Comparative genomics and toxin regulation in the cyanobacterium *Cylindrospermopsis raciborskii*

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Nucleotide sequence based analyses exhibited strain clustering as per geographic location. Toxigenicity and taxonomic identity were not found to impact strain divergence. We report for the first time a varying spatial arrangement of the *cyr* biosynthesis gene cluster isolated from Chinese strains, compared to Australian strains. We, therefore hypothesize, that the arrangement of this cluster is influenced by the geographic location of isolates and not by overall strain relatedness as considered earlier.

Additionally, we examined the effect of nitrogen (N) and phosphorus (P) on growth and ecotype-related changes in production of cylindrospermopsins by *C. raciborskii*. Significantly higher growth rates were observed, only when N and P were co-supplied. In contrast, toxin cell quotas increased significantly in treatments irrespective of whether N was supplied. These increased toxin quotas correlated with an increase in the proportion of *cyrA* to 16S genes in the *C. raciborskii* population, therefore, attributing changes in ecotype dominance as the most likely factor driving changes in toxin production between treatments.

Finally, studies to gauge the changes in the transcriptional regulation of the *cyr* gene cluster under different intensities of light, low (10 µE) and high (100 µE) and pCO_2, 500 ppm (L-CO_2) and 1300 ppm (H-CO_2), revealed that cylindrospermopsin production is constitutive and is not affected by the physiological parameters of light intensity and pCO_2.

Overall, this thesis highlights the influence of environmental drivers and location, on the divergence of *C. raciborskii,* thus, cautions water managers against the rampant threat of this cyanobacterium to water bodies worldwide.

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Abbreviations

AAA	ATPases associated with diverse activities
BWA	Burrows-Wheeler Aligner
CYN	Cylindrospermopsins
DIN	Dissolved inorganic nitrogen
dCYN	7-deoxy-cylindrospermopsin
HABs	Harmful algal blooms
IAD	Innovation amplification divergence
IMG	Integrated microbial genomes
JM	Jaworski medium
NRPS	Non-ribosomal peptide synthetase
P	Phosphorus
PKS	Polyketide synthase
QPCR	Quantitative real-time PCR
Q_{CYNS}	Cell quotas of CYNs
RAST	Rapid annotations subsystems technology
RAMP	Random associated mysterious protein
STX	Saxitoxins
SNPs	Single nucleotide polymorphisms
TN	Total nitrogen
TP	Total phosphorus
U	Urea
VBPOs	Vanadium dependent bromopeptidases

List of Publications

Sinha, R., L. A. Pearson, T. W. Davis, M. A. Burford, P. T. Orr, and B. A. Neilan. 2012. Increased incidence of *Cylindrospermopsis raciborskii* in temperate zones, Is climate change responsible? Water Research 46:1408-1419.

Sinha, R., L. A. Pearson, T. W. Davis, M. A. Burford, and B. A. Neilan. 2014, Comparative genomics of cylindrospermopsin producing *Cylindrospermopsis raciborskii* strains. BMC Genomics 15(83):1471-2164.

Burford, M. A., T. W. Davis, P. T. Orr, R. Sinha, A. Willis, B. A. Neilan. 2014. Nutrient related changes in ecotype dominance drives toxicity of field blooms of the cyanobacterium, *Cylindrospermopsis raciborskii,* Accepted, FEMS microbiology, ecology.

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Chapter 1

Introduction

1.1 Cyanobacteria

Cyanobacteria, also referred to as 'blue-green algae' are gram negative, oxygenic phototrophs that are considered to be progenitors of the higher plant plastids. Cyanobacteria have inhabited Earth for over 2.8 billion years [160] and thrive in a wide range of environments, including fresh and marine waters, hot springs [133], hyersaline ponds [144] and polar deserts [188], to name a few.

Cyanobacteria may be unicellular, colonial or filamentous. They have been classified into five distinct categories based on their morphology, where by, sections 1 and 2 (Chroococcales and Pleurocapsales) are unicellular, while sections 3 to 5 (Oscillatoriales, Nostocales and Stigonematales) are filamentous [146]. Sections 4 and 5 also produce specialised cells responsible for nitrogen fixation referred to as heterocysts. Heterocysts are formed when a thick polysaccharide layer is produced, which isolates the cell from external oxygen thereby activating the enzymatic nitrogenase complex. This mechanism is responsible for the conversion of atmospheric nitrogen into ammonium, the nitrogen source readily consumed by cyanobacteria [101]. Further, some cyanobacterial species produce akinites or spore like cells, which help them survive unfavourable conditions. Some cyanobacterial species also produce motile filaments called hormogonia, which travel away from the main cyanobacterial biomass to bud and form new colonies.

1.2 Harmful algal blooms and cyanotoxins

Cyanobacteria can also proliferate into blooms commonly known as harmful algal blooms or HABs. HABs significantly impact water quality by the formation of surface scums, water discolouration and an unpleasant odour. Some of these bloom-forming species also produce potent cyanotoxins that can have adverse health effects on plants, animals and humans [25]. Detrimental effects on humans range from mild skin and eye irritants to severe effects such as respiratory or digestive tract problems, cancers and death, to mention a few. HABs also lead to the mass losses of aquatic life [135]. On the economic front, losses in tourism, health and fisheries have been estimated to over $ 46 million, and are a grave concern [62].

Cyanotoxins are produced by over 40 cyanobacterial species in approximately 20 genera [120]. These cyanotoxins are thought to be secondary metabolites [120], and their function to the producing organism is unknown. Cyanotoxins are usually classified by their mechanism of activity; microcystins and nodularins are cyclic hepatotoxic peptides [25] and cylindrospermopsins (CYN) and Anatoxin-a[s] are cytotoxic alkaloids [67, 124]. Saxitoxins (Stx) are a neurotoxic organophosphate [158]. Finally, lyngbyatoxins are dermatoxins [129]. Biosynthesis pathways responsible for toxin production have been elucidated for most of these toxins, and they emphasize the fact that each toxin gene cluster is constituted of similar genes [104, 184, 77].

1.3 *Cylindrospermopsis raciborskii*

C. raciborskii is an invasive, oxygenic phototroph, which belongs to the order 4 (Nostocales) of the cyanobacterial classification system [146]. It is a filamentous diazotroph, with terminal heterocysts, which help it fix dinitrogen in the atmostphere, to a more readily usable nitrogen source, ammonium. Trichomes of *C. raciborskii* exhibit varying morphologies curved, coiled or straight. The trichomes are hightly variable in length, exhibiting lengths ranging from 10 to 120 um [21]. This cyanobacterium is found in water bodies worldwide.

1.3.1 Occurrence and geographical distribution

C. raciborskii is found in many tropical/subtropical regions of the world including northern Australia [54, 99, 153], Thailand [90], South America [44] and Africa [51, 105]. However, the scientific evidence for its occurrence in temperate regions is more recent [20, 111]. Observations of *C. raciborskii* in temperate climatic zones have increased over the past two decades (Figure 1.1; [21, 37, 53]. From 1950 to 1990, there were reports *of C. raciborskii* from 10 different temperate locations worldwide. These regions included the European countries, Slovakia [64], Spain [96], Hungary [92, 125], Greece [95] and Germany [84]; the North American states: Kansas [140], Texas [177] and Minnesota [60]; Cuba [80] as well the Australian states of New South Wales and Queensland [136] (Figure 1.1a). In the past two decades, *C. raciborskii* blooms have been shown to be persistent throughout Europe, including Hungary [122], Greece [95] and Germany [51], while new reports have come from France [49], Portugal [122], Austria [36], Serbia [156], Poland [79], and Italy [103]. In North America, *C. raciborskii* blooms have been reported from Florida [198], Michigan [63], Indiana [74] as well as Canada [53] and Mexico [49].

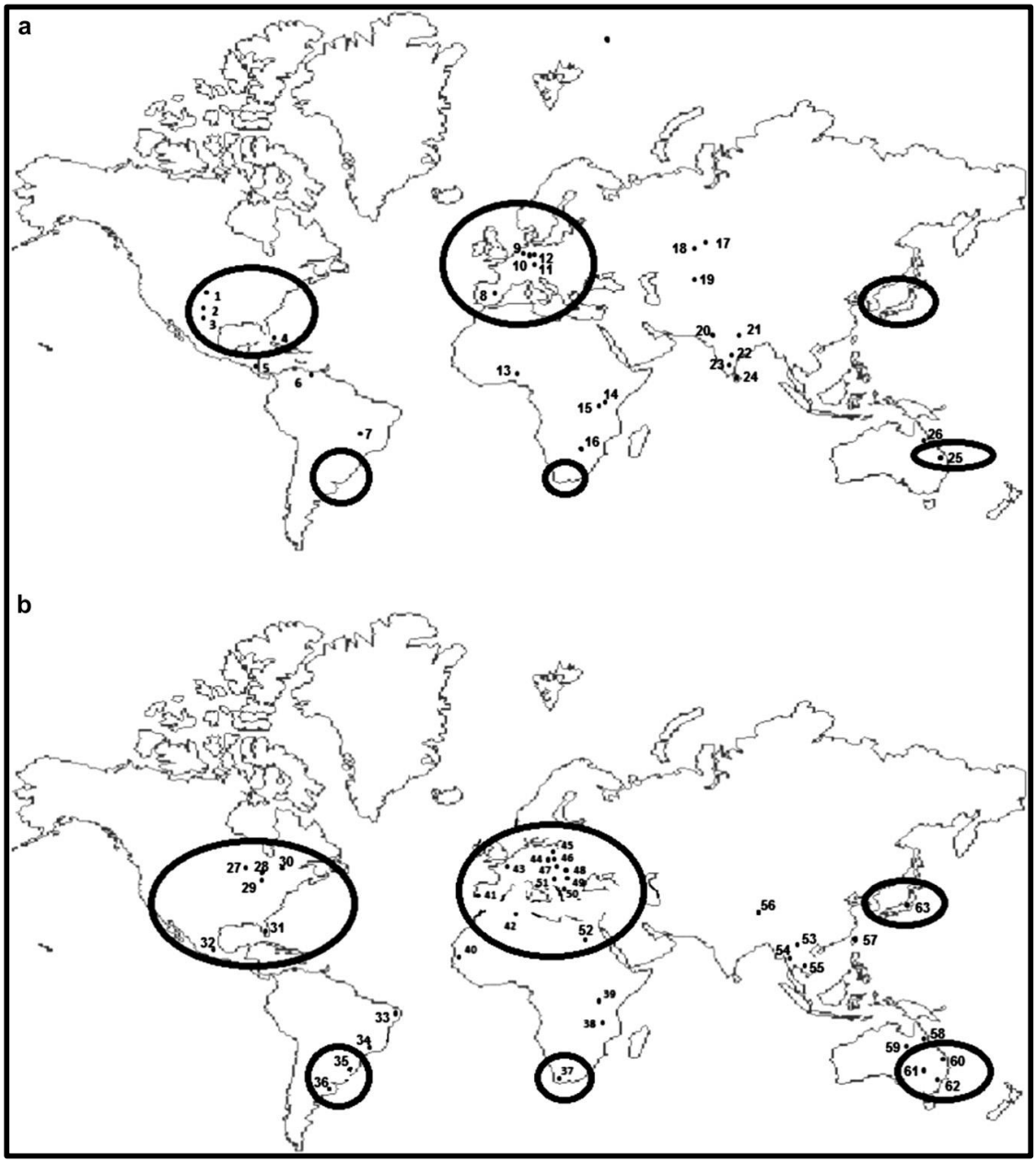

Figure 1.1 Each point represents a country or state of occurrence. (a) Occurrence of *C. raciborskii* from 1950 to 1990. (b) Occurrence of *C. raciborskii* from 1990 to 2010. Circled areas indicate temperate climatic zones where *C. raciborskii* had been found to occur. Information regarding precise geographical location, the first reported observation of a *C. raciborskii* bloom and the corresponding references are provided in Supplementary Table 1.1

Blooms of this cyanobacterium are still prevalent in Australia [151, 172], and have recently been detected in Japan [28], and Algeria [16], Figure 1.1b. The increasing number of reports of *C. raciborskii* in temperate regions of the world suggests that the organism could be expanding its geographical range. However, whether this is due to increased monitoring or true invasiveness of the species is a complex question.

While increased interest in this organism caused by the characterization of cylindrospermopsin by Ohtani et al., in 1992, could have resulted in increased monitoring and detection, global warming [155] could also have played a key role in driving the spread of *C. raciborskii.* Studies have demonstrated a link between increasing temperatures and growth initiation of *C. raciborskii* [192]. *C. raciborskii* was also found to be tolerant to a range of nitrogen and phosphorus conditions [150, 186]. Here we put forward the assertion that the robust and opportunistic nature of *C. raciborskii* has enabled this harmful cyanobacterium to capitalise on climatic changes, further explaining its potential to survive, and in some cases, thrive, in these novel environments.

1.3.2 Invasiveness of *C. raciborskii*

Several theories have been proffered for the invasiveness of *C. raciborskii.* Padisak [130] attributed the spread from tropical/subtropical zones to temperate zones to two main evolutionary centres. *C. raciborskii* acquired several adaptation mechanisms at these evolutionary centres that have aided its survival outside its original native range, thereby giving it a competitive edge over other phytoplankton species. These physiological adaptations include akinete formation and a tolerance to low phosphorus and nitrogen availability. This combination of physiological adaptations together aids survival in unfavourable conditions. African lakes have been suggested as the primary geographic origin for the evolution of *C. raciborskii* [130]. This is attributed to the occurrence of the highest diversity of strains within the species at this location. Thus, it was suggested that *C. raciborskii* adapted to the lower nitrogen and phosphorus conditions in African lakes and then dispersed to tropical regions of Central America and Indonesia [130]. Australia has been suggested as the secondary centre for the evolution of *C. raciborskii* [130]. Many rivers in Australia exhibit a

fluctuation in flow that results in periodic drying and hence salinity flux [191]. Thus, Padisak [130] hypothesized that the salinity tolerance of *C. raciborskii* evolved in this environment.

Importantly, there appears to be a correlation between the geographical distribution of *C. raciborskii* strains and the toxins they produce. For example, Australian [55, 124] and New Zealand [194] strains produce cylindrospermopsin, while strains occurring in South America produce saxitoxins [85]. In contrast, no strains of *C. raciborskii* isolated from Europe and North America have been found to produce cylindrospermopsin to date [42, 77, 122, 198]. It is likely that different selective pressures in these diverse environments directed these tertiary centres of evolution of *C. raciborskii* 'toxitypes'.Anthropogenic factors, such as dispersal through the ballast of ships, or inappropriate transfer of scientific samples, and dispersal by birds have also been suggested as potential mechanisms contributing to the spread of *C. raciborskii* [111, 130]. However, Gugger et al. [49] proffered an alternative hypothesis for the current geographical distribution of this cyanobacterium. They suggested that extinction of *C. raciborskii* occurred during the pleistocene in most geographical locations, and advocated its survival only in warmer climatic conditions on each continent, excluding Antarctica [50]. Increasing temperatures within the temperate zones has since allowed recolonisation and favoured further spread of the species on the European and the American continents.

1.3.3 Nutrient availability as a selection for invasiveness and dominance

Phosphorus (P) is an important driver in the dominance of *C. raciborskii*. This cyanobacterium has been found to dominate phytoplankton communities when dissolved inorganic phosphorus concentrations are below detectable limits [23, 186].

This is due to a higher phosphate uptake capacity and a higher phosphorus affinity compared to other cyanobacteria [69, 186]. *C. raciborskii* can regulate its uptake of, and bio accumulate, inorganic P and has therefore been termed a 'phosphorus opportunist' [186]. Consistent with this, Padisak and Reynolds [131] found that a decrease in the P load at Lake Balaton, Hungary caused a decrease in the abundance of other nitrogen fixing cyanobacteria but there was no change in *C. raciborskii* cell concentrations [131]. Additionally, Branco and Senna [18] observed that bioaccumulation of inorganic P by *C. raciborskii* was linked to its occurrence in the Paranoa Reservoir in Brazil. Furthermore, because of the rapid rate of P uptake by *C. raciborskii*, it can outcompete other phytoplankton during pulses of P-rich water originating from sediments. For example, blooms of *C. raciborskii* were observed during the summer months of 1982, 1992 and 1994 on the eastern side of Lake Balaton, where the concentration of phosphorus was below detectable levels, however, phosphorus content in sediments was elevated [131]. Field experiments conducted in subtropical reservoirs in Queensland, Australia showed that addition of dissolved inorganic phosphate (DIP) to algal communities dominated by *C. raciborskii*, led to a greater dominance of the species compared to other cyanobacteria [139]. Furthermore, laboratory studies found that *C. raciborskii* could also utilise organic sources of P, further expanding its flexibility in P uptake and utilisation. Overall, these studies show that *C. raciborskii* can tolerate a wider range of P concentrations compared to other phytoplankton. This diverse P tolerance could facilitate survival and growth during future climate shifts that are likely to increase storm events increasing nutrient loads into systems but also during times of drought when P inputs could potentially be lower.

Nitrogen also plays an important role in the growth of, and toxin production in

cyanobacteria [57, 127]. Due to the energy demands in reducing NO3 and NO2 to NH4, the highest growth rate in *C. raciborskii* was found in the presence of NH4 [153], the preferred source of dissolved inorganic nitrogen (DIN) [58, 115]. Additionally, the highest intracellular toxin levels were found when DIN sources were depleted [152, 154]. The appearance of heterocysts within the trichome in response to nitrogen limitation is an indicator of nitrogen fixation [123]. Studies prior to the early 1990's showed that nitrogen limitation resulted in the growth and dominance of *C. raciborskii* in tropical/subtropical lakes, including Lake Malawi, eastern Africa [134], Lake Samsonvale, Queensland Australia [54] and Lake Victoria, Uganda [82]. Other researchers [23, 107] have also shown that the rate of uptake of DIN was greater than the nitrogen fixation rate in the presence of DIN. Burford et al.,[23] postulated that the flexible strategy of using DIN when available, and switching to energetically expensive nitrogen fixation when DIN is limiting, gives this species a competitive advantage. Moisander et al., 2008 [107] also showed that in a mixed community of *C. raciborskii* and *Anabaena* spp., *C. raciborskii* was able to outcompete Anabaena spp. by maintaining a positive growth rate when DIN was present. This result has also been found in other field and laboratory based studies [21, 78]. Even though the relationship between different nutrients is complex, these studies indicate that *C. raciborskii* can proliferate in a wide range of nitrogen conditions and can outcompete other cyanobacteria that typically co-occur which has the potential to further its prevalence in aquatic systems that will ultimately be subject higher nutrient loads during future climatic conditions.

1.3.4 Implications of global warming for *C. raciborskii*

Two studies found that *C. raciborskii* thrived between temperatures of 20 to 35 °C [20, 28]. This tolerance to broad temperatures would readily support the survival of *C.*

raciborskii in the temperate zones [63]. Of particular note, *C. raciborskii* has been found in temperate regions during the summer months, when the temperatures were at their highest [53]. Records also show that large bloom populations occur permanently in warm waters, such as those found in Paranoa Lake, Brazil [18]. McGregor and Fabbro [99], showed that *C. raciborskii* persisted in tropical areas all year round, while it was limited to the summer months in the subtropical areas [17, 22, 24]. Finally, it is also possible that *C. raciborskii* is adapting to lower temperatures, thus increasing its temperature tolerance range, and has been found to proliferate between 15 and 18 °C in Alte Donau, Austria [36]. However, this has been the lowest temperature ever recorded for *C. raciborskii* growth.

C. raciborskii also forms spore-like cells (akinetes) which can be dispersed to temperate climatic zones [108 2005, 109 2005, 110 2005, 130] and only germinate in favourable temperatures, [131]. In temperate regions globally, shallow lakes can reach temperatures of 22 °C during the summer months, which would support the germination of *C. raciborskii* akinetes [130]. Padisak and Reynolds [131] found that blooms of *C. raciborskii* had occurred in the summer months of 1982, 1992 and 1994 in Lake Balaton, Hungary, when the average 30 year temperature showed an increase of 2 °C. Hamilton et al. [53] found that blooms of *C. raciborskii* did not occur in Lake Constance, when the water temperatures were recorded to be less than 22 °C. Likewise, blooms of *C. raciborskii* observed in Mona Lake, Michigan in the months of August and September, occurred when a significant increase was recorded in surface and bottom temperatures [63]. Surface temperatures were recorded at 25.7 °C and 23.7 °C for the years 2002 and 2003, while bottom temperatures were recorded at 21.7 °C and 21.8 °C, respectively. Hong et al. [63] thus, hypothesized that the absence of *C. raciborskii* from lake Mona during spring and early summer, was due to unsuitable

water temperature. They observed that bloom formation, as reported by Hamilton et al. [53], occurred only when the bottom temperatures approached 22 °C. Similarly, a study by Mohamed [105], reported blooms of *C. raciborskii* in Egyptian waters during the summer months. Findings from this study suggest that these recent blooms occurred partially as a result of climate change, when the bottom temperatures reached significantly higher temperatures than are usually recorded for their system. This in turn, facilitated the germination of akinetes. This study also stated that regular monitoring showed the absence of *C. raciborskii* blooms prior to April 2002, when the water temperatures were relatively lower.

Furthermore, principle component analysis by Briand et al., in 2002 showed that climatic parameters such as air and water temperatures were responsible for the growth and proliferation of *C. raciborskii* blooms. Temperatures between 21 °C and 26 °C were reached in France, in July 1998 and August 1999, when trichome concentrations of 2.0 **x** 10^6 trichomes L^{-1} were observed. The decrease in water temperatures from 23 °C to a low 14 °C decreased trichome concentrations by an order of magnitude (1.8 **x** 10^5 trichomes L^{-1}). Finally, the appearance of *C. raciborskii* in Florida lakes coincided with increases in water temperatures above 22 °C [26]. The authors also showed that the temperatures of the lakes reported in their study were higher than those reported from the same systems during the 1960s [26].

Although *C. raciborskii* blooms are known to have occurred at temperatures below 20 °C in some locations, it is rare and the density of the blooms are low [36]. A study by Saker and Griffiths [152] showed that a maximum growth rate of 1.2/day was achieved at temperatures above 25 °C, while growth rate varied between 0.3 and 0.5/day for temperatures around 20 °C. Overall, the recent expansion of *C. raciborskii* could be partially attributed to a number of factors which include increasing temperatures. The

current spread of *C. raciborskii* may also be due to its ability to form akinetes. These specialised cells can remain dormant for long periods of time and thus as systems begin to warm, akinetes will germinate and since *C. raciborskii* is a strong competitor for both N and P this will allow the establishment of this cyanobacterium in systems previously devoid of it.

1.4 Cylindrospermopsin

CYN is a cytotoxin that was responsible for the poisoning of 149 people in Palm Island, Australia, in 1971 [48, 56]. Its occurrence has also been associated with cattle mortality in Queensland [150]. Exposure to CYN leads to the inhibition of protein synthesis [46]. Further, it causes the interruption of biosynthetic processes leading to the formation of glutathione [149]. Additionally, the purine nature of CYN is thought to cause irreversible damage to human DNA on exposure, and lead to cancer [162].

Structurally, CYN is a polycyclic uracil derivative with a functional guanidine group. It was characterised by Ohtani et al., in 1992 and contains a hydroxyl group on the uracil bridge at C7 and a pyrimidine ring [175], which may be responsible for the toxicity of the compound [48]. Two analogues of CYN have been reported to date, the first, 7-epicylindrospermopsin, that produce toxins [9], while the other, deoxy-cylindrospermopsin (doCYN), lacks the hydroxyl group on carbon 7 and is non-toxin producing [27].

C. raciborskii is one of many cyanobacterial species that produces cylindrospermopsin (CYN; [56]). Other species that produce CYN are *Aphanizomenon ovalisporum* [8], *Raphidiopsis curvata* [89], *Oscillatoria* [98], *Anabaena lapponica* [169], *Lyngbya wollei* [161], *Umezakia natans* [180], *Raphidiopsis mediterranea* [99] and *Anabaena bergii* [159]. Interestingly, to date, CYN- producing *C. raciborskii* are only found in

certain parts of the world including Australia, Asia, and New Zealand (see review by Sinha et al., 2012[166].

The gene operon responsible for the production of CYN in *C. raciborskii* was identified from two Australian strains of *C. raciborskii* strain AWT205; [104] and Strain CS-505 [173]. It encompasses 43KB of the *C. raciborskii* genome, and encodes 15 ORFs. The CYN biosynthetic gene cluster (*cyr*) was comprised of five NRPS/PKS genes (*cyrB, cyrC, cyrD, cyrE, cyrF*), several genes that are responsible for tailoring reactions (*cyrI, cyrJ, cyrN),* and other genes with specific functional roles (amidinotransferase *cyrA*, uracil ring *cyrH*, transport *cyrK*, regulator *cyrO*). It also contains two transposase genes (*cyrM, cyrL*; [104]. The *cyr* gene cluster has also been elucidated in *Aphanizomenon ovalisporum* [173] and *Oscillatoria* sp. [98]

1.4.1 CYN Regulation in *C. raciborskii*

Research on the expression levels of genes involved in CYN biosynthesis has been scanty. A study investigated the relationship of the transcription levels of *cyrB*, *cyrI*, *cyrJ* and *cyrK* to nitrogen source and light intensity (Stucken, 2010; PhD thesis) and found that transcriptional levels of these genes did not correspond to CYN production.

Differential expression of genes within the *cyr* gene cluster has also been observed in other cyanobacteria. Research with *Aphanizomenon ovalisporum* another invasive, CYN producing cyanobacterium, belonging to the order Nostocales showed differential regulation of the individual genes within the *cyr/aoa* cluster in response to nitrogen starvation, although concentration of CYN remained stable [94]. This study also investigated transcription in response to light (85 µmol photon m^{-2} s^{-1}) and

showed an initial decrease in the transcript levels of *aoaA* (=*cyrA*) and *aoaC* (=*cyrC*), at 8 h ours, recovery at 24 h and eventually doubling at 48 hours in response [94].

Thus, extensive research on gene expression and regulation of the *cyr* genes is needed, in order to understand the factors, which facilitate changes in gene levels, and are related to subsequent changes in toxin production.

1.5 Aims of study

The overall aim of this study was to understand the drivers for the divergence, growth and toxigenicity of *C. raciborskii,* against the backdrop of global warming and geographic location. The spread of *C. raciborskii* is attributed to its ability to survive under varied nutrient and temperature conditions. *C. raciborskii* is a robust and invasive cyanobacterium and it is imperative to understand all aspects of the organism, its functioning at various levels, whether genomic or at the transcript level and further, the drivers that aid its spread. This is the first study of *C. raciborskii* strains from different geographic locations using a comparative genomics approach. Furthermore, there is limited knowledge on the impact of various nutrient drivers on the toxicity of *C. raciborskii*.

The specific aim of chapter 2 was to compare three *C. raciborskii* strains of varying morphologies and toxin profiles, from the same geographical location, namely Queensland, Australia to gain a better understanding of the degree of similarity between these strains of common geographical origin, and what the primary differences between these strains might be. Additionally, we hoped to ascertain why some cyanobacteria possess the *cyr* biosynthetic gene cluster and produce toxin, while others do not.

Chapter 3 is an extension of chapter 2, and encompasses the study of *C. raciborskii* and *Raphidiopsis* sp. genomes from different geographic locations, across the world. We investigated the role of toxicity, speciation and geographic occurrence on the divergence of *C. raciborskii* and *Raphidiopsis* species. A total of nine genomes were sequenced using Illumina next generation technology, and compared using comparative bioinformatics approaches. Our research extensively studied the primary metabolic genes and pathways in these strains, as well as the varying arrangement of the *cyr* gene cluster in strains of *C. raciborskii* and *Raphidiopsis* sp.

Chapter 4 was aimed to determine the effect of nutrients on growth and ecotype-related changes in the production of cylindrospermopsins (CYNs) by the cyanobacterium, *C. raciborskii* (Woloszynska) Seenaya et. Subbaraju. Short-term mesocosm experiments were conducted with mixed phytoplankton populations in a subtropical reservoir and treatments were allocated nitrate, urea and phosphate addition alone or in combination.

Chapter 5 aimed to study the effects of varying light intensities and pCO_2 levels on the gene expression of *cyrA,* and *cyrK*, respectively, and endeavoured to study the effect of these physiological parameters on the cellular production of the toxic metabolites CYN and doCYN in the Australian isolate *C. raciborskii* CS-506. Light experiments were conducted under low (10uE) and high (100uE) light intensities, while pCO_2 levels of 1300 ppm and 500 ppm were used.

1.6 Supplementary

Number on Figure 1	Strain	Place of isolation	Reference
1	NA	Minnesota	(Hill, 1970)
2	NA	Kansas	(Prescott & Andrews,1955)
3	NA	Texas	(Lind, 1984)
4	NA	Cuba	(Komarek, 1984)
5	NA	Nicargua	(Hooker,1993)
6	NA	Venezuela	(Lewis & Wiebezahn,1976))
7	NA	Uruguay	(Hecky & Kling,1987)
8	NA	Brazil	(Branco & Senna, 1994)
9	NA	Spain	(Romo & Miracle, 1994)
10	NA	Germany	(Krienitz, 1996)
11	NA	Hungary	(Olah & ElSamra, 1981)
12	NA	Greece	(Hindak, 1988)
13	NA	Slovakia	(Komarek, 1979)
14	NA	Nigeria	(Imevbore, 1969)
15	NA	Kenya	(Kalff & Watson, 1986)
16	NA	Central Africa	(Hecky & Kling,1987)
17	NA	Uganda	(Komarek, 1991)
18	NA	Russia	(Vinogradska, 1974)
19	NA	Kazahstan	(Ergashev, 1969)
20	NA	Tashkent	(Kutsharova, 1963)
21	NA	India	(Hortobagyi, 1969)
22	NA	India	(Gupta & Kumar, 1968)
23	NA	India	(Singh, 1962)
24	NA	India	(Seenayya & Subba, 1972)
25	NA	SriLanka	(Rott, 1983)
26	NA	Australia	(Padisak, 1997)
27	NA	Australia	(Padisak, 1997)
28	NA	Uruguay	(Vidal & Kruk, 2008)
29	NA	Michigan	(Hong et al., 2006)
30	NA	Indiana	(Jones, 2005)
31	NA	Canada	(Hamilton et al., 2005)
32	Fas-C1	Florida	(Yilmaz & Phlips, 2011)
33	PMC99.02	Mexico	(Gugger et al., 2005)
34	Braz1	Brazil	(Lagos et al., 1999)
35	ITEP-83	Brazil	(Haande et al., 2008)
36	NIVACYA 511	Uganda	(Haande et al., 2008)
37	NA	South Africa	(Harding, 1996)
38	NA	Africa	(Komarek & Kling, 1991)
39	NA	East Africa	(Patterson & Kanchanjika, 1993)
40	PMC115.02	Senegal	(Gugger & Hoffmann,2004)
41	NA	Spain	(Romo & Miracle, 1994)
42	NA	Algeria	(Bouaïcha & Nasri, 2004)
43	PMC99.12	France	(Gugger et al., 2005)
44	ZIE05CR	Germany	(Haande et al., 2008)
45	NA	Poland	(Kokocinski et al., 2010)
46	NA	Austria	(Dokulil, 1996)
47	BAL5	Hungary	(Neilan et al., 2003)
48	NA	Serbia	(Sanja, 2011)
49	NA	Greece	(Figueredo & Giani, 2009)
50	NA	Italy	(Messineo et al., 2010)
51	4799	Portugal	(Neilan et al., 2003)
52	NA	Egypt	(Mohamed, 2007)
53	HAB151	China	(Wu et al., 2010)
54	DMKU51011	Thailand	(Chonudomkul et al., 2004)
55	DMKU51018	Thailand	(Chonudomkul et al., 2004)
56	DMKU51004	Thailand	(Chonudomkul et al., 2004)
57	NA	Taiwan	(Wu et al., 2010)
58	CS-505	Australia	(Saker & Griffiths, 2000)
59	CS-509	Australia	(Saker &Griffiths, 2000)
60	CS-511	Australia	(Saker &Griffiths, 2000)
61	CYP-023	Australia	(Stucken et al., 2009)
62	CS-506	Australia	(Saker & Griffiths, 2000)
63	AWT205	Australia	(Saker & Griffiths, 2000)
64	NIES-993	Japan	(Chonudomkul et al., 2004)

Chapter 2

Comparative genomics of *Cylindrospermopsis raciborskii* strains with differential toxicities

Author contributions

Rati Sinha carried out the molecular genetics experiments, the bioinformatics analyses, genome assembly and annotation and genome comparisons, as well as the drafting of the manuscript. Dr. Aaron Jex supervised all the bioinformatics analyses and performed SNP analyses and multiple alignments/comparisons between genomes. Ryanbi Pratama participated in the NRPS/PKS cluster detection and analyses. Prof. Brett Neilan and Prof. Michele Burford designed the project and helped in its co-ordination. Dr. Leanne Pearson, Dr. Timothy W. Davis and Dr. Julia Muenchhoff participated in the analyses and interpretation of results and helped draft the manuscript.

2.1 Abstract

Cylindrospermopsis raciborskii is an invasive filamentous freshwater cyanobacterium, some strains of which produce toxins. Sporadic toxicity may be the result of gene deletion events, the horizontal transfer of toxin biosynthesis gene clusters, or other genomic variables, yet the evolutionary drivers for cyanotoxin production remain a mystery. Through examining the genomes of toxin producing

and non-toxin producing strains of *C. raciborskii*, we hoped to gain a better understanding of the degree of similarity between these strains of common geographical origin, and what the primary differences between these strains might be. Additionally, we hoped to ascertain why some cyanobacteria possess the cylindrospermopsin biosynthesis (*cyr*) gene cluster and produce toxin, while others do not. It has been hypothesized that toxicity or lack thereof might confer a selective advantage to cyanobacteria under certain environmental conditions.

In order to examine the fundamental differences between toxin producing and non-toxin producing *C. raciborskii* strains, we sequenced the genomes of two closely related isolates, CS-506 (CYN^+) and CS-509 (CYN^-) sourced from different lakes in tropical Queensland, Australia. These genomes were then compared to a third (reference) genome from *C. raciborskii* CS-505 (CYN^+). Genome sizes were similar across all three strains and their G+C contents were almost identical. At least 2,767 genes were shared among all three strains, including the taxonomically important *rpoc1, ssuRNA, lsuRNA, cpcA, cpcB, nifB and nifH,* which exhibited 99.8-100% nucleotide identity. Strains CS-506 and CS-509 contained at least 176 and 101 strain-specific (or non-homologous) genes, respectively, most of which were associated with DNA repair and modification, nutrient uptake and transport, or adaptive measures such as osmoregulation. However, the only significant genetic difference observed between the two strains was the presence or absence of the cylindrospermopsin biosynthesis gene cluster. Interestingly, we also identified a cryptic secondary metabolite gene cluster in strain CS-509 (CYN^-) and a second cryptic cluster common to CS-509 and the reference strain, CS-505 (CYN^+).

Our results confirm that the most important factor contributing to toxicity in *C. raciborskii* is the presence or absence of the *cyr* gene cluster. We did not identify any other distally encoded genes or gene clusters that correlate with CYN production. The fact that the additional genomic differences between toxin producing and non-toxin producing strains were primarily associated with stress and adaptation genes suggests that CYN production may be linked to these physiological processes.

2.2 Introduction

Cyanobacteria are photosynthetic prokaryotes that thrive in a wide variety of habitats. Their occurrence in aquatic environments is of particular interest due to their ability to form dense and potentially toxic blooms under certain environmental conditions [147], [157]. Some of the toxins produced include cyclic hepatotoxic peptides such as microcystin and nodularin [25], alkaloids such as the cytotoxic cylindrospermopsin (CYN) [124], homoanatoxin-a and anatoxin-a(s) [67] and the neurotoxic saxitoxin (STX) [158]. These cyanotoxins, produced by over 40 species from 20 genera of cyanobacteria [120], have adverse health effects on humans and animals, and are a public health and environmental concern [25].

The toxin CYN first came into recognition following the poisoning of 149 people on Palm Island in 1979 [48]. Since then several animal poisonings have been recorded, including cattle mortalities [182]. Interestingly, several distantly related cyanobacterial species produce CYN, including *Aphanizomenon ovalisporum* [8], *Raphidiopsis curvata* [89], *Oscillatoria* sp. PCC6506 [98], *Anabaena lapponica* [169], *Lyngbya wollei* [161], *Umezakia natans* [180], *Raphidiopsis mediterranea* [99], *Anabaena bergii* [159] and *Cylindrospermopsis raciborskii* [56].

C. raciborskii, a filamentous diazotrophic cyanobacterial species, is a known producer of both cylindrospermopsin and saxitoxin [56], with the type of toxin produced apparently linked, in part, to geographic distribution. Although STX-producing *C. raciborskii* strains have been reported in South America [171], based on the current understanding, the most broadly distributed toxigenic members of this species produce CYN [130], with CYN producers (CYN^{+}) having been reported in Australia, Asia, and New Zealand [194]. Additionally, *C. raciborskii* strains containing some *cyr* and *sxt* (saxitoxin biosynthesis) genes have also been found in South America, although these strains were found to be non–toxin producing (72). There is mounting evidence for the global emergence of *C. raciborskii*, with the species exhibiting an increasingly cosmopolitan distribution. *C. raciborskii* was initially identified in tropical climatic regions, however, reports of its occurrence in temperate zones have increased drastically in the last decade [166].

Although the production of CYN and STX by cyanobacteria is well characterized and relates primarily to the presence of toxin-specific biosynthetic gene clusters [77, 104], the mechanism for acquisition/loss of toxin gene clusters between and among closely related strains of *C. raciborskii* or a variety of distantly related cyanobacteria species [172] is not understood. It is also not known, which, if any, genes associated with the *cyr* gene cluster might be lost without loss of toxigenicity. Although a representative genome has been published for a toxigenic strain of *C. raciborskii* [171] there has been no large-scale characterization of the genome of other members of the species, or, specifically between and among toxin producing and non-toxin producing strains. Such comparisons may provide insight into how toxin clusters are shared/inherited, whether the differences between being toxin producing or non-toxin producing strains relates to a loss (e.g. through gene loss or mutational change) or

gain (e.g. through cluster acquisition) of function, and what additional differences may exist between the genomes of toxin producing and non-toxin producing *Cylindrospermopsis* strains.

In the present study we conducted genome-wide comparisons of two closely related, but toxicologically distinct strains, CS-506 (CYN^+) and CS-509 (CYN^-), of *C. raciborskii* isolated from the same geographical region of Queensland, Australia and compared these with the published genome of the CYN^+ *C. raciborskii* CS-505 [171]. A primary aim of this study was to elucidate the minimal set of genes required for CYN production and explore whether or not this genomic locus extends beyond the *cyr* cluster. We also hoped to ascertain if toxin production influences the overall physiology of CS-506, by examining the putative metabolic roles of strain-specific genes. Our results are discussed within the context of the evolution and ecophysiology of these closely related cyanobacterial strains.

2.3 Methods

2.3.1 Cyanobacterial strains and culturing

C. raciborskii strains CS-506 and CS-509 were originally isolated from the Queensland water bodies Solomon Dam (8.7242°S 146.594°E) in 1996 and Lake Julius (20.1315°S 139.723°E) in 1995, respectively. Detailed toxin and morphotype analyses of strains CS-506 and CS-505 were conducted in a previous study in which they were referred to as 'form1' and 'form 2', respectively [153]. For the present study, strains were obtained from the Australian National Algae Culture Collection (ANACC), CSIRO Marine and Atmospheric Research, Hobart, Tasmania. Non-axenic cultures of these strains were grown in 250 ml culture flasks in Jaworski medium (JM;

[66] at 25°C, under a light intensity of 25-30 µmol photons m^{-2} s^{-1} with a 12 h light/dark cycle.

2.3.2 DNA . and quality control

To harvest cells, dense 250 ml cultures were filtered onto 3 µm pore size nitrocellulose membranes and washed with 2 volumes of JM to reduce contaminating bacteria to undetectable levels by 16S PCR (see below). High molecular weight DNA was extracted as previously described by Morin *et al.* (2010). Briefly, cell walls were disrupted mechanically, lyzed enzymatically, extracted and precipitated before washing, air drying and resuspending in sterile DNAse free MilliQ water (Millipore, USA). DNA quality was assessed spectrophotometrically and by gel electrophoresis. Only high molecular weight pure DNA samples were used for sequencing. DNA samples with a 260/280 nm absorbance ratio of 1.8-2.0, and a 260/230 nm absorbance ratio of 1.8–2.0 were considered pure.

The DNA samples were further quality-checked by amplification of the16S rRNA gene by PCR. Primers specifically targeting the cyanobacterial 16S rRNA gene (27FL/809R), and bacterial 16S rRNA gene (27FL/1494R) [119] were used to this end. PCR was performed using the following conditions: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, then a final extension at 72 °C for 30 s. The resulting amplicons were sequenced using the BigDye Terminator kit (Invitrogen) and analysed using Bioedit [184]. DNA samples that yielded pure *C. raciborskii* 16S rRNA gene sequences were finally submitted for genome sequencing. These steps ensured that all *C. raciborskii* DNA samples used for genome sequencing were free from contaminating heterotrophic bacterial DNA.

2.3.3 Genome sequencing and comparative analyses

Paired-end indexed libraries were prepared from purified DNA fragments of approximately 320 bp in length. Genome sequencing was performed using the Genome Analyzer IIx sequencing platform and TruSeq SBS v4 GA kit. The raw reads generated were 100 bp in length. Raw read quality was visualized using FastQC software (Babraham Bioinformatics) with default settings. All raw reads were filtered for quality (mean phred > 20) and end-trimmed (10 bp at 5' and 3') using custom Perl scripts. Paired-reads passing these quality filters were used to estimate the genome size for each strain using the program khmerfreq [68] using kmer = 17 and then assembled using SOAPdenovo software [68] with different kmer lengths [57-64], with the final assembly selected based on overall assembly size, number of contigs and contig size.

The optimal assemblies for *C. raciborskii* CS-506 and CS-509 were annotated using homology-based Integrated Microbial Genomes (IMG; [97] and Rapid Annotations Subsystems Technology (RAST; [5] and predictive modeling, Glimmer [34], Genemarks [12] approaches. We used the published genome of the CYN-producing *C. raciborskii* strain CS-505 [171], also isolated from Solomon Dam, Queensland, Australia, as a reference genome for comparative assessment of these annotation methods. Glimmer 3 and Genemarks vastly overestimated gene number, when compared to the previously published reference genome for *C. raciborskii* strain CS-505. For ease of annotation, and to be consistent with the prediction of CS-505 genes, RAST was used for the final analysis. NRPS-PKS predictor [3] and antiSMASH [100] software were used to identify non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) domains. Additionally, further annotation of genes

encoding hypothetical proteins was conducted using the program InterProScan [142] or the pfam database [141].

Following annotation of each genome, we conducted pair-wise comparisons of the gene-sets predicted for each of strain using BLASTn [2]. Genes were considered common to two taxa in pair-wise comparisons if reciprocal BLAST hits had (a) an e-value $\leq 1*10^{-5}$, (b) $\geq 90\%$ nucleotide identity and (c) a difference between query and alignment length of ≤ 20 bp. Genes not meeting these criteria in any pair-wise comparison were considered strain-specific. To ensure that genes 'missing' from each strain based on these BLASTn comparisons were not absent due to gaps in the assembly or misannotation, we mapped the filtered reads from both CS-506 and CS-509 to the published CS-505 sequence using the program SOAPAligner [88]. The bioinformatic prediction of these strain-specific genes was further tested using PCR for a subset of sequences (n = 13; Supplementary Table 2.1). Briefly, primers targeting genes that were absent from only one of the three *C. raciborskii* strains, CS-505, CS-506 or CS-509, were designed using primer-BLAST (NCBI). PCR was performed using the following conditions: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 mins. Suitable positive and negative controls were used in all PCR experiments and approximately 20 ng of template DNA was used in each reaction. Amplicons were visualized using an agarose gel electrophoresis unit. 1% agarose in TAE gels were stained in ethidium bromide and subsequently viewed using a UV transilluminator. Amplification of an expected size PCR product was used to confirm the presence of a gene. Further, 10 randomly picked amplicons were sequenced and the presence of the genes was confirmed.

Structural and overall nucleotide variation between and among *C. raciborskii* CS-505, CS-506 and CS-509 genomes was assessed at the whole genomic level by comparative alignment using the '-nucmer' (--maxmatch) and 'dnadiff' packages of the program Mummer 3 [35]. Specific nucleotide variation between and among protein-encoding genes and within non-coding regions was assessed by comparative alignment of each genome using the Smith-Waterman alignment algorithm of the program Burrows-Wheeler Aligner (BWA; [87]. Genes common to all three *C. raciborskii* strains were each aligned as orthologous clusters using Muscle [40] and the alignments assessed for synonymous, non-synonymous and indel mutations. To ensure only high-confidence single nucleotide polymorphisms (SNPs) were included in our analysis, we mapped the raw reads for each sample to its respective assembly (using Bowtie2) [86] and filtered the initial SNP calls using the Neighbor-Quality Scoring method [189] in which only SNPs covered to a depth ≥ 5 with reads having a phred mapping quality of ≥ 20 and flanked to the 5' and 3' by at least 3 bases with a phred mapping quality of ≥ 15 were retained for subsequent analysis. Read mapping quality was assessed using Samtools v 0.1.19 [88]. Using these SNP data, various population genetic metrics (e.g. segregating sites, synonymous and non-synonymous SNPs per bp) were calculated using custom-perl scripts.

The data sets supporting the results presented in this manuscript are available in the following repository: Integrated Microbial Genomes [97] repository, unique persistent identifier 12992 and 12991 and NCBI short read archive under accession numbers: SRR1042336 and SRR1041118 for *C. raciborskii* strains CS-506 and CS-509, respectively.

2.4 Results & discussion

2.4.1 Genomic structure

The genomes of *C. raciborskii* strains CS-506 and CS-509 were sequenced via the bridge amplification method on an Illumina genome analyzer. Both genomes were sequenced to a depth of ~828 and 459 fold, respectively. The total assembly size for each genome was 4.1 Mb (N50 = 25,000 bp; total scaffolds 698) for strain CS-506 and 4.0 Mb (N50 = 56,411 bp; total scaffolds 319) for CS-509 (Table1.1), which is comparable to that of the reference strain, CS-505 (3.9 Mb). The largest scaffolds assembled were 67,497 bp for CS-506 and 188,708 bp for CS-509. Overall, the draft assemblies for strains CS-506 and CS-509 yielded 103 and 65 scaffolds >10 kb, 8 and 19 scaffolds >40 kb, and 0 and 5 scaffolds over 100 kb, respectively. The G+C content of both genomes was similar (approximately 40.9%) and comparable to that of the reference strain (40.2%), as well as other filamentous cyanobacterial genomes i.e. *Raphidiopsis* sp. and *Anabaena* sp. presently available in the public databases (40-41.5%) (NCBI). In the absence of a physical map for these genomes, structural variation among the assemblies was interpreted with some caution..

Genome characteristics	*C. raciborskii* strain		
	CS-506	CS-509	CS-505
Size (Mb)	4.1	4	3.9
G+C content (%)	41.1	40.7	40.8
N50 (bp)	25,000	56,411	NA
N90 (bp)	412	268	NA
Largest scaffold (bp)	67,497	1,88,708	2,59,000
Scaffolds > 10 kb	103	65	NA
Scaffolds > 40 kb	8	19	NA
Scaffolds > 100kb	0	5	NA
CDS*	3,268	3,416	3,452
Unique CDS*	176	101	181
Common CDS*	2,767	2,767	2,767
rRNA genes*	8	6	9
tRNA genes*	44	40	42
Genes with functional annotations (%)	36	34	55

Table 2.1: Genome assembly statistics of the three strains. N50, largest contig size, and other information relevant to the assembly of the genomes. NA = Data not available, CDS = coding sequence, *Total number.

Nonetheless, based on pair-wise comparisons, CS-505 (CYN^+) and CS-509 (CYN^-) show a higher level of structural synteny as compared to CS-506 (CYN^+). Overall the CS-509 assembly could be aligned in syntenic blocks with >95% of the CS-505 assembly, whereas CS-506 showed alignment synteny with only 93.6% of this genome. The CS-506 assembly, relative to the CS-505 genome, also contained a larger number of breakpoint (8,315), translocation (1,111), insertion (2,846) and tandem insertion (9) events than the pair-wise alignment of CS-509 and CS-505, further evidence of the greater similarity between the CS-505 and CS-509 genomes, compared to the similarity between the toxic strains CS-505 and CS-506. The CS-509 assembly, in comparison contained 8,036 breakpoint, 927 translocation, 2,539 insertion and 5 tandem insertion events.

CS-505 and CS-509, contrary to their different toxin producing phenotypes, were also significantly more similar in sequence than CS-505 and CS-506, with 8,200 SNPs (72.8% in coding regions) and 13,405 SNPs (75.8% in coding regions) observed between the genomes of these pairs respectively. Based on multiple pair-wise alignment of the three *C. raciborskii* genomes, we established an orthologous relationship among all three strains for 2,917 of the 3,418 protein coding genes annotated for CS-505 [171]. Comparative alignment of these assemblies revealed 99.5% nucleotide identity (representing 3,599,169 alignable bases) among all three strains. For these orthologous genes, we detected 9,460 (3.3 per kb) and 4,766 (1.8 per kb) SNPs between CS-505 and CS-506 and between CS-505 and CS-509, respectively, relating to a mean nt identity of 99.6 and 99.8%, respectively. Notably, among the genes found to be identical among all three strains were *rpoc1* (RNA polymerase subunit), *cpcA* and *cpcB* (phycocyanin alpha and beta subunits) and *nifB* and *nifH* (nitrogen fixation proteins), all of which are widely utilized for phylogenetic analyses of cyanobacteria. In a separate analysis, we also noted 99.8% sequence identity among *ssuRNA* and *lsuRNA* (small and large subunits of ribosomal RNA). Hence, although these loci have proven useful for the molecular classification of cyanobacteria to the species level, our results suggest that they are not useful for differentiating *C. raciborskii* strains. Some of the more variable genes under low mutational selective pressure based on the accumulation of coding (i.e., non-synonymous) to non-coding (i.e., synonymous) SNPs for both CS-506 and CS-509 that may be worthy of further exploration as intra-specific markers for *Cylindrospermopsis* are a putative Random Associated Mysterious Protein (RAMP) superfamily protein, CRC_01868 (3.7 and 4.1% nt variability for CS-506 and CS-

509, respectively), a hemolysin A homolog, CRC_02719 (3.4 and 4.9% variability) and a HAD-superfamily hydrolase, CRC_00377 (3.4 and 2.1% variability).

Interestingly, a large proportion of the differences between CS-506 and CS-509 in the shared, orthologous genes related to non-synonymous (NS) mutations, with these being more than twice as common in the former (n = 5,971 SNPs) than the latter (n = 2,419) relative to CS-505. By contrast, synonymous (S) SNPs in these orthologous genes were relatively equal in number between the two strains (2,495 and 2,054 for CS-506 and CS-509, respectively). The higher rate of NS SNPs in these genes between CS-506 and CS-505 in comparison to CS-509 and CS-505 may be suggestive of significant differences in the ecological niches of or selective pressures on CS-505 and CS-506 despite their shared toxin producing phenotype and the close proximity of their geographic origin.

In addition to these alignment-based comparisons, we independently assessed the gene composition and putatively identified functional differences between the three strains RAST [5]. This approach also allowed identification of novel genes encoded in either CS-506 or CS-509 but not found in CS-505. Based on RAST prediction, the CS-506 and CS-509 assemblies were predicted to encode 3,268 and 3,416 protein encoding genes, respectively (see Table 2.1), compared to the 3,452 currently annotated for CS-505 [171]. With RAST identifying 2,767 of these CDS to be shared as homologs among all three *C. raciborskii* strains, CS-506, CS-509 and CS-505 (Figure 2.1, 2.2), representing 82%, 84.8% and 81.8% of all predicted CDS, respectively.

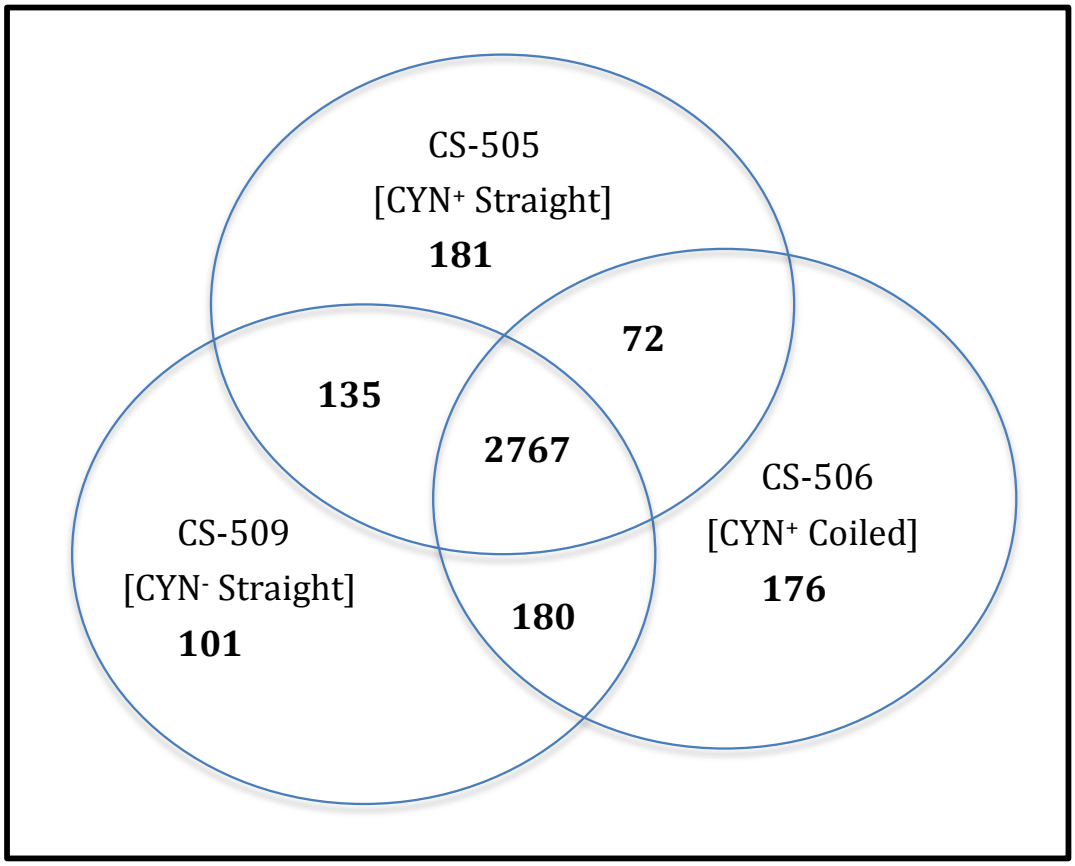

Figure 2.1: Number of exclusive and shared genes among *C. raciborskii* strains CS-505, CS-506 and CS-509. Major phenotypic differences, including toxicity ($CYN^{+/-}$) and morphology (straight/coiled) are also indicated. 297, 173 and 233 genes respectively were excluded from the study.

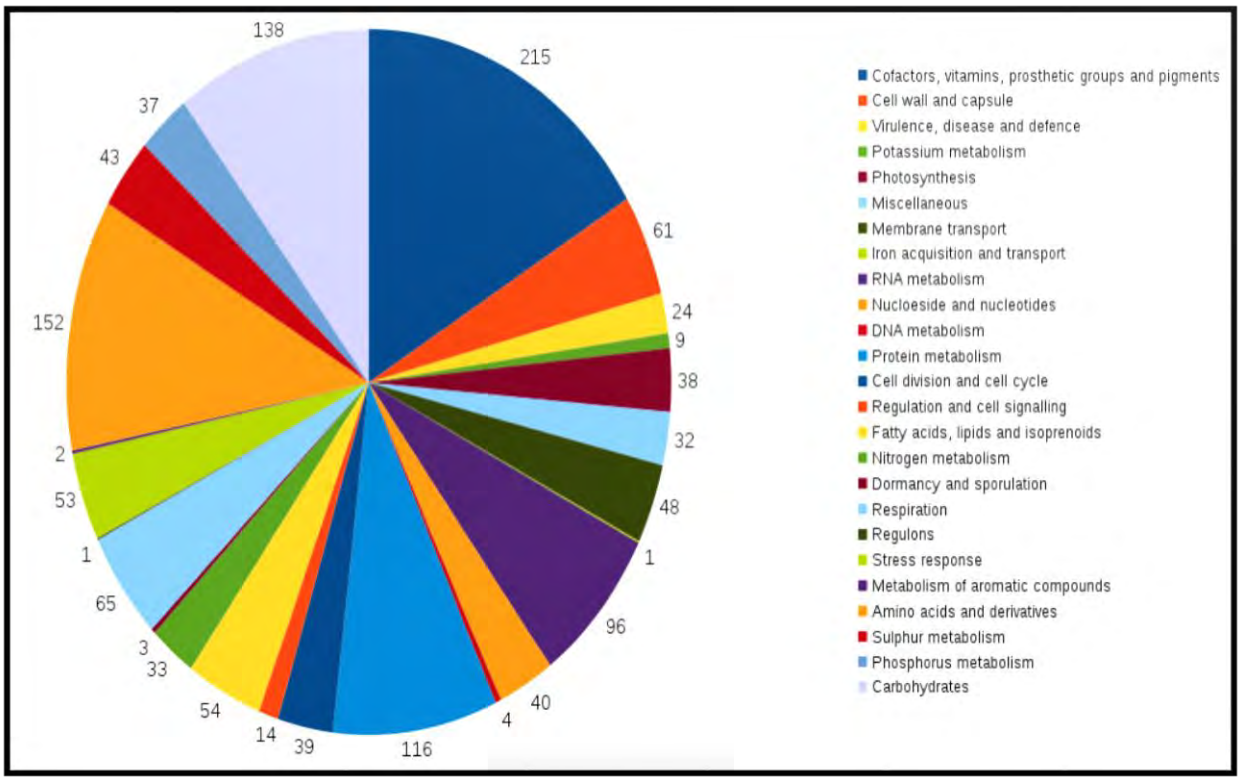

Figure 2.2: Classification of functionally annotated common genes between strains CS-505, CS-506 and CS-509. Fifty-eight percent of genes in this 'core' genome are hypothetical and are not represented in the figure.

2.4.2 Conservation among *C. raciborskii* predicted metabolomes

The genes shared between/among all three *C. raciborskii* strains included those associated with key metabolic pathways, such as photosynthesis, nitrogen and phosphorus metabolism. Many of these pathways appear to be highly conserved across the three *C. raciborskii* strains-and are also similar to primary metabolic pathways identified in other species of cyanobacteria [101, 163, 174]. Although all core genes required for photosynthesis in cyanobacteria [163] were present in each *C. raciborskii* strain, these genes were distributed among eight distinct operons (e.g. *psaAB, psbCD*, *petCA*, *petBD, atpIHGFDAC, coxBAC, chlDHI* and *chlNBL*). This arrangement of photosynthesis genes as eight separate operons is also found in other cyanobacteria, including *Nostoc punctiforme* ATCC 29133 [101], *Raphidiopsis*

curvata, and *Raphidiopsis mediterranea* but distinct from related, non-cyanobacterial species, such as *Rhodobacter sphaeroides*, which organize their photosynthesis genes into a single operon [117]. It is possible that one long continuous photosynthesis gene cluster is ancestral in the bacteria, with subsequent genomic re-arrangement-into separate operons occurring more recently in some taxa.

Nitrogen metabolism genes, including those for nitrogen fixation, ammonium, nitrate and nitrite assimilation and heterocyst development were also conserved among CS-505, CS-506, and CS-509. These genes clustered into several distinct operons, including those for nitrogenase (*nifB, fdxN, nifS, nifU, nifH, nifD, nifK, ORF, nifE, nifN, nifX, ORF, ORF, nifW, hesA, hesB, fdxH*), and nitrite/nitrate uptake and reduction (*nirA, nrtA, nrtB, nrtC, nrtD, narB*). Such operons have been described in other cyanobacteria, including *N. punctiforme* ATCC 29133*, Anabaena PCC* 7120, *A. variabilis* ATCC 29413 [181], and *A. variabilis* PCC 7120 [57].

We also observed the presence of an identical set of phosphorus metabolism genes, displaying high synteny in all three strains. These comprise the *pho* regulon (*phoU, phoR, phoB, pstA, pstB, pstC* and *pstS*), inorganic phosphatase, transhydrogenases (subunits alpha and beta) and alkaline phosphatases, and are required for phosphorus metabolism [174]. The genes *pstA, pstC and pstB* form one cluster while the histidine kinase *phoR* and the transcriptional regulator *phoB* form another. A similar arrangement of phosphorus metabolism genes has been observed in the genomes of *Microcystis aeruginosa* PCC 7806 and *Raphidiopsis mediterranea.*

2.4.3 Substantial differences in transporters among *C. raciborskii* strains

ABC transporters facilitate the translocation of ions or macromolecules across biological membranes, including the export of substances toxic to the cell [93] . Relative to CS-506 and CS-509, CS-505 was enriched for genes involved in transport, export, and nutrient uptake. The CS-505 genome encodes for a large number of ATP Binding Cassette (ABC) transport-related genes, comprising 4.1% of its total genome. In contrast, ABC-transporter genes comprised only 2.3 and 2.4% of the total genes in CS-506 and CS-509, respectively. The enrichment of ABC transporters in CS-505 was mainly limited to those responsible for the transport of glycerophospholipids [68, 179], with 24 of these genes present exclusively within this strain. The strain-specific nature of these specialised ABC transporters suggests a specific ecological adaptation that is not explicitly linked to CYN production, but may relate to membrane structure and permeability. The number of ABC transporters in other cyanobacteria was found to be significantly lower, for example in *Nostoc punctiforme* ATCC 29133, ABC transporters comprise only 0.03% of the genome [101]. Similarly, RAST analyses revealed that in *M. aeruginosa* ABC transporters comprise a mere 0.002% of the genome (48). Other transport-related genes identified in our study strains include amino acid, N-acetylglucosamine related, energy-coupling bacterial, tripartite ATP-independent periplasmic (TRAP) transporters and multidrug and toxic compound extrusion (MATE)-type transporters, all of which were found in consistent, but low, numbers (<1%). MATE transporters belong to the bacterial drug transporter family, and have been previously associated with cyanotoxins. In specific, two genes found in the paralytic shellfish toxin biosynthesis gene cluster are known to be MATE transporters [168]. However, no genes in the cyr biosynthesis gene cluster have been associated with MATE transporters.

2.4.4 Strain-specific genes

Five percent of all genes in CS-506 were specific to this toxin producing strain (although 4.1% of these were annotated as hypothetical proteins). Similarly, 2.9% and 5.2% of all genes in CS-509 (CYN^-) and CS-505 (CYN^+), respectively, were strain-specific (1.7% and 3.5% of these were hypothetical). The strain-specific genes identified seemed to be largely associated with environmental response and adaptation, particularly for phage counteraction, recombination, DNA repair, transport and nutrient uptake, and stress (Figure 2.3). Interestingly, although a similar number of DNA repair genes were present in all the three strains (approximately 0.9 - 1% of their genomes), the three gene sets involved in this process were not identical. Approximately 15% of the total DNA repair related genes in CS-509 were strain-specific, whereas 7.2% of all DNA repair-related genes in strains CS-505 and CS-506 were unique to those strains. Many of these genes were found in multiple loci. The genome of the CYN-producing strain CS-505 was also enriched with interspaced short palindromic repeats (CRISPR) related genes, which comprised 0.6% of its total genome. CRISPR systems are defence machineries used by bacteria and archaea against virus and phage [66]. In comparison, CS-506 and CS-509 contained between 0.3-0.46% CRISPR related genes in their genomes. Strain CS-505 also contained an additional set of *cas1,2* genes containing a CRISPR locus, not present in its counterparts.

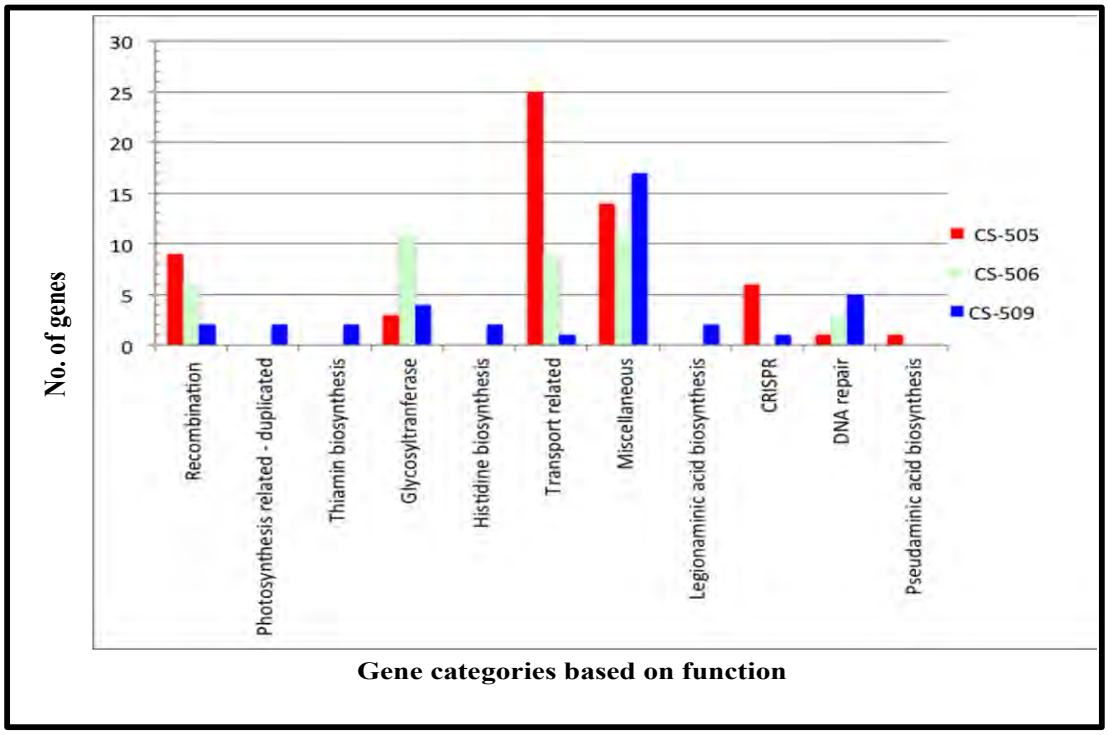

Figure 2.3: Classification of strain-specific genes in the study strains possibly associated with ecophysiological adaptations. Miscellaneous category represents peptidases, proteases, methyltransferases, and and folate, thiamin and cell wall biosynthesis.

The presence of strain-specific genes and proteins has been observed in other cyanobacterial genomes and proteomes. For example, a comparative proteomic analysis of six toxin producing and non-toxin producing *Microcystis aeruginosa* strains reported a large diversity in the protein expression profiles of each strain, with a significant proportion of the identified proteins appearing to be strain-specific. The study found that strains of *M. aeruginosa* species differ in adaptation-related processes, rather than metabolic ones. Additionally, no protein produced exclusively by toxin producing or non-toxin producing strains was found, including the Mcy proteins responsible for microcystin biosynthesis [1]. The observed proteome diversity led to the conclusion that *M. aeruginosa* strains are ecotypes adapted to survival in a particular environmental niche rather than phylogenetically distinct subgroups. Likewise, our data suggests that strains CS-505, CS-506 and CS-509 are ecotypes

adapted to specific ecological niches that exist within the same broader geographic location.

2.4.5 Strain-specific genes found in toxin producing strains

We identified 72 genes (Figure 2.4) common to the CYN-producing strains (CS-505 and CS-506), but absent from the non-toxin producing strain (CS-509). Of these, 34 were annotated as hypothetical. The *cyr* gene cluster (which has already been elucidated in strains AWT205 [104] and CS-505 [171] was identified in CS-506, but not CS-509. The cyr gene cluster encompasses 43 kb and encodes 15 ORFs. It comprises genes responsible for the complex biosynthesis of the CYN, namely an amidinotransferase (*cyrA*), a NRPS/PKS hybrid gene (*cyrB*), four PKS genes (*cyrC, cyrD, cyrE* and *cyrF*), amidohydrolases (*cyrG* and *cyrH*), as well as genes for tailoring reactions (*cyrI, cyrJ*, and *cyrN*), putative transport (*cyrK*), and regulation (*cyrO*). It also contains two transposase genes (*cyrM* and *cyrL*; [104]), indicating the potential for the horizontal transfer of toxicity.

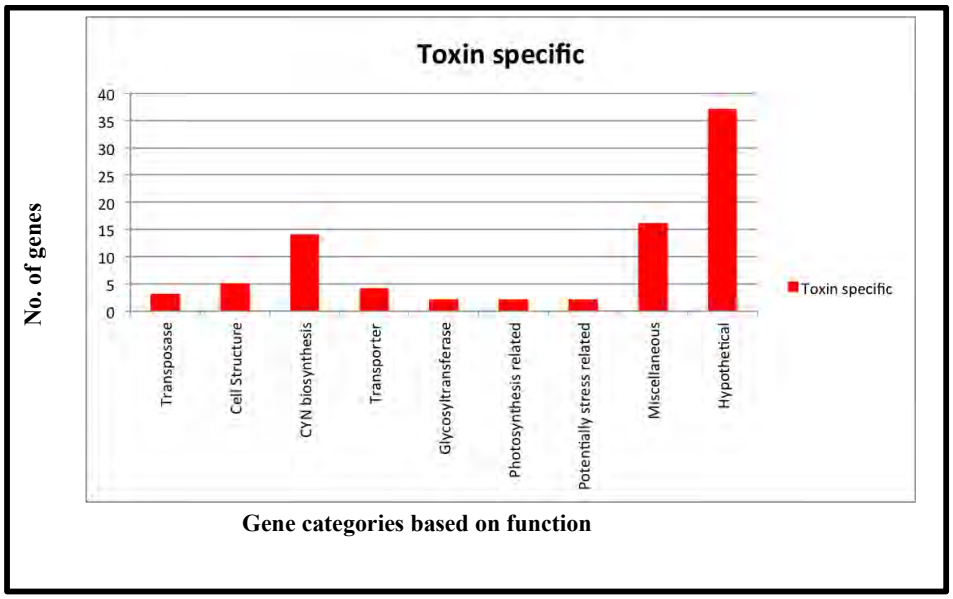

Figure 2.4: Classification of toxin producing strain (CS-505 and CS-506)-specific genes. The miscellaneous category represents genes responsible for protein processing, sialic acid metabolism and cell division.

Although the genes comprising the cyr cluster appear largely conserved among CYN-producing cyanobacteria [72, 170], [104], their location and arrangement differs between/among genera [72]. The *cyr* cluster in *C. raciborskii* CS-506 is flanked by *hyp* (hydrogenase pleitrophy) genes (Figure 5), which play a critical role in the maturation of hydrogenases (i.e., NiFe metalloenzymes) [178] and cluster with 20 other genes, which together are responsible for the expression of the active iron metalloenzyme. This appears to be typical for CYN-producing *Cylindrospermopsis* strains (e.g. AWT205; [104] and CS-505 [171]. In contrast, the *cyr* gene cluster in *Aphanizomenon* sp. is flanked by a transposase and a putative transcriptional attenuator gene [170]. The differential genomic location of the *cyr* cluster in various CYN-producing species suggests that the cluster may be a mobile genetic element capable of jumping between and within cyanobacterial genomes. In further support of this hypothesis, the G+C contents of the *cyr* gene clusters in CS-505 and CS-506 (44-49%, except cyrI 37%) were much higher than the G+C contents of the flanking genes (38-40%) or of the overall genome (40-40.2%). This finding is consistent with previous studies which showed that the G+C content of the *cyr* gene cluster in *Aphanizomenon* sp. 10E6 was >44%, and significantly higher than for neighbouring genes [173].

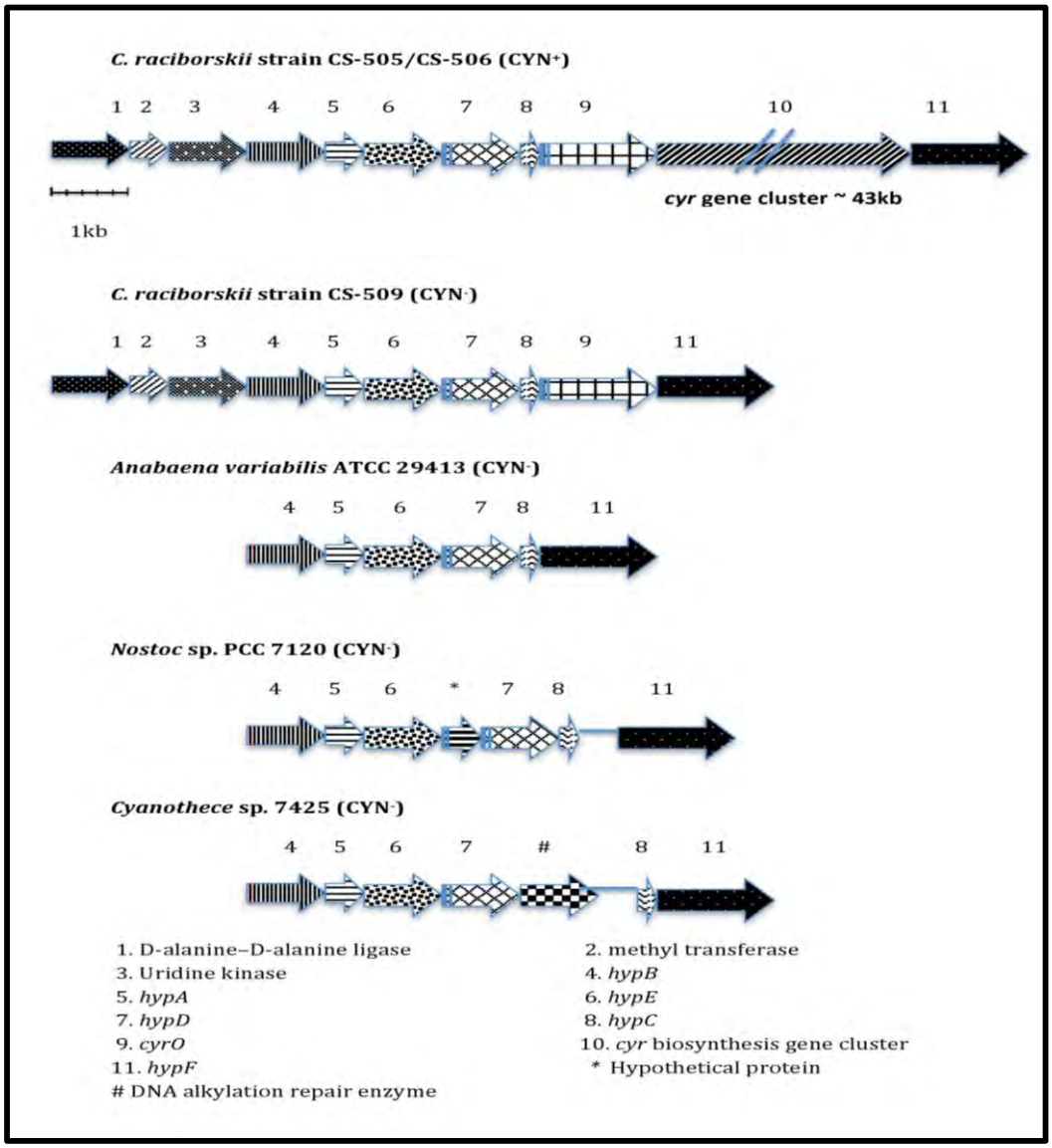

Figure 2.5: Arrangement of genes present in the *cyr* cluster neighbourhood or equivalent genomic location in *C. raciborskii* strains CS-505 and CS-509, *Anabaena variabilis* ATCC 29413, *Nostoc punctiforme* PCC 7120 and *Cyanothece* sp. 7425. This figure highlights the absence of the *cyr* gene cluster in the non-CYN producing *C. raciborskii* strain.

Although the *cyr* cluster was missing from CS-509, a single *cyr* gene, *cyrO* was present between the *hyp* genes. The precise function of CyrO remains to be determined, as does its relationship to the *cyr* cluster. CyrO has low homology to WD repeat proteins, which have diverse regulatory signal transduction roles as well as to ATPases associated with diverse activities (AAA) family proteins which participate in chaperone like functions such as the assembly, operation and disassembly of protein

complexes [104]. The G+C content of *cyrO* is found to be 43, consistent with the *cyr* gene cluster, and higher than the flanking genes, suggesting that it may have been acquired by horizontal gene transfer or has moved from a high GC region of the genome. In contrast to its location in the *C. raciborskii* genome, *cyrO* in *Raphidiopsis curvata* D9 is separate from and distally located to the *cyr* gene cluster [72]. Likewise, no clear orthologs of this gene were found in CYN-producing *Aphanizomenon* sp. 10E6 [172]. The absence of *cyrO* from the *cyr* gene cluster or genomes of other CYN-producing species, suggests that this putative CYN regulatory protein may have an alternative function, or at least is not essential for CYN-production.

Although the presence of the complete *cyr* gene cluster was the most obvious toxin producing strain-specific trait identified in this study, a few other genes were common to CS-505 and CS-506, but absent from CS-509. These included genes putatively involved in transport and protein processing. The production of cyanotoxins is energetically expensive to the cell therefore the expression of additional ABC transporters may facilitate the uptake of nutrients required by toxin producing strains. Single genes responsible for cell division, cell wall capsule biosynthesis, and DNA repair were also found. Another gene, whose function in cyanobacteria has not yet been defined, was also found to be specific to the toxin producing strains. This gene was homologous to vanadium dependent bromopeptidases (VBPOs), which play a role in the hydrogen peroxide-dependent oxidization of halides in eukaryotes [73]. VBPOs can also act as antioxidants, removing hydrogen peroxide, a byproduct of photosynthesis detrimental to cells. In cyanobacteria, VBPOs are thought to be associated with organic compounds that infer allelopathic attributes [73] and therefore selective advantages to toxin producing strains.

2.4.6 Potential mechanism for transfer, acquisition and/or loss of the *cyr* cluster

A recent study comparing over twenty strains of non-toxin producing, STX and CYN-producing *C. raciborskii*, found no correlation between phylogeny and toxicity [172]. Stucken et al. 2010 suggested that the absence of toxicity in some strains of *C. raciborskii* was due to the absence or loss of the *cyr* cluster, rather than to point mutations or partial deletions [172]. Our results support this hypothesis and suggest that *cyr* genes were acquired by horizontal transfer in CS-505 and CS-506, and lost in total from the CS-509 genome.

Evidence of acquired toxicity via horizontal gene transfer (HGT) and its subsequent loss has been documented for other species of cyanobacteria. A recent study by Moustafa et al., 2009 hypothesized that saxitoxin production was either gained independently via HGT in STX-producing strains, or that it was gained by a common ancestor, and lost after several generations from non-STX producers. Genomic analysis of STX-producing *Anabaena circinalis* ACBU02 and a non-STX-producing ACFR02 showed that the latter strain contained four of the 26 sxt biosynthetic pathway genes, advocating the occurrence of genetic deletion. Another study examining the loss of microcystin production in *Plankothrix* species found that certain non-toxin producing strains had lost up to 90% of the *mcy* gene cluster [29]. These strains, however, still contained the genes flanking the mcy gene cluster along with remnants of the transposable regions.

2.4.7 Presence of novel metabolite clusters

Two additional secondary metabolite clusters were detected in the *C. raciborskii* genomes examined in this study. These clusters, designated NRPS1 and NRPS2, are

functionally uncharacterized at this stage (Figure 2.6). Cyanobacteria are producers of an array of bioactive secondary metabolites [121]. Such compounds are often produced by non-ribosomal peptide synthetases (NRPS), and polyketides synthases (PKS) and are of interest due to their toxin producing or therapeutic properties, including antimicrobial, antifungal, or antitumor properties [121].

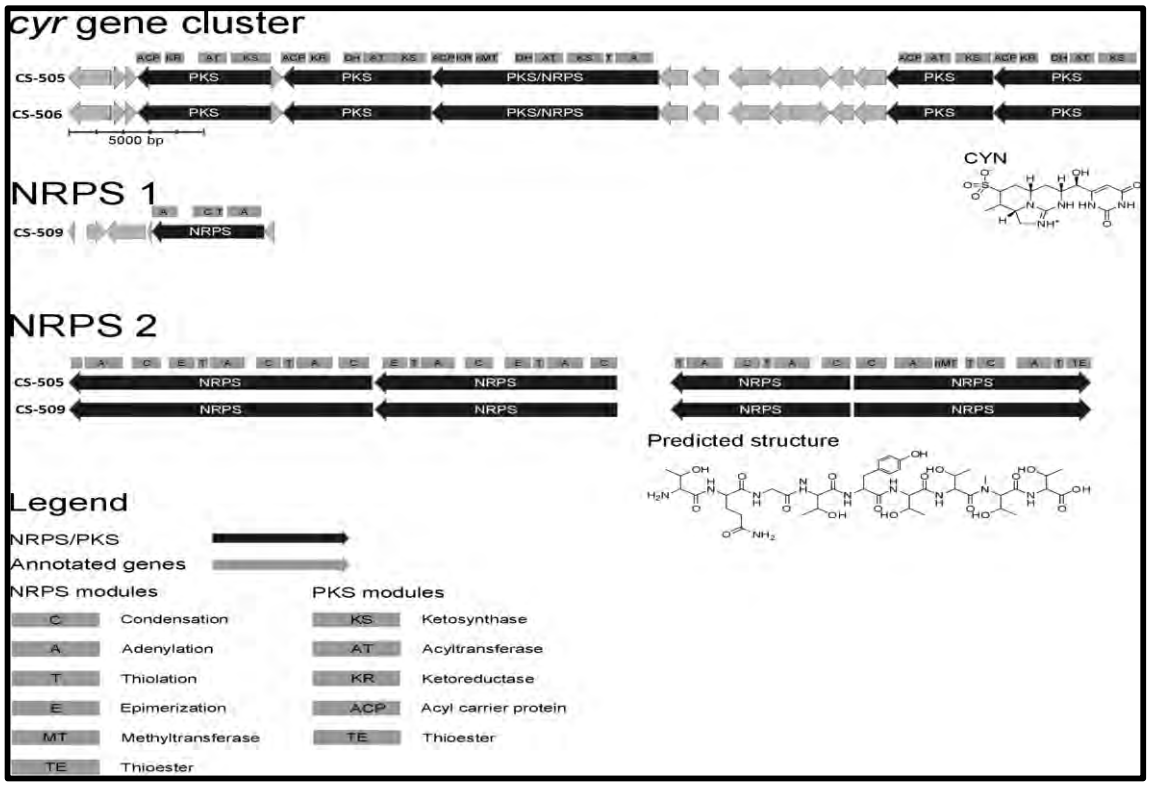

Figure 2.6: Secondary metabolite clusters identified in the CS-505, CS-506 and CS-509 genomes. The structure prediction was performed using AntiSMASH program [100], utilising the monomer prediction of NRPSPredictor 2 [3].

The cryptic NRPS1 gene cluster identified in the non-toxin producing *C. raciborskii* strain CS-509 (ORF 2370-2375) is 7,000 bp long and encompasses 6 ORFs, including a 4,209 bp NRPS, a hypothetical protein (ORF 2373), a major family facilitator (MFS_1 gene, ORF 2372), and a sterol desaturase (ORF 2374). An incomplete NRPS1 cluster was also identified in the reference strain CS-505. However, in this cluster, the NRPS gene exists as a truncated (600 bp) fragment and the MFS_1 gene is missing.

Sequence-based analyses suggest that the NRPS and MFS_1 genes in the CS-509 NRPS1 cluster were acquired via HGT from a common ancestor of *Anabaena variabilis* ATCC 29413 and *Cyanothece* spp., which possess genes with 82% and 86% similarity to NRPS and MFS_1, respectively. The peptide sequence of NRPS1 is also 92% similar to an NRPS in *Raphidiopsis brookii* D9, the function of which is unknown. The *R. brookii* D9 NRPS, however, possesses an additional thiolation domain, putatively required for the production of a dipeptide.

The second cryptic cluster identified in this study, NRPS2 occurs in the straight morphotypes, CS-505 and CS-509, but not in the coiled strain CS-506. NRPS2 is approximately 25 kb in length and comprises 8 ORFs (numbered 2680-2687 in CS-505 and 727-734 in CS-509), including a probable hydroxylase and an acyl carrier protein reductase. This cluster is present in the same location in both CS-505 and CS-509 and is flanked by identical genes in both genomes. AntiSMASH analysis suggests that the NRPS2 cluster is involved in the biosynthesis of an octapeptide, which could possibly be the unidentified *C. raciborskii* toxin reported by Falconer et al. 1999 [41]. However, further experimentation, including mutagenesis of the biosynthesis genes or heterologous expression of the complete cluster, combined with chemo-analytical studies are required to verify this.

2.4.8 Genetic divergence and plasticity

We observed numerous instances of genome duplication in the three study strains, often in multiloci positions. These ranged from photosynthesis-related genes in CS-509 to a unique transposase gene, present as ORFs 10 and 487 in CS-506. Further, two identical gene clusters, each comprising five genes encoding hypothetical proteins, which bear >20% nucleotide similarity to kinases and a gene encoding a DUF324

protein of unknown function, were also found in the CS-509 genome. The genes that constitute these twin gene clusters also show a high degree of similarity (92%) to a cluster of hypothetical proteins in *Cyanothece* sp. PCC 7424.

Nasvall et al. 2012 proposed and subsequently validated the innovation-amplification-divergence (IAD) model, based on a study conducted on over 3,000 generations of the bacterium *Salmonella enterica* [40]. This theory proffers that genes initially amplify to a higher copy number, following which, one of the extra copies suffer mutations. This eventually leads to divergence of the strain from its co-strains. It is possible that the same phenomenon is in play here, and the different strains of *C. raciborskii* are gradually diverging through gene duplication, mutation and deletion events.

Further, the highly plastic nature of the *C. raciborskii* genomes is highlighted by the presence of transposase genes, which are found in close proximity to genes with G+C contents highly deviant from the average G+C content of the *C. raciborskii* genomes. Additionally, G+C rich genes were found amidst genes of lower G+C content. This along with the presence of strain-specific genes and integrases reiterates the polymorphic nature of the *C. raciborskii* genome. Numerous other instances, such as the varying G+C content of the *cyr* gene cluster compared to the flanking *hyp* gene cluster and the presence of transposase genes adjacent to CRISPR arrays, advocate construction of these genomes via HGT events.

Other cyanobacteria also display evidence of genome plasticity. For example 6.8% of the *Microcystis aeruginosa* PCC 7806 genome encodes putative transposases as well as a large number of atypical genes not found in other cyanobacteria. Similarly, a large proportion of the *Nostoc punctiforme* ATCC 29133 genome encodes unique proteins (29%), insertion sites and multilocus repeat sequences [50].

2.4.9 Genes associated with morphological variation between CS-505, CS-506 and CS-509

In addition to differing in toxin-production, CS-505, CS-506 and CS-509 differ in physical morphology; CS-505 and CS-509 are straight, while CS-506 has a coiled trichome. The differing morphologies may be associated with different survival strategies. For example coiled CS-506 have been observed to dominate over straight CS-505 in environmental blooms, likely in relation to the preference among predators, such as Daphnia, for straight, rather than coiled morphotypes [153]. To better understand the genetics underpinning cell morphology in this species, we examined the three *C. raciborskii* genomes for the presence of morphotype-specific genes. Around 200 genes were common to the straight morphotypes (CS-505, CS-509) but absent from the coiled strain (CS-506). As expected, numerous genes related to cell wall and capsule biosynthesis were exclusive to the straight morphotypes. These include genes responsible for the biosynthesis of capsular and extracellular polysaccharide and murein hydrolases, which play a role in the regulation of cell wall growth in bacteria [190]. Additionally, while the *Mre* gene that is responsible for cell shape in bacteria was found in all three strains, we observed a total of five SNPs in the gene sequences, three of which were common to the straight morphotypes. The inactivation of the *Mre* gene in *Anabaena* sp. PCC 7120 has previously been shown to convert the rod-like shape of the cell to a spherical form [66]. While, further experimentation is required to validate whether the *Mre* genes in the coiled *C. raciborskii* strains are active or not, or whether the presence of the SNPs in the straight morphotypes affects the translation of these genes in some way, it is possible that this gene plays a role in *C. raciborskii* morphology. We also found several genes exclusive to the straight morphotypes, many of which are putatively involved in cell division,

transport, DNA repair, recombination and stress response. Whether or not these genes play a role in cell morphology remains to be determined.

2.5 **Conclusions**

Our results suggest that CS-505, CS-506 and CS-509 represent distinct ecotypes of *C. raciborskii*, with subtle genetic differences resulting from the niche selective pressures of their specific but geographically similar environments.

This is the first example of genome comparison between closely related toxin producing and non-toxin producing *C. raciborskii* strains. As expected, the genomes of these strains were very similar. However, subtle genomic differences alluding to the adaptability of the species were identified. Moreover, numerous examples of strain-specific genes, genes with disparate G+C contents, duplicated and/or rearranged genes, as well as transposases and integrases were observed. Taken together, these results underpin the plasticity of the *C. raciborskii* genome and its potential to evolve in the face of selective pressures. This ability to adapt may help explain the recent invasion of *C. raciborskii,* spanning the last 2 decades [166], from tropical to temperate climates around the globe.

Most significantly, we demonstrated that toxicity in this species is dependent on possession of the *cyr* gene cluster, as no other candidate secondary metabolite gene clusters were positively correlated with toxicity. While previous attempts to mutate and thus confirm the role of *cyr* genes have been unsuccessful, we can now conclude with a high degree of certainty that the *cyr* cluster is in fact responsible for CYN biosynthesis. Additionally, the non-toxin producing CS-509 strain lacked the complete suite of *cyr* genes, but possessed a unique cryptic secondary metabolite gene cluster, NRPS1, the function of which is unknown.

The description of two closely related, but toxigenically different *C. raciborskii* genomes can be considered a starting point for further molecular studies into the regulation of CYN production and its native role in this species. For example, transcriptomic and proteomic analyses examining the effects of chemophysical parameters, such as light, nutrients, and trace elements, on toxin producing and non-toxin producing strains can provide insight into the environmental drivers for toxin production. Since the occurrence of this potentially toxic cyanobacterium is on the rise due to increased eutrophication and global warming [132, 192], it is imperative to gain a better understanding of the various aspects of its physiology and the integral role played by CYN production. This will further facilitate better prediction and management of harmful cyanobacterial blooms.

2.6 Supplementary

		CS-505 vs. CS-509		CS-505 vs. CS-506	
Sequences		[REF]	[QRY]	[REF]	[QRY]
	Total seqs	93	2517	93	3050
	Aligned seqs	93	1367	92	1480
		-100.00%	-54.31%	-98.92%	-48.52%
	Unaligned seqs	0	1150	1	1570
		0.00%	-45.69%	-1.08%	-51.48%
Bases	Total bases	3879030	4034670	3879030	4183928
	Aligned bases		3698640		3631332
			-95.35%		-93.61%
	Unaligned bases		180390		247698
			-4.65%		-6.39%
Feature estimates	Breakpoints	8036	2367	8315	2372
	Relocations	12	22	9	21
	Translocations	927	77	1111	91
	Inversions	0	1	0	1
	Insertions	2539	1193	2846	1043
	Insertion sum	523389	311431	594538	262072
	Insertion avg	206.14	261.05	208.9	251.27

Table 2.1: Structural and overall nucleotide variation between *C. raciborskii* CS-505, 506 and 509. Genomes were assessed at the whole genomic level by comparative alignment using the '-nucmer' (--maxmatch) and 'dnadiff' packages of the program Mummer 3.

Comparison of CS-506 and CS-509 relative to CS-505	
Total number segregating sites for whole genome	19154
Total number segregating sites for coding regions	14302
Total number segregating sites for noncoding regions	4852
Total number SNPs for whole genome	21603
Total number SNPs for coding regions	16128
Total number SNPs for noncoding regions	5475

Table 2.2: SNP data comparisons of CS-506 and CS-509 relative to CS-505

Strain	Coding	SegSi	Syn	NonSyn	Stop	Ambig
CS-506	3233398	10143	2671	6391	204	542
CS-509	3233398	5970	2459	3137	37	299

Table 2.3: SNP data comparisons of CS-506 and CS-509 relative to 505; SegSi = segregating sites, variable positions which in this case are equivalent to total SNPs; Syn = synonymous; NonSyn = non-synonymous; Stop = mutations which appear to have introduced a premature stop codon in a gene; Ambig = ambiguous mutations that usually occur where the reference has a non-standard IUPAC nucleotide code (R, Y, etc.)

Chapter 3

Unravelling the *Cylindrospermopsis* and *Raphidiopsis* genome complex

Author Contributions

Rati Sinha carried out the molecular genetics experiments, the bioinformatics analyses, genome assembly, annotation, genome comparisons; NRPS/PKS cluster detection and analyses, as well as the drafting of the manuscript. Prof. Brett Neilan designed the project and helped in its co-ordination. Dr. Leanne Pearson and Dr. Jason Woodhouse participated in the analyses and interpretation of results and helped in the writing of the manuscript.

3.1 Abstract

Cylindrospermopsis and *Raphidiopsis* are filamentous, invasive, phototrophic cyanobacteria that belong to the order Nostocales of the botanical classification system. The two genera are remarkably similar in genetic constitution, morphology, and their ability to produce cyanotoxins. In this study, the impact of speciation and geographic distribution on the genomic composition of *C. raciborskii* and *Raphidiopsis* species was evaluated. The genomes of nine strains, taxonomically classified as either *C. raciborskii* or *Raphidiopsis* were sequenced and compared using various bioinformatics approaches. We describe intra-generic rearrangement of the cylindrospermopsin gene cluster amongst strains of *C. raciborskii* and *Raphidiopsis mediterranea* and further

reinforce the exclusion of two previously annotated genes. Rearrangements within the *cyr* gene cluster appear to correlate with geographic location, and not by taxonomic delineation as previously inferred. Functional and sequence based analyses of the entire genome reveal clustering based on geographic influence, independent of either toxicity or taxonomic identity, however, this was not supported statistically. Together, these findings provide evidence of potentially ongoing inter-genetic recombination *between C. raciborskii* and *Raphidiopsis.*

3.2 Introduction

C. raciborskii is a heterocystous nitrogen fixing cyanobacterium with a cosmopolitan distribution, but is particularly prevalent in tropical and sub-tropical freshwater bodies [166]. *C. raciborskii* is frequently reported within the environment coexisting with the non-heterocystous cyanobacterium *Raphidiopsis* [105]. The genera *C. raciborskii* and *Raphidiopsis* exhibit high genetic and morphological similarity, to the extent that several studies have discussed the possibility that *Raphidiopsis* represents a non-heterocystous life cycle stage of *C. raciborskii* [81, 113] The two genera are found free-floating, solitary, straight or coiled, and readily form terminal akinites.

Attempts to distinguish the genera based on single gene based phylogenetic analyses have been unsuccessful [91]. Common phylogenetic markers such as the 16S-23S intergenic spacer (ITS), 16S rRNA and rpoc1 were used for such analyses. These studies largely supported close phylogenetic proximity of *Raphidiopsis* sp. with some strains of *C. raciborskii,* but not with others [51]. A recent multi locus sequence type (MLST) phylogenetic analyses of seven concatenated gene loci proposed the paraphyletic origin of *C. raciborskii*, further suggesting the congenetic origin of the two genera [196] A genome wide comparison of *C. raciborskii* CS505 and

Raphidiopsis brookii D9 identified 2539 (72.5%) genes common between the two species [171]. This high level of conservation is remarkable, considering that amongst three *C. raciborskii* strains from Queensland, Australia only 2797 (82.2%) genes were common [165].

Raphidiopsis and *C. raciborskii* are both known to produce the toxins Cylindrospermopsin (CYN; [104] and Saxitoxin (STX; [77], although no strain has been reported with the genetic capacity to produce both. Strains of *Raphidiopsi*s are also known to produce analogues of homo-anatoxin [116]. Instances of loss of human and animal life as a consequence of CYN and STX poisoning have been reported in the last decade [118], [14]. Additionally, the rampant spread of *C. raciborskii* from the tropical to the temperate regions of the world is a matter of increasing concern [20], [166]. To date, the occurrence of CYN and STX producing strains is limited to Australasia and South-America respectively. However, given the invasiveness of the organism and a rapidly changing climate, there is no guarantee that this will continue to be the case in the future. The potential of toxin producing strains to invade new ecological niches remains a possibility. As such, it is imperative the factors that support the geographic distribution of these organisms are elucidated.

This study explores the genomic basis for the distribution of *Cylindrospermopsis* and *Raphidiopsis* species, with particular focus on the composition and arrangement of cylindrospermopsin biosynthesis genes. We also examine the metabolic capacity of each strain and discuss possible causes and impacts of gene rearrangements and deletions.

3.3 Materials & methods

3.3.1 Sample collection

Strains of *C. raciborskii* and *Raphidiopsis* spp. were obtained from different geographic locations, including China, Brazil and Australia (Table 3.1). Uni-cyanobacterial but non-axenic cultures of these strains were grown in 250 ml culture flasks in JM medium [66] at 25°C under a light intensity of 25-30 μmol photons m^{-2} s^{-1} with a 12 hour light/dark cycle.

Strain	Morphology	Toxicity	Isolated in	Climate	Reference
*CS-505	Straight	CYN	Qld, Australia	Tropical	[153]
*CS-506	Coiled	CYN	Qld, Australia	Tropical	[153]
*NPD	Straight	CYN	Qld, Australia	Tropical	[33]
*CS-509	Straight	Non-toxic	Qld, Australia	Tropical	[153]
*AWT205	Straight	CYN	Sydney, Australia	Temperate	[55]
*Hab151		Non-toxic	Kunming, China,	Temperate	[195]
*N3	Straight	CYN	China	Tropical	
*T3	Straight	Stx	Rio de Janiero, Brazil	Tropical	[138]
^CHAB 114	Curved	Do-CYN	Qichun, Hubei	Tropical	
#FSS1-150/1	Straight	Do-CYN/ CYN	Qld, Australia	Tropical	[108]
“D9	Straight	Stx	Brazil	Tropical	[171]

Table 3.1: The morphology, location and climate of occurrence, and toxin profiles of strains used in this study. * denotes *C. raciborskii*, ^ denotes *R. curvata*, # denotes *R. mediterranea*, “ denotes *R. brookii.*

3.3.2 DNA extraction, genome sequencing and comparative analyses

Filtration, high molecular weight DNA extraction and confirmation of identity was performed as previously described [165]. Genome sequencing was performed using Illumina platforms at the Clive and Vera Ramaciotti Center for Gene Analysis, UNSW Australia. Processing of raw reads, genome assembly, gene calling and annotation was performed as per Sinha et al., 2014 [165]. Further, a *Cylindrospermopsis-Raphidiopsis* pangenome was created using pairwise blastn comparison of gene sets, using criteria elucidated in Sinha et al., 2014. A pangenome comprises of core and variable gene sets. The core gene sets contain all genes common to all the sample genomes in that study, while variable gene sets contain genes missing from one or more sample genomes. Genes common to all eleven genomes were designated as 'the core genome', while all other genes were allocated to the variable genome.

3.3.3 Statistical analyses

Average nucleotide similarity amongst the *Cylindrospermopsis-Raphidiopsis* core genome was determined by blast and used to create similarity matrix amongst strains. For the functional similarity, cog profiles for each strain were normalized and square root transformed. The relationship between genomes isolated occurring in tropical and temperate environments was investigated using redundancy analysis (RDA, Legendre & Anderson, 1999). Bray-Curtis similarities between each strain were calculated and used to create a similarity matrix. Cluster dendrograms of each strain based on the resultant similarity matrices for each analysis were generated (Figures 3.3 and 3.4). Further, unrestricted permutational MANOVA (PERMANOVA) was utilized to test the whether strains could be differentiated based on either the presence of the *cyr* gene cluster, their country of origin or species.

3.4 Result

Genome size, amongst the eleven strains, ranged from 2.8 -3.8 Mb for *Raphidiopsis* sp. and 3.6 - 4.1 Mb for *C. raciborskii* strains, with GC contents between 40.1 – 41.7% [Table 3.2]. The pangenome, as identified by pairwise blastn analysis, consisted of 1124 genes with a combined size of ~ 1 Mb, common to all 11 genomes. This core genome represented approximately 25-35% of the total genome depending on the total size.

The variable genome, on the other hand, contained 31,587 genes. The *Cylindrospermopsis* sp. and *Raphidiopsis* sp. cores individually contained 1524 and 2103 genes respectively.

Strain	Size (Mb)	GC %	CDS	RNA
*C. raciborskii*CS-505	3.9	40.80	3452	51
*C. raciborskii*CS-506	4.1	41.10	3268	52
*C. raciborskii*CS-509	4.0	40.16	3416	46
*C. raciborskii*AWT205	4.4	40.80	3144	37
*C. raciborskii*HAB151	3.7	41.20	2572	39
*C. raciborskii*N3	4.1	41.40	4062	39
*C. raciborskii*T3	3.7	40.90	3271	42
*C. raciborskii*NPD	3.6	41.50	3080	43
*R. curvata*CHAB 114B	3.9	41.00	3280	53
*R. mediterranea*skuja FSS1-150/1	2.8	40.90	3122	49
*R. brookii*D9	3.2	40.90	3088	51

Table 3.2: Genome assembly statistics of the study strains

Genes identified as contributing to the core genome were annotated as being involved in primary metabolic functions such as, but not limited to, protein, carbohydrate, DNA, RNA, sulphur and phosphorus metabolism. Not all genes involved in these primary metabolic pathways were found within the core genome. Amongst genes involved in

DNA repair the MutL-MutS system, the uvrABC system and the bacterial uvrd genes were found in the pangenome, however genes for base excision, recFOR pathway and recA DNA repair systems were not. Genes involved in photosynthesis, nitrogen metabolism, gram-negative cell wall biosynthesis, potassium metabolism, stress response osmotic and periplasmic stress responses were also missing from the core genome.

3.4.1 Primary metabolism, signaling and transport

Genes and pathways encoding essential primary metabolic functions were, as expected, present in all our study strains (Figure 3.1). However, the number of genes in each metabolic category varied from strain to strain.

3.4.1.1 Carbohydrate and amino acid metabolism

An in depth sequence analysis of the carbohydrate metabolism machinery in the study strains revealed the consistent presence of essential pathways needed for the survival of cyanobacteria. These ranged from the Calvin Benson Cycle comprising the rubisco operon [11], the TCA cycle, responsible for the conversion of acetate to carbon dioxide and energy, to the Entner Doudoroff pathway, which catabolizes glucose to pyruvate using mechanisms different from glycolysis and the pentose phosphate pathway. *C. raciborskii* strains Hab151 (CYN-) and AWT205 (CYN+), however, contained fewer carbohydrate metabolism related genes than the other strains in this study.

Similar trends were observed in the amino acid metabolism and biosynthesis machinery. While genes responsible for the synthesis of typtophan, DAHP, chorismate and cysteine synthesis, were consistently present in all 11 genomes, strains *C. raciborskii* Hab151 (CYN-) and AWT205 (CYN+) contained fewer amino acid

metabolism and biosynthesis genes than their counterparts. Minimal gene sets required for the synthesis of essential amino acids and their derivatives were identified, including genes involved in the biosynthesis of histidine, glycine and arginine, aromatic amino acids and their derivatives (e.g. tryptophan and chorismate), and branched amino acid (e.g. leucine) to name a few. However some genes associated with the glycine cleavage system, tryptophan and DAHP synthesis were absent from both strains.

3.4.1.2 Iron, nitrogen and phosphorus metabolism

Genes encoding heme oxygenase and coproporphyrinogen 3 oxidase, responsible for the uptake and utilization of heme and hemin, were found in all strains of *C. raciborskii.* While coproporphyrinogen 3 oxidase was also consistently present in the *Raphidiopsis* strains, heme oxygenase was not. The two genes were found adjacent to each other and next to typtophan synthesis genes in *C. raciborskii*, while the orphan coproporphyrinogen 3 oxidase gene in *Raphidiopsis* sp. was located next to the sialic acid metabolism cluster.

Nitrogen metabolism related genes responsible for the uptake of nitrate and nitrite, the assimilation of ammonia, and the hydrolysis of cyanates were present in all study strains. Genes responsible for nitrogen fixation were also identified in all *C. raciborskii* strains, but, were, as expected, missing from all *Raphidiopsis* strains.

Finally, identical gene sets responsible for phosphorus metabolism, including those encoding the pho regulon and alkylphosphonates utilization were present in all study strains.

3.4.1.3 Signal Transduction

Environmental and intracellular parameters in bacteria are measured by a complex mechanism of interacting components that together form the signal transduction machinery [47]. Genes associated with this machinery range from histidine and serine/threonine kinase signaling genes and intracellular signaling using GAF sensor domains, to genes encoding periplasmic signaling proteins. Signal transduction genes comprised ~ 0.2% genes in the study strains.

3.4.1.4 Transport

Cyanobacteria contain a complex transport system, which facilitates the translocation of nutrients, ions and macromolecules, across biological membranes, and the export of toxic substances outside the cell [148]. These can be diversified into seven broad categories, namely, ABC transporters, cation transporters, tripartite ATP independent transporters (TRAP), energy-coupling factor (ECF) transporters, ton and tol transport systems and the protein and nucleoprotein secretion systems. Consistent numbers of genes were identified across all the transporter categories except ABC and cation transporter groups. ABC transporters comprised 0.2-0.6% of the genomes of the study strains and were particularly prevalent in *C. raciborskii* CS-505 (CYN+), AWT205 (CYN+), N3 (CYN+) and *R. mediteranea* skuja FSS1-150/1 (CYN+) (0.5-0.6%). ABC transporter genes are responsible for the translocation of amino acids, vitamins, carbohydrates and metal chelate complexes.

Cation transport genes comprised approximately 0.37% of the genomes of *C. raciborskii* strains T3 (STX+), *R. brookii* D9 (STX+) and *R. mediterranea* skuja FSS1-150/1 (CYN+), however they were less abundant (~ 0.2%) in the genomes of the

remaining strains. All studied genomes contained genes for the transport of nickel and cobalt, while the genomes of *C. raciborskii* T3, *R. brookii* D9 and *R. mediterranea* skuja FSS1-150/1 additionally contained genes for the transport of magnesium, copper and blue copper. *C. raciborskii* AWT205 (CYN+) also contained a greater number of ton and tol transport system genes, associated with the uptake of rare nutrients, iron and vitamins B1 and B12.

3.4.2 Phage counteraction

CRISPR systems are defence machineries used by bacteria and archaea against viruses and phages [70]. Varying degrees of sophistication were observed in the CRISPR machinery of our study strains. The genome of reference strain *C. raciborskii* CS-505 was the most enriched with CRISPR related genes, which comprised 0.6% of its total genome. In comparison, CRISPR related genes comprised 0.08-0.4% of the genomes of the remaining strains. Specifically, *C. raciborskii* strains N3 (CYN+) and Hab151 (CYN-) from China, T3 (STX+) and *R. mediterranea* skuja FSS1-150/1 (CYN+) from Australia contained the minimal *Cas1* and *Cas2* genes needed for the functioning of the CRISPR machinery in cyanobacteria. These strains also contained fewer CRISPR (Cas RAMP module) *cmr* clusters.

3.4.3 Osmotic adaptation

Genes for the uptake and biosynthesis of trehalose, a characteristic of low-salt tolerant cyanobacteria, were identified in all study strains. However, no genes encoding trehalase (responsible for the breakdown of trehalose) were identified.

Further, we detected identified betaine biosynthesis and uptake genes and choline uptake genes. Glucosylglycerol genes, commonly associated with halotolerance were not found in any of the study strains.

3.4.4 Metal resistance

Cyanobacteria possess mechanisms to regulate the accumulation of metal ions, and prevent metallic toxicity. This includes the regulation of metals that are always detrimental to the organism, such as mercury and cadmium, and metals that are beneficial in trace amounts but toxic at higher concentrations [164].

All of the study strains contained genes for mercury, cobalt, zinc, cadmium and fluoroquinolone resistance, while some strains *C. raciborskii* T3 (STX+), N3 (CYN+) and CS-505 (CYN+) contained additional genes for aluminium and tellurite resistance.

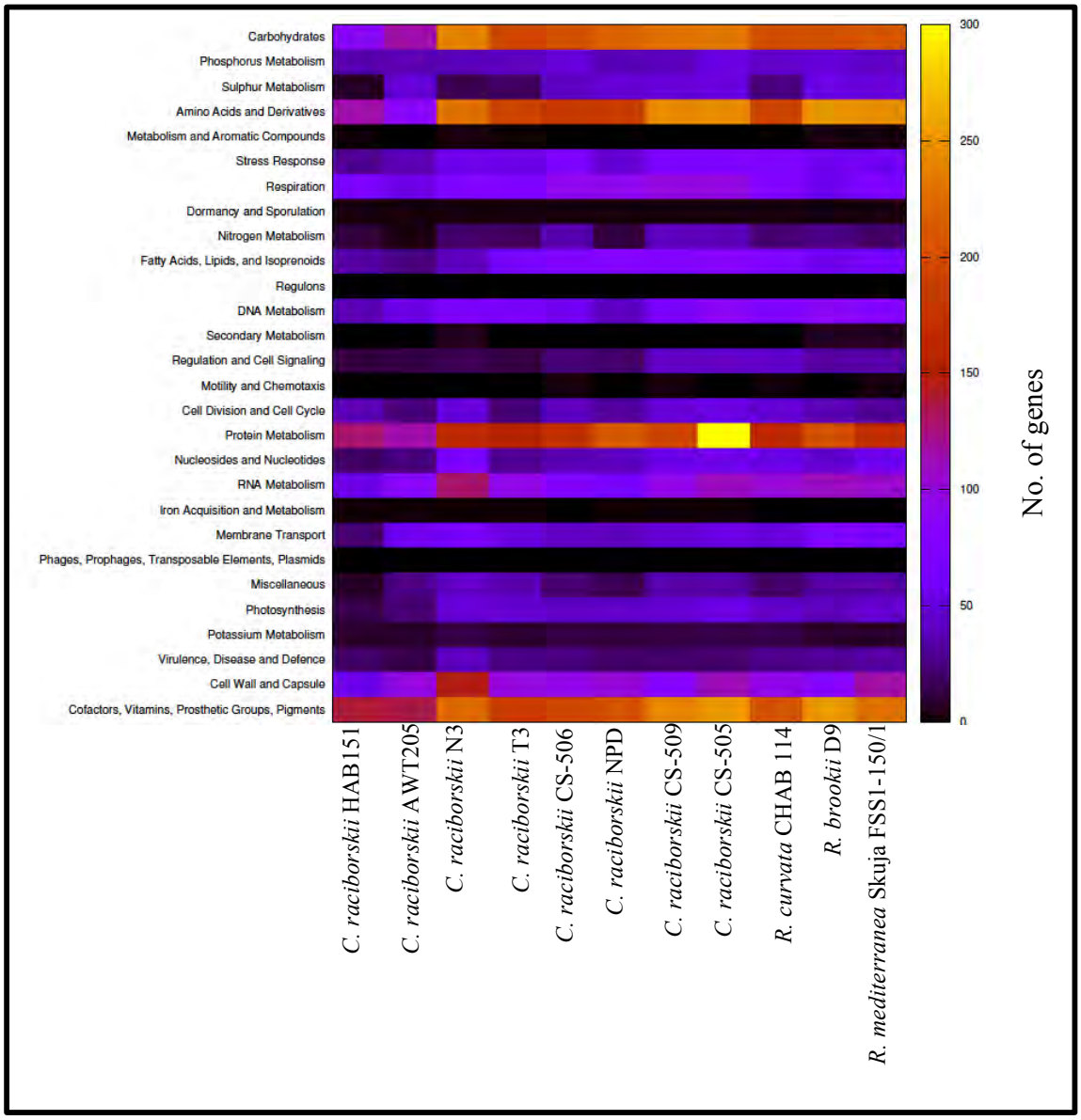

Figure 3.1: Heat map depicting the distribution and abundance of functional subsystems in the study strains. The right hand column indicates gene functional category. The scale on the left hand side indicates number of genes.

3.4.5 Natural product biosynthesis

Genes involved in cylindrospermopsin biosynthesis were present in the *C. raciborskii* strains CS-505, CS-506, NPD, N3 and AWT205, as well as *R. curvata* CHAB114, and *R. mediterranea* skuja FSS1-150/1. Amongst these seven strains two arrangements were observed within the genome assemblies and further confirmed by PCR.

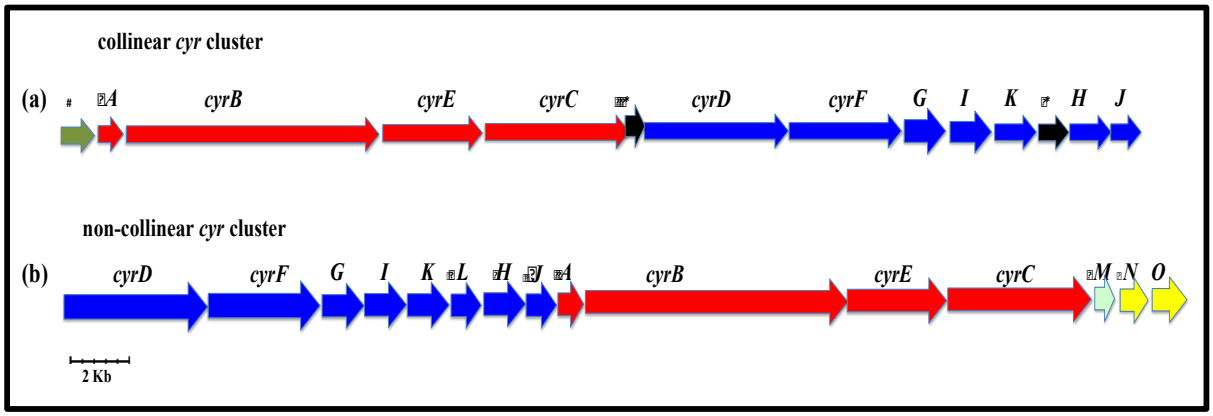

Figure 3.2: Varying arrangements of the *cyr* gene cluster were found within strains of the two genera. (a) Collinear *cyr* cluster in *R. curvata* CHAB 1150, *R. curvata* CHAB114 and *C. raciborskii* N3. (b) Non-collinear *cyr* cluster in *C. raciborskii* AWT205, CS-505, CS-506 and *R. mediterranea* skuja FSS1-150/1. *Tranposases or vestiges of *cyr* genes. #Hypothetical protein. Genes in red and blue colour codes depict gene operons that have been found clustered together in both arrangements. Light green and yellow colour coded genes depict the transposome and tailoring genes respectively, while dark green depicts a hypothetical protein.

C. raciborskii AWT205, CS-505 and CS-506, and *R. mediterranea* skuja FSS1-150/1 exhibit the typical *C. raciborskii* non-colinear arrangement (Figure 3.2). Average nucleotide similarity between genes in these strains was between 99-100%. *C. raciborskii* N3 and *R. curvata* CHAB114 exhibited the typical *Raphidiopsis* co-linear arrangement (Figure 3.2a). The *cyr* cluster in *R. curvata* CHAB114 lacked *cyrN* and *cyrO*, although *cyrO* was identified elsewhere within the genome.

Genomic context was evaluated by identifying genes flanking the *cyr* gene clusters amongst each of the seven strains. The non-colinear *cyr* gene clusters from strains AWT205, CS-505 and CS-506, and *R. mediterrannea* skuja FSS1-150/1 were flanked by the *hyp* gene cluster (Figure 3.3). However, the colinear *cyr* gene clusters in *C. raciborskii* N3 and *R. curvata* CHAB114 were localized between two hypothetical proteins. Within the genome of *C. raciborskii* N3 a single *cyrO* gene was found flanked by the *hyp* gene cluster. This interruption was additionally observed within the genome of the non-CYN producing *C. raciborskii* CS-509. Amongst the saxitoxin producing *Raphidiopsis* D9, the *hyp* gene cluster was interrupted by two hypothetical proteins,

which exhibited no similarity to any genes present within the *cyr* operon. In *R. curvata* CHAB114 nine genes, including *cyrN* and *cyrO* were localised within the *hyp* operon.

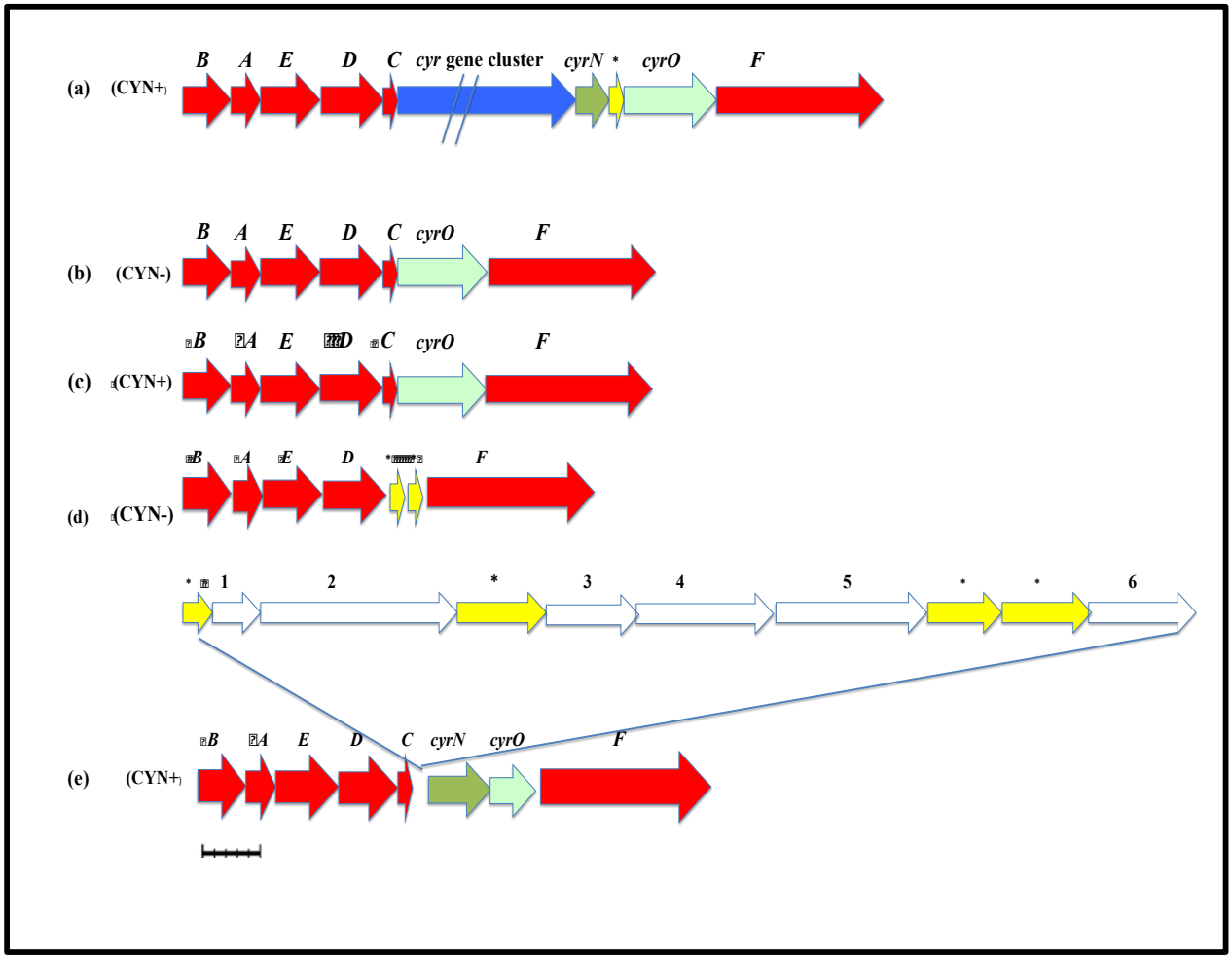

Figure 3.3: High plasticity of the *hyp* gene cluster, and its existence as an insertion site in (a) *C. raciborskii* CS-505/CS-506/AWT205, (b) *R. mediterranea* skuja FSS1-150/1, (c) *C. raciborskii* CS-509, (d) *R. brookii* D9 (e) *R. curvata* CHAB 114. Genes inserted within the *R. curvata* CHAB 114 *hyp* cluster encode an amidohydrolase (1), Cap1p (2), sulphotransferase (3), lipid A exporter (4), amidohydrolase (5), and a sulphite reductase (6). Colour code red indicates *hyp* genes, dark blue, dark green and light green indicate *cyr* genes, and yellow indicates hypothetical genes and light blue indicates genes inserted within *R. curvata* CHAB 114.

Two additional cryptic NRPS clusters (NRPS1 and NRPS2; [165], exhibited sporadic distribution amongst *C. raciborskii* and *Raphidiopsis* strains. Specifically, a cryptic NRPS cluster (NRPS1), previously identified in the non-toxin producing strain CS-509, was additionally observed in all three *Raphidiopsis* species. A truncated fragment of this enzyme was found in *C. raciborskii* strains CS-505, CS-506 and AWT205. A

second 25 kb cluster (NRPS2) was limited to the genomes of *C. raciborskii* N3, Hab151, *C.* AWT205, CS-505 and NPD, as well as *R. curvata* and *R. mediterranea*.

3.4.6 Divergence influencing factors

Nucleotide sequence based analyses revealed the tight clustering of strains based on their geographical location (Figure 3.4). *R. mediterranea* clustered with Australian isolates *C. raciborskii* CS-505, NPD and CS-509, CS-506. The two *C. raciborskii* strains from China clustered together, while the *Raphidiopsis* D9 and *C. raciborskii* T3 from Brazil grouped together, despite belonging to different genera. Clusters of orthologous genes (COG), based annotation takes into account the functional evolution the genome. Principal components analysis, utilising COG annotation, revealed the clustering of the *Raphidiopsis* sp. with C. *raciborskii* strains CS-505, CS-506, CS-509, NPD and N3.

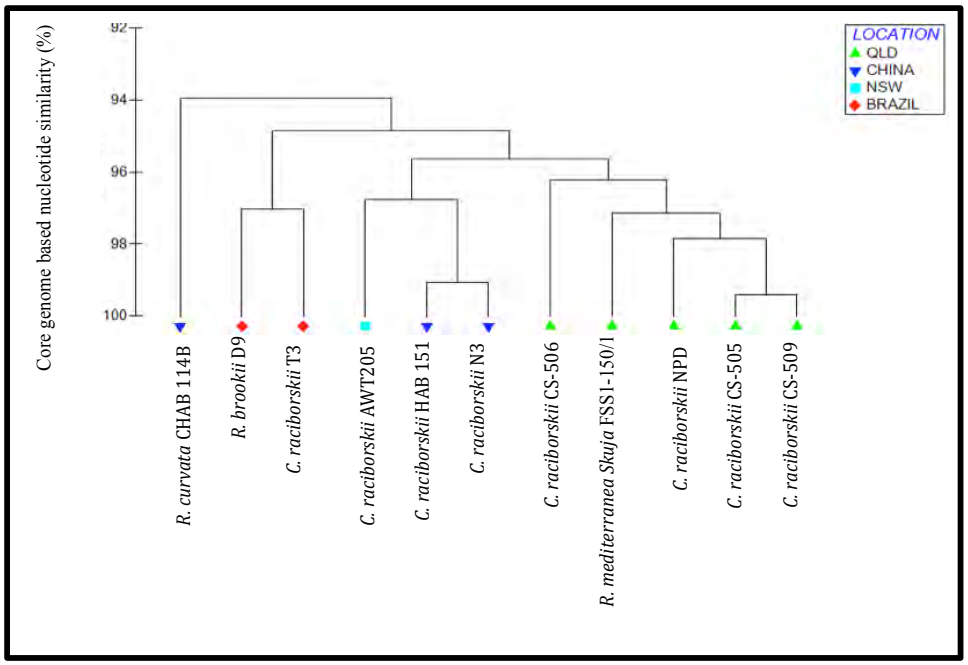

Figure 3.4: Genomic dendrogram based on core nucleotide sequence similarity (%) between study strains. Tight clustering of Australasian versus South American strains is shown (with the exception of *R. curvata* CHAB114B). For detailed information on strain origin refer to Table 3.1. NSW denotes New South Wales; QLD denotes Queensland.

Redundancy analysis was performed to test whether climatic occurrence, taxonomic identity, or toxigenicity could explain the observed clustering (Figure 3.4, 3.5). Neither geographical location (*p* value = 0.228, F = 1.4404), toxicity (*p* value = 0.184, F = 1.555) nor species (*p* value = 0.337, F = 1.0747), were reflected within the functional composition of the genomes. Clustering of strains revealed tight grouping of strains isolated from tropical/subtropical areas to each other, than to strains found in temperate (*C. raciborskii* strains Hab151, AWT205) areas. This again, was not supported statistically.

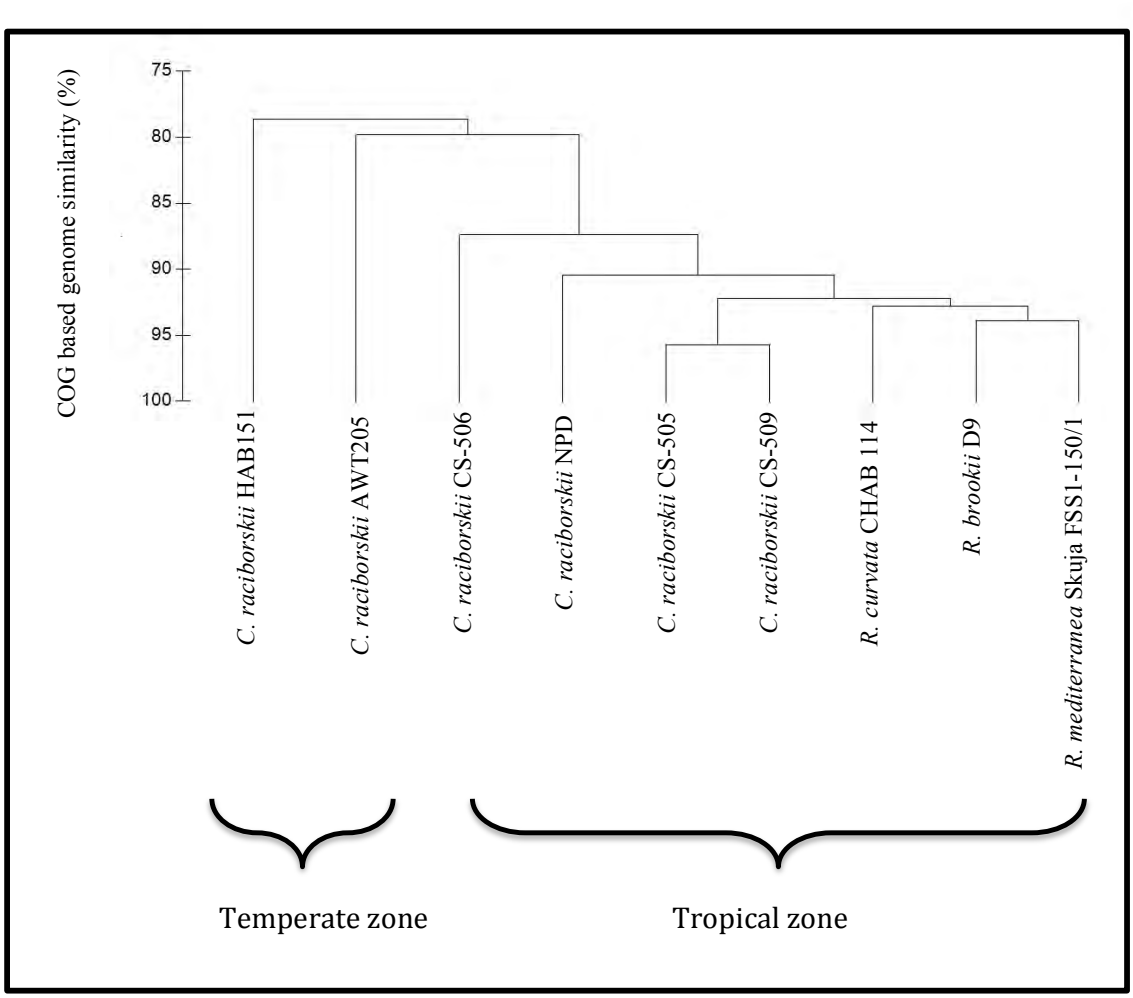

Figure 3.5: COG based genome wide similarity (%) of study strains showing clustering of tropical *Raphidiopsis* spp. and C. *raciborskii* strains CS-505, CS-506, CS-509, NPD and N3 (tropical/subtropical).

3.5 Discussion

3.5.1 The *Cylindrospermopsis-Raphidiopsis* genome complex

The *Cylindrospermopsis-Raphidiopsis* pangenome was found to conform to the 'distributed-genome hypothesis', wherein no single isolate or strain contained the complete compliment of genes (172). This phenomenon has been previously observed for the marine picocyanobacteria, *Synechcoccus* and *Prochlorococcus* (172).

At a functional level the core genome of the *Cylindrospermopsis-Raphidiopsis* complex contained a reduced subset of primary metabolic genes, suggesting either significant genome reduction [38], or rather a level of nucleotide divergence between the strains. Variation within the marine picoplankton pan-genome occurred largely within genes linked to the organisms' interaction with the environment, specifically outer-membrane proteins and transporters (7). Amongst the *Cylindrospermopsis-Raphidiopsis* complex a similar observation was made with the variable genome comprised of over 850 genes involved in phage counteraction, stress response and DNA repair. In summary, while primary metabolism appears to be conserved across the strains examined in this study, genes responsible for niche adaptation were highly variable and may allude to the existence of ecotypes within the *Cylindrospermopsis-Raphidiopsis* complex.

3.5.2 Primary metabolism, signaling and Transport

Our results confirmed the ability of the *C. raciborskii* and *Raphidiopsis* strains to carry out all essential metabolic functions, in spite of differences in toxigenicity, morphology and geographic origin. We did, however, observe the obvious loss of several metabolic genes in *C. raciborskii* strains AWT205 and Hab151, and suggest that it is unlikely that these deletions will significantly affect the metabolism of these strains. Therefore, the

deletions in carbohydrate or amino acid metabolism, or subsequent gene losses in signal transduction may not affect the functional profiles of these strains at all.

A comparative genomics study of twelve strains of *Prochlorococcus* sp. and six strains of *Synechococcus* sp. reported the loss of several genes in these organisms, which were distributed across the genomes and related to several functional categories. The study hypothesized that large losses in small effect genes would not affect the functional profile of the organisms [176]. Our results concur with this study. Strong genome wide selection and other selection pressures could possibly have resulted in the loss of genes with superfluous functions. Genome reduction is an evolutionary strategy, engaged by cyanobacteria to maximize genomic efficiency and counteract selective pressures [171]. It is also possible that the acquisition of new, more useful genes combated these gene losses, and that this accounted for the minimal change in genome size among our study strains.

Further, the genetic composition of the strains could be influenced by different selective pressures in their respective niche environments. The existence of strain specific genes or strain to strain genetic variations have been suggested by various cyanobacterial studies [165], [1]. A proteomic study on *Anabaena circinalis* AWQC131C and AWQC310F revealed that proteomic profiles of the two strains were a consequence of their ecological niches [31]. Similarly, the presence of additional ABC and cation transporters in strains *C. raciborskii* CS-505, AWT205, N3 and *R. mediterranea* skuja FSS1-150/1 could be a possible response to a more phosphate or copper rich environment. The ability to acquire genes depending on environment has been the focus of a previous study, and reiterates the plasticity of the *Cylindrospermopsis–Raphidiopsis* genomes [165].

3.5.3 Phage counteraction, metal resistance and osmotic adaptation

Phage counteraction, metal resistance and osmotic adaptation mechanisms were found to be functional in our study strains. Gene constitution for these processes again seemed to be dependent on the ecological niche.

When a phage attacks a bacterium, phage nucleic acids proliferate in the bacterial cell. This results in the death of most bacteria. A very small number of bacteria however, acquire phage-derived spacers, which confer immunity against the phage or virus. The mechanism by which phage DNA is transferred into the spacer region has not been elucidated. The immunity conferred against a phage occurs in two steps. The CRISPR RNA (Pre-crRNAs) is transcribed from the CRISPR locus, with the help of RNA polymerase. The Pre-crRNAs are then converted to small CRISPR RNAs (crRNAs) by Cas proteins. This is followed by the cleavage step, in which the crRNAs that have high similarity to an incoming foreign invasive phage DNA bind with it and cause the cleavage of the phage DNA [13].

Varying degrees of sophistication were observed in the CRISPR machinery of our study strains. The strains *C. raciborskii* Hab151 and AWT205 were found to contain a minimum number of *cas* genes and a single CRISPR *cmr* cluster, while the remaining strains contained from one to three *cas* gene sets and CRISPR *cmr* clusters. This highlights the under-developed CRISPR machinery in strains Hab151 and AWT205, compared to others that contain a more sophisticated machinery. The fact that strains Hab151 and AWT205, were both isolated from temperate geographical locations could possibly suggest lower phage infestation in these areas.

Similarly the presence of excessive aluminium and tellurite content in certain environments would compel residing strains to acquire genes to counteract metal toxicity. Mechanisms employed by cyanobacterial species to counteract metal toxicity have been the focus of previous studies [197]. Further, all the strains in this study contained trehalose encoding genes. Trehalose is found in low salt tolerant cyanobacteria [126]. The presence of trehalose biosynthesis genes, but the absence of trehalase genes suggests that trehalose is possibly used as a storage compound [83],[112] Further, salinity related betaine genes explain the recorded survival of *C. raciborskii* in up to 4 g L(-1) NaCl [106]. These genes were found in all *C. raciborskii* and *Raphidiopsis* strains.

3.5.4 Divergence influencing factors

Several contrasting ecological strategies have allowed cyanobacteria to proliferate in a wide range of habitats and ecosystems. For example, closely related but genetically distinct *Microcystis* strains known as ecotypes have evolved for survival in specific ecological niches [29]. In the present study, strains from common geographical locations were genetically similar, suggesting that the environment plays a role in shaping the genotypes of these cyanobacteria. Redundancy analyses, however, did not support the observed clustering based on either geographic location, toxigenicity or species. This may be a limitation of the small data set, and more intensive data, representing locations from all parts of the world should be collected and examined towards this end. The presence of strain-specific genes in our study group suggests that *Cylindrospermopsis* and *Raphidiopsis* strains differ in adaptation-related processes, rather than metabolic ones. A similar theory has been proposed for *M. aeruginosa* strains [1].

COG based strain clustering, on the other hand, revealed, that on a functional level, strains with dissimilar genes still have similar functional profiles. Thus, strains may acquire genes according to their geographical niche, yet, the overall function of these genes, irrespective of their geographical origin, is the same. This was strongly supported by our results, which displayed the clustering of strains of *Raphidiopsis* spp. of varying toxicities, isolated from different geographical locations of the world, within themselves, and with *C. raciborskii* strains from Australia. Similarly, nucleotide based genome clustering of study strains showed grouping of *Raphidiopsis-Cylindrospermopsis* strains based on geographic location, irrespective of which of the two species they belonged to, thereby, suggesting that *Raphidiopsis* is possibly an ecotype of *C. raciborskii.* Finally, clustering patterns also suggest the effect of climatic conditions as a factor driving strain divergence.

The two clades, *Raphidiopsis* and *C. raciborskii*, show high degrees of genetic and morphological similarity and are indistinguishable on the basis of common phylogenetic markers such as ITS, rpoc1 and 16S rRNA genes [112]. Studies have suggested the possibility of *Raphidiopsis* being a morphotype of *C. raciborskii,* and have questioned the current taxonomic classification of these organisms [112]. Our results concur with these studies, and emphasize the likelihood of *Raphidiopsis* being a morphotype of *C. raciborskii*, thus, not a separate species.

3.5.5 Rearrangement and plasticity of the *cyr/hyp* gene clusters

The *cyr* gene cluster, responsible for the biosynthesis of cylindrospermopsin (CYN) has been elucidated in *C. raciborskii, Aphanizomenon* sp.*, Oscillatoria* sp. and

Raphidiopsis sp., revealing differential arrangement of *cyr* genes in different genera [72], [172]. This is consistent with studies on *Microcystis* spp. and *Plankothrix* CYA 126, which have reported the formation of novel *mcy* cluster variants with varying gene contents, arrangement and specific domain sequence [29, 148]. Further, the absence of the *cyr* gene cluster has been reported from some strains of *C. raciborskii*. This is consistent with deletion events reported in other cyanobacteria, such as *Planktothrix* sp. and *Anabaena* sp., in which certain non-toxin producing strains lost the majority of their cyanotoxin gene clusters, retaining, in some instances, only flanking genes and remnants of transposable regions [29].

Our study reports, for the first time, the differential arrangement of genes within the *cyr* clusters of *Cylindrospermopsis* and *Raphidiopsis* strains. This observation reveals new insights into the evolution of the *cyr* gene cluster in these two genera. The *cyr* gene cluster elucidated previously comprises genes responsible for the complex biosynthesis of the CYN, namely an amidinotransferase (*cyrA*) , a NRPS/PKS hybrid gene (*cyrB*), four PKS genes (*cyrC, cyrD, cyrE* and *cyrF*), amidohydrolases (*cyrG* and *cyrH*), as well as genes for tailoring reactions (*cyrI, cyrJ*, and *cyrN*), putative transport (*cyrK*), and regulation (*cyrO*). It also contains two transposase genes (*cyrM* and *cyrL*; [104]), indicating the potential for the horizontal transfer of toxicity [104].

Genomic analysis, undertaken here, was unable to differentiate between these two genera, although it is clear that a common ancestor exists [171]. It is likely that this common ancestor more closely resembled *Cylindrospermopsis*, in that it would have possessed terminal heterocysts and the *nif* operon. However, whether acquisition of, and rearrangements within the *cyr* gene cluster occurred prior to its separation into two distinct genera is not clear. The presence of differential arrangements of the cluster in both *Raphidiopsis* and *Cylindrospermopsis* support inter-generic transfer of these large

genomic fragments between the two organisms, an event made possible by their continuing co-occurrence within water bodies. Such a mechanism would not only support the evolution of these clusters within these organisms, but more generally, the large level of homology between the genomic compositions of these organisms.

Regarding the specific arrangement of the *cyr* cluster, there is insufficient evidence to conclude which arrangement was the progenitor. Co-linearity, where the order of the genes within the operon mirrors the order that the enzymes are utilized during biosynthesis, is frequently observed amongst biosynthetic pathways. Typically, it would be suggested that clusters would evolve towards co-linearity to streamline expression of multiple components of the pathway at critical times. However, multiple recombination events in the *cyr* gene cluster are permissible assuming no phenotypic response is observed. After examining the *hyp* gene cluster, which flanks the *cyr* gene cluster in most strains studied so far, we have found evidence of an insertion event, preceding arrival of the *cyr* cluster into this locus. In *Raphidiopsis curvata* CHAB 114, nine genes including *cyrN* and *cyrO* were inserted into the *hyp* cluster. Subsequently, as evident in *C. raciborskii* strains CS-505, CS-506, NPD and *R. mediterranea*, skuja FSS1-150/1, seven of the nine genes present in the insertion site were replaced by the *cyr* gene cluster. Again it is unclear whether in this instance the *cyr* cluster was translocated to the insertion site from another site within the genome or acquired horizontally. This also supports the inference that *cyrN* and *cyrO* are not part of the *cyr* gene cluster, but merely remnants of the previous insertion event. The presence of *cyrO* within the *hyp* operon in *C. raciborskii* N3 (CYN+), CS509 (CYN-) and T3 (STX+) could have been a second recombination event in which *cyrO* was inserted independently of the *cyr* gene cluster or any other genes. Thus, the plasticity of the *hyp*

and *cyr* gene clusters could possibility be attributed to a combination of genetic events that include horizontal gene transfers, deletions and translocations.

3.6 Conclusion

C. raciborskii and *Raphidiopsis* sp. are invasive, toxin-producing organisms, prevalent worldwide. In order to curb and regulate their spread and toxin production, it is imperative to understand the genetic makeup of these strains, and identify the drivers that influence them. Our study highlights the conservation of minimal primary metabolism gene sets, required for the efficient functioning of these strains. We also discuss gene losses and how these may not significantly affect the functional profiles of these organisms. Our study is the first to show the geographical clustering of *C. raciborskii* and *Raphidiopsis* spp. based on whole genome nucleotide identity. We further show tight COG based clustering of strains of *Raphidiopsis* spp. of varying toxicities, which are isolated from different regions worldwide, thereby, highlighting the magnitude of their functional similarity. Our results also suggest the influence of climatic zones of origin as a factor driving strain divergence. Although, nucleotide and COG based clustering was not supported statistically, we recommend this analyses be repeated with larger datasets. This study also shows the extent of plasticity of the *Cylindrospermopsis-Raphidiopsis* genomes, in particular, the *cyr* gene cluster, and its tendency to rearrange without the loss of toxicity. Thus, we emphasize the importance of studying the genomic framework of the two species and caution water managers against the severe threat they pose to water bodies worldwide.

Chapter 4

Nutrient-related changes in ecotype dominance drives toxicity of field blooms of the cyanobacterium *Cylindrospermopsis raciborskii*

Author contributions

Rati Sinha carried out the DNA extractions, primer design and validation, qpcr experiments and data analyses, and wrote certain sections of the manuscript. Dr. Michele Burford, Dr. Phil Orr and Dr. Brett Neilan designed the experiment and helped in its co-ordination. Dr. Michele Burford and Dr. Phil Orr also contributed to the writing of the manuscript. Dr. Timothy W. Davis set up the mesocosm apparatus, while Dr. Anusuya Willis helped in the analyses of results.

4.1 Abstract

Nutrients have the capacity to change cyanobacterial toxin loads via growth-related toxin production, or shifts in the dominance of toxin producing and non-toxin producing strains [127], [32]. This study examined the effect of nitrogen (N) and phosphorus on growth and strain-related changes in production of the toxins, cylindrospermopsins (CYNs) by the cyanobacterium, *Cylindrospermopsis raciborskii*.

Two short-term experiments were conducted with mixed phytoplankton populations dominated by *C. raciborskii* in a subtropical reservoir where treatments had nitrate (NO3), urea (Ur) and inorganic phosphorus (P) added alone or in combination. Growth rates of *C. raciborskii* were only statistically higher than the control on day 5 when Ur and P were co-supplied. In contrast, cell quotas of CYNs (Q_{CYNS}) increased significantly in treatments where P was supplied, irrespective of whether N was supplied, and this increase was not necessarily related to growth rates. Increased Q_{CYNS} did correlate with an increase in the proportion of the *cyr*A toxin to 16S genes in the *C. raciborskii* population. Therefore changes in strain dominance are the most likely factor driving changes in toxin production between treatments. Our study has demonstrated differential effects of nutrients on growth and strain dominance reflecting a *C. raciborskii* population with a range of strategies in response to environmental conditions.

4.2 Introduction

Much of our understanding of the effect of nutrient loading on the proliferation of toxin producing cyanobacterial species has focussed on the ubiquitous genus, *Microcystis,* which can produce microcystins. Nutrient addition may promote production of microcystins by stimulating growth [127], by causing growth-related changes in the cell quota of microcystins, or by changing the relative concentrations of toxin producing and non-toxin producing strains within mixed bloom populations [76], [32].

There is limited information on the effect of N and P loading on the production of toxins by the N-fixing cyanobacterium *C. raciborskii.* This species dominates lakes

and reservoirs in temperate and tropical environments including Australia [56], North America [26], [63], South America [44], Europe [42], [20], Africa [105] and Asia [28] and New Zealand. There are indications of range expansion in response to increasing global temperatures.

C. raciborskii can potentially produce a suite of harmful metabolites called cylindrospermopsins (CYNs) and saxitoxins (STXs), although the ability to produce them is not universal. Some Australian strains of *C. raciborskii* produce CYNs, while some South American strains produce STXs [122]. CYNs have been linked to a major intoxication incident on Palm Island, Queensland, Australia in 1979 when 149 people became ill following ingestion of drinking water contaminated with CYNs. Most were children, and many required hospitalisation [14], [56]. Conversely there is little evidence of the northern hemisphere strains producing toxins, although recently CYNs have been correlated with *C. raciborskii* occurrence in an Italian lake [103], and a strain from Portugal was found to be toxic by mouse bioassay, but didn't produce any of the known cyanotoxins [43].

Previous laboratory studies have shown that production of CYNs is affected by nutrients. For example, a study by Saker and Neilan (2001) found highest CYNs concentrations when *C. raciborskii* was grown in the absence of a fixed N source, and lowest concentrations when grown on ammonium, even though growth rates were highest on the ammonium source. The transcriptional response of the *cyr* genes, responsible for CYNs production, was examined in response to four different N sources. Transcript levels remained consistent during the experiment indicating constitutive expression under these conditions [171]. Studies of another CYNs producer, *Aphanizomenon ovalisporum* (Forti) found that P-starvation decreased

CYNs cell quotas [6]. In contrast, Bar-Yosef *et al.* (2010) showed increased Q_{CYNS} production in *A. ovalisporum* under P starvation. Therefore, there is inconsistency in findings relating to the effect of nutrients on CYNs production between and within species, and this highlights the need for further examination of the role of nutrients in affecting toxin production.

Therefore, the present study examined the effect of added N in two forms (NO3, Ur), and P on growth, Q_{CYNS}, as well as the relative proportion of the CYNs gene (*cyr*A vs. 16s) in mixed phytoplankton populations dominated by *C. raciborskii*. The aim was to determine which nutrient forms affect growth and CYNs production, and the relative importance of two mechanisms in driving changes in bloom toxicity – specifically, growth-related impacts on CYNs production at the cellular level, and shifts in strain dominance at the population level.

4.3 Materials & methods

4.3.1 Study sites

The study was conducted at North Pine Reservoir (Lake Samsonvale, 27° 15′ S, 152° 55′ E), which is located in subtropical, southeast Queensland, Australia. This reservoir is a source of drinking water for the city of Brisbane and several regional towns and cities close by. It has an area of 21.8 km^2 and 215,000 ML at full supply volume. It is a warm, monomictic reservoir that is stratified in the summer months with a surface mixed layer of about 5 m, and is well mixed during the winter months following overturn in autumn [24]. The watershed for this reservoir is typically rural with some peri-urban development, and is dominated by cattle grazing pasture (>50%), with approximately 20% natural vegetation [24]. An artificial destratification system was

installed in the lower reaches of the reservoir and began operating in 1995. It is typically switched on in spring and runs over the summer each year when the reservoir is stratified.

4.3.2 Experiments

Two mesocosm experiments were conducted over two 5 d periods during the austral summer (December 2010) in a protected inlet in the central region of the reservoir using the mesocosm design and protocol described by Muhid *et al.* (2013). The mesocosms consisted of 0.5 m × 0.5 m × 3 m deep polyethylene bags with a total volume of approximately 800 L and supported by 2 m × 2 m frames at the surface. Bird netting was placed over the tops of the frames to prevent birds from disturbing the experimental apparatus.

At the commencement of each experiment, each mesocosm was filled using a submersible pump with water from 0.5 m below the surface but within the surface mixed layer [4]. There were six treatment mesocosms (n=3): 1. control; 2. daily additions of P (9.3 μg L^{-1} potassium dihydrogen phosphate, AR grade); 3. daily additions of Ur (35 μg L^{-1} urea, AR grade); 4. daily additions of NO3 (70 μg L^{-1} potassium nitrate AR grade); 5. Ur + P (35 + 9.3 μg L^{-1}); and 6. NO3 + P (70 + 9.3 μg L^{-1}). These concentrations were chosen as they were two to three times higher than the mean background nutrient concentrations in surface waters of the reservoir during the summer stratified period [23]. The mesocosms were operated as semi-continuous cultures with nutrients added after sampling each day, and mixed gently with a Secchi disc.

4.3.2.1 Sampling

Physico-chemical profiles were measured mid-morning each day in each mesocosm and in the adjacent ambient water using a calibrated data SONDE (YSI 6920). The parameters measured were water temperature, dissolved oxygen, pH, conductivity and turbidity. Secchi depth was measured manually.

A 5 L subsample of water from each mesocosm was collected daily after physico-chemical profiling using a 3 m depth-integrated hose-pipe sampler. The hose-pipe sampler was rinsed with adjacent water between each mesocosm to ensure that there was no carryover of nutrients between different treatments. A subsample of water was collected for total N (TN) and total phosphorus (TP). Another subsample was filtered through a 0.45 μm membrane filter (MILLEX-HA/HV), and filtrate were immediately frozen at -20°C for later analysis of total dissolved N (TDN) and P (TDP), as well as P, ammonium, NO3 + nitrite, and Ur.

Subsamples of water were also stored on ice until returned to the laboratory for a range of analyses. Water was filtered through glass fibre filters (ADVANTEC, GF75) using gentle filtration for analysis of chlorophyll a concentrations. The filters were stored frozen at -80^{o}C until analysed. Sub-samples for DNA analysis were collected on 47 mm polycarbonate filters (2 μm pore size), snap-frozen in liquid N and stored at -80 until analysed. Sub-samples for CYNs analysis were filtered onto glass fibre filters (ADVANTEC, GF75) using gentle filtration, and filters frozen for CYNs analysis. Sub-samples were also fixed with Lugol's iodine solution (2% final concentration) for algal community identification and enumeration.

4.3.2.2 Sample analyses

Samples for P, ammonium, NO3 + nitrite concentrations were analysed using a Discrete Chemistry Analyser (SmartChem 200, WESTCO Scientific Instruments Inc., Brookfield) (American Public Health Association, 1995). TDN and TDP samples were digested using a simultaneous persulfate digestion method for N and P [65] before being analysed on the flow injection analyser (LACHAT 8000QC). Particulate P (PP) and N (PN) were determined by subtracting TDP and TDN from TP and TN respectively. Dissolved organic P (DOP) was determined by subtracting the inorganic form of P from TDP. Dissolved organic N (DON) was determined by subtracting the inorganic forms of N (ammonium, NO3 + nitrite) from TDN.

Chlorophyll a concentrations were determined spectrophotometrically by sonicating the glass fibre filters in 90% acetone on ice to extract the pigments. The sonicated extracts were kept at -20 °C for 24 h to ensure all pigments had eluted into the acetone. After this time, the samples were spun in a desktop centrifuge for 5 min at 800 x g (~2,000 rpm; Eppendorf, Germany) allowed to warm to room temperature in the dark, filtered through a glass fibre filter filters (ADVANTEC, GF75) to remove any particulates and absorbances were measured at 750, 665, 664, 647 and 630 nm [71].

Enumeration of *C. raciborskii* and total cell counts were conducted microscopically using a Sedgewick Rafter cell under a compound microscope (LEICA DM 4000) with 400× magnification [193]. Enumeration ceased after a minimum of 23 units (trichomes/filaments or colonies) or a maximum of 100 squares or 100 units were counted. Heterocysts, which were almost exclusively present on *C. raciborskii*, were also quantified microscopically. The total phytoplankton and *C. raciborskii* biovolumes for the common phytoplankton species found in Lake Samsonvale were

calculated using data from an adjacent reservoir where cell dimensions had been measured for multiple cells, and the average biovolume calculated using published equations [61]. The cell specific division rates (μ_c) were calculated over the five days of the study using first order rate kinetics:

$$\mu_c = \frac{\ln(C_5) - \ln(C_0)}{T_5 - t_0}$$

where C_0 and C_5 are the cell concentrations at the beginning and after five days respectively, and T_5 and T_0 are days 5 and 0 respectively.

4.3.2.3 CYNs sample processing

Samples for CYNs (cylindrospermopsin and deoxycylindrospermopsin) analysis were prepared as described previously by Orr *et al.* (2010). Briefly, filters containing particulate CYNs were sonicated in 10 mL of 50 mmol L^{-1} aqueous acetic acid and centrifuged. The extracted samples were filtered through a 0.45 µm filter (MILLEX-HA/HV) to remove any particulate matter. A 1 mL subsample of the filtrate was analysed for CYNs using a Shimadzu Prominence HPLC – MS/MS (Shimadzu Corp., Kyoto, Japan) using an AB/Sciex API4000Q (Applied Biosystems, Foster City, CA) mass spectrometer equipped with an electrospray (TurboV) interface (modified from Eaglesham *et al.*, 1999). The retention times for CYN and deoxy-CYN were 6.8 and 7.0 min respectively. The limit of detection was <0.2 µg L^{-1} and the response is linear to 500 µg L^{-1} using a 60 µL injection volume. CYNs concentrations were divided by the cell concentrations of *C. raciborskii* to determine Q_{CYNS}. There were either low densities or no other known producers of CYNs in the treatments so it was assumed that CYNs were only produced by *C. raciborskii.*

4.3.3 Molecular methods

Prior to DNA extraction, filtered samples were supplemented with 10 µg pGem plasmid, which served as an internal control for PCR inhibition and extraction efficiency. High molecular weight DNA was extracted as previously described by Morin *et al.* (2010). Briefly, cell walls were disrupted mechanically, lyzed enzymatically, extracted and precipitated before washing, air drying and resuspending in sterile DNAse free MilliQ water (Millipore, USA). DNA samples showing 260/280 and 260/230 nm absorbance values ranging from 1.8 -2.0 were considered pure and used for qPCR experiments.

The quality of the DNA samples was checked by PCR amplification of the 16s rRNA gene, using cyanobacterial 16S specific primers 27FL/809R (Table 1). PCR was performed on a MyCycler^{TM} Thermal Cycler (Bio-Rad, USA) in standard 20 µL volumes. Template genomic DNA of approximately 5 ng μL^{-1}, 10 picomoles of the forward and reverse primers, 0.2 U of Taq DNA Polymerase (Bioline, USA), 2.5 mmol L^{-1} magnesium chloride and 200 µmoles L^{-1} dNTPs was also used. PCR was performed using the following conditions: initial denaturation 94°C for 2 min, subsequently followed by 30 cycles of denaturation of 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min. Appropriate amplification of PCR amplicons was confirmed by visualization using agarose gel electrophoresis, where bands of 800 bps were detected.

4.3.4 Primer validation

Primers targeting the *cyrA* gene (Table 4.1) of the CYNs biosynthesis cluster, in all CYNs producing cyanobacteria were designed using Primer Blast (NCBI). Primers

were checked for primer dimer formation and cross dimer formation using Primer 3. Primer validation was carried out by PCR.

Target gene	Primer	Sequence	Reference
cyrA	cyrAF	TTGATTGTACTTTTGTCCCCTT	This study
cyrA	cyrAR	GCGAAAGGGAATTGGATAG	This study
pGEM	M13F	CCCAGTCACGACGTTGTAAAAACG	(Coyne et al., 2005)
pGEM	pGEMR	TGTGTGGAATTGTGAGCGGA	(Coyne et al., 2005)
16s *rRNA*	JRT-16SF	AGCCACACTGGGACTGAGACA	(Al-Tebrineh et al., 2010)
16s *rRNA*	JRT-16SR	TCGCCCATTGCGGAAA	(Al-Tebrineh et al., 2010)
16s *rRNA*	27FL	AGAGTTTGATCCTGGCTCAG	(Neilan et al., 1997)
16s *rRNA*		GCTTCGGCACGGCTCGGGTCGATA	(Jungblut et al., 2005)

Table 4.1: Primers used for PCR and qPCR analyses.

PCR was performed on a MyCycler^{TM} Thermal Cycler (Bio-Rad, USA) in standard 20ul volumes. Template genomic DNA of approximately 5ng μL^{-1}, 10 picomoles of the forward and reverse primers, 0.2 U of Taq DNA Polymerase (Bioline, USA), 2.5 mmol L^{-1} magnesium chloride and 200 µmoles L^{-1} dNTPs was also used. Isolates with and without the *cyrA* gene, i.e. positive (CS-505, CS-506) and negative (CS-509, CS-510) controls were used. These were strains of *C. raciborskii* obtained from the CSIRO culture collection in Hobart, Tasmania, Australia. Cultures were maintained in Jaworski Medium (JM) at 28 °C on a 12:12 light:dark cycle at 10 µmol $m^{-2}s^{-1}$ using a cool white fluorescent light. PCR was performed using the same conditions as outlined above.

PCR amplification yielded amplicons of expected size of 280 bps, in the toxin producing strains CS-505 and CS-506, but showed no amplification in non-toxin strains CS-509 and CS-510. A negative control also showed no amplification. This showed the specificity of the primer set, and the absence of unspecific amplification

and binding. Sanger sequencing was performed on the amplicons to confirm that the amplicon was *cyrA*.

4.3.5 Quantitative real-time PCR (qpcr)/ molecular analyses

Two-step real-time PCR experiments were performed in a 72-well Rotor gene 3000 system (Corbett Life Sciences), in order to investigate the proportions of 16s vs. *cyrA* genes from *C. raciborskii* cells. Primers were developed that were specific for CYNs producing cyanobacteria, this enabled relative quantification of non-toxin producing and CYNs producing *C. raciborskii* within the mesocosms (Table 4.1).

Standard curves of each primer set (*cyrA, pGEM,* 16S *rRNA*) were constructed in order to confirm comparable amplification efficiencies of the target and the reference genes respectively. PCR products generated by the amplification of *C. raciborskii* CS505 DNA were precipitated with 2 volumes of ethanol and washed with 70% ethanol, and used as template for the standard curve. This purified genomic DNA was diluted to 3ng μl^{-1}, and further 8 fold serial dilutions were made. Triplicate qPCR reactions consisted of 5 µL SensiFast TM Sybergreen No-ROX Mix 2x (Bioline pty. Ltd), 10 pmol of forward and reverse primers for each primer set, and made up to 10 µL with sterile MQ were used. For unknown samples 5 ng of template DNA was used for each reaction. Cycling conditions *for cyrA,* 16S and *pGEM* primers included an initial denaturation of 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s,

followed by a melt-curve cycle of 0.5 °C increases at 15 s intervals, as per the Corbett Life Sciences pre-set program.

The relative change in proportion of toxin producing *C. raciborskii* was calculated using the ΔΔCT method of relative quantification.

4.3.6 Statistical analyses

Data were tested for normality and transformed as required. One-way ANOVAs were performed using the statistical program, SAS (Version 9.2), to determine significant effects of the treatments on physicochemical parameters, nutrients, chlorophyll *a,* *C. raciborskii* cell concentrations, Q_{CYNS} and the proportion of toxin genes ($\Delta\Delta C_T$ *cyr*A vs. 16s). Where significant ($P<0.05$), a Fischer's post-hoc test was used to test for treatment differences.

4.4 Results

4.4.1 Physicochemical parameters

Mean morning water temperatures remained relatively constant over the five days of each experiment at 26.7 ± 1.0 and 25.9 ± 0.8 °C for Experiments 1 and 2 respectively. Similarly, conductivity was also constant at 0.18 ± 0.01 and 0.17 ± 0.02 mS cm-1 respectively (Table 2). In both experiments, dissolved oxygen concentrations, pH, and turbidity were typically higher and Secchi depths were statistically lower in the NO3 + P and Ur + P treatments compared to the control and other treatments over the duration of the experiment. TN concentrations were statistically higher in the NO3, Ur, NO3 + P and Ur + P treatments.

Expt	Treatment	Temp. (°C)		Cond. (mS cm^{-1})		DO (mg L^{-1})		pH		Turbidity (NTU)		Secchi (m)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	Ambient	26.5	1.1	0.17	0	8.7	0	8.7	1	3.5	1	1.1	0
	Control	26.6	1	0.17^{B}	0	8.4^{B}	1	9.0^{C}	0	2.9^{D}	0.3	1.0^{A}	0
	NO3	26.5	1	0.18^{A}	0	8.9^{B}	1	9.1^{C}	0	3.6^{CD}	0.6	$0.9A^{B}$	0
	Ur	26.7	1	0.18^{B}	0	8.8^{B}	1	9.1^{BC}	0	3.7^{B}	0.8	0.9^{BC}	0
	P	26.7	0.9	0.17^{B}	0	8.4^{B}	1	9.1^{BC}	0	3.2^{BC}	0.5	0.9^{BC}	0
	NO3+P	26.8	1	0.18^{A}	0	10.0^{A}	1	9.4^{A}	0	4.8^{A}	1.4	0.8^{C}	0
	Ur +P	26.7	1.1	0.18^{A}	0	9.7^{A}	1	9.4^{AB}	0	5.0^{A}	1.4	0.8^{C}	0
2	Ambient	26	0.8	0.17	0	7.4	1	8.6	0	3.7	0.3	0.9	0
	Control	25.9	0.8	0.17^{C}	0	8.0^{A}	1	9.2^{A}	0	4.1^{D}	0.2	0.8^{A}	0
	NO3	25.9	0.8	0.17^{BC}	0	8.4^{A}	1	9.3^{AB}	0	4.5^{CD}	0.7	0.7^{AB}	0
	Ur	25.9	1.3	0.17^{C}	0	8.4^{A}	1	9.3^{AB}	0	5.0^{BC}	0.8	0.7^{AB}	0
	P	25.9	0.8	0.17^{C}	0	8.2^{A}	1	9.3^{AB}	0	4.6^{CD}	0.7	0.7^{AB}	0
	NO3+P	25.9	0.8	0.17^{A}	0	9.3^{B}	1	9.5^{B}	0	5.8^{AB}	1.6	0.6^{B}	0
	Ur +P	25.9	0.8	0.17^{AB}	0	9.4^{B}	1	9.5^{B}	0	6.0^{A}	1.5	0.6^{B}	0

Table 4.2: Mean (SD) of physico-chemical parameters measured mid-morning in the treatments across the five days and the adjacent water (Ambient) on day 0 in all three experiments. Cond. = specific conductivity, DO = dissolved oxygen and Temp. = water temperature. A, B, C denotes where there are statistical differences between treatments ($P<0.05$).

Expt	Treat	Day	TN mg L^{-1}		TP mg L^{-1}		Ammonium mg L^{-1}		P mg L^{-1}		NO3/nitrite mg L^{-1}		U mg L^{-1}		DON mg L^{-1}		DOP mg L^{-1}		DN:P ratio	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	Ambient	0	0.53	0.1	0.02	0	0.01	0.001	0.01	0	0.01	0	0.01	0	0.29	0.02	bd		14.9	3.1
	Control	5	0.52	0	0.02	0	0.01	0.003	0	0	0.02	0.01	0.01	0	0.3	0.01	bd		7.7	
	NO3	5	0.84	0	0.02	0	0.01	0.001	0	0	**0.2**	0.03	0.01	0	0.3	0.02	bd		115.5	53
	Ur	5	0.82	0.1	0.02	0	0.09	0.011	0	0	0.01	0	0.04	0	**0.33**	0	bd		94.2	17
	P	5	0.58	0	0.07	0	0.01	0.001	0	0	0.01	0	0.01	0	0.28	0.02	bd		9.6	1.8
	NO3+P	5	0.89	0	0.07	0	0.01	0.001	0	0	0.01	0	0.01	0	0.32	0.01	0.01	0	9.2	1.4
	Ur +P	5	0.83	0.1	0.06	0	0.01	0	0	0	0.01	0	0.01	0	0.33	0.01	0	0	9	0.3
2	Ambient	0	0.53	0.1	0.02	0	0.01	0.001	0	0	0.01	0	0.01	0	0.27	0.02	bd		13.3	3.3
	Control	5	0.58	0	0.02	0	0.01	0.001	0	0	0.01	0	0.01	0	0.27	0.02	bd		11.1	
	NO3	5	0.85	0.1	0.02	0	**0.02**	0.015	0	0	**0.14**	0.03	0.01	0	0.29	0.02	bd		91.9	17
	Ur	5	0.77	0.1	0.02	0	0.08	0.021	0	0	0.01	0	0.01	0	0.26	0.03	bd		56	17
	P	5	0.66	0.1	0.06	0	0.01	0.001	**0.01**	0	0.01	0	**0.01**	0	0.26	0.02	bd		9.1	2.8
	NO3+P	5	0.89	0	0.06	0	0.01	0.001	0	0	0.01	0	0.01	0	0.27	0.03	bd		9.1	0.8
	Ur +P	5	0.86	0.1	0.07	0	0.01	0.001	0	0	0.01	0	0.01	0	0.28	0.06	bd		11.7	2.7

Table 4.3: Mean (SD) nutrient concentrations (mg L-1) in all treatments on day 5 and adjacent waters (Ambient) in day 0 in both experiments. Treat = treatment, bd = below detection. Detection limit for dissolved nutrients is 0.002 mg L-1. DN = ammonium + NO3 + Ur concentrations. Bold designates significantly different from control ($P<0.05$)

compared with the control in both experiments by day 5 (Table 4.3). TP concentrations were statistically higher in the P, NO3 + P and Ur + P treatments compared with the control in both experiments. Ammonium concentrations were close to the detection limit (0.002 mg L^{-1}) in most treatments by day 5, but substantially higher in the U treatments in both experiments, and also in the NO3 treatment in Experiment 2 (Table 4.3). NO3 concentrations were highest in the NO3 treatment on day 5. Urea concentrations were close to the detection limit (0.002 mg L^{-1}) with the exception of the Ur treatment in Experiment 1 on day 5. P and DOP concentrations were also near detection limits (0.002 mg L-1). DON concentrations (including Ur) were similar between treatments, except in the Ur treatment in Experiment 1 where it was higher on day 5. Dissolved N:P ratios were very high compared with Redfield (1958) atomic ratio of 7.2:1 in the NO3 and Ur only treatments in both experiments on day 5 while dissolved N:P ratios were similar and lower than Redfield (1958) ratios in the P only, Ur + P and NO3 + P treatments.

4.4.2 Chlorophyll a, biovolume and particulate nutrients

Chlorophyll a concentrations were significantly higher ($P<0.05$) in the NO3, Ur and P treatments compared with the control, and higher still in the NO3 + P and Ur + P treatments in both experiments by day 5 (Figure. 4.1). Chlorophyll *a* concentrations were highly correlated with the total biovolume of the phytoplankton community, as calculated from the cell count data ($R^2 = 0.68$). Based on changes in chlorophyll *a* concentrations, the phytoplankton population growth rates over the study were calculated to be highest in the Ur + P and NO3 + P treatments, i.e. 0.20 to 0.22 d^{-1} in Experiments 1 and 2 respectively.

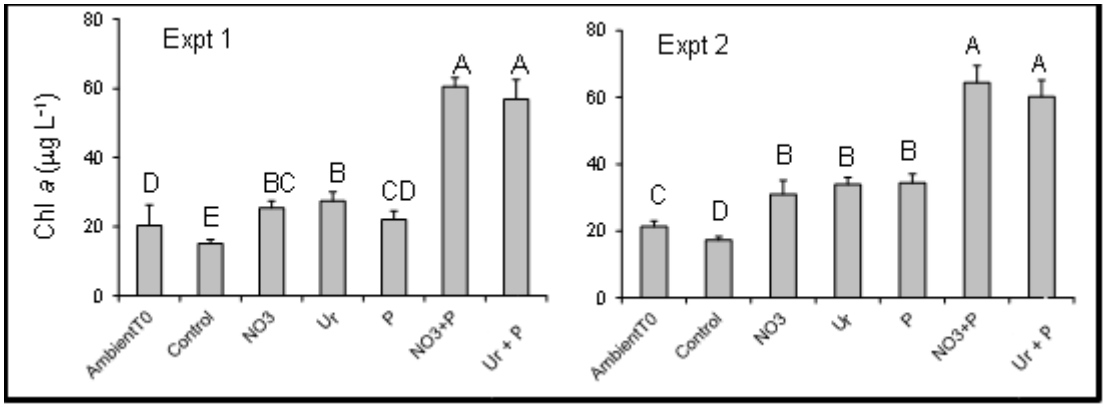

Figure 4.1: Mean (+ SD) chlorophyll *a* (Chl *a*) concentrations (µg L^{-1}) in adjacent waters on day 0 (AmbientT0) and treatments on day 5 in two experiments. A, B, C, D denote statistical differences ($P< 0.05$).

Chlorophyll *a* concentrations increased as PN concentrations increased, while PP concentrations in the P treatment increased without a commensurate increase in chlorophyll *a* concentrations (Figure 4.2). Only in the both Ur + P and NO3 + P treatments did PP, PN and chlorophyll *a* concentrations increase. The PN:PP ratio in the Ur + P and NO3 + P treatments were higher than Redfield's (1958) atomic ratio of 7.2:1. The PN:PP ratios in the P treatment were similar or slightly lower than 7.2:1, while the ambient, control and N only treatments all had substantially higher ratios population measures and CYNs.

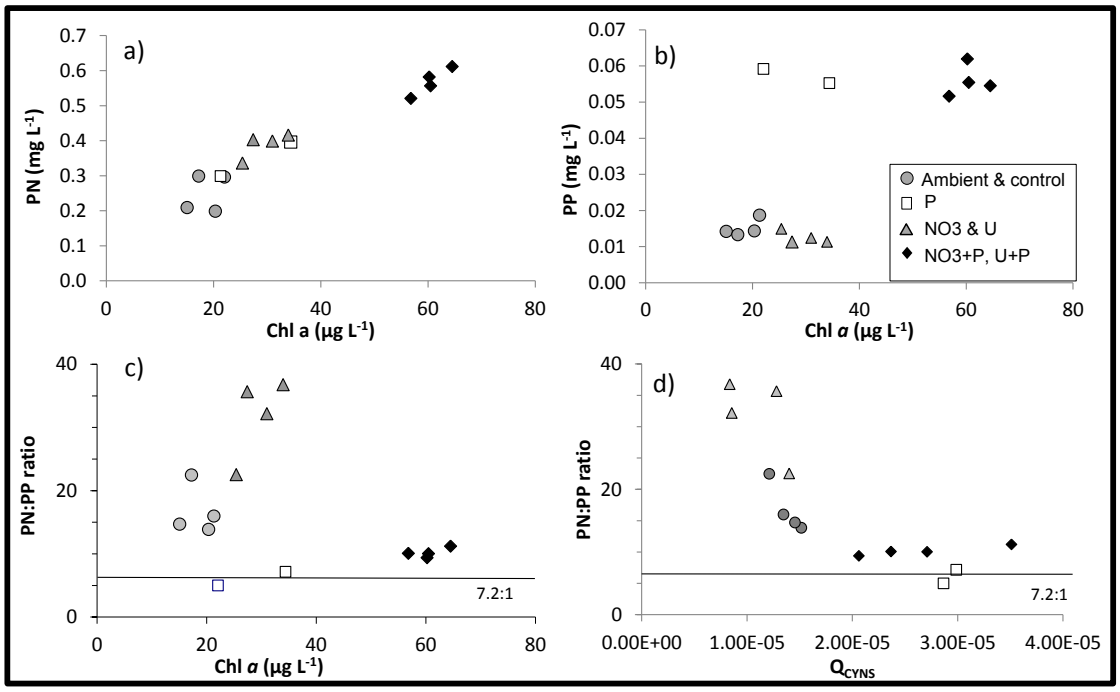

Figure 4.2: Chlorophyll *a* concentrations (µg L^{-1}) versus a) PN, b) PP, c) PN:PP ratios, and d) Q_{CYNS} vs. PN:PP ratios across all treatments for both experiments after 5 d.

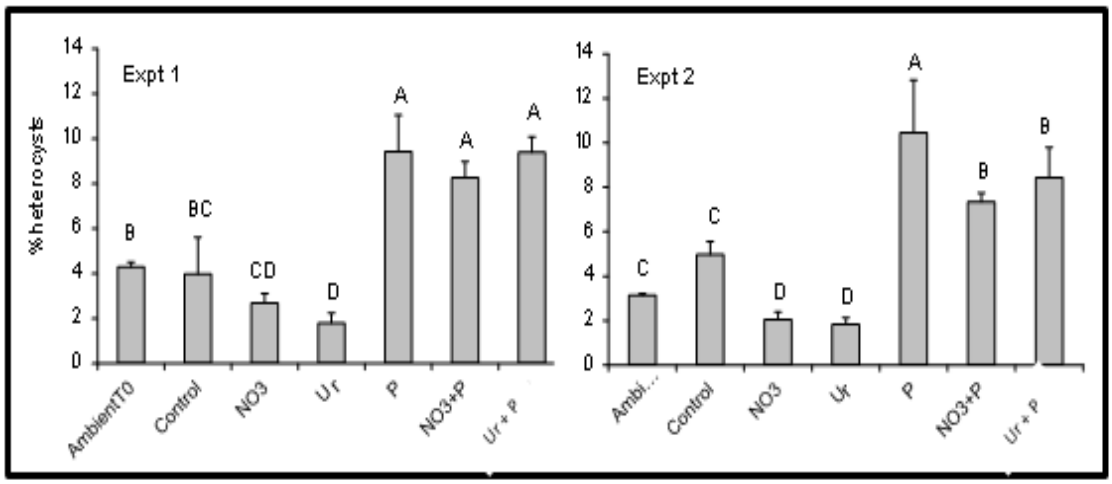

Figure 4.3: Mean (+ SD) cell concentrations (cells ml^{-1}) and growth rates of *C. raciborskii* (d^{-1}), in adjacent waters on day 0 (AmbientT0), and ambient and treatments on day 5 in two experiments. A, B, C, D denote statistical differences ($P < 0.05$).

Growth of *C. raciborskii* occurred in all treatments over the five days in both experiments. Cell specific division rates ranged from 0.04 to 0.13 d^{-1} in the control and Ur + P treatments respectively in Experiment 1, and from 0.09 to 0.19 d^{-1} in the control and Ur + P treatments respectively, in Experiment 2 (Figure 4.3). This resulted in significantly higher cell concentrations in the Ur + P treatment. However, cell concentrations were not significantly higher in the NO3, Ur or P alone, or NO3 + P treatments compared with the control after 5 d (P>0.05, Fig. 3). In Experiment 2, once again only the Ur + P treatment was higher than the control after five days.

The proportion of heterocysts in the *C. raciborskii* cell population was statistically higher in the P alone, NO3 + P and Ur + P treatments compared with the control by day 5 (P>0.05, Figure 4.4). The Ur treatment had a lower proportion of heterocysts than the control after 5 days in both experiments, as well as the NO3 treatment in the second experiment.

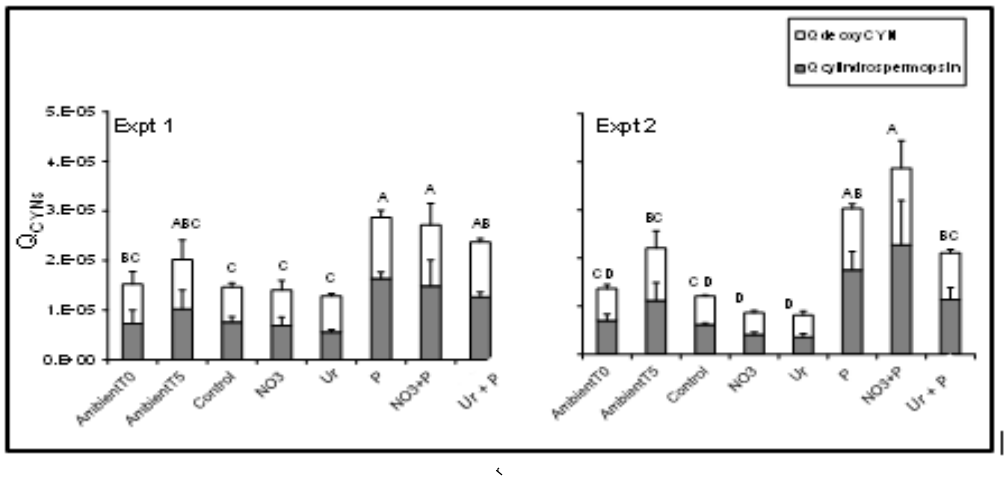

Figure 4.4: Mean (+ SD) percentage heterocysts on filaments of *C. raciborskii* in adjacent waters on day 0 (Ambient) and treatments on day 5 in two experiments. A, B, C, D denote statistical differences (P< 0.05).

It was the species with the highest phytoplankton biovolume at the start of the experiment, constituting 30 - 33% of the biovolume (Table 4.4). This increased to 42-

45% in the control by day 5. In Experiment 1, only the P only treatment on day 5 had a significantly higher percentage of *C. raciborskii* than on day 0 (47% compared with 30% respectively). In Experiment 2, the control, Ur, NO3 and P alone and Ur + P treatments on day 5 were significantly higher than day 0, as high as 51%. The other dominant genera at the start of the experiment were diatoms, e.g. *Synedra, Achnanthidium,* as well as another cyanobacterium, *Planktolyngbya,* and a range of genera in other classes, i.e. chlorophytes, chrysophytes and cryptophytes (Table 4.4). Other than *C. raciborskii,* the chlorophyte, *Monoraphidium,* the diatoms, *Achanthidium* and *Synedra,* and the cyanobacterium, *Planktolyngbya* were the dominant genera with the highest growth rates across both experiments, i.e. 0.01 – 0.27, 0.27, 0.16 – 0.17 and 0.14 - 0.23 d^{-1} respectively (Table 4.4). Highest growth was typically in the Ur + P treatment.

Mean Q_{CYNS} in the P, NO3 + P and Ur + P were statistically higher than for the control and N-only treatments in both experiments by day 5. There were equal proportions of the two dominant CYNs (cylindrospermopsin and deoxycylindrospermopsin), in all treatments and in both experiments. Q_{CYNS} was compared with cell-specific division rates of *C. raciborskii* for all treatments in both experiments and there was no statistically significant correlation between Q_{CYNS} and division rates ($R^2 < 0.2$). Q_{CYNS} was compared with PN:PP molar ratios across both experiments (Figure 4.4). Across all the treatments, Q_{CYNS} was highest in treatments that were stoichiometrically balanced.

		Biovolume (μm^3 mL^{-1})		Max. growth rates (d^{-1})	
Genus	**Class**	**Expt 1**	**Expt 2**	**Expt 1**	**Expt 2**
Monoraphidium	Chlorophyte	466,600	2,630,100	0.27 (Ur +P)	0.01 (Ur)
Achnanthidium	Bacillariophyte	958,700	1,677,500	0.27 (Ur +P)	0.27 (NO3+P)
Aulacoseira granulata	Bacillariophyte	564,600	412,700	0.10 (Ur)	0.03 (Ur +P)
Synedra	Bacillariophyte	1,965,200	1,708,700	0.16 (Ur+P, NO3+P)	0.17 (Ur+P, NO3+P)
Urosolenia	Bacillariophyte			0	0
Unidentified Chrysophytes	Chrysophyte	423,100	377,900	0.03 (Ur)	0.17 (Ur)
Cryptomonas spp.	Cryptophyte	329,500	838,100	0	0.02 (Ur+P, NO3+P)
Cylindrospermopsis raciborskii	Cyanophyte	4,529,600	4,592,300	0.13 (Ur +P)	0.19 (Ur +P)
Planktolyngbya	Cyanophyte	404,900	576,200	0.23 (Ur +P)	0.14 (Ur +P)
Total		**12,089,400**	**14,922,900**		

Table 4.4: Most common algal genera, by biovolume (μm^3 mL^{-1}) at the commencement of the two experiments, and their maximum growth rates, and treatments in which this occurred, over five days.

4.4.3 *cyrA* gene proportions

$\Delta\Delta C_T$ qPCR was used to calculate the relative proportion of *cyr*A genes (toxin CYNs producing cells) to 16s genes (total *C. raciborskii* cell equivalents), to demonstrate the change in proportion of the toxic cell equivalents in the population over the study. In Experiment 1 there were no statistical significant changes (P>0.05) between the beginning and end of the experiment (Figure 4.5).

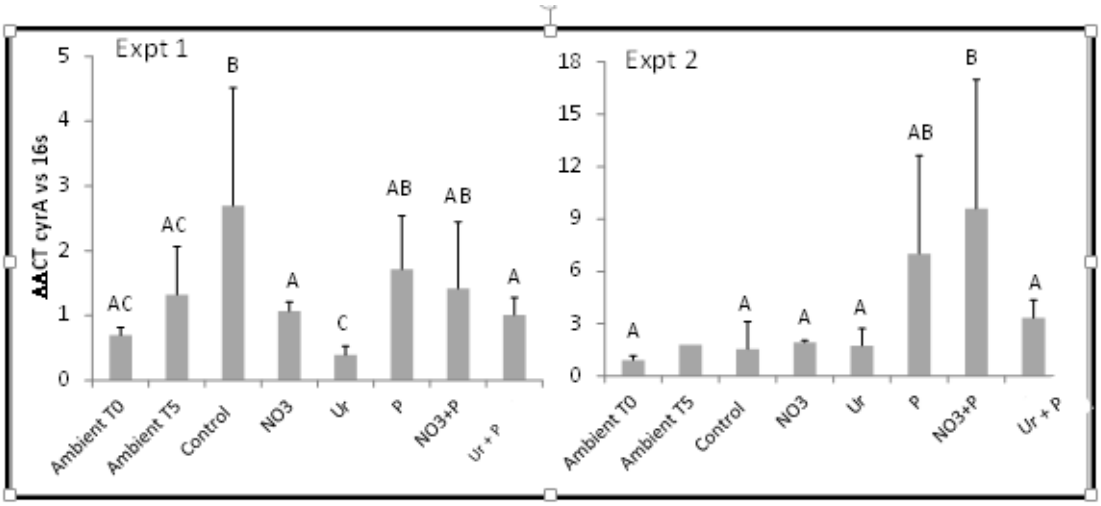

Figure 4.5: Mean (+ SD) proportion of *cyr*A toxin genes to 16s genes ($\Delta\Delta C_T$ *cyr*A vs. 16s) in all treatments on day 5, and adjacent water (Ambient) on days 0 and 5 for both experiments. A, B and C denote statistical differences (P<0.05).

However, there was a high level of variability between replicates. This is likely to be due to the small amount of DNA extracted. In Experiment 2, only the NO3 + P treatment was statistically higher than all other treatments, and again there was a high level of variability between replicates. In both experiments, the relative proportion of *cyr*A genes (CYNs producing cells) to 16s genes was highly correlated with the Q_{CYNS} of each treatment, i.e. $R^2 = 0.71$ and 0.72 in Experiments 1 and 2 respectively (Figure 4.6).

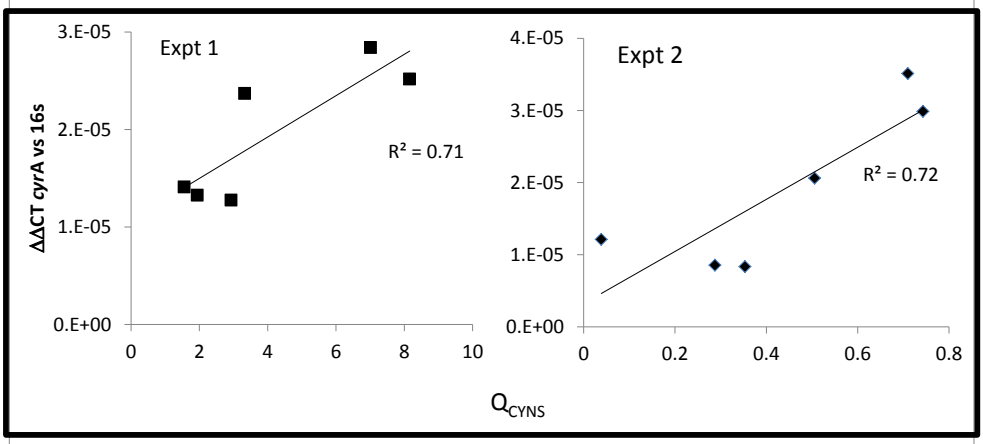

Figure cross all treatments for both experiments.

4.5 Discussion

Our study showed that Q_{CYNS} were highest when P was added, either alone or with NO3 in two mesocosm experiments dominated by *C. raciborskii*. The proportion of *cyr*A genes, relative to the 16S genes, in the *C. raciborskii* population was also higher in treatments with higher Q_{CYNS} in both experiments. This suggests that toxin producing strains responded more quickly than non-toxin producing strains to the addition of P over the five days of the experiment. Since there were low *C. raciborskii* population growth rates, there must have been differential growth of toxin producing strains compared with non-toxin producing strains over the five days. The importance

of shifts in strain dominance of *C. raciborskii* has previously been proposed by Orr *et al.* (2010), based on a study of three reservoirs, including the reservoir in our study. They compared cell quotas of CYNs, the *cyrC* toxin gene and the *C. raciborskii* specific gene (*rpoC1*) in a population, and proposed that the proportion of toxin producing and non-toxin producing strains could explain the differences in bloom toxicity.

Although an increase in Q_{CYNS} and a shift towards more toxic strains of *C. raciborskii* was correlated with P addition in our study, the explanation for this is unclear. Another study in a Saudi lake also found higher Q_{CYNS} when P concentrations were higher, but the mechanisms were not identified. Consistent with our study, other studies have shown that cyanobacterial species can have multiple strains that exhibit differences in physiological function, and responses to environmental conditions. For example, studies by Davis *et al.* (2014) have shown that two Australian strains of *C. raciborskii* grown under identical conditions had different Q_{CYNS}. Additionally, studies of another toxin producer - the cyanobacterium *Microcystis* - have shown that strains which were toxigenic can out-compete non-toxic ones at elevated N and P concentrations [32]. One study has shown that toxigenic strains of *Microcystis* had higher nutrient requirements than non-toxic species [187], and Briand *et al.* (2012) demonstrated the higher energetic costs of microcystin production by *Microcystis.* Additionally, bloom phase can affect the proportion of toxic and non-toxic strains [19]. It appears that the presence of multiple strains increases the flexibility of a species to adapt to variation in environmental conditions. Strain complexity and dynamics may therefore be related to the degree of variability in environmental conditions within a system, and the physiological plasticity of different strains.

There was no evidence in our study that *C. raciborskii* population growth rate was an important factor affecting CYNs production, since Q_{CYNS} were higher in some treatments even though growth was not high. Previous studies have also shown that the CYNs gene cluster is constitutively expressed, and hence growth phase does not affect CYNs production, as expressed by Q_{CYNS} [33].

C. raciborskii populations grew slowly, i.e. growth rates < 0.2 d^{-1}, in all treatments in both experiments over five days suggesting that even in the control, sufficient nutrients were available for some growth. However, when compared with the control on day 5, cell concentrations in both experiments were only statistically higher in the treatments where Ur + P was supplied, suggesting that both N and P were needed for maximum growth. In contrast, the whole phytoplankton community, as measured by chlorophyll *a* concentrations and the growth rates of individual dominant genera, increased with NO3, Ur and P alone, although the greatest response was with Ur + P addition. In contrast, a previous short-term microcosm study in our reservoir found an increase in *C. raciborskii* cell concentrations in response to P addition alone, but not to N + P addition [139]. Additionally, a mesocosm study in an adjacent reservoir also found that P addition alone increased *C. raciborskii* cell concentrations, [114]. In contrast to our study, P concentrations at the commencement of these studies were at or below detection limits (0.002 mg L^{-1}) and DN:P ratios were higher. This highlights the importance of antecedent conditions in determining growth responses to nutrient additions. Toxin concentrations were not measured in either study so Q_{CYNS} could not be calculated.

Interestingly, there was no statistically significant increase in *C. raciborskii* cell concentrations from the co-addition of NO3 + P, suggesting a preference for Ur over

NO_3^-. A mesocosm study by Finlay *et al.* (2010) also found stimulation of cyanobacterial growth with Ur addition. One explanation for the preference for U is that Ur also contains carbon which is enzymatically cleaved and released as carbon dioxide [45]. Release of carbon dioxide reduces cyanobacterial reliance on the energetically expensive process of bicarbonate at high pH [7]. The pH in our study was high, typically > 9, meaning that utilization of urea may have increased carbon dioxide availability for growth.

Although the addition of P did not increase *C. raciborskii* population growth rates, there was accumulation of phosphorus in cells, as measured by PP concentrations, equivalent to that in the N + P treatments. This is consistent with a strategy of 'storage adaptation' by the phytoplankton community, providing cells with a buffer against periods of low P availability [145]. The net effect of this storage was stoichiometrically balanced PN:PP ratios i.e. 7.2:1. Interestingly highest Q_{CYNS} ratios occurred at stoichiometrically balanced PN:PP ratios although it is not clear whether this is coincidental or reflects an optimal growth state of CYNs producing strains.

Heterocyst production was highest in the P only treatment suggesting that P availability limited N fixation. Plominsky *et al.* (2013) showed that N fixation in *C. raciborskii* occurs exclusively in the heterocysts. Moisander *et al.* (2011) also found higher N fixation rates with P only addition, compared with a control, in *C. raciborskii*-dominated phytoplankton communities. P is an important nutrient for DNA transcription and synthesis of enzymes used to fix N. Surprisingly, N+P addition also stimulated heterocyst production. It appears to reflect either the population, or strains within the population, utilizing N for heterocyst production as insurance against

future N limitation, at the expense of growth. This may be a physiological adaptation to fluctuating N availability.

Previous studies in this reservoir have shown that dissolved forms of N are preferred by the C. raciborskii-dominated community throughout the year, with N fixation only occurring periodically when dissolved inorganic N concentrations were sufficiently low, i.e. 0.01 – 0.05 mg L^{-1} [23]. Fixing N_2 is a more energy demanding process than metabolizing dissolved forms of N so it is not surprising that this species preferentially uses available sources of dissolved N [58]. This supports the findings of Lewis and Wurtsbaugh (2008) who calculated that N_2 fixation rates are usually insufficient to support a substantial component of the N uptake requirements of phytoplankton in lakes. *C. raciborskii* has been described as a generalist with regard to N source and fluctuations in supply, in contrast to another diazotroph, *Anabaena*, where growth was higher under low inorganic N concentrations [107].

In our study, we have assumed that *C. raciborskii* is the only CYNs producer. It certainly dominates. However, just as we postulate that P promotes an strain shift from less to more toxic strains, another explanation for increases in Q_{CYNS} may be an increase in the proportion of other CYNs-producing species. However, this is unlikely because concentrations of other species known to produce CYNs such as *A. ovalisporum* (Forti) and *Raphidiopsis mediterranea* Skuja, were less than 2% of the *C. raciborskii* cell concentrations. This does not preclude the presence of previously undescribed or unknown CYNs producers.

In summary, this study found that P replete treatments had higher Q_{CYNS} in phytoplankton populations dominated by *C. raciborskii,* irrespective of whether N was also added. Changes in Q_{CYNS} were not linked to population-based cell division rates,

but correlated with changes in the proportion of the *cyr*A gene to 16S gene in both experiments, and this strongly suggests a shift in strain dominance to toxic producers. Therefore, it appears that nutrient regimes play an important role in driving changes in the dominance of strains with and without CYNs production capacity. The different effect of N vs. P on cell division and CYNs production suggests plasticity in the population that enables individual strains as well as the population as a whole to adapt to a range of prevailing environmental conditions.

Chapter 5

Effect of light and elevated pCO_2 on cylindrospermopsin biosynthesis in *Cylindrospermopsis raciborskii*

Author contributions

Rati Sinha carried out the RNA extractions, primer design and validation, qpcr experiments and data analyses for the light-intensity samples. Mattia Pierangelli grew the cultures under both the light and pCO_2 conditions, and harvested pellets at specific time points. Dr. Anusuya Willis carried out the RNA extractions, primer design and validation, qpcr experiments and data analyses for the pCO_2 samples. Dr. Michele Burford, Dr. J. Beardall and Dr. Brett Neilan designed the experiment and helped in its co-ordination. Rati Sinha, Mattia Pierangelli and Dr. Anusuya Willis contributed to the writing of the manuscript.

5.1 Abstract

Cylindrospermopsin (CYN) and 7-deoxy-cylindrospermopsin (dCYN) are potent cytotoxins produced by numerous genera of cyanobacteria, including the freshwater cyanobacterium *Cylindrospermopsis raciborskii* (Nostocales). *C. raciborskii* is an invasive species, and thus, the understanding of environmental parameters which drive its toxicity has received significant interest from water health authorities. Light and pCO_2 deeply affect cell growth and eco-physiological characteristics in photosynthetic organisms and are thus major environmental parameters regulating cyanobacterial proliferation and possibly, the production of cyantoxins. In this study, we investigated

the effects of varying light intensities and pCO_2 levels on the CYN and dCYN production and gene expression of *cyrA,* under varying light intensities, and both *cyrA* and *cyrK,* under varying pCO_2 conditions in *C. raciborskii* ecotype CS-506. For cells exposed to different light intensities (10 and 100 μmol photons m^{-2} s^{-1}) we observed that the variation of total CYN (CYN + dCYN) cell quota was uncoupled with the cell growth characteristics suggesting that CYN production is not affected by light conditions and is a constitutive process. Besides, the lack of correlation between changes in transcript levels (*cyrA*) and cell quotas suggests that the production of intracellular CYN and dCYN are regulated at the protein level. Under elevated pCO_2 (1300 ppm), we observed a slight increase of dissolved portions of CYN and dCYN, however there was no change in the gene expression of *cyrA* and *cyrK*. Thus, differently from light, the level pCO_2 in the environment could play a role in regulation of *C. raciborskii* and water toxicities. Lastly, our results suggest also that CYN has different physiological role(s) with respect to other cyanobacterial toxins.

5.2 Introduction

Cylindrospermopsin (CYN) is a toxic polyketide-derived alkaloid which is produced by several cyanobacterial genera, including *Aphanizomenon*, *Oscillatoria, Anabaena* and *Cylindrospermopsis* [75]. The cyanobacterium *Cylindrospermopsis raciborskii* has received particular attention in recent decades due to its ability to form severe Harmful Algal Blooms (HAB), combined with its invasive capacity and recent latitudinal expansion (5, 30). In *C. raciborskii*, CYN and a natural variant, 7-deoxy-cylindrospermopsin (dCYN) are produced in differing amounts by ecotypes found in Australia, New Zealand and South Africa [90], [94]. Both CYN and dCYN are water-soluble. They are produced intracellularly and are thought to be released into

the surrounding water body via cell lysis [33]. These toxins can accumulate in the water body as they have high solubility and are resistant to degradation [27], causing serious environmental and human health issues [14]. Thus, it is imperative to understand what factors, presumably environmental, can influence the regulation and production of these toxins.

Several theories h*ave* been proffered for the regulation of CYN production by numerous environmental factors*,* including nitrogen source, light intensity, sulfate, pCO_2 and phosphate availability, to name a few [171], [186], [139]. Studies of CYN production by *C. raciborskii* in mesocosms have found that phosphate addition can increase production of CYN, without a commensurate increase in growth, and this was ascribed to shifts in strain dominance rather than growth (Burford et al. submitted). The effect of the atmospheric pressure of CO_2 (pCO_2) on CYN production, on the other hand has not been studied to date. However, associations between the production of another cyanotoxin, microcystin, and pCO_2 have been observed. Increased microcystin levels were recorded in *M. aeruginosa* PCC 7806 under both high nitrate and pCO_2 conditions [176]. The effect of light on the production of CYN as well as other cyanotoxins, on the other hand, has been extensively explored. The highest microcystin levels in both batch and continuous culture studies in strains of *Anabaena* spp. were detected under light exposures of 20-44 µmol photons m^{-2} s^{-1} [47], [93], [11]. Contradictory findings in microcystin-producing *Microcystis aeruginosa* PCC 7806 batch studies revealed an increase in microcystin content ranging from 1.2 to 3.8 fold, with the highest microcystin levels being recorded at 20 µmol photons m^{-2} s^{-1} in some studies, and 142 µmol photons m^{-2} s^{-1} for others [83], [164], [126]. With relevance to CYN, a study by Dyble et al. (2006) [39] found a positive correlation between light intensity (18-140 µmol photons m^{-2} s^{-1}) and cylindrospermopsin

production. In contrast, Preubel et al. (2009) found little correlation between light and cylindrospermopsin production in two strains of *A. flos-aquae*.

Finally, the CYN and dCYN biosynthesis gene cluster, which has been elucidated in *C. raciborskii* AWT205 [104], CS-505 [171] and CS-506 [165], is flanked by *hyp* (hydrogenase pleitrophy), genes that are under the regulation of the global nitrogen regulator *ntcA*. These studies therefore, provided evidence that CYN production may be linked to one or more environmental drivers. However, whether production is a constitutive process or affected by changes in cell physiological status is still unknown.

This study investigates how increases in photon flux and pCO_2 regulate the gene expression of CYN biosynthesis genes *cyrA* and *cyrK,* and cellular production of the toxic metabolites CYN and dCYN in *C. raciborskii* ecotype CS-506. Stark increases of over 30% in atmospheric pCO_2 have been recorded, and these are predicted to double by 2100 [167], thereby, knowledge on the effect of pCO_2 on CYN production in the already invasive *C. raciborskii* is critical.

Lastly, in order to increase our understanding of the factors promoting production of these toxic metabolites, greater knowledge of their cellular role is necessary. In particular we need to understand how production is linked to cell physiology. Light and CO_2 are the energy source and C substrate, respectively, for photosynthesis and as such are important environmental parameters driving physiological processes in photoautotrophic organisms. Previous studies by Pierangelini *et al.* (2014a) [137] showed that the growth rate of *C. raciborskii* ecotype CS506 varied under different photon fluxes and pCO_2, and the observed changes indicated that ecophysiological performance was influenced by the environment. The long-term effect of increasing

pCO_2 levels may drive changes in toxin production by *C. raciborskii* and photon flux can have seasonal as well as diel and depth effects. These two environmental drivers were chosen to help predict future responses and possible geographic range expansion of *C. raciborskii* populations.

5.3 Materials and methods

5.3.1 *C. raciborskii* and culture conditions

The *C. raciborskii* toxic ecotype CS-506 was provided by the Australian National Algae Culture Collection, Hobart, Tasmania, Australia. The strain was originally isolated from Solomon Dam, on Palm Island, Queensland, Australia in 1996 (8.7242°S, 146.594°E).

CS-506 was exposed to photon fluxes of 10 and 100 μmol photons m^{-2} s^{-1} in order to investigate the effects of light on growth and CYN biosynthesis. For the experiments, the *C. raciborskii* ecotype was grown in, at least triplicate, batch cultures using Jaworski's Medium (JM) (Culture Collection of Algae and Protozoa, Argyll, United Kingdom), incubated at 25 ºC and with a 12:12-h light:dark photoperiod. Before starting the experiments, the batch cultures were acclimated at the experimental condition for at least 15 d. To ensure homogeneous exposure of the cells to light, the flasks were gently shaken every day.

Physiological analyses were carried out at different stages (log and stationary phases) of the growth curve, with sampling performed at set cell concentrations (Figure 5.1). Experimental samples were collected at the same time of the day to avoid effects related to the light:dark cycle. The investigation on the effects of different pCO_2 on cellular CYN biosynthesis was carried out on cells in continuous cultures (T 25 ºC; 80

μmol photons m^{-2} s^{-1}; 12:12-h light: dark photoperiod) maintained in an exponential growth regime and exposed to pCO_2 of 500 ppm (L-CO_2) and 1300 ppm (H-CO_2).

5.3.2 Cell biovolume and concentration

Cell biovolume was measured during the experiments using an optical microscope (Zeiss Axioskop optical microscope, Zeiss, Gottingen, Germany). Cell concentration for the duration of the experiment was determined by combining microscopic cells count and spectrophotometric reading of the optical density (λ=750 nm) of algal suspension in the culture [137].

5.3.3 CYN and dCYN samples extraction and quantification

A known volume of culture was filtered onto glass fibre filters (25 mm Whatman GF/C filters), the retained cells were dried at 60ºC and then stored frozen until used for the particulate CYN and dCYN quantification. The filtrated supernatant was lyophilised and used to determinate the proportion of dissolved CYN and dCYN.

Samples for the quantification of both particulate and dissolved CYN and dCYN quotas were resuspended in 1 mL of 50 mmol L^{-1} acidic acetic and then probe sonicated for 30 s, while kept on ice and in dim light. The extracted samples were then centrifuged (30 min; 10,000 *g*) to remove any particulate matter prior to HPLC analysis. A 400 μL subsample was taken and analysed for CYNs using HPLC – MS/MS [52] using an AB/Sciex API4000Q (Applied Biosystems, Foster City, CA) mass spectrometer equipped with an electrospray (TurboV) interface coupled to a Shimadzu Prominence HPLC system (Shimadzu Corp., Kyoto, Japan). The limit of detection was <0.2 μg L^{-1}. The total CYN produced by *C. raciborskii* CS-506 was calculated as the sum of both particulate and dissolved CYN and dCYN, as:

total CYN = CYN $_{(Particulate + Dissolved)}$ + dCYN $_{(Particulate + Dissolved)}$.

5.3.4 RNA extraction

RNA extraction and cDNA synthesis. A known volume of culture was filtered through a polycarbonate membrane filter (25 mm, 2.0 µm pore size, Sterlitech Corporation, WA) and placed in 1 mL RNA*later* ® (Ambion, TX, USA) and stored frozen at -80°C until analysed.

RNA*later* was removed prior to extraction, and the cells were snap-frozen in liquid nitrogen in order to break open the cells. Cell culture grown and harvested under different photon flux was extracted using the Qiagen RNA Easy Plant extraction kit (Qiagen), according to the manufacturer's instructions. Similarly, pCO_2 exposed cell samples were extracted using the Bioline Isolate RNA mini kit (Bioline).

The extracted RNA was treated with 3 U of Turbo DNase (Ambion, TX) for 4 h at 37°C. RNA quality and purity was assessed using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). CDNA synthesis for photon flux related samples, was carried out using the first Strand cDNA synthesis kit (Invitrogen, Life Technologies), whereas pCO_2 exposed samples were processed using the Tetro cDNA synthesis kit (Bioline), as per the manufacturer's instructions. One hundred ng of RNA was used as template for cDNA synthesis. The cDNA was subsequently precipitated with 2 volumes of ethanol, and then further washed with 70% ethanol. It was further assessed for quality and purity using the Nanodrop spectrophotometer. The yield and quality of cDNA was found to be consistent using both the methods. The two expression experiments were carried out independently of each other and the results are reported against internal controls.

5.3.5 Amplification primer validation

In order to study changes in the transcription levels of toxin-producing *cyrA* and *cyrK* genes under the influence of light and pCO_2, suitable primers were designed (Supplementary Table 5.1). The primers were checked for cross-dimer and primer-dimer formation respectively, using Primer 3 [10]. Primer validation was performed on a MyCyclerTM Thermal Cycler (Bio-Rad). PCR conditions used were as follows: initial denaturation 94 ºC for 2 min, subsequently followed by 30 cycles of denaturation of 30 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min and a final extension at 72 ºC for 7 min. Final reaction volumes of 20 µL were used, which comprised 10 pmol L−1 of the forward and reverse primers, 0.2U of Taq DNA polymerase (Bioline, USA), 2.5 mmol L−1 magnesium chloride, 200 pmol L-1 dNTPs, and 5 ng L−1 of DNA template. Appropriate controls (positive and negative) were used. These primers amplified expected amplicons of 171 and 187 bp, respectively, from CYN producing ecotypes C. raciborskii CS-505, CS-506 but not from *C. raciborskii* CS-509, a non-toxic ecotype. These ecotypes of *C. raciborskii* were obtained from the CSIRO culture collection in Hobart, Tasmania. Further, there was no amplification in the negative controls, confirming the specificity of the primer set, and highlighting the absence of any cross-dimer formation or unspecific binding. Finally, Sanger sequencing was performed on the amplicons to confirm that the presence of *cyrA* and *cyrK* gene fragments, respectively.

5.3.6 Quantitative real-time PCR (qPCR)

Two-step qPCR experiments for photon flux experiment samples were performed in a 72-well Rotor gene 3000 (Corbett Life Sciences, Valencia, CA). All reactions were performed in biological and technical triplicates. Total reaction volumes of 10 µL final volumes were used, which comprised 5 µL 2x SensiFast TM Sybergreen (Bioline), 10

pmol L−1 forward and reverse primers for each primer set and sterile MQ water. Five ng µL-1 of template DNA was used for unknown samples in each reaction. The cycling conditions for all the primer sets were as follows: an initial denaturation of 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s, followed by a melt-curve cycle of 0.5 ºC increases at 15 s intervals.

For the pCO_2 experiment samples, triplicate qPCR reactions of 20 µL final volume using SYBR greenER qPCR SuperMix Universal (Life Technologies) were set up. The concentration of primers and unknown samples was consistent with the photon flux experiment. The qPCR reactions were run on StepOnePlus Real-Time PCR system (Applied Biosystems), with the cycle: initial 50 °C for 2 min, 95 °C for 10 min 40 cycles of 95 °C 15 s and 60 °C for 30 s, followed by a melt-curve cycle of 0.5 °C increases at 15 s intervals, as per the StepOne software pre-set program.

Standard curves of each primer set were performed, spanning a concentration of 3 ng to 3 fg. Linear regression analysis was used to compare the reaction efficiencies of the target and the reference genes, respectively. The cDNA generated from a laboratory culture of *C. raciborskii* CS-506 was used as template for the standard curves. Finally, transcript levels were normalized to reference genes 16S rRNA and rpoc1 genes, respectively. The relative change in proportion of the *cyrA* and *cyrK* genes was calculated using the ΔΔCT method of relative quantification [75].

5.3.7 Statistical analyses

Significance of differences between the different phases of the growth cycle of cells exposed at 10 and 100 µmol photons m^{-2} s^{-1} were evaluated by Two-ways ANOVA. The variation among means in relation to time was tested by using One-way ANOVA. The comparison of two data points was performed by one-tailed *t*-test. All the analyses

were carried out using the software statistical software GraphPad Prism 6 setting a level of significance $P < 0.05$.

5.4 Results

5.4.1 Growth

The growth rate of *C. raciborskii* ecotype CS-506 was higher at a photon flux of 100 compared with 10 µmol photons m^{-2} s^{-1} (Figure 5.1). In contrast, maximum cell concentrations were twice as high at a photon flux of 10 µmol photons m^{-2} s^{-1} (20×10^6 cells mL^{-1}) compared with 100 µmol photons m^{-2} s^{-1}. The μ_c of *C. raciborskii* ecotype CS-506 grown under different pCO_2, were previously calculated by Pierangelini *et al.* (2014b) and were 0.44 d^{-1} and 0.48 d^{-1} at low CO_2 and high CO_2, respectively.

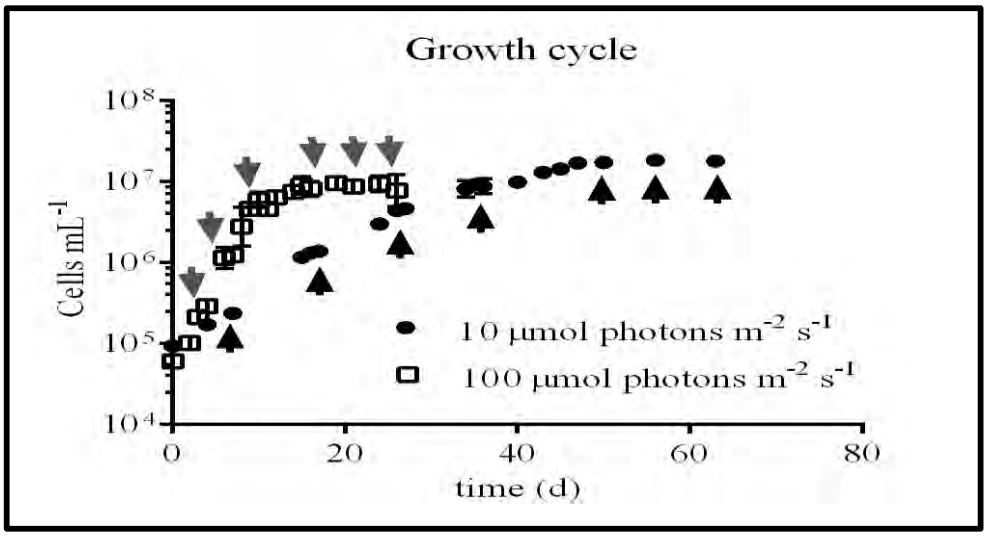

Figure 5.1: Increase of number of cells mL^{-1} in batch culture of *Cylindrospermopsis raciborskii* ecotype CS-506 exposed at 10 and 100 µmol photons (PAR) m^{-2} s^{-1}. Arrows indicate the cell concentrations of the growth cycle when sampling for CYN and dCYN quantification was performed. Vertical bars indicate standard deviations. Data are from at least three replicate cultures.

5.4.2 CYN and dCYN production

Due to the variation in cell size of *C. raciborskii* between the two treatments, low CO_2 and high CO_2 (Pierangelini *et al.* 2014b; Unpublished data), the amounts of both particulate and dissolved CYN and dCYN measured under the different light and pCO_2 experimental conditions were normalised to quantified cell biovolume. We measured that the dCYN was only a minor proportion of the total (less than 10%) .

Comparing the log phase of the growth cycle of cells exposed at the two light conditions, the total CYN cell quota was higher in cells at 10 than 100 µmol photons m^{-2} s^{-1} (Figure 5.2A). On the other hand, at the end of stationary phase the total CYN cell quota was higher under 100 compared with 10 µmol photons m^{-2} s^{-1}. No differences were observed in total CYN during the middle of the growth cycle.

Similar observations were also recorded for the particulate CYN and dCYN (Figure 5.2B, C). However, comparing the stationary phase, whereas similar cell quotas for particulate CYN were observed between cells exposed at 10 and 100 µmol photons m^{-2} s^{-1}, the particulate dCYN was higher under the 100 µmol photons m^{-2} s^{-1}. There was an increase in the dissolved portions of CYN and dCYN (Figure 5.2D, E), at the end of stationary phase under 100 µmol photons m^{-2} s^{-1}. This indicated that there was an increase in production of these two compounds, which were then released outside the cell.

The ratio between the CYN (particulate + dissolved) and dCYN (particulate + dissolved) (Figure 5.2F) showed no variation between log and stationary phases for cells exposed at 100 µmol photons m^{-2} s^{-1}. On the other hand, under a photon flux of 10 µmol photons m^{-2} s^{-1}, the CYN: dCYN increased as cells moved from log to

stationary phase, reflecting a change in the balance of CYN and dCYN production under this experimental condition.

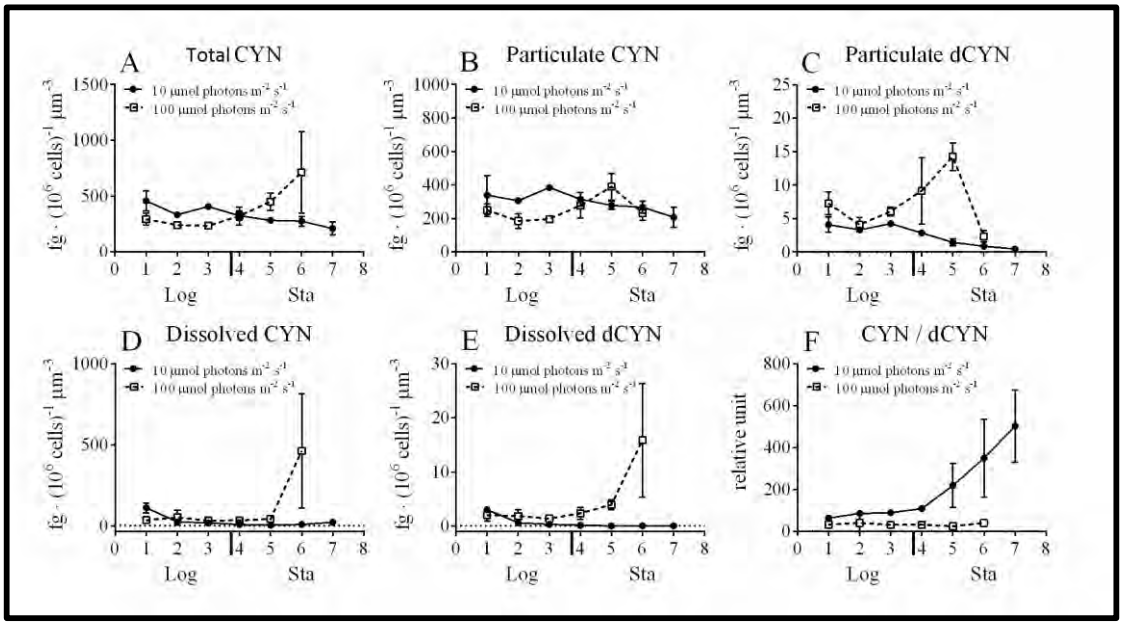

Figure 5.2: Variations of *Cylindrospermopsis raciborskii* CS-506 cell toxicity during the growth cycle of cultures exposed at light intensity of 10 and100 µmol photons (PAR) m^{-2} s^{-1}. (A) Total CYN = CYN $_{(Particulate + Dissolved)}$ + dCYN $_{(Particulate + Dissolved)}$. CYN and dCYN are shown as both particulate; (B, C) and dissolved (D, E) portions. (F) CYN / dCYN = CYN (particulate + dissolved) / dCYN (particulate + dissolved). Data are from at least three replicate cultures.

The effects of different pCO_2 on CYN production in *C. raciborskii* CS-506 cell are reported in Table 5.1 and 5.2. To emphasizes the importance of the normalization factor in quantifying cell toxins contents, results of CYN and dCYN under L-CO_2 and H-CO_2 were expressed as both cell biovolume [fg $(10^6$ cells$)^{-1}$ µm^3] and for each individual cell [fg cell^{-1}]. When the total CYN produced was normalized per cell biovolume, no differences were found between cells exposed at both L-CO_2 and H-CO_2 (Table 5.1). Conversely, when total CYN production is calculated from each individual cell, an increase is observed under H-CO_2 (Table 5.1), which seems to be an artefact due to the larger cell size observed under this experimental condition (Pierangelini, 2014b;). This discrepancy highlights that choice of the normalization

factor is important in order to compare the CYN production capabilities under experimental conditions, which might affect the overall cell morphology. The same results were also observed for the particulate portion of both CYN and dCYN (Table 5.2). On the other hand, an increase of the both dissolved CYN and dCYN was observed under H-CO_2 in despite of the normalization factor (Table 5.2).

pCO_2	Total CYN	
	fg $(10^6$ cells$)^{-1}$ μm^3	fg $cell^{-1}$
L-CO_2	416(67)	19(3)[a]
H-CO_2	447(69)	26(4)[b]

Table 5.1: Total CYN (= $CYN_{Particulate + Dissolved}$ + $dCYN_{Particulate + Dissolved}$) in *Cylindrospermopsis raciborskii* CS-506 exposed to Low and High CO_2. Values are normalized both as cell biovolume [fg $(10^6$ cells$)^{-1}$ μm^3] and for each individual cell [fg $cell^{-1}$]. Significant differences are indicated by different letters (a, b).

pCO_2	CYN	dCYN	CYN	dCYN
	fg $(10^6$ cells$)^{-1}$		fg $cell^{-1}$	
L-$CO_{2\ Particulate}$	312(53)	10(3)	14(2.38)[a]	0.45(0.13)[a]
H-$CO_{2\ Particulate}$	306(38)	11(2)	18(2.24)[b]	0.65(0.14)[b]
L-$CO_{2\ Dissolved}$	89(12)[a]	5.4(0.8)[a]	4(0.54)[a]	0.25(0.04)[a]
H-$CO_{2\ Dissolved}$	123(37)[b]	7.7(1.3)[b]	7.2(2.17)[b]	0.46(0.08)[b]

Table 5.2: Particulate and dissolved forms of CYN and dCYN in *Cylindrospermopsis raciborskii* CS-506 exposed to Low and High CO_2. Values are normalized both as cell biovolume [fg $(10^6$ cells$)^{-1}$ μm^3] and for each individual cell [fg $cell^{-1}$]. Significant differences are indicated by different letters (a, b).

5.4.3 CYN genes expression analysis

The expression of the *cyrA* gene, the first step in the CYN biosynthesis pathway, and the *cyrK* gene, the putative multidrug exporter, were investigated by RT-qPCR. The expression of *cyrA* and *cyrK* showed no change from L-CO_2 to H-CO_2 conditions (Figure 5.3).

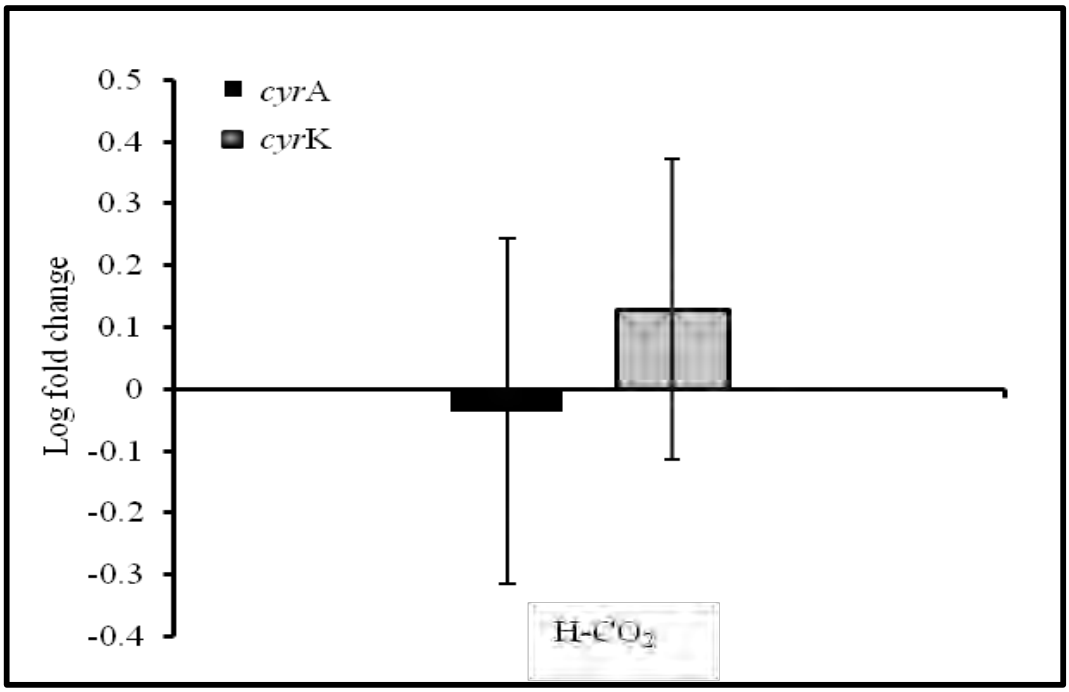

Figure 5.3: Changes in transcript levels of gene *cyrA* and *cyrK* versus the reference gene *RpoC1*, from continuous cultures grown under H-CO_2 conditions compared to L-CO_2 (calibrator) conditions.

The expression of *cyrA* showed a transitionary increase at the initial stage of stationary phase (phase 4) of cells exposed to 10 µmol photon m^{-2} s^{-1} and during the log phase (phase 2-3) of cells exposed at 100 µmol photon m^{-2} s^{-1} after which the expression level decreased (Figure 5.4). However, these changes in transcription (Figure 5.4) did not correlate with the observed change in total CYN cell quota. Further, the changes in *cyrA* versus the 16S *rRNA* and *rpoC1* reference genes were not uniform for both the low and high light treatments.

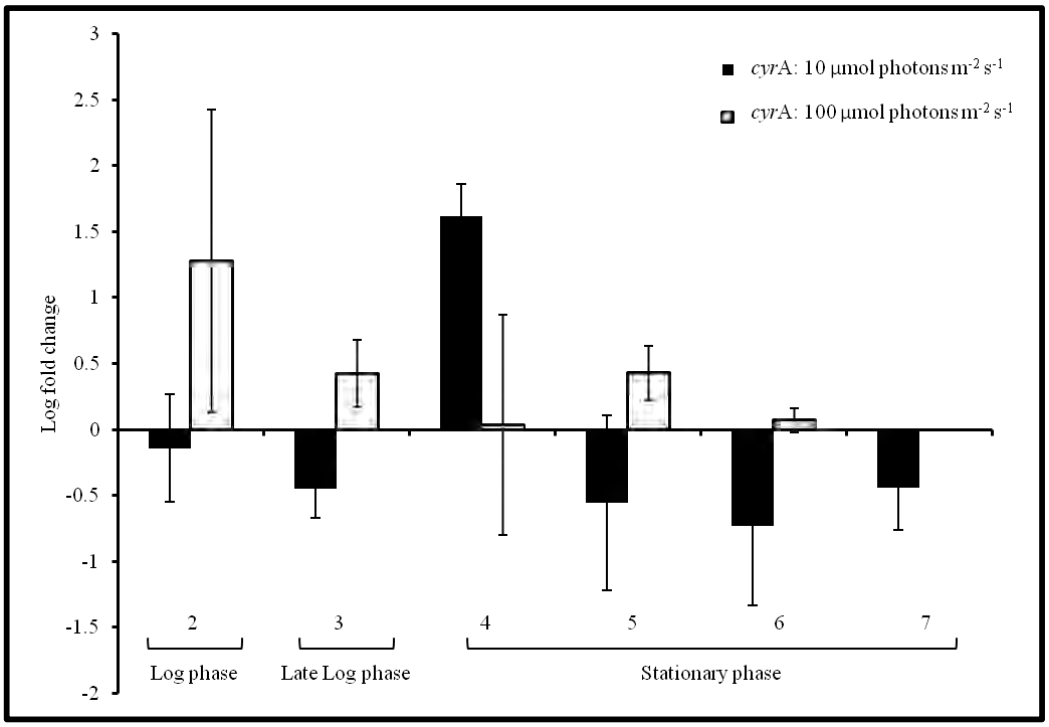

Figure 5.4: Changes in the transcript levels of gene *cyrA,* under light intensity of 10 umol photon m^{-2} s^{-1}, and 100 umol photon m^{-2} s^{-1}, versus reference gene *Rpo*C1, from batch cultures, with samples collected throughout the growth phase. Growth phase 1 was used as the calibrator.

5.5 Discussion

Global warming has led to significant changes to the ecophysiology of the Earth's biosphere. Increases in temperature, light, and pCO_2 are some of the many factors that will possibly show the greatest levels of change. Variations in these factors have been known to impact the proliferation of cyanobacterial blooms, and possibly their toxin profiles [166].

Here, we demonstrated that despite the significant growth response of *C. raciborskii* to photon flux and CO_2 the cellular production of the toxic metabolites CYN and dCYN remained mostly unchanged, and there was no correlation with the transcriptional level of the genes *cyrA* and *cyrK.* The changes in *cyrA* versus the 16S rRNA and *rpoC1* reference genes were also not uniform for both the low and high light treatments. This could be attributed to varied expression of reference genes, and suggests care should

be taken when using 16S rRNA under varying light conditions (Supplementary Figures. 1 and 2). The dissolved proportion of CYN and dCYN increased slightly under elevated pCO_2, however, no increase in the transcriptional level of *cyrK* was observed to support an increase in active transport. Thus, CYN cellular biosynthesis can be considered a constitutive process with cell quotas conserved under these different environmental conditions. This is consistent with a previous study of intra- and extracellular CYN in *C. raciborskii* by Davis et al. (2013), which also advocated the production of CYN and dCYN to be constitutive. Moreover, the uncoupling of the total CYN production rate with any of the physiological parameters measured by Pierangelini *et al.* (2014a, 2014b) also indicate that the CYN is unlikely to be involved in any major processes relating to cellular photosynthetic metabolism.

This is the first study to investigate the effects of increased pCO_2 on CYN and dCYN biosynthesis. Pierangelini *et al.* (2014b; unpublished data) observed profound changes in the photosynthetic characteristics in *C. raciborskii* cells exposed to H-CO_2. Despite these physiological changes, our results show no changes in total and particulate CYN and dCYN production in *C. raciborskii* cells. However, an increase in both dissolved CYN and dCYN did occur under H-CO_2, suggesting that an increase in production of CYN occurred, and while the cellular proportion of particulate CYN was maintained, the excess CYN was released into the water column.

Cultures under both L-CO_2 and H-CO_2 were maintained in exponential growth regime and thus were not prone to cell-death or lysis. Therefore, the increased portions of dissolved CYN and dCYN under H-CO_2 are likely to be a result of an active transport system through the cell membranes. Mihali *et al.* (2008) [104] postulated the *cyrK* is a transporter of CYN based on its homology, however, this has not been established

experimentally and the current study did not measure an increase in *cyrK* expression, although an increase in dissolved CYN was observed.

Previous studies on the impact of CO_2 on cyanotoxin production, however, contradict these results. A positive association was shown between the production of microcystin and low CO_2 conditions in *Microcystis aeruginosa* PCC 7806 [185]. This study also found that toxic strains of *Microcystis aeruginosa* were able to dominate at low CO_2 conditions, while the non-toxic strains dominated under high CO_2 conditions. A second study on the dinoflagellate *Alexandrium tamarense*, found that cellular levels of saxitoxin were higher in low CO_2 treatments. Results of these two studies suggest that higher pCO_2 levels would reduce toxin production and promote the dominance of the non-toxic cyanobacterial strains. Our results however, do not concur with these trends, and it is possible that the differnce may be attributed to the putative role or response of CYN in *C. raciborskii*, as opposed to these other toxins.

A putative hypothesis for the function of CYN is a sink for carbon. It has been proposed that microbial secondary metabolites can function as excretion products of unwanted compounds or reserve pools [30]. CYN could contain up to 6% of the total *C. raciborskii* intracellular carbon pool [128]. Under H-CO_2 Pierangelini *et al.* (2014b) speculated that an increased amount of CO_2 is fixed into molecular pools, which could be either stored in the cell (e.g. carbohydrates) or excreted externally. Therefore, it seems a reasonable speculation that CYN and dCYN could act as a potential sink for the allocation of CO_2 when environmental perturbation of pCO_2 occurs. Their toxicity to mammals would then be seen as an inadvertent secondary affect.

There have been contradictory findings of environmental conditions affecting CYN production. Hawkins *et al.* (2001) found a strong correlation between the increase of

specific cell division rate and increase of internal CYN production for exponentially growing cells. Dyble *et al.* (2006) [39] showed an increase in *C. raciborskii* CYN cell production under higher light conditions in culture studies, and nitrogen source has also been reported to influence CYN cell quotas [151]. However, in other studies, no correlation between growth and light regimes with CYN production from *C. raciborskii* were observed [143], [33]. Our results are consistent with these latter studies, with no difference in *C. raciborskii* CYN and dCYN cell quotas with respect to the changes in growth and light regimes. Thus, the increasing light intensities of the Earth's surface may not impact CYN production. The discrepancies between these and previous studies may arise from differences in CYN cell quota measurements, as our results show calculating CYN by biovolume or cell number can lead to different interpretations.

Expression levels of genes involved in CYN biosynthesis in *C. raciborskii* were used to investigate the relationship of the transcription levels of *cyrB*, *cyrI*, *cyrJ* and *cyrK* to nitrogen source and photon flux (Stucken, 2010). It was found that transcriptional levels of these genes did not correspond to CYN production. Our findings, with respect to transcription results from cells under different photon flux, concur with the differential expression of genes and lack of correlation between changes in the transcript levels and toxin concentration. This possibly suggests that the production of intracellular CYN levels are regulated at the protein level [15]. Post-transcriptional regulation (and/or accumulation of RNA) may represent a physiological advantage for the cell to avoid CYN production and/or transport when environmental conditions don't require it, but still allow prompt triggering of its biosynthesis or extracellular release if needed.

Differential expression of genes within the *cyr* gene cluster has also been observed in other cyanobacteria. Research with *Aphanizomenon ovalisporum,* another invasive CYN-producing cyanobacterium, belonging to the order Nostocales, showed differential regulation of the individual genes within the *cyr/aoa* cluster in response to nitrogen starvation, although the concentration of CYN remained stable [94]. This study also investigated transcription in response to light intensity (85 µmol photon m^{-2} s^{-1}) and showed an initial decrease in the transcript levels of *aoaA* (=*cyrA*) and *aoaC* (=*cyrC*), at 8 h, recovery at 24 h and eventually doubling at 48 h in response [94]. Our study also saw an increase in transcription of *cyrA* under higher light in exponential phase, however, no increase in CYN production was observed in our study.

Interestingly, the present study found that both CYN and dCYN cell production increased in the stationary phase of the *C. raciborskii* under 100 µmol photons m^{-2} s^{-1}, and is mostly released outside the cells. This is consistent with recent observations on *Oscillatoria,* another CYN-producing cyanobacterium [15] and can be partially attributed to the death and lysis of the cells, as has been observed with *C. raciborskii* [33].

The results of this study differ from similar studies with *Microcystis aeruginosa*, which produces the toxic metabolite, microcystin. For example Orr and Jones (1998) [127] found a linear relationship between intracellular microcystin and μ_c, implying a direct role of the primary metabolism of the cell. Further studies suggest that microcystin has a role in the light-adaptation process in a mutant of *Microcystis aeruginosa* deficient in microcystin, Hesse *et al.* (2001) [59] observed a different specific light absorbance and pigment composition compared to the wild-type. Further

investigation has shown that a significant portion of microcystin binds to protein under high light conditions, potentially protecting the function of these molecules [102]. Therefore, as previously suggested [15], it seems that CYN and microcystin have different physiological functions in the cell although currently, the exact role of these cyanotoxins is still unknown.

In summary, our study has shown that the cell quota of CYN remains stable under elevated light conditions and that small differences in the transcription level of *cyrA* under elevated light, and *cyrK* under pCO_2 conditions have no effect on CYN levels inside or outside the cell. Notably, the increase in the proportion of dissolved CYN and dCYN, under pCO_2 conditions have consequences for future environmental management of drinking water supplies. The effect of environmental factors, especially pCO_2, on cyanotoxins rpoduction warrants further research.

Primer name	Primer sequence	Size	Target	Positively amplified	Negatively amplified	Reference
Q*cyrA* F	TGGTTGGCGATGAATG	290	*cyrA*	CS-505, CS-506	CS-509, T3	This study
Q*cyrA* R	GGATAGACCTCAAAGCCG	290	*cyrA*	CS-505, CS-506	CS-509, T3	This study
JRT16SF	AGCCACACTGGGACTGAGACA	80	16s *rRNA*	CS-505, CS-506		(Al-Tebrineh et al., 2010)
JRT16SR	TCGCCCATTGCGGAAA	80	16s *rRNA*	CS-505, CS-506		(Al-Tebrineh et al., 2010)
rpoC1F	GACATGGTTTTGGGAGCCTA	181	*rpoC1*	CS-505, CS-506		This study
rpoC1R	CGTTATCCGGTTGTCCTGTT	181	*rpoC1*	CS-505, CS-506		This study
rpoC2F	CGTCGTTCTGGGGAAGTAAA	176	*rpoC2*	CS-505, CS-506		This study
rpoC2R	AATGGTTGACCAGCTTCCAC	176	*rpoC2*	CS-505, CS-506		This study
16SF	CCAGCATTAAGTTGGGCACT	184	16S *rRNA*	CS-505, CS-506		This study
16sR	GCCTTCGATCTGAACTGAGC	184	16S *rRNA*	CS-505, CS-506		This study
cyrAF	TGATTTTTGACGCAGCAGAC	171	*cyrA*	CS-505, CS-506	CS-510	This study
cyrAR	AATCAATGTGGGATGGGAAA	171	*cyrA*	CS-505, CS-506	CS-510	This study
cyrKF	CACCCTATTGGTGGGATTGT	187	*cyrK*	CS-505, CS-506	CS-510	This study
cyrKR	CATTTGTGCCCCTTCGTAGT	187	*cyrK*	CS-505, CS-506	CS-510	This study

Table 5.1: Primers used in this study

5.6 Supplementary

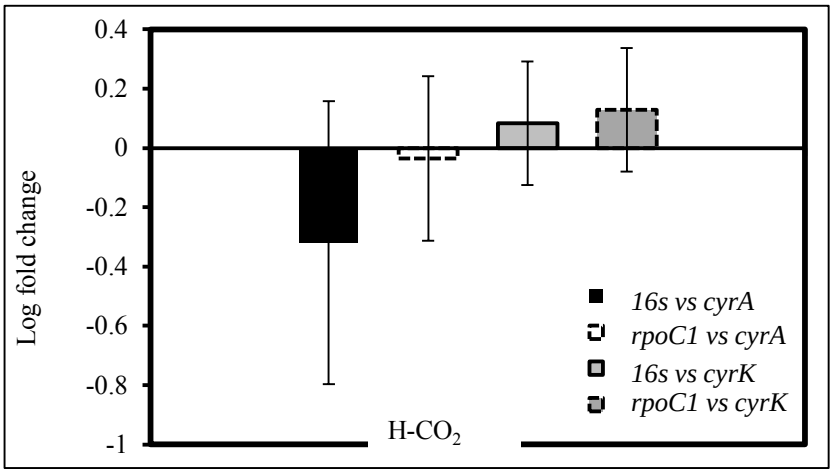

Figure 5.1: Change in transcription level of *cyrA* and *cyrK* vs *rpoC1* and 16S.

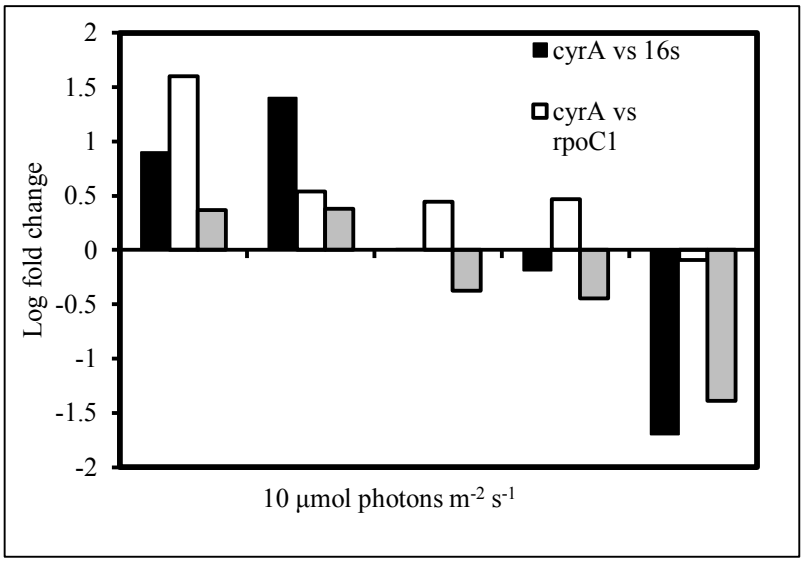

Figure 5.2a: Change in transcription level of *cyrA* and *cyrK* vs *rpoC1* and 16S (10 µmol photons m^{-2})

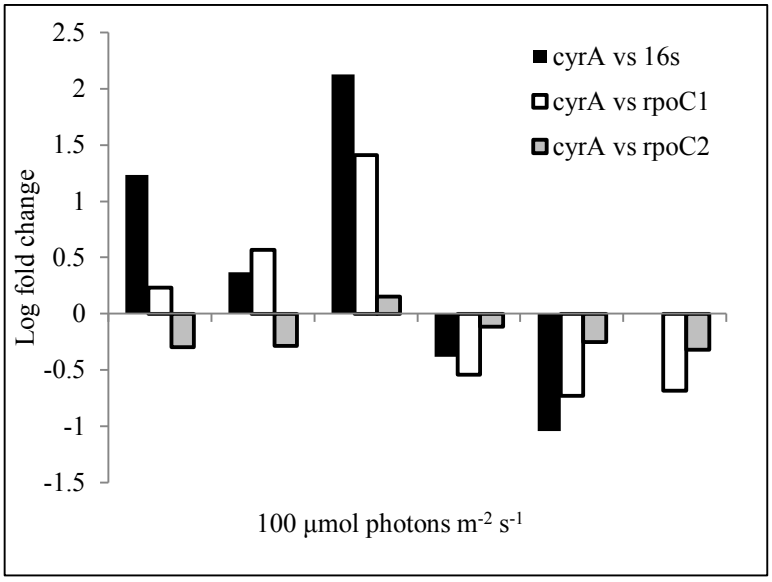

Figure 5. 2b: Change in transcription level of *cyrA* and *cyrK* vs *rpoC1* and 16S (100 µmol photons m^{-2})

Chapter 6

General discussion

C. raciborskii is an invasive cyanobacterium, some strains of which have the ability to produce toxins. The prevalence of this cyanobacterium has increased rampantly in the temperate areas, and this has been the cause of concern to water managers around the world (164). In order to efficiently curb the spread of *C. raciborskii*, and facilitate bloom management, it is imperative to understand the mechanisms that aid the spread of this organism, and help it sustain in unfavourable conditions. The genome composition of an organism may shed light on its genetic capability to react to environmental stimuli. We, therefore, carried out extensive analyses on the genomes of this species. Initial studies included analysing the genomes of *C. raciborskii* strains of varying toxicities and morphologies, isolated from the same geographical location, Queensland, Australia. There after, we looked at the larger picture, encompassing strains from Australia, China and Brazil. Strains of *Raphidiopsis* sp, another CYN-producing organism, thought to be a morphotypic stage of *C. raciborskii,* were also included in these analyses.

Our initial results revealed a remarkable genetic similarity of ~ 97% between three strains from the same geographical location. Nucleotide based clustering studies on eleven genomes, thereafter, showed strains of common geographical locations to group together. The comprehensive genome analyses of eleven strains, thus, reiterated the genetic similarity between strains isolated from the same geographic location. Strains

isolated from the same lake; CS-505 (CYN+, straight) and CS-509 (CYN-, straight) were found to share a larger number of genes, compared to strain CS-506 (CYN+, coiled), which was isolated from the same region. This was an important finding since it revealed that toxic strains were less similar to each other, than co-occurring strains, and, that strains of the same morphology share higher magnitudes of similarity than those strains that have the same toxin profiles. Our analyses further confirmed that toxicity was not a factor of divergence and that the most important factor contributing to toxicity in *C. raciborskii* or *Raphidiopsis* sp. was the presence or the absence of the *cyr* gene cluster. This was consistent with the production of cyanotoxins by several other genera of cyanobacteria such as *Microcystis* sp., [183] *Anabaena* sp. [169] and *Oscillatoria* sp. [98], to name a few. No other distally encoded genes or gene clusters that correlate with CYN production were identified. Since the additional genomic differences between toxic and non-toxic strains were primarily associated with stress and adaptation genes, we suggest that CYN production may possibly be linked to these physiological processes. We further found that the arrangement of the *cyr* gene cluster, responsible for the production of CYN differs by geographic location, and not by genera as considered earlier. This emphasised the critical fact that this toxin-producing cluster can suffer rearrangements and mutations, yet, it preserves its ability to produce toxins. This is consistent with studies on *Microcystis* spp. and *Plankothrix* CYA 126, which have reported the formation of novel *mcy* cluster variants with varying gene content, arrangement and specific domain sequences [29, 148]. More than anything, this reiterates the potential future problems cyanobacteria pose to the water bodies globally.

The inclusion of non-toxic strains; CS-509 (Australia) and Hab151 (China) was done to ascertain if the absence of the *cyr* gene cluster had any impact on strain-relatedness. The two non-toxic strains were not phylogentically related in any of our analyses –

functional or nucleotide based. Surprisingly, strain Hab151 (China) showed maximum association with a CYN-producing strain, AWT205 (Australia), found in a different geographical location that shared identical climatic conditions. Our results thus, showed a clustering trend based on climatic conditions, although there was insufficient statistical support for this.

Further comparative analyses of the essential metabolic pathways of our strains revealed that these species had the ability to perform all necessary metabolic functions. Some strains; AWT205 (CYN+, Australia) and Hab151 (CYN-, China), both isolated from temperate climatic zones, lacked several metabolic genes which their counterparts possessed. These strains, however, contained the minimal gene sets required for the essential metabolic machinery.

Another critical factor was the presence of strain-specific genes that were essentially adaptation related in their functions. Similar mechanisms have also been found in other cyanobacterial genera, such as *Microcystis aeruginosa* [1, 31]. These coupled with phage counteraction genes, are probably acquired by strains based on their specific environmental niche. For instance, some strains have greater metal resistance than their counterparts. This highlighted the probability that the plasticity of the genomes of these species aid them in acquiring other ancillary genes. We also observed the loss of large numbers of genes, however, these losses did not seem to affect strain function in any way. Similar trends have been observed in the cyanobacterium *Prochlorococcus* sp. [176].

Further, the inclusion of *Raphidiopsis* sp. was carried out to ascertain the impact of the loss of diazotrophy on the divergence of these organisms. Divergence was not affected by diazotrophy. This thesis thus, sheds light on the different mechanisms in play, in the

successful invasion of *C. raciborskii* to previously uninhabited ecological niche. This highlights the fact that bloom control models in place must cater to the individual and specific ecosystems of these organisms, and that these models evolve bases on the ecophysiology of the particular water body and its specific location.

The second half of this thesis investigated the effect of nutrients and environmental conditions on population growth and toxin production in *C. raciborskii*. These experiments have shed light on the association between toxin production and environmental factors, including nutrition, and will help water managers ascertain which factors are most detrimental to toxin production in *C. raciborskii* infested water bodies.

Firstly, we performed a mesocosm study, which investigated the effect of nitrogen (N) and phosphorus on growth and toxin production of a mixed population of cyanobacterium, containing the CYN-producer *C. raciborskii*. Our study showed that Q_{CYNS} were highest when P was added, either alone or with NO_3 in two mesocosm experiments dominated by *C. raciborskii*. The proportion of *cyr*A genes, relative to the 16S genes, in the *C. raciborskii* population was also higher in treatments with higher Q_{CYNS} in both experiments. This suggests that toxic strains responded more quickly than non-toxic strains to the addition of P over the five days of the experiment. However, although an increase in Q_{CYNS} and a shift towards more toxic strains of *C. raciborskii* was correlated with P addition in our study, the explanation for this is unclear. Our results, therefore, advocate that changes in strain dominance are the most likely factor driving changes in toxin production between treatments.

Finally, we endeavoured to study the effects of varying light intensities and pCO_2 levels on the gene expression of two *cyr* genes, and studied the effect of these physiological

parameters on the cellular production of the toxic metabolites CYN and dCYN in the Australian isolate *C. raciborskii* CS-506. Results revealed that CYN production is constitutive and not affected by physiological parameters, such as light. Experiments to study the effect of pCO_2 on CYN production and transcript levels of *cyr* genes, showed an increase in the gene expression of *cyrA* and *cyrK* under HCO_2 conditions. This correlates with an increase in dissolved CYN and dCYN levels under HCO_2, thus, highlights the likelihood of pCO_2 affecting CYN biosynthesis.

This thesis highlights the interdependency of strain selection, bloom formation and toxin release due to global warming, eutrophication and the inevitable rise in the carbon dioxide concentrations of the earth, and warn of the serious consequences of the release of domestic and industrial pollutants into our rivers and streams. We have demonstrated, yet again, the possible association between phosphate and toxin production, and highlight that phosphate effluents form large deposits of industrial waste, which in many countries is released into water bodies, possibly affects the production of CYN.

6.1 Future directions and conclusion

Next generation genome sequencing has led to in-depth knowledge of the genomes of *C. raciborskii* and other cyanobacteria. The distribution of strains sequenced around the world, however, is severely limited. Sequenced genomes of *C. raciborskii* strains have been isolated from China, Australia and Brazil. No representative strains from America, or Europe, for instance have been sequenced. A wider genome pool of *C. raciborskii* would help compare the degree of similarity and the main differences between strains isolated from different parts of the world and possibly highlight important trends. Finally, gene regulation studies, looking at the changes in transcript levels of CYN,

should be carried out under culturing and nutrient conditions not studied before, such as in chemostats with iron limitation.

This thesis highlights the association between global warming and its indirect effects on cyanobaterial ecophysiology. We emphasise how the changing global climate can influence the spread of a toxin-producing cyanobacterium. We caution water managers worldwide about this microorganism, and urge the facilitation of further research so as to truly understand the mechanisms involved in the functioning of *C. raciborskii*, and hence curb its spread.

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