Short-Term Physiological and Transcriptomic Analysis of Saxitoxin-Producing and Nonproducing *Raphidiopsis raciborskii* (Cyanobacteria) under an Extremely High CO$_2$ Environment

Ricardo Rogers Paranhos [1,2,*,†], Mauro Vilar [2,†], Luísa Hoffmann [3], Thayane Bottaro [2], Rosane Silva [2], Renato C. Pereira [4,5] and Sandra M. F. O. Azevedo [2,*]

[1] Programa de Pós-Graduação em Dinâmica dos Oceanos e da Terra, Universidade Federal Fluminense, Niterói 24210-346, RJ, Brazil

[2] Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro 21941-170, RJ, Brazil; maurovilar@biof.ufrj.br (M.V.); thayanebottaro@biof.ufrj.br (T.B.); silvaros@biof.ufrj.br (R.S.)

[3] Federal Institute of Education, Science and Technology of Rio de Janeiro (IFRJ), Rio de Janeiro 20270-021, RJ, Brazil; luisa@biof.ufrj.br

[4] Department of Marine Biology, Federal Fluminense University, (UFF), Niterói 24001-970, RJ, Brazil; rcrespo@id.uff.br

[5] Rio de Janeiro Botanical Garden Research Institute, Rio de Janeiro 22460-030, RJ, Brazil

* Correspondence: biorogers@gmail.com (R.R.P.); sazevedo@biof.ufrj.br (S.M.F.O.A.)

† These authors contributed equally to this work.

Abstract: Findings about CO$_2$ dynamics in the Earth's ancestral atmosphere have suggested much higher concentrations in past eras. Along this line, cyanobacteria are an early evolved photosynthetic group that is suggested to have experienced both high and low CO$_2$ availability since their Precambrian origin, and therefore, it is reasonable to assume that these microbes have the potential to cope with these scenarios by rapidly adjusting to various carbon dioxide levels. Thus, in this work, we performed a short-term (72-h) investigation of the physiological parameters (cell growth, photosynthesis and saxitoxin production) of toxic and nontoxic strains of the cyanobacterium *Raphidiopsis raciborskii* challenged by an extremely high pCO$_2$ (40,000 ppm). Additionally, the transcriptomic profile (regarding the carbon concentrating mechanism and photosynthesis) of selected toxic and nontoxic strains is also presented. We found that short-term exposure to extremely elevated CO$_2$ concentrations did not affect *R. raciborskii* physiology regardless of toxin production. However, transcripts related to bicarbonate transporters and the RuBisCO enzyme indicated the upregulation of CCM and downregulation of the Calvin cycle, respectively. According to our findings, at least at the initial growth phase, *R. raciborskii* was able to cope with a very high CO$_2$ level, which shed light on the understanding that this species might have the potential to cope with carbon dioxide in water above the predicted levels.

Keywords: CCM; saxitoxins; climate change; transcriptome; cyanobacteria

1. Introduction

Usually, CyanoHABs (cyanobacterial harmful algal blooms) are correlated with artificial eutrophication in inland and coastal waters impacted by anthropogenic activities. However, in recent decades, these phenomena have become more frequent and persistent worldwide due to the rise in CO$_2$, which directly affects global temperatures and results in extreme climate changes [1,2]. According to the International Panel on Climate Change (IPCC), a threefold increase in the current CO$_2$ concentration (410 ppm) is expected in the next hundred years [3], worsening global warming even more. Moreover, in addition to

increasing global surface temperature, CO_2 enhancement can also affect the water chemistry (e.g., pH and dissolved inorganic carbon dynamics) in both freshwater and marine environments, which together may impact the ecophysiology of several HAB species [4].

Over the past geological eras, the planet Earth has experienced many changes in atmospheric gas composition [5], with special emphasis on carbon dioxide. The highest CO_2 concentrations date from the Precambrian and Paleozoic eras [6] and estimates suggest that carbon dioxide concentrations could have reached 6000 ppm during the Cambrian period and decreased until the Permian to reach the current values. However, more recently, Lehmer et al. [7] suggested an even higher estimated CO_2 concentration by modeling iron oxidation of micrometeorites by CO_2. The authors suggested that 2.7 billion years ago, our atmosphere could have been composed of a much higher concentration of carbonic gas (approximately 70%) [7]. These findings coincide with estimates of both the emergence of cyanobacteria and the atmospheric oxygenation around 2.7–3.8 billion years ago [8]. Therefore, these oxyphotosynthetic microbes must have coped with an extremely variable CO_2 scenario up until the present conditions.

As a key enzyme in the photosynthetic process, CO_2-fixing ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) has evolved in different phytoplankton groups in terms of CO_2/O_2 affinity [9]. For instance, in cyanobacteria, to sustain maximum CO_2 fixation, RuBisCO was allocated to cellular microcompartments called carboxysomes [10]. In early evolved phytoplankton, such as cyanobacteria and dinoflagellates, RuBisCO enzymes had a low CO_2 affinity since these microorganisms dated from a scenario with higher CO_2 availability [9]. However, as the CO_2 concentration declined, phytoplankton evolved carbon concentrating mechanisms (CCMs) [11] to allow a higher inorganic carbon (C_i) accumulation at the active site of carboxilation. CCMs consist of uptake systems (membrane transporters and enzymes) that enable cyanobacteria and some phytoplankton to take up C_i when CO_2 levels are low. Five different C_i uptake systems have been described in cyanobacteria to date: two for CO_2 uptake (NDH-I_3 and NDH-I_4) and three for HCO_3^- uptake (BCT1, BicA and SbtA) with differential bicarbonate affinity and flux rate [12,13]. Moreover, the enzyme carbonic anhydrase (CA) also plays a role in CCM functioning by catalyzing the interconversion between CO_2 and HCO_3^- and preventing C_i leakage out of the cell [14].

Among bloom-forming cyanobacteria, *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*) [15] stands out as an N_2-fixing filamentous cyanobacterium known to tolerate a range of environmental conditions [16,17], in addition to being considered an invasive microbe, with recent expansion to temperate regions [18,19]. This cyanobacterium is also assumed to be a bicarbonate-user species with a CCM more suitable to acquire C_i as HCO_3^- [20,21], and its adaptive success has been related to high phenotypic plasticity [22]. Furthermore, some strains of *R. raciborskii* produce toxic metabolites, including bioactive alkaloids such as cylindrospermopsins (CYNs) or saxitoxins (STX—also referred to as paralytic shellfish toxins). STXs are guanidinium-containing neurotoxic alkaloids that block cellular ionic channels encompassing more than 50 analogs, according to the degree of sulfation (e.g., saxitoxin, gonyautoxins and C-toxins) and decarbamoylation (e.g., dicarbamoyl-saxitoxin) [23]. The CYN analogs (7-epicylindrospermopsin and 7-deoxycyclicylindrospermopsin) are tricyclic alkaloids of cytotoxic potential whose mechanism of action is the inhibition of protein synthesis, and genotoxic, immunotoxic and oxidative damage effects have been described [24].

In South America, toxic strains of *R. raciborskii* have been reported to be STX producers [21,25,26], while no strain has been characterized as producing CYN to date, although this alkaloid has been found in environmental samples [27]. In addition, strains lacking genes encoding STXs or CYN [28] can also compose *R. raciborskii* blooms, cooccurring with toxic blooms.

Studies regarding the effects of increasing CO_2 on *R. raciborskii* usually focus on the scenario predicted by the IPCC (a threefold increase in 100 years or ~1300 ppm) and have demonstrated responses to high CO_2 conditions by examining different parameters, such as

photosynthesis [29], cellular Ci transporters [20], toxin production [30] and metabolism [31]. In general, the data suggest that this species can cope with the future CO_2 scenario. However, little is known regarding extremely high CO_2 concentrations, as reported for past geological eras, since the studies are restricted to the levels predicted for the future. Furthermore, there is a gap in the comprehension of the overall early responses underlying exposure to extreme CO_2 levels, which are likely to determine the upcoming physiological responses. In addition to several studies that have examined only toxic strains, it is also important to assess whether the ability to produce toxins is a significant trait regarding the response to increasing CO_2. Thus, we aimed to evaluate the physiological traits and the overall gene expression (transcriptome) of saxitoxin-producing and nonproducing strains of *R. raciborskii* under short-term exposure to extremely high CO_2 conditions (40,000 ppm). Considering the CO_2 scenario from which cyanobacteria have evolved, we tested the hypothesis that *R. raciborskii* can potentially cope with extreme CO_2 conditions, regardless of toxin production.

2. Materials and Methods

2.1. Cyanobacterial Strains and Culture Conditions

Four *Raphidiopsis raciborskii* strains were used in this study: the saxitoxin-producing LETC-CY-05 (formerly CYRF-01) and T3, which produce mainly the nonsulfated analogs saxitoxin (STX) and neosaxitoxin (neoSTX), and their decarbamoylated variants [32,33]. The nonproducing strains were LETC-CY-01 (formerly CYLP-01) and LETC-CY-02 (formerly NPCS-1) [34]. These cyanobacterial strains were isolated from waterbodies from different Brazilian regions: LETC-CY-05 (Funil Reservoir, Rio de Janeiro, southeastern Brazil), T3 (Billings Dam, São Paulo, southeastern Brazil), LETC-CY-01 (Paranoá Lake, Federal District, midwestern Brazil) and LETC-CY-02 (Custódia Reservoir, Pernambuco, northeastern Brazil). Batch cultures were established in sterile ASM-1 medium [35] under constant aeration ($\cong 0.041\%$ CO_2, air level) provided by an air injection device, initial pH 8.0, 23 ± 1 °C, light intensity of 40–50 µmol photons $m^{-2} \cdot s^{-1}$ and a 12:12 h light:dark cycle. The stock cultures were kept at the exponential growth phase through weekly medium renovation with fresh liquid ASM-1.

2.2. Experimental Setup

Batch cultures ($n = 4$) of the *R. raciborskii* strains were inoculated to an initial cell concentration of approximately 10^5 cells mL^{-1} ($\cong 15$ $mm^3 \cdot L^{-1}$) in 2-L Erlenmeyer flasks filled with sterile ASM-1 culture medium. The cyanobacteria were grown in an extremely high CO_2 environment obtained through a Thermco 8500 gas mixer (Thermco Instrument Corp, La Porte, IN, USA) coupled to a 100% CO_2 cylinder adjusted to provide 4% CO_2-rich air bubbling ($\cong 40,000$ ppm). The CO_2-rich air bubbling system consisted of a cotton plug crossed by a glass pipette immersed in the culture medium and connected to a silicone hose, which in turn was attached to a 5 mL syringe filled with hydrophobic cotton as a filtration device through which the compressed air passed. Controls ($n = 4$) consisted of cultures established at air level CO_2 conditions ($\cong 410$ ppm or 0.041% provided by an air compressor). The experiment was run over 72 h to examine the physiological responses and CCM and photosynthesis-related gene expression underlying cyanobacterial acclimation to the different CO_2 environments. Samples were taken every 24 h to evaluate physiological parameters (growth and photosynthesis) and after 72 h for cyanotoxins and transcriptome analysis.

2.3. Growth and Photosynthesis Measurements

Cyanobacterial samples were preserved with 1% acetic Lugol's solution, and cell counting was performed on a Fuchs–Rosenthal hemocytometer using an optical microscope (Olympus Trinocular Microscope BX series, Olympus, Center Valley, PA, USA). Given that *R. raciborskii* is a filamentous species, the measurement of at least 30 random trichomes and cells of each strain was carried out to establish the average cell number per trichome.

Cell measurements were also used to estimate the mean cell volume (μm^3) according to its respective geometric form and, together with the cell concentration, used to estimate the biovolume ($mm^3 \cdot L^{-1}$) [36,37]. The specific growth rate ($\mu \cdot d^{-1}$) was calculated according to Reynolds [38], and the growth yield (fold change) was determined by the ratio between the final and the initial biovolume.

The photosynthetic yield (photosystem II quantum yield; *Fv'/Fm'*) was measured for each *R. raciborskii* strain by a fluorimeter Phytoplankton Analyzer equipped with a PHYTO-EDF sensor (Walz PHYTO-PAM- Heinz Walz, Effeltrich Germany).

2.4. Transcriptomic Profile of CCM and Photosynthesis-Related Genes

We examined *R. raciborskii* LETC-CY-05 and LETC-CY-01 (saxitoxin-producing and nonproducing, respectively) transcriptomes to verify the effect of extremely high CO_2 on the expression of genes related to the carbon concentration mechanism and photosynthesis in both toxic and nontoxic strains.

2.5. Sample Processing and RNA Extraction

A total of 500 mL samples (*n* = 3) were harvested after 72 h of incubation from both control and extremely high CO_2 cultures. The samples were centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments, Wilmington, DE, USA) (10 min, $9148.2 \times g$). The supernatant was discarded, and the pellet was immediately frozen in a Shell Freezer (Labconco, Kansas City, MO, USA). Frozen material was lyophilized (Labconco, Kansas City, USA), and RNA extraction was performed with freeze-dried cell biomass. Total RNA extraction was performed by adding 3 mL of TRIzol (Ambion, Austin, TX, USA) to the freeze-dried biomass and processed according to the manufacturer's instructions. Ribosomal RNA depletion of the samples was obtained using a Ribo-Zero™ rRNA Removal kit (Epicenter, Denver, CO, USA).

2.6. Library, Sequencing and Gene Expression Analysis

Library preparation for massively parallel sequencing was performed using the Ion Total RNA-seq Kit v2 protocol and barcodes (RNA-Seq Barcode 1–16 kit, Thermo Fisher, Massachusetts, USA) to allow multiplex sequencing. Sequencing was performed using an Ion PI Hi-Q Sequencing 200 kit in a high-potency Ion Proton™ sequencer (Thermo Fisher, USA) on an Ion PI™ V3 semiconductor chip (100 gigabases of capacity). Sequencing reads were exported from Ion Server (Thermo Fisher, USA) in FASTQ format and uploaded to CLC Genomics Workbench v. 8.5 to proceed to sequence data analysis. Quality control was initially applied by removing low quality and size (<25 bp) sequences. Reads were mapped against the *R. raciborskii* reference genome CS-505, available in public databases. To identify genes in which transcripts varied significantly (fold change < −1.5 > 1.5; *p* value < 0.05), empirical analysis of differential gene expression (EDGE) was used [39]. To improve the confidence of comparative analysis between CO_2 conditions, datasets were log-transformed [40]. Moreover, in this study, differential gene expression analysis focused on some aspects of inorganic carbon acquisition-related transcripts. Complete transcriptome data will be provided in a further publication (data not shown).

2.7. Saxitoxin Extraction and HPLC-FLD Analysis

After 72 h of incubation, 200 mL culture samples of each STX-producing strain (T3 and LETC-CY-05) were harvested for saxitoxin analysis. For STX extraction, the samples were freeze-dried, resuspended in 500 mM acetic acid solution and incubated for 1 h. Subsequently, the extract was centrifuged at 8 °C and $1937 \times g$ for 10 min, and the supernatant was preserved. The procedure was repeated twice. The pooled supernatants were filtered through a 0.45 μm polyvinylidene fluoride (PVDF) Whatman filter and stored in 1.5 mL vials at −20 °C until the subsequent cyanotoxin analysis.

Saxitoxin content was analyzed using high-performance liquid chromatography equipment (Shimadzu, Kyoto, Japan) coupled to a fluorescence detector (HPLC-FLD). Toxin

separation was performed in a silica-based reversed-phase column (125.0 mm × 4.0 mm, 5 μm; Lichrospher 100 Reversed-Phase C18), and the analysis was carried out for nonsulfated saxitoxins (neosaxitoxin and saxitoxin) according to Oshima [41] under the following conditions: mobile phase of 2 mM heptanesulfonate in 30 mM ammonium phosphate buffer plus 5% acetonitrile running under isocratic conditions and at a flow rate of 0.8 mL/min. STX detection was performed using an RF-10 Alx fluorescence detector (Shimadzu, Japan) at excitation and emission wavelengths of 330 nm and 390 nm, respectively. The STXs were identified and quantified by comparison with STX and neoSTX standard solutions purchased from the Institute of Marine Bioscience, National Research Council of Canada (Halifax, NS, Canada). Total STX data (neoSTX + STX) were obtained in volumetric concentration ($\mu g \cdot L^{-1}$) but thereafter expressed as the total STX cell quota (fgSTX cell^{-1}) for toxin production evaluation.

2.8. Statistical Analysis

A two-way analysis of variance (ANOVA) was applied to compare strain-specific responses to the CO_2 effects on biovolume and photosynthetic yield. Student's *t*-test was used to perform comparisons of the CO_2 effect on the *R. raciborskii* growth rate, growth ratio and toxin production. All analyses and graphs were performed in GraphPad Prism 8.0 software, and the statistical significance assumed was a *p* value < 0.05.

3. Results

Incubation under an extremely high CO_2 environment (or high partial CO_2 pressure; pCO_2) did not affect the growth of most of the tested *R. raciborskii* strains (Figure 1). However, only the nontoxic LETC-CY-01 showed a significant decrease in growth under the higher pCO_2 (RM ANOVA, $F_{(1,6)}$ = 13.88; $p < 0.01$). In general, regardless of the ability to produce toxins and pCO_2 effects, *R. raciborskii* displayed an intraspecific variability in growth pattern. For instance, although the toxic *R. raciborskii* T3 was accidentally inoculated at an approximately twofold higher initial cell concentration (Figure 1), this strain displayed either a growth ratio (biomass yield) or a specific growth rate similar to the nontoxic LETC-CY-02, which in turn was different from the nontoxic LETC-CY-01 (Figure 2).

The decreased biovolume concentration shown by *R. raciborskii* LETC-CY-01 in response to the extremely high CO_2 (Figure 1) also resulted in a significantly decreased biomass production and specific growth rate (*t*-test; $p < 0.05$), demonstrating by the fact that this cyanobacterium grew half as much in comparison to itself when grown under air-level CO_2 concentration (Figure 2).

Furthermore, under a high pCO_2, LETC-CY-01 also displayed a significant decrease in the quantum yield of photosystem II (Fv'/Fm') after 24 h of incubation (Bonferroni's test, $p < 0.0001$), accompanied by a slight but still significant increase (RM ANOVA, $p < 0.05$; Table 1). Overall, the CO_2 environment had no impact on the photosynthetic yield of the other tested *R. raciborskii* strains (Table 1).

Regarding the STX-producing *R. raciborskii* strains, T3 was characterized as displaying a relatively higher total saxitoxin production (reaching ~12-fold; STX + neoSTX) than LETC-CY-05. Both toxic strains displayed significant changes in toxin production after 72 h of incubation (Figure 3; pairwise *t*-test, $p < 0.05$). However, saxitoxin production was not affected when these strains were challenged by extremely high pCO_2.

The transcriptome of the nontoxic LETC-CY-01 and the toxic LETC-CY-05 was examined for transcripts of CCM and photosynthesis-related genes by using the EDGE method. According to the transcriptomic analysis, both strains displayed an upregulation in genes encoding bicarbonate transport in response to the extremely higher pCO_2 (Table 2). However, genes encoding NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase, RuBisCO and related to CCM activity were downregulated in LETC-CY-01 after 72 h of incubation under a 40,000 ppm CO_2 concentration (Table 2).
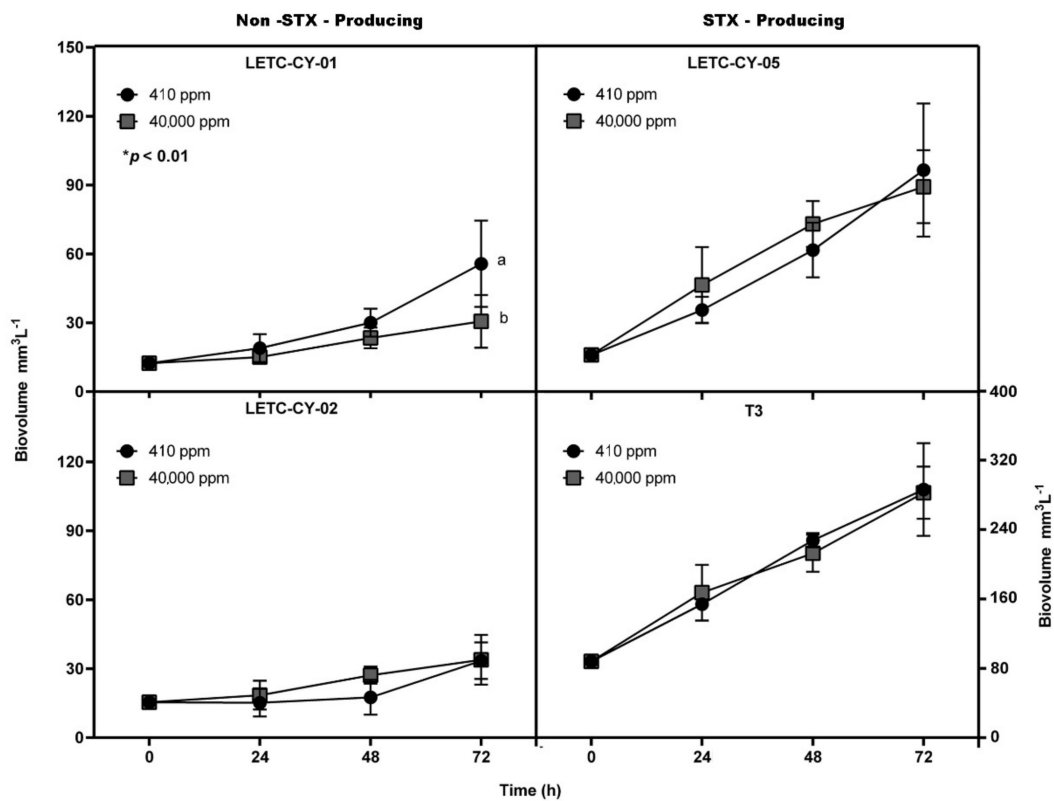
Figure 1. Growth curves estimated by biovolume ($mm^3 \cdot L^{-1}$) for LETC-CY-01, LETC-CY-02, LETC-CY-05 and T3 strains of *Raphidiopsis raciborskii* exposed to extremely high pCO$_2$ concentrations ($\cong$40,000 ppm) and under control conditions (current pCO$_2$ concentration $\cong$410 ppm). Different letters indicate significant differences ($p < 0.05$).

Table 1. Photosynthetic yields *(Fv′/Fm′)* for LETC-CY-01, LETC-CY-02, LETC-CY-05 and T3 strains of *Raphidiopsis raciborskii* exposed to extremely high pCO$_2$ concentrations ($\cong$40,000 ppm) and under control conditions (current pCO$_2$ concentration; $\cong$410 ppm). Different letters indicate significant differences ($p < 0.05$).

| | Non-STX Producing | | | | STX Producing | | | |
| | LETC-CY-01 | | LETC-CY-02 | | LETC-CY-05 | | T3 | |
Time	410 ppm	40,000 ppm	410 ppm	40,000 ppm	410 ppm	40,000 ppm	410 ppm	40,000 ppm
0 h	0.53 ± 0.00 [a]	0.53 ± 0.00 [a]	0.46 ± 0.00	0.46 ± 0.00	0.63 ± 0.00	0.63 ± 0.00	0.53 ± 0.00	0.53 ± 0.00
24 h	0.45 ± 0.05 [a]	0.33 ± 0.01 [b]	0.52 ± 0.02	0.49 ± 0.04	0.59 ± 0.02	0.60 ± 0.01	0.48 ± 0.01	0.49 ± 0.01
48 h	0.48 ± 0.03 [a]	0.36 ± 0.02 [b]	0.54 ± 0.01	0.51 ± 0.01	0.57 ± 0.01	0.54 ± 0.02	0.53 ± 0.01 [a]	0.46 ± 0.03 [b]
72 h	0.53 ± 0.02 [a]	0.45 ± 0.02 [b]	0.47 ± 0.03	0.48 ± 0.01	0.57 ± 0.02	0.54 ± 0.03	0.55 ± 0.01	0.53 ± 0.02
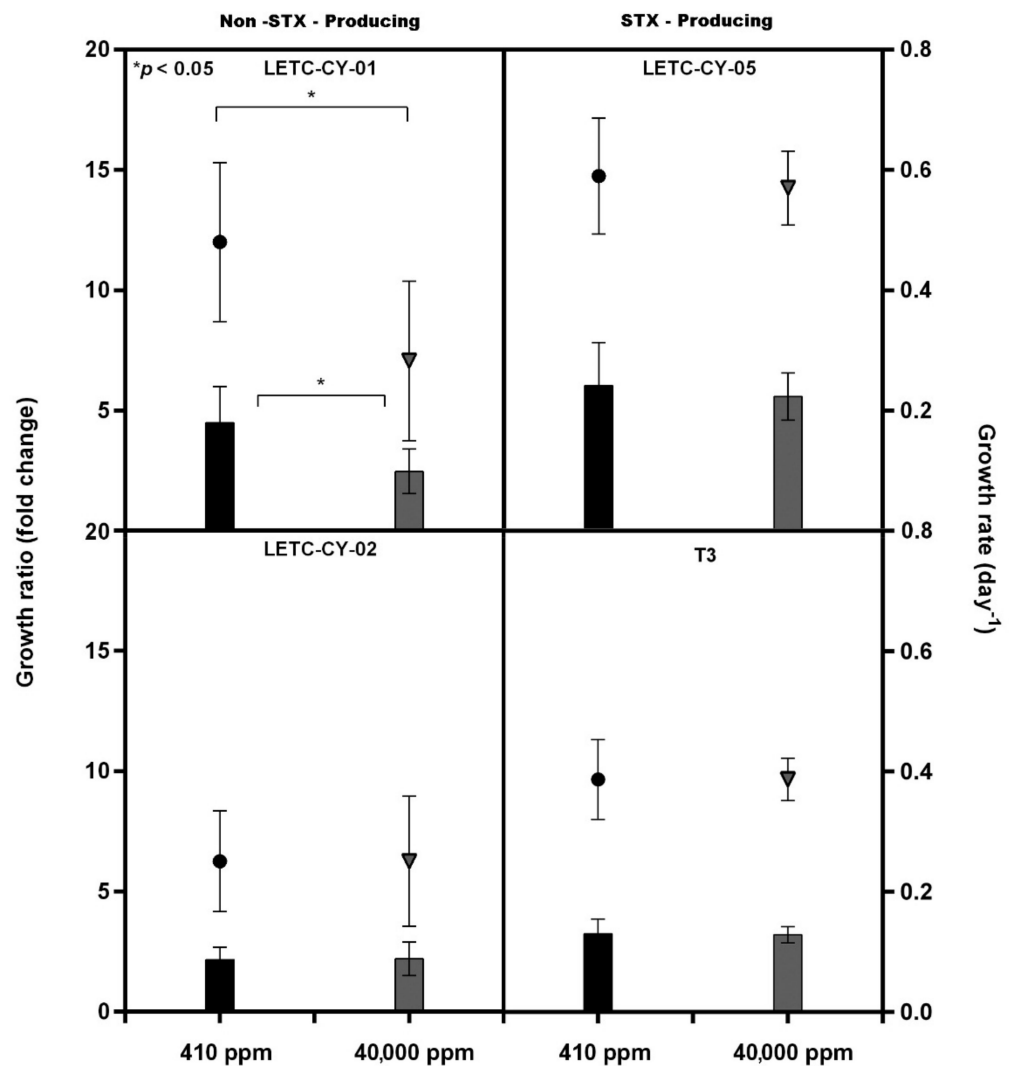
Figure 2. Growth rates (diamond and circle; day^{-1}) and growth ratio (bars; fold change) estimated by biovolume for LETC-CY-01, LETC-CY-02, LETC-CY-05 and T3 strains of *Raphidiopsis raciborskii* exposed to extremely high $p\text{CO}_2$ concentrations ($\cong$40,000 ppm) and under control conditions (current $p\text{CO}_2$ concentration $\cong$410 ppm). Significant differences (*) = $p < 0.05$, Student's *t*-test.

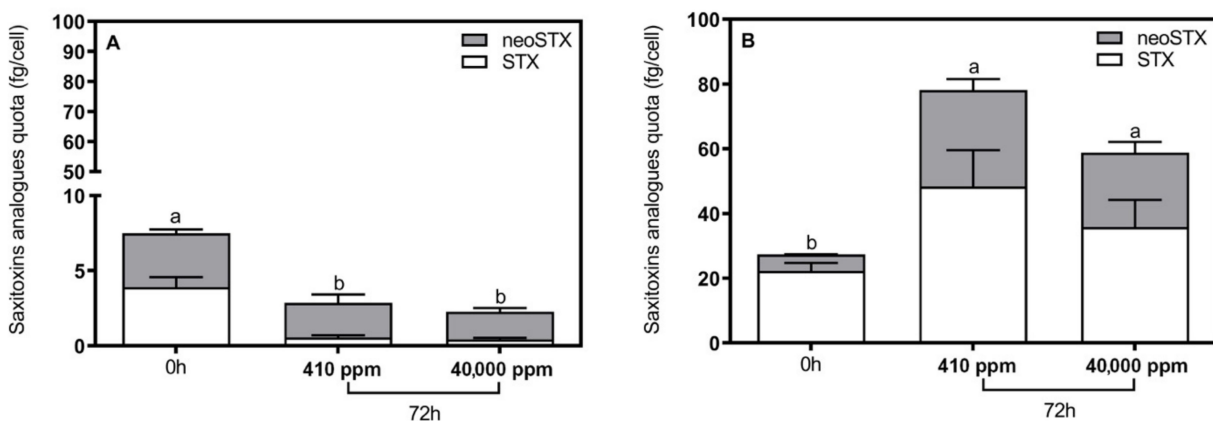**Figure 3.** Total saxitoxin quota (fg/cell) of *Raphidiopsis raciborskii* (**A**) LETC-CY-05 and (**B**) T3 exposed to extremely high $p\text{CO}_2$ concentrations ($\cong$40,000 ppm) and under control conditions (current $p\text{CO}_2$ concentration, $\cong$410 ppm) at 0 and 72 h. [a,b] Significant differences = $p < 0.05$, ANOVA.

Table 2. Differential gene expression (EDGE) of *R. raciborskii* strains LETC-CY-01 and LETC-CY-05 under extremely high $p\mathrm{CO_2}$ ($\cong$40,000 ppm).

Group	Annotation	Fold Change	*p*-Value	Feature ID
LETC-CY-01				
Membrane transport	Possible carbon dioxide concentrating mechanism protein CCM Activity K	−1.8	0.01	CRC_00971
	Phosphoribulokinase (EC 2.7.1.19)	−1.79	0.01	CRC_00573
	TRAP dicarboxylate transporter, DctQ subunit, unknown substrate 6	−1.78	0.03	CRC_02038
	Carbon dioxide concentrating mechanism protein CCM Activity O	−1.76	0.001	CRC_00967
	Carboxysome protein CCM Activity N	−1.58	0.01	CRC_00968
	Bicarbonate transporter, bicarbonate binding protein	1.79	0.04	CRC_00146
	DevC protein	1.56	0.05	CRC_01107
	ABC-transporter DevC-like protein	2.1	0.01	CRC_00437
	Phospholipid-lipopolysaccharide ABC transporter	3.5	0.002	CRC_01570
RuBisCO regulation	Ribulose-1,5-bisphosphate carboxylase/oxygenase	−1.61	0.03	rbcS
	Ribulose-phosphate 3-epimerase (EC 5.1.3.1)	−1.54	0.03	CRC_01082
	Protein CP12, regulation of Calvin cycle via association/dissociation of PRK/CP12/GAPDH complex	−1.52	0.04	CRC_01224
LETC-CY-05				
Membrane transport	Bicarbonate transport system permease protein	3.36	0.04	CRC_00145
	Bicarbonate transporter, bicarbonate binding protein	5.02	0.01	CRC_00146

4. Discussion

In this study, we investigated the short-term physiological and transcriptomic responses of STX-producing and nonproducing *R. raciborskii* to an extremely high carbon dioxide concentration. In general, when assessing physiological parameters, such as growth, photosynthesis and saxitoxin production, most of the *R. raciborskii* strains were not affected by increased $\mathrm{CO_2}$. However, transcriptomic analysis revealed an upregulation of CCM activity proteins, such as bicarbonate transporters, and downregulation of proteins related to the Calvin cycle, such as RuBisCO.

Overall, regardless of $\mathrm{CO_2}$ challenge and the ability to produce STXs, the strains maintained their growth (increased biovolume) during the examined three-day cycle. *R. raciborskii* LETC-CY-01 was the unique strain that showed a reduction in biomass production and growth kinetics when subjected to the extremely high $\mathrm{CO_2}$ concentration. Although most of the studies usually assess cyanobacterial growth over a longer cycle (1–4 weeks), the response to increased $\mathrm{C_i}$ availability can be rapidly evidenced in the first few days (<4 days), as reported by Holland et al. [20] and Vilar and Molica [21]. These authors have shown *R. raciborskii* to perform better under a high $\mathrm{CO_2}$ environment but much lower than the $p\mathrm{CO_2}$ tested in our experimental setup.

Furthermore, despite not being affected by increasingly high $p\mathrm{CO_2}$, most of the *R. raciborskii* strains displayed different growth rates, in addition to toxin production, which suggests intraspecific variability. Willis et al. [42] reported different growth rates and toxin quotas in 24 *R. raciborskii* strains. The authors highlighted that, in nature, several subpopulations constitute a 'populational mosaic' by occurring as different ecotypes. This rationale has also been pointed out by Saker and Neilan [43]. Indeed, subpopulations at different abundances and physiological traits at different levels (e.g., chlorophyll cell quota) may occur and have divergent environmental preferences, such as depth, light intensity, nutrient and organic matter sources [44–46].

In general, *R. raciborskii* photosynthesis was not affected by the extreme CO_2 level. The efficient maintenance of photosynthesis under the different CO_2 concentrations could be a result of a very adjustable photosynthetic apparatus. In a study with one *R. raciborskii* strain, the plasticity of photosystem II (PSII) was observed when the cyanobacterium was cultured under two distinct CO_2 conditions (current and 1300 ppm) [29]. Exposed cells reorganized their PSII when submitted to higher concentrations of CO_2, while the carbon concentration mechanism of *R. raciborskii* had a better performance under lower CO_2 conditions [29]. Moreover, in a meta-analysis study with 20 phytoplankton strains, it was demonstrated that earlier groups (cyanobacteria and dinoflagellates) had a higher CO_2 concentrating mechanism (CCM) plasticity because they may have evolved to compensate for the low specificity of RuBisCO for CO_2 by diversifying their carbon transporters [9]. In fact, in our findings, although an extremely high CO_2 concentration was introduced in the cultures, the slight mean pH difference observed between the experimental conditions (from 9.5 under 410 ppm to 8.2 under 40,000 ppm) (data not shown) was still in the range of bicarbonate availability; inorganic carbon has been assumed as the more suitable to be used by *R. raciborskii* [20]. A similar study did not find relevant variations in pH values when cultures were submitted to 400 and 1000 ppm CO_2 [31]. Studies also demonstrated a diversity of CCM genes in the genomes of different *R. raciborskii* strains [31,47]. Here, we first report preliminary results of the transcriptomic response of *R. raciborskii* to extremely high pCO_2.

Additionally, in our comparative transcriptomics analysis, we found an upregulation of the BicA transporter and downregulation of RuBisCO, carboxysome formation (*ccmO* and *ccmN* transcripts) and proteins related to the Calvin cycle after 72 h under extremely high pCO_2. Although BicA is characterized as having a relatively low affinity for bicarbonate ($K_{0.5}$ ~70–350 μmol·L^{-1}) in contrast to a high flux rate [12], under increased C_i availability, a higher affinity to bicarbonate is less essential for Ci acquisition than a higher flux rate. However, Sandrini et al. [48] found a downregulation of bicarbonate transporters and no significant change in RuBisCO and carboxysome protein expression for *Microcystis* PCC 7806 immediately after 2 h when cultured under a sixfold higher pCO_2. However, the authors demonstrated an upregulation of bicarbonate transporters at low C_i availability, which is argued to be induced CCM activity for better CO_2 acquisition by the cyanobacterium. These short-term variations in transcript levels indicate that cyanobacteria can rapidly respond to CO_2 dynamics with a likely physiological adjustment to cope with the pressure exerted by the environment.

The increased pCO_2 had no significant effect on saxitoxin production by either *R. raciborskii* T3 or LETC-CY-05. This finding demonstrates that toxin production is probably not involved in immediate cellular responses to changes in CO_2 concentration, at least in the first 72 h. However, the significant differences between the STX cell amounts displayed by the strains demonstrated that they have different potential effects on toxin production.

Vilar and Molica [21] first reported the effect of C_i availability on *R. raciborskii* saxitoxin production and evidenced a decreased STX amount in contrast to a higher biomass produced under a high C_i level. In contrast to our experimental setup, the authors provided a higher C_i environment by culturing the cyanobacterium in an aerated medium enriched with sodium bicarbonate (0.2 mM $NaHCO_3$). In an approach similar to our study, Pierangelini et al. [30] analyzed the cylindrospermopsin pool size in *R. raciborskii* under light and CO_2 variations, concluding that the production of this toxin is constitutive and is not affected by light or high CO_2 (1300 ppm). We observed a similar effect with two STX-producing *R. raciborskii,* even when challenged by a much greater CO_2 concentration (40,000 ppm). Additionally, the remarkable differences in the regular toxin production by the *R. raciborskii* strains compared to the absence of an effect induced by the experimental challenge suggest that, to a certain extent, the intraspecific variability in toxin production might overlap with the changes promoted by different CO_2 levels.

Furthermore, short-term exposure (72 h) was important to evaluate a likely genetic background to rapidly respond to a challenging CO_2 environment and enable cells to maintain their growth. Strain acclimatization to the higher pCO_2 prior to the experiment

was not made as we aimed to observe any shift in parameters involved with the plasticity of *R. raciborskii*, since this species is known for its small genotype variability and high environmental plasticity [28].

In addition, this study evaluated the direct impacts of a greenhouse gas. Studies encompassing indirect impacts, such as dissolved inorganic carbon dynamics in water, and global climate changes, such as temperature increases (e.g., [21,31]), cannot be disregarded once, e.g., warming can allow *R. raciborskii* the optimum conditions for expansion and occurrence in environments where this species usually does not occur [49]. Similarly, a continental-scale study with water bodies in Europe demonstrated that temperature is indeed involved in the distribution of cyanotoxins or toxic cyanobacteria [50]. Additionally, Willis et al. [31] demonstrated that temperature is more important than pCO_2 concentration (750 and 1000 ppm) for two *R. raciborskii* strains. The authors also observed contrasting strain responses for the two tested CO_2 levels.

Impacts similar to those found for freshwater cyanobacteria were also observed for marine phytoplankton, where elevated CO_2 concentrations did not affect marine cyanobacteria [51,52], emphasizing the rationale that solely increased CO_2 is not as relevant for predicting future impacts on aquatic environments, especially if considering the different levels of CO_2 with which cyanobacteria have coped over the past geological eras [5–7] as well as the extensive framework of genes selected by evolutionary forces due to its prevalence and ancient origin [8]. Additionally, a recent review by Ma and Wang [53] evidenced, by analyzing field data, that phytoplankton biomass was correlated with CO_2 rise, but there was a lack of correlation when considering only cyanobacterial biomass. The authors highlight the complexity of simultaneous phenomena occurring in aquatic environments, such as competition, stressors, nutrient cycling and grazing, and compared them to laboratory results to demonstrate the gaps and predictability between the different approaches [53]. Additionally, it has been demonstrated that speculations about CCMs functioning under saturation of CO_2 being able to affect cyanobacterial growth are not corroborated by field observations as cyanobacteria have displayed a good performance under any CO_2 condition [53].

5. Conclusions

According to our findings, at least at the initial growth phase, *R. raciborskii* was able to cope with a very high CO_2 level, which shed light on the understanding that this species might have the potential to cope with carbon dioxide in water above the predicted levels.

Additionally, STX production was not altered in the presence of extremely high pCO_2, nor there was difference in the production of the two types of STX analyzed (neoSTX and STX). Different concentrations were observed only between different strains regardless of pCO_2 conditions.

Finally, only LETC-CY-01, a nontoxic strain, had its growth altered in the presence of an extremely high pCO_2 concentration. Our findings extend our comprehension of the plasticity of the cyanobacterium *Raphidiopsis raciborskii*.

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