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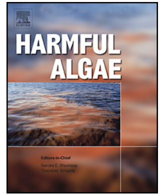

Constitutive toxin production under various nitrogen and phosphorus regimes of three ecotypes of *Cylindrospermopsis raciborskii* ((Wołoszyńska) Seenayya et Subba Raju)

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ABSTRACT

Cylindrospermopsis raciborskii is a global invasive cyanobacterium, with some ecotypes (i.e. strains) producing the toxin cylindrospermopsin, CYN. Multiple ecotypes can co-exist, complicating prediction of toxin concentrations based on cell concentrations. This study examined the growth response and toxin production of three Australian ecotypes of *C. raciborskii*, two toxic (CS-505, CS-506) and one non-toxic (CS-510), to a range of nitrogen (N) and phosphorus (P) concentrations. CYN cell quota was constant under all N:P ratios and concentration conditions, indicative of a constitutive response, yet the CYN cell quota was 6-fold higher in CS-506 compared to CS-505. The ecotypes differed in response to dissolved N depletion: there was a 4-fold difference in the number of cells heterocyst mL^{-1} between CS-505 and CS-510, while CS-506 did not produce any heterocysts and was unable to grow in N deplete conditions. Growth rates were lower for all ecotypes as [P] increased, indicative of a species with a strategy of P storage rather than increased growth. Presumably this is an adaptation to low and fluctuating P conditions. However, the negative effect of increasing [P] on growth is surprising. In contrast, increasing [N] resulted in higher growth rates across ecotypes. This study highlights the importance of understanding differences in growth and toxin production between ecotypes in response to environmental conditions in order to more effectively predict blooms and toxin yields.

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1. Introduction

Cylindrospermopsis raciborskii (Wołoszyńska) Seenayya et Subba Raju, is a globally occurring cyanobacterium that can dominate in freshwater lakes and reservoirs (Padisák, 1997). It is a solitary, filamentous diazotroph with the potential to produce harmful compounds. Australian ecotypes produce the alkaloid cytotoxin, cylindrospermopsin (CYN), which is harmful to humans and animals (Falconer et al., 1999), and summer blooms of *C. raciborskii* cause problems, particularly in tropical water reservoirs (McGregor and Fabbro, 2000; Neilan et al., 2003). The most significant account of human poisoning is associated with an incident on Palm Island in 1979, where 148 people suffered from hepatoenteritis (Byth, 1980; Hawkins et al., 1985).

The number and severity of harmful algae blooms, including cyanobacteria, has been increasing and the combined effects of eutrophication and climate change are predicted to result in an even greater frequency of blooms (O'Neil et al., 2012; Paerl and Paul, 2012; Sinha et al., 2012). *C. raciborskii* is considered an invasive species as its habitat range has increased in recent decades (Sinha et al., 2012). The ability of this species to proliferate in new environments has been attributed to its high phenotypic plasticity (Bonilla et al., 2011). This plasticity of the species may actually be caused by the existence of multiple ecotypes (i.e. strains) each with their own optimal physiological requirements (Chonudomkul et al., 2004; Piccini et al., 2011).

Ecotypes which differ in their CYN cell quotas (Q_{CYN} – the amount contained in an individual cell) can have major effects on the amount of CYN produced during a bloom (Orr et al., 2010;

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Mohamed and Al-Shehri, 2013; Somdee et al., 2013). Multiple ecotypes of *C. raciborskii* have been identified worldwide and throughout Australia with Q_{CYN} ranging from 0 to 55.8 fg cell^{-1}. This can result in blooms of up to 180 μg L^{-1} CYN (e.g. Saker et al., 1999; Orr et al., 2010). The presence of multiple ecotypes, with differing Q_{CYN}, which vary in dominance due to a range of poorly understood environmental drivers makes prediction of CYN concentrations during a bloom difficult (e.g. Bittencourt-Oliveira et al., 2012; Fathalli et al., 2011; Burford et al., 2014).

Nutrients are one of the main environmental drivers of cyanobacterial blooms. In particular, nitrogen (N) and phosphorus (P) play major roles affecting *C. raciborskii* dominance in freshwater systems (for reviews see Burford and Davis, 2011; O'Neil et al., 2012). European strains of *C. raciborskii* have been shown to have a high affinity and storage capacity for dissolved inorganic phosphorus (DIP) (Isvánovics et al., 2000). It can also fix atmospheric dinitrogen (N_2), but it has a 4-fold higher growth rate when dissolved inorganic nitrogen (DIN) is available (Plominsky et al., 2014). These combined features allow *C. raciborskii* to respond within minutes to nutrient inputs (Amaral et al., 2014), and may be key factors in its worldwide success and dominance in many ecosystems (Padisák, 1997).

We examined the growth of three Australian ecotypes of *C. raciborskii* under different ratios and concentrations of N and P in laboratory culture experiments. The aim of this experiment was to determine the difference in magnitude of the ecotypes' growth response and CYN production to different N and P concentrations.

2. Materials and methods

2.1. C. raciborskii *ecotype cultures*

Three non-axenic ecotypes (i.e. strains) of *C. raciborskii* were obtained from the Australian National Algae Culture Collection, CSIRO Marine and Atmospheric Research, Hobart, Australia. The ecotypes originated from tropical North Queensland, Australia, toxic ecotypes CS-505 and CS-506 were isolated in 1996 from Solomon Dam (18°43′27″ S, 146°35′38″ E), and non-toxic CS-510 was isolated in 1997 from North Queensland, possibly from McKinlay Dam (21°16′ S, 141°18′ E). Ecotypes CS-505 and CS-510 have straight trichomes, and CS-506 has coiled trichome morphology. Cultures of all ecotypes were maintained in Jaworski Medium (JM; Anderson, 2005) at 28 °C on a 12 h:12 h light:dark cycle at 10 μmol photon (PAR) m^{-2} s^{-1} using a cool white fluorescent light (Crompton). *C. raciborskii* is low-light adapted species (Bonilla et al., 2011) and the light level of 10 μmol photon (PAR) m^{-2} s^{-1} ensured growth rates under nutrient replete conditions were light limited (Pierangelini et al., 2015b). Cultures were gently manually shaken three to four times per week during incubation.

2.2. Growth experiments

In order to optimize the experimental conditions, preliminary experiments were conducted to determine:

1. Sampling frequency (supplementary data); growth was monitored daily under both JM+ and JM− culture.
2. Effect of centrifugation; cells were sub-cultured with and without centrifugation and washing to ascertain that there was no effect on growth rate (data not shown).
3. Number of days to starve cells of N and P (i.e. time until $QP \leq QP_{min}$); as each ecotype required a different starvation time non-starved cells were used.
4. Minimum N and P requirements for 10 days growth; determined by a pilot growth experiment using ecotypes CS-506 (coiled morphotype) and CS-510 (straight morphotype). Concentrations of N and P in JM were reduced logarithmically to ½, ¼, 1/8, and 1/16 and inoculated with the ecotypes (1:10 dilution) and culture density was monitored spectrophotometrically for 15 days. This pilot study indicated that 1/8 of JM concentration of N and P was the minimum required for growth, and no difference in maximum culture density was seen from full strength to ½ strength. Therefore the concentrations for the growth experiment were based on ½, ¼ and 1/8 reductions in N and P concentrations (Table 1).

Table 1
Concentrations of nitrogen (μmol L^{-1}) and phosphorus (μmol L^{-1}) of modified Jawoski's Medium for growth experiments.

Treatment	$NaNO_3$ (μmol L^{-1})	KH_2PO_4 + Na_2HPO_4 (μmol L^{-1})	Molar ratio N:P
Control +N941.+P200	941	200	4.7
Control N0.P0	0	0	–
N0.P200	0	200	–
N941.P0	941	0	–
N470.P100 (1/2 + 1/2)	470	100	4.7
N470.P25 (1/2 + 1/8)	470	25	18.8
N118.P100 (1/8 + 1/2)	118	100	1.18
N118.P25 (1/8 + 1/8)	118	25	4.7
N235.P50 (1/4 + 1/4)	235	50	4.7

Each ecotype was grown in batch culture with full JM media for 10 days before starting the experiment: each ecotype was subcultured in 500 mL tissue culture flasks with an inoculation of 250 mL culture and grown until mid-exponential growth phase (10 days). To prepare N and P free JM all combined N and P was removed (replacing $Ca(NO_3)_2$–$4H_2O$ with $CaCl_2$–$2H_2O$ and $(NH_4)_6MO_7O_{24}$–$4H_2O$ with Na_2MoO_4–$2H_2O$, equivalent concentrations). Modified JM was prepared by the addition of $NaNO_3$ and KH_2PO_4:Na_2HPO_4 at the required treatment concentrations). Cells were harvested by centrifugation for 5 min at 800 × *g* (Eppendorf AG, Hamburg, Germany), washed once with sterile N and P-free JM and re-suspended in a minimum of 1350 mL N and P-free JM medium or further diluted to achieve equal culture density. 50 mL of re-suspended cells were transferred to 250 mL culture flasks containing 200 mL of modified JM with the various N and P concentrations and with final culture density for all ecotypes of OD750 = 0.01 ± 0.002, which is equivalent to CS-505 = 800 × 10^3, CS-506 = 600 × 10^3, and CS-510 = 350 × 10^3 cells mL^{-1}. The N and P concentrations were a combination of high, medium and low N and P based on 1/2, 1/4 and 1/8th concentration of N and P in full JM media (Table 1), the treatments are labeled according to the concentration of the treatments, e.g. N470:P100, where treatment is [N] = 470 μmol L^{-1} and [P] = 100 μmol L^{-1}. All treatments were in triplicate and incubated in the culture conditions described above. All glassware used in media preparation was washed with 10% (v/v) HCl to prevent N and P contamination.

2.3. Sample collection

Subsamples from each flask were collected under sterile conditions at 5-day intervals for 30 days. A 5 mL subsample was used for optical density measurements at every time point, with cell enumeration. A second 5 mL subsample was collected and stored at −20 °C for CYN_{total} (particulate + dissolved; cylindrospermopsin (CYN) + deoxy-cylindrospermopsin (dCYN)) analysis.

2.4. Optical density and growth calculations

The growth of the cultures was monitored by measuring the optical density at 750 nm (OD750) using a spectrophotometer (Novaspec II, Pharmacia Biotech). The OD750 was measured

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