Harmful Algae 60 (2016) 131–138

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

The role of heterocytes in the physiology and ecology of bloom-forming harmful cyanobacteria

Lilen Yema[a,*], Elena Litchman[b], Paula de Tezanos Pinto[a,b]

[a] Laboratorio de Limnología (Lab. 44, 4to piso), Dpto. de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales (Pab. II), Univ. de Buenos aires (UBA), IEGEBA (CONICET-UBA), Intendente Güiraldes 2620, C1428EHA, Buenos Aires, Argentina
[b] Department of Integrative Biology, Ecology, Evolutionary Biology and Behavior Program, W.K. Kellogg Biological Station, Michigan State Univ., 3700 East Gull Lake Dr., Hickory Corners, MI 49060, USA

ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form 7 October 2016
Accepted 11 November 2016
Available online xxx

Keywords:
Nostocales
Heterocyte
Phosphorus
Nitrogen fixation
Dolichospermum
Cylindrospermopsis

ABSTRACT

Dolichospermum flos-aquae and *Cylindrospermopsis raciborskii* are two cyanobacteria species which cause harmful blooms around the world. Both these species share the capacity to fix atmospheric nitrogen in heterocytes (cell where fixation occurs). While *Dolichospermum* can express heterocytes at rather regular intervals across the filament, *Cylindrospermopsis* can only express heterocytes at the end of the filament. The aim of this study was to experimentally assess the role of heterocyte position in the eco-physiological responses of these bloom forming cyanobacteria. Replicated monocultures of each species were grown at different eutrophication scenarios (limiting and sufficient nitrogen and phosphorus concentrations, in factorial design). *Dolichospermum* reached high biomass regardless of the nitrogen (and phosphorus) provided, suggesting that this species could bloom in situations with and without nitrogen limitation. In contrast, *Cylindrospermopsis* reached high biomass only when nitrogen supply was high; its biomass was 15-20 times lower when relying on nitrogen fixation. Hence, despite its ability to fix nitrogen, blooms of *Cylindrospermopsis* would be expected only under high total nitrogen availability. In *Dolichospermum* heterocytes occurred only in the scenarios without supplied nitrogen while in *Cylindrospermopsis* heterocytes occurred regardless of nitrogen availability. Yet, in both species nitrogen fixation occurred (heterocytes were functional) only when nitrogen was limiting, and nitrogen fixation increased significantly at higher phosphorus concentration. Finally, in the absence of supplied nitrogen, filament length in *Dolichospermum* was the longest, while filaments in *Cylindrospermopsis* were the shortest (up to 13 times shorter than at nitrogen sufficiency). Therefore, heterocyte expression in *Dolichospermum*, and filament length in *Cylindrospermopsis* seem good proxies of nitrogen fixation. The eco-physiological responses recorded here help understand the distribution of these species along nutrient gradients in nature.

1. Introduction

Cyanobacteria blooms are a worldwide problem as they cause serious ecological, economic and health problems in water bodies around the globe. These blooms are usually mono-specific or composed of only a few species, though it is difficult to predict which species will bloom. A better characterization of the physiologies of the bloom-forming genera will help predict which species may bloom under certain conditions. Addressing this prediction is becoming urgent because cyanobacterial blooms are increasing in frequency and intensity around the world (reviewed in O'Neil et al., 2012), mostly due to increased eutrophication (Heisler et al., 2008; reviewed in O'Neil et al., 2012) and climate change (Paerl and Huisman, 2008; Paerl and Paul, 2012).

Many bloom-forming cyanobacteria belong to the genera *Dolichospermum* and *Cylindrospermopsis*. Furthermore, *Cylindrospermopsis* is considered an invasive species in temperate regions (Padisák, 1997; Sinha et al., 2012), hence, there is an increasing concern in understanding its performance in nature.

Species within those two genera share several traits that provide high fitness in a wide range of environments, including the capacity to fix atmospheric nitrogen (in heterocytes), and to produce toxins and dormant cells (akinetes).

Both *Dolichospermum* and *Cylindrospermopsis* share a monophyletic origin (Rajaniemi, 2005; Tomitani et al., 2006; Werner

* Corresponding author.
E-mail addresses: lilen.y@ege.fcen.uba.ar (L. Yema), litchman@msu.edu (E. Litchman), paulatezanos@ege.fcen.uba.ar (P. de Tezanos Pinto).

http://dx.doi.org/10.1016/j.hal.2016.11.007
1568-9883/

et al., 2012; Komárek, 2013) where all species within the clade have the ability to fix nitrogen in specialized cells named heterocytes. This ability usually increases cyanobacterial fitness under nitrogen deficit (Schindler, 1977; Tilman et al., 1986; de Tezanos Pinto and Litchman, 2010). At the same time, these genera are also able to grow on nitrogen compounds dissolved in water. However, it is unclear if the reason why heterocytous nitrogen fixing cyanobacteria dominate in eutrophic lakes is because they have the ability to fix nitrogen (Ferber et al., 2004).

Heterocytes differentiate from a vegetative cell after undergoing major morphological and physiological changes (Komárek, 2013). All species within the *Dolichospermum* genus express heterocytes at rather regular intervals across the filament (Komárek, 2013). Hence, a single filament can have many heterocytes. This pattern of heterocyte distribution is common across most heterocytous nitrogen fixers (e.g., genera *Dolichospermum*, *Aphanizomenon*, *Anabaena*, *Sphaerospermopsis*). Species within the genera *Cylindrospermopsis*, however, differentiate heterocytes only in a terminal position (Komárek, 2013). Thus, a maximum of two heterocytes can occur per filament, one at each end. The hypothesis proposed in this work is that differences in heterocyte position in the filament between *Dolichospermum* (intercalar position) and *Cylindrospermopsis* (terminal position) result in key differences in their physiology. This, in turn, may scale up to the population level and may shape the niche of these species.

At the individual level, the hypothesis is that the contrasting heterocyte position in a filament constrains the maximum number of vegetative cells in the filament. Empirical evidence shows that one heterocyte develops every 10–20 vegetative cells under nitrogen limitation (reviewed in Wolk et al., 1994; Zhang et al., 2006; Kumar et al., 2010). This may suggest that there might be a particular heterocyte to vegetative cell ratio, indicating the number of vegetative cells that can thrive on nitrogen fixed by a single heterocyte. Hence, under nitrogen-fixing conditions, the number of vegetative cells in a given filament in *Dolichospermum* should be proportional to the number of heterocytes. In the same line of thought, it could be assumed that in *Cylindrospermopsis* filaments would reach a maximum of about 20–40 vegetative cells. Under sufficient nitrogen conditions, however, the number of vegetative cells in *Cylindrospermopsis* should be much higher than during nitrogen fixation. Also, the density of heterocytes within a population is proportional to the rate of nitrogen fixation (de Tezanos Pinto and Litchman, 2010). It is accepted that heterocyte expression reflects nitrogen fixation, but, several studies show presence of heterocytes without nitrogen fixation (e.g. Kenesi et al., 2009).

There is an acknowledged trade-off between nitrogen fixation and phosphorus requirements, where nitrogen fixation increases phosphorus demand (Stewart and Alexander, 1971; Howarth et al., 1988). This trade-off has been observed in both *Cylindrospermopsis* (Kenesi et al., 2009) and *Dolichospermum* (Stewart and Alexander, 1971). The cause leading to this trade-off remains unclear, yet it may suggest a physiological constraint. For example, there is a higher need for ATP under nitrogen fixation (16 ATPs are hydrolyzed per N_2 fixed) (Simpson and Burris, 1984). Also, in heterocytes there is evidence of a high expression of two enzymes of the oxidative penthose pathway, which contain phosphorus in their structure (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), compared to vegetative cells (reviewed in Wolk et al., 1994). Because heterocyte expression seems linked to increased phosphorus needs, it can be argued that *Cylindrospermopsis* has lower phosphorus requirements during fixation (which at maximum expresses two heterocytes per filament) than in *Dolichospermum* (which expresses several heterocytes per filament). Another hypothesis is that the higher the availability of phosphorus, the higher the density of heterocytes and the amount of nitrogen fixed. In the current scenarios of increased eutrophication it is of particular relevance to understand how phosphorus availability shapes nitrogen fixation, and how it may scale to population levels and render a bloom formation.

In this study the role of heterocyte position and expression was experimentally assessed in two harmful cyanobacteria that often bloom in water bodies around the world – *Cylindrospermopsis raciborskii* and *Dolichospermum flos-aquae*. These cyanobacteria were exposed to different eutrophication scenarios and their eco-physiological responses assessed in terms of filament length, heterocyte density, amount of nitrogen fixed and biomass.

2. Materials and methods

2.1. Experimental design

Two species of heterocytous nitrogen fixing cyanobacteria were employed: *Dolichospermum flos-aquae* (Brébisson ex Bornet and Flahault) Wacklin, Hoffmann and Komárek 2009 and *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju 1972. The species were isolated from Michigan (USA) lakes and kept in monocultures. Before the experiment, each monoculture was preconditioned during two weeks in nitrogen-free and low phosphorus-medium, to achieve the exhaustion of cellular stored nutrients. At the onset of the experiment each species was exposed to two contrasting nitrogen concentrations (N = 0 to promote fixation and N-surplus −1000 μM− to prevent nitrogen fixation, as fixation is negligible at high N concentrations) and two phosphorus levels (low P = 1 μM and high P = 20 μM, mimicking both mesotrophic and hypereutrophic status, respectively, OECD, 1982), in a factorial design. Hence, the four treatments assessed were: N-fixing low P, N-fixing high P, N-uptake low P and N-uptake high P. The different nutrient treatments were obtained by modifying the nitrogen (nitrate) and phosphorus concentrations in regular WC medium (Guillard, 1975). To prevent carbon or iron limitation, extra HCO_3^- and trace metals were aseptically added (×2 and ×1.5 the standard concentration, respectively) after autoclaving the modified WC media. The experiment was conducted in 250 ml Erlenmeyer flasks filled with 200 ml of medium containing cyanobacteria (species were inoculated at low abundances, approximately 500 filaments mL^{-1}). Each treatment was run in triplicate (4 treatments* 2 species *3 replicates = 24 flasks). The experimental units were maintained in conditions suitable for cyanobacteria growth: constant photoperiod (14 h of light: 10 h of dark), irradiance (100 μmol photon $m^{-2}\,s^{-1}$) and temperature (25 °C), in a semi-continuous regime (daily dilutions of 0.2 d^{-1}). Every day, flasks were swirled and randomly re-arranged within the environmental chamber. The experiment lasted 28 days; based on our previous experience this duration was found sufficient to allow biomass saturation and to observe nitrogen fixation. The experimental units were sampled with a weekly frequency (total of 5 samplings). On each sampling date filament density and nutrient concentration (total and dissolved inorganic fractions of nitrogen and phosphorus) were measured. Samples for density estimation were preserved with Lugol's solution and counted in Palmer cell using a light microscope (400×). The counting unit was the filament, and density was expressed as number of filaments per milliliter. Nutrient availability was measured as follows: total nitrogen (Bachmann and Canfield, 1996), nitrate (Crumpton et al., 1992), total phosphorus (digested before measurements) and phosphate on a nutrient analyzer (Lachat Quik Chem 8500, Method 10-115-01-1-F, Acid Persulfate Digestion Method). The amount of nitrogen fixed at the end of the experiment was indirectly estimated by solving the total nitrogen dynamics equation at equilibrium: Nfix = a (N-Nin). Where Nfix is the amount of nitrogen

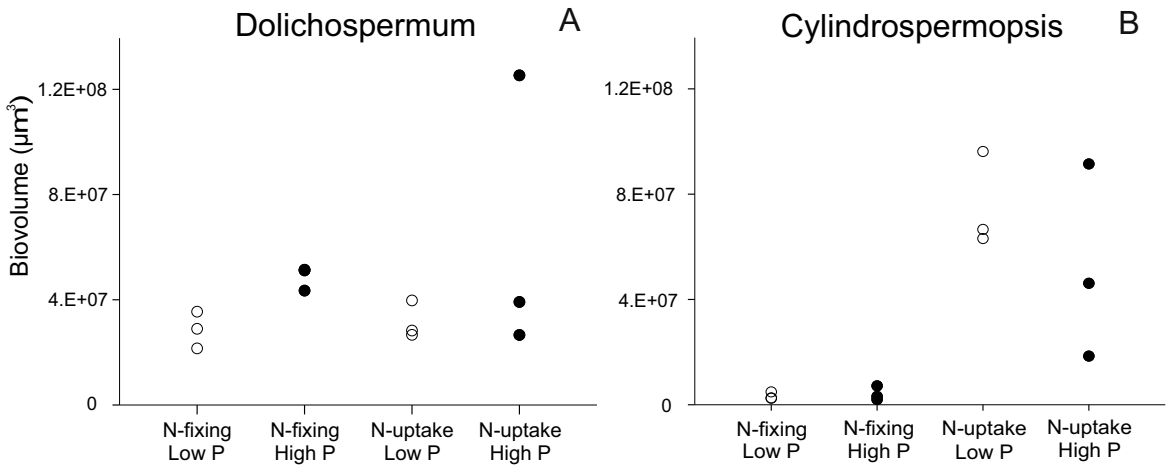

Fig. 1. Biovolume of: a) *Dolichospermum* and b) *Cylindrospermopsis* at the end of the experiment (day 28). 3 replicates per treatment (n = 12 per species). Note that black colour represents treatments with high P and white colour treatments with low P.

fixed ($\mu M\ day^{-1}$), "a" is the dilution rate (day^{-1}), N is total nitrogen measured in the system (μM), and Nin is total nitrogen supplied in the medium (μM). This N mass balance method is used as a common indirect method to estimate N fixation, along with another indirect methods such as acetylene reduction. While it has its limitations, it is used in many studies (e.g. de Tezanos Pinto and Litchman, 2010), including the terrestrial N fixation (Cleveland et al., 2010), because it is inexpensive, relatively straightforward methodologically and can be carried out easily. N fixation was also expressed per heterocyte and per biovolume.

At the end of the experiment, the following morphological traits were also assessed in both species: filament length and width (in μm) and number of heterocytes per filament. These traits were measured in 25 filaments, for each species and treatment (25 measures* 3 replicates* 4 treatments* 2 species = 600 measurements). The density of heterocytes was computed based on the density of filaments and the average number of heterocytes in each replicate. The filament volume was calculated following Hillebrand et al. (1999), using filament length and width. Next, the biovolume of each treatment was calculated computing filament density multiplied by volume.

2.2. Statistical analysis

At the end of the experiment (when cultures usually reach steady growth) the following statistical analyses were used:

1) A Kruskal-Wallis non parametric test (as the homogeneity of variances assumption was not met, even after transforming the variables) to test for differences among treatments for the variables of biovolume and filament length, for each species. The main factor was the treatment with four levels: N-fixing low P, N-fixing high P, N-uptake low P and N-uptake high P. Whenever the test rendered significant, a Mann-Whitney non parametric test was run for pairwise comparisons. For the latter the Bonferroni correction (p-value/number of contrasts) was used to avoid inflating the Type I error.
2) A two- way ANOVA to assess the effect of phosphorus concentrations on nitrogen fixation between nitrogen fixing treatments (N-fixing low P, N-fixing high P). The factors were: i) species (with two levels: *Dolichospermum* and *Cylindrospermopsis*), and ii) phosphorus (with two levels: low P, high P). The assumptions of normality and homogeneity of variances were tested.
3) Curve fitting (linear, logarithm, power or exponential) to test for the best descriptor of the trait–trait relationships between i) the rate of nitrogen fixation and ii) the ratio of heterocyte to vegetative cells density (which corrects possible biomass effects) based on the significance of the ANOVA and the coefficient of determination (R^2).

3. Results

Biovolume of *Dolichospermum* at the end of the experiment was similar across treatments (median of N-fixing treatment = $3.95\ 10^7$ vs. median of N-uptake treatment = $3.37\ 10^7\ \mu m^3$, H = 4.8, df = 3, p = 0.183) (Fig. 1a). Conversely, the biovolume of *Cylindrospermopsis* differed significantly among treatments (H = 8.8, df = 3, p = 0.034) (Fig. 1b): in the N-fixing treatments (Median = $2.9.10^6\ \mu m^3$, Mean rank = 3.5, n = 6) it was one order of magnitude lower than in the N-uptake treatments (Median = $6.5.10^7\ \mu m^3$, Mean rank = 9.5, n = 6) ($U = 0.00$, $z = -2.88$, $p = 0.04$). In addition, *Cylindrospermopsis* biovolume in N-fixing situation was one order of magnitude lower than *Dolichospermum* biovolume, while in N-uptake treatments *Cylindrospermopsis* biovolume was almost two times higher than in *Dolichospermum* (Fig. 1ab).

Dolichospermum cultures reached high density (mean 2×10^4–$1.2 \times 10^5\ ind\ mL^{-1}$) after the second week of the experiment (Fig. 2a). In contrast, *Cylindrospermopsis* cultures reached high density (mean $6 \times 10^4\ ind\ mL^{-1}$) only in the N-uptake situations; values were more than five times lower in the N-fixing treatments (Fig. 2b). Final densities of *Cylindrospermopsis* in the N-fixing treatments were about three times lower than in *Dolichospermum* (Fig. 2ab). Both species showed almost lack of density increase during the first week of the experiment (Fig. 2ab). In *Cylindrospermopsis*, this apparent lag lasted two weeks in the N-fixing treatments (Fig. 2b).

At the end of the experiment (day 28) both species showed wide plasticity in filament length across treatments (Fig. 3a–b). Filament length in *Dolichospermum* was significantly shorter at N-uptake and high P supply than in both N-fixing treatments, but similar to N-uptake low P (Fig. 3a, Table 1). Conversely, the filament length of *Cylindrospermopsis* in the N-fixing scenarios was significantly shorter –between 3 and 13 times- than in N- uptake treatments (Fig. 3b, Table 1). In the N-uptake scenarios the contrasting phosphorus levels rendered similar responses in *Cylindrospermopsis* filament length (Table 1). In contrast, in the

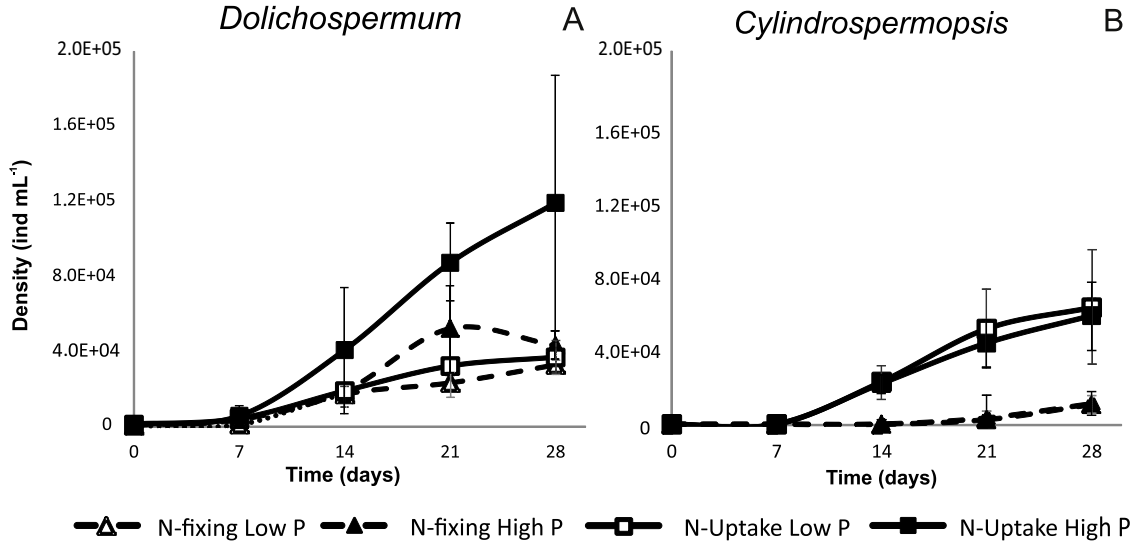

Fig. 2. Filament density throughout the experiment in: a) *Dolichospermum* and b) *Cylindrospermopsis*. Bars represent standard deviation of the mean.

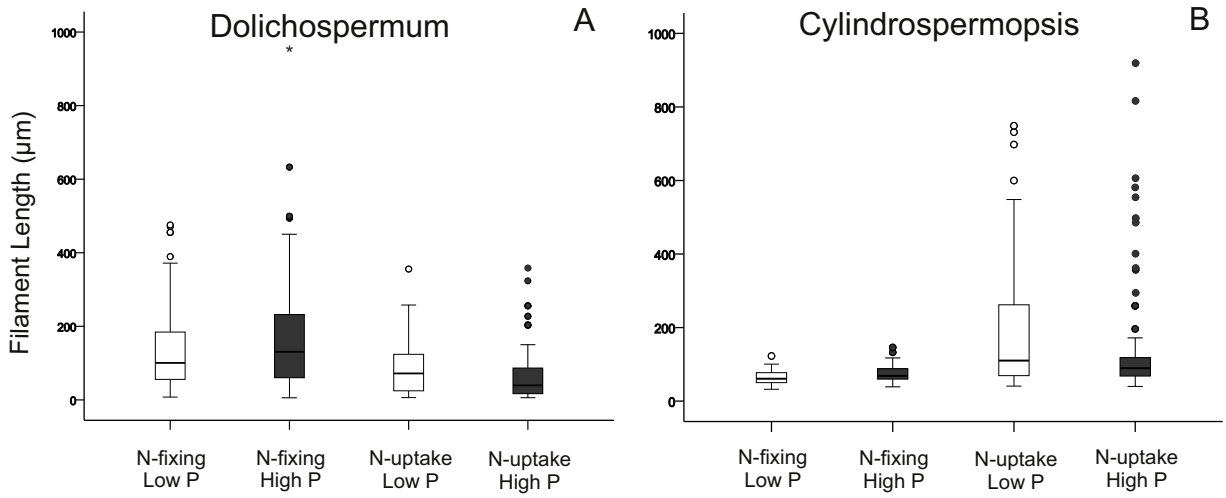

Fig. 3. Box plots of filament length in: a) *Dolichospermum* and b) *Cylindrospermopsis* at the end of the experiment (day 28). Circles correspond to outliers (more than 1.5 times the IQR-Interquartile range- than the rest of the scores). Note that black colour represents treatments with high P and white colour treatments with low P.

N-fixing treatments, phosphorus availability had a significant effect on filament length: at high P filaments were longer than at low P (Fig. 3b, Table 1). In the N-fixing treatments, regardless of the phosphorus levels, *Cylindrospermopsis* filament length showed little dispersion of data (Fig. 3b). In N-uptake treatments, in contrast, filament length presented high variability (Fig. 3b).

In both species total nitrogen (TN) was high in N-uptake treatments (ca. 1100 µM) throughout the experiment and similar to the concentration supplied (Fig. 4a). Conversely, TN in the N-fixing treatments was undetectable at the onset of the experiment, increased during the first two weeks and then remained at similar concentrations until the end of the experiment (Fig. 4b). In the N-fixing treatments TN at the end of the experiment was about twofold higher in the *Dolichospermum* cultures compared to the *Cylindrospermopsis* cultures (Fig. 4b). For both species, the dissolved inorganic nitrogen (DN, nitrate) in the N-uptake treatments was high (about 800 µM) throughout the experiment (Fig. 4c), whereas it remained undetectable in the N-fixing treatments, despite the increase in TN (Fig. 4d).

Throughout the experiment, total phosphorus concentrations remained within the range of the values supplied in the low P and high P treatments, respectively (Fig. 4f and 4e). Dissolved phosphorus (DP) decreased in all treatments throughout the experiment (Fig. 4g and h). In the high P treatments, final DP concentrations were high in both species (range 6–15 µM), and was consumed more by *Dolichospermum* than by *Cylindrospermopsis* (Fig. 4 g). In the low P treatments, DP was undetectable in both species by the end of the experiment: *Dolichospermum* consumed most of DP during the first week of the experiment, whereas in *Cylindrospermopsis* it remained detectable until the third week of the experiment (Fig. 4 h). Throughout the experiment the DP concentration was, in both species, higher in the N-fixing than in the N-uptake scenarios.

In *Dolichospermum*, when nitrogen supply was high (N-uptake treatments), a lack of heterocytes was found and a non-detectable nitrogen fixation rate was measured (Fig. 5a). In contrast, in the absence of supplied nitrogen (N-fixing treatments), a high heterocyte density (mean 5×10^4–1.1×10^5 het mL^{-1}) and a high nitrogen fixation rate (mean 20–25 µmol N L^{-1} d^{-1}) was observed (Fig. 5a). In *Cylindrospermopsis*, both at N-fixing and N-uptake treatments heterocyte density was high (mean 1.5×10^4–6×10^4 het mL^{-1}) (Fig. 5b), but the mean percentage of cells that were

Table 1
Mann Whitney Contrasts for filament length in a) *Dolichospermum* and b) *Cylindrospermopsis*. Pairwise contrasts are significant (*) whenever $p < 0.008$ as the Bonferroni correction ($\alpha = 0.05/n = 6$ contrasts) was used to avoid inflating the type I error. First row depicts the p value, second row depict the value of the Mann Whitney statistic.

Dolichospermum

	Treatments	N-fixing low P	N-fixing high P	N-uptake low P	N-uptake high P
N-fixing low P	*p*	–	0,149	0,029	**3.24×10^{-6}***
	Statistic	–	2429	2232	1574
N-fixing high P	*p*	–	–	**0,001***	**3.62×10^{-8}***
	Statistic	–	–	1909	1347
N-uptake low P	*p*	–	–	–	0,02
	Statistic	–	–	–	2192
N-uptake high P	*p*	–	–	–	–
	Statistic	–	–	–	–

Cylindrospermopsis

	Treatments	N-fixing low P	N-fixing high P	N-uptake low P	N-uptake high P
N-fixing low P	*p*	–	**0,006***	**1.37×10^{-9}***	**3.94×10^{-8}***
	Statistic	–	2080	1200	1351
N-fixing high P	*p*	–	–	**2.39×10^{-6}***	**2.39×10^{-4}***
	Statistic	–	–	1557	1835
N-uptake low P	*p*	–	–	–	0,151
	Statistic	–	–	–	2430
N-uptake high P	*p*	–	–	–	–
	Statistic	–	–	–	–

heterocytes was much higher in the N fixing (10.9-10.6%) than in the N uptake treatments (3.2–6.9%).

Nitrogen fixation rate was high only in the N-fixing treatments (Fig. 5b). In the N-fixing treatments the number of vegetative cells per heterocyte was of 24.6 in *Dolichospermum* and 9.1 in *Cylindrospermopsis*.

Significant differences were observed when assessing the effect of phosphorus and species identity on nitrogen fixation rate (F: 18.46, df = 3, p = 0.0006). Regarding phosphorus, nitrogen fixation rate was significantly higher when phosphorus availability was high (F: 15.51, df = 1, p = 0.004). Regarding species, the nitrogen fixation rate was significantly higher in *Dolichospermum* than in *Cylindrospermopsis* (F: 39.34, df = 1, p = 0.002). The interaction term (species*phosphorus), however, was non-significant (*F:* 0.53, df = 1, p = 0.48). When assessing N-fixation per heterocytes and biovolume, *Cylindrospemopsis* fixed significantly more nitrogen than *Dolichospermum* (F: 33.522, df = 1, p = 0.00041 per heterocyte and F: 25.611, df = 1, p = 0.000976 per biovolume) regardless of the phosphorus concentration assayed ($p > 0.05$). The interaction term (species * phosphorus) was not significant both for N-fixation per heterocyte and biovolume.

Finally, nitrogen fixation rate showed a significant positive linear relationship ($y = 104\ x + 4.6$, $p = 0.033$, R^2: 0.191) with the ratio of heterocyte density to vegetative cell density.

4. Discussion

Our results suggest that heterocyte position and expression influence the eco-physiology of the bloom-forming cyanobacteria *Dolichospermum flos-aquae* and *Cylindrospermopsis raciborskii*. *Dolichospermum* reached high and similar biomass regardless of nitrogen and phosphorus concentrations. In terms of standard nutrient limitation bioassays, like in Kolzau et al. (2014), these results suggest that *Dolichospermum* biomass seems not limited by nitrogen availability (or by phosphorus concentrations $\geq 1\ \mu M$). Indeed, many field studies show that heterocytous N-fixers blooms in situations with low nitrogen (Ferber et al., 2004; Wood et al., 2010; Dolman et al., 2012). But also, blooms of heterocytous N-fixers can often occur at high nitrogen availabity (Dolman et al., 2012; O'Farrell et al., 2012; reviewed in Li et al., 2016) with almost lack of heterocyte expression (Jacobsen and Simonsen, 1993).

Regardless of the phosphorus level, *Cylindrospermopsis* reached high biomass at high nitrogen concentration but very low biomass when relying on nitrogen fixation. The high difference in biomass across nitrogen treatments —one order of magnitude— suggests that *Cylindrospermopsis* is strongly affected by nitrogen limitation (but not by phosphorus concentrations $\geq 1\ \mu M$). Kenesi et al. (2009) also observed low biomass in nitrogen fixing situations. *Cylindrospermopsis* is described as an invasive species (Padisák, 1997; Sinha et al., 2012). Nevertheless, based on our results, it can be argued that it would be unable to invade environments with low total nitrogen concentration as nitrogen fixation seem to provide little fitness gain to this species. Hence, blooms of *Cylindrospermopsis raciborskii* would be expected under high total nitrogen and at a wide range of total phosphorus concentrations. Indeed, several studies found that *Cylindrospermopsis* grows better when depending on dissolved nitrogen sources (ammonium, nitrate, urea) than upon fixation, and at low phosphate concentrations (reviewed in Burford et al., 2016). Also, field studies assessing *Cylindrospermopsis* distribution in nutrient gradients found similar results to those proposed here (Dolman et al., 2012; Kokociński and Soininen, 2012).

Although *Cylindrospermopsis* had higher N fixation values per heterocyte and per biovolume, *Dolichospermum* showed higher N fixation rate per liter probably because its higher heterocyte density. This again is in concordance with *Cylindrospermopsis* preference of environments with high nitrogen availability.

The generally accepted knowledge that heterocyte density is a proxy for nitrogen fixation (Lehtimäki et al., 1997; Ferber et al., 2004; de Tezanos Pinto and Litchman, 2010) is supported by our study only for *Dolichospermum*. In *Dolichospermum* heterocyte expression occurred only at low nitrogen availability and heterocyte density was proportional to the amount of nitrogen fixed. This result agrees with previous studies (de Tezanos Pinto and Litchman, 2010) and hence, in *Dolichospermum* heterocyte density seems a good proxy of nitrogen fixation. In *Cylindrospermopsis*, however, heterocytes were present both at high and low nitrogen availability, though nitrogen fixation occurred only when nitrogen

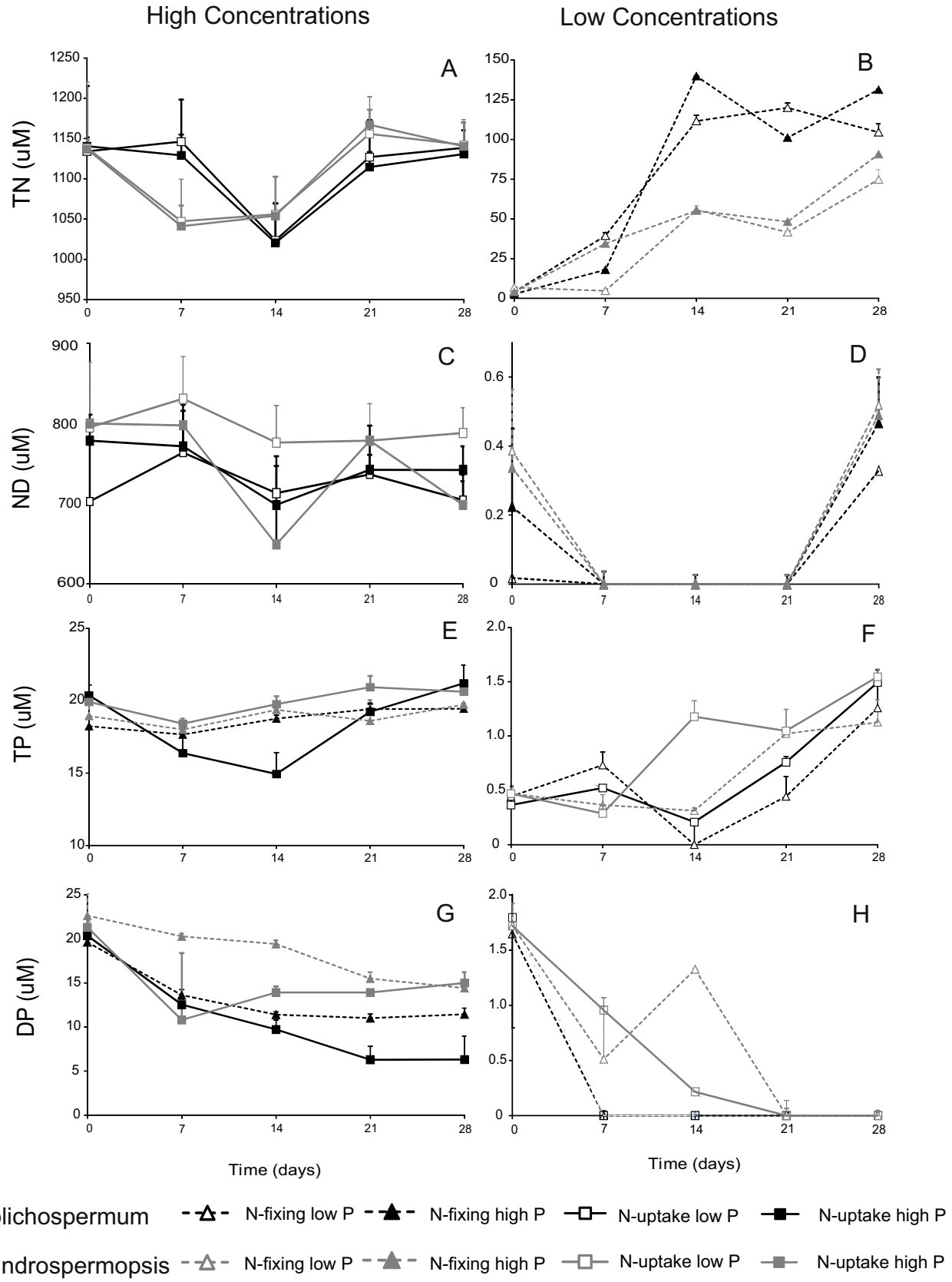

Fig. 4. Total and dissolved nitrogen and phosphorus availability throughout the experiment. The left column encompass treatments with high TN (N-uptake) and high TP, while the right column treatments with low TN (N-fixing) and low TP. Note that axes have different scales.

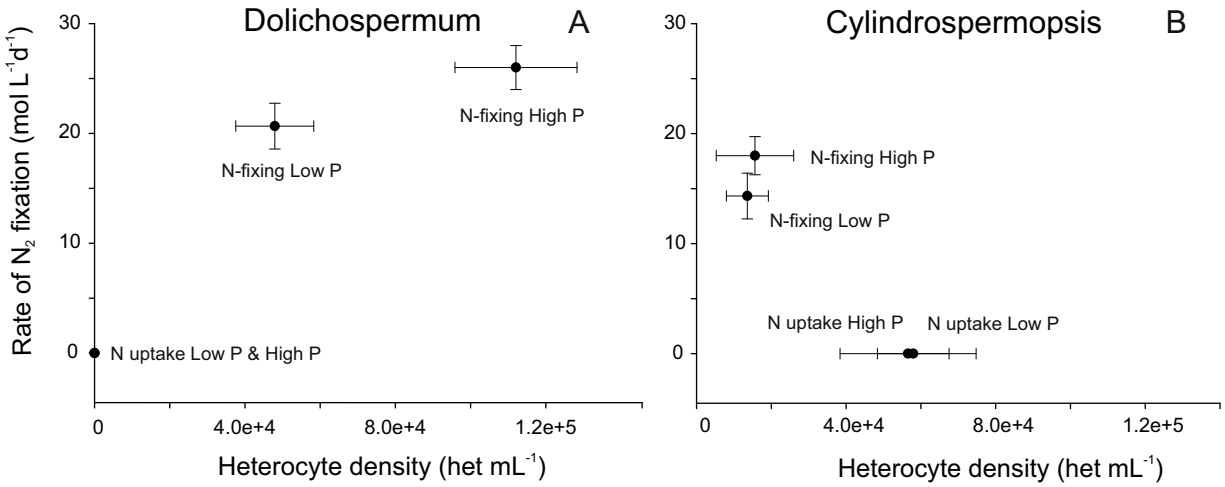

Fig. 5. The relationship between heterocyte density (heterocytes mL^{-1}) and rate of nitrogen fixation (μmol/L.d N_2) at the end of experiment (day 28). Bars denote the standard deviation of the mean for each trait. Note that in *Dolichospermum* there is absence of heterocyte expression in both N- uptake treatments (in all 6 replicates).

concentrations were low. Therefore, in *Cylindrospermopsis*, heterocyte presence does not imply heterocyte functioning (nitrogen fixation), as also seen by Kenesi et al. (2009). In this species, heterocyte development seems less influenced by the environmental trigger of nitrogen scarcity. Counter intuitively, heterocyte density in *Cylindrospermopsis* was higher under nitrogen sufficient conditions, compared to the nitrogen deficient conditions. This happened because its biomass was much higher (15–20 times) under high nitrogen compared to nitrogen-free situations, and because heterocytes were developed despite the high nitrogen availability. Therefore, in this species, heterocyte presence is a poor proxy for nitrogen fixation. Finally, in *Cylindrospermopsis*, the percentage of cells that were heterocytes was much higher (about one and a half to three times) in the N fixing than in the N uptaking situations, evidencing that more cells differentiate into heterocytes when nitrogen availability is scarce.

Despite the differences observed in the two species, remarkable similarities in heterocyte functioning were found: a) both species fixed nitrogen only when nitrogen availability was low and b) increased phosphorus resulted in significantly higher rate of nitrogen fixation. This finding supports the general knowledge that nitrogen fixation requires higher phosphorus availability (Stewart and Alexander, 1971; Kenesi et al., 2009) and may be related to the increased ATP demands for nitrogen fixation (Simpson and Burris, 1984) and/or increased synthesis in the heterocyte of enzymes that contain phosphorus in their structure (reviewed in Wolk et al., 1994). This underscores the risk of phosphorus enrichment in nitrogen-limited systems.

Both species showed a wide plasticity in filament length, from very short to very long filaments. In *Dolichospermum*, filaments were the longest under N-fixing conditions (and high phosphorus availability) and the opposite happened in *Cylindrospermopsis* (where filaments were 3 to 13 times shorter under nitrogen scarcity, compared to nitrogen sufficiency). The latter probably occurs because few vegetative cells can grow on the nitrogen fixed by (up to) two heterocytes, which are located at the end of the filament. Under nitrogen fixing conditions, *Cylindrospermopsis* had an average of nine vegetative cells per heterocyte, which would imply a filament of about 18 vegetative cells. This constraint on the maximum number of vegetative cells was reflected in much lower flexibility in filament length during nitrogen fixation (compared to a wide plasticity at high nitrogen). Kenesi et al. (2009) also found that *Cylindrospermopsis* filaments were longer under N-sufficient conditions. In a field study assessing the morphological plasticity of *Cylindrospermopsis* in a shallow lake in South America, filaments were very short at low total nitrogen, and much longer and variable at high total nitrogen (Sarthou Suárez, 2016). In light of our results, for *Cylindrospermopsis*, it is proposed that filament length could be used as a proxy of nitrogen fixation. Hence, when most filaments in the population are short and with little variation in its length, a strong reliance of nitrogen fixation can be inferred, and the opposite trend (long filaments with wide variation in length) for high nitrogen availability.

Further, phosphorus needs in *Cylindrospermopsis* were found to be lower than in *Dolichospermum*. Previous studies show high biomass of *Cylindrospermopsis* in low phosphorus scenarios (Kenesi et al., 2009; Bonilla et al., 2012). This species has high affinity for phosphate uptake and storage capacity (Isvánovics et al., 2000) and the ability to use dissolved organic phosphorus (Bai et al., 2014). In both species, the dissolved phosphorus was higher under N-fixing conditions (lack of N addition), probably because of the limitation by nitrogen and reduced uptake of phosphorus. Both species were able to grow well at low P concentrations (1 μM), so, at least when growing in monocultures, this concentration seems sufficient to support growth.

Of all the highly diverse planktonic heterocytous nitrogen-fixers (Nostocales) all genera except *Raphidiopsis* share the trait of nitrogen fixation within a heterocyte. The high conservatism of this trait in Nostocales may reflect the high fitness this trait (nitrogen fixation within a heterocyte) affords under nitrogen deficiency. *Raphidiopsis* is morphologically identical to *Cylindrospermopsis* except for the ability to develop heterocytes (Komárek, 2013) and to fix nitrogen, as it lacks part of the genes involved in heterocyte and nitrogen fixation (Stucken et al., 2010). Based on the results of this study it may be argued that *Raphidiopsis* could have arisen from *Cylindrospermopsis*, as a consequence of natural selection on a trait that provided little fitness advantage in *Cylindrospermopsis*.

Because *Raphidiopsis* and *Cylindrospermopsis* are morphologically identical in the absence of heterocyte differentiation (Komárek, 2013), it complicates their identification in natural samples. But, in light of our results that show that *Cylindrospermopsis* develops heterocytes regardless of nitrogen availability in the environment, it may be much easier to differentiate blooms of *Cylindrospermopsis* (presence of heterocytes in at least part of the population) from those of *Raphidiopsis* (complete absence of heterocyte in all the population).

Results show that although the two harmful cyanobacteria from the genera *Dolichospermum* and *Cylindrospermopsis* share

similarities in terms of nitrogen fixation physiology, they differ considerably in their fundamental niches. Our findings, at least partially, explain the distribution of these species in nature, and provide tools for forecasting their occurrences based on nutrient availability.

Acknowledgements

We want to thank Ruben Lombardo and Melina Devercelli for statistics advice and Inés O'Farrell for comments on the draft. This work was funded by: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, PIP 1142010100236; CONICET-AVCR; NSF grants DEB 0845932 and CBET-1134215. PTP thanks the financial support of Fulbright-CONICET grant. We also want to express our gratitude to the two anonymous reviewers that helped to improve this paper. This is a W.K. Kellogg Biological Station contribution nº 1976.[CG]

References

Bachmann, R.W., Canfield, D.E.J., 1996. Use of an alternative method for monitoring total nitrogen concentrations in Florida lakes. Hydrobiologia 323, 1–8.

Bai, F., Liu, R., Yang, Y., Ran, X., Shi, J., Wu, Z., 2014. Dissolved organic phosphorus use by the invasive freshwater diazotroph cyanobacterium, *Cylindrospermopsis raciborskii*. Harmful Algae 39, 112–120. doi:http://dx.doi.org/10.1016/j.hal.2014.06.015.

Bonilla, S., Aubriot, L., Soares, M.C.S., González-Piana, M., Fabre, A., Huszar, V.L.M., Lürling, M., Antoniades, D., Padisák, J., Kruk, C., 2012. What drives the distribution of the bloom-forming cyanobacteria *Planktothrix agardhii* and *Cylindrospermopsis raciborskii*? FEMS Microbiol. Ecol. 79, 594–607. doi:http://dx.doi.org/10.1111/j.1574-6941.2011.01242. x.

Burford, M.A., Beardall, J., Willis, A., Orr, P.T., Magalhaes, V.T., Rangel, L.M., Azevedo, S.M.F.O.E., Neilan, B.A., 2016. Understanding the winning strategies used by the bloom-forming cyanobacterium *Cylindrospermopsis raciborskii*. Harmful Algae 54, 44–53. doi:http://dx.doi.org/10.1016/j.hal.2015.10.012.

Cleveland, C.C., Houlton, B.Z., Neill, C., Reed, S.C., Townsend, A.R., Wang, Y.P., 2010. Using indirect methods to constrain symbiotic nitrogen fixation rates: a case study from an Amazonian rain forest. Biogeochemistry 99, 1–13.

Crumpton, W.G., Isenhart, T.M., Mitchell, P.D., 1992. Nitrate and organic N analyses with second-derivative spectroscopy. Limnol. Oceanogr. 37, 907–913. doi:http://dx.doi.org/10.4319/lo.1992.37.4.0907.

de Tezanos Pinto, P., Litchman, E., 2010. Interactive effects of N:P ratios and light on nitrogen-fixer abundance. Oikos 119, 567–575. doi:http://dx.doi.org/10.1111/j.1600-0706.2009.17924.x.

Dolman, A.M., Rücker, J., Pick, F.R., Fastner, J., Rohrlack, T., Mischke, U., Wiedner, C., 2012. Cyanobacteria and cyanotoxins: the influence of nitrogen versus phosphorus. PLoS One 7, e38757. doi:http://dx.doi.org/10.1371/journal.pone.0038757.

Ferber, L.R., Levine, S.N., Lini, a., Livingston, G.P., 2004. Do cyanobacteria dominate in eutrophic lakes because they fix atmospheric nitrogen? Freshw. Biol. 49, 690–708. doi:http://dx.doi.org/10.1111/j.1365-2427.2004.01218.x.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chantey, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum, New York, pp. 29–60.

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., Suddleson, M., 2008. Eutrophication and harmful algal blooms: a scientific consensus. Harmful Algae 8, 3–13. doi:http://dx.doi.org/10.1016/j.hal.2008.08.006.

Hillebrand, H., Dürselen, C.-D., Kirschtel, D., Pollingher, U., Zohary, T., 1999. Biovolume calculation for pelagic and benthic microalgae. J. Phycol. 35, 403–424.

Howarth, R.W., Marino, R., Lane, J., Cole, J.J., 1988. Nitrogen fixation in freshwater estuarine, and marine ecosystems. 1. Rates and importance. Limnol. Oceanogr. 33, 669–687.

Isvánovics, V., Shafik, H.M., Présing, M., Juhos, S., 2000. Growth and phosphate uptake kinetics of the cyanobacterium, *Cylindrospermopsis raciborskii* (Cyanophyceae) in throughflow cultures. Freshw. Biol. 43, 257–275.

Jacobsen, B.A., Simonsen, P., 1993. Disturbance events affecting phytoplankton biomass, composition and species diversity in a shallow, eutrophic, temperate lake. Hydrobiologia 249, 9–14. doi:http://dx.doi.org/10.1007/978-94-017-1919-3_2.

Kenesi, G., Shafik, H.M., Kovács, A.W., Herodek, S., Présing, M., 2009. Effect of nitrogen forms on growth, cell composition and N_2 fixation of *Cylindrospermopsis raciborskii* in phosphorus-limited chemostat cultures. Hydrobiologia 623, 191–202. doi:http://dx.doi.org/10.1007/s10750-008-9657-9.

Kokociński, M., Soininen, J., 2012. Environmental factors related to the occurence of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyta) at the north-eastern limit of its geographical range. Eur. J. Phycol. 47 (1), 12–21. doi:http://dx.doi.org/10.1080/09670262.2011.645216.

Kolzau, S., Wiedner, C., Rücker, J., Köhler, J., Köhler, A., Dolman, A.M., 2014. Seasonal patterns of nitrogen and phosphorus limitation in four German lakes and the predictability of limitation status from ambient nutrient concentrations. PLoS One 9, e96065.

Komárek, J., 2013. Cyanoprokaryota 3. teil/3rd part: heterocytous genera. In: Büdel, B., Gärtner, G., Krienitz, L., Schagerl, M. (Eds.), Süwasserflora Von Mitteleuropa/Freshwater Flora of Central Europe 19/3. Springer Spektrum, Berlin, Heidelberg (1130 pp.).

Kumar, K., Mella-Herrera, R.A., Golden, J.W., 2010. Cyanobacterial heterocysts. Cold Spring Harb. Perspect. Biol. 2 (a00315).

Lehtimäki, J., Moisander, P.H., Sivonen, K., Kononen, K., 1997. Growth, nitrogen fixation, and nodularin production by two baltic sea cyanobacteria. Appl. Environ. Microbiol. 63, 1647–1656.

Li, X., Dreher, T.W., Li, R., 2016. An overview of diversity, occurrence, and toxin production of bloom-forming *Dolichospermum* (*Anabaena*) species. Harmful Algae 54, 54–68. doi:http://dx.doi.org/10.1016/j.hal.2015.10.015.

O'Neil, J.M., Davis, T.W., Burford, M.A., Gobler, C.J., 2012. The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. Harmful Algae 14, 313–334. doi:http://dx.doi.org/10.1016/j.hal.2011.10.027.

O'Farrell, I., Bordet, F., Chaparro, G., 2012. Bloom forming cyanobacterial complexes co-occurring in a subtropical large reservoir: validation of dominant eco-strategies. Hydrobiologia 698, 175–190. doi:http://dx.doi.org/10.1007/s10750-012-1102-4.

OECD, 1982. Eutrophication of Waters, Monitoring, Assessment and Control. Organisation for Economic Cooperation and Development, Paris.

Padisák, J., 1997. *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. Arch. Hydrobiol./Suppl Monogr. 107 (4), 563–593.

Paerl, H.W., Huisman, J., 2008. Climate. Blooms like it hot. Science 320, 57–58. doi:http://dx.doi.org/10.1126/science.1155398.

Paerl, H.W., Paul, V.J., 2012. Climate change: links to global expansion of harmful cyanobacteria. Water Res. 46, 1349–1363. doi:http://dx.doi.org/10.1016/j.watres.2011.08.002.

Rajaniemi, P., 2005. Phylogenetic and morphological evaluation of the genera *Anabaena, Aphanizomenon, Trichormus* and *Nostoc* (Nostocales, cyanobacteria). Int. J. Syst. Evol. Microbiol. 55, 11–26. doi:http://dx.doi.org/10.1099/ijs.0.63276-0.

Sarthou Suárez, F.V., 2016. Floraciones de cianobacterias: efectos de la eutrofización y la variabilidad climática. Tesis de Maestría en Geociencias-PEDECIBA. Universidad de la República, Uruguay.

Schindler, D.W., 1977. Evolution of phosphorus limitation in lakes. Science 195, 260–262.

Simpson, F.B., Burris, R.H., 1984. A Nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science 224, 1095–1097.

Sinha, R., Pearson, L.A., Davis, T.W., Burford, M.A., Orr, P.T., Neilan, B.A., 2012. Increased incidence of *Cylindrospermopsis raciborskii* in temperate zones – is climate change responsible? Water Res. 46, 1408–1419. doi:http://dx.doi.org/10.1016/j.watres.2011.12.019.

Stewart, W.D.P., Alexander, G., 1971. Phosphorus availability and nitrogenase activity in aquatic blue-green algae. Freshw. Biol. 1, 389–404.

Stucken, K., John, U., Cembella, A., Murillo, A.A., Soto-Liebe, K., Fuentes-Valdés, J.J., Friedel, M., Plominsky, A.M., Vásquez, M., Glöckner, G., 2010. The smallest known genomes of multicellular and toxic cyanobacteria: comparison, minimal gene sets for linked traits and the evolutionary implications. PLoS One 5 (2), e9235. doi:http://dx.doi.org/10.1371/journal.pone.0009235.

Tilman, D.G., Kiesling, R., Sterner, R.W., Kilham, S.S., Johnson, F.A., 1986. Green, bluegreen and diatom algae Taxonomic differences in competitive ability for phosphorus, silicon and nitrogen. Arch. Hydrobiol. 106, 473–485.

Tomitani, A., Knoll, A.H., Cavanaugh, C.M., Ohno, T., 2006. The evolutionary diversification of cyanobacteria: molecular-phylogenetic and paleontological perspectives. Proc. Natl. Acad. Sci. U. S. A. 103, 5442–5447. doi:http://dx.doi.org/10.1073/pnas.0600999103.

Werner, V.R., Laughinghouse, I.V., Fiore, H.D., Sant'Anna, M.F., Hoff, C.L., de Souza, C., Santos, K.R., Neuhaus, E.B., Molica, R.J.R., Honda, R.Y., Echenique, R.O., 2012. Morphological and molecular studies of *Sphaerospermopsis torques-reginae* (Cyanobacteria, Nostocales) from South American water blooms. Phycologia 51, 228–238. doi:http://dx.doi.org/10.2216/11-32.1.

Wolk, P.C., Ernst, A., Elhai, J., 1994. Heterocyst metabolism and development. In: Bryant, D.A. (Ed.), The Molecular Biology of Cyanobacteria. Kluwer Academic Publishers, The Netherlands, pp. 769–823.

Wood, S.A., Prentice, M.J., Smith, K., Hamilton, D.P., 2010. Low dissolved inorganic nitrogen and increased heterocyte frequency: precursors to *Anabaena planktonica* blooms in a temperate, eutrophic reservoir. J. Plankton Res. 32, 1315–1325. doi:http://dx.doi.org/10.1093/plankt/fbq048.

Zhang, C.-C., Laurent, S., Sakr, S., Peng, L., Bédu, S., 2006. Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. Mol. Microbiol. 59, 367–375. doi:http://dx.doi.org/10.1111/j.1365-2958.2005.04979.x.