Physical and chemical processes promoting dominance of the
toxic cyanobacterium *Cylindrospermopsis raciborskii*

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Abstract

The freshwater cyanobacterium, *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenayya and Subba Raju is a common species in lakes and reservoirs around the world. In some areas of the world it can produce cyto- and hepatotoxins (cylindrospermopsins, saxitoxins), making blooms of this species a serious health concern for humans. In the last 10-15 years, there has been a considerable body of research conducted on the ecology, physiology and toxin production of this species and this paper reviews these studies with a focus on the cylindrospermopsin (CYN)-producing strains. *C. raciborskii* has low light requirements, close to neutral buoyancy, and a wide temperature tolerance, giving it the capacity to grow in many lentic waterbodies. It also has a flexible strategy with respect to nitrogen (N) utilisation; being able to switch between utilising DIN and N fixation as sources of N fluctuate. Additionally this species has a high phosphate (DIP) affinity and storage capacity. Like many cyanobacteria, it also has the capacity to use dissolved organic phosphorus (DOP). Changes in nutrient concentrations, light levels and temperature have also been found to affect production of the toxin CYN by this species. However, optimal toxin production does not necessarily occur when growth rates are optimal. Additionally, different strains of *C. raciborskii* vary in their cell quota of CYN, making it difficult to predict toxin concentrations, based on *C. raciborskii* cell densities. In summary, the ecological flexibility of this organism means that controlling blooms of *C. raciborskii* is a difficult undertaking. However, improved understanding of factors promoting the species and toxin production by genetically capable strains will lead to improved predictive models of blooms.

Introduction

The cyanobacterium, *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenayya and Subba Raju dominates lakes and water reservoirs in temperate and tropical environments including Australia (Hawkins et al., 1985), North America (Chapman and Schelske, 1997; Hong et al., 2006), South America (Branco and Senna, 1996; Bouvy et al., 2006; Figueredo and Giani, 2009), Europe (Fastner et al., 2003; Briand et al., 2004), Africa (Dufour et al., 2006; Mohamed, 2007) and Asia (Chonudomkul et al., 2004). It is a solitary, filamentous diazotroph that appears to be invading more freshwaters across a diversity of habitats. There are recent accounts of the appearance of this species in temperate areas of the world including northern Europe (Briand et

al., 2004; Saker et al., 2003; Wiedner et al., 2007), New Zealand (Wood and Stirling, 2003), and northern USA (Hamilton et al., 2005; Hong et al., 2006).

C. raciborskii can produce a suite of potentially harmful compounds, although the ability to produce them is not universal. Some Australian strains of *C. raciborskii* produces an alkaloid cytotoxin, CYN, while some Brazilian strains produces paralytic shellfish poisoning (PSP) toxins (Neilan et al., 2003). Furthermore, strains isolated from Portuguese lakes show toxicity in mice bioassays but do not produce any of the known cyanotoxins (Saker et al., 2003). CYN has been linked with a major poisoning incident on Palm Island, Queensland, Australia in 1979 when 149 people became ill, many requiring hospitalisation (Hawkins et al., 1985). Conversely there is little evidence of the northern hemisphere strains producing toxins, although recently CYN concentrations have been correlated with *C. raciborskii* densities in an Italian lake (Messineo et al., 2010).

In 1997, Padisák reviewed the state of knowledge of *C. raciborskii* distribution and ecology. Our review builds on work by focussing on the nutrient and physical conditions (light, mixing, temperature) that will promote *C. raciborskii* dominance and production of the toxin, CYN, with an emphasis on publications since 1997.

Factors affecting *C. raciborskii* ecology

Light and mixing

Reynolds et al. (2002) has proposed that low light and optically deep mixing increase *C. raciborskii* dominance. Maximum cell concentrations have been measured when the mixed depth:euphotic depth ratios were greater than one (McGregor and Fabbro, 2000; Saker and Griffiths, 2000). Additionally, *C. raciborskii* was found to dominate in deeper reservoirs (> 15 m) and where stratification was >5°C.

Laboratory studies have shown that *C. raciborskii* has low optimal light requirements for growth (Shafik et al., 2001; Briand et al., 2004, Dyble et al., 2006). O'Brien et al. (2009) also found primary productivity was higher at low light levels in a *C. raciborskii*-dominated reservoir. These experiments involved incubating water samples in a range of fluctuating light conditions and demonstrated why deep mixing favours *C. raciborskii*. Therefore, when the mixing depth exceeds the euphotic depth,

cells will be dark-acclimated. This will increase their rate of production when they are circulated through the euphotic zone. This is consistent with studies of buoyancy of *C. raciborskii*, which have shown that this species is not positively buoyant, but they have very low rates of sinking (Kehoe, 2010).

Artificial mixing

Artificial destratifiers mix surface waters into deeper waters, breaking down thermal stratification and reducing light availability for algal growth. Several studies have used artificial destratification to reduce populations of potentially toxic species, including *Microcystis* (e.g. Visser et al., 1996; Lindenschmidt, 1999).

North Pine Reservoir, a drinking water reservoir in southeast Queensland has had severe summer blooms of *C. raciborskii* since the 1980s with CYN frequently being detected. An analysis of this data in the mid 1990s suggested that *C. raciborskii* blooms dominated during of low rainfall, and when the water column was more stable (Harris and Baxter, 1996). It was concluded that vertical stratification was likely to provide the competitive advantage for *C. raciborskii* over other algal species. The installation of a destratification unit in North Pine Reservoir in the mid 1990s designed to reduce stratification and sediment remineralisation of nutrients, and hence reduce *C. raciborskii* dominance, had the opposite effect. However, blooms of *C. raciborskii* now commence earlier and are more sustained (Antenucci et al., 2005; Burford and O'Donohue, 2006).

Antenucci et al. (2005) suggested that the dominance of *C. raciborskii* in North Pine Reservoir prior to installation of the destratification unit was due to its ability to compete for DIP, and increased dominance post-destratification due to the ability to compete for light. Another study which compared North Pine Reservoir with two adjacent naturally mixed reservoirs suggested that the dominance of *C. raciborskii* post-destratification was also linked to a superior DIP scavenging ability (Burford and O'Donohue, 2006). However, the research of O'Brien et al. (2009) provides the mechanism for why artificial destratifiers have been ineffective at controlling this species, i.e. dark adaptation via mixing increases primary production during periods of exposure to light in a species adapted to low light levels.

Temperature effects

Historically, *C. raciborskii* was considered a tropical and subtropical species. McGregor et al. (2000) in a study of subtropical and tropical reservoirs in Australia found the species in highest abundance and with year-round presence in the tropics. In the subtropics, *C. raciborskii* dominates in the summer months (Bouvy et al., 2000; McGregor et al., 2000; Burford and O'Donohue, 2006; Burford et al., 2007). However, in more recent years it appears to have expanded its range into more temperate areas around the world.

The growth response of *C. raciborskii* to temperature has been examined using multiple strains isolated from both temperate and tropical areas (Briand et al., 2004; Chonudomkul et al., 2004). Net growth was found in a temperature range from 20 to 35°C, with maximum growth at 30°C. The broad temperature tolerance may explain the capacity of *C. raciborskii* to invade temperate areas of the world (e.g. Chapman and Schelske, 1997; Fastner et al., 2003; Briand et al., 2004; Hong et al., 2006; Messineo et al., 2010). Studies have also found that *C. raciborskii* dominates in temperate systems at higher temperatures (Hamilton et al., 2005; Conroy et al., 2007).

It has been proposed that cyanobacteria will increasingly dominate freshwater systems due to global warming (Padisák, 1997; Paerl and Huisman, 2008). Indeed, Wiedner et al. (2007) has shown that growth initiation of *C. raciborskii* is controlled by temperature in German lakes and has proposed that the invasion of *C. raciborskii* into these lakes is the result of global climate change.

Nutrients

C. raciborskii is a diazotroph which allows this species to fix N when DIN sources are depleted. However, is low DIN a requirement for *C. raciborskii* to dominate? A microcosm experiment was conducted to determine competition between *C. raciborskii* and another diazotroph, *Anabaena* spp. under a range of DIN concentrations (Moisander et al., 2008). This study found that *C. raciborskii* was a stronger competitor than *Anabaena* when DIN was present. Studies have shown that DIN uptake rates were higher than N fixation rates for *C. raciborskii*-dominated waters when DIN was available (Présing et al., 1996; Burford et al., 2006).

Energetically, DIN is a more efficient source for growth of *C. raciborskii* than gaseous N. Therefore it is not surprising that DIN is preferentially used, when available. Laboratory studies have confirmed that growth rates were fastest when N was supplied as ammonia, followed by nitrate, followed by urea (Saker et al., 1999; Hawkins et al., 2001; Saker and Neilan, 2001). Spröber et al. (2003) proposed that N fixation was activated depending on the N content of the cells. Therefore it is likely that *C. raciborskii* has a flexible strategy with respect to N: namely when DIN concentrations are sufficient, this source is used, and during short-term periods of depletion, N fixation can be deployed.

Phosphorus (P) also appears to play an important role in the dominance of *C. raciborskii*. It dominates in reservoirs and lakes when phosphate concentrations are below detection limits (Padisák and Istvánovics, 1997; Burford and O'Donohue, 2006). Istvánovics et al. (2000) showed that a cultured European strain of *C. raciborskii* has a high P affinity and storage capacity, and suggests that the species is opportunistic with regard to DIP. Therefore *C. raciborskii* dominance may be related to rapid DIP uptake in natural environments (Padisák, 1997; Shafik et al., 2001). In laboratory cultures, *C. raciborskii* also has the capacity to utilise DOP, giving it an advantage over eukaryotes in low DIP environments (Posselt, 2010). Additions of DIP in lake bioassay experiments dominated by *C. raciborskii*, resulted in increased dominance of this species compared with the rest of the algal community (Posselt et al., 2009). In contrast, the addition of N and P did not promote dominance of *C. raciborskii*. In contrast, Quiblier et al. (2008) found that P and N+P preferentially promoted *C. raciborskii*. Recent research in laboratory culture experiments has shown that *C. raciborskii* grows faster under P limitation when there is sufficient supply of DIN (Kenesi et al., 2009). They suggest that this is because P requirements for heterocyte production are higher during DIN limitation. The relationship between P and N utilisation, and *C. raciborskii* dominance is, therefore, not straightforward.

Cylindrospermopsin (CYN) production by *C. raciborskii*

CYN is a potent hepatotoxin produced by cyanobacterial species of the orders Oscillatoriales (*Lyngbya wollei*; Seifert et al., 2007) and Nostocales which includes *Aphanizomenon ovalisporum* (Banker et al., 1997), *Aphanizomenon flos-aquae*

(Preußel et al., 2006), *Umezakia natans* (Harada et al., 1994), *Raphidiopsis curvata* (Li et al., 2001a), and *Cylindrospermopsis raciborskii* (Hawkins et al., 1985; Ohtani et al., 1992). CYN is an alkaloid hepatotoxin (Runnegar et al., 1994; 1995; 2002) which also has neurotoxic effects (Kiss et al., 2002) and could potentially be carcinogenic (Humpage et al., 2000). Although, *C. raciborskii* can be found on almost every continent, the ability to produce CYN is not universal. Lagos et al. (1999) found that Brazilian *C. raciborskii* strains do not produce CYN although some strains do produce the neurotoxin, saxitoxin. Also, previous studies have found that European and Asian *C. raciborskii* strains can be toxic to mice but do not contain any of the known cyanotoxins (Fastner et al., 2003; Saker et al., 2003). Furthermore, no North American strains have been found to produce CYN or contain the necessary genes to do so (Neilan et al., 2003; Kellmann et al., 2006; Yilmaz et al., 2008). To date, only Australian (Hawkins et al., 1985; Ohtani et al., 1992), New Zealand (Wood and Stirling, 2003) and some Asian (Li et al., 2001b; Chonudomkul et al., 2004) strains of *C. raciborskii* have been found to produce CYN.

In order to produce CYN, strains of *C. raciborskii* must contain the CYN biosynthesis gene cluster (*cyrA* – *cyrO*; Mihali et al. 2008). The CYN biosynthesis gene cluster spans 43 kb and 15 open reading frames (Mihali et al., 2008). Like other cyanotoxins, such as microcystin (Tillet et al., 2000), nodularian (Moffitt and Neilan, 2004), and jamaicamides (Edwards et al., 2004), CYN is synthesized by a complex series of non-ribosomal peptide synthetases (NRPS), polyketide synthetases (PKS) and mixed NRPS/PKS systems (Mihali et al., 2008). Australian strains of *C. raciborskii* produce the CYN and the CYN analogue, deoxy-cylindrospermopsin (Norris et al., 1999).

Very few studies have investigated the environmental factors that promote the production of CYN by *C. raciborskii*. To date, only the effects of temperature (Saker and Griffiths, 2000), N (Saker et al., 1999; Hawkins et al., 2001; Saker and Neilan, 2001), and light (Saker, 2000; Dyble et al., 2006) on CYN production in laboratory experiments have been investigated. Temperature has been found to play a key role in the production of CYN. However, there seems to be a disconnect between optimal growth temperature and optimal CYN production temperature for *C. raciborskii*. Saker and Griffiths (2000) found that while CYN production was highest at 20°C, optimal growth temperatures were between 25 and 30°C, a temperature range similar

to other cyanobacteria (Robarts and Zohary, 1987). Saker and Griffiths (2000) found that there was a negative correlation between temperature and CYN production between 20 and 35°C. Furthermore, they found that CYN-producing strains ceased production of CYN at 35°C although these strains still grew well at this temperature. Cells restarted CYN production if incubated at lower temperatures. The results of the aforementioned suggest that there is not a linear correlation between growth and CYN production with respect to temperature. The implications of this is that although maximum *C. raciborskii* growth rates are at higher temperatures (25 - 35°C; Saker and Griffiths, 2000; Briand et al., 2004) potentially resulting in high densities of *C. raciborskii*, these blooms may have low toxicity. Since many studies examining the role of environmental factors on growth and toxin production are conducted in the range of temperatures for maximum growth, rather than maximum toxin production, this may confound interpretation of the key factors driving toxin production.

Light is another environmental factor that has been found to affect CYN production. Dyble et al. (2006) studied a range of light intensities between 18 and 140 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$, a range similar to that found at depths where *C. raciborskii* grows well (Havens et al., 1998; Bouvy et al., 1999; O'Brien et al., 2009). This study found that there was a positive relationship between light intensity and the production of CYN, with highest CYN production ($192 \mu\text{g CYN L}^{-1} \text{ d}^{-1}$) occurring at the highest light intensity. This finding may be important as, typically, the highest light intensities occur during the summer months when *C. raciborskii* is abundant (Fabbro and Duivenvoorden, 1996; Bouvy et al., 2000; Mc Gregor and Fabbro, 2000; Hamilton et al., 2005; Weidner et al., 2007; Figueredo and Giani, 2009). However, it should be acknowledged that the effect of higher light levels, such as those in outdoor conditions, is unknown. Additionally, little is known of the interactions between higher temperatures and higher light intensities, and their impact on the CYN production rate. Dyble et al. (2006) incubated at 25°C which is within the range of optimal growth temperatures for *C. raciborskii* but not at optimal CYN production temperatures, according to Saker and Griffiths (2000). Preußel et al. (2009) investigated the interactive effects of light and temperature on CYN production on two temperate strains of the cyanobacterium, *Aphanizomenon flos-aquae*. Their study

found that the interaction of light and temperature had very little impact on CYN production and hypothesized that this could be because strains were isolated from temperate, rather than tropical lakes. Therefore, further work is needed to elucidate any potential interactive effects of these parameters on CYN production by *C. raciborskii*.

Surprisingly, very little is known about the effect of nutrients (N, P) on CYN production. Saker et al. (1999) and Saker and Neilan (2001) investigated the impact of different sources of DIN on CYN production by Australian isolates of *C. raciborskii*. Interestingly, these studies found that the highest intracellular CYN concentrations (reported as % of freeze-dried weight) were found in the cultures that were devoid of a fixed form of N and lowest in cultures grown with saturating concentrations of ammonium. These results were similar to the results found by Saker (2000) who found the highest CYN content in cells grown in DIN-free media (up to 0.84% of cellular dry weight). This contrasts with the findings for growth, i.e. highest growth rates occurred in the cultures grown with NH_4^+ and the lowest growth rates were found in the treatments lacking a fixed N source (Saker et al., 1999; Saker and Neilan, 2001).

Mihali et al. (2008) hypothesized that the reason for the increased CYN concentrations in the absence of fixed N was potentially due to the placement of the CYN biosynthesis gene cluster in the *C. raciborskii* genome. This cluster is flanked by the *hyp* gene homologs that are associated with the maturation of hydrogenases. As the *hyp* gene cluster in another cyanobacterium, *Nostoc* spp., is under the regulation of the global N regulator (*NtcA*; activates the transcription of the N assimilation genes) that it is plausible that the *hyp* genes and, therefore, the CYN biosynthesis gene cluster are under the same regulation in *C. raciborskii*. Other studies have found that CYN concentrations have been positively correlated with both total N and total P concentrations (Wiedner et al., 2008). Furthermore, recent research has suggested that CYN production rates are positively correlated to *C. raciborskii* growth rates in P-limited cultures during exponential growth (P. Orr pers. comm.). To date, however, no studies have investigated the individual species of N

(nitrate, ammonia and urea) or the potential interactive effects of N and P on CYN production rates in *C. raciborskii*.

Another complicating factor in the study of CYN production by *C. raciborskii* is the issue of active export of this compound. Previous studies have found that up to 80% of total CYN concentrations can be in the dissolved pool (Saker and Griffiths, 2000; Hawkins et al., 2001; Dyble et al., 2006; Preußel et al., 2009), although similarly to other toxin-producing cyanobacteria (*Microcystis*; Orr and Jones, 1998) greater than 80% of CYN is kept within the cell during exponential growth (Saker and Griffiths, 2000; Hawkins et al., 2001). It seems unlikely that *C. raciborskii* actively exports CYN as this compound is energy-intensive to synthesize. CYN is N-rich compound (5 atoms N per molecule). Toxic *C. raciborskii* strains will also have additional N requirements associated with the enzymes involved in the synthesis of microcystin (Mihali et al., 2008). Furthermore, although the CYN molecule does not contain P, it is indirectly linked to CYN production as the DNA and RNA required for this process require P. Therefore, as this molecule requires a significant nutrient sources and energy to synthesize it seems unlikely that it would be actively exported by *C. raciborskii*. However, more studies need to be conducted to further understand if the CYN found in the dissolved pool is a result of cell lysis, cell leakage, or active transport in aged or stressed *C. raciborskii* cells.

CYN quotas can vary between strains, further adding to the complexity of investigating CYN production. Saker and Griffiths (2000) found that there was a four order of magnitude difference between the most toxic and least toxic isolates of *C. raciborskii*. Orr et al. (2009) found that spatially and temporally diverse natural populations of potentially toxic *C. raciborskii* differed in their CYN quotas by up to an order of magnitude. Furthermore, this study also found that CYN quotas could be as high as 60 fg cell⁻¹, and other studies have shown that CYN content could be as high as 0.8% of total cellular dry weight in some strains (Saker, 2000). These differences highlight the fundamental question: Why do different strains yield varying CYN quotas? Does producing less CYN allow for these strains to dominate under certain conditions that would not be suitable for strains with higher CYN quotas? Are the different CYN production rates simply a function of varying growth rates between strains? Clearly, more research is needed to answer these questions.

The characterization of the CYN biosynthesis pathway (Schembri et al., 2001; Mihali et al., 2008) and subsequent development of *C. raciborskii*-specific real-time PCR assays (Rasmussen et al. 2008) has allowed for field studies to distinguish between co-occurring CYN-producing and non-CYN producing strains of *C. raciborskii* (Orr et al., 2010). This is important as CYN-producing and non-CYN-producing strains of *C. raciborskii* are indistinguishable using traditional light microscopy. Therefore, results of traditional field experiments are limited to broad generalizations regarding the total *C. raciborskii* community although CYN-producing and non-CYN-producing strains could react differently to various environmental conditions. This molecular quantification technique has been used in multiple cyanobacterial field studies to date (e.g. Rinta-Kanto et al., 2005; Rinta-Kanto and Wilhelm, 2006; Oberholster et al., 2006; Hotto et al., 2008; Davis et al., 2009; Ha et al., 2009; Rinta-Kanto et al., 2009a, b; Ye et al., 2009; Moisander et al., 2009a, b; Baxa et al., 2010; Orr et al. 2010). Furthermore, this technique is becoming increasingly common and will allow for future studies to investigate the response of CYN-producing versus non-CYN-producing strains of *C. raciborskii* to varying environmental conditions leading to a more detailed understanding of how CYN production by genetically-capable strains will vary during field and laboratory experiments.

Finally, the interactions between potential environmental controls will be extremely important in understanding the production of CYN by *C. raciborskii*. All studies to date have focused on one variable, which has provided valuable insights into the impacts these single variables have on CYN production, however, as Vézé et al. (2001) demonstrated, studies investigating the interactions between variables (such as N and P) also valuable as cells would be subject to these types of interactions in the natural environment. Their study found that high concentrations of N and P favoured the growth and toxin production of microcystin-producing strains of *Microcystis* over their non-microcystin-producing counterparts. Furthermore, Davis et al. (2009) found that the additive effects of increased temperature and increased P concentrations yielded the highest growth rate for potentially microcystin-producing strains of *Microcystis* of any populations monitored in 75% of the systems studied. These examples highlight the importance of conducting factorial experiments with multiple variables in order to gain a better understanding of how future environmental changes

(i.e. increased temperature and/or eutrophication) will impact the production of CYN by genetically-capable strains of *C. raciborskii* and, ultimately, the toxicity of future bloom events.

Conclusions

In conclusion, research in recent years has established that key reasons for the dominance of the cyanobacterium *C. raciborskii* are low light requirements, close to a neutral buoyancy, a wide temperature tolerance, flexible strategy with respect to N and a high uptake affinity and storage of P. Optimal growth occurs in a different suite of light, temperature and nutrient conditions to toxin production. Additionally, there is considerable variability in the response between strains. However, an improved understanding of the factors promoting growth and toxin production will lead to improved predictive models of blooms.

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