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Initial growth phases of two bloom-forming cyanobacteria (*Cylindrospermopsis raciborskii* and *Planktothrix agardhii*) in monocultures and mixed cultures depending on light and nutrient conditions

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Abstract – Proliferations of cyanobacteria have detrimental effects on ecosystem functioning, and on the global freshwater food chain. Many studies have focused on the "*in situ*" dynamics of bloom-forming cyanobacteria, including *Cylindrospermopsis raciborskii* and *Planktothrix agardhii*. Few have used experimental assays to explore the fast-growing ability of naturally co-occurring species. Here we investigated the growth of these species when exposed separately (*i.e.*, in monocultures) to a range of light and nutrient conditions, plus their interactive performances in mixed cultures in a short-time experiment (6 days). The use of microplates made it possible to carry out multiple measurements of *in-vivo* fluorescence (IVF), and to monitor species-dependent biovolumes. No allelopathic effect was significantly observed for any target species, while significantly lower growth rates were obtained in mixed cultures, which may reflect other interference interactions between the species. We showed that *Planktothrix* grew faster with low light intensity and high nutrient concentrations, and was drastically inhibited by nitrogen deprivation, in contrast to *Cylindrospermopsis*. However, *Cylindrospermopsis* outgrew *Planktothrix* at high NH_4^+ concentrations, suggesting that this species may be a serious competitor for the native species in many water systems.

Key words: Bloom-forming cyanobacteria / interactions / growth rate / mixed cultures

Introduction

Cyanobacterial proliferations have attracted considerable attention in recent decades, as their frequent and intensive occurrence in freshwater has increased worldwide, due in part to anthropic activities (Paerl and Huisman, 2009). The prevalence of such bloom-forming species is of a worldwide concern, as they are able to produce toxic compounds that contaminate drinking waters, becoming a real threat to human health (Chorus and Bartram, 1999). One of the most common freshwater cyanobacterial species, *Planktothrix agardhii* (Gomont) Anagnostidis and Komárek (1988), has undergone considerable investigation as it is capable of producing microcystins (MCs) and many other secondary metabolites in temperate areas (Keil *et al.*, 2002). A second species, *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju (1972), has also become a cause of increased concern (Padisák, 1997). In addition to its bloom-forming abilities (Sinha *et al.*, 2012), this species (Order: Nostocales) is also able to produce cyanotoxins, such as cylindrospermopsin (CYN) and/or paralytic shellfish poisoning (PSP) toxins, which have detrimental effects on aquatic organisms (Kokocinski *et al.*, 2009). *C. raciborskii* was originally known as a tropical to subtropical species (Komárek and Anagnostidis, 2005), and initially assigned to the S_N (*i.e.*, located in warm and mixed layers and can tolerate light and nitrogen (N) deficiencies) phytoplankton functional groups (Reynolds *et al.*, 2002); whereas *P. agardhii* was assigned to the S_1 group (*i.e.*, in turbid and mixed layers and can tolerate high light). However, recent studies of ecological preferences, geographical distribution and other

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factors that may be implied in their current prevalence, tend to indicate that both these species occupy wide ecological ranges, which contributes to their successful distribution and worldwide expansion that has recently been confirmed for *C. raciborskii* (see O'Neil *et al.*, 2012, for review). However, blooms are complex events, involving multiple environmental factors simultaneously (Heisler *et al.*, 2008), which have led to some inconsistencies in the literature, related to the environmental parameters tested (Posselt *et al.*, 2009). The main shortcoming is that many studies have provided specific information about the environmental requirements of the two species occurring separately (McGregor and Fabbro, 2000), while very few of them have recently reported the co-occurrence of *C. raciborskii* and *P. agardhii* in lakes and reservoirs (Stefaniak and Kokocinski, 2005; Kokocinski *et al.*, 2009). For example, in the Tunisian Bir M'Cherga reservoir, from which the strains used in this study were obtained, *P. agardhii* occurs as a perennial biomass and dominates the phytoplankton community, whereas *C. raciborskii* is favored by higher temperatures and light intensities that only occur for a short period each year (Jenhani *et al.*, 2012). One might expect that a period of increased water surface temperature (due to climate change) would favor the growth of *C. raciborskii* and enable it to outcompete *P. agardhii*, as recent studies tend to indicate that two main factors, eutrophication and rising temperatures, are indeed promoting the successful expansion of *C. raciborskii* (Padisák, 1997; Paerl and Huisman, 2009; Sukenik *et al.*, 2012). The wide ecological ranges of these species suggest that they are both capable of predominating in water bodies, and so competitive interactions for resources between *C. raciborskii and P. agardhii* seem to be inevitable. Hence, it is important to consider how these two species interact when confronted by the same changing factors, and whether the success of *C. raciborskii* in spreading through extensive aquatic systems may be due to particular physiological capacities, such as an ability to grow faster than other phytoplankton species. We therefore investigated in laboratory settings, the initial growth response by determining the growth of each species separately (in monocultures), and when both were present together (in mixed cultures). The short time-scale involved (6-days) was expected to provide data on the rapid growth of *C. raciborskii* that makes it such a competitor, and show whether there were any interactive effects when the two species were exposed together to the same conditions. Although many investigations have referred to the ecological requirements and preferences of species in their natural habitats, very few have included "*in vitro*" experimental assays using physiological data about the species and their relationships to various environmental factors (Bonilla *et al.*, 2012). Fewer still have looked at the interactions and growing performances of two species in mixed cultures.

The present study was carried out using microplate assays, which offer an appropriate alternative to flasks (Pavlic *et al.*, 2006), as this allowed us to test a large number of samples and several conditions simultaneously, and to obtain an accurate estimation of the optimum growth conditions (Skjelbred *et al.*, 2012).

Consequently, we set out to determine: (i) the initial growth phases of *P. agardhii* and *C. raciborskii* occurring separately (*i.e.*, in monocultures), when confronted by stressful conditions, such as high light conditions, three levels of N sources (NH_4^+ and NO_3^-), including starvation; (ii) the possibility of allelopathic effect of released compounds by each species to the other target strain; (iii) the interference and/or interactive effects between the two species in mixed cultures; (iv) the fast-growing performance of these two species over a short period of time, using equal biovolumes to identify the more effective competitor and/or the possible prevalence of one species over the other. To the best of our knowledge, this is the first study to report the possible interactions of two bloom-forming species in mixed cultures, initially containing equal biomasses (*i.e.*, biovolumes), and exposed to a range of light and nutrient conditions over a 6-d period.

Materials and methods

Strains and culture conditions

C. raciborskii and *P. agardhii* strains were collected from the Tunisian Bir M'Cherga reservoir in 2009, and were deposited in the Paris Museum Collection (PMC) under the designations PMC702.10 and PMC684.10, respectively. After isolating a single filament, the strains of *C. raciborskii* (PMC702.10) and of *P. agardhii* (PMC 684.10) were maintained under non-axenic conditions in the Z8 liquid medium (Kotai, 1972), at 25 and 20 °C, respectively. Cyanobacterial cultures were illuminated with 20 ($\pm$ 2) µmol photons $m^{-2}.s^{-1}$ under white light (Osram white FM 11W/730 universal white), using a light:dark cycle of 16:8 h.

Toxicity tests

As both cyanobacteria are potentially toxic species, tests for MCs and CYNs were carried out on *P. agardhii* and *C. raciborskii* strains using the MCs-ADDA ELISA (Abraxis) and CYN ELISA (Abraxis) kits, respectively (the saxitoxin was not analyzed here). All samples were previously sonicated four times on ice for 30 s using an ultrasonic probe to destroy the cells and release the toxins into the liquid medium. Neither strain (PMC702.10 and PMC 684.10) was toxic. The detection of genes involved in toxin production was checked by PCR on fresh samples using primers targeting the *mcy* A, *mcy* B and *mcy* E genes for MCs and on *ps* and *pks* genes for CYN (according to Berger *et al.*, 2006). No PCR product was obtained.

Allelopathic assay

Although neither MCs nor CYN was detected in these strains, many other compounds (*i.e.*, secondary

Table 1. Experimental conditions used in this study.

Nitrate (mg L^{-1})	NO_3-free = 0	$Z8^a{}_{10}$-NO_3 = 5.26	Z8-NO_3 = 52.60
Ammonium (mg L^{-1})	NH_4-free = 0	$Z8^a{}_{10}$-NH_4 = 0.53	Z8-NH_4 = 5.34

[a]$Z8_{10}$ corresponding to dilution to tenth of the NO_3 and NH_4 concentrations in a Z8 culture medium.

metabolites) can be produced by each species, which may have adverse effects on the co-occurring species when placed in the same environment (*i.e.*, mixed culture). Consequently an allelopathic test was realized before the experimental growth conditions.

The cultures of PMC702.10 and PMC684.10 were maintained in optimal conditions (see below) and were collected during the exponential growth phase (after 8 days) at a $OD_{750} = 0.8$, and filtered through a 1.2 µm cellulose filter (Whatman, Millipore). The filtration was handled with care to avoid cell lysis and to collect only the chemical compounds released from the living cells (*i.e.*, allelochemicals) according to Rice (1984). The cell-free filtrate, including potential allelopathic substances from *P. agardhii* was incorporated (100 µL) to the *C. raciborskii* culture (100 µL) in a 96-well microplate. The growth of the cells exposed to the filtrate was tracked at different exposure times (0, 2, 4, 12, 24, 48, 96, 120 h) using a chlorophyll-*a in-vivo* fluorescence (IVF) as described below, and compared to the control (*i.e.*, growth of the strain without treatment). The same experiment was applied for each species (*i.e.*, *P. agardhii* exposed to the cell-free filtrate of *C. raciborskii*; and *C. raciborskii* exposed to the cell-free filtrate of *P. agardhii*) in triplicate.

Experimental setup

Precultures of PMC702.10 and PMC684.10 were maintained in 250-ml batch cultures containing 100 ml of the Z8 medium and exposed to continuous light (25 µmol photons $m^{-2}.s^{-1}$) at 25 °C with regular shaking. Observations from initial experiments showed that both species grew best at 25 µmol photons $m^{-2}.s^{-1}$. This light intensity was therefore used for experiments investigating nutrient conditions. Each preculture was used to inoculate 96-well microplates with an initial OD_{750} set at 0.1. In order to reduce evaporation, which can influence the growth of the controls and of the samples in neighboring wells, the border lines and columns (*i.e.*, lines A & H, Colum: 1 & 12) were filled with MilliQ water. Line B was a control line; each well contained 200 µl of the Z8 medium. The other lines included the different samples in five replicates for each of the experimental conditions in mono- and mixed cultures.

The cultures were exposed to several experimental conditions, including two light intensities (25 and 75 µmol photons $m^{-2}.s^{-1}$), two N sources (NO_3^--N and NH_4^+-N) at three concentrations (Table 1). Prior to the light experiments, the precultures were acclimatized to the light intensity (*i.e.*, 25 or 75 µmol photons $m^{-2}.s^{-1}$) for 8 days under continuous lighting. The adapted cells were then transferred into the 96-well microplate for the experiment. The nutrient concentrations (*i.e.*, NO_3^- and NH_4^+ sources) were previously selected according to the Z8 culture medium composition (Rippka, 1988) and modified with respect to our N source selection (NH_4^+ or NO_3^-). The Z8 medium contains several N sources: as $NaNO_3$ and $Ca(NO_3)_2$ $4H_2O$ and NH_4Cl. Consequently for the N-starvation experiment (*i.e.*, NH_4^+-free and NO_3-free) all the N sources were removed and replaced by the same charged elements (*e.g.*, $Ca(NO_3)_2$ $4H_2O$ was changed by $CaCl_2$ $2H_2O$). Thus for the NH_4^+ experiment, two levels of NH_4^+ were used: $Z8_{10}$-(NH_4^+) corresponding to the 1/10 dilution of the Z8 culture medium (Table 1), and Z8-(NH_4^+), corresponding to the Z8 medium, in the absence of any other N source (Table 1). The NO_3^- experiment was performed in the same way (Table 1). All the modified media (and the N sources) were buffered and checked to ensure that the pH was maintained (*i.e.*, that of the Z8 medium, pH = 7.4). This test was intended to determine the highest affinity and/or the best assimilated N source for each species.

Measurements

The growth kinetics of mono and mixed cultures were performed using *in vivo* chlorophyll-*a* fluorescence (IVF) at an excitation wavelength of 460 nm; with an emission wavelength of 660–780 nm, and quantification with a microplate reader (Modulus™, Turner Biosystems, France). The maximum growth rate (μ_{max}) was calculated as described by Guillard (1973). $\mu = (\ln \times 2 - \ln \times 1/t_2 - t_1)$, where t_1 (*i.e.*, beginning of the exponential phase: T_2) and t_2 (*i.e.*, T_6: end of the experiment) are the measurement times, and x is the fluorescence (expressed in IVF) at time t.

A previous positive correlation was obtained between the IVF values and the biomass of each species ($y = 1.7238x + 4.2426$; $r^2 = 0.959$ for *P. agardhii*; and $y = 17.299x + 25.752$; $r^2 = 0.844$ for *C. raciborskii*; $n = 15$, Fig. S1, online material available at: www.limnology-journal.org), which conduced us to select the both measurements in mixed cultures, as the "global" IVF values did not discriminate the between species in mixed cultures. In order to determine the specific growth and potential dominance of each species in the mixed cultures, the assessment of biovolumes was preferred for this study, because the cell density (cells.mL^{-1}) would have been inaccurate, as the filaments of *Planktothrix* are about four times larger (average size: 7.5 ($\pm$ 0.3) µm wide) than those of *Cylindrospermopsis* (average size, 1.9 ($\pm$ 0.2) µm wide). Consequently an equal biovolume ($mm^3.L^{-1}$) was determined for the initial T_0 experiment with the appropriate dilutions. The biovolumes were calculated on the basis of a geometric model (*i.e.*, cylinder shape) according to Sun and Liu (2003) for 30 randomly selected distinct individuals, according to the Utermöhl' technique

(Utermöhl,1958), using a microscope coupled to a digital sight DS-LI image acquisition system (Nikon Inc., Melville, USA). An initial biovolume of 12.41 $mm^3.L^{-1}$ of each species were then used as the inoculum, providing a total biovolume of 24.82 $mm^3.L^{-1}$ (*i.e.*, 2X biovolumes of each species) for all experiments.

Statistical analyses

The statistical analyses were conducted using the Statview III software (Roth *et al.*, 1995) after checking the normality and the homogeneity of the variance tests for each data set. For the allelopathic assay, each target species was in contact with filtrates of the other species (during a 6-d incubation period) and compared to the control (*i.e.*, species without filtrate) by a one-way analysis of variance (ANOVA) in triplicate. The cyanobacterial growth of *Planktothrix* and *Cylindrospermopsis* (*i.e.*, dependent variable) from the both cultures (mono *versus* mixed) was evaluated under different nutrient and light conditions (*i.e.*, factors) by ANOVA. A *post-hoc* Fisher test (Protected Least Significance Difference (PLSD) analysis) was performed when significant differences ($P < 0.05$) were found. All the experiments were carried out in triplicate.

Results

Allelopathic effects

Figure 1 showed no inhibitory effect of the cell-free filtrate on the growth (IVF values) of any co-occurring target species ($P > 0.05$). The growth of *P. agardhii* was not significantly affected by the cell-free filtrate of *C. raciborskii* (ANOVA; d.f. = 1; $F = 0.22$; $P = 0.88$), whatever the time period ($n = 8$) and instead, has shown a slightly positive growth after a 24 h exposure time (Fig. 1(a)). While *C. raciborskii* showed a less pronounced but not significant growth (ANOVA; d.f. = 1; $F = 0.21$; $P = 0.64$) when exposed to the cell-free filtrate after 48 h of exposure (Fig. 1(b)), in comparison to the control.

Light intensity experiments

At low light intensity (25 µmol photons $m^{-2}.s^{-1}$) in monocultures, the IVF curves of *Cylindrospermopsis* and *Planktothrix* showed similar patterns, with an exponential growth phase from T_4 to T_6. Consequently, the μ_{max} was significantly higher for *P. agardhii* than for *C. raciborskii* as shown in Table 3. In mixed cultures, under low light condition, IVF values were slightly lower than that in any of the monocultures (Fig. 2(a)), which corroborated the μ_{max} differences obtained between the growth of *P. agardhii* in monocultures *versus* mixed cultures (Tables 2 and 3). Moreover, the observation of the biovolumes revealed that *P. agardhii* grew faster from T_0 to

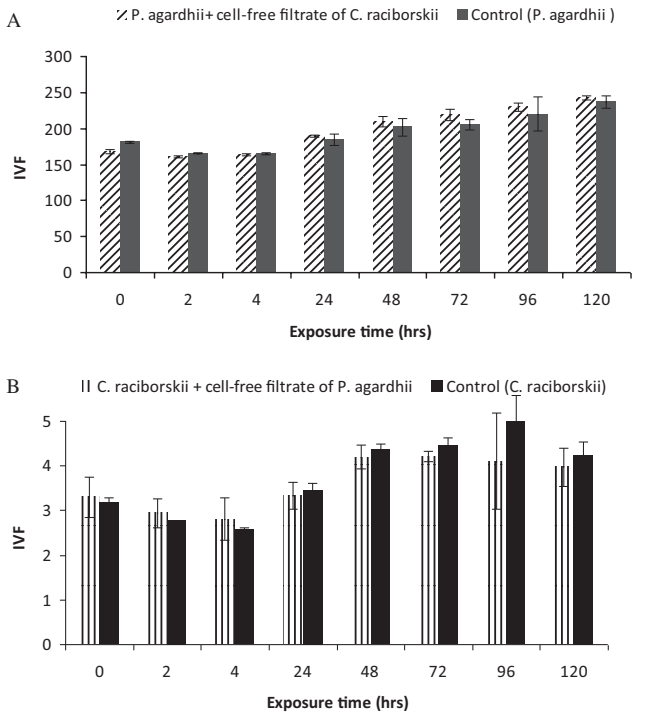

Fig. 1. Allelopathic effects of one strain to the growth (IVF values) of target species. (a) Cell-free filtrate of *Cylindrospermopsis raciborskii* on *Planktothrix agardhii* growth (dashed blocks) compared to the control (black blocks). (b) Cell-free filtrate of *P. agardhii* on *C. raciborskii* growth (dashed blocks) compared to the control (black blocks). ± Standard deviation ($n = 3$).

T_6 (57% of biomass), whereas *C. raciborskii* started to grow later (T_2), but then progressively increased to reach a similar biovolume (~ 43% of biomass) (Fig. 2(a)).

At the higher light intensity (75 µmol photons $m^{-2}.s^{-1}$) in monocultures, the IVF values were threefold lower for *P. agardhii* and twofold lower for *C. raciborskii* compared to those at low light intensity (see above). The decrease in growth rate, between the two light-level experiments, was more marked in *P. agardhii* than in *C. raciborskii* (from 0.656 to 0.496 d^{-1} and from 0.459 to 0.415 d^{-1}, respectively) suggesting that *Planktothrix* is more sensitive to moderate light intensity. In mixed cultures, the growth rate (μ_{max}) was significantly different ($P < 0.05$; Table 3) to that in monocultures, and fell to a μ_{max} of 0.240 d^{-1} (Table 2). Considering the biovolume curve, *P. agardhii* grew very little from T_0 to T_3, but surprisingly had increased at T_6 to correspond to > 80% of biomass, whereas *C. raciborskii* remained at a low biomass level (close to the initial value) throughout the experiment (Fig. 2(b)).

N-source experiments

In monocultures for the NH_4^+ experiment, the both IVF curves differed from each other, with a complete growth inhibition of *Planktothrix* under NH_4^+-free and

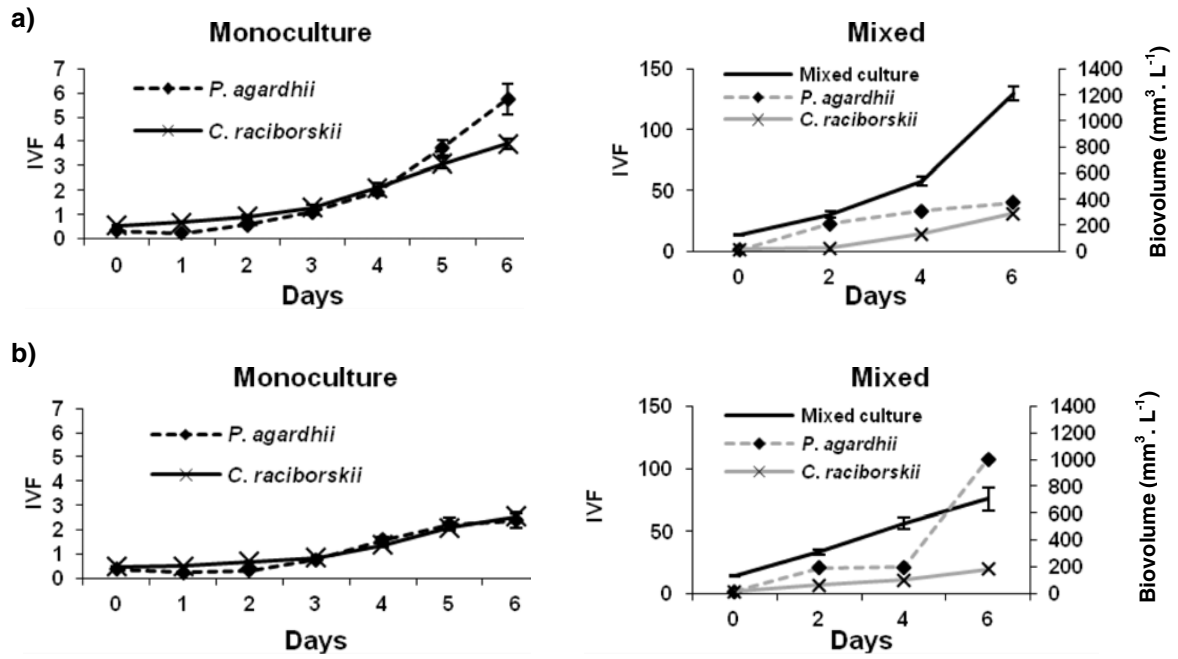

Fig. 2. (a, b) Growth dynamics of *Cylindrospermopsis raciborskii* and *Planktothrix agardhii* in monoculture and mixed culture at two light intensities obtained using biovolumes (gray lines) and IVF (10^3) (*in vivo* fluorescence) (black line). The dotted line corresponded to the IVF values of *P. agardhii;* and the solid black line, the IVF values of *C. raciborski*. (a) 25 µmol photons $m^{-2}.s^{-1}$; (b) 75 µmol photons $m^{-2}.s^{-1}$.

at a low NH_4^+concentration (*i.e.*, $Z8_{10}$-NH_4^+), whereas *C. raciborskii* was still able to grow under both these stressful conditions (Fig. 3; Tables 2–4). At higher NH_4^+ level, the IVF value was still very low (Fig. 3(a)), and significant differences between the *P. agardhii* and *C. raciborskii* growth rates were obtained (Tables 3 and 4). This may suggest that NH_4^+ is a drastically limiting factor for *P. agardhii* growth (Table 2) for which it is the sole source of N, but only a moderately assimilated N-source for *C. raciborskii*. In mixed cultures, according to the IVF values, no growth rate was detected under NH_4^+ starvation (NH_4^+-free); and very low μ_{max} values were obtained for both the other NH_4^+ concentrations (Fig. 3(b); Table 2), which may re-emphasize the unfavorable effect of NH_4^+ as the source of N combined with possible competitive interactions between the species ($P < 0.001$ within mono *versus* mixed cultures Table 4).

The biovolumes curve revealed low growth for both species during the 6-d NH_4^+ starvation period, but a more complex growth pattern was found for a low level of NH_4^+. At T_2, *P. agardhii* had grown faster than *C. raciborskii*, reaching 61% of the total biomass, but it rapidly declined from T_3 to T_6, whereas the *C. raciborskii* biomass progressively increased from T_3 to T_6, to reach 83% of total biomass at T_6 (Fig. 3(b)). An even more marked pattern was observed under the high NH_4^+ concentration (*i.e.*, Z8-NH_4), where the *P. agardhii* biovolumes rapidly declined from T_2 to T_3, whereas *C. raciborskii* constantly increased, and became the prevalent species at T_6 (making up 91% of the total biomass – Fig. 3(b)). This tends to suggest that *C. raciborskii* has a higher affinity to NH_4^+ as the sole N source, and this gave it a competitive edge over *P. agardhii*.

In monocultures for the NO_3^- experiment, the nutrient level has a significant effect on the growth (IVF curve) and μ_{max} of *Planktothrix* and of *C. raciborskii* (Table 4). No growth was obtained under NO_3^- depletion for *P. agardhii*, as suggested by growth rates (Table 2), whereas *C. raciborskii* grew steadily (between-species differences: monoCyl *versus* monoPlk; Table 4). NO_3^- as the N source provided a higher growth rate value for both species ($P < 0.001$; Table 4) than NH_4^+ did, showing that both species preferred NO_3^- as N source ($\mu_{max} = 0.672\ d^{-1}$ for *P. agardhii* and $\mu_{max} = 0.480\ d^{-1}$ for *C. raciborskii*), but that *P. agardhii* had greater affinity to a high NO_3^- source (Table 2 and Fig. 4(a)).

With the NO_3^- source in mixed cultures, the IVF values and biovolume curve of *P. agardhii* were quite different. While the IVF values showed a very low and significant reduced growth for both species (Fig. 4(b) and Tables 2–4) under NO_3^- starvation; the biovolume curve revealed a greater increase of *P. agardhii* under low NO_3^- concentration (*i.e.*, $Z8_{10}$-NO_3) from T_2 to T_3. However, probably due to the high biomass in the wells, which may have led to nutrient depletion, a rapid decline in both species was noted at T_6 (Fig. 4(b)), corresponding to the high differences ($P < 0.001$) between the mono *versus* mixed cultures tests (Table 4). In contrast, at a higher NO_3^- concentration (*i.e.*, Z8-NO_3), the prevalence of *P. agardhii* was significant at T_6, and *C. raciborskii* remained at a very low biomass level throughout the experiment. This finding showed the greater affinity of *P. agardhii* to the NO_3^- source,

Table 2. Maximum growth rate (μ_{max}) of *Cylindrospermopsis raciborskii* and *Planktothrix agardhii* strains exposed to different conditions of light and nutrients in monoculture and mixed cultures.

Conditions	Monoculture*		Mixed culture*
Light intensity (µmol photons $m^{-2}.s^{-1}$)	*P. agardhii*	*C. raciborskii*	*P. agardhii* + *C. raciborskii*
25	0.656 ± 0.06	0.459 ± 0.08	0.403
75	0.496 ± 0.09	0.415 ± 0.04	0.240
Nutrient sources			
NH_4^+-free	0	0.360 ± 0.06	0
$Z8_{10}$ -(NH_4^+)	0	0.370 ± 0.07	0.050
Z8-(NH_4^+)	0.464 ± 0.10	0.384 ± 0.08	0.100
NO_3^--free	0	0.304 ± 0.03	0
$Z8_{10}$ -(NO_3^-)	0	0.184 ± 0.002	0.100
Z8-(NO_3^-)	0.672 ± 0.10	0.480 ± 0.08	0.552

*μ_{max} was estimated during the exponential growth phase ($n = 15$ for monocultures or $n = 5$ for mixed cultures) ± SD.

Table 3. The results of ANOVA comparing the effect of different conditions of light and nutrients on the maximum growth rate (μ_{max}) of *Cylindrospermopsis raciborskii* (Cyl) and *Planktothrix agardhii* (Plk) exposed to both culture conditions (Mono versus Mixed).

Factor	Source of variation	d.f.	MS	*F*	*P* value
Light:	Mono (Cyl/Plk) versus mixed growth				
25 µmol		2	0.057	15.9	0.0039**
75 µmol		2	0.052	14.229	0.053 ns
Nutrient: NH_4^+	Mono (Cyl/Plk) versus mixed growth				
NH_4^+-free		2	0.117	263.43	< 0.001***
$Z8_{10}$-(NH_4^+)		2	0.121	3951.22	< 0.001***
Z8-(NH_4^+)		2	0.102	33.142	0.06 ns
Nutrient: NO_3^-	Mono (Cyl/Plk) versus mixed growth				
NO_3^--free		2	0.093	1099.2	< 0.001***
$Z8_{10}$-(NO_3^-)		2	0.025	646.17	< 0.001***
Z8 – (NO_3^-)		2	0.027	5.649	0.047*

d.f. = degree of freedom; MS = mean of square; *F* = Fisher test value. The statistical significances are indicated with asterisks: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, NS, not significant.

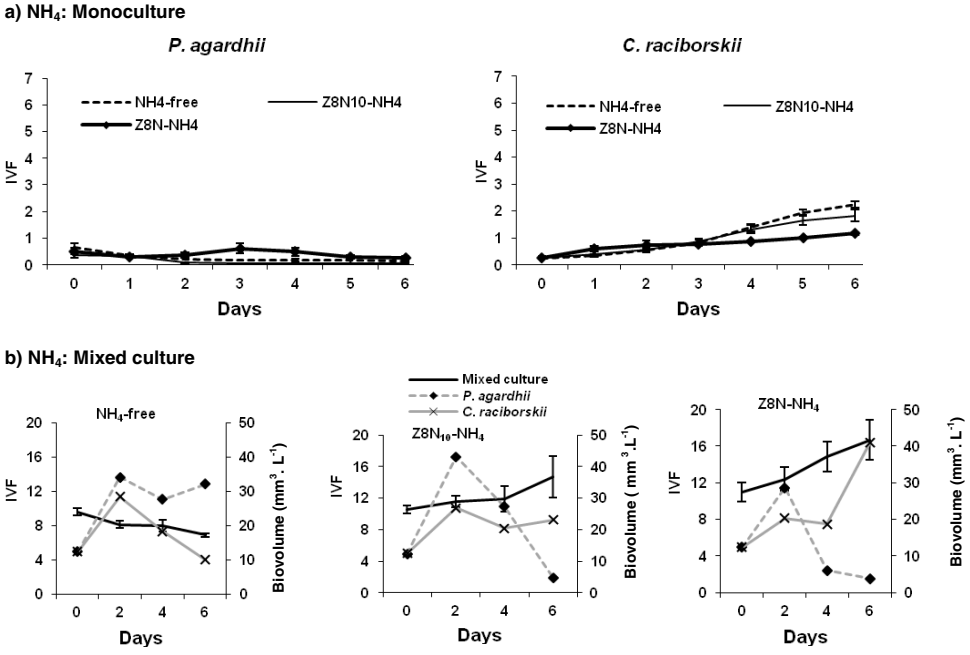

Fig. 3. (a, b) Growth dynamics of *Cylindrospermopsis raciborskii* and *Planktothrix agardhii* in monoculture (a) and mixed culture (b) at different ammonium concentrations, obtained using IVF (10^3) (*in vivo* fluorescence) (black line). The dotted and solid black lines indicated the different NH_4^+ concentrations in (a). The biovolumes recorded in mixed cultures (b) are indicated in gray lines.

Table 4. Fisher PLSD *post-hoc* tests, showing significant differences within-groups, corresponding to the growth rate of *Cylindrospermopsis raciborskii* (Cyl) and *Planktothrix agardhii* (Plk) in mono- and mixed cultures, when exposed to various conditions (light and nutrients).

Factor	Groups	Significance
Light:	Mono-Cyl × mono-Plk	0.0041**
25 µmol	Mono-Cyl × mixed	0.506
	Mono-Plk × mixed	0.002**
75 µmol	Mono-Cyl × mono-Plk	0.151
	Mono-Cyl × mixed	0.0117*
	Mono-Plk × mixed	0.002**
Nutrient: NH_4^+-free	Mono-Cyl × mono-Plk	< 0.001***
	Mono-Cyl × mixed	< 0.001***
	Mono-Plk × mixed	nd
$Z8_{10}$-(NH_4^+)	Mono-Cyl × mono-Plk	< 0.001***
	Mono-Cyl × mixed	< 0.001***
	Mono-Plk × mixed	nd
Z8-(NH_4^+)	Mono-Cyl × mono-Plk	0.124
	Mono-Cyl × mixed	0.001***
	Mono-Plk × mixed	0.002**
NO_3^--free	Mono-Cyl × mono-Plk	< 0.001***
	Mono-Cyl × mixed	< 0.001***
	Mono-Plk × mixed	nd
$Z8_{10}$-(NO_3^-)	Mono-Cyl × mono-Plk	< 0.001***
	Mono-Cyl × mixed	< 0.001***
	Mono-Plk × mixed	< 0.001***
Z8-(NO_3^-)	Mono-Cyl × mono-Plk	0.0161*
	Mono-Cyl × mixed	0.2825
	Mono-Plk × mixed	0.0766

The mean difference is significant at the 0.05 level: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$. nd, not determined.

which provided a better and faster growth, leading it to outcompete *C. raciborskii* during this period of time. *C. raciborskii* seemed to lose its advantage in the presence of the high NO_3^- level, as the sole N source, and was more subject to competitive interactions with co-occurring *P. agardhii*, at least under this initial growing phase and specific conditions.

Discussion

Our study has highlighted differences in the behavior of each species: (i) maintained in monocultures and (ii) in comparison to the mixed cultures (mono *versus* mixed). The highest differences of growth rate (μ_{max}) between *P. agardhii* and *C. raciborski* in monocultures, concerned the low light intensity ($P < 0.01$) and starvation and/or low nutrient concentration ($P < 0.001$). With regard to light intensity, *P. agardhii* had a higher μ_{max} (0.65 d^{-1}) at low light than at 75 µmol photons $m^{-2}.s^{-1}$, which corroborated previous studies (Davis and Walsby, 2002; Oberhaus *et al.*, 2007), and highlighted the high affinity of this species for low irradiance (Mur and Beydorff, 1978; Bright and Walsby, 2000). Consequently, *P. agardhii* grew faster and outcompeted *C. raciborskii* with regard to biomass (IVF and biovolumes), especially at day 6 in our study, which are consistent with Bonilla *et al.* (2012) findings, who reported that *P. agardhii* had a higher capacity for growth than *C. raciborskii* under low light intensity. An unexpected result was the lower μ_{max} value of C. *raciborskii* at higher light intensity, as the species is known to tolerate a wide range of light intensities including optimal irradiances up to 75 µmol photons $m^{-2}.s^{-1}$ around the world (Shafik *et al.*, 2001; Briand *et al.*, 2004; O'Neil *et al.*, 2012). This may be due to a special feature of the strain selected, which was collected from a low irradiance (< 40 µmol photons $m^{-2}.s^{-1}$) sampling site (data not shown) and could be acclimated to such specific conditions. Further investigations would be provided on different strains to confirm this hypothesis.

For N-source experiments, under starvation and low nutrient concentration, the growth of each species significantly differed, suggesting a species-specific response when exposed to stress conditions. Our results showed that under N-source starvation, monocultures of *P. agardhii* were unable to grow when NH_4^+ or NO_3^- was depleted (*i.e.*, $\mu_{max} = 0$), whereas the growth of *C. raciborskii* seemed unaffected under NH_4^+ and/or NO_3^- depletion and low concentration. The growth curves (IVF) recorded here, tend to suggest that *C. raciborskii* had a higher affinity for NH_4^+, as previously reported in Saker and Neilan (2001) and Kokocinski *et al.* (2010) and other studies, who found that *C. raciborskii* grew faster when N was supplied in the form of ammonia, followed by nitrate, and then urea. In contrast, *P. agardhii* exhibited a clear preference for NO_3^- for the first 3 days, as suggested by the growth curves in the two N-source experiments and the highest μ_{max} found in *P. agardhii* at a high NO_3^- concentration, which corroborated the findings of Løvstad (1984) and Nicklisch (1994). These results tend to suggest the high requirement of *P. agardhii* for substantial N-source levels (especially NO_3^-) for maintaining its growth; in contrast to the low requirements and the ability of *C. raciborskii* to take up different N sources, as this species can shift between the DIN uptake modes and diazotrophy (Moisander *et al.*, 2008).

These specific preferences for either NH_4^+ or NO_3^- sources were also recorded when the both species co-occurred in mixed cultures, which could explain the increasing biomass (*i.e.*, biovolumes) of *C. raciborskii* at high NH_4^+ concentration, and thus outcompeted *P. agardhii* by day 6 of incubation. In contrast, the high affinity for NO_3^- might explain the rise of *P. agardhii* in mixed cultures, where it inhibited the growth of *C. raciborskii* throughout the study. However, overall the co-culture experiments showed a constant lower growth rate value in comparison to the monocultures under stress nutrient conditions, which may enhance the presence of interferences and/or competitive interactions between both species in these small volumes. Meanwhile, the allepotathic assay carried out in this study tend to suggest that any neither compound nor allelochemical released from the living cells (according to Rice, 1984), repressed the other species (target species), at least during this time of exposure (120 h). More investigations are needed regarding the putative effect of these chemical substances,

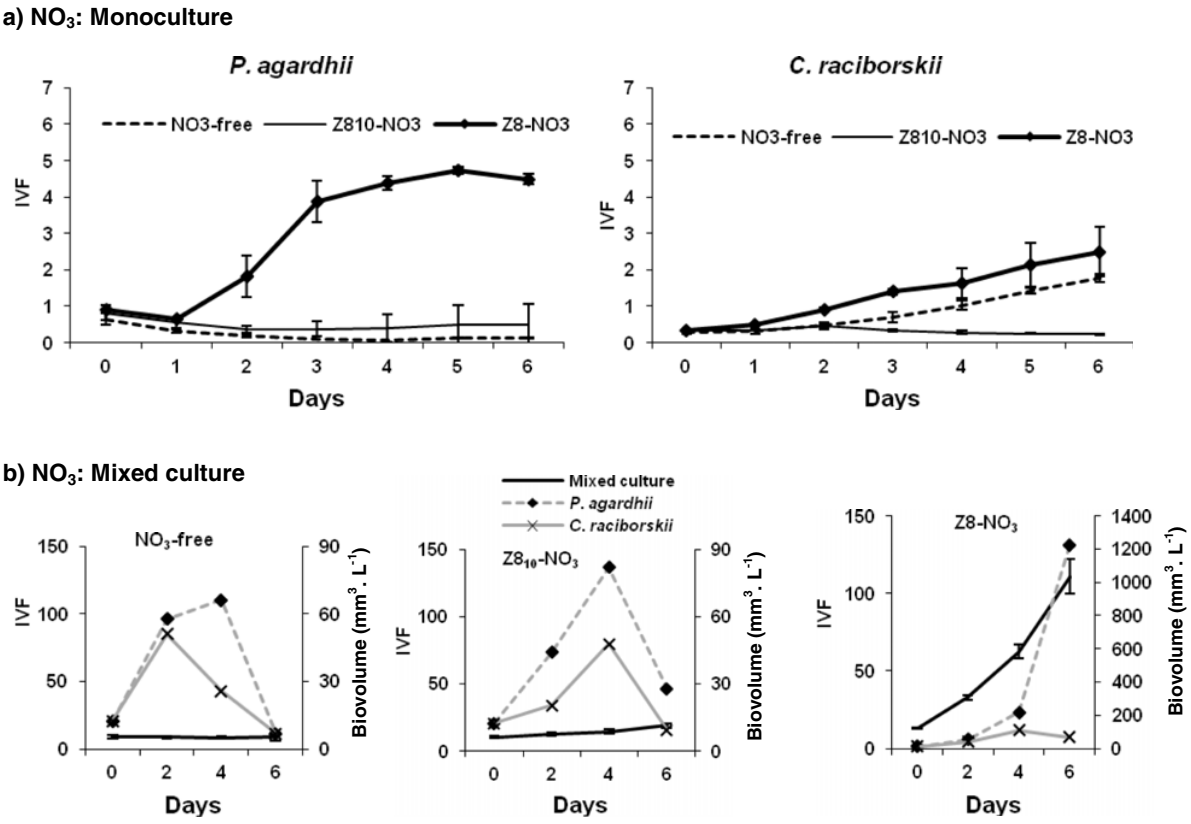

Fig. 4. (a, b) Growth dynamics of *Cylindrospermopsis raciborskii* and *Planktothrix agardhii* in monoculture (a) and mixed culture (b) at different nitrate concentrations, obtained using IVF (10^3) (*in vivo* fluorescence) (black line). The dotted and solid black lines indicated the different NO_3^- concentrations in (a). The biovolumes recorded in mixed cultures (b) are indicated in gray lines.

as they can be closely associated with competition for limiting nutrient resources and/or under altered nutrient changes (Graneli *et al.*, 2008). Furthermore, we cannot rule out the possibility that other types of interaction may occur between the two species, which could explain why the value found for μ_{max} was always lower in mixed cultures than in monocultures, and also some unexpected results, such as those found under NH_4^+ or NO_3^- starvation conditions and under moderate light intensity at day 6.

Finally, the experiments performed here suggested not only the nutrient preferences of each species, but also the growth strategy adopted in terms of speed and/or capacity, which differed in the two species during the initial growth phase (0 to 3-d experiments). This is a key factor, as a rapid growth capacity in one species subsequently impairs the growth of the less fit species. In fact, C. *raciborskii* often displays delayed growth, or a lag-phase, for the first 2 days of incubation. Such drawback may be intensified in the mixed culture, where C. *raciborskii* rarely dominated in all the experiments, despite its known nutrient tolerance and preferences for various environmental factors (Moisander *et al.*, 2008; O'Neil *et al.*, 2012). In contrast, *P. agardhii* displayed a fast-growing response during the first 2 days and also kept growing until the end of experiment. Its rapid expansion may be further promoted by the size of its filaments, which are around four times bigger than those of *C. raciborskii*. This means that the ability of *P. agardhii* to grow faster increases its relative surface area much faster, which enables it to outgrow slow-growing, co-occurring species (Li and Li, 2012).

However, it is difficult to extrapolate these results to predict physiological abilities of species, as our analysis focused on one strain for each species and may provide a higher strain-specific response than a species-specific one. Further investigations need to be extended to several strains to confirm their growth capacity, as the both species (especially *C. raciborski*) have a great phenotypic plasticity, enabling them to cope with environmental changes and thrive under new conditions (Bonilla *et al.*, 2012; Sinha *et al.*, 2012). Likewise, as our investigation involved a short-time period, it would be useful to extend the time-scale up to 21 days, in order to determine more precisely the interactions, such as competition and/or interference that may occur between these two bloom-forming species when they are both present. For this purpose, the flask method would be required here (*i.e.*, >200 mL of cultures), as the microplate is a valid and an appropriate alternative method for use over a short-time of incubation (Eisentraeger *et al.*, 2003). The positive correlation between IVF values and biomass within the microplate assays (data not shown), led us to choose this method as an alternative way to

analyze the daily IVF measurements to assess growth rates of filamentous cyanobacteria, as it is quick, readily reproducible and only requires small volumes of material (Satoh *et al.*, 2005). However, the small test volume, which is one of the advantages of this method, can also be a limitation because of the rapid turnover of nutrients, which makes it necessary to select a short-time-scale in order to minimize redox changes and evaporation, especially during the exponential growth phase of the bacteria (Eisentraeger *et al.*, 2003; Gregor *et al.*, 2008).

In conclusion, further experiments are needed to find out whether the species that prevails over the short time period investigated here (*i.e.*, *P. agardhii*) also does so over longer periods of time. This investigation may also show whether *C. raciborskii* is able to outcompete *P. agardhii* despite the initial fast-growth potential of the latter suggested here, or whether its long-term survival is likely to be jeopardized by interference and/or effective interactions, which over a longer time-scale could lead to an exclusive competition under specific conditions. While the experiments were carried out in laboratory settings, which make them hazardous to extrapolate to natural conditions; these data may find direct correspondence to challenging questions about the interactions between environmental changes and global expansion of few cyanobacterial species. These findings are of particular relevance albeit are difficult to gauge without direct exposure, which can be gained from initial and controlled *in vitro* experiments.

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