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

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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

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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