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Efeitos fisiológicos, químicos e moleculares de altas concentrações de CO_2 em *Cylindrospermopsis raciborskii* (Cianobactéria)

TESE DE DOUTORADO

Ricardo Rogers Paranhos

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Universidade Federal Fluminense

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Dinâmica dos Oceanos e da Terra da Universidade Federal Fluminense, como parte dos requisitos necessários à obtenção do título de Doutor em Ciências.

Orientadores: Dr. Renato Crespo Pereira

Drª. Sandra Maria Feliciano de Oliveira e Azevedo

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Ata de Defesa Tese de Doutorado

Ao(s) 15 dia (s) do mês de Fevereiro de 2017, às 09:00 horas, no Departamento de Geologia e Geofísica/LAGEMAR, da Universidade Federal Fluminense, reuniu-se a Banca Examinadora designada para argüir a Defesa da Tese de Doutorado do (a) aluno (a) **Ricardo Rogers Paranhos** sob o título:

"Efeitos fisiológicos, químicos e moleculares de altas concentrações de CO 2 em Cylindrospermopsis raciborskii (Cianobactéria). "

A banca constituída pelos professores:
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15 de Fevereiro de 2017

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*“Não há nada que eu admire mais do que alguém que planta uma árvore cuja sombra talvez nunca venha a se deitar.***“**

(Fala de uma das personagens do filme Exótico Hotel Marygold 2)

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RESUMO

O CO_2 é um gás que vem recebendo grande atenção nos últimos anos, pois é o principal causador do efeito estufa e seu elevado aumento está relacionado aos processos de industrialização. Estima-se que no final deste século as concentrações atuais (400 ppm) triplicarão, causando impactos ainda mais sérios no clima e consequentemente prejudicando o ambiente e as diferentes espécies. As cianobactérias são um dos grupos de organismos mais antigos (3,8 bilhões de anos) e, portanto, evoluiram frente ao grande número de mudanças ocorridas até o momento. Um desses eventos foi a alta das concentrações de CO_2 no período Paleoceno atingindo teores 10 vezes mais elevados que os atuais (4000 ppm). Desta maneira, o presente estudo avaliou, em laboratório, respostas fisiológicas, incluindo a síntese de saxitoxina e cianopeptídeos e transcriptomas de cepas de *Cylindrospermopsis raciborskii*, produtoras e não-produtoras de saxitoxina (SXT), frente à concentrações de 400 e 4000 ppm de CO_2, buscando compreender as respostas adaptativas dessas linhagens e os futuros riscos de um cenário com maiores concentrações de CO2 como potencial estimulador de dominância de cianobactérias nos ambientes aquáticos continentais. Através da análise dos resultados foi observado que linhagens produtoras de saxitoxina (CYRF e T3), de maneira geral, tiveram crescimento e produção da toxina estáveis. Contudo ao analisar as taxas de crescimento das linhagens não-produtoras (CYLP e NPCS-1) e seus transcriptomas, observou-se que os grupos expostos ao alto CO_2 tiveram menores taxas de crescimentos e regulação do RNA alterada. A análise comparativa dos extratos de cianopeptídeos permitiu obsevar que CYLP e T3 variaram a produção daquelas moléculas de maior peso molecular quando cultivados sob alto CO_2. Foi observado que as principais alterações ocorreram entre cepas, variando fisiologia e metabolismo de formas diferentes, chamando atenção para o fato de diferentes linhagens responderem de forma diferente à mudança gasosa. Os resultados também chamam atenção para a necessidade de aprofundar o conhecimento acerca do melhor conhecimento dos cianopeptídeos e seu papel ecológico e aprofundamento das técnicas transcriptomicas aplicadas às cianobactérias.

Palavras-chave: *cianobactérias; cianometabolitos; efeito estufa*

ABSTRACT

CO_2 is a gas that has received great attention in recent years, since it is the main cause of the greenhouse effect and its high increase is related to industrialization processes. It is estimated that by the end of this century the current concentrations (400 ppm) will triplicate, causing even more serious impacts on the climate and consequently harming the environment and its organisms. Cyanobacteria is one of the oldest group of organisms (3.8 billion years old) and therefore have evolved in face of the great number of environmental changes that happened so far. One of these events was the increase of CO2 concentrations in the Paleocene period reaching levels 10 fold higher than current (4000 ppm). Though, the present study evaluated the physiological, chemical (saxitoxin and cyanopeptides) and transcriptomic responses of toxin producing and non-producing *Cylindrospermopsis raciborskii* strains, against concentrations of 400 and 4,000 ppm of CO_2, in order to understand this harmfull algal bloom former would behave. Saxitoxin producing lineages (CYRF and T3), in general, had a stable growth and toxin production. However, when analyzing the growth rates of non-producing strains (CYLP and NPCS-1) and their transcriptomes, it was observed that the groups exposed to high CO_2 had lower rates of growth and increased RNA regulation. The comparative analysis of the extracts of cyanopeptides allowed to observe that CYLP and T3 varied the production of those molecules of greater molecular weight under higher CO_2. It was observed that the main variations occurred among strains, varying physiology and metabolism in different ways, drawing attention to the fact that different strains respond differently to stress. The results also call attention to the need to improve knowledge on cyanopeptides and their ecological roles and also the need to deepen expertise on transcriptomic techniques applied to cyanobacteria.

Keywords: *cyanobacteria; cyanometabolites; greenhouse gas*

SUMÁRIO

LISTA DE FIGURAS

LISTA DE TABELAS

1. Introdução Geral

O planeta Terra, desde sua formação até a atualidade, vem sendo marcado por profundas alterações, mudanças geográficas e em especial climáticas. Nossa atmosfera já passou por resfriamentos e aquecimentos e diversos processos geológicos alteraram sua composição gasosa diversas vezes ao longo de bilhões de anos de transformações (Brandes *et al*., 1998; Tian, 2005; Marty, 2012; Kasting and Howard, 2016). No curso destas transformações, os gases oxigênio (O_2) e carbônico (CO_2) foram aqueles que propiciaram as condições necessárias à propagação das diferentes formas de vida celulares. Acredita-se que a combinação de uma grande disponibilidade de CO_2, abundância de nutrientes como fósforo e nitrogênio, além de metais pesados oriundos de intensa atividade vulcânica, somadas a uma grande penetração da luz solar criaram as condições ideais para a expansão e dominância de organismos de organização celular simples, capazes de realizar a fotossíntese: as primeiras cianobactérias (Shopf, 1974; Alterman, 2007).

Estes organismos ancestrais, com origem estimada de 3,8 bilhões de anos (Altermann, 2007) tem sido considerados os mais antigos a habitarem o planeta e criaram as condições de O_2 necessárias à vida das formas aeróbias. As condições que promoveram sua expansão tendo sido capazes de alterar a dominância do gás carbônico para o gás oxigênio, durante a fase que foi chamada de “período das algas azul-esverdeadas” (tradução livre de “age of blue-greenalgae” – Schopf, 1974), no Pré-cambriano (2,5 – 0,54 bilhões de anos).

Devido às intensas mudanças no clima planetário e ao aparecimento de mais e mais formas de vida com diferentes necessidades e estratégias, os mecanismos de sobrevivência das cianobactérias foram se modificando de forma que foram selecionados aqueles que as permitissem lidar com a grande competição por recursos (nutrientes, luz e espaço). Dentre as principais estratégias utilizadas estão a fixação do nitrogênio atmosférico através de células especializadas (heterocito), fototropismo alterando a flutuabilidade através do aerocisto, o desenvolvimento dos

mecanismos concentradores de carbono (do inglês CCMs - Badger & Price, 2003), importante estratégia adotada pelo grupo das cianobactérias para aumentar a eficiência da captação de CO_2 quando a atmosfera começou a ficar pobre neste gás. A presença de metabolitos secundários com atividade anti-proteases (Welker *et al*., 2006; Chipala *et al*., 2011; Silva-Stenico *et al*., 2012; 2015) e produção de toxinas com variadas propriedades ecológicas. Outras estratégias comuns aos procariontes também favoreceram as bactérias fotossintetizantes, como a transferência lateral de genes, aumentando a variabilidade e trazendo adaptações ao meio (Jain *et al*., 1999; Boucher *et al*., 2003), além da alta plasticidade fenotípica que lhes permite resistir às mais diversas variações ambientais (Stucken *et al*., 2009; Carneiro *et al*., 2013; Soares *et al*., 2013[a]; Sinha *et al*., 2014; Rangel *et al*., 2016).

A ordem Nostocales reúne espécies de cianobactérias que agregam todas as habilidades adaptativas citadas e cuja espécie *Cylindrospermopsis raciborskii* vem chamando muita atenção na atualidade (Figura 1). Com conjuntos de células que formam filamentos, esta espécie de cianobactéria está adaptada a viver sob condições de pouca luminosidade sendo capaz de regular sua profundidade (Shafik *et al*., 2003). Nas últimas décadas começou-se a identificar um padrão de distribuição global de *C. raciborskii* que agora é encontrada em rios, reservatórios e lagos de água doce do mundo inteiro (Neilan *et al*., 2003) e, assim como outras cianobactérias, foi observado que em regiões de maior densidade populacional, onde o impacto antrópico é maior, ocorre uma expressiva dominância dessa espécie (Huszar *et al*., 2000; Soares *et al*., 2013[b]). Todo esse sucesso adaptativo gera muita preocupação acerca da qualidade da água dos ambientes aquáticos continentais, pois já foi demonstrado que linhagens da espécie podem produzir ao menos dois grupos de cianotoxinas prejudiciais á saúde humana, bem como demais espécies aquáticas: a cilindrospermopsina e as saxitoxinas.

Figura 1. Culturas de *Cylindrospermopsis raciborskii*. Filamentos e heterocito. Extraído de: (https://ncma.bigelow.org/ccmp1973?___SID=U#.WiugzhSjfJM); acervo do LETC e (http://www.paho.org/par/index.php?option=com_content&view=article&id=773:analizan-rasil-aguas-lago-ypacarai-ante-aparicion-cianobacteria-cylindrospermopsis-raciborskii-temid=258), respectivamente.

No Brasil, a maioria as linhagens de *C. raciborskii* que foram isoladas até o momento são produtoras de saxitoxinas (SXTs), incluindo neo-saxitoxina (Neo-SXT) e goniautoxinas (GTX) (REFERÊNCIA). Essas moléculas são alcalóides de esqueleto carbamatos, que atuam como toxinas em mamíferos, se ligando às bombas de prótons, especialmente os receptores de sódio e cálcio nos neurônios e bloqueando sua atividade. Esse processo pode levar a uma série de consequências fisiológicas em mamíferos como alterações na transmissão do estímulo nervoso em sinapses, fundamentais em diversas atividades do corpo (Tanino *et al*., 1977).

Contudo, a verdadeira preocupação reside na presença de altas densidades de cianobactérias na água. Este fenômeno conhecido como floração de cianobactérias ocorre quando as condições que propiciaram a dominância destes organismos, se tornam disponíveis.

Considerando como essênciais para o crescimento desses micro-organismos a luz, a disponibilidade de CO_2 e nutrientes inorgânicos, com exceção da principal fonte de luz que ainda é o Sol, atualmente as principais fontes de nutrientes provêm de origem antrópica. São os esgotos despejados sem tratamento (domésticos, industriais e rurais) diretamente nos rios e lagos que, associados à presença de micro-organismos, geram um acelerado processo de eutrofização dos corpos d'água. Somado a essas conhecidas causas, nas últimas décadas uma outra

preocupação, já em estágio avançado, começou a ser detectada. A elevação das concentrações de CO_2 atmosférico, em consequência da intensa industrialização dos últimos cem anos (IPCC, 2014). Sendo o principal causador do efeito estufa, o este gás, oriundo principalmente de fontes antrópicas, já causou elevação da temperatura dos oceanos em pelo menos um grau e diversas consequências à vida marinha foram observadas, com impacto indireto sobre a economia (REFERÊNCIA). Nas regiões do planeta onde não ocorreu aumento na temperatura outros tipos de mudanças climáticas vêm sendo observados, como mudança nos padrões de circulação atmosférica e oceânica, que influenciam diretamente os climas em escala continental (IPCC, 2014). Há ainda uma previsão de aumento em até três vezes (de 400 para 1200 ppm) nas concentrações do CO_2 atmosférico, o que levaria ao agravamento das mudanças que já estão sendo vivenciadas.

Dentro deste cenário, cianobactérias e outros organismos fotossintetizantes poderiam estar se beneficiando tanto do aumento da temperatura (Castro *et al*., 2004), quanto do aumento da disponibilidade do gás carbônico. Os Mecanismos Concentradores de Carbono de cianobactérias (MCCs ou CCMs do inglês Carbon Concentrating Mechanisms) são diversos e atuam amplificando a eficiência na concentração do CO_2 intracelular em torno da enzima RUBISCO (ribulose 1-5-bifosfato carboxilase oxigenase). Esta possui papel fundamental na fixação do carbono na sua forma inorgânica, na forma do fosfoglicerato, dando início ao ciclo de Calvin, gerando então moléculas orgânicas essenciais à célula. Os MCCs das cianobactérias são os mais eficazes dentre os organismos fotossintetizantes, aumentando até cem vezes a concentração de carbono inorgânico para a fotossíntese (Badger & Price, 2003). Eles estão distribuídos na membrana plasmática e podem transportar ativa ou passivamente, com ou sem custo energético, seletivamente ou não, o carbono em sua forma inorgânica (Badger *et al*., 2006). Este é acumulado principalmente na forma do íon bicarbonato (HCO3-) ou gás carbônico. Foi demonstrado que *C. raciborskii* utiliza o íon bicarbonato de forma

mais eficiente que o CO_2 e também proposto que em um ambiente onde a forma gasosa vem emergindo, espécies melhor adaptadas a essa forma estariam em desvantagem adaptativa (Holland *et al*., 2012).

Por outro lado, ao relacionar a expansão destes organismos com o atual panorama das mudanças climáticas obtém-se o padrão inverso (Sinha *et al*., 2012). Ainda mais quando considerada a alta plasticidade encontrada nos MCCs de *C. raciborskii*, capaz de ativar e inativar os diferentes transportadores em função da condição de carbono existente (Pierangelini *et al*., 2014). Sobremaneira, os reais benefícios ou malefícios do aumento do CO_2 ainda produzem resultados controversos sobre as respostas fisiológicas de cianobactérias em geral.

Ainda também carente de conhecimento é o papel ecológico das saxitoxinas produzidas por *C. raciborskii*. Devido a grande preocupação com os efeitos nocivos e propriedades farmacológicas dessa toxina, pouco interesse houve na sua importância ambiental e ao assumí-la como uma toxina, pouco foi avaliado sobre sua relação com suas células produtoras e possíveis papéis de sinalização, proteção ou outros tipos de interação molecular importantes do ponto de vista ecológico. Conhecem-se alguns fatores que regulam a produção de saxitoxina, como o aumento da condutividade da água, onde através da quantidade e qualidade dos sais existentes, *C.raciborskii* produziu maiores concentrações dessas moléculas na fase estacionária e tabém variou a produção diária relacionada com maiores concentrações nos períodos de luz (Carneiro *et al*., 2009; 2013). Pomati e colaboradores (2004) relacionou o aumento da produção de SXT em cultivos onde a condutividade da água era maior, com sua atividade bloqueadora de canais de sais. Ele entãopropôs que essa toxina atuaria contribuindo para a homeostase protegendo as células produtoras do diferencial osmótico criado reduzindo a entrada passiva de sais através dos canais transportadores.Esta então foi a principal teoria apresentada para uma função celular da SXT até o presente estudo.

Apesar de relevantes, as saxitoxinas representam uma ínfima porção da diversidade de metabolitos produzidos pelas cianobactérias. Além das clássicas cianotoxinas, *Cylindrospermopsis raciborskii* e cianobactérias em geral produzem diferentes peptídeos de origem não-ribossomal com grande variedade estrutural, chamadas de cianopeptídeos (Marahiel *et al*., 1997). Esses constituem a maior parte dos metabólitos secundários produzidos por bactérias fotossintetizantes (Agha & Quesada, 2014) e estima-se que entre 68% e 90% deles estejam concentrados no meio intracelular (Ferreira, 2006). Alguns dos principais cianopeptídeos já descritos são as aeruginosinas, cianopeptolinas, anabaenopeptinas, microgininas e microviridinas e, devido à sua atividade como inibidores de protease, podem interferir em diversos processos fisiológicos como progressão do ciclo celular, digestão de alimentos, angiogênese, coagulação sanguínea, regulação da pressão arterial, apresentação do antígeno, inflamação e apoptose. (Welker *et al*., 2006; Chipala *et al*., 2011; Silva-Stenico *et al*., 2012; 2015). Em decorrência do interesse pelo seu potencial farmacológico, vários estudos já foram feitos e sabe-se hoje que essas moléculas possuem, por exemplo, atividade antibacteriana, antiviral, antifúngica e anticâncer (Burja *et al*., 2001; Singh *et al*., 2005; Welker *et al*., 2006[a]; Tan, 2007; Gademann & Portmann, 2008; Villa & Gerwick, 2010; Singh *et al*., 2011).

Explorando tal potencial, diversos grupos de pesquisa já se mobilizam há mais de uma década para conhecer a diversidade desses metabólitos (Welker *et al*., 2006[a]; Silva-Stenico *et al*., 2011; Carneiro *et al*., 2012; Sanz *et al*., 2015; Briand *et al*., 2015). Graças a esses esforços já são conhecidas ao menos 50 variantes estruturais para cada classe de cianopeptídeos descrita (Ferreira, 2006), o que a priori somaria mais de 500 tipos de moléculas, considerando todos os cianopeptídeos já identificados, cujas as atividades biológicas ainda são pouco estudadas. Essas moléculas já foram descritas em diferentes linhagens de *Synechococcus elongatus*, *Microcystis panniformis*, *Fischerella sp*. (Silva-Stenico *et al*., 2011), *Woronichinia naegeliana* (Bober *et al*., 2014), *Chroococcidiopsis sp*.,

Pleurocapsa sp., Leptolyngbya sp., Oculatella sp., Nostoc sp., Desmonostoc sp., Brasilonema sp. (Sanz *et al*., 2015), *Sphaerocavum brasiliense* (Silva-Stenico *et al*., 2015) e *Microcystis* (Briand *et al*., 2015). Na espécie *Cylindrospermopsis raciborskii* microgininas com atividade antibacteriana foram encontradas (Silva-Stenico *et al*., 2011).

Uma interpretação mais voltada para o papel desse grupo de moléculas no ambiente foi apresentada pelo trabalho de Agha e Quesada (2014) que propuseram que oligopeptídeos são potenciais biomarcadores de químiotipos de cianobactérias e o conhecimento da diversidade desses cianopeptídeos poderia contribuir para o conhecimento acerca da estrutura e ecologia das diferentes populações. Pesquisas explorando a relevância dos cianopeptídeos para os ambientes aquáticos tem sido realizadas. Rohrlack e colaboradores (2003) mostraram que a microviridina J prejudicou indiretamente o processo de ecdise de *Daphinia sp.* ao inibir a síntese de quitina, açúcar importante na formação do exoesqueleto em artrópodos. Saqrane e colaboradores, em 2007, mostrou que a exposição crônica de *Lemna gibba* a microcystina provocou uma redução no crescimento e na produção de clorofila da planta. Peptídeos cíclicos isolados de uma *Oscillatoria sp.* inibiram crescimento de algas verdes em uma atividade considerada alelopática (Leão *et al*., 2010). Já Schatz e colaboradores (2007), observaram que a lise de células de *Microcystis sp.* provocou o aumento das concentrações de microcistina e sugeriram que o aumento de peptídeos como microviridinas e microgininas, além da microcistina, atuariam em processos de sinalização para a produção de defesas contra predadores, pela colônia.

Por outro lado, cianopeptídeos não foram estudados do ponto de vista da resposta a fatores abióticos e uma vez que cianobactérias são organismos capazes de condicionar o meio, prevalece uma lacuna no que diz respeito ao comportamento do metabolismo dessas moléculas frente variações de elementos de grande preocupação, como o gás carbônico.

Ao passo que as ferramentas de análise química nos permitem compreender melhor as variações qualitativas e quantitativas dos produtos fim no metabolismo celular, ao longo dos últimos anos, a aplicação de técnicas transcriptômicas vem permitindo compreender melhor os mecanismos genéticos envolvidos nessas respostas em cianobactérias (Pinto *et al*., 2009; Hark & Gobler, 2013; Sandrini *et al*., 2014; Stucken *et al*., 2014; Makower *et al*., 2015; Pierangelini *et al*., 2015). Atualmente as técnicas de transcriptoma quantitativo facilitam a elucidação das mudanças fisiológicas durante os processos adaptativos (D'Agostinho *et al*., 2016). Contudo, as técnicas ômicas (genômica, transcriptômica e proteômica) ainda encontram-se em fase inicial de desenvolvimento, o que tem expandido seu uso como ferramentas no estudo de micro-organismos, permitindo o estudo dos diferentes grupos como compartimentos moleculares isolados e entendendo os genes como parte de uma rede complexa (Souza *et al*., 2014)

As espécies de cianobactérias para as quais existe maior conhecimento nessa área são principalmente *Microcystis aeruginosa*, *Prochlorococcus sp*., *Synechococcus sp.* e *Synechocystis sp*. (Zinser *et al*., 2009; Thompson *et al*., 2011; Hernandez-Prieto and Futschik, 2012; Ludwig and Bryant, 2012[a]; 2012[b]; Harke and Gobler, 2013; Sandrini *et al*., 2014; Makower *et al*., 2015). Apenas dois estudos utilizando ferramentas genéticas, relacionados à *C. raciborskii*, tentaram compreender, por exemplo, como fatores como CO_2, luz e nitrogênio atuam na biossíntese de toxinas (Stucken *et al*., 2014; Pierangelini *et al*., 2015). É importante observar que nenhum desses trabalhos envolveu cepas brasileiras produtoras de SXT. Dado que chama atenção, dadas as dimensões continentais do país e a grande dominância da espécie em reservatórios e lagos ao longo de toda nossa extensão (Huszar *et al*., 2000; Soares *et al*., 2013).

O primeiro registro da ocorrência de *C. raciborskii* no Brasil foi feito para o Lago Paranoá (Brasília-DF), em 1969 (Branco & Senna, 1991) e a partir da década de 80 muitos relatos foram emitidos (ver Schaker, 2012). Muitas linhagens passaram

a ser estudadas quanto à produção de toxina (Molica *et al*., 2002; Ferrão-Filho *et al*., 2010; Hoff-Risseti *et al*., 2012) e utilizadas em diversos estudos relacionados à espécie (Costa *et al*., 2006; Ferrão-Filho *et al*., 2008; 2009; *Carneiro et al*., 2009; Clemente *et al*., 2010; Silva *et al*., 2011; Carneiro *et al*., 2013; Costa *et al*., 2013). Todavia, como já proposto por Agha e Quesada (2014), as diferentes cepas de cianobactérias possuem perfis tão distintos que uma mesma espécie poderia ser separada em diferentes ecotipos, cada um com um perfil metabólico, morfológico e fisiológico distinto.

Uma vez considerada a alta plasticidade destes organismos, a história evolutiva de cada lago que habitam, suas condições limnológicas distintas, populações de micro e macro organismos, além das diferentes variáveis a que estão expostas, é de extrema importância conhecer as características que as unem como espécie e separam como linhagens. Só assim é possível tecer extrapolações em nível mais amplo do conhecimento.

Sendo assim, é prudente o uso de estudos que abranjam a ecofisiologia das cianobactérias em diferentes níveis de resposta, como fisiológico e molecular. Uma vez que este tipo de análise permite melhor percpepção da convergência dessas respostas. Baseando-se nesse conceito, o presente trabalho tenta agregar maior conhecimento sobre os processos envolvidos no sucesso de *C. raciborskii* frente às mudanças climáticas que vem ocorrendo, comparadas com um evento pretérito, de milhões de anos, através de experimentos em laboratório com variações das concentrações de CO_2, observando as respostas fisiológicas, químicas e genéticas de quatro diferentes cepas brasileiras.

2. Objetivos Gerais

Esta tese está divida em dois capítulos que representam os objetivos gerais que compõem este estudo. Cada um dos capítulos está organizado como um manuscrito a ser publicado em revista científica.

1. Avaliar os efeitos fisiológicos de altas concentrações de CO_2 em distintas cepas tóxicas e não-tóxicas de *Cylindrospermopsis raciborskii* .

2. Avaliar e comparar os transcriptomas de uma cepa tóxica e uma não-tóxica de *Cylindrospermopsis raciborskii* cultivadas sob duas concetracões de CO_2.

3. Chapter 1: Analysis of physiological and chemical effects of high CO_2 over toxic and non-toxic *Cylindrospermopsis raciborskii* (Cyanobacteria) Brazillian strains.

Rogers R., Plouguerné E., Brandão L., Pereira R.C., Azevedo S.M.F.O.

Abstract

Understand the ancient evolutionary history of cyanobacteria, considered the older photosynthetic linving organisms (3.8 Ga), may be the pathway for understanding the future of such organisms in a changing climate. Those prokaryots have faced a variety of environmental changes including a 10 fold higher CO_2 concentration (4,000 ppm) during the Paleoce (65 - 55 million years ago). It is estimated that until the year of 2,100 this gas will reach 3 fold higher concentrations than current (400 ppm) generating great concern once cyanobacteria form harmful algal blooms. Therefore the saxitoxin production and cyanopeptides variability of toxic and non-toxic strains of *Cylindrospermopsis raciborskii* under current and elevated CO_2 (400 and 4,000 ppm respectively), together with physiological parameters, was evaluated. Results showed that non-toxic strains had lower growth rates under high CO_2. No alterations in chlorophyll concentrations and photosynthetic efficiency suggest that alterations didn't occour during the clear phase of photosynthesis. The toxic strains had a non-significative decrease in saxitoxin production after 72h and cyanopeptides profiles varied in one toxic and one non-toxic strain under high CO_2. Despite general observations, it was verified differential growth intensities, chlorophyll concentrations and peptide profiles for each strain, under elevated CO_2.

3.1. Introduction

Cyanobacteria are undoubtely ancient organisms and can be considered the elementary photosynthetic live beings. There is still no concensus about the period that cyanobacteria appeared, but there are evidences from 3.8 billion years (Ga) ago (Altermann, 2007). What is well accepted is that Precambrian period was “the age of blue-green algae” (Schopf, 1974) and the period between 2.5 and 0.54 Ga ago was called Proterozoic era due to the change of atmosphere from anoxic to oxygenated thanks to cyanobacteria photosynthesis (Shopf and Walter, 1982).

Along the geological history, our planet has witnessed many changes in atmospheric gas composition (Brandes *et al*., 1998; Pavlov, 2005; Marty, 2012; Kasting and Howard, 2016). Probably the most important shift for life was the increase of O_2 disponiblity in the atmosphere as a result of abundant light and high CO_2 concetrations necessary for photosynthesis. While cyanobacteria is supposed to have emerged billion years ago, until now scientific evidence of the highest CO_2 concentration refers to Paleocene (Fanerozoic), dated of aproximately 65,5 million years ago (Pearson and Palmer, 2000). Data points to concentrations 10 times higher than current (400 ppm) and recent photosynthetic procarionts probably descended from the ones who were able to manage such concentrations.

Predictions of the International Panel on Climate Change (IPCC) points to three-fold increase of CO_2 concentration over the current, reaching aproximately 1,200 ppm in the next century (IPCC, 2014). Damages of recent increases already elevated the temperature of the planet and started processes like oceans acidification as well as a plenty of colateral effects caused by so-called climate changes by scientists (IPCC, 2014). There is a concern about the possible correlation between those climatic effects and the spread of cyanobacteria into new areas (Sinha *et al*., 2012). Also their physiological capabilities are considered to be adapted to the emerging environmental changes as well the conditions created as a

result of antropogenic behavior, creating conditions to Harmfull Algal Blooms (HAB) formation (Paerl and Huisman, 2009).

Cyanobacteria synthetize chlorophyll-*a* and perform photosynthesis by capturing carbon ions from water and releasing oxygen. The carbonic gas used by RUBISCO in photosynthesis must be aquired by very evolved mechanisms named as CCM (CO_2 Concentrating Mechanism), a very important strategy in a poor CO_2 atmosphere (Badger and Price, 2003).

Cylindrospermopsis raciborskii (Woloszynska) Seenayya & Subba Raju (1972) is a filamentous cyanobacterium species of the Nostocales order, found in lakes and reservoirs of all continents (Neilan *et al*., 2003; Sinha *et al*., 2012; Antunes *et al*., 2015). *Cylindrospermopsis raciborskii* is adapted to live under low light conditions (20 - 30 µE m^{-2} s^{-1}), regulate buoyance (Kehoe, 2010), store phosphorus and regulate nitrogen uptake (Isvánovics *et al*., 2000; Burford *et al*., 2006) and also can grow at different temperatures (Chonudomkul *et al*., 2004). Many studies have demonstrated that phenotypic plasticity is also an important adaptative strategy of this organism. Wild strains of these cyanobacterium can be non-toxic or produce different toxins, such as cylindrospermopsin or saxitoxins - SXT (Pearson *et al*., 2010; Zegura *et al*., 2011; Buford *et al*., 2016; Pearson *et al*., 2016), forming HABs (Pearl and Huisman, 2009).

Beyond toxins and their relevance to human and aquatic organisms, cyanobacteria produce a sort of metabolites of possibly equal ecological or physiological importance but still poorly understood. Cyanopeptides are a very diverse group of molecules known by it protease inhibitory activity (Welker *et al*., 2006; Chipala *et al*., 2011; Silva-Stenico *et al*., 2012; Silva-Stenico, *et al*., 2015) and diversity probably associated to the non-ribosomal synthesis pathway (Marahiel *et al.*, 1997). In an ecological and evolutionary context, this high variability of cyanopeptide molecules could propitiate better adaptative advantages for cyanobacteria by improving their plasticity (Welker and Dörhen, 2006). Other

exmaples of ecological activities are protection against heterotrophic consumers (Bagchi *et al*., 2012), better ability to compete for space and resources against other photosynthetic organisms (Leão *et al*., 2010) and to serve as chemical cues that signals to increase the production of metabolites by population of *Microcystis* sp. (Schatz *et al.*, 2007).

Notwithstanding, during the 10 years of research with cyanopeptides, major effort has been directed to characterization of new metabolites and focused on which organism produces which group of molecules (Welker *et al*., 2006; Silva-Stenico *et al*., 2011; Carneiro *et al*., 2012; Sanz *et al*., 2015; Briand *et al*., 2015). There is no doubt that this knowledge is fundamental to embassy further works with focus on understanding the importance of these metabolites for cells, why they are concentrated in cytoplasm, their cellular function and their possible ecological role.

Increasing in concentrations of atmospheric CO_2, associated to cyanobacteria evolutionary history, their ability to adapt and worldwide propagation are of great concern due to risks to human health associated to the expansion of these organisms. The aim of this study was to evaluate the physiological responses of strains of *C. raciborskii* to similar Paleocene carbonic gas concentrations (4,000 ppm – Pearson and Palmer, 2000) in comparison to current conditions. The behavior of saxitoxins and cyanopeptides are also investigated. Here, we observed the chemical profile variation of cyanopeptides among different strains, reinforcing the idea that each one is an ecotype belonging to different aquatic ecosystems each with its own biotic characteristics.

3.2. Material and Methods

3.2.1. Growth under different CO_2 conditions

Two saxitoxin producers (CYRF-01 and T3) and two non-producer (CYLP and NPCS-1) strains of *C. raciborskii* from culture collection of LETC/IBCCF (Laboratory of Cyanobacterial Ecophysiology and Toxicology) cultivated in ASM-1 medium

(Gorham *et al.*, 1964), with constant aeration provided by the same air compressor, pH = 8.0, temperature of 23±1 °C, light intensity of 40–50 µE m^{-2} s^{-1} and a 12:12 h light:dark cycle. The CYRF and T3 strains have been reported as saxitoxins producers (Ferrão-Filho *et al.*, 2007), while the CYLP and NPCS-1 are non-toxin producer strains. About the two toxin producers, CYRF-1 was isolated from Funil reservoir, Resende (RJ, Brazil) and T3 strain was isolated from a branch of Billings water reservoir, called Taquacetuba in 1996 (Lagos *et al*., 1999). The CYLP strain was isolated from Lago Paranoá (Brasília, D.C.) and NPCS-1, was isolated from Custódia (PE, Brazil). The cultures were kept in exponential growth phase through the replacement of medium once a week until to be used as inoculum for the experiments.

Four strain replicates were used for each experimental condition starting with a inoculum of approximately $2x10^5$ Cell / mL in a volume of 2L of medium. The experiments have lasted 72 h, in which controls were mantained in current atmospheric CO_2 conditions (400 ppm) and treatments to Paleocenic´s CO_2 estimated concentration of 4,000 ppm. Both conditions were obtained through a Thermo Fisher gas mixer coupled in a 100% CO_2 cylinder (Figure 2).

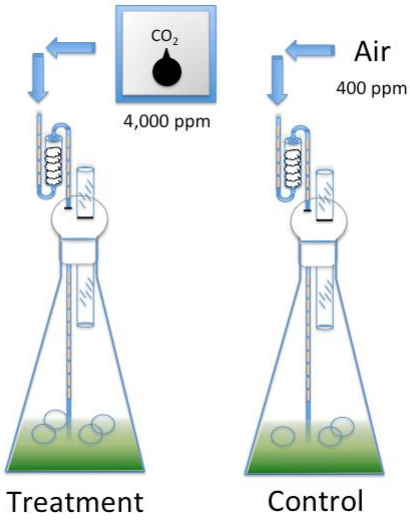


Figure 2. Representative scheme of the experimental condition. For each one of the 4 strains four replicates of *C. raciborskii* was exposed to CO_2 400 ppm (current atmospheric) and 4,000 ppm (Paleocenic´s CO2 estimated concentration).

To ensure that CO_2 gas form was really being introduced inside the medium, a pilot experiment with T3 strain was performed to monitor pH during inoculation of the gas in experimental conditions followed of total alkalinity measures. The pH was obtained by monitoring an erlenmeyer under CO_2 400 and 4,000 ppm with the electrode introduced fulltime inside the medium, without opening the cover and making reads during the day period. Alkalinity was assayed using 3 replicates of cultures under both CO_2 conditions and by colecting samples of 30 mL using a syringe connected to a silicone tube that was in constant contact with the cultures. Values were obtained following the described in Standard Methods for the Examination of Water and Wastewater (Federation, W. E., & American Public Health Association, 2005).

To evaluate the physiological parameters, cell density, chlorophyll concentration and photosynthetic yield of the corresponding cultures was measured from small volumes (5mL) collected from erlenmeyers in intervals of 24h, since the beginning of the experiment. Parameters such as pH and dissolved oxygen (D.O.) were measured in intervals of 12 h directly in erlenmeyers through an access made with a cutted falcon tube. The cover was kept threaded when not sampling.

The cell counts were performed on a Fuchs-Rosenthal hemocytometer using an optical microscope (Olympus Trinocular Microscope BX series). But since *C. raciborskii* is a filamentous species, it was carried out a measurement of at least 30 random cells in order to establish the average cell size necessary for density counts of these populations. Counts were estimated for each strain by measuring filaments total length and dividing the value by the mean cell length obtained. Finally, the correction formule for Fuchs-Rosenthal chamber was applyed to calculate cell concentrations (cell / mL). Growth rate was calculated as proposed by Reynolds (2006) and cell yield determined by the final cell density divided by the initial density.

Chlorophyll concentration (ug/cell/mL) and photosynthetic efficiency (yield/Chlorophyl/Cell/mL) were obtained in a Phytoplankton Analyzer (Walz PHYTO-

PAM), pH was measured with a Digimed´s portable meter and concentration of dissolved oxygen was determined with a Ysi Pro oxymeter.

At the end of the experiments, 200 mL was sampled from SXT producer strains (T3 and CYRF) for quali- and quantitative analysis by HPLC and 500 mL of each of four strains tested were sampled for cyanopeptides analysis by LC-MS. All sampled material was freeze-dried and stored in a freezer.

3.2.2. Saxitoxins extraction

The proportion of solvent used was 1 mL of acetic acid (500 mM) for each 100 mL of sampled culture. After addition of solvent, samples were left in a shaker for 1h. The mixture was then centrifuged at 8ºC, 3,800 rpm, for 10 minutes, and the supernatant was reserved and the procedure repeated twice. At the end, the pellet was discarded and the crude extract obtained was kept in a freezer. Before HPLC analysis the crude extract was filtered in a 0.45 µm PVDF Whatman´s filter.

3.2.3. Saxitoxins analysis (SXT + NeoSxt)

The presence and amount of saxitoxin and neosaxitoxin were analyzed according to Oshima (1995), using a Shimadzu HPLC system with a silica-base reversed phase column (125 mm × 4.0 mm, 5 µm; Lichrospher 100 Reversed Phase C18). Samples were eluted using a mobile phase A (2 mM heptanesulfonate in 30 mM ammonium phosphate), and a mobile phase B (6% acetonitrile, pH 7.1) under isocratic condition using a flow rate of 1 mL/min. The saxitoxins were detected using a fluorometric detector with an excitation at 330 nm and an emission at 390 nm. The toxins were identified and quantified by comparison with known retention times and integrated areas of STX standard purchased from the Institute of Marine Bioscience, National Research Council of Canada (Halifax, NS, Canada).

3.2.4. Cyanopeptide extraction

Cyanopeptides extraction and analysis were based on Silva-Stenico *et al*. (2011) and Isaacs *et al*. (2014). Extractions were performed by collecting 100 mg fromfreeze-dried cultures. Twelve milliliters of a solution of methanol:Mili-Q H2O + acetic acid (5%), 1:1 was added to freeze dried material and kept under agitation during 1 h. The extract was then centrifuged at 4,000 rpm, during 15 minutes under 4 ^{o}C. Supernatant was separated and the procedure was repeated twice using the pelllet. Supernatants were pooled and the methanolic portion was evaporated under nitrogen stream, at 50 ^{o}C.

3.2.5. Purification

This procedure was realized using Strata X 33µ (500 mg/6 mL) cartridges pre-activated with 10 mL of methanol 100%, followed by 20 mL of Mili-Q ultra pure water in order to remove the organic solvent. Samples were passed through the cartridge, and then the cartridges were eluted using 10 mL of ultra pure Milli-Q water followed by 10 mL of methanol 10%. Finally, extracts were eluted with 20 mL of methanol 100% and concentrated under nitrogen stream to a 1.0 mL volume.

All extracts were filtered using 0.22 mm PVDF membrane before injections for analysis by LC/MS.

3.2.6. Cyanopeptide LC/MS analysis

Comparative analysis were carried out on an Agillent Technologies 1,200 Liquid Chromatography system coupled to an hybrid triple quadrupole/Linear Ion Trap mass spectrometer (Applied Biosystems/MDS SCIEX 3,200 Q TRAP® LC-MS/MS System). Separations were achieved using a Luna C18 column (150.0 mm x 4.6 mm, inner diameter 5 µm) (Phenomenex, Torrance, CA, USA). Samples (40µL) were eluted using a mobile phase A (5 mM amonium acetate in water, 0.1% formic acid)

and a mobile phase B (5 mM amonium acetate in acetonitrile, 0.1% formic acid). Elution in isocratic mode (1/1) was performed for 90min at a flow of 1 mL/min. The ionization source conditions were as follows: positive ionization, capillary potential of 3,500 V, temperature and flow of drying gas (nitrogen) of 1 mL/min and 325 ^{o}C. Mass spectra were acquired using electrospray ionization in the positive mode over the range of m/z from 400 to 1200. All samples were run in triplicate.

3.2.7. Statistical analysis

Cell growth, chlorophyl concentration, photosynthetic efficiency, pH and dissolved oxygen data of both CO_2 conditions were compared using Factorial ANOVA (Statsoft STATISTICA). Saxitoxins production at time zero and CO_2 400 and 4,000 ppm at 72 h were compared using ANOVA. Whenever normality and homogeneity of variances assumptions were not attended, the non-parametric equivalent test was applied; α = 5% was assumed.

3.3. Results and Discussion

In general, results evidenced that when submitted to ten times CO_2 current concentrations (400 ppm x 10) *C. raciborskii* cells kept growing, dindn´t altered photosynthesis when compared between conditions, produced chlorophyll and maintained the secondary metabolites production during the three days of experiment. Considering all *C. raciborskii* tested strains (CYLP, NPCS-1, CYRF and T3), the non-toxic strains under high CO_2 (4,000 ppm) suffered expressive alterations experiencing a decrease in growth rates. Additionally very diverse peptides profile was obtained through LC-MS analysis. Factorial analysis of variance evidenced that main differences are between strains (p < 0,001).

Results address attention for a diversityof *C. raciborskii* that respond differently to the same abiotic stressor under laboratory conditions.

Results of pilot experiment demonstrated that under higher CO_2 concentration the pH decreased at least 1 unit. When alternated the same culture flask between CO_2 400 and 4,000 ppm the immediate variation of the hydrogen potential could be obseved (Figure 3).

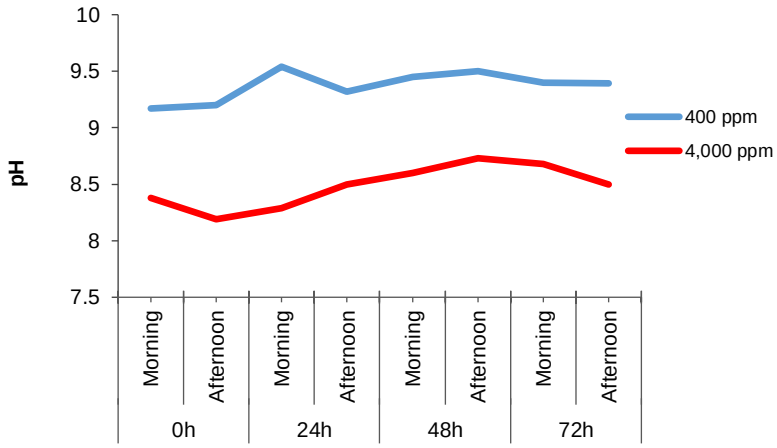


Figure 3. pH measured directly in cultures of T3 in the morning and in the afternoon during 72h under CO_2 400 ppm and 4,000 ppm.

Measures of alkalinity demonstrated an increase in carbon assimilation during the 72h of experiment but differences between cultures under CO_2 400 and 4,000 ppm were only observed at 24h of experiment. At this point a higher alkalinity was observed in cultures receiving more CO_2 (Figure 4).

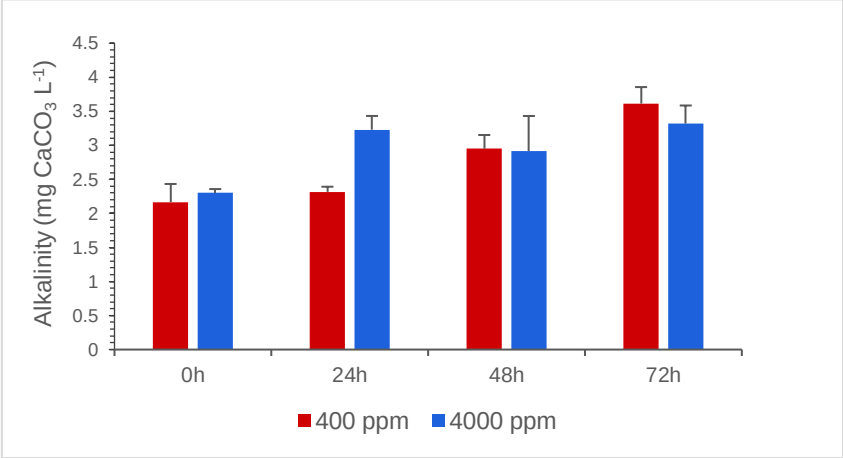


Figure 4. Total alkalinity of T3 cultures submitted to CO_2 at 400 and 4,000 ppm during 72h. n = 3.

3.3.1. Cell Density

All four strains (CYLP, NPCS-1, CYRF and T3) grew during the 72 h of experiment, either at 400 (control) and 4,000ppm (treatment) concentrations of CO_2 (Figure 5). Cultures were started with an inoculum of 10^5 cells^{-mL}, and CYLP and CYRF reached 10^6 cells^{-mL}, demonstrating a great density increase in 72 h, while NPCS-1 and T3 strains reached densities close to $9x10^5$ cells^{-mL}. The growth rate (μ) (Table 1) suggests that exponential phase lasted basically from the moment of the inoculum to the end of experiment. The NPCS-1 strain, which started to grow slowly from 24 to 48h rapidly matched the same cell density of the higher CO_2 condition during the last day. Other specific variations have occurred for CYLP that grew better under control condition during the 72 h, presenting a higher cell density at the end (1.35×10^6 cells^{-mL}) when compared to experimental condition (7.39×10^5 cells^{-mL}). For the two toxic strains (CYRF and T3), growth rates did not differ between tested conditions, but differed between strains. In contrast, the two non-toxic strains (CYLP and NPCS-1) had a twice-bigger growth rate in current CO_2 condition when compared to the higher 4,000ppm CO_2 situation. Nevertheless, these *C. raciborskii* strains exhibited very similar growth rates when maintained under the current CO_2 conditions. Three different groups could be identified according to this parameter: **(a)** CYLP and NPCS-1, μ = 0.42 to 0.48, **(b)** CYRF, μ = 0.58 and **(c)** T3, μ = 0.39. The higher specific difference in cell rate occurred for CYLP strain which treatment group was about half of control group (2.46 and 4.49 respectively). Cell rates of each strain are summarized on table 1.

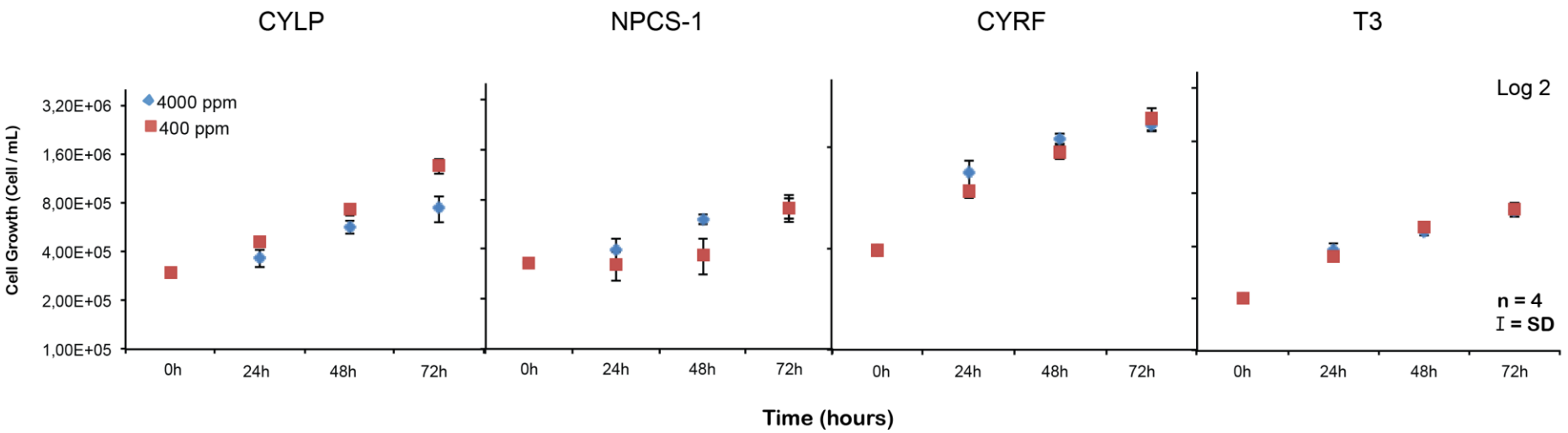


Figure 5. Growth (cell density) of *C. raciborskii* strains CYLP, NPCS-1, CYRF and T3, along 72 h growing under two CO_2 conditions. Red squares represent 400 ppm and blue rhombs represent 4,000 ppm CO_2 conditions, respectively. n = number of replicates; SD = standard deviations.

Table 1. Growth rates and Cell rates calculated for the 72 h experiments with CYLP, NPCS-1, CYRF and T3 *C. raciborskii* strains.

Strain	**CYLP**		**NPCS-1**		**CYRF**		**T3**	
CO_2 Condition	**400 ppm**	**4,000 ppm**	**400 ppm**	**4,000 ppm**	**400 ppm**	**4,000 ppm**	**400 ppm**	**4,000 ppm**
Growth Rate (μ)	0.48 ± 0.1	0.28 ± 0.04	0.42 ± 0.20	0.18 ± 0.1	0.59 ± 0.09	0.57 ± 0.05	0.39 ± 0.03	0.39 ± 0.06
Cell Rate ($T_{72\,h}$ / $T_{0\,h}$)	4.49 ± 1.5	2.46 ± 0.90	2.19 ± 0.51	2.17 ± 0.84	6.03 ± 1.80	5.57 ± 0.98	3.35 ± 0.60	3.21 ± 0.34

Growth results observed for T3 in control conditions match with the ones found by Carneiro *et al.* (2009) that exposed this strain to different light conditions. In that work, a very similar growth response was found at 100 µmol photons m^{-2} s^{-1} and current CO_2 concentration. The growth rate value of 0.301 ± 0.02 was calculated demonstrating great similarity of growth response with our results with the same strain, 7 years later, maintained at same laboratorial conditions.

Price *et al.* (1998, 2008) in studies with *Synechococcus* and *Synechocystis* strains suggested that cyanobacteria actively makes use of both CO_2 and HCO_3^- mechanisms to increase cytosolic inorganic carbon levels. Holland *et al.* in 2012 demonstrated the high efficiency of *C. raciborskii*'s CO_2 concentrating mechanism (CCM) proving that this species gets most of its carbon from HCO_3^- form and suggesting that in a growing atmospheric CO_2 scenario this species would be in competitive disadvantage.. The four strains of the same species used in our experiments were submitted to a ten times higher CO_2 concentration than current and the cells still managed to grow. We don't know how it would behave in a competitive situation but ability to concentrate carbon as an isolated factor didn't seem to be limiting when observed the physiological responses obtained for *C. raciborskii*.

3.3.2. Chlorophyll concentration and photosynthetic efficiency

In general, the comparison of chlorophyll concentrations (Chl/Cell) between control and treatment conditions showed similar results (Figure 6). Variations of orders of magnitude were found when comparing the strains (CYLP = ± 2.14 x 10^{-2}; NPCS-1 = ± 9.00 x 10^{-4}; CYRF = ± 3.30 x 10^{-2}; T3 = ± 1.50 x 10^{-3}). Chlorophyll concentrations in both conditions increased slightly during the 72 hours of experiment, but under control conditions there was an increase of about 1 µg Chl/Cell more than treatment groups.

At the last day of experiment, all strains increased chlorophyll concentrations when compared to the first day and the groups submitted to high CO_2 produced less this pigment compared to natural CO_2 treatment. Following an inverse pattern, photosynthetic efficiency of cultures under high CO_2 was lower at the same moment (Figure 7).

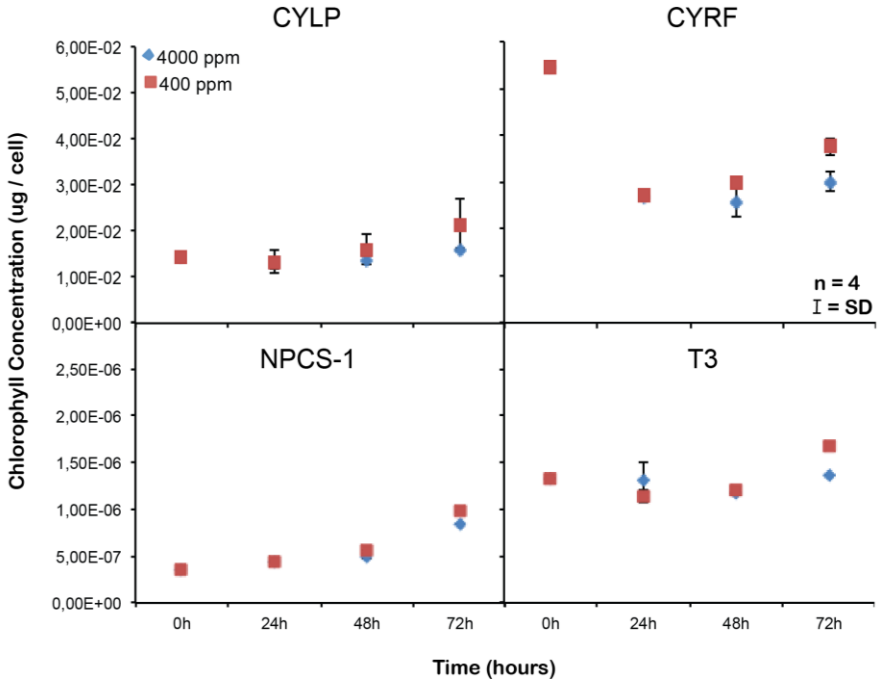


Figure 6. Chlorophyll concentration (µg/Cell) of *C. raciborskii* strains (CYLP, NPCS-1, CYRF and T3), along 72 h growing under two CO_2 conditions. Red squares and blue rhombs represent 400 and 4,000 ppm CO_2 conditions, respectively. n = number of replicates; SD = standard deviations.

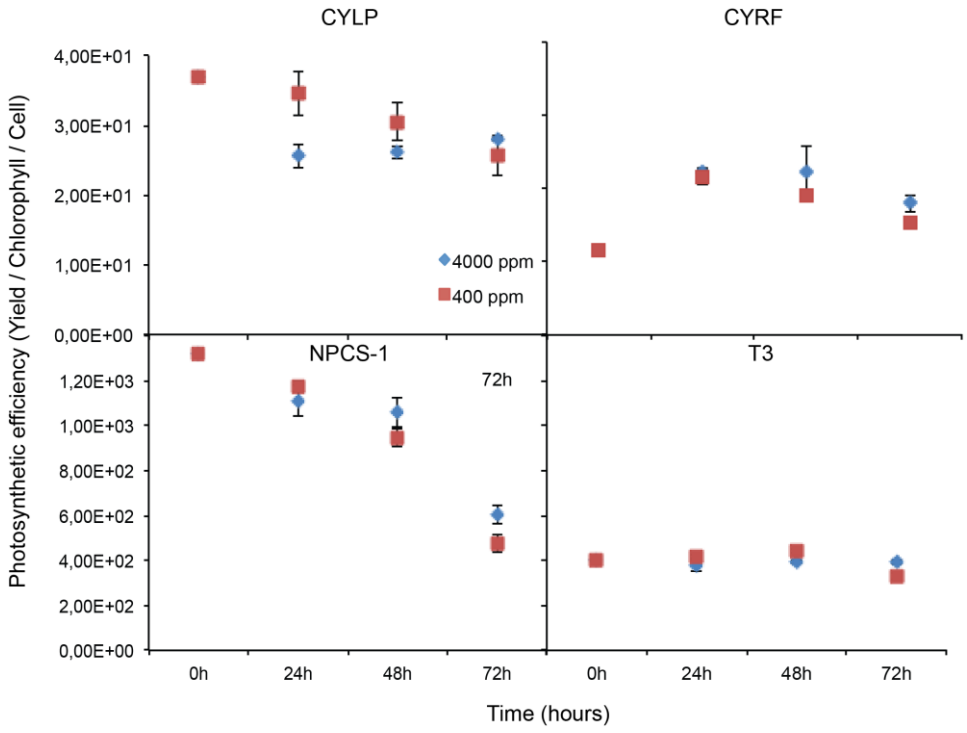


Figure 7. Photosynthetic efficiency of *C. raciborskii* strains (CYLP, NPCS-1, CYRF and T3), along three days growing under two CO_2 conditions. Red squares and blue rhombs represent 400 and 4,000 ppm CO_2 conditions, respectively. n = number of replicates; SD = standard deviations.

Pierangelini *et al*. (2014) evaluated the impact of low and high CO_2 (500 and 1,300 ppm respectively) over the photosynthetic apparatus of a *C. raciborskii* strain from Queensland (Australia). They concluded that under an elevated CO_2 condition a major reorganisation of photosynthetic apparatus occour in paralallel with alterations in CCM activity response. It was demonstrated that the higher concentration has increased cellular division rate, biovolume and provoked alterations in PSII. Authors suggest activation of less effective and less energy expensive CCM's in higher CO_2 condition which could be beneficial for allocation of energy in other synthesis processes. In a previous study it was also demonstrated that cyanobacteria's CCMs requires energy from photosynthesis to operate (Beardall *et al*., 1998). Data obtained by Kranz *et al.* (2010) about the balance between CO_2 and light adjustment in cultures of *Thricodesmium* sp. another cyanobacteria species, brought the idea that high CO_2 levels, when combined to high light, can stimulate growth rates in

cyanobacteria. Contradicting what was expected here the light and very high CO_2 conditions tested didn´t provide such a differential environment for growth when compared to control conditions. In both conditions tested, cells of each strain responded similarly to the attempted stress caused, each one with its own metabolic characteristic. The PSII and CCM plasticity found by Pierangelini *et al*., (2014) could be the reason for such similar responses between our controls and treatments as an effort to maintain *C. raciborskii* cell stability.

Though our results demonstrate variability in chlorophyll concentration response among strains, they agree with the idea that *Cylindrospermopsis raciborskii* can regulatephysiology to reach specific responses common to the species.

3.3.3 Dissolved oxygen and pH

These two factors were monitored to observe how high CO_2 would step in respiratory processes and if there would happen significant pH changes known to be selective in *C. raciborskii* CCM strategies.

Oxygen concentrations in culture mediums varied in a range of 8 – 9 ppm in almost all strains no matter the gas condition (Figure 8).

The ASM-1 medium was adjusted to pH 8.0 before the experiments. At time zero, pH was around 7.2 ± 0.39 and at 72 hours it was about 7.6 ± 1.0 in culture medium for all experimental conditions (Figure 9). Diel variations for each strain separately was not higher than ± 0.5, even in control or treatment.No statistical difference was detected ($p > 0.05$).

Dissolved oxygen in Brazilian tropical reservoirs with predominance of *C. raciborskii* can vary from 4.4 to 14.6 mg L^{-1} (Bouvy *et al*., 2000). Values found in tested cultures represent the average values found in those habitats as well as average pH values found, which were equal to 8.2 in nature and 7.7 in our laboratory findings. Those values are also conformable with the ones found for a southeastern Brazilian reservoir (Souza and Truzzi, 1998). Data found for cultures demonstrated

great similarity between environmental parameters and controlled condition. No statistical difference was detected for D.O. data ($p > 0.05$).

To allow sampling data without compromising the progress of experiments the airflow had to be interrupted. This procedure allowed the measure of pH and D.O. parameters inside the laminar flow, through a hole in the cultures hold avoiding contamination along the days of experiment. Once interrupted the constant CO_2 source, pH could have increased immediately making it impossible to measure correctly such parameter. When measured directly in cultures under airflow, contamination by fungus interrupted experimental attempts due to intense manipulation necessary to sample experimental units.

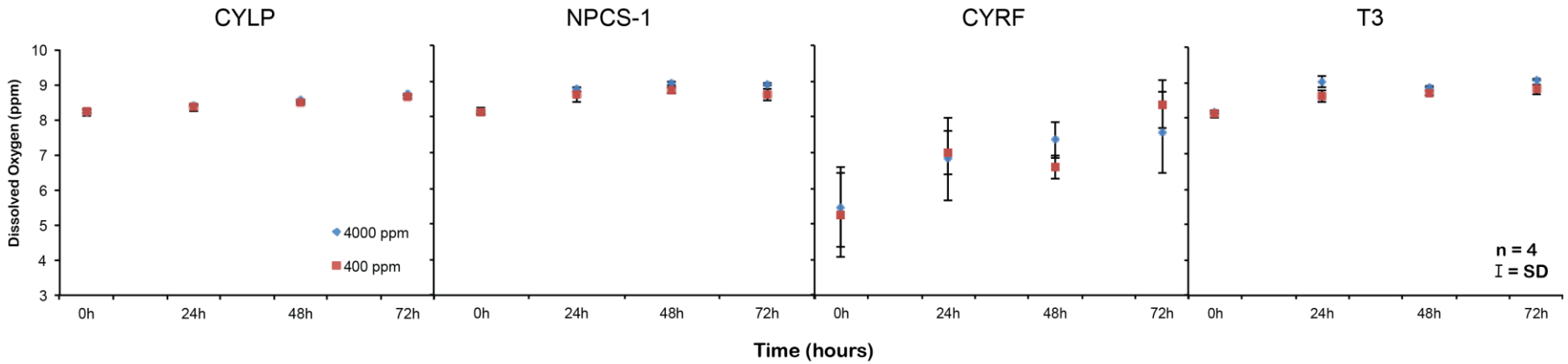


Figure 8. Dissolved oxygen in culture medium of *C. raciborskii* strains (CYLP, NPCS-1, CYRF and T3), along three days growing under two CO_2 conditions. Red squares and blue rhombs represent 400 and 4,000 ppm CO_2 conditions, respectively. n = number of replicates; SD = standard deviations.

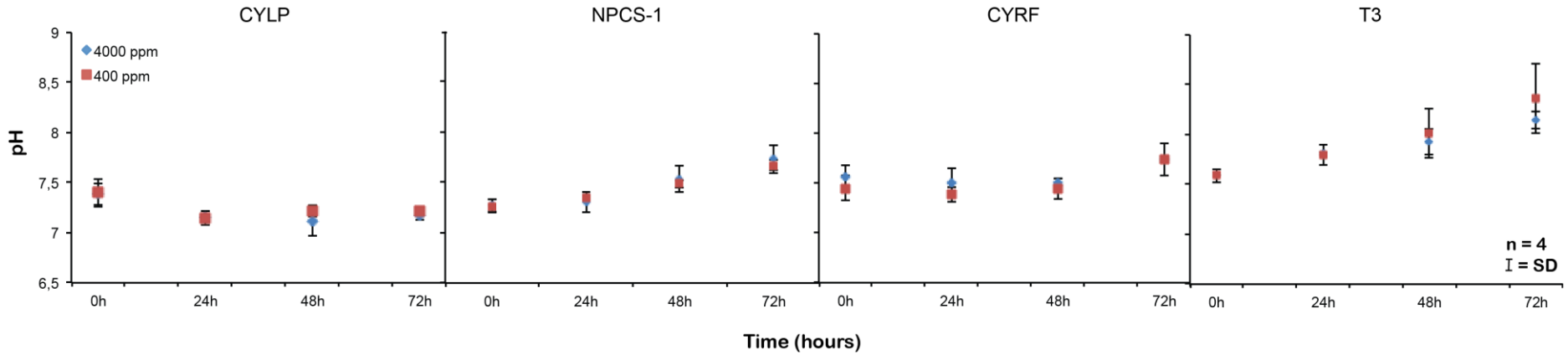


Figure 9. pH values in culture medium of *C. raciborskii* strains CYLP, NPCS-1, CYRF and T3, along three days growing under two CO_2 conditions. Red squares and blue rhombs represent 400 and 4,000 ppm CO_2 conditions, respectively. n = number of replicates; SD = standard deviations.

Studies working directly with the CO_2 gas form were performed in a diversity of themes that comprise survival strategy associated to the carbon forms (Holland *et al*., 2012), changes in photosynthetic apparatus in response to carbonic gas partial pressure (Pierangelini *et al.*, 2014), CCM strategies and toxin production (Caraco and Miller, 1998; Kranz *et al*., 2010; Pierangelini *et al*., 2015). The experiments performed here focused on the metabolic response associated to physiological parameters.

Influence of land water use, rainfall regime, and temperature oscillations, coupled with diel dynamics of dissolved CO_2, are considered important factors in pH variations in lakes (Psenner and Schimidt, 1992). The CO_2 gas form exchanges between the atmosphere and water in a dynamic equilibrium. When the atmospheric levels of CO_2 increase the dissolution and concentration of this gas into the water increases. This process can alter the equilibrium of ions, such as HCO_3^- and CO_3^{2-}, and lower the pH. However, change in alkalinity can also be a result of the abiotic action cited above, combined with biotic metabolism of secreted molecules. No alterations could be observed ($p > 0.05$). Mostly because of the interruption of airflow at the moment of the sampling once pH monitored directly with the airflow demonstrated a decrease in pH under CO_2 at 4,000 ppm. Whatever processes involved, pH level was in the optimal range for cyanobateria (7-9) and physiological responses observed reflected that.

3.3.4 Total saxitoxin production

Once molecules of saxitoxin and neo-saxitoxin can be interconverted (Wright, 1995), the total saxitoxins production considered was the sum of concentrations from both molecule-types in each experimental sample.

Total saxitoxins production by CYRF and T3 strains was measured at the beginning and after 72 hours of experiments by HPLC method (Figure 10). Values found at time zero were similar to that control conditions after 72 h for both strains

(7.1_x10^{-6} ng/cell; $7,81_x10^{-6}$ ng/cell, respectively for T3 and $8,37_x10^{-6}$ ng/cell; $8,71_x10^{-6}$ ng/cell for CYRF, respectively). Cultures maintained under CO_2 at 4,000 ppm suffered a decrease of 2.0_x10^{-6} ng/cell in total saxitoxin production at 72 h. No statistical difference between CO_2 coditions was detected (p > 0,05). However ANOVA detected statistical differences between the two toxin producers strains (p < 0.05).

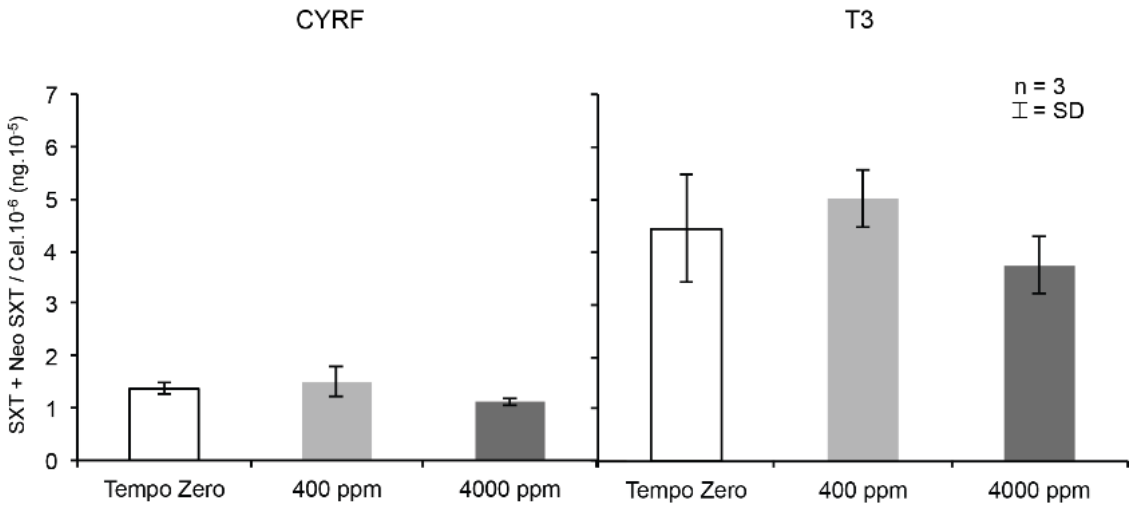


Figure 10. Saxitoxin + neo-saxitoxin concentrations (ng/cell) found for the day the experiment was conducted (white bars) and after 72 hours for both conditions (grey and black bars). n = number of replicates; SD = standard deviation.

Due to the toxicological importance of saxitoxins, this group of carbamate molecules represents a great concern of scientists and authorities and has been studied since 1975 (Schantz *et al.*) with focus on producing organisms, toxicological effects, biosynthesis and action mechanisms (Pearson *et al*., 2010; 2016). A relevant physiological response was already found for *C. raciborskii* and was described by Pomati *et al*. (2004). Authors had linked the intracellular increases in SXT concentrations in T3 strain in response to extracellular accumulation of Na^+ ions and alkalinity increase. The authors suggested that this action would regulate toxin production also playing role in cell homeostasis. Since then this is the closer

approach to saxitoxin cellular function. Later, Carneiro *et al*. (2013) observed that T3 cells had better fitness in medium with increased water hardness ($MgCO_3$ and Na_2CO_3). Cells also increased SXT cell quota when cultivated with Na_2CO_3 (5 mM) and $MgCO_3$ (10 mM). In 2009, Carneiro and co-authors observed that during the light period *C. raciborskii* T3 strain exposed to 100 µmol photons m^{-2} s^{-1} produced higher levels of SXT.

The same light intensity used by Carneiro *et al*. (2009) was employed in the present study and values of total saxitoxin concentrations in cultures at current CO_2 levels (zero hour and 72 h at 400 ppm) varied similarly. Non-statistically difference ($p > 0.05$) has occurred in cultures exposed to high CO_2. In the other hand, this is the first report of differential SXT cell quota between two different strains ($p < 0.05$). In 2013, Neilan and colaborators emphazised the almost 10 years of poor understanding of SXT functions and 4 years later it is still the same. In experiments performed here, the absence of competitors and the optimum growth conditions offered could have influence in the allocation of resources. Considering Carbon Nutrient Balance Hypotesis (CNBH – McClintock *et al*., 2001) that predicts that when carbon is available but nutrients are abundant, organisms should invest resources in growth. If carbon is limiting, it should been allocated to growth and the production of carbon-based defenses should decrease. However, cultures maintained under natural CO_2 levels have been exposed to the same conditions except gas concentration. The higher concentrations didn´t increased growth neither toxin production in both producer strains. On the other hand, non-producer strains also grew during experiments and despite the different phenotypes, results demonstrate no correlation with saxitoxins production. The idea that saxitoxin really play a role on cell homeostasis seems embracing if we observe that the only consensus in response found was related to conductivity changes (Pomati *et al*. 2004; Carneiro *et al*., 2013). Due to high photosynthetic energy required by CCM's activated in the presence of high CO_2 (Pierangelini *et al*., 2014), the production of secondary

metabolites could be compromised as well, which was not observed in outr experimetns. The experiment should be repeated for a longer period to better monitor the toxin production under high CO_2. For both strains a 2 ng toxin concentration decrease was observed under high CO_2. Comparisons between exponential and stationary phases would be interesting, once production of saxitoxin is lower during the growth phase.

3.3.5. Cyanopeptides

Due to the diversity of cyanopeptides to be explored and the comparative nature of this work, no compound characterization was done until the moment. However, since the extraction procedure until the LC-MS analysis, all techniques performed were based on a study of the literature about cyanopeptides Welker *et al*., 2006[a]; Silva-Stenico *et al*., 2011; Carneiro *et al*., 2012; Sanz *et al*., 2015; Briand *et al*., 2015). Based on those studies, we believe that mass spectrometry profiles found are peptide rich and represent the diversity of these molecules production found in experimental conditions (CO_2 at 400 vs. 4,000 ppm). Molecular characterization techniques require a deeper investigation and it will be optimized and performed in future steps of this study. It is important to emphasize that until present days only a microginin peptide was described in a Brazilian *C*. *racbiborskii* strain (Silva-Stenico *et al*., 2011; 2015; Sanz *et al*., 2015).

Results showed that for CYRF and NPCS-1 strains, m/z Da spectrum didn't varied between the two CO_2 conditions (Figures 13 and 12 respectively). However, for CYLP and T3 (Figures 11 and 14 respectively) samples mass spectra obtained indicated a great variation in secondary metabolites produced under different carbonic gas levels. Data presented shows that under CO_2 levels of 4,000 ppm, T3 cells only produced metabolites of mass values lower than 850 mz, Da. When cultured under 400 ppm levels, a wider spectrum with masses ranging from 400 to 1.200 mz, Da was found. An inverse profile was found for CYLP, which produced a

broader variety of metabolites under high CO_2 condition and metabolites of mass lower than 800 Da under natural CO_2 condition. NPCS-1 *C. raciborskii* strain showed a diverse metabolic profile with masses ranging from 400 to 1,100 Da in both CO_2 conditions. The higher number of the most representative masses of metabolites of CYRF ranged from 400 to 690 Da with the presence of two metabolites with molecular weight between 900 and 970 Da.

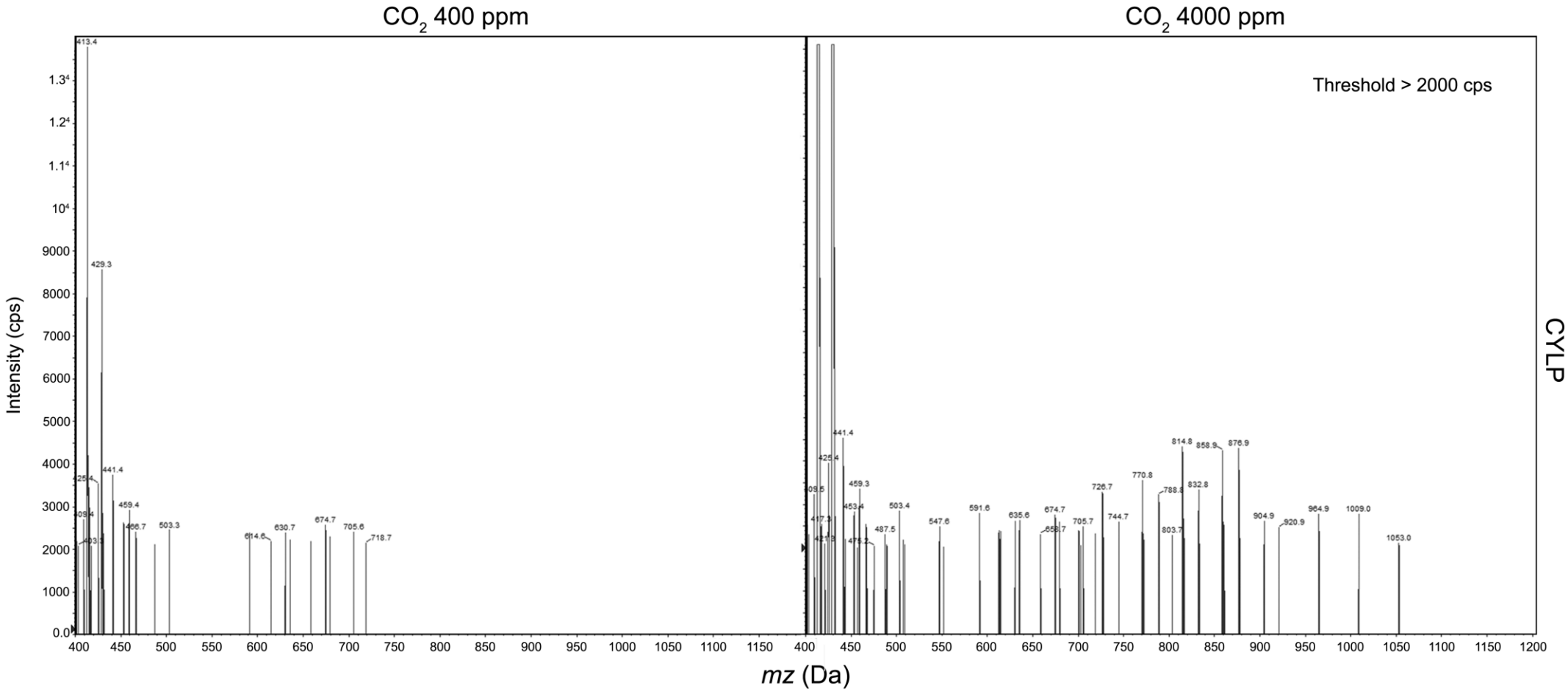


Figure 11. LC-MS mass spectrum from methanol/H_2O (1:1) + acethic acid 5% extracts of *C. raciborskii* CYLP strain tested under CO_2 at 400 ppm and 4,000 ppm. Threshold was applied to select the masses with higher intensity (cps) values.

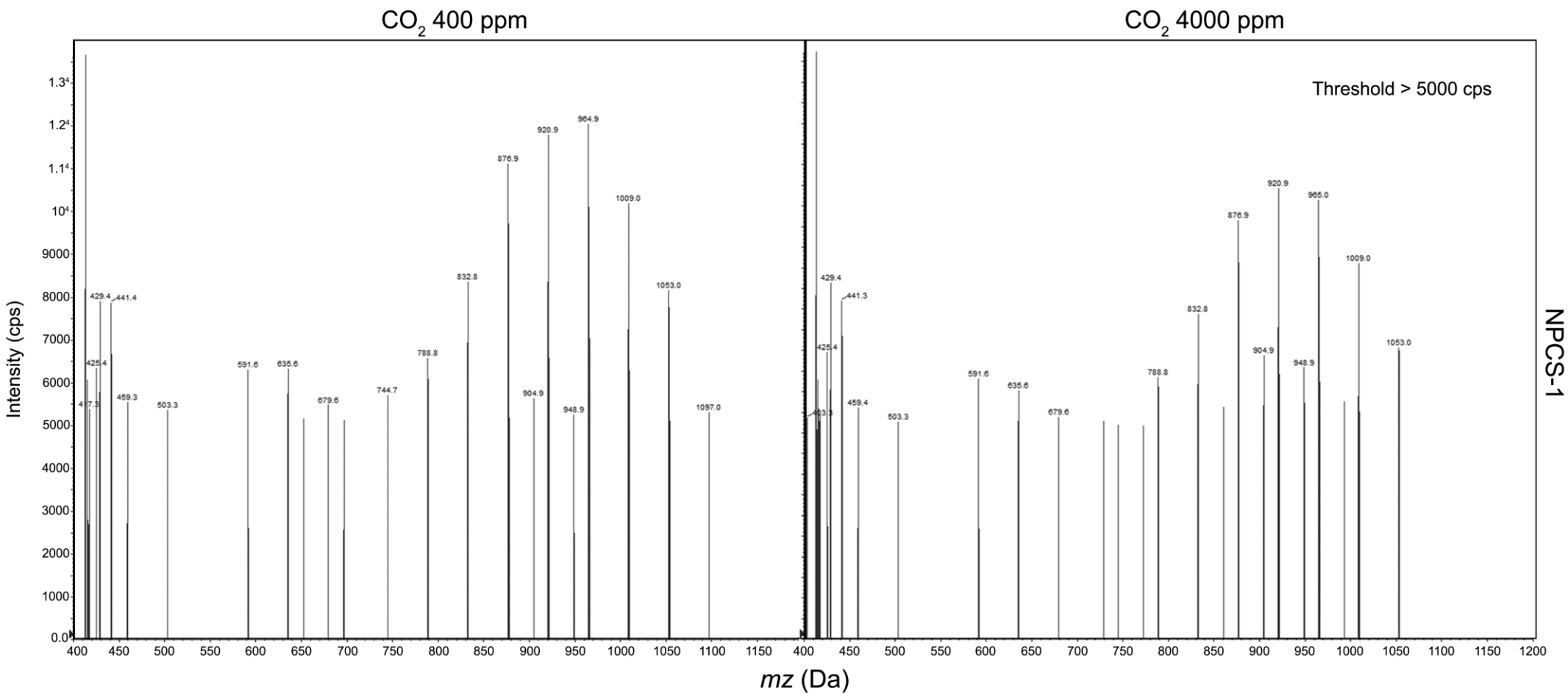


Figure 12. LC-MS mass spectrum from methanol/H_2O (1:1) + acethic acid 5% extracts of *C. raciborskii* NPCS-1 strain tested under CO_2 at 400 ppm and 4,000 ppm. Threshold was applied to select the masses with higher intensity (cps) values.

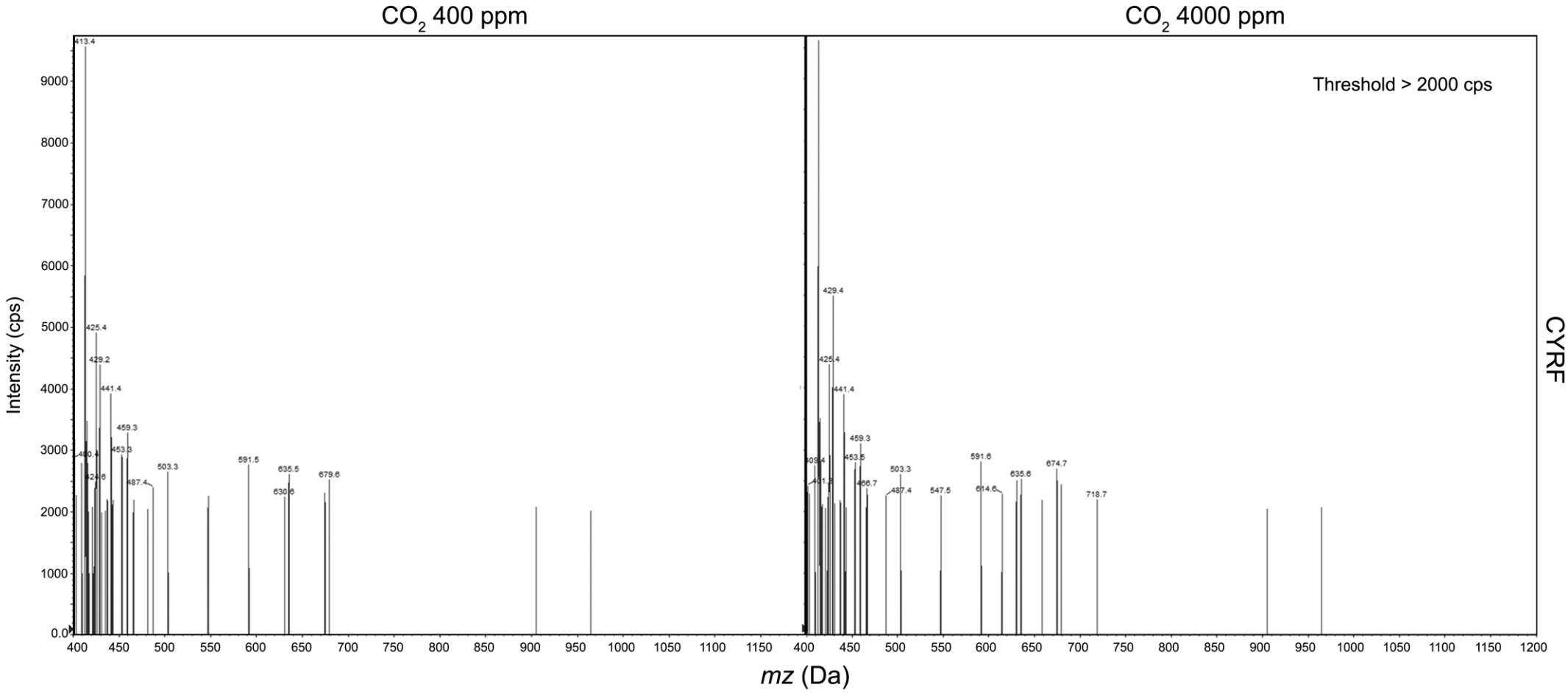


Figure 13. LC-MS mass spectrum from methanol/H_2O (1:1) + acethic acid 5% extracts of *C. raciborskii* CYRF strain tested under CO_2 at 400 ppm and 4,000 ppm. Threshold was applied to select the masses with higher intensity (cps) values.

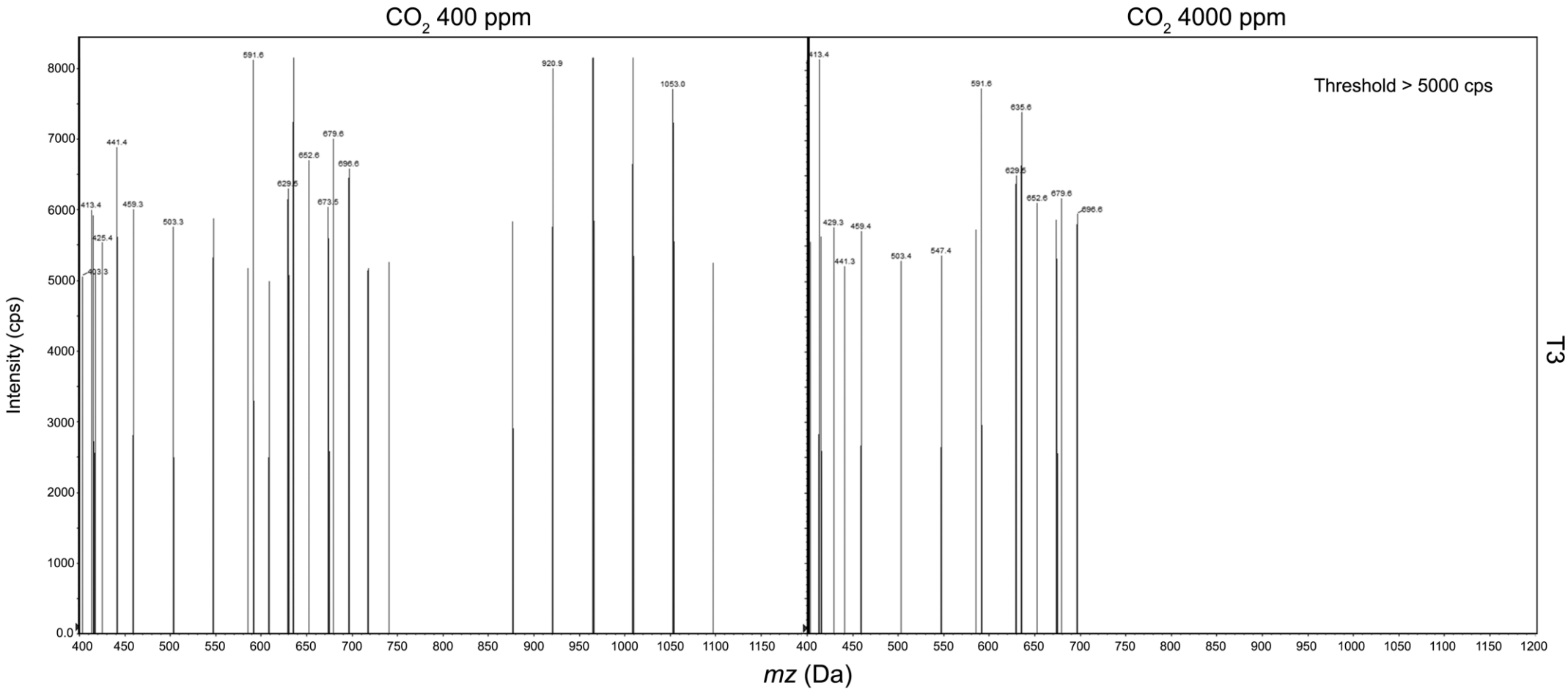


Figure 14. LC-MS mass spectrum from methanol/H_2O (1:1) + acethic acid 5% extracts of *C. raciborskii* T3 strain tested under CO_2 at 400 ppm and 4,000 ppm. Threshold was applied to select the masses with higher intensity (cps) values.

Strains studeid were isolated from different aquatic environments and different regions at different periods, all of them included in culture collection of the Laboratory of Ecophysiology and Toxicology of Cyanobacteria at Carlos Chagas Filho Biophysics Institute (UFRJ) at least since 2006. It represents 10 years of cell division and culture adaptation under the same laboratory conditions. A scenario where an adaptative convergence could be expected. However, distinct metabolic profiles were observed when compared mass spectrometric results from different strains. Differences in signal intensity (cps) and mass diversity were found and demonstrate variable metabolic responses for the four strains. This result may be indicative of a possible conservative biochemical functions in strains. Future isolations of new strains should monitore either physiology and metabolic profile since the remove from the natural environment until the stablishment in laboratory.

3.3.6. General considerations

In table 2, the results obtained with the four tested strains were summarized. Statistical analysis revealed that differences lay between strains and not between CO_2 concentrations.

After 72 h of experiment CYLP and CYRF strains had a general higher growth (1.35×10^6 and 2.34×10^6 $Cell^{-mL}$) than NPCS-1 and T3 (7.07×10^5 and 6.5×10^5 $Cell^{-mL}$). Among growth rates, under current CO_2 CYRF strain had the higher average rate (0.59) and T3 strain had the lowest (0.39). When cultivated under high CO_2 CYLP and NPCS-1 strains showed a twice-lower **GR** values (0.28 and 0.18). However statistical analysis did not confirm differences ($p > 0.05$) when compared to high CO_2 groups. Power analysis was employed (software G*Power - Faul *et al*., 2007) to test if experimental conditions were enough to detect differences whether they exist. Test result point to a very low analytical power with the possibility of a type II error where the null hypothesis is accepeted when it is false. To confirm this result an experiment with more replicates should be employed.

Chlorophyll concentrations found also diverge in orders of magnitude, since CYRF and CYLP are more efficient producers (3.76×10^{-2} and 2.14×10^{-2} μg^{-Cell}) than T3 and NPCS-1 (3.29×10^{-3} and 9.74×10^{-4} μg^{-Cell}). The efficiency of photosystems inversely followed the chlorophyll production.

Examining the pH values obtained, it was observed that CYLP strain was the one who managed the lower concentrations (7.22) and T3 managed the most alkaline condition (8.36).

Table 2. Physiological data obtained for *C. raciborskii* strains (CYLP, NPCS-1, CYRF and T3) maintained under 400 and 4,000 ppm levels of CO_2 during 72 h of experiment. NP = non-producer.

	CO_2 400 ppm at 72 h				**CO_2 4,000 ppm at 72 h**			
Physiological parameters and culture conditions	**CYLP**	**NPCS-1**	**CYRF**	**T3**	**CYLP**	**NPCS-1**	**CYRF**	**T3**
Cell Density ($Cell^{-mL}$)	1.35×10^{6}	7.07×10^{5}	2.34×10^{5}	6.50×10^{5}	7.39×10^{5}	7.16×10^{5}	2.16×10^{6}	6.41×10^{5}
Growth Rate (μ)	0.48	0.42	0.59	0.39	0.28	0.18	0.57	0.39
Cell Rate ($T_{72\ h}$ / $T_{0\ h}$)	4.49	2.17	6.03	3.25	2.46	2.19	5.57	3.21
Chlorophyll (μg Chl / $Cell^{-mL}$)	2.14×10^{-2}	9.74×10^{-4}	3.76×10^{-2}	1.68×10^{-3}	1.60×10^{-2}	8.40×10^{-4}	3.01×10^{-2}	1.36×10^{-3}
Photosynthetic Efficiency (Yield / μg Chl^{-Cell})	2.57×10	4.77×10^{2}	1.52×10	3.29×10^{2}	2.81×10	6.04×10^{2}	1.80×10	3.92×10^{2}
pH	7.22	7.66	7.73	8.36	7.17	7.74	7.74	8.14
Dissolved Oxygen (ppm)	8.64	8.61	8.35	8.82	8.72	8.92	8.91	9.08
Total Saxitoxin (SXT + neoSXT ng / Cell)	NP	NP	8.71×10^{-6}	7.81×10^{-6}	NP	NP	6.97×10^{-6}	5.87×10^{-6}

Results demonstrated differences in metabolic responses of *Cylindrospemopsis raciborskii* under current CO_2 concentration. Low growth rates (2x smaller) of non-toxic strains under high CO_2 adress attention to possible roles of secondary metabolites as a molecular response to gas variation. Despite CO_2 condition studied, under current concentrations each strain metabolized different amounts of chlorophyll, had different photosynthetic activity, grew in different rates and densities and had different metabolic profiles. The species *C. raciborskii* seems to have great genetic heterogeneity and genome that defines very well the different strains (Wilson *et al.*, 2000). Such interspecific differences could be evidence of different adaptative strategies and can help to explain the dominance success of this species. Genetically, other species were demonstrated to not only to differ in an environmental scale (Johnson *et al*., 2006), but also in a local scale (Kashtan *et al*., 2014). Authors Agha and Quesada (2014) explored deeply the idea of chemotypes in cyanobacteria species. Indeed, diversity of toxic metabolites produced by cyanobacteria is one of the first example of stablishment of ecotypes and this diversity is a probable fruit of evolutionaty history (Neilan *et al*., 2003).

The non-toxic/toxic character as example of evolutionary adaptation was well demonstrated by Rangel *et al*. (2016), who observed that in a non-saxitoxin producer strain of *C. raciborskii*, long filaments were less ingested by copepods while short filaments of a saxitoxin producer strain produced more toxin and were less consumed. Plasticity of cyanobacteria is also well represented in physiological level for *C. raciborskii* by the ability to modify the photosynthetic apparatus under elevated CO_2 concentration, activating different CCM's according to the main availability of carbon source (Pierangelini *et al*., 2014). Therefore, *C. raciborskii* can alter mechanisms of energy capture to better respond to environmental changes. In this study, small and non-statistical relevant changes were observed in saxitoxin production by CYRF and T3 when in the presence of high concentration of CO_2 but great differences between these strains were detected ($p < 0.05$). There is still doubt

about how *C. raciborskii*"s SXT produciton would behave in a long scale experiment. Until now, this molecule was only proposed to play role in ion regulation by blocking sodium channels (Pomati *et al*., 2004). Here, the first report with evidence of changes possibly caused by CO_2 open new questions about the importance of saxitoxin for cell homeostasys. In a very similar attempt, Pierangelini *et al.* (2015) analyzed cylindrospermopsin pool size in *C. raciborskii* under light and CO_2 variations. However, for this cyanotoxin, no difference between gas conditions was observed and cells grew normally.

Phenotypic plasticity can be considered a great characteristic presented by bacteria as well. Those organisms evolved to survive under an enormous variety of natural conditions, such as cold, hot, alkaline, acid, salty, aquatic, terrestrial and symbiotic conditions. It has been demonstrated for CCM's (Badger *et al*., 2006), cyanopeptides (Ishida *et al*., 2009), temperature tolerance (Soares *et al*., 2013[a]), chloroplasts adaptation (Hüner *et al*., 2012), nutrient absorbtion (Berman-Frank *et al*., 2007; Saxton *et al*., 2012), light use (Baulina, 2012) and is expressed in organism's genome (Frangeoul *et al*., 2008). Paleocene's carbonic gas concentrations (4,000 ppm) tested here probably stimulated molecular and physiological adaptations in strains studied allowing them to rapidly adapt to the new condition employed. In the past the gradual elevation of CO_2 concentrations may have allowed the selection of predecessors of *C. raciborskii* fulled of genes for a wide range of environmental conditions. Events of such high CO_2 levels were not a constant in geological history of the planet, but high concentrations of the gas can be found even in lakes with intense and constant algal blooms acting as donnors of the gas resultant from respiration to the atmosphere (Cole *et al*., 1994).

LC-MS analysis allowed to observe variation of metabolic profiles under high CO_2, absence of variation and distinct profiles between strains under current CO_2. In the first case, CYLP produced more metabolites under high CO_2 and T3 produced less metabolites. So if gas intensity has influence on secondary metabolites

production it can alter the metabolic profile of some strains. The signal intensity that could be a indirect quantitative measure, didn't varied between CO_2 conditions but varied between strains. Agha and Quesada (2014) affirm that due to the distinct qualitative oligopeptide composition, these molecules are suitable biomarkers of cyanobacterial subpopulations. The non-ribosomal peptide synthesis mechanism allows the occurrence of occasional modifications by epimerization, methylation, or heterocyclization (Konz and Marahiel, 1999; von Döhren *et al*., 1997), thus generating a great diversity of metabolites with the same core skeleton and terminal variations. This diversity would improve the chances of *C. raciborskii* to produce metabolites with ecological function as protection.

Beyond the potential for bioprospecting of metabolites produced by cyanobacteria is the poorly known ecological relevance of these molecules. Results demonstrate how variable they are in lineages of a same species and that they vary or not when a single environmental factor is changed. Evidences draw attention for the importance to improve studies on functionalities of cyanopeptides for cyanobacteria itself with potential to be ecological and evolutionary key drivers granting diverse adaptative advantages. A great number of secondary metabolites became important for more complex organisms as seaweeds and vascular plants providing, for example, defense against consumers, ability to compete for space, chemical signaling and U.V. protection (McClintock, 2001). Cyanobacteria have emerged long time before more complex photosynthetic organisms thus being the basis for evolution of uni- and multicellular eukaryotic cells. However, studies on chemistry of secondary metabolites of algae are in a forward stage of knowledge (Amsler, 2008). In contrast, until 2007 the majority of natural products discovered were derived from microorganisms isolated from marine sponges (Blunt *et al*., 2009). This increase was a result of discoveries that metabolites believed to be original from invertebrates were in truth produced by microorganisms (König *et al*., 2006). Therefore past experiences summed to recent discoveries would help to lead the

way aquatic researchers will focus their effort to bring new advances in freshwater chemical ecology.

Freshwater species received great attention on specific toxins due to medical importance and due to the elevated frequency that HABs occour in lakes and resservoirs once they are much more limited and unstable compartments when compared to the oceans. Other aggravating is the proximity with the human species and as well the euthrophyzation caused by our species, generating ideal conditions to development of blooms, generaly toxic. In addition freshwater cyanobacteria physiology is very well known once there is concern in managing species in potable water sources but its ecology and interaction with other micro and macro organisms still require attention. In the other hand, marine cyanobacteria form less frequent toxic blooms but their ecological relationship is very important for a number of benthic life forms, acting as symbionts (Paul *et al*., 2014). This relastionship will reflect the structure of entire coastal environments organized over reef forming species like corals, which demand the symbiotic relastioship with photosynthetic bacteria (Charpy *et al*., 2012). This is of extreme importance for fishering once primary productivity is increased (Pereira & Soares-Gomes, 2009). As ecological roles are more studied in this group, the natural products profile and molecular diversity is better described (Blunt *et al*., 2009; Paul *et al*., 2014). Notwithstanding fresh and marine water cyanobacteria share common observations like expansion in a climate change scenario (Paerl and Paul, 2012) and as observed at the present study, they don't suffer great impact under elevated CO_2 concentrations (Paul *et al*., 2014). Maybe within the group there are more common characteristics that can explain their evolutionary processes than scientists can imagine and a macro scale watch over the group of cyanobacteria, which encompass marine, freshwater and other habitats, would be a prolific source of new information and way to interpret their worldwide expansion and the conquest of almost all environments.

Not only experiments with more strains would allow a better comprehension of the group cyanobacteria but a better look at the similarities between behaviors of different classes, orders, genera and species in a same changing world.

4. Chapter 2: Transcriptomic analisys of the effects of high CO_2 of toxic and non-toxic strains of *Cylindrospermopsis raciborskii* (Cyanobacteria)

Rogers R., Hoffman L., Bottaro T., Pereira R.C., Ürményi T.P., Silva R., Azevedo M.F.O.

Abstract

Cyanobacteria are ancient organisms (3,8 Ga) that faced a countless number of environmental changes selecting charateristics that allowed the dominance of all kind of aquatic and terrestrial ecosystems during almost all geological events. One of those events was the CO_2 elevation to 4,000 ppm during the Paleocene (65 – 55 million years) and after decreasing to current concentrations (< 400 ppm) it is now predicted a new elevation to 3 fold higher until the year of 2,100. Transcriptomes of one toxic (CYRF) and one non-toxic (CYLP) strain of *Cylindrospermopsis raciborskii* cultivated under current and elevated CO_2 concentrations (400 and 4,000 ppm) during 72 h were performed to investigate the RNA expression of those organisms under previous atmospheric conditions. Sequences analysed at the end of experiment revealed a higher number of CYLP genes regulated in high CO_2 condition when compared to CYRF. Both strains had a similar number of genes up and downregulated . When submitted to the same experimental condition the different strains expressed completely different RNAm. The CYRF strain also had a smaller number of statiscally different genes regulated under high CO_2 when compared to CYLP. This work could demonstrate that two different strains of *C. raciborskii* altered RNA expression when submitted to CO_2 at 4,000 ppm and that different strains had completely different RNA profile when submitted to the same experimental condition.

4.1. Introduction

From evidences of origin at 3.8 billion years ago (Ga) (Alterman, 2007), time enough to tell the history of the life on Earth, cyanobacteria figures in the list of the most well succeeded life forms habiting the planet. Adapted to live in cold and hot, alkaline and acidic, marine, freshwater, saline, terrestrial and symbiotic environments (Badger *et al*., 2006), the plasticity of the group is the probable key to their evolutionary triumph. Along the eras, temporal and spatial variations in temperature, habitat composition, O_2 and CO_2 levels, as well as light intensity were the environmental forces which driven selection of a variety of primordial genes that guarantee the group dominance (Badger *et al*., 2006).

High plasticity of cyanobacteria and bacteria in general (Stucken *et al*., 2009; Carneiro *et al*., 2013; Soares *et al*., 2013; Sinha *et al*., 2014; Rangel *et al*., 2016) as a result of many ages of evolutive processes like lateral gene transfer (Boucher *et al*., 2003) and short generation time can be adressed as efficient adaptation mechanisms of these organisms. Lateral gene transfer processes were also demonstrated to be constant (Jain *et al*., 1999) and assumed to perform complex transformations in basic biology of cells (Boucher *et al*., 2003). Additionaly, enzymatic processes of non-ribosomal pathways realized by peptide synthetases, shared by the primary metabolism, are also responsible for creating a diversity of molecules with same molecular core altered by slight variations in such processes (Welker and Döhren, 2006). Those molecules of protease inhibitory activity (Welker *et al*., 2006; Chipala *et al*., 2011; Stenico *et al*., 2012; Silva-Stenico, *et al*., 2015) were described to play important ecological roles (Schatz *et al.*, 2007; Leão *et al*., 2010; Bagchi *et al*., 2012).

Due to the toxicological focus on microcystin, one well-studied cyanobacteria species is *Microcystis aeruginosa*. In this species was observed homeostasis between nutrient regulation by iron, nitrogen and toxin production (Kaneko *et al*., 2007; Frangeul *et al*., 2008; Ginn *et al*., 2010; Alexova *et al*., 2011) and as a

transcriptomic signal (Makower *et al*., 2015). Nevertheless alterations in secondary metabolism found are correlated with environmental factors, which demonstrates an evolutive importance of such molecules in assisting cyanobacteria cells to respond to natural phenomena.

Cohabiting with Microcystis, *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju (1972) is a filamentous cyanobacteria of the order *Nostocales*, that during the two last decade is being reported to widespread over new habitats of all continents (Neilan *et al*., 2003; Bonilla *et al*., 2012). Strains of this cyanobacteria can be non-toxic or produce extremely danger toxins, such as cylindrospermopsin and saxitoxins - SXT (Pearson *et al*., 2010; Zegura *et al*., 2011; Pearson *et al*., 2016) also forming Harmful Algal Blooms (HABs) (Pearl and Huisman, 2009). Saxitoxins were proposed to play role in cellular homeostasis (Pomati *et al*., 2004) and SXT gene cluster was described in *Cylindrospermopsis raciborskii* strain. Results indicated a mosaic structure with many similarities to other cyanobacteria and dinoflagellates (Kellman *et al*., 2008). Beyond the saxitoxin genes, molecular characterization of different strains from Australia could demonstrate a great level of genetic heterogeneity between different strains of *C. raciborskii* associated to polymorphic DNA genes and that they group very well the specieswhen analysed by the *rpoC1* (Wilson *et al*., 2000). This result draw attention for the existence of ecotypes depending on the environment that those organisms habit and such differences can be detected inside DNA.

Advances in transcriptomic techniques have allowed researchers to observe changes in RNA expression under different conditions and studies were applied to cyanobacteria (D'Agostino *et al*., 2016). Such knowledge increased understanding about gene regulation under a variety of stimuli, making possible to glimpse the key drivers of specific metabolic responses. Environmentally, metatranscriptomes techniques were applied, for example, to observe the predominance of gene activity during dial variations, but D'Agostino and co-authors (2016) address the

interpretation of data against the background of unstable environmental conditions as a great challenge. Another important challenge is the improvement of cyanobacterial RNA extraction techniques due to the scarcity of papers and lack of standardized detailed information (Harke & Gobler, 2013; Sandrini *et al*., 2014; Stucken *et al*., 2014; Pierangelini *et al*., 2015). Nevertheless, it is not surprise since RNA molecules have a short lifetime and extraction demands a great amount of cellular biomass. This biomass is more easily obtained during natural blooms, but this type of samples has more analytical bias due the presence of a great diversity of life forms. In laboratory, difficulty remains in conciliate production of biomass with reproduction of a natural scenario inside a limited space.

In geological scale, more recently, cyanobacteria crossed the highest CO_2 levels reported by scientists, of a scale of 4,000 ppm, ten times current concentrations, during the Paleocene (Pearson and Palmer, 2000). Further, CO_2 has decreased and stabilized until the beginning of the first industrial revolution (IPCC, 2014). Current CO_2 levels are increasing and this gas is addressed as the villain of one of the last decade major social, politic and scientific concern: the climate changes (IPCC, 2014). It is also predicted by the IPCC that until the year of 2,100 greenhouse gas will reach 3 times actual levels (1,200 ppm). Implications of this phenomenon to cyanobacteria are being studied and it was also discussed that the spread of cyanobacteria to new areas can correlate with climatic alterations (Sinha *et al*., 2012). Moreover their physiological capabilities are considered to be adapted to the emerging environmental changes as well the conditions created as a result of antropogenic impact, propitiated conditions to occurrence of HABs (Paerl and Huisman, 2009).

Concerned to the predicted scenario, the aim of this study was to analyse transcriptomes of one saxitoxins producer and one non-producer strain of *C. raciborskii* submitted to Paleocene and current CO_2 concentrations in an attempt to

through past conditions understand how cyanobacteria should deal with future climate.

4.2. Material and Methods – Transcriptomes experiment

4.2.1. Growth under different CO_2 conditions

One toxic (CYRF) and one non toxic (CYLP) strains of *C. raciborskii* from culture collection of LETC/IBCCF (Laboratory of Ecophysiology e Toxicology of Cyanobacteria, UFRJ) were cultivated in ASM-1 medium (Gorham *et al.*, 1964), with constant aeration, pH = 8.0, temperature of 23±1 °C, light intensity of 40–50 μE m^{-2} s^{-1} and a 12:12 h, light: dark cycle. The CYRF strain was reported to produce saxitoxins (Ferrão-Filho *et al.*, 2007), while the CYLP and NPCS-1 are non-toxin producer strains. The cultures were kept in exponential growth phase by the replacement of medium once a week until they were used as inoculum for the experiments.

Four strain replicates were used for each experimental condition. The experiments have lasted 72 h, in which controls were submitted to current atmospheric CO_2 conditions (400 ppm) and treatments to Paleocenic´s CO_2 estimated concentration (4,000 ppm). Both conditions were obtained through a Thermo Fisher gas mixer coupled in a 100% CO_2 cylinder (Figure 15).

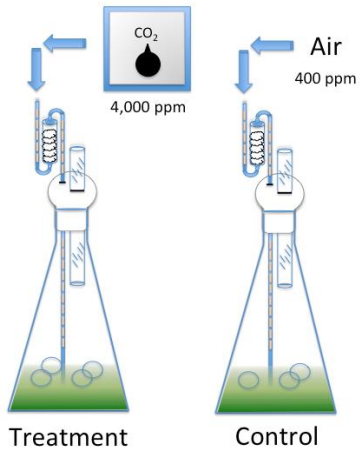


Figure 15. Representative scheme of the experimental condition. For each one of the 2 strains four replicates of *C. raciborskii* was exposed to CO_2 400 (current atmosphere) ppm and 4,000 ppm (Paleocenic´s CO_2 estimated concentration).

In order to evaluate the physiological performance of the two strains of *C. raciborskii* under stress condition, cell density, chlorophyll concentration and photosynthetic yield of these corresponding cultures were measured in intervals of 24 h, since the beginning of the experiment, while parameters such as pH and dissolved oxygen (D.O.) were measured in intervals of 12 h.

The cell counts were performed on a Fuchs-Rosenthal hemocytometer using an optical microscope (Olympus Trinocular Microscope BX series). But since *C. raciborskii* is a filamentous species, it was carried out a measurement of at least 30 random cells in order to establish the average cell size necessary for density counts of these populations. Counts were estimated for each strain by measuring filaments total length and dividing the value by the mean cell length obtained. Finally, the correction formule for Fuchs-Rosenthal chamber was applyed to calculate cell concentrations (cell/mL).

Chlorophyll concentration (ug/cell) and photosynthetic efficiency (yield/Chlorophyl/Cell) were obtained in a Phytoplankton Analyzer (Walz PHYTO-PAM), pH was measured with a Digimed´s portable meter and concentration of dissolved oxygen was determined with a Ysi Pro oxymeter.

4.2.2. Comparative Transcriptomes

To obtain RNA for gene expression comparisons, one toxic (CYRF) and one non-toxic (CYLP) strain were assessed under the same experimental conditions described in item *4.2.1* (volume, light, aeration and CO_2 concentration). Volumes of 500 mL were sampled at the end of 72 h of CO_2 exposure and RNA extraction was proceded at maximum time of 30 minutes under constant refrigeration to assure nucleic acids integrity. Three replicates for control and treatment for each strain were performed.

4.2.3. RNA extraction

Immediately after sampled, cultures were centrifuged in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments) for 10 minutes, under 7,500 rpm, at 4ºC. Supernatant was then carefully separated and pellet was immediately frozen in a Shell Freezer (Labconco) during 5 min. After that, frozen material was lyophilized (Labconco) until completely dried, in an approximate period of 24 h. For total RNA extraction, 3 mL of Trizol (Trizol, Ambion) was added and extraction protocol was performed according to manufacturer's instructions. Nucleic acid was quantified using RNA Broad Range (BR) Assay kit (Invitrogen) in a Qubit® fluorometer (Invitrogen). In order to eliminate DNA, 5 µg of RNA was treated with TURBO DNA-free™ kit (Ambion) and quantified again.

Ribosomal RNA depletion was realized using Ribo-Zero™ rRNA Removal kit (Epicentre). Aliquots of total and depleted RNA were analyzed using RNA pico kit (Agilent) by microfluidic electrophoresis in Bioanalyzer (Agilent).

4.2.4. Library sequencing preparation

Ion Total RNA-seq Kit v2 protocol was used to prepare libraries for massively parallel sequencing. Samples were linked to barcodes (RNA-Seq Barcode 1-16 kit, Thermo Fisher) to allow simultaneously sequencing and posteriorly differentiation. Libraries size was verified with microfluidic electrophoresis in Bioanalyzer (Agilent).

4.2.5. Clonal amplification and enrichment

Libraries clonal amplification by emulsion PCR was performed with Ion One Touch 200 Template kit V2, using the Ion OneTouch2 equipment (Thermo Fisher). Ion Sphere Particles (ISPs) amplification was quantitatively evaluated using Ion Sphere Quality Control kit in a Qubit® 2.0 fluorometer (Invitrogen) and subsequentely enriched in Ion OneTouch ES equipment (Thermo Fisher).

4.2.6. Massively parallel sequencing

All samples (controls and treatments) from both strains (CYRF and CYLP) were added to a Ion PI™ V3 semiconductor chip (100 gigabases of capacity). Sequencing was performed using Ion PI Hi-Q Sequencing 200 kit in a high potency Ion Proton™ sequencer (Thermo Fisher).

4.2.7. Transcriptomic data analysis (Bioinformatics)

Data generated was analyzed using CLC Genomics v.8.5 software (Qiagen). Reads were exported from Ion Server (Thermo Fisher) in FASTA format and imported to CLC.

4.2.8. Quality control of reads and mapping on reference genome

Quality control was initially applied by removing low quality and size (< 25 bp) sequences. Reads were mapped against the *C. raciborskii* reference genome CS-505, available in public databases.

4.2.9. Sample normalization

Comparison between gene expression values for the same sample was done through normalization by reads per kilobase per million of total reads (RPKM) (Bolstad *et al*., 2003; Allison *et al*., 2006; Mortazavi *et al*., 2008). This normalization corrects by coding region size of the gene and allows the comparison between genes with different sizes, since longer mRNA originates a greater number of reads. RPKM also corrects for differences in total reads obtained from different samples, since maintain in the same proportion the number of reads for each transcript.

4.2.10. Differencial gene expression

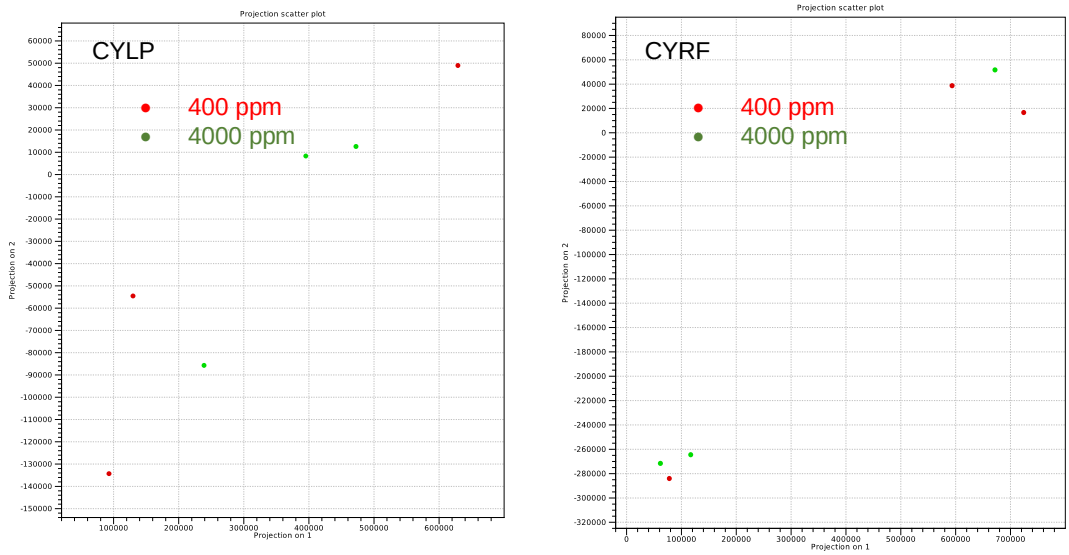
To allow a reliable comparative analysis between samples, data was transformed to logarithms through Baggerly´s t test (Baggerly *et al*., 2003).

To identify genes in which transcripts varied with statistically significance, empirical analysis of differential gene expression (EDGE) was used since it was developed to analyze a lot of transcripts in different experimental conditions (Robinson & Smyth, 2008).

Differential gene expressions with fold changes lower or higher than 1.5 and with *p* value < 0.05 were considered statistically significant and consequently used. Results were organized in cellular functional categories according to the function of the gene expressed.

4.3. Results

A 96% fill of Ion Proton chip was obtained with 8.43 G total bases and 91,705,719 total reads from which 64% were usable (Figure 16). The read lenght had an average value of 92 bp. Figure below shows component analysis of experimental replicates of CYRF and CYLP.



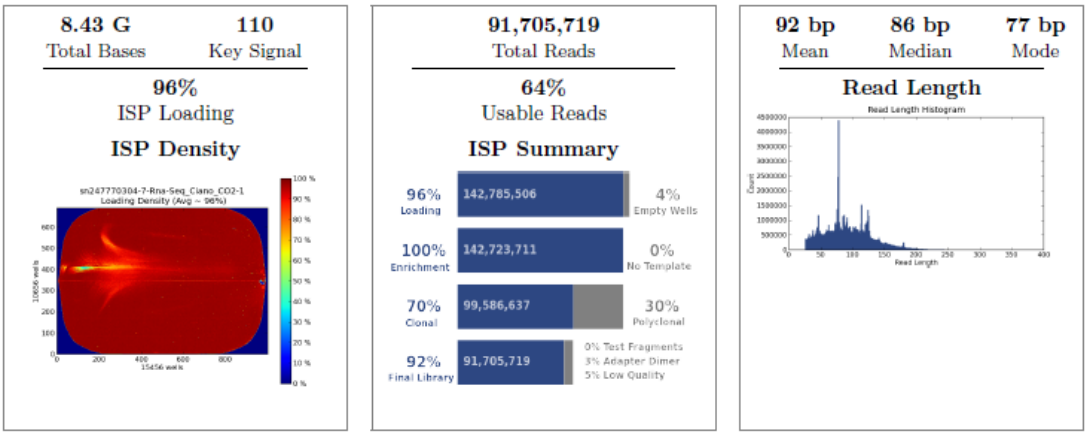


Figure 16. Component analysis of experimental replicates used in transcriptomes. Red spots repesent CO_2 400 ppm and green sposts CO_2 4000 ppm. Ion Proton sequence run log. Chip fill containing 3 control replicates (CO_2 400 ppm) and 3 treatment replicates (CO_2 4,000 pppm) within 72 h, indicating quantity, quality and reads length.

Transcriptomic responses varied greatly between the studied strains and EDGE-*test* detected statistically signficant diferences (Tables 3 and 4) between experimental conditions in each strain (CO_2 at 400 and 4,000 ppm). In short, different strains of the same species (toxic CYRF and non-toxic CYLP) responded differently to high CO_2 condition (Figures 17 and 18).

The number of downregulated and upregulated genes in each studied strain was near 50% in both lineages. Similarities in response between strains occurred only in a similar nucleoside-diphosphate-sugar epimerase (Feature ID: CRC_00684) and a bicarbonate transporter/bicarbonate binding protein (Feature ID: CRC_00146) (Tables 3 and 4). Statistically different gene expression of CYRF summed 28 and CYLP summed 181 sequences, from a total of about 3,500 when exposed to 4,000 ppm levels of CO_2.

Functional groups were determined using online genetic libraries (NCBI, KEGG, BRENDA, ENZYME, UNIPROT) and when expectations were not joined, a research in the literature was performed.

4.3.1. CYRF gene expression under high CO_2

In CYRF, response of genes inolved in general cell activity like methilation, hidrolization, oxidation had the higher fold-change values of downregulation (-26.31) and the highest upregulation value found (9.17) was of enzymatic activity of Alpha-ketoglutarate-dependent taurine dioxygenase, responsible for the utilization of taurine (2-aminoethanesulfonic acid) as an alternative sulfur source (UNIPROT). The cell division inhibitior (CRC_00655) had a fold-change of -10.02, in fact, cells grew constantly during all the experiment. Ribosome activity was downregulated as well and it is represented in the form of one acetyltransferase and one ribonuclease (fold-change: -7.22 and 3.47 respectively). The photosynthetic activity suffered both a downregulation and upregulation (fold-change: -5.88 and 3.40/4.07 respectively). Negative response was correlated to a ferredoxin, an electron transporter, and positive regulation was correlated to NADH and piruvate activity. This second related to the dark phase of photosynthesis. Membrane transporters were both up and down regulated in transporter genes with negative fold-changes of -5.66 and -3.26 compared to the positive found (7.79 and 2.88).

Oligopeptide transporter (permease) was downregulated under high CO_2 (fold-change: -3.82), while a gene regulator of protein synthesis (Parvulin-like peptidyl-prolyl-isomerase) was upregulated (fold-change: 4.60). Downregulation of a CRISPR associated gene was detected as well. Other downregulated functional groups were correlated with RNA activity (fold-change: -3.01), membrane receptor (fold-change: -3.81), plasmid regulation (fold-change: -5.70) and Zn transport (fold-change: -8.88).

General upregulated expression referred to epimerase activity (fold-change: 2.82), GTP translational fator (fold-change: 3.38), RNA activity (fold-change: 3.91) and alcoholic fermentation (fold-change: 2.86). Upregulation of carbon concentrating mechanisms (CCMs), specific for bicarbonate, was observed with fold changes equal to 3.36 and 5.02. Average pH of the ASM-1 medium was around 8.0, a value found

in cyanobacteria habitats (Souza and Truzzi, 1998). Once *C. raciborskii* was described to better capture CO_2 from bicarbonate form (Holland *et al*., 2012), expression of such genes should be expected.

The upregulation of a isopentenyl-diphosphate delta-isomerase gene related to Mevalonate pathway (fold-change: 3.54), the main terpenoidic synthesis route, adresses atention to possible secondary metabolite production under high CO_2 concentrations.

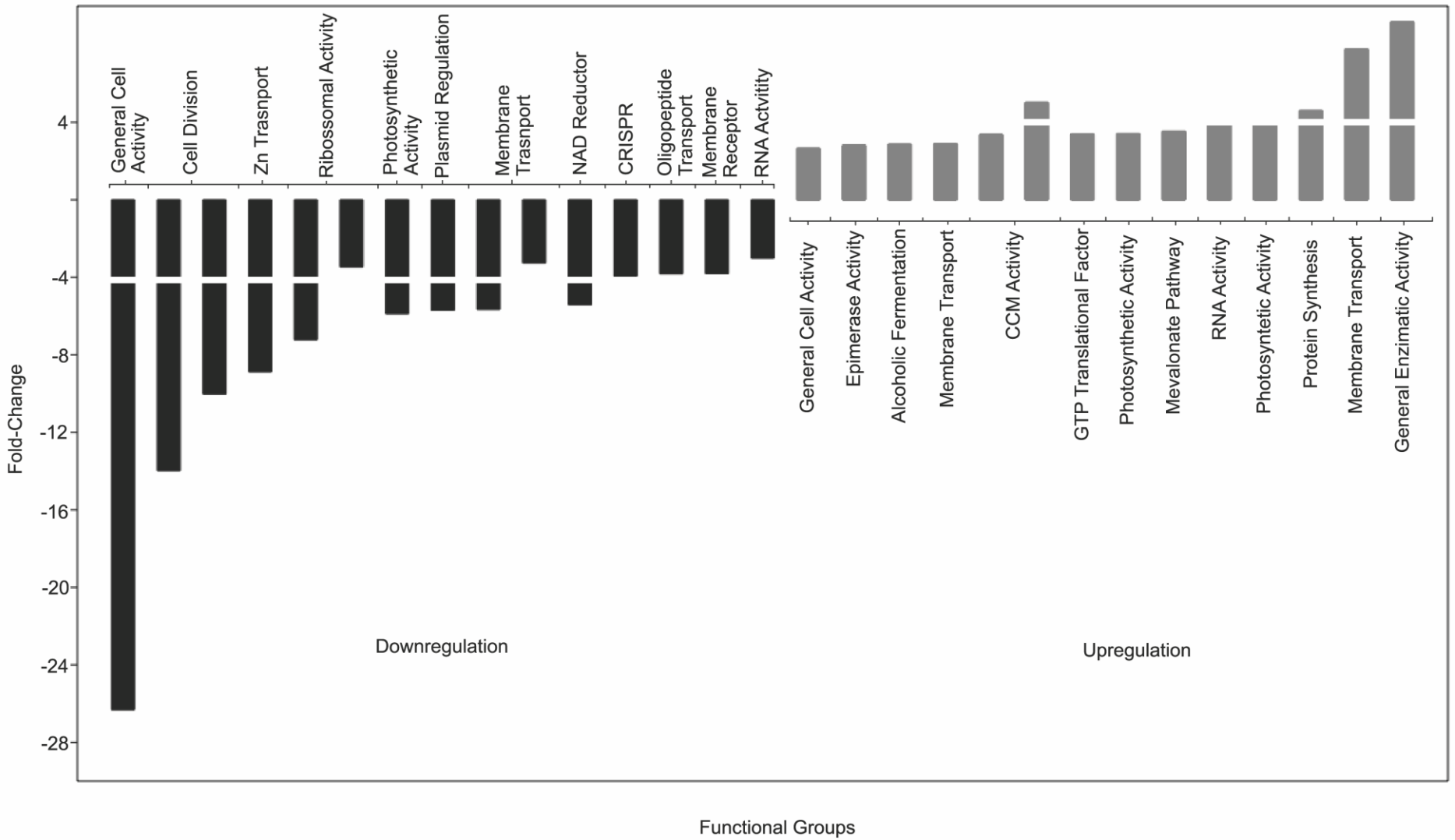


Figure 17. Gene regulation of CYRF strain according to EDGE-*test* statistics based on fold-changes (down and upregulation) and functional groups. Each bar represent fold-change of a single regulated gene. White horizontal lines represent an arbitrary threshold of the highest fold-changes found.

4.3.2. CYLP gene expression under high CO_2

CYLP strain had a higher statistically significant gene regulation in face of high CO_2 concentration. Expressive downregulation occurred in functional groups involved in pigment synthesis, with fold-changes ranging from -2.06 to -3.7, all phycocyanin and allophycocyanin subunits belonging to ***cpc*** and ***apc*** genes. Another representative downregulation occurred in a group of 22 genes responsible for the photosynthetic activity, the ***psb*** and ***psa*** genes that regulates photosystems I and II. Fold changes ranged from -1.53 to -2.29. Also, genes related to glucose synthesis in dark phase of photosynthesis and heterocyst formation ***patA*** were downregulated togheter with a NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase. In the sequence of negative regulation, a group of 14 enzymes of general function among nucleotidases, transferases, phosphatases, reductases and synthases had fold-changes, 1.51 -2.73. Together with this group, it was found a thioredoxin regulator (***trxA*)**, present in almost all life forms and important antioxidants (Navarro *et al*., 2000). In the same way, heme oxygenase, a gene of oxidative stress response had a fold change equal to -2.35. Ribosomal activity was supressed as well in a group of 7 genes including the ***rpl21*** that regulates the ribosomal protein L21.

The up and downregulation was observed for a set of genes from a variety of functional groups. The group encompassing general enzymes had 18 genes upregulated with fold-changes ranging between 1.52 and 19.76, all of them encoding nitrogenases, phosphoesterases, hydrogenases, oxidases, methyltransferases, dehydrogenases, epimerases, desulfurases, transferases and decarboxylases. The higher fold-change found in this functional group belonged to a Butyryl-CoA dehydrogenase enconding gene. The protons pump group had a set of 7 ATP synthase genes (***atp1, atpA, atpC, atpD, atpH*** and another two without annotation) negatively regulated and a potassium-transporting ATPase C chain positively regulated. Fold changes were -1.63 to -2.09 and 1.83, respectively The activity of CCMs had 3 factors downregulated while a bicarbonate binding protein has been

expressed in the high CO_2 treatment. Photosynthetic activity was upregulated through the expression of 6 genes with one of them identified as ***coxB***, a cytochrome C oxidase. Fold-changes ranged from 1.54 to 3.67. Increased expression of nutrient absortion through ***nifB***, ***nifH***, ***nifE*** and ***hesA*** nitrogen (N) fixation and ABC transporters plus cobalt (Co) and molybdenium (Mo) regulators was detected in contrast to supression of magnesium (Mg) absorption regulation. Three cell division related genes including ***ftsW*** were downregulated while three cell division proteins (FtsX, Ftn6 and FtsH) were upregulated. Membrane activity represented by membrane transport, membrane transporters and membrane proteins suffered intense up and downregulation enconding a variety of genes. A fatty acid desaturase related to plasmatic membrane maintanance presented a high fold-change of 17.88.

Functional groups of genes exclusively upregulated, include a CRISPR associated gene, similar to the one found in CYRF strain results. A heat shock proteins (HSP) class I was positively expressed as well, even with cultures under a controlled temperature of 24 ^{o}C. Omega 3 ***pfaA*** and ***pfaB*** subunits important for membrane structure had average fold-changes of 2.00. Another membrane related positive regulation was of one possible porine. An iron-sulfur regulation was expressed by SufB, SufC, SufD and SufR. Finally a permease of the drug/metabolite transporter (DMT) superfamily, which is adressed to metabolite transport was upregulated in levels of 4,000 ppm of CO_2.

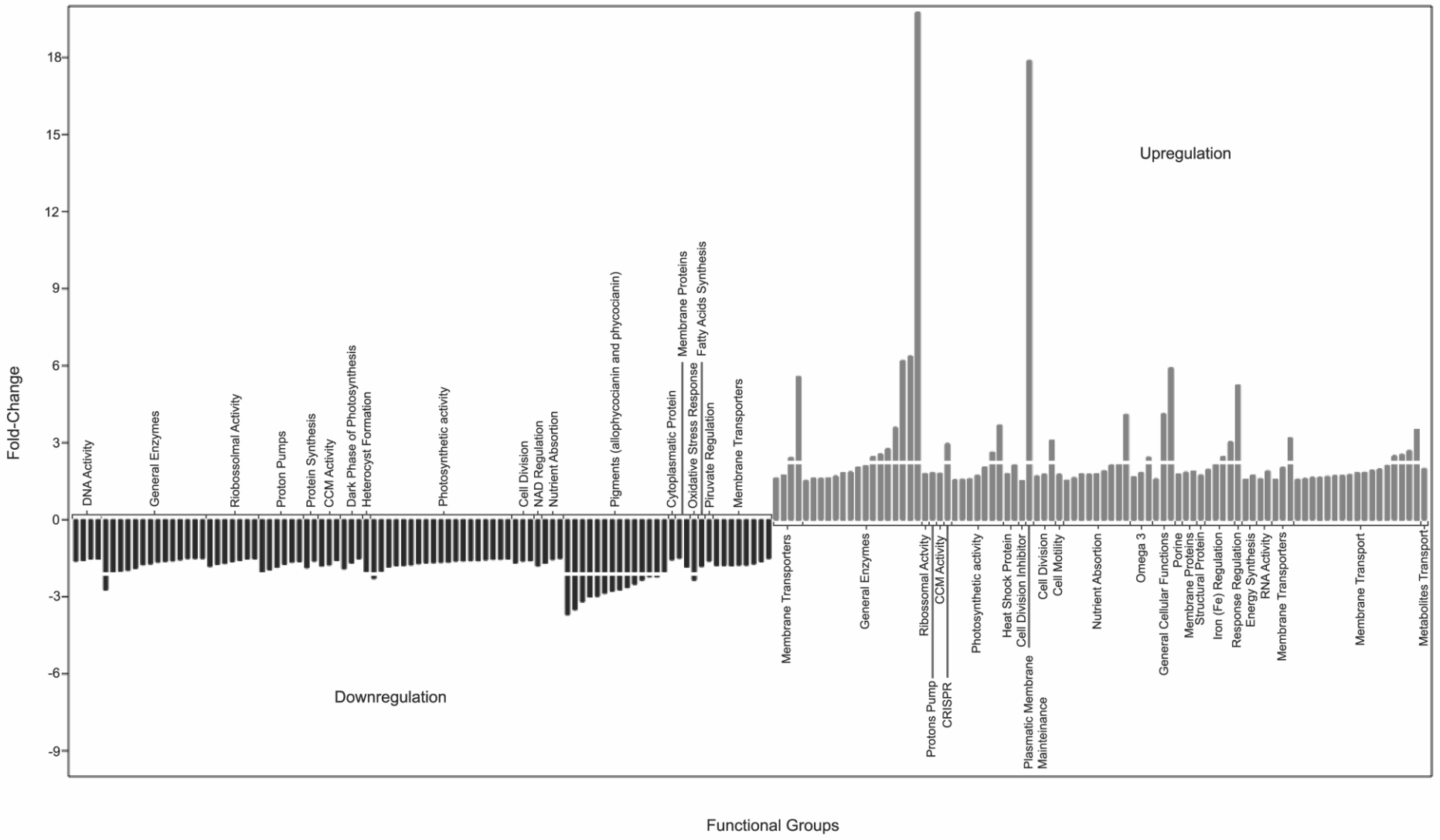


Figure 18. Gene regulation of CYLP strain according to EDGE-*test* statistics based on fold-changes (down and upregulation) and functional groups. Each bar represent fold-change of a single regulated gene. White horizontal lines represent an arbitrary threshold of the highest fold-changes found.

4.4. Discussion

The cell density of cultures continued to increase during the days of experiment and no difference between cultures under different conditions was detected as well as the photosynthetic activity. The option for a short duration experiment was made to test the cell mechanisms ready to respond a gas alteration acquired along evolution of these organisms. Cyanobacteria have faced similar greenhouse gas concentrations during the paleocene (65.5 million years ago) (Pearson and Palmer, 2000) and selective or not, such environmental force was presente during the survivorship of *C. raciborskii* in evolutionary history. Nevertheless, differential RNA expression was observed between cultures under CO_2 at 400 ppm and 4,000 ppm, as well as between two different strains of a same species. Tough the two different ecotypes (toxic and non-toxic strain) presented differential gene responses that were grouped in similar functional groups - CCMs, cell division, ribosomal and photosynthetic activity, membrane transport and receptors, CRISPR, RNA activity, protein synthesis and general enzymes activity. This organization was an attempt to better try to understand results found in an ecophysiological perspective. The results can then represent a convergent response mechanism of two different ecological types reacting to the same gas change. Both strains expressed different amounts and classes of genes but both kept growing during the exposure to the 4,000 ppm CO_2 concentration. On the other hand, a study with toxic and non-toxic strains of *Microcystis aeruginosa* demonstrated that different lineages may have differential responses to changes in CO_2 levels (Van de Wall *et al*., 2011). However, this study analyzed the behavior of strains when co-habiting the same culture and shifts were observed where the toxic strain got benefited from low CO_2 concentration and non-toxic dominated in a high CO_2 level.

The most studied metabolites of *C. raciborskii* are the cylindrospermopsin and saxitoxins and much less known for this species, the cyanopeptides (Pearson *et al*., 2010; 2016; Silva-Stenico *et al*., 2011; 2015; Sanz *et al*., 2015). A previous study

with cylindrospermopsin, another toxin produced by *C. raciborskii*, monitored ***cyrA*** and ***cyrK*** genes expression under high CO_2 and no alterations in genes nor toxins were observed (Pierangelini *et al*., 2015). Here, none of the ***sxt*** gene family was and PKS regulation factors responsible by toxin and cyanopeptides synthesis were statistically significant during regulation under high CO_2. Nevertheless, evidences of upregulation of metabolites activity was detected in both strains. For CYRF was the detection of a isopentenyl-diphosphate delta-isomerase present in the most important terpenoidic route, the Mevalonate pathway and for CYLP was the detection of a permease belonging to DMT transporters superfamily, involved with metabolites and drug tranport. Some of the terpenes described in cyanobacteria are geosmin (*Nostoc punctiforme*), 2-methylisoborneol (*Pseudoanabaena* sp.), ambiguine (*Fischerella ambigua*) and welwitindolinone (*Hapalosiphon welwitschii* and *Westiella intricata*) (Dittman *et al*., 2015). Majority of terpenes known were isolated from terrestrial and marine plants, liveworths and funghi, but Yamada and co-authors (2015) evidenced the existence of a variety of terpene synthases producing mostly odoriferous terpenes in bacteria of the genus *Streptomices*. Results found may motivate more detailed investigations on freshwater cyanobacteria metabolic profiles with ecological and bioprospection interests. In marine habitats terpenes are evolutionary Keys to a great diversity of organisms (McClintok *et al*., 2001).

Focusing in the expected response to carbonic gas increase the CCM regulation was expected once they are the main mechanisms of carbon assimilation (Badger and Price, 2003). The significant upregulation of CCM genes in CYRF strain when submitted to high CO_2 concentration raise the question of why those cells activated such mechanism when the availability of the gas is great. The CYLP strain indeed had a downregulation of genes from this functional group. A recent research (Sandrini *et al*., 2016) demonstrated that transcriptional regulators of ***ccmR*** and ***ccmR2*** increased expression until the beggining of night (19 h) and are downregulated during the night in current CO_2 atmosphere. In the present work, RNA

extractions here were performed during the light period of the day (about 10:00 am) and cultures received a 10 x greater CO_2 concentration. Despite ***ccmR*** genes not being monitore nor observed in the presente study not allowing direct comparisons, in conditions studied

Intense regulation of genes involved in photosynthetic activity regulation in CYLP was observed but was not physiologically detected. The high up and downregulation of genes could suggest great activity of photosyntheic apparatus in this strain. However only the light phase was monitored with no alteration in the highest CO_2 condition leading to questions about how the dark phase behaves in such conditions. Once analyzed RNA corresponded to the end of experiment it was not possible to identify the moment those genes started to transcribed. However, RNA from 0 and 24 hours intervals were extracted for future sequencing and analysis.

In a transcriptomic study with *Phrocloroccocus* strains, an iron stress was caused and after 48 and 53 hours of experiment each for a specific strain and in both cases transcriptomic responses were observed in less than 24 h (Thompson *et al*., 2011). The results observed in our study also corroborate with the ones found by Pierangelini *et al*. (2014), that analyzed alterations in photosynthetic apparatus caused by elevated CO_2 concentrations (1,300 ppm). In contrast, this study assumed that the dark phase of photosynthesis was not affected. However, in our results genes of Calvin-cycle were downregulated.

Probably asssociated to photosynthetic activity but not restricted to it was the upregulation under high CO_2 of iron-sulfur clusters (Suf) involved with electron transport, cytochromes and ferredoxin. In a *Synechocystis* strain ***suf*** operon was overexpressed under oxidative stress conditions (Wang *et al*., 2004), which was in fact proposed to play a role in photosyntesis homeostasis and redox stress in cyanobacteria (Balasubramanian *et al*., 2006).

Genes involved in plasmatic membrane regulation demonstrated high activity as well and like photosynthesis an intense up and downregulation was detected. In addition, intense membrane activity should represent intense exchange with the extracellular medium. Specially because genes involved regulates transporters, proteins, structure, signaling and protection. Under elevated CO_2 regulation of membrane modifications increasing important structural elements as heat shock proteins, nutrient transporters (ABC family), omega 3 polyunsaturated fatty acids, low molecular weight membrane proteins and porines was started.

The idea of ecotypes was discussed in a review by Agha and Quesada (2014) and what they propose is that the distinct qualitative oligopeptide composition of cyanobacteria molecules are suitable biomarkers of cyanobacterial subpopulations. Notwithstanding, great differences between transcriptomic responses of the two strains tested here were observed and in truth they are already known to be different ecotypes in relation to toxin production. What was seen is that instrinsic characteristics of different strains can be exposed by the transcriptomes. Fact oberved when CYLP and CYRF transcriptomes obtained are compared by the same CO_2 concentrations. That is an interesting observation since *C. raciborskii's* strains seems to share great similarity differing only between some secondary metabolites genes as ***cyr*** cluster (Sinha *et al*., 2014). Other previous studies already demonstrated this differential response in cyanobacterial strains in species of *Microcystis aeruginosa* and *Prochloroccocus* (Thompson *et al*., 2011; Van de Waal *et al*., 2011; *Voigt et al*., 2014).

The RNA extraction protocol used in this work made it possible to go ahead with sequencing procedures and obtain interesting results, however it doesn't mean that adjustments are not necessary. Analysis with RNA pico kit (Agilent) by microfluidic electrophoresis had a very satisfactory result and a 96% fill of the Ion Proton chip was obtained. However, the volume of culture necessary to obtain enough mRNA from an experiment in a cell density as closer as possible of a natural

HAB condition (10^6 cells / mL) was elevated. In addition, extraction procedures must be performed in an interval of maximum 30 minutes to avoid RNA total degradation (Selinger *et al*., 2003). Complementary *C. raciborskii* is very sensible to centrifugation and slow speeds for long periods must be used but even so, pellet formed was not consistent and some losses probably occurred during the removal of the supernatant. Though improvements in an attempt to reduce as much as it possible protocol intereferences from transcriptomic analysis of cyanobacteria are necessary. Nevertheless, this is the first report on the use of transcriptomic techniques using massively parallel sequencing to analyze a *C. raciborskii* environmental response and one of the few with cyanobacteria.

Despite the fact that extrapolations between toxic and non-toxic profiles cannot be done differences between the responses of the two strains cultivated under the same experimental conditions could be observed. The relevance of each gene overexpressed or not for the chemical and physiological processes controlled by them remains in doubt for this study. This paper expected to contribute with the knowledge about the ability of different ecotypes of a same species of cyanobacteria to respond to a new environmental condition which in this case was the CO_2 concentration.

4.5. Suplementrary Matherial

Table 3. Resume of CYRF strain transcriptome results after EDGE-*test* (under 4,000 ppm CO_2) organized by functional groups and ascendant fold-change.

Group	Anotation	Fold change	*p*-value	Feature ID
Cell Division	Cell division inhibitor	**-10,02**	0,01	CRC_00655
CRISPR	CRISPR-associated RAMP Cmr4	**-3,94**	0,04	CRC_01868
General Cell Activity	Undecaprenyl-phosphate galactosephosphotransferase (EC 2.7.8.6)	**-26,31**	0,000428	CRC_01094
	Glycolate dehydrogenase (EC 1.1.99.14); iron-sulfur subunit GlcF	**-13,98**	0,00321	CRC_03104
Membrane Receptor	Long-chain-fatty-acid-CoA ligase (EC 6.2.1.3)	**-3,81**	0,04	CRC_01133
Membrane Transport	NAD kinase (2.7.1.23)	**-5,66**	0,02	CRC_00275
	Ammonium transporter	**-3,26**	0,02	CRC_00109
NAD Reductor	NAD-reducing hydrogenase subunit HoxF (EC 1.12.1.2)	**-5,41**	0,02	CRC_01119
Oligopeptide Transport	Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	**-3,82**	0,04	CRC_02340
Photosynthetic Activity	Ferredoxin	**-5,88**	0,04	CRC_01763
Plasmid Regulation	Plasmid encoded restriction endonuclease Per	**-5,70**	0,04	CRC_00874
Ribosome Activity	Ribosomal-protein-S18p-alanine acetyltransferase (EC 2.3.1.-)	**-7,22**	0,01	CRC_01918
	Ribonuclease P protein component (EC 3.1.26.5)	**-3,47**	0,03	CRC_02850
RNA Activity	Transcriptional regulator, MecI family	**-3,01**	0,03	CRC_02663
Zn Trasnport	Zinc ABC transporter, periplasmic-binding protein ZnuA	**-8,88**	0,04	CRC_01523
Alcoholic Fermentation	Alcohol dehydrogenase (EC 1.1.1.1); acetaldehyde dehydrogenase (EC 1.2.1.10)	**2,86**	0,02	CRC_02984
CCM Activity	Bicarbonate transport system permease protein	**3,36**	0,04	CRC_00145
	Bicarbonate transporter, bicarbonate binding protein	**5,02**	0,01	CRC_00146
Enzymatic Activity	Alpha-ketoglutarate-dependent taurine dioxygenase (EC 1.14.11.17)	**9,17**	0,05	CRC_02394
Epimerase Activity	Similar to nucleoside-diphosphate-sugar epimerases	**2,82**	0,04	CRC_00685
General Cell Activity	D-amino-acid-oxidase (EC 1.4.3.3)	**2,65**	0,04	CRC_02512
GTP Translational Factor	GTPase probable translation factor	**3,38**	0,04	CRC_00201
Membrane Transport	Polyphosphate kinase (EC 2.7.4.1)	**2,88**	0,02	CRC_00233
	Sulfate and thiosulfate binding protein CysP	**7,79**	0,00155	CRC_02726
Mevalonate Pathway	Isopentenyl-diphosphate delta-isomerase, FMN-dependent (EC 5.3.3.2)	**3,54**	0,05	CRC_00876
Photosynthetic Activity	Enoyl-(acyl-carrier-protein) reductase (FMN) (EC 1.3.1.9), inferred for PFA pathway	**3,40**	0,04	CRC_02051
	Pyruvate decarboxylase (EC 4.1.1.1); alpha-keto-acid decarboxylase (EC 4.1.1.-)	**4,07**	0,01	CRC_02060
Protein Synthesis	Parvulin-like peptidyl-prolyl-isomerase	**4,60**	0,05	CRC_02567
RNA Activity	Transcriptional regulator, PadR family	**3,91**	0,05	CRC_02218

Table 4. Resume of CYLP strain transcriptome results after EDGE-*test* (under 4,000 ppm CO_2) organized by functional groups and ascendant fold-change.

Group	Anotation	Fold change	*p*-value	Feature ID
CCM Activity	Possible carbon dioxide concentrating mechanism protein CCM Activity K	**-1,8**	0,01	CRC_00971
	Carbon dioxide concentrating mechanism protein CCM ActivityO	**-1,76**	0,00108	CRC_00967
	Carboxysome protein CCM Activity N	**-1,58**	0,01	CRC_00968
Cell Division	Plasmid maintenance system antidote protein	**-1,68**	0,02	CRC_00508
	Cell division protein	**-1,61**	0,00826	ftsW
	Bacterial cell division membrane protein	**-1,59**	0,00561	CRC_02872
Dark Phase of Photosynthesis	Fructose-1,6-bisphosphatase, GlpX type (EC 3.1.3.11) / Sedoheptulose-1,7-bisphosphatase (EC 3.1.3.37)	**-1,91**	0,00389	CRC_00285
	Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	**-1,68**	0,02	CRC_01551
	Protein CP12, regulation of Calvin cycle via association/dissociation of PRK/CP12/GAPDH complex	**-1,52**	0,04	CRC_01224
DNA Activity	Adenine specific DNA methyltransferase	**-1,61**	0,02	CRC_02335
	Ribonuclease I precursor (EC 3.1.27.6)	**-1,58**	0,02	CRC_01635
	DNA-binding protein HU	**-1,53**	0,03	CRC_01160
	Translation elongation factor Ts	**-1,53**	0,03	CRC_00546
Energy Synthesis	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes	**-1,61**	0,03	CRC_02582
Fatty Acids Synthesis	Acyl carrier protein	**-1,82**	0,00269	CRC_03011
General Enzymes	5'-nucleotidase (EC 3.1.3.5)	**-2,73**	0,0000143	CRC_01418
	4'-phosphopantetheinyl transferase (EC 2.7.8.-)	**-2,08**	0,00731	CRC_01556
	Light-independent protochlorophyllide reductase iron-sulfur ATP-binding protein ChlL (EC 1.18.-.-)	**-2,01**	0,00248	CRC_02607
	Protein serine/threonine phosphatase(EC:3.1.3.16)	**-1,97**	0,00494	CRC_02187
	Uroporphyrinogen III decarboxylase (EC 4.1.1.37)	**-1,89**	0,00384	CRC_00624
	Cobalamin synthase	**-1,75**	0,00402	CRC_03169
	Glutamine synthetase inactivating factor IF7	**-1,72**	0,00333	CRC_02159
	Ketol-acid reductoisomerase (EC 1.1.1.86)	**-1,65**	0,01	CRC_02299
	Glyoxalase family protein	**-1,62**	0,04	CRC_03165
	Histidine triad (HIT) nucleotide-binding protein, cyanobacterial subgroup	**-1,61**	0,03	CRC_01053
	IMP dehydrogenase/GMP reductase	**-1,6**	0,00541	CRC_02461
	Alkyl hydroperoxide reductase subunit C-like protein	**-1,56**	0,02	CRC_00162
	Thioredoxin	**-1,51**	0,04	trxA
	Probable dioxygenase	**-1,51**	0,03	CRC_02271
	Methylenetetrahydrofolate dehydrogenase (NADP+) (EC 1.5.1.5) / Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)	**-1,51**	0,01	CRC_03264
Heterocyst Formation	Response regulator	**-2,03**	0,00309	patA
Membrane Receptor	Outer membrane vitamin B12 receptor BtuB	**-1,84**	0,00398	CRC_01303
Membrane Transport	MscS Mechanosensitive ion channel	**-1,72**	0,00713	CRC_02481
	Guanylate kinase (EC 2.7.4.8)	**-1,63**	0,00137	CRC_00264

	Zinc ABC transporter, periplasmic-binding protein ZnuA	**-1,51**	0,04	CRC_01523
Membrane Transporters	Phosphoribulokinase (EC 2.7.1.19)	**-1,79**	0,01	CRC_00573
	Peptidyl-prolyl cis-trans isomerase	**-1,78**	0,01	CRC_01238
	TRAP dicarboxylate transporter, DctQ subunit, unknown substrate 6	**-1,78**	0,03	CRC_02038
	Melibiose carrier protein	**-1,77**	0,04	CRC_00336
	Phosphoglycerate kinase (EC 2.7.2.3)	**-1,77**	0,00956	CRC_02799
NAD Regulation	NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13)	**-1,8**	0,00881	CRC_03494
Nutrient Absortion	Mg protoporphyrin IX monomethyl ester oxidative cyclase (aerobic) (EC 1.14.13.81)	**-1,69**	0,02	CRC_00914
	Cobalt-precorrin-8x methylmutase (EC 5.4.1.2)	**-1,55**	0,01	CRC_03195
	Cobalt-precorrin-2 C20-methyltransferase (EC 2.1.1.130)	**-1,51**	0,04	CRC_03194
Oxidative Stress Response	Heme oxygenase (EC 1.14.99.3)	**-2,35**	0,000000295	CRC_03007
Photosynthetic Activity	Photosystem II	**-2,29**	0,0000346	psbY
	Photosystem I subunit IV (PsaE)	**-2,01**	0,00171	CRC_00113
	Subunit of photosystem I	**-1,84**	0,00822	psaD
	Photosystem II manganese-stabilizing protein (PsbO)	**-1,79**	0,00571	CRC_03278
	subunit of photosystem I	**-1,78**	0,00179	psaX
	Cytochrome C553 (soluble cytochrome f)	**-1,76**	0,00774	CRC_01185
	Subunit of photosystem I	**-1,71**	0,03	psaJ
	Subunit of photosystem I	**-1,68**	0,02	psaL
	Photosystem II	**-1,67**	0,02	psbZ
	Cytochrome b6-f complex subunit, apocytochrome f	**-1,66**	0,03	CRC_02349
	Light-independent protochlorophyllide reductase subunit B (EC 1.18.-.-)	**-1,65**	0,01	CRC_02269
	Ribulose-1,5-bisphosphate carboxylase/oxygenase	**-1,61**	0,03	rbcS
	Photosystem II	**-1,6**	0,03	psbV
	Subunit of photosystem I	**-1,59**	0,04	psaF
	Photosystem II	**-1,59**	0,01	psbU
	Cytochrome b6-f complex iron-sulfur subunit PetC1 (Rieske iron sulfur protein EC 1.10.99.1)	**-1,56**	0,03	CRC_02348
	Ribulose-phosphate 3-epimerase (EC 5.1.3.1)	**-1,54**	0,03	CRC_01082
	Cytochrome b6-f complex subunit, cytochrome b6	**-1,54**	0,04	CRC_02197
	Glycogen synthase, ADP-glucose transglucosylase (EC 2.4.1.21)	**-1,53**	0,03	CRC_02999
Pigments	Phycocyanin	**-3,7**	0,0000199	cpcA
	Phycocyanin	**-3,5**	0,0000648	cpcB
	Phycocyanin	**-3,19**	0,0000545	cpcF
	Phycocyanin	**-3**	0,000113	cpcG1
	Phycocyanin	**-2,98**	0,000394	cpcC
	Phycocyanin	**-2,86**	0,0000282	cpcG4
	Phycocyanin	**-2,78**	0,000383	cpcD
	Phycocyanin	**-2,73**	0,000542	cpcE
	Allophycocyanin	**-2,64**	0,00076	apcB
	Phycocyanin	**-2,51**	0,00117	cpcG2_1

	Allophycocyanin	**-2,35**	0,0026	apcA
	Allophycocyanin	**-2,22**	0,00093	apcC
	Allophycocyanin	**-2,22**	0,00381	apcE
	Allophycocyanin beta chain	**-2,06**	0,00095	CRC_02161
Proteína citoplasmática	Putative cytoplasmic protein	**-1,56**	0,05	CRC_01637
Protons Pump	ATP synthase	**-2,09**	0,0023	atpH
	ATP synthase	**-1,95**	0,04	atp1
	Glucose-1-phosphate adenylyltransferase (EC 2.7.7.27)	**-1,87**	0,00834	CRC_00827
	ATP synthase	**-1,85**	0,00664	atpA
	ATP synthase	**-1,74**	0,02	atpD
	ATP synthase	**-1,65**	0,04	atpC
	ATP synthase beta chain (EC 3.6.3.14)	**-1,63**	0,03	CRC_01155
Ptotein Membrane	YbbM seven transmembrane helix protein	**-1,5**	0,04	CRC_02465
Ribosome Activity	Teichoic acid export ATP-binding protein TagH (EC 3.6.3.40)	**-1,74**	0,02	CRC_03035
	LSU ribosomal protein L31p	**-1,7**	0,00817	CRC_01376
	SSU ribosomal protein S18p @ SSU ribosomal protein S18p, zinc-independent	**-1,63**	0,02	CRC_03038
	Ribosomal subunit interface protein	**-1,58**	0,00317	CRC_00122
	SSU ribosomal protein S10p (S20e)	**-1,53**	0,01	CRC_00261
	Encoding the plastid r-protein L21	**-1,52**	0,04	rpl21
	LSU ribosomal protein L20p	**-1,82**	0,0021	CRC_02517
CCM Activity	Bicarbonate transporter, bicarbonate binding protein	**1,79**	0,04	CRC_00146
Cell Division	Cell division protein FtsX	**1,69**	0,01	CRC_02559
	Cell division protein Ftn6	**1,77**	0,03	CRC_02893
	Cell division protein FtsH (EC 3.4.24.-)	**3,08**	0,01	CRC_02692
Cell Division Inhibitor	Septum site-determining protein MinC	**1,51**	0,00498	CRC_03004
Cell Motility	Type IV pilin PilA	**1,76**	0,04	CRC_03415
CRISPR	CRISPR-associated protein Csc1	**2,95**	0,02	CRC_01441
DNA Activity	Amino-terminal intein-mediated trans-splice / DNA polymerase III alpha subunit (EC 2.7.7.7)	**1,62**	0,01	CRC_00420
	DNA gyrase subunit A (EC 5.99.1.3)	**1,72**	0,04	CRC_00487
	Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase) (EC 3.6.1.-)	**2,41**	0,000000129	CRC_01190
	Type I restriction-modification system, specificity subunit S (EC 3.1.21.3)	**5,56**	0,03	CRC_03302
Energy Synthesis	Pyruvate formate-lyase activating enzyme (EC 1.97.1.4)	**1,56**	0,04	CRC_02985
	Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), ADP-binding subunit DhaL	**1,72**	0,01	CRC_03063
General Enzymes	Mo-dependent nitrogenase-like protein	**1,52**	0,03	CRC_01814
	Putative pathogenesis related protein	**1,58**	0,00279	CRC_03259
	PHP family metal-dependent phosphoesterase	**1,6**	0,00663	CRC_02273
	Uptake hydrogenase large subunit (EC 1.12.99.6)	**1,61**	0,05	CRC_02737
	Sulfite oxidase and related enzymes	**1,62**	0,00637	CRC_02115
	Cobalamin biosynthesis protein CbiG / Cobalt-precorrin-3b	**1,69**	0,0063	CRC_0181

	C17-methyltransferase		6	5
	Uptake hydrogenase small subunit precursor (EC 1.12.99.6)	**1,82**	0,0098 1	CRC_0273 6
	Pentapeptide repeat family protein	**1,85**	0,01	CRC_0298 6
	Homoserine dehydrogenase (EC 1.1.1.3)	**2,03**	0,0015 3	CRC_0261 5
	Similar to nucleoside-diphosphate-sugar epimerases	**2,09**	0,03	CRC_0068 4
	Cysteine desulfurase (EC 2.8.1.7), SufS subfamily	**2,44**	0,0000 603	CRC_0180 7
	Cyanophycinase (EC 3.4.15.6)	**2,54**	0,0004 3	CRC_0198 7
	Glycosyl transferase, family 2	**2,75**	0,02	CRC_0204 1
	Putative serine protease	**3,58**	0,0079 5	CRC_0269 3
	PhnG protein	**4,11**	0,0093 8	CRC_0199 8
	Mobile element protein	**5,91**	0,02	CRC_0324 8
	Probable benzoylformate decarboxylase (EC 4.1.1.7)	**6,19**	0,0000 247	CRC_0046 8
	Arginine decarboxylase (EC 4.1.1.19) / Lysine decarboxylase (EC 4.1.1.18)	**6,36**	0,03	CRC_0140 7
	Butyryl-CoA dehydrogenase (EC 1.3.99.2)	**19,76**	0,02	CRC_0239 8
Heat shock protein	Molecular chaperone (small heat shock protein)	**1,78**	0,0035 6	CRC_0007 3
	Heat shock protein, class I	**2,13**	0,0000 204	CRC_0091 6
Iron (Fe) Regulation	Iron-sulfur cluster regulator SufR	**1,95**	0,0006 08	CRC_0180 3
	Iron-sulfur cluster assembly protein SufD	**2,16**	0,0000 0437	CRC_0180 6
	Iron-sulfur cluster assembly protein SufB	**2,44**	0,0000 221	CRC_0180 4
	Iron-sulfur cluster assembly ATPase protein SufC	**3,01**	0,0000 303	CRC_0180 5
Membrane Transport	DevC protein	**1,56**	0,05	CRC_0110 7
	Phosphate transport system regulatory protein PhoU	**1,57**	0,05	CRC_0080 1
	Serine/threonine kinase	**1,59**	0,0083 3	CRC_0277 9
	YbhB and YbcL - Raf kinase inhibitor-like protein	**1,64**	0,02	CRC_0180 1
	Urea carboxylase-related ABC transporter, periplasmic substrate-binding protein	**1,64**	0,02	CRC_0209 1
	Urea carboxylase-related ABC transporter, ATPase protein	**1,68**	0,0089 6	CRC_0208 9
	Serine/threonine kinase with WD-40 repeat	**1,7**	0,03	CRC_0306 7
	Molybdenum transport system permease protein ModB (TC 3.A.1.8.1) / Molybdenum transport ATP-binding protein ModC (TC 3.A.1.8.1)	**1,72**	0,0042	CRC_0211 1
	heterocyst specific ABC-transporter, membrane fusion protein DevB homolog	**1,74**	0,0049 7	CRC_0043 8
	ABC-type bacteriocin/lantibiotic exporter, contains an N-terminal double-glycine peptidase domain	**1,82**	0,04	CRC_0000 6
	putative membrane protein	**1,83**	0,04	CRC_0028 1
	Phosphonate ABC transporter ATP-binding protein (TC 3.A.1.9.1)	**1,91**	0,0024 3	CRC_0188 3
	Urea carboxylase-related ABC transporter, permease protein	**1,96**	0,04	CRC_0209 0
	Anhydro-N-acetylmuramic acid kinase (EC 2.7.1.-)	**2,03**	0,0017 9	CRC_0218 5
	ABC-transporter DevC-like protein	**2,1**	0,01	CRC_0043 7
	Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	**2,48**	0,04	CRC_0301 8
	Toxin secretion ABC transporter ATP-binding protein	**2,53**	0,0001 2	CRC_0256 6
	Cobalt-zinc-cadmium resistance protein CzcA; Cation	**2,68**	0,04	CRC_0001

	efflux system protein CusA			9
	Putative multicomponent Na+:H+ antiporter subunit B	**3,17**	0,00291	CRC_02306
	Phospholipid-lipopolysaccharide ABC transporter	**3,5**	0,00246	CRC_01570
Metabolites Transport	Permeases of the drug/metabolite transporter (DMT) superfamily	**1,99**	0,0027 8	CRC_03185
Nutrient Absortion	Cobalt-precorrin-6y C15-methyltransferase [decarboxylating] (EC 2.1.1.-)	**1,52**	0,05	CRC_03471
	Nitrogen fixation	**1,63**	0,01	nifB
	Nitrogen fixation	**1,77**	0,00406	nifH
	Nitrate ABC transporter, ATP-binding protein / Nitrate ABC transporter, nitrate-binding protein	**1,77**	0,02	CRC_00144
	Zn-ribbon-containing, possibly RNA-binding protein and truncated derivatives	**1,77**	0,01	CRC_00674
	Nitrate ABC transporter, ATP-binding protein	**1,89**	0,0086	CRC_00143
	Nitrogen fixation	**2,12**	0,01	nifE
	Nitrogen fixation	**2,24**	0,05	hesA
	Molybdenum cofactor biosynthesis protein MoaC	**4,09**	0,00186	CRC_00386
Omega3	Omega-3 polyunsaturated fatty acid synthase subunit, PfaA	**1,66**	0,00344	CRC_03021
	Omega-3 polyunsaturated fatty acid synthase subunit, PfaA	**1,83**	0,00189	CRC_02047
	Omega-3 polyunsaturated fatty acid synthase subunit, PfaB	**2,42**	0,02	CRC_02049
Photosynthetic Activity	Heme A synthase, cytochrome oxidase biogenesis protein Cox15-CtaA	**1,54**	0,02	CRC_00761
	Cytochrome c oxidase	**1,57**	0,02	coxB
	Diflavin flavoprotein Sll0550	**1,58**	0,04	CRC_01002
	Cytochrome c oxidase polypeptide I (EC 1.9.3.1)	**1,71**	0,04	CRC_03084
	CAB/ELIP/HLIP superfamily of protein	**2,03**	0,01	CRC_02565
	Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	**2,62**	0,00257	CRC_03085
	Photosystem II protein D1 (PsbA)	**3,67**	0,000152	CRC_01419
Plasmatic Membrane Maintainance	Fatty acid desaturase	**17,88**	0,04	CRC_00606
Porine	Possible porin	**1,76**	0,000714	CRC_00456
Protons Pump	Potassium-transporting ATPase C chain (EC 3.6.3.12) (TC 3.A.3.7.1)	**1,83**	0,00481	CRC_02184
Membrane Protein	Deoxyribose-phosphate aldolase (EC 4.1.2.4)	**1,84**	0,00133	CRC_01061
	27kDa outer membrane protein	**1,88**	0,02	CRC_00958
Regulação de resposta	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain	**5,23**	0,03	CRC_00495
Ribosome Activity	HlyD family secretion protein	**1,78**	0,00386	CRC_02560
RNA Activity	Cyanobacteria-specific RpoD-like sigma factor, type-1	**1,58**	0,00621	CRC_02179
	Cyanobacteria-specific RpoD-like sigma factor, type-2 @ Group 2 RNA polymerase sigma factor	**1,89**	0,000391	CRC_01188
Structural Protein	Conserved Membrane Protein	**1,73**	0,04	CRC_00533

5. Discussão geral

Os experimentos realizados no presente estudo tiveram como objetivo principal avaliar como cianobactérias respondem a mudança na concentração do gás carbônico para condições antes encontradas por seus ancestrais. Tanto sob a forma de respostas moleculares, quanto fisiológicas. O primeiro tipo de resposta representaria a primeira etapa de respostas envolvendo a expressão de genes regulatórios e produção de metabólitos que auxiliariam no processo adaptativo. Já o segundo tipo de resposta representaria o sucesso deste ajuste expresso em crescimento e atividade fotossintética, ou forneceria informação sobre a existência de algum estresse às linhagens estudadas. Esta sequência então poderia representar a existência de um arcabouço adaptativo pré-existente pronto para ser utilizado quando estas cianobactérias fossem expostas a uma condição ambiental conhecida. Embora já tenham se passado milhares de anos desde o Paleoceno, quando as concentrações de CO_2 chegaram a 4.000 ppm e um longo processo de seleção natural venha ocorrendo desde então, o último registro científico de elevada quantidade de gás carbônico na atmosfera remete a este período. A possível ocorrência de cianobactérias em períodos com maiores concentrações deste gás apenas demonstraria o quão bem adaptados estão estes organismos às variações na composição da atmosfera terrestre.

A teoria vigente sobre como cianobactérias dominaram o planeta diz que estes organismos se beneficiaram de disponibilidade de CO_2, luz e nutrientes em abundância, permitindo que expandissem rapidamente e dominassem os mais diversos ambientes. Contudo, ao longo destes bilhões de anos, as mudanças na atmosfera terrestre, também foram forças seletivas

certamente envolvidas no desenvolvimento de eficientes mecanismos concentradores de carbono (MCCs), importantes para o predomínio de cianobactérias em ambientes com concentrações de gás carbônico similares às atuais. Os resultados obtidos neste estudo mostram que, apesar de pertencerem à uma mesma espécie, diferentes cepas de *Cylindrospermopsis raciborskii,* respondem de forma diferente às mudanças provocadas pelo aumento da concentração de CO_2. Este aspecto foi constatado nas respostas de cepas tóxicas e não tóxicas às condições a elas imposta. Quando comparadas as taxas de crescimento, as cepas de *C. raciborskii* produtoras de saxitoxina diferiram das não produtoras, onde as primeiras responderam mantendo a taxa de crescimento igual a dos seus respectivos controles, enquanto as cepas não tóxicas sofreram oscilações. O que foi obtido então foi um padrão diferenciado de resposta ao estresse de CO_2 para cada ecotipo estudado. Rantala e colaboradores (2004) sugerem para a espécie de cianobactéria *Microcystis sp.* que inicialmente todas eram produtoras de toxinas e que não produzí-las seria fruto de uma deleção genética ao longo da evolução. Ou seja, o caráter não tóxico teria sido fruto de deleções nos processos de formação das linhagens e espécies e não fruto de transferências laterais de genes seguidos de novos processos seletivos, como inicialmente pensado. Apesar das diferentes respostas encontradas, as análises de toxina (SXT) não demonstraram aumento nem redução significativa em sua produção. De toda forma, muitas vezes a vantagem adaptativa poderia estar na presença do metabólito e não apenas na sua concentração. Entretanto, nas análises transcriptômicas não foi identificada a expressão aumentada de nenhum gene do cluster ***sxt,*** portanto não é

possível afirmar que estas toxinas possuem relação direta com as respostas encontradas.

Os resultados também evidenciaram que, apesar das diferenças na produção de clorofila entre as cepas, nenhuma linhagem estudada sofreu alterações no conjunto de respostas associadas à fase clara da fotossíntese (produção de clorofila e eficiência fotossintética). Por outro lado, no transcriptoma da cepa que não produz SXT (CYLP) foi detectada uma repressão na transcrição de uma frutose-bifostafato aldolase responsável por associar ou dissociar o complexo PRK/CP12/GAPDH regulando o ciclo de Calvin. Essas observações chamam a atenção para processos celulares pouco explorados, mas que tem íntima relação com a biossíntese de moléculas derivadas dos metabolismos primário e secundário, incluindo o metabolismo energético de organismos fotossintetizantes. Tomar conhecimento da participação desta aldolase na modulação da resposta ao aumento do gás carbônico abre novas portas no que diz respeito ao estudo da fase escura em um ambiente mais rico em CO_2.

O transcriptoma é um tipo de análise complexa onde é possível observar a resposta celular a um estímulo como um todo. O intervalo de 72 horas foi escolhido, pois do ponto de vista fisiológico as células já teriam dado início aos processos celulares envolvidos com a manutenção celular sob condição de alto teor de CO_2. Sendo assim, o que estaríamos observando seria um retrato dos processos de regulação gênica empregados pelas células de *C. raciborskii* em um ambiente rico em gás carbônico e para o qual evolutivamente já estariam preparadas. De maneira geral, ao compararmos os resultados das cepas CYRF e CYLP, fica evidente a relação

entre a diferença no tipo de expressão encontrada em cada uma das linhagens.

Apesar de diferenciadas pela produção de toxina, tanto as respostas fisiológicas quanto as respostas encontradas no perfil metabólico rico em cianopeptídeos e na transcrição gênica chamam a atenção para a existência de grandes diferenças. Corroborando ainda os resultados gerais obtidos para CYRF e CYLP, a primeira linhagem não alterou seu perfil de metabolitos secundários quando exposta ao alto CO_2 enquanto a segunda passou a produzir, ou aumentou a produção, de moléculas de peso molecular superior a 700 Da. Ou seja, mais uma vez o metabolismo de CYLP foi alterado na condição experimental (CO_2 à 4.000 ppm). Ao observar os resultados das análises dos extratos ricos em cianopeptídeos foi possível concluir que a cepa não-tóxica lançou mão de um novo grupo de metabólitos para responder à condição testada. Já a linhagem tóxica não alterou, nem o metabolismo de forma geral, nem mesmo parece ter alterado o metabolismo da síntese da toxina, exceto por uma redução não significativa ($p > 0,05$) na produção da SXT. Contudo, os resultados com as outras duas linhagens, uma tóxica (T3) e uma não tóxica (NPCS-1) apresentaram padrões inversos aos obtidos para CYRF e CYLP. Desta forma, os resultados obtidos com as análises dos extratos brutos, apesar de corroborarem os transcriptomas, não permitem tecer padronizações em relação aos ecotipos estudados. O caráter qualitativo dos extratos não foi explorado à fundo, contudo nas tabelas 5 a 8, as massas com maior intensidade de sinal (cps) para cada cepa, foram registradas e comparadas com a literatura (Fewer *et al*., 2009; Silva-Stenico et al., 2011; 2015; Carneiro *et al*., 2012; Sanz *et al*., 2015; Briand *et al*.,

2015). Estas tabelas então apresentam possíveis cianopeptídeos a serem explorados através de técnicas de LC-MS/MS, para identificação e confirmação daqueles efetivamente produzidos por *C. raciborskii* e para abrir as portas para futuros estudos ecológicos sobre o papel desses metabólitos, respeitando que este é o espectro inicial de busca e que muito trabalho ainda precisa ser feito nessa direção. Na espécie estudada, apenas um tipo de cianopeptídeo foi identificado até o momento: uma microginina na linhagem 339-T3 (Silva-Stenico *et al*., 2011).

Tabela 5. Relação das massas de maior intensidade de sinal e seus possíveis cianopeptídeos, encontradas nos extratos brutos MeOH : H_2O (1:1) dos cultivos da cepa CYLP.

Cianopeptídeo	m/z	intensidade (CPS)
Aeruginosin9	879	1,03E+04
Anabaenopeptin5	878	1,08E+04
Cyanopeptolin1014	1015	2,20E+04
Cyanopeptolin978	965	1,41E+04
Microginin299B	921	1,59E+04
Microginin711	712	1,16E+04
Microginin91A	575	1,47E+04

Tabela 6. Relação das massas de maior intensidade de sinal e seus possíveis cianopeptídeos, encontradas nos extratos brutos MeOH : H_2O (1:1) dos cultivos da cepa NPCS-1.

Cianopeptídeo	m/z	intensidade (CPS)
AerucyclamideA	533	1,11E+04
AerucyclamideC	979	1,16E+04
AerucyclamideD	535	1,65E+04
Aeruginopeptin917SB	1059	1,59E+04
Aeruginosin1	753	1,02E+04
Aeruginosin10	849	2,15E+04
Aeruginosin11	893	1,24E+04
Aeruginosin2	767	1,21E+04
Aeruginosin3	737	1,25E+04
Aeruginosin4	823	1,12E+04
Aeruginosin5	837	1,69E+04
Aeruginosin6	851	1,07E+04
Aeruginosin669	669	1,24E+04
Aeruginosin691	691	1,70E+04
Aeruginosin724	725	1,08E+04

Aeruginosin9	879	1,28E+04
Aeruginosinamide	561	1,56E+04
Anabaenopeptin1	850	2,09E+04
Anabaenopeptin11	849	2,15E+04
Anabaenopeptin13	856	1,66E+04
Anabaenopeptin14	883	1,03E+04
Anabaenopeptin15	863	1,11E+04
Anabaenopeptin16	897	5,19E+04
Anabaenopeptin5	878	2,27E+04
Anabaenopeptin6	906	2,04E+04
Cianopeptolin10	1012	1,26E+04
Cianopeptolin6	984	1,15E+04
Cryptophycin 326	675	3,83E+04
Cyanopeptolin1014	1015	1,06E+04
Cyanopeptolin914	957	1,51E+04
Cyanopeptolin962	949	2,88E+04
Cyanopeptolin963A	943	1,41E+04
Cyanopeptolin964	963	2,06E+04
Cyanopeptolin972	973	1,02E+04
Cyanopeptolin978	965	5,91E+04
CyanopeptolinA	946	1,05E+04
CyanopeptolinS	926	2,57E+04
FerintoicacidA	674	3,12E+04
Fischerellin A	409	4,43E+04
Microginin299B	921	2,87E+04
Microginin51A	917	2,32E+04
Microginin51B	931	1,11E+04
Microginin91A	575	2,36E+04
Microginin91B	609	1,22E+04
Microginin91C	704	1,72E+04
Microginin91E	772	1,33E+04
Microginin99A	773	1,99E+04
Microginin99B	806	3,31E+04
MIcroginin AL584	585	7,29E+04
MicrogininFR4	742	1,11E+04
MicrogininFR5	726	1,18E+04
MicrogininFR6	740	1,40E+04
MicrogininFR9	751	1,05E+04
MicrogininT2	698	1,13E+04
MicropeptinB	959	1,09E+04
SpumiginA	613	6,06E+04
SpumiginB	627	2,32E+04
SpumiginF	597	1,43E+04

Tabela 7. Relação das massas de maior intensidade de sinal e seus possíveis cianopeptídeos, encontradas nos extratos brutos MeOH : H_2O (1:1) dos cultivos da cepa CYRF.

Cianopeptídeo	m/z	intensidade (CPS)
Cyanopeptolin978	965	1,94E+04
Fischerellin A	409	1,06E+04
Microginin91A	575	1,04E+04

Tabela 8. Relação das massas de maior intensidade de sinal e seus possíveis cianopeptídeos, encontradas nos extratos brutos MeOH : H_2O (1:1) dos cultivos da cepa T3.

Cianopeptídeo	m/z	intensidade (CPS)
AerucyclamideC	979	1,07E+04
AerucyclamideD	535	3,30E+04
Aeruginopeptin917SB	1059	1,05E+04
AeruginosamideB	603	1,20E+04
Aeruginoside126A	715	5,60E+04
Aeruginosin1	753	1,07E+04
Aeruginosin10	849	1,03E+04
Aeruginosin11	893	1,28E+04
Aeruginosin205A&B	805	1,39E+04
Aeruginosin3	737	1,13E+04
Aeruginosin4	823	1,12E+04
Aeruginosin5	837	1,28E+04
Aeruginosin6	851	1,23E+04
Aeruginosin684	637	1,50E+04
Aeruginosin691	691	3,67E+04
Aeruginosin89a	637	1,25E+04
Aeruginosin9	879	1,92E+04
Aeruginosinamide	561	1,79E+04
Anabaenopeptin1	850	1,40E+04
Anabaenopeptin11	849	1,03E+04
Anabaenopeptin16	897	1,37E+04
Anabaenopeptin3	864	1,00E+04
Anabaenopeptin4	892	1,77E+04
Anabaenopeptin6	906	1,97E+04
Cianopeptolin10	1012	1,45E+04
Cianopeptolin5	1018	1,01E+04
Cryptophycin 326	675	8,68E+04
Cyanopeptolin1014	1015	1,11E+04
Cyanopeptolin962	949	1,06E+04
Cyanopeptolin963A	943	1,05E+04
Cyanopeptolin964	963	4,16E+04
Cyanopeptolin972	973	1,13E+04
Cyanopeptolin978	965	1,47E+04

CyanopeptolinS	926	1,21E+04
FerintoicacidA	674	1,76E+04
Fischerellin A	409	1,86E+04
Microginin299B	921	9,62E+04
Microginin51A	917	2,08E+04
Microginin51B	931	1,59E+04
Microginin91A	575	1,32E+04
Microginin91B	609	1,10E+04
Microginin99A	773	1,29E+04
MIcroginin AL584	585	3,15E+04
MicrogininGH787	959	1,13E+04
MicropeptinB	959	1,23E+04
SpumiginA	613	5,22E+04
SpumiginB	627	2,05E+04
SpumiginF	597	1,70E+04
SpumiginG	595	2,55E+04

Embora o experimento de curta duração propicie respostas sobre o arcabouço genético desta espéice, ele deixa dúvidas se as diferentes linhagens estudadas continuariam sobrevivendo ao aumento do gás por um maior número de gerações. Foi possível observar que em até 72 horas houve uma resiliência das cepas tóxicas que não apresentaram nenhum tipo de alteração fisiológica até o último dia de experimento e que as cepas não tóxicas apresentaram alterações suas taxas de crescimento (> 50%), contudo todas mantiveram esse crescimento. Ainda não se sabe a capacidade de recuperação dessas cepas afetadas pela presença do alto teor CO_2, ou mesmo quanto do metabolismo secundário poderia mudar significativamente após o período testado. Os resultados apontam para uma melhor resposta imediata ao aumento gasoso pelas cepas tóxicas. Essa resposta diferencial também poderia estar associada a alternâncias de diferentes linhagens no ambiente produzindo ciclos de dominância destes organismos em função de alterações na disponibilidade do gás. Sabe-se que as concentrações de CO_2, na água, devido à processos tanto bióticos quanto abióticos podem superar

as concentrações atmosféricas e criar condições diferenciadas, que em escala microbiológica, podem representar mudanças bruscas no meio que estes organismos habitam.

Devido a quantidade de genes que podem estar envolvidos em diferentes processos celulares como fotossíntese, divisão celular, transporte de membrana, absorção de nutrientes e ainda o longo caminho a ser percorrido na elucidação dos genes totais envolvidos na regulação de cada processo, mesmo a diferença significativa encontrada na expressão dos genes obtidos, não foi suficiente para assegurar o grau de relação entre o que é transcrito e o que é observado em nível celular. Outra questão levantada durante as análises está relacionada ao momento correto para se fazer a avaliação fisiológica em resposta à uma expressão gênica, uma vez que a transcrição é o início da cadeia de resposta celular, seguida da síntese e endereçamento e o quanto o que foi observado realmente foi capaz de refletir um processo já encadeado de resposta molecular. O RNA estudado referia-se ao final do experimento e consequentemente ao final das determinações de clorofila, atividade fotossintética e metabolismo secundário (produção de SXT e cianopeptídeos). O RNA dos intervalos de tempo entre 0 h e 24 h foi extraído e está armazenado para assim que possível construir uma melhor resposta à esta pergunta. Adicionalmente, a aplicação das técnicas transcritptômicas ao estudo das cianobactérias ainda está em fase pioneira de utilização e tanto questões como desafios metodológicos ainda terão que ser enfrentados. Apesar do sucesso na extração e da qualidade atestada pelos diversos protocolos aplicados ao pré-sequenciamento, perguntas com relação à representatividade dos transcritos, à forma mais

adequada de interpretá-los, às técnicas mais adequadas de processamento dos dados, ao intervalo de tempo de resposta celular e aos possíveis erros associados que interfiram na resposta obtida, serão de grande relevância em estudos futuros que utilizem esta técnica.

Da experiência obtida neste trabalho ficou claro que a análise de transcriptomas precisa estar associada a um conjunto de análises fisiológicas e químicas capazes de refletir uma resposta celular global e vice-versa. Monitorar os níveis de RNA mensageiro (mRNA) nos permite avaliar os mecanismos de expressão gênica de um organismo em uma determinada condição. Além disso, o padrão global dos mRNAs é uma aproximação do padrão global de proteínas correspondentes, permitindo uma caracterização preliminar da célula estudada.

Uma espécie de cianobactéria deve ser compreendida através do conhecimento agregado das diferenças entre as linhagens, assim como o metabolismo secundário precisa ser compreendido à partir do estudo das diferentes moléculas encontradas no extrato bruto desses organismos, bem como o entendimento das variações na fase clara da fotossíntese não pode ser desacoplada da fase escura. Sendo assim, o estudo realizado abre perspectivas para estudos dos mecanismos adaptativos de cianobactérias e também mostra o quanto do metabolismo secundário, e não apenas de cianopeptídeos, pode ser explorado do ponto de vista ecológico. Também ficou evidenciado que, embora auxiliem no aprofundamento do conhecimento, o estudo concentrado em apenas uma única cepa ou mesmo em um grupo pequeno delas pode conter vieses e dessa forma dificultar

tomadas de decisão do manejo destes organismos no ambiente, uma vez que são de grande importância econômico-social.

Apesar de todo o avanço feito até a presente data, os estudos mostram que ainda há um grande universo de perguntas a serem respondidas em relação aos processos adaptativos de cianobactérias. Estes organismos continuam a contar muito da história evolutiva das células e os processos de seleção natural que vem moldando a vida no planeta.

Por fim, do ponto de vista prático, quando consideramos que cianobactérias mantém seu crescimento sob condições de CO_2 superiores àquelas previstas pelo IPCC (2014) de aumento até 3x até o ano de 2100, aumenta nossa preocupação sobre o controle destes organismos em nossos reservatórios de água. Especialmente se considerados outros impactos relacionados, como aumento da presença de sais na água resultante de maior evaporação devido ao aumento da temperatura e aumento da disponibilidade de fósforo e nitrogênio proveniente de fontes antrópicas. Considerando que esta é uma espécie de cianobactéria produtora de toxinas extremamente perigosas, tanto para a saúde humana como para a saúde dos ambientes, muito trabalho ainda precisa ser feito no sentido de obter um maior domínio sobre como lidar e se prevenir de futuras florações, que vem se tornando cada vez mais frequentes, procurando antever os cenários ambientais que estão por vir.

O conhecimento sobre cianobactérias é sem dúvidas a maior ferramenta tecnólogica que pode ser produzida para melhorar a qualidade das nossas reservas de água e consequentemente nossa qualidade de vida.

6. Conclusões

Através deste estudo foi possível concluir que diferentes linhagens de *Cylindrospermopsis raciborskii*, mesmo quando apresentam a mesma resposta fisiológica para um mesmo efeito (CO_2), produzem diferentes teores de clorofila e crescem em proporções diferentes, em nível de ordens de grandeza. A mesma variação foi encontrada nos perfis metabólicos de produção de cianopeptídeos.

As cepas CYLP (não-tóxica) e T3 (tóxica) variaram a produção de cianopeptídeos na condição de alto CO_2 enquanto as cepas CYRF (tóxica) e NPCS-1 (não-tóxica) não variaram.

Quanto à produção de SXT pelas cepas tóxicas, foi observado um leve decréscimo na produção, após 72 horas sob alto CO_2. No entanto, do ponto de vista estatístico, este resultado não foi significativo ($p < 0,05$).

As taxas de crescimento das cepas não-tóxicas (CYLP e NPCS-1) foram duas vezes menores sob a condição de alto CO_2 e nas cepas tóxicas não houve variação. Sendo assim, foi possível concluir que, apesar de continuarem crescendo, as linhagens não-tóxicas tiveram seu crescimento prejudicado quando expostas a CO_2 4000 ppm.

Da mesma forma, no transcriptoma da cepa não-tóxica (CYLP), foi observada regulação de um maior número de genes, quando comparados com os transcritos da cepa tóxica (CYRF).

Enzimas de atividade geral, atividade da membrana plasmática e genes relacionados com a fotossíntese foram, dentre os estatisticamente mais regulados, aqueles com maior atividade de transcrição sob a condição de alto CO_2.

Esta tese então foi capaz de agregar novos conhecimentos acerca do metabolismo secundário, crescimento e fotossíntese, além da regulação do RNA de 2 linhagens distintas de *C. raciborskii* cultivadas sob concentrações de 400 e 4.000 ppm de CO_2.

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