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Constitutive Cylindrospermopsin Pool Size in *Cylindrospermopsis raciborskii* under Different Light and CO_2 Partial Pressure Conditions

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Cylindrospermopsin (CYN) and 7-deoxy-cylindrospermopsin (dCYN) are potent hepatotoxic alkaloids produced by numerous species of cyanobacteria, including the freshwater *Cylindrospermopsis raciborskii*. *C. raciborskii* is an invasive cyanobacterium, and the study of how environmental parameters drive CYN production has received significant interest from water managers and health authorities. Light and CO_2 affect cell growth and physiology in photoautotrophs, and these are potential regulators of cyanotoxin biosynthesis. In this study, we investigated how light and CO_2 affect CYN and dCYN pool size as well as the expression of the key genes, *cyrA* and *cyrK*, involved in CYN biosynthesis in a toxic *C. raciborskii* strain. For cells growing at different light intensities (10 and 100 μmol photons m^{-2} s^{-1}), we observed that the rate of CYN pool size production (μ_{CYN}) was coupled to the cell division rate (μ_c) during batch culture. This indicated that CYN pool size under our experimental conditions is constant and cell quotas of CYN (Q_{CYN}) and dCYN (Q_{dCYN}) are fixed. Moreover, a lack of correlation between expression of *cyrA* and total CYN cell quotas (Q_{CYNs}) suggests that the CYN biosynthesis is regulated posttranscriptionally. Under elevated CO_2 (1,300 ppm), we observed minor effects on Q_{CYN} and no effects on expression of *cyrA* and *cyrK*. We conclude that the CYN pool size is constitutive and not affected by light and CO_2 conditions. Thus, *C. raciborskii* bloom toxicity is determined by the absolute abundance of *C. raciborskii* cells within the water column and the relative abundance of toxic and nontoxic strains.

Cylindrospermopsins (CYNs) are a group of related toxic polyketide-derived hepatotoxic alkaloids which are produced by several species of cyanobacteria from genera including *Aphanizomenon*, *Oscillatoria*, *Anabaena*, *Raphidiopsis*, and *Cylindrospermopsis* (1). The cyanobacterium *Cylindrospermopsis raciborskii* (Wołoszyńska, *Nostocales*) Seenaya et Subba Raju has received particular attention in recent decades due to its ability to form harmful algal blooms (HAB), combined with its invasive capacity and recent latitudinal expansion (2–4).

Three CYNs are known to be produced by *C. raciborskii* in Australia (5), namely, cylindrospermopsin (CYN), 7-epi-cylindrospermopsin (7-epi-CYN), and 7-deoxy-cylindrospermopsin (dCYN). Each is known to be produced in differing amounts by different strains found in Australia, New Zealand, South Africa, and Thailand (6, 7). All CYNs are produced intracellularly and are thought to be released into the surrounding water body during cell lysis (8). In Australian strains of *C. raciborskii*, 7-epi-CYN is a minor component (usually less than 5% of the total CYNs), and it is not further considered in this study. CYNs are sensitive to sunlight but rather resistant to temperature, artificial light, and pH changes (9). Because of these natural properties, CYNs can accumulate in a water body, presenting serious environmental and human health risks (10). Thus, it is imperative to understand what factors influence the regulation and biosynthesis of CYNs.

The CYN biosynthesis gene cluster (*cyr*), which has been elucidated in *C. raciborskii* strains AWT205 (11), CS505 (12), and CS506 (13), is comprised of 15 open reading frames, spanning a total of 43 kb, and is flanked by *hyp* (hydrogenase pleitrophy) genes which are thought to be under the control of the global nitrogen regulator gene *ntcA* (11). These studies, therefore, have provided evidence that CYN pool size may be linked to one or more environmental drivers. However, whether cellular pool size is constitutive or is affected by changes in cell physiological status is unclear.

Several studies have been carried out to investigate the effects of environmental factors such as light intensity, temperature, nitrogen and phosphate availability, and culture age on CYN production by *C. raciborskii* and other CYN-producing cyanobacteria (8, 14–21). On the other hand, while the effects of elevated CO_2 on production of other cyanotoxins (such as microcystins and saxitoxins) by other cyanobacteria and dinoflagellates have been studied (22, 23), the effect of CO_2 on CYN pool size by *C. raciborskii* has not.

The levels of light and CO_2 in natural aquatic environments vary on a daily, seasonal, and annual scale (24–27). In addition, changes in incident irradiance (e.g., due to cloud cover) and CO_2 in natural aquatic environments will also be brought about in future years as a consequence of global climate change (28–30). Variations of both light and CO_2 are expected to impact the growth of cyanobacteria, their bloom formation, and their toxicity (2, 4, 31, 32). Light and CO_2 are the energy source and C substrate, respectively, for photosynthesis and as such are important envi-

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ronmental parameters driving physiological processes in photoautotrophic organisms. Thus, to understand how variations of light and CO_2 regulate the changes in *C. raciborskii* cell and bloom toxicity, we need to understand how environmental variables actually control the CYN pool size and the physiological process of CYN biosynthesis. It is still unclear if biosynthesis of CYN is a constitutive process, or if its levels are affected posttranslationally by changes in cell physiological status.

Previous studies by Pierangelini et al. (33, 34) showed that the specific cell division rate (μ_c) of *C. raciborskii* CS506 varied under different light intensities and CO_2 levels. To expand our knowledge about the effects of environmental parameters on the intracellular CYN pool size of *C. raciborskii* (strain CS506), we investigated how different light and CO_2 regimens regulated the expression of genes involved in CYN biosynthesis, such as *cyrA* (amidinotransferase, the first step in CYN biosynthesis) and *cyrK* (putative transport), and the impact these factors have on (i) the rate of production, (ii) CYN cell quotas for CYN (Q_{CYN}) and dCYN (Q_{dCYN}), and (iii) the extracellular volumetric concentrations of CYN and dCYN.

MATERIALS AND METHODS

Two experiments were carried out to test the impacts of light and CO_2 on CYN pool size and CYN gene expression on a toxic strain of *C. raciborskii*. For both experiments, the toxic strain *C. raciborskii* (CS506) was provided by the Australian National Algae Culture Collection, CSIRO, Hobart, Tasmania, Australia. This strain was originally isolated from Solomon Dam, Palm Island, Queensland, Australia (18.7242°S, 146.594°E) in 1996.

Culture conditions. (i) Light. *C. raciborskii* was grown in nonaxenic batch cultures ($n \geq 3$) using Jaworski's medium (JM; Culture Collection of Algae and Protozoa, Argyll, United Kingdom) at 25°C with a 12-h:12-h light:dark photoperiod. To investigate the effects of light regimen on growth, CYN pool size, and gene expression, cultures were grown under subsaturating (10 μmol photons [photosynthetically active radiation, or PAR] m^{-2} s^{-1}) and potentially saturating (100 μmol photons [PAR] m^{-2} s^{-1}) light intensities. A previous study had shown that the cell division rate (μ_c) was saturating at the higher light level and was subsaturating at the lower level (33). Cultures were preacclimated under the experimental conditions for at least 15 days prior to the experiment. To ensure homogeneous exposure of the cells to light, the flasks were gently shaken every day.

Subsamples were collected for analysis of particulate (intracellular) and dissolved (extracellular) CYN and dCYN and for gene expression levels at different stages of the cell cycle (Fig. 1). Samples were collected at the same time of the day to avoid photoperiod effects.

(ii) CO_2. The investigation of the effects of different CO_2 partial pressure (pCO_2) on CYN pool size and gene expression was carried out in nonaxenic cultures maintained in log-phase growth and in equilibrium with a gas phase having ~500 ppm CO_2 (low CO_2, or L-CO_2) or 1,300 ppm CO_2 (high CO_2, or H-CO_2), as described in reference 34. These levels of pCO_2 were sufficient to bring dissolved CO_2 in the medium to ~11 μmol $liter^{-1}$ and ~30 μmol $liter^{-1}$, respectively (34). Cells were also grown at 25°C at a light intensity of 80 μmol photons (PAR) m^{-2} s^{-1} with a 12-h:12-h light:dark photoperiod.

Cell concentration, growth, and biovolume. Culture growth was measured spectrophotometrically at 750 nm, while cell concentration was measured microscopically (33). During log phase, there was a linear correlation between the optical density at 750 nm (OD_{750}) and cell concentration over the range of cell concentrations measured ($r^2 > 0.98$). The OD_{750} was used to estimate cell concentrations on days when cell count subsamples were not collected. Cell biovolume was measured during the experiments by using an optical microscope (Axioskop optical microscope; Zeiss, Göttingen, Germany) as described by Pierangelini et al. (33).

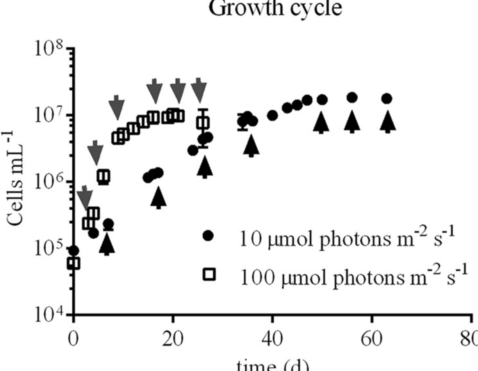

FIG 1 Cell concentration curves for batch cultures of *C. raciborskii* CS506 grown at 10 or 100 μmol photons (PAR) m^{-2} s^{-1}. Arrows indicate sampling events for CYN, dCYN, and *cyrA* quantification. Vertical bars indicate standard deviations. Data are from a minimum of three replicate cultures.

CYN and dCYN extraction and quantification. An aliquot of culture (5 to 20 ml for the light experiment; 8 ml for the CO_2 experiment) was filtered under subdued light onto glass fiber filters (25-mm Whatman GF/C). The filters were dried at 60°C (maximum of 24 h for light experiment; 8 to 10 h for CO_2 experiment) and then stored frozen until immediately prior to extraction for CYN and dCYN analyses. The supernatant was stored frozen until lyophilized and used to determine the volumetric concentration of extracellular CYN and dCYN in the cultures.

Samples were resuspended in 1 ml of 50 mmol $liter^{-1}$ acetic acid and then probe sonicated for 30 s on ice in dim light. The samples were centrifuged (30 min; 10,000 × *g*; Beckman Microfuge E) prior to high-performance liquid chromatography (HPLC) analysis. A 400-μl subsample was taken and analyzed for CYNs. Standard methods for CYN analysis were used as described by Eaglesham et al. (35). This method used HPLC-tandem mass spectrometry (MS/MS) (12, 35) with an AB/Sciex API4000Q mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray (TurboV) interface coupled to a Shimadzu Prominence HPLC system (Shimadzu Corp., Kyoto, Japan), with an Altima C_{18} (150- by 4.6-mm, 5-μm) column (Alltech Associates, Baulkam Hills, NSW, Australia) at 40°C. The limit of detection was less than 1.2 pg on-column.

The total volumetric CYN concentration (i.e., μg CYN $liter^{-1}$ of culture) was calculated as the sum of the extracellular and intracellular volumetric CYN and dCYN concentrations, as follows:

$$\text{total CYN} = \text{CYN}_{(\text{intra+extra})} + \text{dCYN}_{(\text{intra+extra})}$$

As described by Anderson et al. (36) and Orr and Jones (37), we used first-order rate kinetics to measure the specific rates of (i) cell division (μ_c) and (ii) total CYN pool size (μ_{CYN}), where C_1 and C_2 are cell or CYN concentration at times t_1 and t_2; thus, the following equation was used:

$$\mu = [\ln(C_2) - \ln(C_1)]/(t_2 - t_1)$$

The total CYN volumetric concentration (in μg $liter^{-1}$) was also converted to the cell quota (Q_{CYN}, in fg $cell^{-1}$) by dividing the volumetric concentration by the cell concentration (cells $liter^{-1}$):

$$Q_{CYN} = [(\text{total CYN concentration})/(\text{cell concentration})] \times 10^9$$

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). Samples were stored frozen at −80°C until analyzed.

RNA*later* was removed prior to extraction, and the cells were snap-frozen in liquid nitrogen in order to break open the cells. Cultures grown and harvested under different light intensities were extracted using a Qiagen RNA Easy Plant extraction kit (Qiagen), according to the manufac-

turer's instructions. Similarly, for the CO_2 treaments, cell samples were extracted using the Bioline Isolate RNA minikit.

The extracted RNA was treated with 3 U of Turbo DNase (Ambion, TX) for 4 h at 37°C. RNA quality and purity were assessed using a spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA synthesis for light intensity-related samples was carried out using the First-Strand cDNA synthesis kit (Invitrogen, Life Technologies), whereas CO_2-exposed samples were processed by using the Tetro cDNA synthesis kit (Bioline), per the manufacturer's instructions. One hundred nanograms of RNA was used as a 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 by using the Nanodrop spectrophotometer. The yield and quality of cDNA were found to be consistent using both methods. The two expression experiments were carried out independently of each other, and the results are reported against internal controls.

Amplification primer validation. Suitable primers were designed to study changes in the transcription levels of the *cyrA* and *cyrK* genes responsible for biosynthesis of CYNs under the influence of light and CO_2. The primers were checked for cross-dimer and primer-dimer formation, respectively, by using Primer 3 (38). Primer validation was performed on a MyCycler thermal cycler (Bio-Rad). PCR conditions used were as follows: initial denaturation 94°C for 2 min, followed by 30 cycles of denaturation of 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Final reaction volumes of 20 μl were used, which comprised 10 pmol $liter^{-1}$ of the forward and reverse primers, 0.2 U of *Taq* DNA polymerase (Bioline, Taunton, MA), 2.5 mmol $liter^{-1}$ magnesium chloride, 200 pmol $liter^{-1}$ deoxynucleoside triphosphates, and 5 ng $liter^{-1}$ of DNA template. Appropriate controls (positive and negative) were used. These primers amplified expected amplicons of 171 and 187 bp, respectively, from *C. raciborskii* CYN-producing strains CS505 and CS506 but not from the non-CYN-producing strain, CS509. 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 the presence of *cyrA* and *cyrK* gene fragments.

qPCR. Two-step real-time quantitative PCR (qPCR) experiments for samples from the light experiment were performed in a 72-well Rotor Gene 3000 apparatus (Corbett Life Sciences, Valencia, CA). All reactions were performed in biological and technical triplicates. Total reaction volumes of 10 μl (final volume) were used, which comprised 5 μl 2× SensiFast Sybergreen (Bioline), 10 pmol $liter^{-1}$ forward and reverse primers for each primer set, and sterile Milli-Q water (Merck Millipore, Darmstadt, Germany). Template DNA at 5 ng μl^{-1} was used for unknown samples in each reaction mixture. The cycling conditions for all the primer sets were as follows: (i) an initial denaturation of 95°C for 10 min, (ii) 40 cycles of 95°C for 15 s and 60°C for 30 s, and (iii) a melt-curve cycle of 0.5°C increases at 15-s intervals.

For samples from the CO_2 experiment, triplicate qPCRs were set up with a 20-μl final reaction volume and using SYBR greenER qPCR SuperMix Universal (Life Technologies). The concentration of primers and unknown samples was consistent with the experiment under different light levels. The qPCRs were run on a StepOnePlus real-time PCR system (Applied Biosystems), with the following cycling conditions: (i) an initial 50°C for 2 min, (ii) 95°C for 10 min, then 40 cycles of 95°C 15 s and 60°C for 30 s, followed by (iii) a melt-curve cycle of 0.5°C increases at 15-s intervals, as per the StepOne software preset program.

Standard curves of each primer set were performed, spanning a concentration of 3 fg to 3 ng. Linear regression analysis was used to compare the reaction efficiencies of the target and the reference genes. The cDNA generated from a laboratory culture of strain CS506 was used as the template for the standard curves. Finally, transcript levels were normalized to the reference genes 16S rRNA and *rpoC1* for *cyrA* and *cyrK*, respectively. The relative change in the proportion of the *cyrA* and *cyrK* genes was calculated using the $\Delta\Delta C_T$ method of relative quantification (1).

Statistical analyses. We performed a Pearson correlation between (i) cell division rates (μ_c) and intracellular CYN production rate (μ_{CYN}) during the batch culture and (ii) the CYN cell quota and expression of the *cyrA* gene. The comparison of two data points between L-CO_2 and H-CO_2, was performed with a two-tailed *t* test. Analyses were carried out using the statistical software GraphPad Prism 6, setting the level of significance at $P < 0.05$.

RESULTS

Growth. The maximal μ_c of *C. raciborskii* CS506, calculated during log phase, was higher for cultures grown at 100 μmol photons (PAR) m^{-2} s^{-1} (0.47 day^{-1} until day 11) than for those grown at 10 μmol photons (PAR) m^{-2} s^{-1} (0.15 day^{-1} until day 28), showing that cultures grown at 10 μmol photons (PAR) m^{-2} s^{-1} were light limited (Fig. 1). Although the measured final cell yield in both treatments was different (1.4×10^7 cells ml^{-1} for the 10-μmol photons [PAR] m^{-2} s^{-1} treatment and 8.5×10^6 cells ml^{-1} for the 100-μmol photons [PAR] m^{-2} s^{-1} treatment), the difference was within the range of statistical counting error (±20%) expected for the number of cells enumerated (39).

The maximal μ_c values of *C. raciborskii* CS506 grown under different CO_2 levels and the same light intensity of 80 μmol photons (PAR) m^{-2} s^{-1} were previously calculated by Pierangelini et al. (34) and were 0.45 day^{-1} and 0.49 day^{-1} for L-CO_2 and H-CO_2, respectively.

CYN and dCYN. In the light experiment, the increase in the intracellular volumetric CYN concentration (mass of intracellular CYN per ml of culture) with either light treatment (Fig. 2A) closely followed the increase in cell concentration in the batch culture. The extracellular concentrations of both CYN and dCYN increased as cells moved from log to stationary phase under 100 μmol photons (PAR) m^{-2} s^{-1} (Fig. 2C and D).

During batch culture, the μ_{CYN} calculated for total cellular CYNs and plotted against the corresponding μ_c are reported in Fig. 3. The high correlations between μ_{CYN} and μ_c for both light treatments ($R^2 = 0.84$ and 0.95) showed that the rate kinetics of μ_c were the same as for μ_{CYN}.

The variation of total CYN cell quotas (Q_{CYN}) during the cell cycle for cultures exposed at 10 and 100 μmol photons (PAR) m^{-2} s^{-1} are shown in Fig. 4.

The Q_{CYN} and cell quotas of each individual CYN (Q_{CYN} and Q_{dCYN}) in cultures grown at different CO_2 pressures are reported in Tables 1 and 2. To emphasize the importance of the normalization factor, results are also expressed on a per-cell biovolume basis. Statistically higher Q_{CYN} values were found in cultures grown under H-CO_2 ($P = 0.0044$) (Table 1). However, on a biovolume basis, no differences were found for total CYN between cells grown under L-CO_2 and those grown under H-CO_2 ($P = 0.4480$) (Table 1). Similar results were observed for intracellular CYN and dCYN and extracellular CYN (Table 2). On the other hand, increases in extracellular dCYN were observed under H-CO_2 despite normalization to biovolume (Table 2).

CYN gene expression analysis. The expression of *cyrA* (Fig. 5), the first step in the CYN biosynthesis pathway, showed a transitory increase at the initial stage of stationary phase (phase 4) in cells grown at 10 μmol photon (PAR) m^{-2} s^{-1} and during the log phase (phase 2 to 3) for cells grown at 100 μmol photon (PAR) m^{-2} s^{-1}, after which the expression levels decreased. However, these changes in transcription did not correlate with the Q_{CYN} shown in Fig. 3 (*cyrA* expression versus 10 μmol photon (PAR)

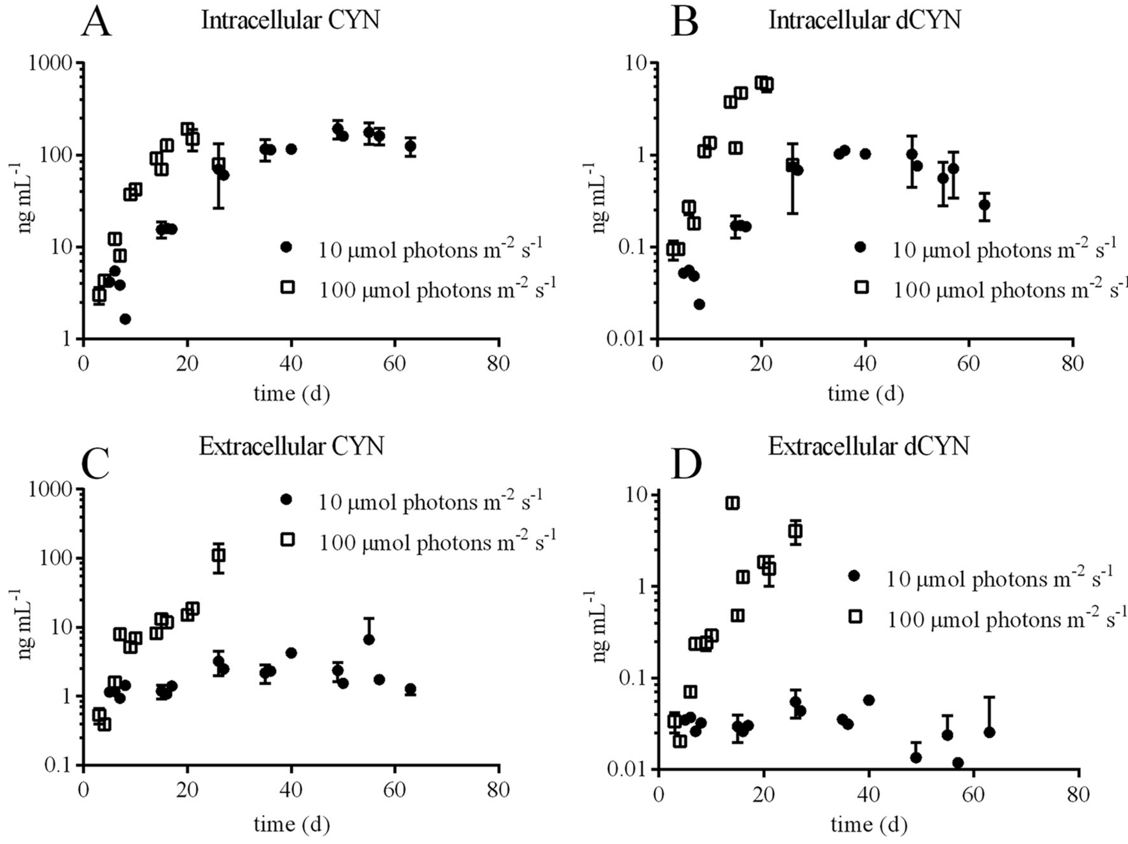

FIG 2 (A and B) The intracellular CYN and dCYN volumetric concentrations during batch culture growth of *C. raciborskii* CS506 under low- or high-light experimental conditions. (C and D) Extracellular CYN and dCYN volumetric concentrations during batch culture growth under low- or high-light experimental conditions. The intra- and extracellular dCYN pool sizes represented only a minor component (<5%) with respect to the intra- and extracellular CYN.

m^{-2} s^{-1}, R^2 = 0.09; *cyrA* expression versus 100 μmol photon (PAR) m^{-2} s^{-1}, R^2 = 0.24). Further, the changes in *cyrA* expression versus that of the 16S rRNA and *rpoC1* reference genes, respectively, were not uniform for either the low- or high-light treatments.

The expression of *cyrA* and *cyrK* (putative multidrug exporter) showed no changes under L-CO_2 compared to H-CO_2 conditions (Fig. 6).

DISCUSSION

In this study, we demonstrated that a toxic strain of *C. raciborskii* exposed to both low- and high-light conditions had a rate of production of CYNs (μ_{CYN}) which matched the variation of μ_c during batch growth. This showed that Q_{CYN} for an individual strain was not affected by light changes, light limitation, or the changes of maximal μ_c *per se* caused by light intensity. The consequence of this is that the total CYN cell quotas (Q_{CYN}) remained mostly constant over the cell cycle, varying by just 1- or 2-fold. We also showed there was no correlation between Q_{CYN} and the transcriptional level of the gene *cyrA*, which is involved in CYN production. This indicated that for *C. raciborskii* (CS506) grown under different light and CO_2 conditions, the CYN pool size is constitutive and that regulation of CYN biosynthesis under these

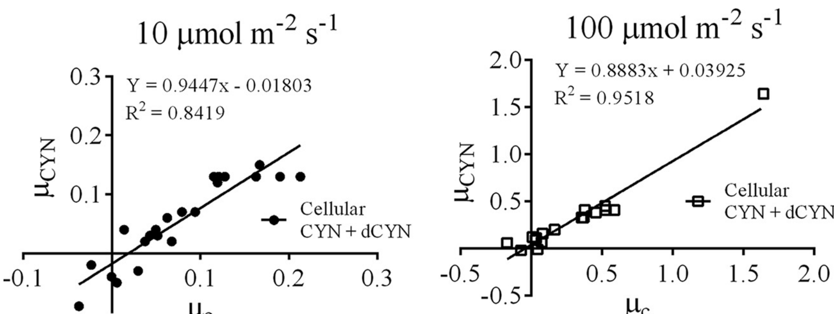

FIG 3 First-order rate kinetics for production of total cellular (intra- plus extracellular) CYN plus dCYN (μ_{CYN}), versus the corresponding first-order rate kinetics for μ_c, for cells exposed to 10 or 100 μmol photons (PAR) m^{-2} s^{-1} during batch culture growth.

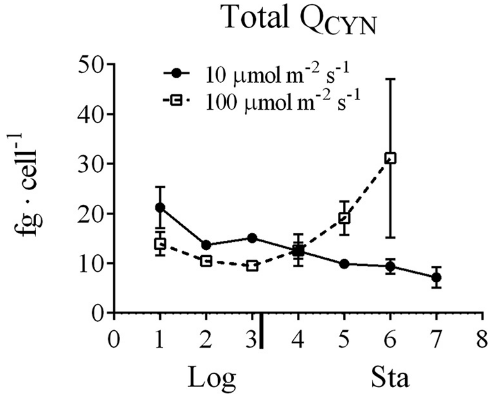

FIG 4 Variations of *C. raciborskii* CS506 total Q_{CYN} during the cell growth cycle of cultures exposed at light intensities of 10 or 100 μmol photons (PAR) m^{-2} s^{-1}. Data are from at least three replicate cultures.

TABLE 2 Intra- and extracellular CYN and dCYN in *C. raciborskii* CS506 cultures exposed to low or high CO_2[a]

CYN location and CO_2 exptl condition	Mean (SD) cell quota (fg/cell)		Mean biovolume concn (10^3 fg/μm^3)	
	Q_{CYN}	Q_{dCYN}	CYN	dCYN
Intracellular				
L-CO_2	14 (2.4)*	0.45 (0.13)*	312 (53)	10 (3)
H-CO_2	18 (2.2)*	0.65 (0.14)*	306 (38)	11 (2.4)
Extracellular				
L-CO_2	4 (0.55)*	0.25 (0.04)*	89 (12)	5.5 (0.9)*
H-CO_2	7.3 (2.17)*	0.46 (0.08)*	123 (37)	7.7 (1.3)*

[a] Intra- and extracellular CYN and dCYN in *C. raciborskii* CS506 cultures exposed to low (~500 ppm) or high (1,300 ppm) CO_2 (L-CO_2 or H-CO_2, respectively). *, significant difference (for L-CO_2 versus H-CO_2 condition for the CYN location) ($P < 0.05$).

conditions occurs at a posttranscriptional (i.e., the physiological) level.

This finding is in direct agreement with that of Hawkins et al. (40), who also measured a linear correlation between specific rates of cell division and CYN production in N-limited batch cultures of *C. raciborskii* (strain AWT205). It is also in full agreement with the findings of a previous study by Davis et al. (8), who concluded that for cultures grown under nutrient-replete but low-light conditions, the production levels of CYN and dCYN were constitutive.

When comparing the effects of light on production of CYNs by *C. raciborskii*, there have been seemingly contradictory findings in the past. For instance, Dyble et al. (16) showed an increase in CYN under higher-light conditions in *C. raciborskii* cultures. In other studies, Carneiro et al. (19) and Davis et al. (8) found no correlation between the phase of the growth cycle or light regimen, with variations in CYN cell quotas. Our results are entirely consistent with these two studies, as we found that μ_{CYN} values were not affected by either low- or high-light conditions. Thus, for instance, during *C. raciborskii* bloom events, the attenuation of light intensity in the water column through changes in self-shading caused by changes in algal abundance will not impact the CYN cell quotas for *C. raciborskii*. A complication of the comparison of the CYN cellular pool size between laboratory and field investigations may arise from the potential CYN degradation due to sun (high) light intensity (9). Despite this, it is interesting that the Q_{CYN} levels measured in the study were comparable to Q_{CYN} levels reported for natural populations of *C. raciborskii* in Australian freshwater environments (32).

As our results show, calculating production as the intracellular volumetric concentration rather than as a ratio against another

TABLE 1 Total CYN in *C. raciborskii* CS506 cultures exposed to low or high CO_2[a]

CO_2 exptl condition	Mean (SD) Q_{CYN} (fg/cell)	Mean (SD) biovolume concn (10^3 fg CYN/μm^3)
L-CO_2	19 (3)*	416 (67)
H-CO_2	26 (4)*	447 (69)

[a] Total CYN [$CYN_{(intracellular + extracellular)}$ + $dCYN_{(intracellular + extracellular)}$] in *C. raciborskii* CS506 cultures exposed to low (~500 ppm) or high (1,300 ppm) CO_2 (L-CO_2 or H-CO_2, respectively). *, the difference between the mean Q_{CYN} for L-CO_2 versus H-CO_2 conditions was statistically significant ($P < 0.05$).

parameter, such as dry weight, biovolume, chlorophyll, or protein (which are variables in their own right) can lead to very different interpretations (and sometimes misinterpretations) about production of metabolites. Treatments may also affect the production dynamics of those other metabolites/parameters, so that any apparent discrepancy between this and previous studies could arise from differences in the way CYN production was measured and interpreted (17, 19). Thus, as explained by Orr and Jones (37), the normalization factor is critically important when interpreting metabolite dynamics (i.e., production and loss) under experimental conditions. Taking this into consideration, all the research results available to date about CYN production by *C. raciborskii* can be unified into a single hypothesis, which indicates that production of CYNs by *C. raciborskii* is a constitutive process.

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* in response to nitrogen source and photon flux (41). They found that transcriptional levels of these genes did not correspond to rates of production of CYNs or cell quotas. Our findings, with respect to transcription results from cells under different light intensities, concur with the differential expression levels of genes and lack of correlation between changes in the transcript levels and toxin production. This suggests perhaps that the cellular CYN pool size is regulated at the posttranscriptional, protein level (41), although we cannot rule out completely the influence of genetic regulation.

The changes in *cyrA* expression versus expression levels of the 16S rRNA and *rpoC1* reference genes were also not uniform for the low- and high-light treatments. This could be attributed to varied expression of reference genes and suggests that care should be taken when using 16S rRNA under different light conditions. The difference under the different CO_2 conditions was minor (see Fig. S1 in supplemental material).

Differential expression of genes within the *cyr* gene cluster has been observed in other cyanobacteria. Research with *Aphanizomenon ovalisporum* (Forti), 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 (42). Shalev-Malul et al. (42) also investigated transcription in response to light intensity (85 μmol photons [PAR] m^{-2} s^{-1}) and showed an initial decrease in the tran-

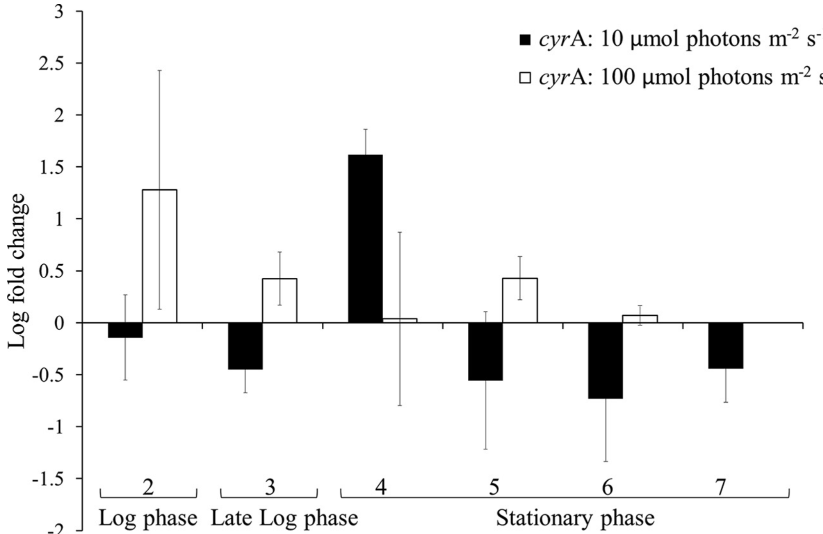

FIG 5 Changes in transcript levels of *cyrA* under a light intensity of 10 or 100 μmol photons (PAR) $m^{-2} s^{-1}$, versus those of the reference gene *rpoC1*, from batch cultures. Samples were collected throughout the growth phase. Growth phase 1 was used as the calibrator.

script levels of *aoaA* (*cyrA*) and *aoaC* (*cyrC*) at 8 h, recovery at 24 h, and an eventual doubling at 48 h. Our study also saw higher transcription of *cyrA* under higher light in exponential phase, but no increase in Q_{CYN} was observed and there was no correlation between Q_{CYN} and increased transcription.

Interestingly, the present study found that the extracellular CYN and dCYN were higher in stationary phase for cultures grown under 100 μmol photons $m^{-2} s^{-1}$ compared to cultures grown under 10 μmol photons $m^{-2} s^{-1}$, probably as a result of external release. This is consistent with recent observations for *Oscillatoria*, another CYN-producing cyanobacterium (20), and can be partially attributed to the death and lysis of cells, as observed previously with *C. raciborskii* by Davis et al. (8).

Pierangelini et al. (34) previously reported changes in the photosynthetic characteristics of *C. raciborskii* cells grown under high-CO_2 conditions (H-CO_2). The CYN results reported here came from that same experiment, and they represent the first attempt to study the effects of elevated CO_2 on CYN cellular pool size and gene regulation. Our results showed a higher Q_{CYN} under H-CO_2. However, this difference was relatively small (less than 2-fold) and well within the range found for a single strain and could be accounted for by less than 1 cell division within the cultures. Moreover, such a difference can be attributed to the larger cell biovolume under H-CO_2 conditions. The uncoupling of Q_{CYN} from the physiological parameters (e.g., photosynthesis, CO_2 acquisition) measured by Pierangelini et al. (34) indicates that CYNs are unlikely to be involved in any major processes related to cellular photosynthetic metabolism.

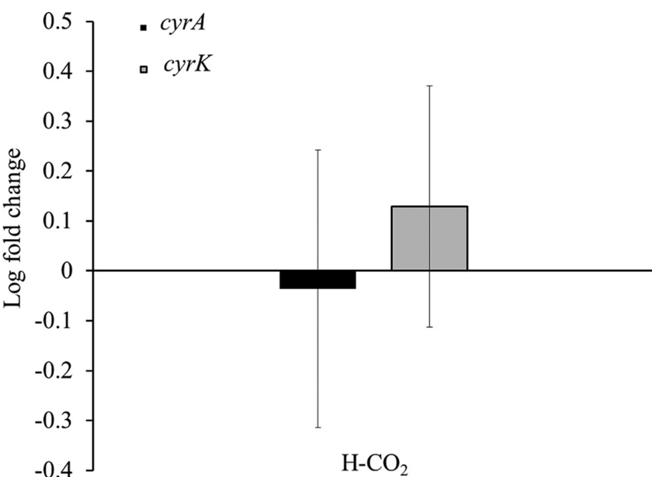

FIG 6 Changes in transcript levels of *cyrA* and *cyrK* versus that of the reference gene *rpoC1*, from continuous cultures grown under H-CO_2 conditions versus L-CO_2 (calibrator) conditions.

Previous studies on the impact of CO_2 on production of other cyanotoxins, however, contradict our results. Greatorex (43) measured lower gravimetric nodularin (NODLN) concentrations (i.e., the amount of NODLN expressed on a per unit of dry weight basis) under elevated CO_2 for *Nodularia spumigena* Mertens ex Bornet & Flahault. Van de Waal et al. (44) showed that toxic strains of *Microcystis aeruginosa* were able to dominate under low-CO_2 conditions, while the nontoxic strains dominated under high-CO_2 conditions. For the dinoflagellate *Alexandrium tamarense* (Lebour) Balech, Van de Waal et al. (23) found that cell quotas of paralytic shellfish poisons (saxitoxins) were higher with low-CO_2 treatments. The results of these two latter studies suggest that higher CO_2 levels would reduce toxin concentrations and promote the dominance of the nontoxic cyanobacterial strains. On the other hand, our results suggest that perturbation of CO_2 in the environment will not affect *C. raciborskii* cell toxicity, which is consistent with the constitutive nature of the CYN pool size. The difference from other cyanobacterial species may be related to the different role(s) attributed to cyanobacterial toxins (45). For instance, the presence of dissolved dCYN in the water column could be beneficial for *C. raciborskii* growth through allelopathic effects on other phytoplankton species (e.g., inducing alkaline phosphatase secretion) (46–48) and zooplankton grazers (49). Their toxicities to mammals would then be seen as an inadvertent secondary effect.

In summary, our study has shown that Q_{CYN} is fixed within a small range (2- to 3-fold) irrespective of the growth conditions. We also showed that differences in the transcription level of *cyrA* under different light intensities, and *cyrK* under different CO_2 conditions, have no control over the rates and timing of CYN pool size production. We therefore conclude that due to the constitutive nature of CYN pool size, the toxicity of a *C. raciborskii* bloom is likely to be controlled by population dynamics and strain-specific susceptibilities of strains (with different CYN cell quotas) to environmental parameters (21, 33, 50–56) rather than by any direct stimulation of the CYN production pathway, including gene transcription. *C. raciborskii* bloom toxicity would also be governed by changes in light and CO_2, which regulate the growth of (toxic) cells in the water column and the faster buildup of a (toxic) population, rather than by their effects on CYN pool size.

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