $V_{max}$ (fmol cell$^{-1}$ h$^{-1}$) | $H_{PO4}$ (μM) | *t* | *P* | $V_{max}/H_{PO4}$ |
|---|---|---|---|---|---|
| *Anabaena* sp.[4] | 0.16 | 24.67 ± 12.44 | 1.98 | 0.07 | 0.006 |
| *C. ovalisporum*[1] | 0.25 | 10.13 ± 2.67 | 2.16 | 0.05 | 0.024 |
| *C. raciborskii*[5] | 0.16 | 44.95 ± 26.17 | 1.72 | 0.11 | 0.004 |
| *Nodularia* sp.[2] | 0.21 | 24.16 ± 17.80 | 1.36 | 0.19 | 0.009 |
| *Phormidium* sp.[3] | 0.24 | 33.64 ± 13.82 | 2.44 | 0.03 | 0.007 |

The exponent on each species name shows the rank as competitor according to the ratio $V_{max}/H$

The conditions for this trade-off are met for *Nodularia* sp. (see Tables 3, 4 and previous global ranking) but not for *Anabaena* and *C. raciborskii*. For *C. ovalisporum*, very similar as a competitor to *Phormidium* sp. (see Tables 3, 4 and previous global ranking) predicting the trade-off with this method is not reliable. Then, we performed experimental runs with very high relative initial abundances of *C. ovalisporum* (data not shown) that could help to resolve this uncertainty. If the trade-off was present, then, *C. ovalisporum* should be able to exclude *Phormidium* sp. However, in these experiments, the

**Table 4** Best parameter estimates for the growth Monod equation obtained from the growth experiment, indicating the statistical significance for the estimated half-saturation constants (students-t value and associated *p* value)

| Species | $\mu_{max}$ ($day^{-1}$) | $K_{PO4}$ (μM) | *t* | P | $\mu_{max}/ K_{PO4}$ |
|---|---|---|---|---|---|
| *Anabaena* sp.[5] | 0.29 | 45.67 ± 25.45 | 1.79 | 0.11 | 0.006 |
| *C. ovalisporum*[3] | 0.19 | 15.13 ± 9.97 | 1.52 | 0.16 | 0.01 |
| *C. raciborskii*[3] | 0.43 | 38.30 ± 15.46 | 2.48 | 0.03 | 0.01 |
| *Nodularia* sp.[1] | 0.29 | 2.11 ± 1.62 | 1.30 | 0.23 | 0.14 |
| *Phormidium* sp.[2] | 0.65 | 28.93 ± 8.96 | 3.23 | 0.01 | 0.02 |

The exponent on each species name shows the rank as competitor according to the ratio $\mu_{max}$/K

trend of the *Phormidium* sp. population was to maintain or even increase in numbers over approximatively 50 days. This means that there is also no condition for this trade-off with *C. ovalisporum*.

## Long-term competition experiments and model simulations

The results of the long-term competition experiments, as well as the corresponding competition model fits, are represented in Fig. 3. In this Figure, the ratios at the top left corner of each panel show the ratio of the initial abundances of the two competing species. The best parameter estimates for the model fits to each experiment are shown in Table 5.

Figure 3 shows a clear and fast exclusion of *Anabaena*, *C. raciborskii* and *C. ovalisporum*. For *C. raciborskii* and *Anabaena* sp. it was not possible to demonstrate that allelopathy and not competition for phosphate was the reason for the exclusion of the toxic species, due to the lack of a trade-off between allelopathy and competitive ability for phosphate. For *C. ovalisporum*, it was not possible to exclude *Phormidium* in experiments employing high relative initial abundances of *C. ovalisporum* (see previous section) suggesting that there is also no trade-off between these two species.

For *Nodularia* sp., in Fig. 3 we have two plots, corresponding to each alternative outcome of the competition. There was a clear exclusion of the allelopathic species (*Phormidium* sp.) when the relative initial abundances favors *Nodularia* sp. (ratio = 0.36) and the exclusion of the best competitor (*Nodularia* sp.) in the opposite situation (ratio = 15.28).

Table 5 shows the best model parameters estimated for these experiments. The competitor rankings (pairwise) did not change with respect to those estimated independently of these competition experiments (see Tables 3, 4 and previous section). In general, the growth of the species in these long-term experiments was more efficient than in the short growth experiments. This is why all the fitted maximum growth rates were larger than those in Table 4, and most of the $K_{PO4}$ values lower.

In order to check whether the results of these experiments could be extrapolated to more combinations of initial relative abundances of the species, we performed 9 million simulations (see Methods) for each of the *Phormidium* sp.-toxic species pairs. The parameters employed were the same as shown in Table 5, except for the simulation with *Nodularia* sp., because we need a single set of parameters that can explain both outcomes obtained. Then, in that case we employed the parameter set fitted for the experiment showing exclusion of *Nodularia* sp. with these changes: reducing the $\mu_{max}$ of *Phormidium* sp. to 0.53 and the allelopathic effect to $0.9 \times 10^{-7}$.

The simulations showed that the *Phormidium* sp. was the winner of the competition in all cases with *Anabaena* sp., *C. raciborskii* and *C. ovalisportum*. The results of the simulations for *Nodularia* sp. are shown in Fig. 4. In this case, there is a region of the grid of relative abundances where *Phormidium* sp. wins and another one where *Nodularia* sp. wins. These regions, however, are slightly off the diagonal, the region where *Phormidium* sp. wins being the larger of the two.

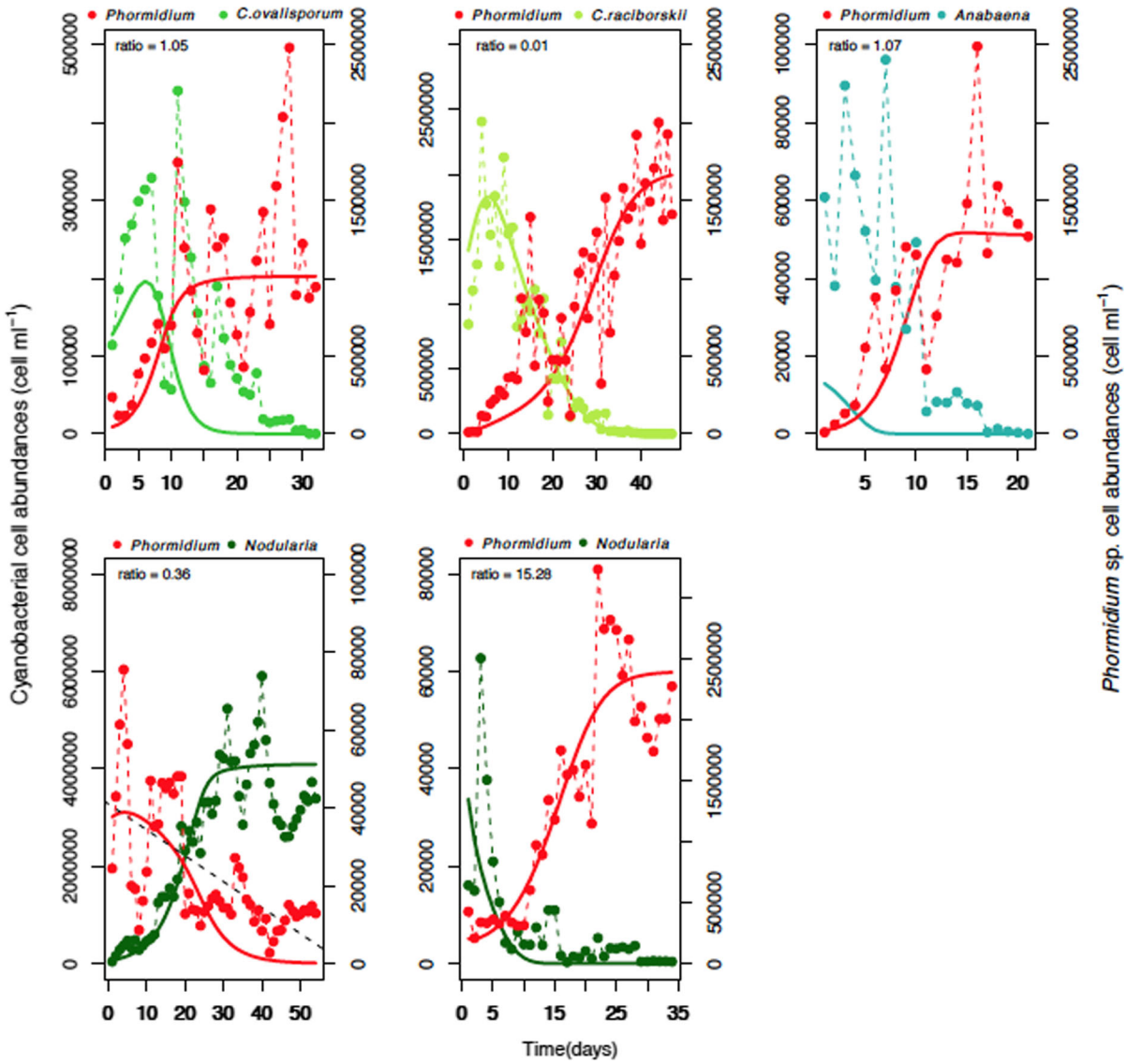


**Fig. 3** Outcomes of long-term competition experiments between *Phormidium* sp. and toxic species. Dots with hyphened lines represent the real data from the experiments, as averages of four independent samples. Solid lines represent the model fits according to the parameter values shown in Table 5. Numbers in the top left corner indicate the ratio of initial *Phormidium*:toxic species as an average over the first three days. Panel 4 plots a linear regression fit for the abundance of *Phormidium* over time (hyphened line), which showed a significant negative slope (-667, $p > 0.001$). (Color figure online)

## Discussion

All the bioassays showed a significant allelopathic effect of *Phormidium* sp. In this kind of experiments, the strength of the effect detected is usually reduced because the source of allelochemical compounds is not renewed or increased during the experiment, due to the absence of the allelopathic species. For this reason, we would expect that, in the long-term experiments, with the physical presence of *Phormidium* sp., the effectiveness of allelopathy will be greater. In fact, in those experiments (Fig. 3) we could see large populations of the target species completely collapsing in a few days. The negative allelopathic effect of this strain of *Phormidium* sp. was well known for certain microalgae, like *Chlorella vulgaris* and *Ankistrodesmus falcatus* (Barreiro and Vasconcelos 2014; Barreiro Felpeto et al. 2018; Leão et al. 2010) but not for

**Table 5** Best parameters fit of the competition model obtained from each of the long-term competition experiments (Ph = *Phormidium* sp.; Chr = *Chrysosporum ovalisporum*; Ana = *Anabaena* sp.; Nod = *Nodularia* sp.; Cyl = *Cylindrospermopsis raciborskii*). The winner of the competition on each trial is shown in bold

| Competition experiment | | $Y$ (cell $\mu M^{-1}$) | $\mu_{max}$ (cell $day^{-1}$) | $K_{PO4}$ ($\mu M$) | $\mu_{max}/ K_{PO4}$ | $mA$ (cell $day^{-1}$) |
|---|---|---|---|---|---|---|
| ***Phormidium* sp.** vs | Ph | 79,731 | 1.14 | 11.27 | **0.1** | $5e^{-06}$ |
| *Anabaena* sp | Ana | 336,947 | 0.51 | 29.13 | 0.02 | |
| ***Phormidium*** sp. vs | Ph | 63,262 | 1.35 | 14.04 | 0.10 | $5.2e^{-07}$ |
| *C. ovalisporum* | Chr | 267,319 | 0.62 | 5.02 | **0.12** | |
| ***Phormidium*** sp. vs | Ph | 159,824 | 1.09 | 24.92 | **0.04** | $2.4e^{-07}$ |
| *C. raciborskii* | Cyl | 276,102 | 0.56 | 18.44 | 0.03 | |
| ***Phormidium* sp.** vs | Ph | 184,438 | 0.78 | 11.24 | 0.07 | $1.2e^{-06}$ |
| *Nodularia* sp. | Nod | 135,478 | 0.46 | 5.88 | **0.08** | |
| *Phormidium* sp. vs | Ph | 208,132 | 0.39 | 7.73 | 0.05 | $4.9e^{-07}$ |
| ***Nodularia* sp.** | Nod | 251,366 | 0.60 | 3.74 | **0.16** | |

The $\mu_{max}/K_{PO4}$ ratio calculated with these parameters is an estimate of the best competitor for nutrient, highlighting in bold the best competitor of each pair

cyanobacteria. It had already been reported as having an effect on the general structure of phytoplanktonic communities, but without clarifying the target species and whether the effects have positive or negative sign (Leão et al., 2012). On the other hand, there were species of microalgae reported to be resistant, such as *Chlamydomonas reinhardtii* and *Selenastrum capricornutum* (Barreiro and Vasconcelos, 2014).

Our finding with these allelopathy bioassays is very interesting because all four species employed can cause toxic blooms in the natural environment, and hence the potential of this strain of *Phormidium* sp. for bioremediation of toxic blooms deserves to be further studied.

Taking into account that our five species are ecologically relevant, the parameter values obtained for phosphate competitive ability are a valuable source for further trait-based ecological studies of plankton dynamics (Litchman and Klausmeier 2008; Wentzky et al. 2020). These values are estimated in laboratory experiments, and only exist for a reduced number of species. When they are unknown, they are inferred from a consideration of phylogenetic relationships and surface-volume relationships (Bruggeman et al. 2009). From the values obtained for our species, keeping in mind the uncertainty due to the statistical inference of some of these parameters, it was very clear that *Nodularia* sp. was a relatively good competitor, *Phormidium* sp. and *C. ovalisporum* had an intermediate rank, and *C. raciborskii* and *Anabaena* sp. were relatively poor competitors. This ranking was further supported by the results of the long-term competition experiments.

It was not possible to demonstrate that allelopathy was the cause of exclusion in the species *C. ovalisporum*, *C. raciborskii* and *Anabaena*. But, especially for the *C. ovalisporum*, which was very close to *Phormidium* sp. in competitive ability for phosphate (Tables 3, 4 and 5) allelopathy seemed to be more important than competition for phosphate. In comparable experiments, exclusion due to resource competition is often much slower (50–70 days)) than that caused by allelopathy (20–30 days) (Barreiro Felpeto et al. 2018). In this case, despite the similar competitive abilities of these two species, exclusion occurred around 30 days, suggesting an important allelopathic effect. The model simulations (Fig. 4) showed that the region of the grid with a dominance of *Phormidium* sp. covers a larger area than the equivalent for N*odularia* sp. This means that the effect of allelopathy by *Phormidium* sp. is stronger than the competitive advantage conferred to *Nodularia* sp. as a better competitor for phosphate. Another explanation could be that the parameter set chosen for this simulation was basically the one from the experiment when *Nodularia* sp. was excluded. However, this explanation should be discarded since, as explained in the results section, we reduced the competitive advantage

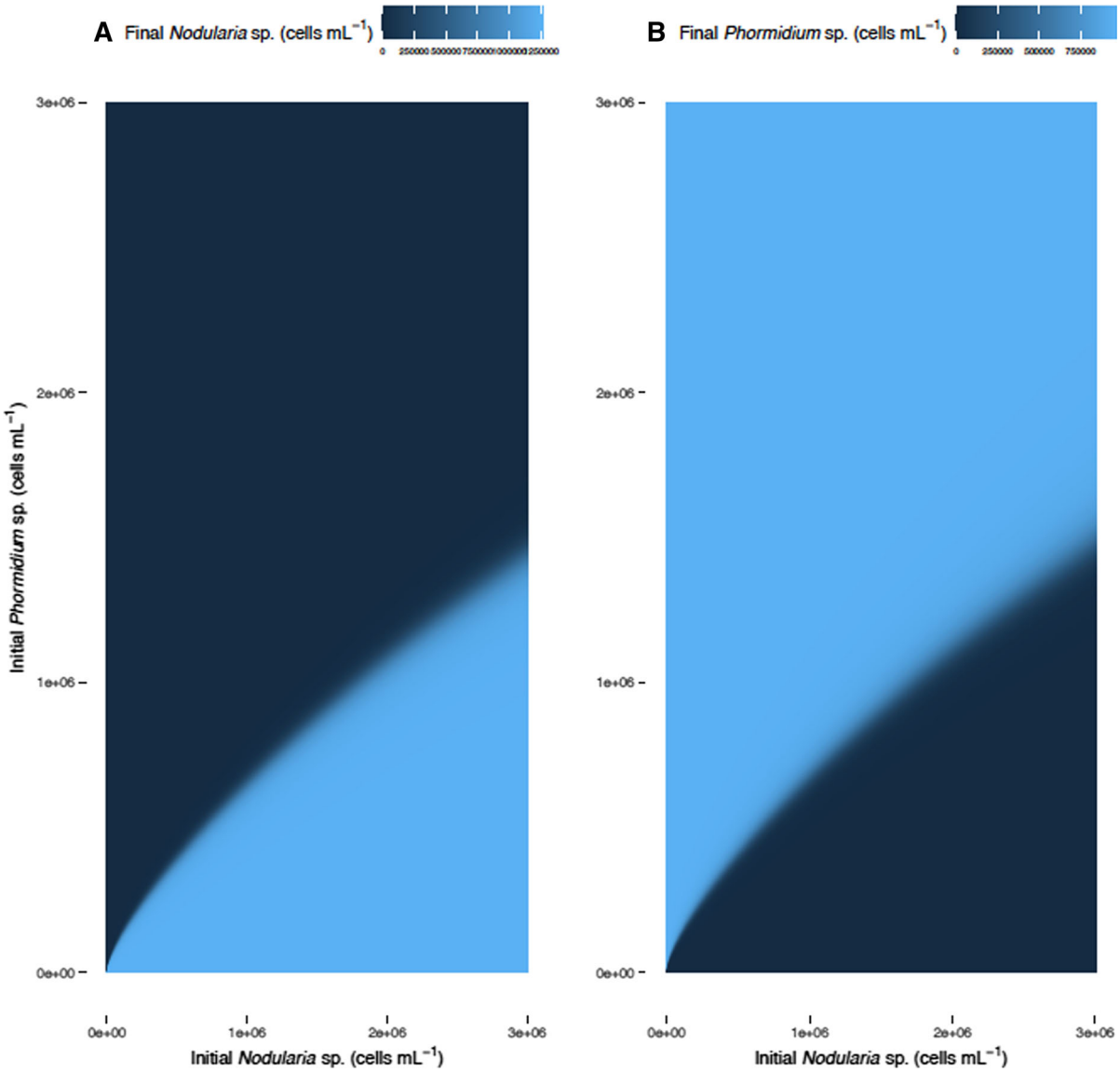


**Fig. 4** **A**—Results of the simulations of the competition model showing the equilibrium abundances of *Nodularia* sp. **B**—Results of the simulations of the competition model showing the equilibrium abundances of *Phormidium* sp. (Color figure online)

of *Phormidium* sp. by reducing $\mu_{max}$ and particularly *mA* as much as one order of magnitude.

Due to the use of non-axenic cultures, we cannot exclude the presence of bacterial allelochemicals in the co-culture experiments. However, according to our experimental design, the hypothetical presence of allelopathic bacteria would be associated with *Phormidium* sp., somehow producing a joint allelopathic effect. Therefore, this aspect does not affect our conclusions. pH could also be a factor responsible for harmful effects. However, cyanobacteria are well known to be tolerant of a high pH (Huisman et al. 2005). In our co-culture systems, although pH was not measured, the biomass developed was lower than in similar experiments with more efficient species, and in which pH was monitored, never showing values above 9 (Barreiro Felpeto et al. 2018).

According to the classical theory of resource competition in Ecology (Tilman 1977), the initial abundance of each competing species is a factor that has no influence in determining the best competitor. This theory was developed for nutrient competition.

However, according to recent works (Roy 2009), this principle does not hold in the presence of an allelopathic interaction in interplay with nutrient competition. In this case, theory predicts that the winner of the competition depends on the initial relative abundances of the species. But, this is only true if there is a trade-off with competition for the limiting nutrient. In these experiments with *Nodularia* sp., this theoretical prediction was demonstrated, showing reversed outcomes of the competition experiments as a function of the initial relative abundance of the competitors (Figs. 3 and 4). Barreiro Felpeto et al. (2018) demonstrated this same principle for this same strain of *Phormidium* sp. and the chlorophyte *Ankistrodesmus falcatus*.

Considering the results obtained here, continuing with the study of the viability of *Phormidium* sp. allelochemicals as a method of bioremediation of toxic cyanobacterial blooms could be promising. It would be necessary to optimize allelochemical production by this strain of *Phormidium* sp., and test their effectiveness in a mesocosm scale experiment. In addition, ecotoxicological experiments should be performed in order to test the potential effect of *Phormidium* sp. allelochemicals on other organisms. Biological measures for control of toxic cyanobacterial blooms are promising as being more environmentally friendly than other methods, since the effects are more targeted, and the compounds potentially more easily degraded. They also have the potential for being produced sustainably. However, it should be considered that some molecules could also be highly toxic to other organisms, and the costs of production could be high.

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