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Use of the cell quota and chlorophyll content for normalization of cylindropermopsin produced by two *Cylindrospermopsis raciborskii* strains grown under different light intensities

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Abstract

The production of cylindrospermopsin (CYN) by *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju (Cyanobacteria) was already shown to be changed by variations in light intensities. Influences of light intensity on CYN production by two different *C. raciborskii* strains (CYP011K and CYP030A) were studied in batch culture conditions. The strains were grown under photon light intensities from 40 to 348 µmol photons m^{-2} s^{-1}. The growth rates were measured by exponential regression of cell number variation along the time. The concentration of chlorophyll-*a* (Chl-*a*) was measured by spectrophotometric methods and the CYN concentration was measured by high-performance liquid chromatographic methods. Data from these CYN analyses were evaluated as cell quota (ng CYN 10^{-6} cells) or as the relative proportion between toxin concentration and chlorophyll-*a* concentration (CYN Chl-a^{-1}). The CYP011K showed higher growth rates than the CYP030A strains. According to the cell quota results, the production of CYN by these two strains of *C. raciborskii* is not affected by the intensity of the surrounding light during growth. However, analysis of the relative proportion of CYN Chl-a^{-1} led to different interpretations, as a negative relation between light intensity and CYN production.

Key words: *Cylindrospermopsis raciborskii*, CYN cell quota, cylindrospermopsin per Chlorophyll-*a*.

Uso da normalização de cylindrospermopsina por quota celular e por clorofila em cepas de *Cylindrospermopsis raciborskii* sob diferentes intensidades luminosas

Resumo

A produção de cilindrospermopsina (CYN) por *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya e Raju Subba já foi associada a mudanças de intensidade luminosa. Neste estudo avaliamos as influências de diferentes intensidades de luz na produção de CYN por duas cepas de *C. raciborskii* (CYP011K e CYP030A) em cultivos fechados. As cepas foram cultivadas em intensidades luminosas de 40 a 348 µmol fotóns m^{-2} s^{-1}. As taxas de crescimento foram medidas por meio de regressão exponencial aplicada à variação do número de células ao longo do tempo. A concentração de clorofila-a (Chl-*a*) foi medida por métodos espectrofotométricos e a concentração de CYN foi determinada por técnicas de HPLC. Os dados das análises de CYN foram avaliados como quota celular (ng CYN 10^{-6} células) ou como proporção relativa entre a concentração de toxina e clorofila-a (CYN Chl-a^{-1}). A cepa CYP011K apresentou maiores taxas de crescimento do que a cepa CYP030A. De acordo com os resultados de cota celulares, a produção de CYN por estas duas linhagens de *C. raciborskii* não é afetada pela intensidade da luz, durante o crescimento. No entanto, a análise da proporção relativa de CYN Chl-a^{-1} levou a interpretações diferentes, como a aparente relação inversa entre o aumento da intensidade luminosa e a produção de CYN. Este estudo reforça o fato de que diferentes formas de normalização de dados levam a diferentes interpretações sobre a toxicologia fisiologia e ecologia de *C. raciborskii*.

Palavras-chave: *Cylindrospermopsis raciborskii*, quota celular, cilindrospermopsina por clorofila-a.

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INTRODUCTION

Cylindrospermopsis raciborskii is a planktonic, nitrogen-fixing, bloom-forming, freshwater cyanobacterium. *C. raciborskii* has become a notorious cyanobacterium, due to its invasive capacity associated with global warming and its worldwide occurrence (Wiedner *et al*. 2007, Haande *et al*. 2008). The increased proliferation of *C. raciborskii* is of particular concern due to the ability to produce toxins. This species was first associated with a human poisoning incident on Palm Island, Australia, in 1979 (Hawkins *et al*. 1985). Subsequently, Ohtani *et al*. (1992) indentified a potent hepatotoxic alkaloid containing a cyclic guanidine named cylindrospermopsin (CYN) from *C. raciborskii* strains. Since 1992, several strains of *C. raciborskii* from Australia and Asia have been frequently described as CYN producers (Griffiths & Saker 2003).

C. raciborskii can grow under different sources of nitrogen and low phosphate concentrations (Padisák 1997, Saker & Neilan 1999). Low light intensities and optically deep mixing tolerance is thought to increase its dominance (Wiedner *et al*. 2007, O'Brien *et al*. 2009). However, to different water bodies, there is not a strong correlation between *C. raciborskii* bloom and CYN concentration samples (Griffiths & Saker 2003). Furthermore, associations between growth and CYN concentrations in environmental samples are variable and unpredictable (Griffiths & Saker 2003).

Light intensity is considered as the growth and metabolism regulator of phytoplankton species (Reynolds 2006). Several laboratory studies on the effects of different photon density flux on toxin production by cyanobacteria have been developed. For example, in *Microcystis aeruginosa* production of microcystins was enhanced with increasing light intensity, with decreasing light intensity or it was not affected by light (Wiedner *et al*. 2003).

Light have major importance for regulating the dynamics of populations of *C. raciborskii* (Wiedner *et al*. 2007, Carneiro *et al*. 2009). Blooms of *C. raciborskii* have occurred under unsteady light intensities until to 830 µmol photons m^{-2} s^{-1} (Bouvy *et al*. 1999, Briand *et al*. 2002) and some strains of this species could acquire advantages both in low and high light intensities (Briand *et al*. 2002). So, light intensity is a regulator of the growth and ability to produce toxins by *C. raciborskii*. However, few studies have been conducted to explain the effects of light intensity on the production of CYN under controlled conditions. Additionally, due the literature use different ways to describe *C. raciborskii* ecophysiology, the present study investigated the differences between CYN data normalization in two *C. raciborskii* strains under different light intensity.

MATERIAL AND METHOD

Strain maintenance

CYP011K and CYP030A were the two strains of *C. raciborskii* used. These strains were kindly provided by Dr. Andrew Humpage (Australian Water Quality Centre, Australia) and their identities were confirmed as CYN producers by high-performance liquid chromatographic (HPLC) techniques. The strains were grown and stored in the culture collection of the Laboratory of Cyanobacterial Ecophysiology and Toxicology (IBCCFº - UFRJ). Nonaxenic batch cultures were grown in ASM-1 medium (Gorhan *et al*. 1964) with aeration (provided by a compressed air pump), at 24 ± 2 ºC, with photoperiods of 12h. These conditions were used for all experiments. Cultures of these strains were kept under photon density flux of 40 µmol photons m^{-2} s^{-1}, provided by ordinary fluorescent light bulbs, and the light intensity was measured with a quanta sensor (QST-100 Box – Biospherical Instruments Inc.).

Selection of the light intensities

The CYP011K and CYP030A strains were grown under different light intensities prior to the experimental tests to verify their susceptibility to high light. The maximum light intensity of 100 µmol photons m^{-2} s^{-1} for CYP011K and 348 µmol photons m^{-2} s^{-1} for CYP030A were selected because the strains formed a lot of cell lysis when grown at higher light intensities. During the stationary phase, cultures of the CYP011K strain were maintained under photon flux densities of 40, 60 and 100 µmol photons m^{-2} s^{-1}, while cultures of the CYP030A strain were maintained under photon flux densities of 100 and 348 µmol photons m^{-2} s^{-1}. The cultures were kept under these conditions for five generations, during which they were able to adapt to their respective photon flux densities. The strains were inoculated in glass balloons, with 1.0 10^6 cells per milliliter of the ASM-1 medium (of which 2 liters were used). Each set of conditions was performed in triplicate. Before the cells were inoculated, each light intensity was measured by immersing the quanta sensor in distilled water (using the same volume as the cultures) at the bottom of a similar glass balloon.

Growth measurements

To determine the rate of cellular growths, under sterilized conditions, samples were harvested daily throughout the duration of the experiment (12 days). Cell measurements were performed under light microscopy using an ocular ruler. A few milliliters were harvested from each culture to determine the average length of a cell by randomly measuring the lengths of ninety cells. At each sampling time, samples were also used to measure the lengths of the filaments, by a Fuchs Rosenthal hemocytometer. The total length of the filaments measured was divided by the average cellular length, giving the number of cells per milliliter of culture obtained from each condition (Carneiro *et al*., 2011). The growth rates of the strains were determined by exponential regression of cell number variation along the time, and are presented as the relative growth rate (µ day^{-1}) (Reynolds, 2006), using the followed formula:

$$N_t = N_0 e^{\mu . t}$$

where N_t and N_0 are the final and initial number of cells (cells mL^{-1}), respectively, and t is the time (day).

Chlorophyll-a measurements

Chlorophyll-*a* (Chl-*a*) were determined in samples harvested under sterile conditions every three days over the course of experiment. Ten milliliters were taken from each culture and filtered using borosilicate filters (13mm diameter – Millipore). The filters were put into test tubes with 5 mL of pure methanol (100%), and the resulting solutions were kept in the dark for 30 min. The samples were then centrifuged (10000 g, 20 min, 4º C) and the supernatant solutions were harvested and used to determine their optical densities in 665 nm (with a turbidity correction at 750 nm). The Chl-*a* concentration was calculated via Mackiney's extinction coefficient (Mackiney 1941), which uses the followed formula:

$$C = [(OD_{665}\text{-}OD_{750}) \cdot v] / (V \cdot k \cdot d)$$

where C is the Chl-*a* concentration (µg L^{-1}), v is the volume of methanol (mL), V is the volume of the culture (L), k is Mackiney's extinction coefficient (74.5 L g^{-1} cm^{-1}), and d is the distance travelled by the light (cm).

CYN Analysis

To determine the CYN concentrations, 500 mL samples were collected. These samples were collected on the sixth and twelfth days of culturing of each strain to represent the exponential and stationary growth phases respectively. These samples were filtered through borosilicate filters (45mm diameter – Millipore). After filtration, the cells contained in the filters were analyzed to CYN measurments. All samples were stored at -20ºC until they were analyzed by HPLC.

The methods used for the extraction of CYN from intracellular samples were modifications of those described by Li *et al*. (2001). The filter from each sample was cut into small pieces, and added to 20 mL of Milli-Q water. The mixtures were shaken for two hours with a magnetic workbench shaker, and were then centrifuged (30 min, 10000 g). The supernatants were then passed through octadecylsilane cartridges (Bond Elut C_{18}). Cartridges were regenerated with 20 mL of pure methanol (100%), and equilibrated with 20 mL of ultrapure water (Milli-Q). Before samples were passed, the cartridges were eluted with 40 mL of Milli-Q water. The eluted fractions were then freeze-dried, liofilized, and resuspended in 2 mL of Milli-Q water. These suspensions were then filtered with 0.45 µm nylon filters (13 mm – Millipore), and the resulting pure samples were analyzed by HPLC.

The intracellular CYN were analyzed by a Shimadzu HPLC system, using a silica-base reversed phase column (125mm x 4.0mm, 5µm; Lichrospher 100 RP 18) with a photo-diode array detector. The chromatography was performed according Welker's method (Welker *et al*. 2002) using a mobile phase with a polarity gradient of 0-50% water:methanol added with trifluoroacetic acid (TFA, 0.05%, V/V) over 20 min. The CYN was detected at 262 nm, and its identity was confirmed by comparison with known retention times and the integrated areas of the standard. This standard was purchased from the Institute of Marine Bioscience – National Research Council of Canada (Halifax, Canada).

The CYN concentrations that had been determined by HPLC were then assessed as cell quota (ngCYN 10^{-6} cells) and as the relative proportion between CYN and Chl-*a* content (CYN Chl-a^{-1}) in order to elucidate the dynamics of the CYN production in the two different strains of *C. raciborskii* with cell number corresponding to a bloom of this species (10^6 cells mL^{-1}). The relative proportion was calculated by dividing the CYN concentration per liter (µg L^{-1}) by the Chl-*a* concentration per liter (µg L^{-1}).

Statistical analysis

All of the data are expressed as mean values ± standard error (SE). To CYP011K strain, one-way analysis of variance (ANOVA) was used to test the differences between the data of each treatment. If the ANOVA results showed that the variations were significant ($p < 0.05$), Tukey's multiple comparison tests were used to compare the values of each treatment used. In comparisons to CYP030A strain data, Student's *t*-tests were performed to determine the differences between the two observations. All tests were performed with 95% of significance ($p<0.05$) using the Statistica for windows (v9.0) application.

RESULTS

Cellular growth

In all light treatments, both strains grew exponentially until the sixth day (Fig 1). The relative growth rates are presented in Table 1. The cells of the CYP011K strain had the highest growth rate under 100 µmol photons m^{-2} s^{-1} (ANOVA, $p<0.05$), and this growth rate represented the highest growth rate across the two strains of *C. raciborskii* (*t*-test, $p<0.05$). Significant differences in growth rates were not seen in the cultures of CYP030A cells (*t*-test, $p>0.05$) submitted to different light intensities.

Chlorophyll-a variation

Chl-*a* concentrations were normalized to 10^6 cells. The CYP011K strain cells under different tested photon density flux has showed the highest Chl-*a* concentrations after the third day of culturing (Fig. 2A, ANOVA, $p<0.05$ to all treatments). In the cell cultures stored under 40 µmol photons m^{-2} s^{-1}, the concentration of Chl-*a* doubled between the third and ninth day of growth (Fig 2A; *t*-test, $p<0.05$). This trend also occurred in the cultures stored under 60 µmol photons m^{-2} s^{-1} between the sixth and ninth days (Fig 2A; *t*-test, $p<0.05$).

The cells of the CYP011Kstrain grown under 100 µmol photons m^{-2} s^{-1} had no change in Chl-*a* concentration for the duration of the growth (Fig 2A; ANOVA, $p>0.05$). This same pattern was also seen in cells of CYP030A strain exposed to the two tested photon density flux (Fig 2B; ANOVA, $p>0.05$ to the two treatments). However, in the results from the ninth day of culturing (early stationary phase), significant

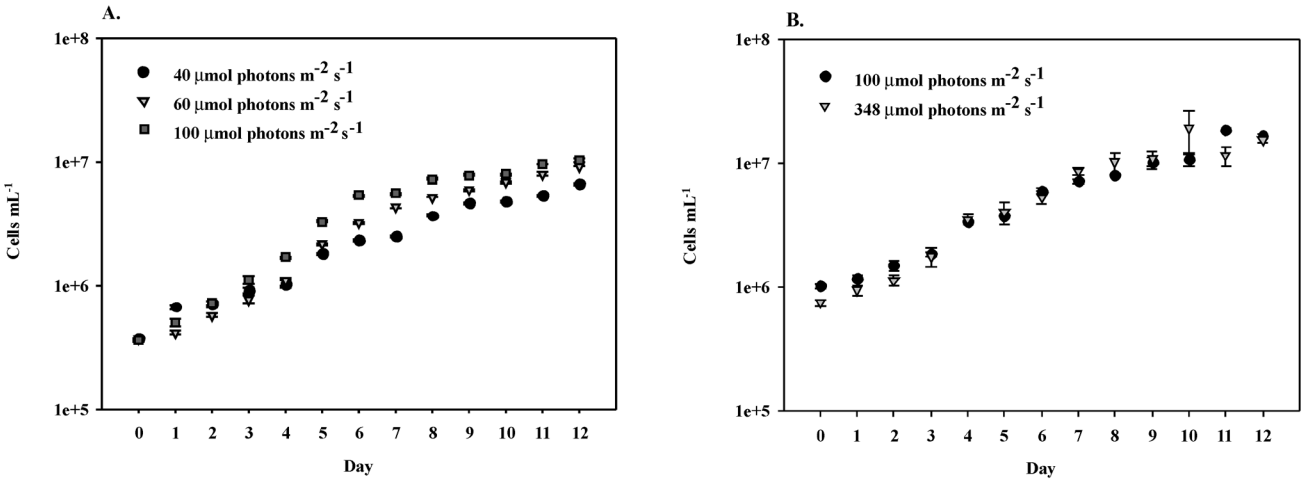

Figure 1 - Growth curves of *C. raciborskii* CYP011K (A) and CYP030A (B) strain cells exposed to three different light intensities. The error bars are standard errors (n=3).

Table 1 – Growth rates and Chlorophyll-*a* concentrations in *C. raciborskii* cells exposed to four different light intensities.

Strain	Treatment[a]	µ,day^{-1}[b]	Chl-*a* (µg 10^{-6} Cells)	
			9th day	12th day
CYP 011K	40	0.313 ± 0.04	0.324 ± 0.01	0.310 ± 0.02
	60	0.477 ± 0.03	0.291 ± 0.01	0.251 ±0.03
	100	0.524 ± 0.02	0.234 ± 0.01	0.210 ± 0.02
CYP 030A	100	0.393 ± 0.03	0.113 ± 0.02	0.108 ±0.05
	348	0.375 ± 0.12	0.277 ± 0.11	0.305 ±0.12

Data is presented with average ± standard error (n=3). [a] The light intensities are given in µmol photons m^{-2} s^{-1}. [b] µ is relative growth·day^{-1}.

differences between two samples from the different strains that were kept under 100 µmol photons .m^{-2} .s^{-1} were observed (Table 1). The Chl-*a* concentration in the CYP011K strain cells was two times that of the CYP030A strain cells (Fig 2B, *t*-test; ninth day, p<0.05, twelfth day, p<0.05).

T*he influence of light intensity on CYN production by C. raciborskii*

The intracellular CYN measurements as cell quota are given in table 2.

In both the exponential and the stationary phase, cells of both strains did not present any significant differences in the CYN cell quota (CYP011K, ANOVA, p>0.05; CYP030A, *t*-test, p>0.05). These results clearly show that the production of CYN by both of these strains of *C. raciborskii* is not affect by the intensity of the light to which they are exposed during growth.

Results of CYN production as the proportion between CYN Chl-*a* $^{-1}$ are given in table 2. These results show a significant decrease in the relative proportion CYN Chl-*a* $^{-1}$ of the CYP011K strain cells kept under 100 µmol photons m^{-2} s^{-1} during the exponential phase, as compared to that of 40 and 60 µmol photons m^{-2} s^{-1} (*Tukey's*-test, p<0.05 for both). Decreases in the relative proportion CYN Chl-a^{-1} were

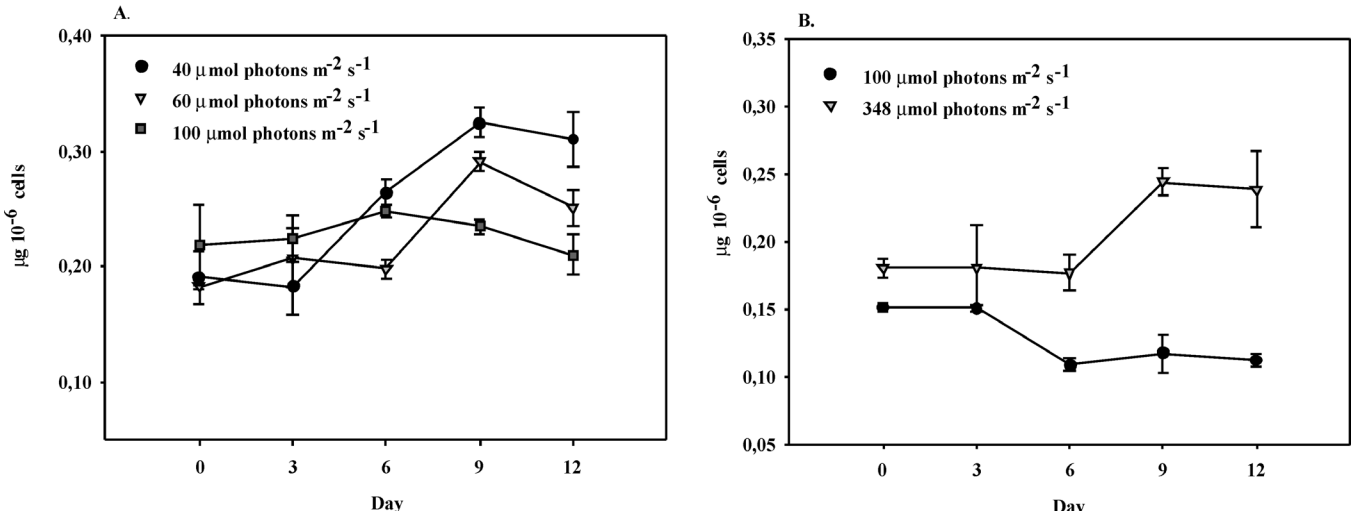

Figure 2 - Chlorophyll-*a* variation in *C. raciborskii* CYP011K (A) and CYP030A (B) strain cells exposed to three different light intensities. The error bars are standard errors (n=3).

Table 2 - Values of CYN data from the two different *C. raciborskii* strains at different phases of growth.

Strain	Treatment[a]	Cell quota		Proportion	
		ng 10^{-6} cells		CYN Chl-a^{-1}	
		6th day	12th day	6th day	12th day
CYP 011K	40	24.687 ± 9.51	17.538 ± 3.06	0.092 ± 0.002	0.056 ± 0.003
	60	18.853 ± 6.00	16.524 ± 1.16	0.094 ± 0.001	0.067 ± 0.006*
	100	12.112 ± 5.55	14.189 ± 2.44	0.049 ± 0.002**	0.068 ± 0.008*
CYP 030A	100	5.731 ± 1.40	5.065 ± 1.04	0.053 ± 0.007	0.106 ± 0.003
	348	3.179 ± 0.67	5.369 ± 2.19	0.021 ± 0.003*	0.016 ± 0.005**

[a] Data are presented as average of CYN ± standard error (n=3). The light intensities are given in µmol photons m^{-2} s^{-1}. * Statistical differences ($p<0.05$), **

also observed in the exponential and stationary phases of the CYP030A strain (*t*-test, $p<0.05$ and $p<0.01$, respectively). However, significant increases in the relative proportion CYN Chl-a^{-1} of the CYP011K strain was observed when cells were kept under 60 and 100 µmol photons m^{-2} s^{-1} and reached to their stationary phases (*Tukey's*-test, $p<0.05$ for both).

These observations showed that the CYN production decreases with increased light intensity for the CYP030A strain. However, the relationship between these two factors was shown to vary according the growth phase in the case of the CYP011K strain.

Variation of CYN production between the different phases of growth

To verify the changes in the production of CYN between the two different phases of growth, the percentages of CYN production in the stationary phase were compared with those of the exponential growth phase for each strain. To simplify the observations, data with statistical tests are listed in Table 3. In summary, the CYN production by the CYP011K strain was reduced in cyanobacteria grown under lower photon density flux (Table 3), as is confirmed by the data in terms of cell quota and the ratio of CYN Chl-a^{-1}. However, the CYN production in the CYP011K strain was higher under 100 µmol photons m^{-2} s^{-1}. This same effect was also observed with the CYP030A strain, although this is only apparent when the data is evaluated as the proportion CYN Chl-a^{-1}. Interestingly, at the highest photon density flux in the CYP030A strain (348 µmol photons m^{-2} s^{-1}), the CYN production can be viewed as both reduced and increased, based on the CYN content normalization.

Comparison of the CYN production between the studied strains

It is clear that the trends in CYN production seen with the CYP030A strain are different from those observed with the CYP011K strain. In fact, at the same light intensity (100 µmol photons m^{-2} s^{-1}), the CYP011K strain produced 2.1 times more CYN per cell than the CYP030A strain (table 3, *t-test*, $p<0.05$) during the exponential phase. This trend continued to the stationary phase, for which the CYN content per cell in the CYP011K strain was 2.8 times higher than that of the CYP030A strain cells (table 3, *t-test*; $p<0.01$).

While these trends are apparent, they must be verified by looking to the CYN Chl-a^{-1} proportion. In the exponential phase, the CYP030A strain's CYN Chl-a^{-1} ratio was 0.06 times higher than that of the CYP011K strain (table 3, *t-test*, $p>0.05$). This difference became greater during the stationary phase, during which the CYN Chl-a^{-1} proportion in the CYP030A strain increased to 1.56 times higher than that of the CYP11K strain (table 3, *t-test*, $p<0.01$).

DISCUSSION

Our results emphasize the fact that different normalizations of the cyanotoxin data can lead to different conclusions about

Table 3 – Percentages between determined CYN at stationary phase in relation to exponential phase of two *C. raciborskii* strains.

Strain	Treatment[a]	Cell quota	Proportion
		ng 10^{-6} cells	CYN Chl-a^{-1}
CYP 011K	40	-19.2% ± 3.8*	-38.8% ± 8.5*
	60	-14.7% ± 2.3*	-29.2% ± 4.8*
	100	23.2% ± 2.2*	39.8% ± 3.2*
CYP 030A	100	-7.5% ± 2.6*	101.3% ± 18.2**
	348	64.1% ± 3.4**	-27.3% ± 4.2*

[a] Data are presented as average of CYN ± standard error (n=3). The light intensities are given in µmol photons mm^{-2} s^{-1}. * Statistical differences ($p<0.05$), ** Statistical differences ($p<0.01$). Negative values represent decreases in the production of CYN (stationary phase).

the physiology and toxin production of a cyanotoxin-producing species. Regarding to the growth of *C. raciborskii* strains, under laboratory conditions, this species was previously reported to have optimal growth at 121 or 240 µmol photons m^{-2} s^{-1} (Dokuli & Mayer 1996), 80 µmol photons m^{-2} s^{-1} (Padisák 1997, Dyble *et al*. 2006) or 118 µmol photons m^{-2} s^{-1} (Saker *et al*. 1999). Wiedner *et al*. (2007) demonstrated that *C. raciborskii* requires light intensities between 80 and 120 µmol photons m^{-2} s^{-1} in order to maximize growth, however, this cyanobacterium can achieve close to these maximum growth rates with light intensities of up 500 µmol photons m^{-2} s^{-1}. In this way, *C. raciborskii* is tolerant of high light intensities. Higher relative growth rates were seen during the exponential growth phase of the CYP011K strain when exposed to higher intensities of light (from 40 to 100 µmol photons m^{-2} s^{-1}). The CYP030A strain had a lower growth rate at 100 µmol photons m^{-2} s^{-1} than that of the CYP011K strain, which seem confirms that the growth rate of *C. raciborskii* could be strain specific. *C. raciborskii* could be efficient at capturing light, which would allow it to survive in environments with low intensities of light (Padisák 1997). Regarding morphology, while *C. raciborskii* CYP011K exhibited a great number of cell lyses at 100 µmol photons m^{-2} s^{-1}, the CYP030A strain showed no negative effects due to the intensity of the light under which they were grown until 348 µmol photons m^{-2} s^{-1}.

In low-intensity environments, increases in Chl-*a* production would be sufficient to maintain a steady growth rate. Higher Chl-*a* concentrations were seen in CYP011K strain cells at lower intensities of light (40 and 60 µmol photons m^{-2} s^{-1}). This confirms Padisák's (Padisák 1997) hypothesis because under intensities of 100 µmol photons m^{-2} s^{-1} and above, CYP030A strain cells showed no significant increases in Chl-*a* concentration at any point in their growth. However, under the lowest intensities of light, the Chl-*a* concentration duplicated during the growth of the CYP011K strain cells. In batch cultures, the uneven spread of light caused by cell shadings in the upper layers is a recurring problem. The consequence of this is the increase in the Chl-*a* concentration of the cells in order to supply the additional energy required due to the decrease in external lighting (Reynolds, 2006). If high intensities of light can maintain normal growth levels and solve the problems associated with cell shadings, increase in the Chl-*a* concentration hardly ever is observed (Reynolds, 2006), as seen in cells of the CYP011K strain that were exposed to 100 µmol photons m^{-2} s^{-1} and in cells of the CYP030A strain that were exposed to 100 and 348 µmol photons m^{-2} s^{-1}.

The relationship between CYN production and light intensity has already been investigated in *C. raciborskii* strains. In a review, Griffiths and Saker (2003) explained that despite the fact that *C. raciborskii* cells can grow better when exposed to intensities of light between 50 and 100 µmol photons m^{-2} s^{-1}, the highest CYN cellular content is obtained when the cells are exposed to higher intensities of light. This observation suggests that the relationship between the CYN production increment and increased light intensity is proportional. More recently, Dyble *et al.* (2006) described a linear relationship between the intensity of the light when cells are exposed to light from 18 to 75 µmol photons m^{-2} s^{-1}. Intracellular CYN production per Chl-*a* content increased at the eighth and sixteenth days of culturing of the *C. raciborskii* strain SDS (which was grown in a nitrogen-free medium). No relationship was seen when the CYN production in each of the two strains studied in this work was evaluated as a cell quota (CYN 10^{-6} cells). From our results, it is possible to confirm that these two strains of *C. raciborskii* (CYP011K and CYP030A) are not affected by the intensity of the light to which they are exposed during growth.

If the CYN production of the two studied strains is evaluated as the CYN Chl-a^{-1} proportion, it often elucidates the negative relationship between CYN production and the intensity of light. This is still true, even taking into account that the CYP030A strain produced about two times less Chl-*a* per cell than the CYP011K strain. The mathematical conversion of the CYN data to a proportion of the Chl-*a* content probably does not reflect the real behavior of the cyanotoxin production. In any case, whether viewing it as a cell quota or as the CYN Chl-a^{-1} proportion, the CYN production by CYP011K and CYP030A are different from those previously reported in the literature. However, cells grown in nitrogen-free media had high CYN contents (on a cell dry-weight basis) as compared with cells grown in media with nitrogen sources (Saker & Neilan 2001, Griffiths & Saker 2003). Therefore, it is possible that different amounts of CYN are produced by *C. raciborskii* based on environmental factors such as the presence of nitrogen in the medium.

According to Hawkins *et al*. (2001), the growth phases of *C. raciborskii* are also important because the CYN could be produced differently during the exponential and stationary phases under different environmental stimuli. CYN concentrations per cell dry-weight of four strains of *C. raciborskii* produced more CYN during the exponential phase (Reynolds, 2006). Dyble *et al*. (2006) described a proportional CYN accumulation (CYN Chl-a^{-1}) from the eighth to the sixteenth days of culturing the cells with the *C. raciborskii* strain SDS that was kept in a nitrogen-free medium, at several different intensities of light (35, 75, 53 and 140 µmol photons m^{-2} s^{-1}). This data suggests the accumulation of CYN during the stationary phase. When analyzing the production of CYN as a cell quota (CYN 10^{6} cells) or as the CYN Chl-a^{-1} proportion, we noted the different variations in CYN production based on the intensity of the light and on the strain (Table 2). In summary, our results clearly demonstrate that different conclusions can be made, depending on the normalization of the data, to explain the effects of light on CYN production by *C. raciborskii*.

Neilan *et al*. (2003) found three separated phylotypes of *C. raciborskii* strains that are CYN producers, in Australia, Europe and North and South America, based in the 16S rRNA gene nucleotide sequences. Chonudomkul *et al*. (2004) explained that toxin the production by twenty-four different strains of *C. raciborskii* were polyphyletic when their similarities in short repetitive sequences in the genes were analyzed. Recently, Stucken *et al*. (2009) showed that there is no correlation

between toxic phenotypes and phylogenetic association in the Australian strains. The results from these previous studies show that different strains of *C. raciborskii* can have different rates of toxin production. These observations agree with our results, as two different strains stored under a same intensity of light (100 µmol photons m^{-2} s^{-1}) produced significantly different CYN concentration per cell. According Reynolds (2006), growth measurements at the different bases (e.g. dry-weight, chlorophyll-*a*, optical density) provide a larger problem and are responsible for the loss of species-specific physiological information. The conclusions formed based on the CYN Chl-a^{-1} proportion are most likely extraneous, for the reasons discussed above.

CONCLUSION

The production of cylindrospermopsin by the two *C. raciborskii* strains represented in this work is not effected by light intensity.

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