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 NH₄⁺ 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* (Wołoszynska 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 S₅ (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 S₁ 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* (Padišák, 1997; Paele and Huismans, 2009; Sukeník 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 (NH₄⁺ and NO₃⁻), 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 biomass (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 (± 2) μmol photons m⁻²s⁻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
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 OD750 = 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 initial OD750 set at 0.1. In order to reduce evaporation, which can influence the growth of the cells exposed to the filtrate, including potential allelopathic substances from *P. agardhii*, precultures 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 (NO₃⁻·N and NH₄⁺·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., NO₃⁻ and NH₄⁺ sources) were previously selected according to the Z8 culture medium composition (Rippka, 1988) and modified with respect to our N source selection (NH₄⁺ or NO₃⁻). The Z8 medium contains several N sources: as NaNO₃ and Ca(NO₃)₂ 4H₂O and NH₄Cl. Consequently for the N-starvation experiment (i.e., NH₄⁺-free and NO₃⁻-free) all the N sources were removed and replaced by the same charged elements (e.g., Ca(NO₃)₂ 4H₂O was changed by CaCl₂ 2H₂O). For thus the NH₄⁺ experiment, two levels of NH₄⁺ were used: Z8₁₀(NH₄⁺) corresponding to the 1/10 dilution of the Z8 culture medium (Table 1), and Z8₀(NH₄⁺), corresponding to the Z8 medium, in the absence of any other N source (Table 1). The NO₃⁻ 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 (µmax) was calculated as described by Guillard (1973), µ = (ln x₂ – ln x₁) / (t₂ – t₁), where t₁ (i.e., beginning of the exponential phase: T₀) and t₂ (i.e., T₀: 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 ($y = 1.7238x + 4.2426$; $r² = 0.959$ for *P. agardhii*; and $y = 17.299x + 25.752$; $r² = 0.844$ for *C. raciborskii*; n = 15). Fig. S1, additional material available at: www.limnology-journal.org, which could be used to select the best 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 (± 0.3) µm wide) than those of *Cylindrospermopsis* (average size, 1.9 (± 0.2) µm wide). Consequently an equal biovolume (mm³.L⁻¹) was determined for the initial T₀ 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.

### Table 1. Experimental conditions used in this study.

<table>
<thead>
<tr>
<th>Nitrate</th>
<th>NO₃⁻-Free = 0</th>
<th>Z8₁₀(NO₃) = 5.26</th>
<th>Z8-NO₃ = 52.60 (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium</td>
<td>NH₄⁺-Free = 0</td>
<td>Z8¹₀(NH₄) = 0.53</td>
<td>Z8-NH₄ = 5.34 (mg L⁻¹)</td>
</tr>
</tbody>
</table>

*Z8₁₀ corresponding to dilution to tenth of the NO₃ and NH₄ concentrations in a Z8 culture medium.*
(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 $t_{\text{max}}$ 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 $t_{\text{max}}$ 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 $T_6$ (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 ($t_{\text{max}}$) was significantly different ($P < 0.05$; Table 3) to that in monocultures, and fell to a $t_{\text{max}}$ 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...
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 $\mu_{\text{max}}$ 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$_4^+$ (Fig. 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$_2$ 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 $\mu_{\text{max}}$ 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 ($\mu_{\text{max}} = 0.962$ d$^{-1}$ for P. agardhii and $\mu_{\text{max}} = 0.480$ 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 (μmax) of *Cylindrospermopsis raciborskii* and *Planktothrix agardhii* strains exposed to different conditions of light and nutrients in monoculture and mixed cultures.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Monoculture*</th>
<th>Mixed culture*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light intensity (μmol photons m⁻²s⁻¹)</td>
<td><em>P. agardhii</em></td>
<td><em>C. raciborskii</em></td>
</tr>
<tr>
<td>25</td>
<td>0.656 ± 0.06</td>
<td>0.459 ± 0.08</td>
</tr>
<tr>
<td>75</td>
<td>0.496 ± 0.09</td>
<td>0.415 ± 0.04</td>
</tr>
<tr>
<td>Nutrient sources</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄⁺-free</td>
<td>0</td>
<td>0.360 ± 0.06</td>
</tr>
<tr>
<td>Z₈₋(NH₄⁺)</td>
<td>0</td>
<td>0.370 ± 0.07</td>
</tr>
<tr>
<td>NO₃⁻-free</td>
<td>0</td>
<td>0.304 ± 0.03</td>
</tr>
<tr>
<td>Z₈₋(NO₃⁻)</td>
<td>0</td>
<td>0.184 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.672 ± 0.10</td>
</tr>
</tbody>
</table>

*μmax 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 (μmax) of *Cylindrospermopsis raciborskii* (Cyl) and *Planktothrix agardhii* (Plk) exposed to both culture conditions (Mono versus Mixed).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Source of variation</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light:</td>
<td>Mono (Cyl/Plk) versus mixed growth</td>
<td>2</td>
<td>0.057</td>
<td>15.9</td>
<td>0.0039**</td>
</tr>
<tr>
<td></td>
<td>25 μmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>75 μmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient: NH₄⁺</td>
<td>Mono (Cyl/Plk) versus mixed growth</td>
<td>2</td>
<td>0.117</td>
<td>263.43</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺-free</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z₈₋(NH₄⁺)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient: NO₃⁻</td>
<td>Mono (Cyl/Plk) versus mixed growth</td>
<td>2</td>
<td>0.093</td>
<td>1099.2</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td></td>
<td>NO₃⁻-free</td>
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</tr>
<tr>
<td></td>
<td>Z₈₋(NO₃⁻)</td>
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<tr>
<td></td>
<td>Z₈ – (NO₃⁻)</td>
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</tbody>
</table>

* = degree of freedom; MS = mean of square; F = Fisher test value. The statistical significances are indicated with asterisks: ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 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 NH₄⁺ 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).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Groups</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light:</td>
<td>Mono-Cyl × mono-Plk</td>
<td>0.0041***</td>
</tr>
<tr>
<td>25 μmol</td>
<td>Mono-Cyl × mixed</td>
<td>0.506</td>
</tr>
<tr>
<td>75 μmol</td>
<td>Mono-Cyl × mixed</td>
<td>0.002**</td>
</tr>
<tr>
<td>Nutrient:</td>
<td>Mono-Cyl × mono-Plk</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>NH₄⁺-free</td>
<td>Mono-Cyl × mixed</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>NO₃⁻-free</td>
<td>Mono-Cyl × mixed</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Z8₁₀(NO₃⁻)</td>
<td>Mono-Cyl × mono-Plk</td>
<td>0.124</td>
</tr>
<tr>
<td>Z₈(NO₃⁻)</td>
<td>Mono-Cyl × mixed</td>
<td>0.001***</td>
</tr>
<tr>
<td>NO₃⁻-free</td>
<td>Mono-Cyl × mixed</td>
<td>0.002**</td>
</tr>
<tr>
<td>Z₈(NO₃⁻)</td>
<td>Mono-Cyl × mixed</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Z₈(NO₃⁻)</td>
<td>Mono-Cyl × mixed</td>
<td>0.001***</td>
</tr>
<tr>
<td>Z₈(NO₃⁻)</td>
<td>Mono-Cyl × mixed</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Z₈(NO₃⁻)</td>
<td>Mono-Cyl × mixed</td>
<td>0.0161*</td>
</tr>
<tr>
<td>Z₈(NO₃⁻)</td>
<td>Mono-Cyl × mixed</td>
<td>0.2825</td>
</tr>
<tr>
<td>Z₈(NO₃⁻)</td>
<td>Mono-Cyl × mixed</td>
<td>0.0766</td>
</tr>
</tbody>
</table>

The mean difference is significant at the 0.05 level: ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 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 NO₃⁻ 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 (μₘₐₓ) between *P. agardhii* and *C. raciborskii* 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 μₘₐₓ (0.65 d⁻¹) at low light than at 75 μmol photons m⁻²s⁻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 μₘₐₓ 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⁻²s⁻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⁻²s⁻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 NH₄⁺ or NO₃⁻ was depleted (i.e., μₘₐₓ = 0), whereas the growth of *C. raciborskii* seemed unaffected under NH₄⁺ and/or NO₃⁻ depletion and low concentration. The growth curves (IVF) recorded here, tend to suggest that *C. raciborskii* had a higher affinity for NH₄⁺, 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 NO₃⁻ for the first 3 days, as suggested by the growth curves in the two N-source experiments and the highest μₘₐₓ found in *P. agardhii* at a high NO₃⁻ concentration, which corroborated the findings of Lovstad (1984) and Nicklisch (1994). These results tend to suggest the high requirement of *P. agardhii* for substantial N-source levels (especially NO₃⁻) 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 NH₄⁺ or NO₃⁻ 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 NH₄⁺ concentration, and thus outcompeted *P. agardhii* by day 6 of incubation. In contrast, the high affinity for NO₃⁻ 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 allelopathic 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,
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 $\mu_{\text{max}}$ was always lower in mixed cultures than in monocultures, and also some unexpected results, such as those found under $\text{NH}_4^+$ or $\text{NO}_3^-$ 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 both species (especially $C.$ raciborskii) 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 experiments. Where the daily integral of growth rates with those calculated from rates of photosynthesis in Planktothrix spp. isolated from Blelham Tarn, English Lake District. New Phytol., 156, 225–239.


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References


