JEAN COUTINHO ODER

PHYSIOLOGICAL AND METABOLIC RESPONSES OF MICROCYSTIN-PRODUCER CYANOBACTERIAL STRAINS TO INORGANIC CARBON

Dissertation presented to the Universidade Federal de Viçosa, as part of the requirements of the Graduate Program in Botany, to obtain the title of *Magister Scientiae***.**

Advisor: Wagner L. Araújo

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ABSTRACT

ODER, Jean Coutinho, M.Sc., Universidade Federal de Viçosa, February, 2022. **Physiological and metabolic responses of microcystin-producer cyanobacterial strains to inorganic carbon.** Adviser: Wagner L. Araújo.

The phylum *Cyanobacteria* (*Bacteria* domain) constitutes a group of gram-negative prokaryotes able to perform oxygenic photosynthesis. These organisms are widely distributed around the globe and can be found in free form or in symbiotic associations with plants, diatoms, fungi, and sponges. Cyanobacteria are very important for the biogeochemical cycles of carbon (C) and nitrogen (N), because they are able to fix inorganic C (Ci) and some genera also perform biological N fixation (BNF). Some cyanobacterial strains also produce toxins (cyanotoxins), such as microcystins (MCs), which have hepatotoxic effects in animals. In this study, we evaluated the effects of different Ci sources on the metabolism of two filamentous heterocytous MC-producer strains (Scytonemataceae CCM-UFV057 and *Neowestiellopsis* sp. CCM-UFV026), characterizing their growth and metabolic responses. For this, the two strains were grown separately in BG-11_0 medium, with 0.02 $g \cdot L^{-1}$ of sodium carbonate [control (C)]; and in BG-11_0 medium, with 0 $g \cdot L^{-1}$ (T1), 0.016 $g \cdot L^{-1}$ (T2), 0.032 $g \cdot L^{-1}$ (T3), 0.16 $g \cdot L^{-1}$ (T4), 0.8 $g \cdot L^{-1}$ (T5) and 1.6 $g \cdot L^{-1}$ of sodium bicarbonate (T6). The growth parameters of strain *Neowestiellopsis* sp. CCM-UFV026 were affected at T1 (0.016 $g \cdot L^{-1}$ of sodium bicarbonate), but were not affected as the Ci concentration increased. The strain Scytonemataceae CCM-UFV057 showed lower growth at T6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate). In the highest sodium bicarbonate concentration, both strains showed decreased pigment contents (chlorophyll *a* and phycobiliproteins). The strain *Neowestiellopsis* sp. CCM-UFV026 had its metabolism and growth rate less impacted at the higher Ci concentration than the strain Scytonemataceae CCM-UFV057, since the latter showed signs of chlorosis in its culture and high values of cellular glycogen.

Keywords: Heterocitous strains. Growth rate. Growth curves. Metabolites contents.

RESUMO

ODER, Jean Coutinho, M.Sc., Universidade Federal de Viçosa, fevereiro de 2022. **Respostas fisiológicas e metabólicas de cianobactérias produtoras de microcistina a carbono inorgânico**. Orientador: Wagner L. Araújo.

O filo *Cyanobacteria* (domínio *Bacteria*) constitui um grupo de procariotas gram-negativos capazes de realizar a fotossíntese oxigênica. Estes organismos estão amplamente distribuídos em todo o mundo e podem ser encontrados em forma livre ou em associações simbióticas com plantas, diatomáceas, fungos e esponjas. As cianobactérias são muito importantes para os ciclos biogeoquímicos do carbono (C) e nitrogênio (N), pois são capazes de fixar C inorgânico (Ci) e alguns gêneros também realizam a fixação biológica de N (FBN). Algumas cianobactérias produzem toxinas (cianotoxinas), como as microcistinas (MCs), que têm efeitos hepatotóxicos em animais. Neste estudo, avaliamos o efeito de diferentes fontes de Ci no metabolismo de duas linhagens heterocitadas produtoras de MC (Scytonemataceae CCM-UFV057 e *Neowestiellopsis* sp. CCM-UFV026), caracterizando suas respostas de crescimento e metabólicas. Para isto, as duas linhagens foram cultivadas separadamente em meio $BG\text{-}11_0$, com 0,02 $g \cdot L^{-1}$de carbonato de sódio [controle (C)]; e em meio $BG\text{-}11_0$, com 0 $g \cdot L^{-1}$ (T1), 0,016 $g \cdot L^{-1}$ (T2), 0,032 $g \cdot L^{-1}$ (T3), 0,16 $g \cdot L^{-1}$ (T4), 0,8 $g \cdot L^{-1}$ (T5) e 1,6 $g \cdot L^{-1}$ de bicarbonato de sódio (T6). Os parâmetros de crescimento da linhagem *Neowestiellopsis* sp. CCM-UFV026 foram menores em T2 (0,016 $g \cdot L^{-1}$ de bicarbonato de sódio), mas não foram afetados em concentrações crescentes de Ci. Já a linhagem Scytonemataceae CCM-UFV057 mostrou menor crescimento em T6 (1,6 $g \cdot L^{-1}$ de bicarbonato de sódio). Sob T6, tratamento com a maior concentração de Ci (1,6 $g \cdot L^{-1}$), ambas as linhagens mostraram menor conteúdo de pigmento (clorofila *a* e ficobiliproteínas). A linhagem *Neowestiellopsis* sp. CCM-UFV026 teve seu metabolismo e taxa de crescimento menos impactados em maiores concentração de Ci do que a linhagem Scytonemataceae CCM-UFV057, uma vez que esta última mostrou sinais de clorose em cultura e altos valores de glicogênio celular.

Palavras-chave: Linhagens heterocitadas. Taxa de crescimento. Curvas de crescimento. Conteúdos metabólicos.

SUMMARY

1. INTRODUCTION

The phylum *Cyanobacteria* (*Bacteria* domain) constitutes a group of gram-negative prokaryotes able to perform oxygenic photosynthesis (Sinha and Häder 2008). These microorganisms are cosmopolitan (Sinha and Häder 2008) and despite being mostly found as free-living forms, they can also form symbiotic associations with a wide range of plants, diatoms, fungi, and sponges (Adams and Duggan 2008). Coupled with photoautotrophy, which relies on the fixation of the inorganic carbon (C) as CO_2 (Zhang et al. 2018, Herrero and Flores 2019), some cyanobacterial genera also perform the biological N fixation (BNF) (Doolittle 1988, Flores and Herrero 2009, Esteves-Ferreira et al. 2017, 2018, Herrero and Flores 2019). Thus, cyanobacteria are important players in the biogeochemical cycles of both C and N.

Members of the *Cyanobacteria* group exhibit remarkable variability in morphology, ranging from unicellular to filamentous (homocytous and heterocytous) forms (Dvořák et al. 2015). For instance, the heterocytous filamentous strains constitute a phylogenetically coherent group, which is classified within the order Nostocales (Komárek et al. 2014). This order presents, besides the vegetative cells, common to the other morphological groups, whose functions are mainly related to oxygenic photosynthesis and asexual reproduction (Castenholz 2001, Flores and Herrero 2009), heterocytes, akinetes, and hormogonia (Komárek 2010). Heterocytes harbors the nitrogenase enzymatic complex and are the sites of the BNF (Esteves-Ferreira et al. 2018). Akinetes, known as resistance spores, are cells with a thickened wall, usually differentiated during the stationary phase, resistant to cold and heat, and they can germinate to produce new filaments under favorable conditions (Meeks and Elhai 2002, Meeks et al. 2002). The hormogonia, on the other hand, constitute special filaments, related to the reproduction and dispersal (Campbell et al. 1993, Meeks et al. 2002, Flores and Herrero 2009).

Cyanobacteria are also a source of several bioactive secondary metabolites, which have important characteristics including toxicity, antiviral activity, photoprotection, and antioxidant activity, among others (Harada 2004, Ehrenreich et al. 2005, Kultschar and Llewellyn 2018). Accordingly, more than 2,000 bioactive metabolites have been identified in distinct cyanobacterial strains to date (Filatova et al. 2021). Often, the production of secondary metabolites is enhanced in response to abiotic or biotic stress conditions (Kultschar and Llewellyn 2018), providing both

protection and aiding in survival, ultimately giving an advantage to cyanobacteria over other species (Gupta et al. 2013). Among the bioactive secondary metabolites, cyanobacteria produce a wide variety of cyanotoxins, compounds that harm target species in their surrounding areas (Kultschar and Llewellyn 2018).

Cyanotoxins production has been drawing increasing attention due not only to its toxicity to humans and other animals, but also because its production can be increased in massive growth events in water bodies, named *blooms* (Wood 2016, Chorus et al. 2021). It has been reported that the input of N and phosphorus (P) by human activities may significantly impact the eutrophication of water bodies (Neumann et al. 2017, Malone and Newton 2020). Despite decades of research and billions of dollars spent on nutrient removal to reduce primary productivity, the blooming of cyanobacteria species in freshwater continues to be a significant global issue (Molot et al. 2014).

Cyanotoxins can be classified by their chemical nature, being cyclic peptides (microcystin and nodularin), alkaloids (anatoxin and saxitoxin), polyketides (apliziatoxin), and amino acid (β-Methylamino-L-alanine) (Ilieva et al. 2019). Cyanotoxins are also classified by their action in the human body, as neuro, hepato, endo, and dermatoxins (Neilan et al. 1999, Dittmann et al. 2013, Bhattacharyya et al. 2015). Among the hepatotoxins, the most common and studied is microcystin (MC), characterized as a small and monocyclic heptapeptide, composed of the general structure: (1) - X (2) - D-MAsp (3) - Z (4) - Adda (5) - D-Glu (6) - Mdha (7), in which X and Z are the most variable groups (Sivonen and Jones 1999) (Figure 1).

The MC have a strong affinity and can bind covalently to proteins from the serine/threonine phosphatases (PPs) group, which can remove phosphate from proteins in many biochemical pathways (Beyer et al. 2012). This inhibition causes the toxin's primary damage to living organisms (MacKintosh et al. 1990, Wu et al. 2018). The non-protein amino acid Adda is essential for the toxicity of the MC molecule. Isomerization and/or oxidation of the Adda dramatically reduces the MC toxicity (Songet al. 2006).

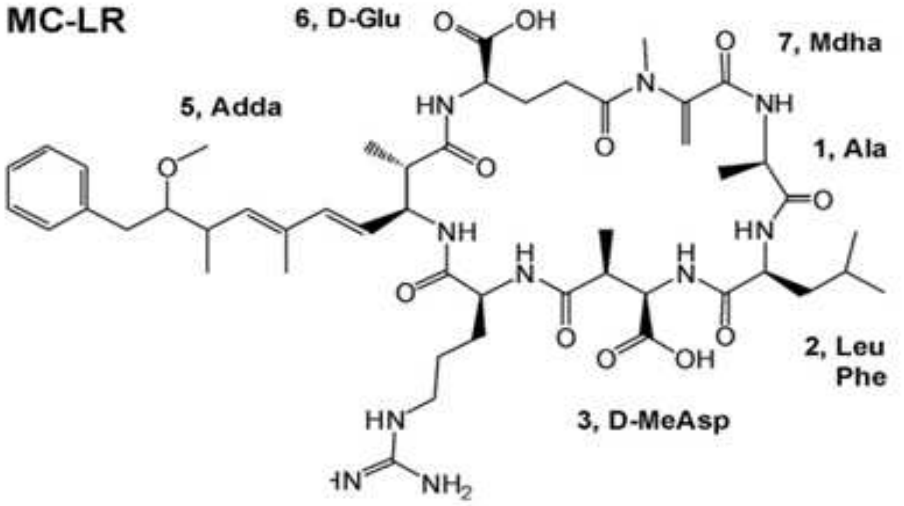

Figure 1. Illustration of the structure of the LR-MC (leucine-arginine), the best studied congener among the MCs variants. Abbreviations: Adda, (2S,3S,8S,9S)-3- amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-(4E,6E)-dienoic acid; D-Glu, glutamic acid; Mdha, N-methyl-a-b-dehydroalanine; Ala, alanine, Leu, leucine, Phe, phenylalanine, D-MeAsp, D-erythro-b-methyl-aspartic acid, Aba, Aminoisobutyric acid, Arg, arginine, Val, valine and Met, methionine (Heck et al. 2018).

The production of different MC isoforms by the same strain is common and, to date, more than 250 structural variants of MC have been described (Schuurmans et al. 2018). MC are synthesized by a non-ribosomal peptide synthase (NRPS) mechanism and the gene cluster responsible for the biosynthesis of MC encodes for peptide synthetases, polyketide synthases and enzymes responsible for tailoring (Tillett et al. 2000). Despite some variations, the MC gene cluster is generally 50 kbp in size, harboring approximately 10 encoding genes named *mcy*A to *mcy*J (Figure 2) (Fewer et al. 2007, 2013, Heck et al. 2018).

It is important to mention, however, that the role of MC in cyanobacterial cells remains uncertain (Briand et al. 2008). This fact aside, compelling evidence demonstrates that different environmental factors may affect MC biosynthesis in cyanobacterial cells (Boopathi and Ki 2014). By using the MC-producing strain *Microcystis aeruginosa* PCC7806 and analyzing the expression of the genes *mcy*B and *mcy*D, Kaebernick et al. 2000 showed that the levels of both transcripts increased under both high light and red light, revealing that the expression of the genes encoding for microcystin synthetase can be regulated by light quality and light intensity.

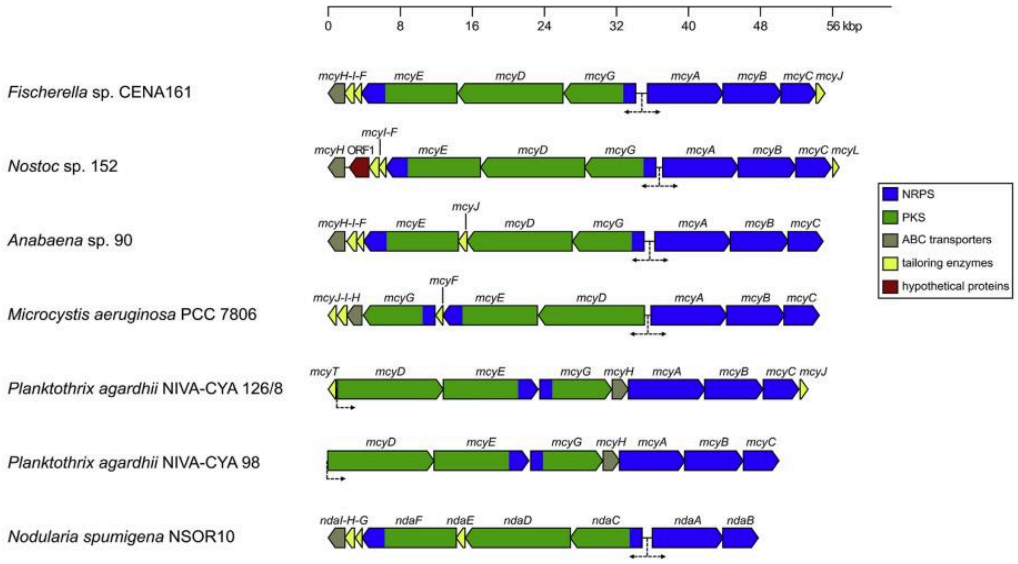

Figure 2. Arrangement of gene clusters coding for the biosynthesis of microcystin in different cyanobacterial strains. Arrows indicate the transcriptional start sites from the putative promoter regions. Abbreviations: NRPS, nonribosomal peptide synthetases; PKS, polyketide synthases (Heck et al. 2018).

By using the same strain (*M. aeruginosa* PCC7806) and its knockout mutant for the gene *mcy*A, Phelan and Downing (2011) proposed that MC may have a photoprotective role since the mutant was characterized by chlorosis at a high light intensity, which did not occur with the wide-type strain. Compelling evidence revealed a covalent interaction of MC with cysteine residues, increasing the fitness of *Microcystis* under oxidative stress conditions (Zilliges et al. 2011).

The effect of light intensity on the strain *M. aeruginosa* PCC7806 and in its *mcy*B-mutant was further analyzed revealing that light interferes with the assembling and the subcellular localization of RuBisCO (Barchewitz et al. 2019). More importantly, the authors also demonstrated that the mutant strain (that does not produce MC) was unable to relocate the subunits of RuBisCO out of carboxysomes under high light, the relocation of Rubisco's subunits is important in times of stress due to excess energy as they allow photorespiration, being a form of energy dissipation, as well as in higher plants. Accordingly, the mutant is more susceptible to the effects of high light (*e.g.,* chlorosis and oxidative stress) than its wild-type.

Not only light but also other stress conditions can impact MC production. Cellular microcystin quotas showed a significant positive correlation with nitrate uptake and consequently, the nitrogen content in *M. aeruginosa.* Thus, the ratio of nitrate uptake was positively correlated to cellular microcystin (Downing et al. 2005). Under inorganic carbon (Ci) deficient conditions, Jähnichen et al. 2007 also investigated the strain *M.*

aeruginosa PCC7806 and its mutant for the gene *mcy*B, and concluded that the wild-type strain adapts better to fluctuating Ci conditions than the mutant, indicating a likely role of MC during this adaptation.

Recent advances in studies concerning the synthesis and possible roles of MCs were mostly carried out with unicellular and non-nitrogen-fixing strains, mainly the strain *M. aeruginosa* PCC7806, most likely due to the development and availability of mutants for the MC production (Dittmann et al. 1997). As expected, therefore, studies with heterocytous filamentous strains are still relatively scarce. This fact aside, Castro (2019) recently demonstrated that MC production in the heterocytous filamentous strain Scytonemataceae CCM-UFV057 is directly influenced by Ci concentration, regardless of the source. More precisely, it was possible to demonstrate that higher Ci concentrations led to lower MC production, without impacts on growth and photosynthesis, revealing the impacts of Ci on the MC production in a nitrogen-fixing heterocytous strain.

It has been recently shown that when there is a decrease in the C/N ratio in MC-producing cyanobacterial cells, an increase in the production of this toxin is also observed (Sandrini et al. 2016, Castro 2019). The aforementioned studies showed that both the unicellular (*M. aeruginosa* PCC7806) and the heterocytous (Scytonemataceae CCM-UFV057) strains respond similarly to Ci deficiency. Taken together, these studies have analyzed how the production of MC can be influenced by environmental factors, including the impact of Ci availability, mainly in unicellular strains. However, the MC production and the factor driving it may be distinct in other filamentous heterocytous strains. Furthermore, our knowledge on the mechanisms of action of Ci on the primary metabolism of cyanobacteria remains rather fragmented. Castro (2019) brings in his work a series of metabolic responses for the microcystin-producer strain Scytonemataceae CCM-UFV057, such as pigment content, photosynthesis and respiration responses and growth parameters, demonstrating that for this strain, the different concentrations of Ci applied did not affect negatively the growth, but other metabolites and their photosynthesis were negatively affected by the treatment with a higher concentration of inorganic carbon, then the questioning arises whether in another strain that is also heterocytous and microcystin-producer if this pattern may be similar between both and in intermediate treatments of those applied in its work. Given the above, the main goal of this work was to investigate, in two morphological distinct heterocytous strains, in response to different Ci concentrations

to analyze how, and to which extent, the decrease in Ci availability affects the primary metabolism, photosynthesis, and respiration of these strains.

2. GOALS

2.1 General Goals

- To investigate how and which extent the metabolism of MC-producing heterocytous strains are affected in response to different concentrations of Ci.

2.2 Specific Goals

- To characterize the metabolic responses (*e.g.,* chlorophyll, amino acid, protein and glycogen content) in two morphological distinct heterocytous strains to different Ci concentrations;
- To verify the growth in optical density of two morphological distinct heterocytous strains when subjected to different Ci treatments.
- To verify the changes in photosynthesis and respiration in two distinct heterocytous strains to different Ci concentrations;

3. MATERIAL AND METHODS

3.1 Strain selection

Two cyanobacterial strains (Scytonemataceae CCM-UFV057 and *Neowestiellopsis* sp. CCM-UFV026), maintained in the Collection of Cyanobacteria and Microalgae at Universidade Federal de Viçosa (CCM-UFV), were used. These strains were previously characterized based on morphological and molecular data (mainly 16S rRNA phylogeny) and were also confirmed as MC-producers based on molecular and chemical tools.

3.2 Light curves

To obtain the compensation and light saturation points and later determine the light intensities for the cultivation, light curves were performed on a Clark electrode (Oxygen Monitoring System, Oxylab+ Hansatec) (Figures 3 and 4). The strains were grown in 50 mL Erlenmeyer flasks containing 20 mL of BG-11_0 culture medium (BG-11 medium without nitrogen) (Rippka et al. 1979), and maintained for three days on an orbital shaker at 100 rpm, 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of light intensity, 16/8-hour photoperiod (light/dark), at 23 ± 2 °C. Before the analysis, the electrode was calibrated using sodium dithionite (aiming for 0 % oxygen saturation in the apparatus). Then, the chamber was rinsed with distilled water. After acclimation, 2 mL of the homogenized culture of each strain, properly acclimatized for 30 min in the dark, were placed inside Clark's electrode chamber at 25 °C and the compensation and light saturation points were determined by analyzing the oxygen evolution generated under continuously increasing of light intensity in the chamber (Nagarajan et al. 2014).

3.3 Pre-inoculum culture conditions

The pre-inoculum cultures were grown in 500 mL Erlenmeyer flasks (containing 250 mL of BG-11_0 medium) and maintained in the culture room (according to the conditions described in section 3.2) to reach enough biomass to perform the experiments. After growth, the biomass was centrifuged for 10 minutes at 11.000 *g*, and the supernatant was discarded. The residual biomass was then inoculated into flasks with the medium corresponding to the treatments with different Ci concentrations.

3.4 Growth curves under different concentrations of inorganic carbon

To identify the different phases of growth (lag, log and stationary) under the treatments, growth curves were performed. For the two strains (CCM-UFV057 and CCM-UFV026) the following treatments were used, always applying sodium bicarbonate ($NaHCO_3$) as the Ci source instead of sodium carbonate -Na_2CO_3- (BG-11_0 standard medium), except the control (sodium carbonate – Na_2CO_3): Control [C] (BG-11_0, 0.02 g·L^{-1}), T1 (0 g·L^{-1}) T2 (0.016 g·L^{-1}), T3 (0, 032 g·L^{-1}), T4 (0.16 g·L^{-1}), T5 (0.8 g·L^{-1}) and T6 (1.6 g·L^{-1}). The experiments were conducted in 125 mL Erlenmeyer flasks containing 50 mL of medium, which were maintained under the conditions described above. The growth of the strains was measured by optical density by spectrophotometer at a wavelength of 750 nm ($O.D._{750nm}$) -Griffiths et al. 2011- for 15 days, with the initial $O.D._{750nm}$ (point 0) = 0.1. Measurements were made daily and in triplicate. After reading in the spectrophotometer, 10 mL of culture from each sample were separated, filtered in nitrocellulose membranes previously dried and weighed (pore size 0.22 µm). The membranes containing the filtered biomasses were then dried and weighed to obtain the dry mass. Next, the material was also calcinated for an ash-free dry mass determination (Obuekwe et al. 2019). After delimiting the log and stationary phases of each treatment, new growth curves were performed, as described above, aiming to collect material for metabolite analysis at two points: the middle of the log phase and the middle of the stationary phase.

3.5 Kinetic parameters of growth

The maximal growth rate (μ_{max}) and generation time (*Gt*) of *Neowestiellopsis* sp. CCM-UFV026 and Scytonemataceae CCM-UFV057 were calculated by applying a linear regression of the data within the logarithmic phase (Giannuzzi 2019, Alvarenga et al. 2020), using the software SigmaPlot® 10. For *Gt*, the following formula was applied $Gt=\ln(2)/\mu_{max}$.

3.6 Biochemical analyses

For biochemical analyses, the biomass present in each repetition was collected for each strain in the middle of the log and stationary phase at 6, 12 and 24 hours of the photoperiod (representing 2 points in light phase and 1 point in the final of dark phase). So, this biomass was centrifuged at 20.000 *g* at 4°C. The supernatant was

discarded and the pellet was transferred to 2 mL microtubes. The material was then frozen in liquid nitrogen and dried in a lyophilizer. After lyophilization, the biomasses were weighed and aliquoted (~5 mg) in 2.0 mL microtubes on precision scales.

For methanolic extraction, 700 µL of methanol (100 %) was added to the microtubes with biomass, which were heated at 80 °C under 500 rpm stirring for 20 min (adapted from Lisec et al. 2006). The methanolic extract was, centrifuged at 15.000 × *g* for 10 min at 4 °C. From the supernatant, chlorophyll *a* and amino acids were quantified. The pellet was used to quantify total proteins and glycogen (Castro, 2019). For the determination of chlorophyll *a*, 100 µL of methanol was added to 100 µL of supernatant; then, the sample´s densities at wavelengths 653 and 666 nm (DO_{653nm} and DO_{666nn}) were determined in a microplate reader (Versa max, Molecular Devices) (Porra et al. 1989). The remaining volume was washed (to remove pigments) with the addition of 375 µL of chloroform and 750 µL of ultrapure water. The material was then centrifuged at 19,000 *g* for 10 min at 4 °C, leading to the formation of a new pellet. The aqueous phase formed (in which the metabolites of interest are found) was transferred to another 1.5 mL microtube for amino acid quantification. For such quantification, 50 µL of the sample was transferred to individual wells in a 96-well microplate, to which 50 µL of sodium citrate buffer (1 M, pH = 5.2) + 0.02 % ascorbate and 100 µL of ninhydrin were added in the dark. The microplates were sealed and heated at 90 °C for 20 minutes and after cooling down to room temperature. The absorbance of samples was determined at 570 nm ($O.D._{570nm}$) (Microplate reader, Versa max, Molecular devices), comparing with a predetermined proline standard curve (Cross et al. 2006).

The first residual pellet was washed with 1 mL of ethanol (70 %) with subsequent addition of NaOH (0.1 M) and heated at 95 °C for protein extraction. In the wells of the microplates, 250 µL of Bradford solution plus 5 µL of sample protein suspension were added, and the absorbance was determined at 595 nm ($O.D._{595nm}$) (Microplate reader, Versa max, Molecular devices) comparing with the predetermined standard curve of bovine serum albumin protein (BSA) (Bradford 1976). For glycogen quantification, the sample was neutralized by adding acetic acid (1M) to the residual pellet. Then, the suspension had its absorbance determined at a wavelength of 340 nm ($O.D._{340nm}$) (Fernie et al. 2001).

3.7 Phycobiliproteins quantification

For extraction and quantification of phycobiliproteins (PBP), approximately 10 mg of freeze-dried biomass collected in the middle of the logarithmic and stationary phase were ruptured by mechanical shock. The procedure was conducted in a Tissue Lyzer (Retsch MM 400), with metal balls for 1.5 minutes and frequency of 24 Hz. . After the addition of the buffer, the contents were homogenized in a vortex and centrifuged at 19000 *g*, for 10 min at 4 °C. The obtained supernatant was transferred and stored in a 15 mL tube. The extraction step described above was repeated until all pigments were removed from the samples. Then, 1.9 mL of sample was transferred to a 2 mL microtube, to which 100 µL of streptomycin sulfate (200 mg mL^{-1}) was added. The samples were kept at 4 °C for thirty minutes and then centrifuged at 18000 *g* for 10 minutes at 4 °C. From the supernatant, 1.4 mL volume was transferred to a new microtube, and 400 µL of DTT solution (1mM) was added. Approximately 200 µL of the supernatant was aliquoted into 96-well microplates and read at DO565nm, DO620nm, and DO650nm in a microplate reader (Versa max, Molecular devices), allowing quantification of phycocyanin, allophycocyanin and phycoerythrin (de Marsac and Houmard 1988).

3.8 Analysis of photosynthesis and respiration

The analyses were conducted using Clark electrode (Oxigen Monitoring System, Oxylab+ Hansatec) and the apparatus was calibrated as described in 3.2. Samples were grown in the treatments with different Ci concentrations and in triplicates were collected in the middle of the log and stationary phase for strains *Neowestiellopsis* sp. CCM-UFV026 and Scytonemataceae CCM-UFV057. In the Clark electrode the respiration of 2 mL of each sample was used to measure the O_2 evolution by keeping them for 5 minutes in the dark and also the photosynthesis was measured at the cultivation intensity of 90 µmol photons·m^{-2}·s^{-1}. The results of oxygen evolution given by the equipment were normalized by ash-free dry mass, according to the procedure described in item 3.4.

3.9 Experimental design and statistical analysis

The experiment was conducted and analyzed following a randomized block design with each block composed of 2 strains and seven treatments with at least three

replications. The data obtained for growth, physiological and metabolic parameters were subjected to an analysis of variance (ANOVA, $P<0,05$), and the means were compared using the Tukey's test at 5% probability. All analyses were performed on STATISTICA software (StatSoft), and Principal Component Analysis (PCA) was performed on MiniTab18 software.

4. RESULTS

4.1 Light curves of *Neowestiellopsis* sp. CCM-UFV026 and Scytonemataceae CCM-UFV057

The data obtained from the analysis carried out on the Clark electrode showed, for both strains (*Neowestiellopsis* sp. CCM-UFV026 and Scytonemataceae CCM-UFV057), that when the pre-inoculum is cultivated in a standard BG-11_0 culture medium with 0.02 $g \cdot L^{-1}$ of sodium carbonate (Ci), the light saturation points (*I*s) was approximately 120 µmol photons$\cdot m^{-2} \cdot s^{-1}$ (Figures 3 and 4). Interestingly, the compensation points (*I*c) were 15 and 35 µmol photons$\cdot m^{-2} \cdot s^{-1}$ for the strains CCM-UFV026 and CCM-UFV057, respectively (Figure 3 and 4). Accordingly, following these results, we decided to grow the strains under a light intensity of 90 µmol photons$\cdot m^{-2} \cdot s^{-1}$ in the next experiments.

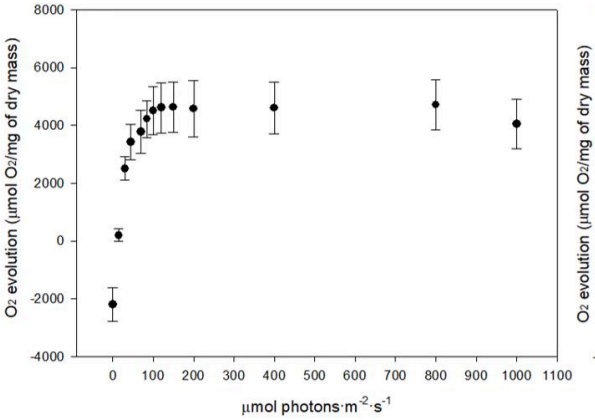

Figure 3. Light curve of *Neowestiellopsis* sp. CCM-UFV026 based on oxygen evolution at Clark electrode. For the analysis, 2 mL of sample ($O.D._{750nm}$ = 0.8), on the fourth day of cultivation, previously cultivated in standard $BG\text{-}11_0$ culture medium [with 0.02 $g \cdot L^{-1}$ of sodium carbonate (Ci)] were acclimated for 20 minutes in the dark and then introduced into the Clark electrode chamber. The sample was left for another 5 minutes in the dark and subjected to the light intensities indicated in the graph for 3 minutes. Values represent means ± standard error (n = 4).

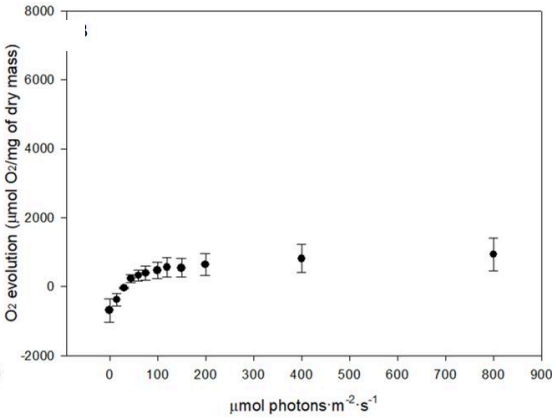

Figure 4. Light curve of Scytonemataceae CCM-UFV057 based on oxygen evolution at Clark electrode. For the analysis, 2 mL of sample ($O.D._{750nm}$ = 0.8), on the fourth day of cultivation, previously cultivated in standard $BG\text{-}11_0$ culture medium [with 0.02 $g \cdot L^{-1}$ of sodium carbonate (Ci)] were acclimated for 20 minutes in the dark and then introduced into the Clark electrode chamber. The sample was left for another 5 minutes in the dark and subjected to the light intensities indicated in the graph for 3 minutes. Values represent means ± standard error (n = 4).

4.2 Differential growth of the strains in response to Ci levels

The growth curves measured by optical density showed similar patterns within the same strains in the different treatments. Lag phases were not observed regardless of the strains or treatments (Figures 5 and 6). The logarithmic (log) phase ranged from day 1 to 8, for CCM-UFV026 (Figure 5) and from day 1 to 7, for the strain CCM-UFV057 (Figure 6). A pattern of growth deceleration (end of log phase) was observed from day 6 to 8, for both strains. Based on this, we were able to select the sampling points for subsequent biochemical analyses, which were the middle of the log phase and the middle of the stationary phase. Accordingly, for both strains, the sampling points were on day 4 (log phase) and day 10 (stationary phase), as indicated by the blue arrows (Figures 5 and 6).

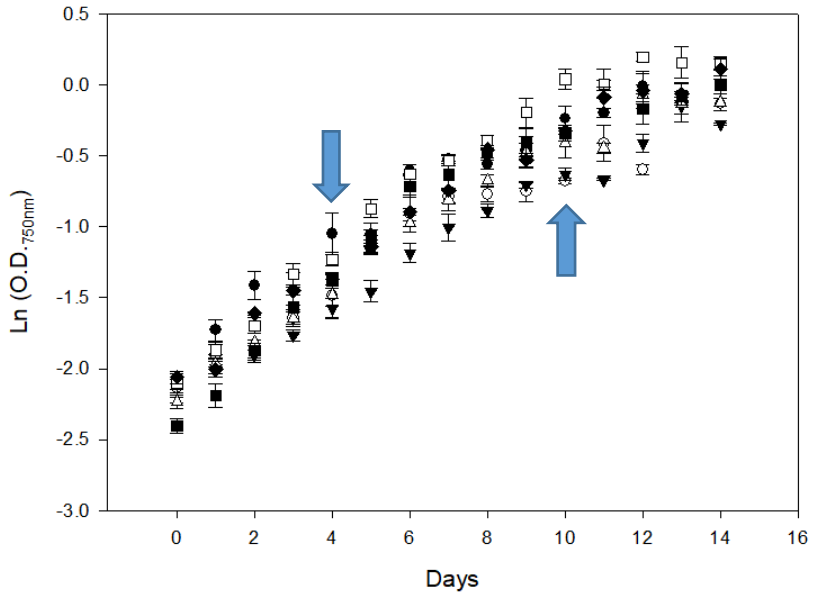

Figure 5. Growth curves based on optical density measurements (O.D.$_{750nm}$) of *Neowestiellopsis* sp. CCM-UFV026 grown in culture media supplied with different concentrations of Ci. Growth was monitored at 24 hours intervals for 15 days. Treatments: Control -0,02 g·L^{-1} of sodium carbonate- (black circles); T1 -0 g·L^{-1} of sodium bicarbonate- (white circles); T2 -0,016 g·L^{-1} of sodium bicarbonate- (black triangles); T3 -0,032 g·L^{-1} of sodium bicarbonate- (White triangles); T4 -0,16 g·L^{-1} of sodium bicarbonate- (black squares); T5 -0,8 g·L^{-1} of sodium bicarbonate- (white squares); T6 -1,6 g·L^{-1} of sodium bicarbonate- (black lozenge). Blue arrows indicate the collection days for the metabolism analyses (days 4 and 6 for log and stationary phase, respectively). Values represent means ± standard error (n = 3).

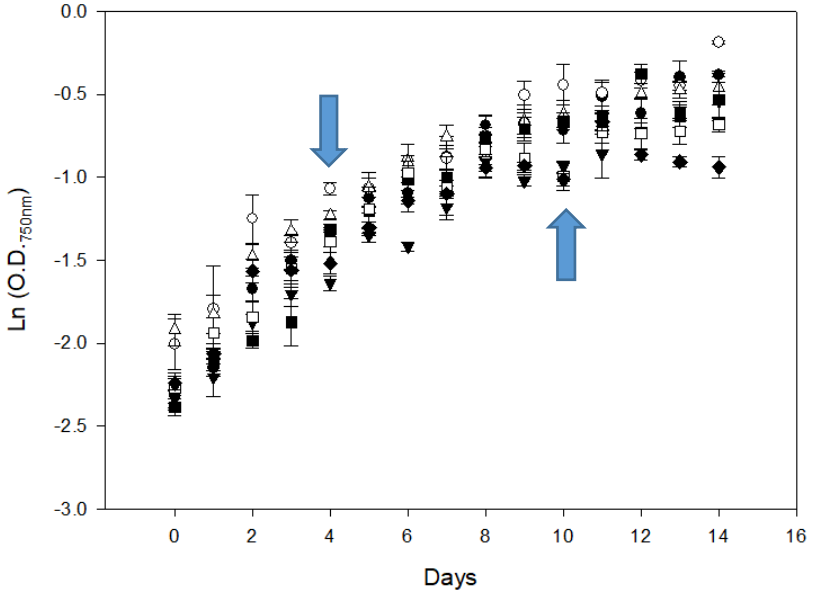

Figure 6. Growth curves based on optical density measurements (O.D.$_{750nm}$) of Scytonemataceae CCM-UFV057 grown in culture media supplied with different concentrations of Ci. Growth was monitored at 24 hours intervals for 15 days. Treatments: Control -0,02 g·L^{-1} of sodium carbonate- (black circles); T1 -0 g·L^{-1} of sodium bicarbonate- (white circles); T2 -0,016 g·L^{-1} of sodium bicarbonate- (black triangles); T3 -0,032 g·L^{-1} of sodium bicarbonate- (White triangles); T4 -0,16 g·L^{-1} of sodium bicarbonate- (black squares); T5 -0,8 g·L^{-1} of sodium bicarbonate- (white squares); T6 -1,6 g·L^{-1} of sodium bicarbonate- (black lozenge). . Blue arrows indicate the collection days for the metabolism analyses . Values represent means ± standard error (n = 3).

4.3 Kinetic parameters of growth

By analyzing the growth parameters of both strains in the log phase, a similar pattern was noted (Tables 1 and 2). Both strains (*Neowestiellopsis* sp. and Scytonemataceae CCM-UFV057) showed the higher values of μ_{max} (0.2786 ± 0.0157 and 0.2347 ± 0,0171, respectively) in treatment T4 (0.16 $g \cdot L^{-1}$ of sodium bicarbonate). Accordingly, this response caused a decrease in generation time (*Gt*) that reached values of 59.69 ± 3.41 and 70.86 ± 5,54 hours for *Neowestiellopsis* sp. CCM-UFV026 and Scytonemataceae CCM-UFV057, respectively, but only significant to Scytonemataceae CCM-UFV057. On the other hand, the treatments responsible for lower μ_{max} and higher *Gt* were different for the analyzed strains. *Neowestiellopsis* sp. CCM-UFV026 and Scytonemataceae CCM-UFV057 showed the lower μ_{max} values in T2 (0.016 $g \cdot L^{-1}$ of sodium bicarbonate) and T6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate), respectively (Tables 1 and 2), leading to the higher *Gt*, 113 and 108 hours. Interestingly, the variation of Ci concentrations promoted more changes in the growth parameters (table 2) of the strain CCM-UFV057 when compared to the strain CCM-UFV026.

Table 1. Values of maximum growth rates (μ_{max}) and generation time (*Gt*) obtained for the strain *Neowestiellopsis* sp. CCM-UFV026.

Treatments	μmax	*Gt* (hours)
Control	0.2258 ± 0.018 bc	73.65 ± 5.64 b
T1	0.1997 ± 0.0077 bc	83.28 ± 3.14 b
T2	0.1469 ± 0.0200 c	113.21 ± 14.91 a
T3	0.224 ± 0.0123 bc	81.52 ± 4.67 b
T4	0.2786 ± 0.0157 a	59.69 ± 3.41 b
T5	0.2459 ± 0.0010 ab	67.63 ± 0.27 b
T6	0.1991 ± 0.0072 bc	83.53 ± 3.15 b

Values are presented as means ± standard error (n = 3). Means followed by the same letter for an individual parameter do not differ by 5% of probability (Tukey's test).

Table 2. Values of maximum growth rate (μ_{max}) and generation time (*Gt*) obtained for the strain Scytonemataceae CCM-UFV057.

Treatments	μmax	*Gt* (hours)
Control	0.1971 ± 0.0102 ab	84.38 ± 4.37 ab
T1	0.1768 ± 0.0207 ab	94.07 ± 10.49 ab
T2	0.1948 ± 0.0153 ab	85.37 ± 7.27 ab
T3	0.1714 ± 0.0089 ab	97.03 ± 5.20 ab
T4	0.2347 ± 0.0171 a	70.86 ± 5.54 b
T5	0.208 ± 0.0083 ab	79.96 ± 3.21 ab
T6	0.1533 ± 0.0081 b	108.49 ± 5.53 a

Values are presented as means ± standard error (n = 3). Means followed by the same letter for an individual parameter do not differ by 5% of probability (Tukey's test).

4.4 Biochemical analysis

4.4.1 Chlorophyll *a*

Overall, the highest values of chlorophyll *a* were observed during the stationary phase for both strains and under treatments with low Ci concentration (Figures 7 and 8). Accordingly, the higher values were found for the strain Scytonemataceae CCM-UFV057, under the treatments T1 and T2 (0 $g \cdot L^{-1}$ and 0.016 $g \cdot L^{-1}$ of sodium bicarbonate) (Figure 8B). Interestingly, regardless the strain, growth phase or sampling time, the lower contents of chlorophyll *a* were achieved in T6, which has the highest Ci (1.6 $g \cdot L^{-1}$ of sodium bicarbonate).

A decrease in chlorophyll *a* content in the stationary phase for both strains were also observed in samples obtained in treatments 4, 5 and 6 (0.016, 0.8 $g \cdot L^{-1}$ and 1.6 $g \cdot L^{-1}$ sodium bicarbonate) compared to other concentrations of Ci. This pattern was also especially remarkable at 12 h (log phase) and 24 h (stationary phase) for the strain *Neowestiellopsis* sp. CCM-UFV026 (Figure 7). Similarly, reductions in chlorophyll *a* were noticed at 24 h (log phase) and at all points collected during the stationary phase for the strain Scytonemataceae CCM-UFV057 (Figure 8).

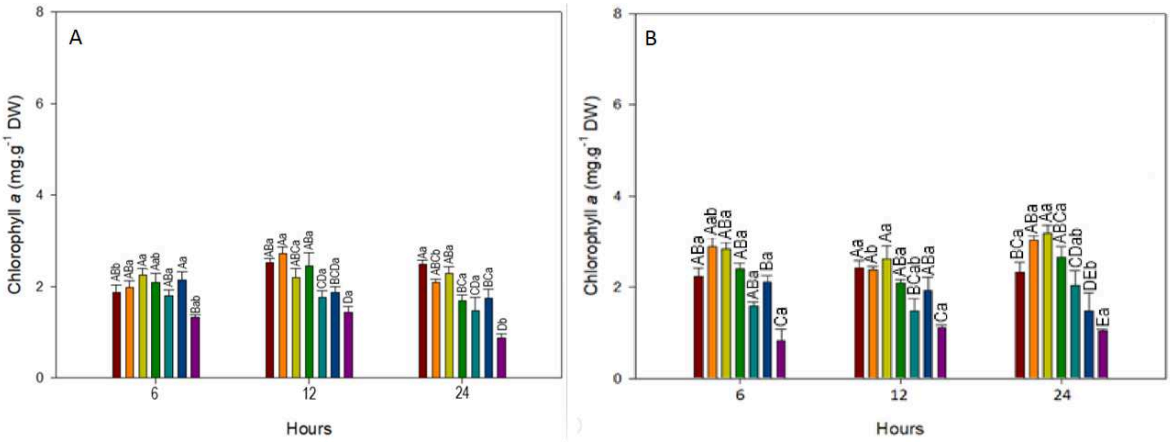

Figure 7. Variation in the chlorophyll *a* content for the strain *Neowestiellopsis* sp. CCM-UFV026 in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars); T2 (yellow bars); T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments and lowercase letters represent statistical differences in the same treatment through the time.

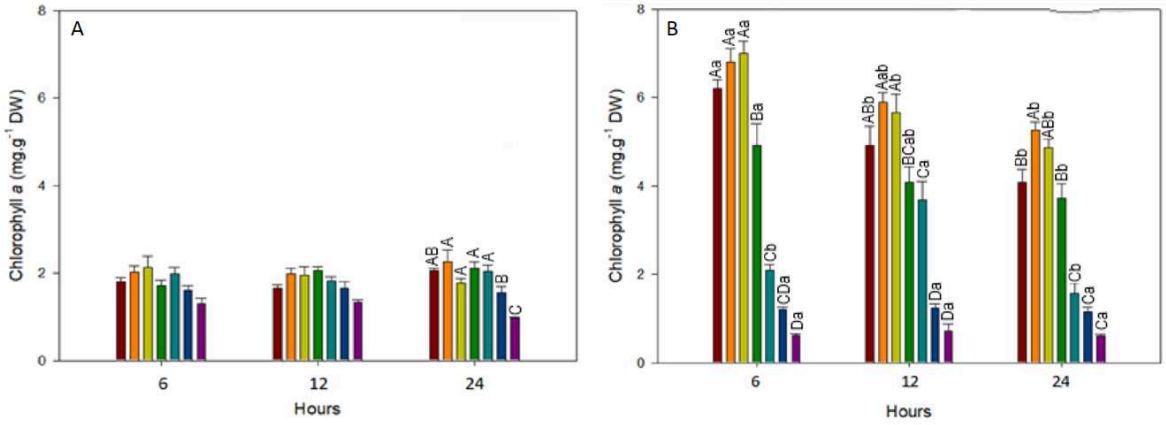

Figure 8. Variation in the chlorophyll *a* content for the strain Scytonemataceae CCM-UFV057 in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars); T2 (yellow bars); T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments and lowercase letters represent statistical differences in the same treatment through the time. *On Log phase the time factor doesn't present statistical difference.

4.4.2 Total amino acids

In the log phase, the strain *Neowestiellopsis* sp. CCM-UFV026 showed neither differences among the collection times for were observed nor for the 6h and 12h points among the treatments (Figure 9A), yet the treatment 6 (1.6 $g.L^{-1}$ of sodium bicarbonate) presented the highest values of amino acids after 24h (Figure 9A).

In the stationary phase, the *Neowestiellopsis* sp. strain CCM-UFV026 did not show a clear pattern of increase or decrease in amino acid content according to the Ci concentrations (Figure 9B). However, there was an increase in the values in treatment 1 (0 $g.L^{-1}$ of sodium bicarbonate) and treatment 2 (0.016 $g.L^{-1}$ of sodium bicarbonate) in the dark period comparing, with the previous time (12h) (Figure 9B).

In the log phase of Scytonemataceae CCM-UFV057, on the 6 h samples, the treatments 4 (0.16 $g.L^{-1}$ of sodium bicarbonate), 5 (0. $g.L^{-1}$ of sodium bicarbonate) and 6 (1.6 $g.L^{-1}$ of sodium bicarbonate) showed the highest values for amino acid content (Figure 10A). Whereas at the 24h point (dark), treatments 4 (0.16 $g.L^{-1}$ of sodium bicarbonate) and 5 (0.8 $g.L^{-1}$ of sodium bicarbonate) showed no significant differences with other treatments, but the treatment 6 (1.6 $g.L^{-1}$ of sodium bicarbonate) showed the lowest values at this time and between the other sample points (Figure 10A).

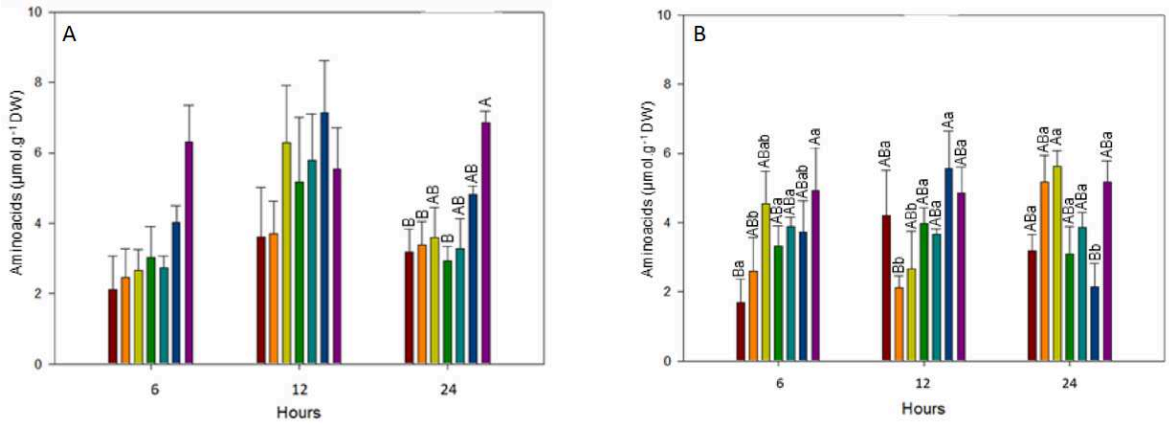

Figure 9. Variation in the amino acids content for the strain *Neowestiellopsis* sp. CCM-UFV026 in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars); T2 (yellow bars); T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments and lowercase letters represent statistical differences in the same treatment through the time. * Over the log phase, only the treatments on 24h presented statistical differences.

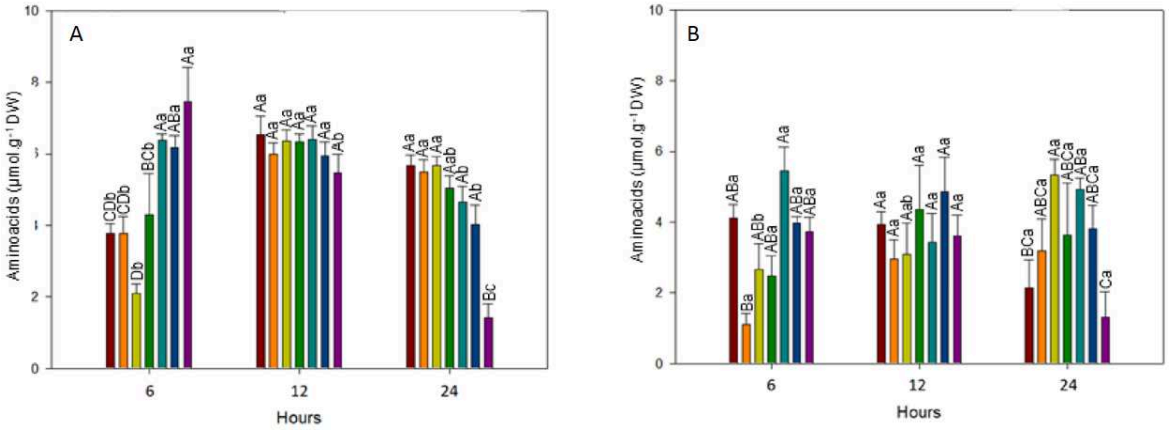

Figure 10. Variation in the amino acids content for the strain Scytonemataceae CCM-UFV057 in three sampling points over log (A) and stationary (B) phases. Variation in the amino acids content for the strain in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars); T2 (yellow bars); T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments and lowercase letters represent statistical differences in the same treatment through the time.

4.4.3 Total soluble proteins

For the strain *Neowestiellopsis* sp. CCM-UFV026 no differences were observed for the contents of total soluble proteins, regardless the treatments, growth phases or sampling points (Figure 11). Similar pattern was observed for the strain Scytonemataceae CCM-UFV057 at stationary phase (Figure 12B). However, at log

phase, this strain was characterized by changes in total soluble proteins, mainly in the treatment T3 (0.032 $g \cdot L^{-1}$ of sodium bicarbonate) and T5 (0.8 $g \cdot L^{-1}$ of sodium bicarbonate), that lead to the lowest contents at times 6 and 24 h, respectively (Figure 12A). It is also important to note that the strain *Neowestiellopsis* sp. CCM-UFV026 had the higher values of total proteins.

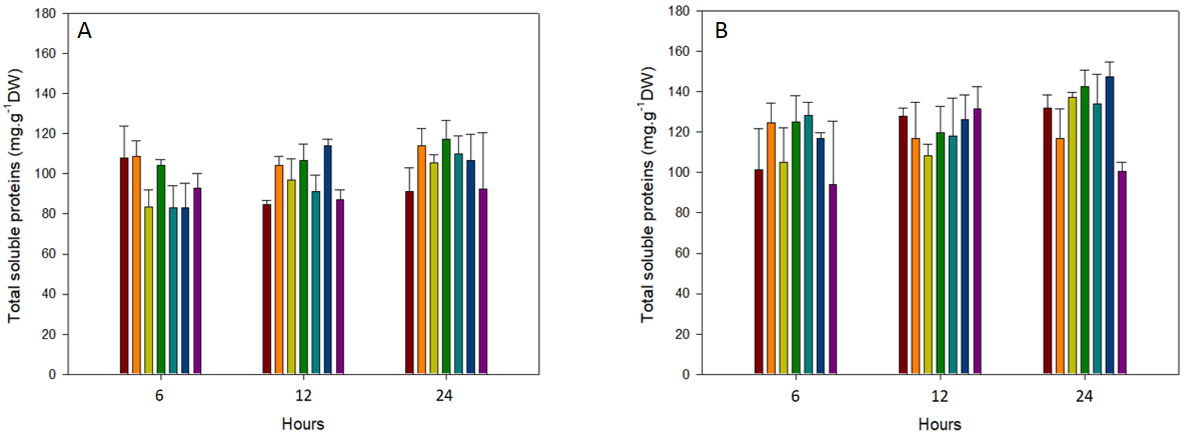

Figure 11. Variation in the total soluble proteins content for the strain *Neowestiellopsis* sp. CCM-UFV026 in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars); T2 (yellow bars); T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments, and lower-case letters demonstrate statistical differences along time. *The samples in both phases did not present statistical differences.

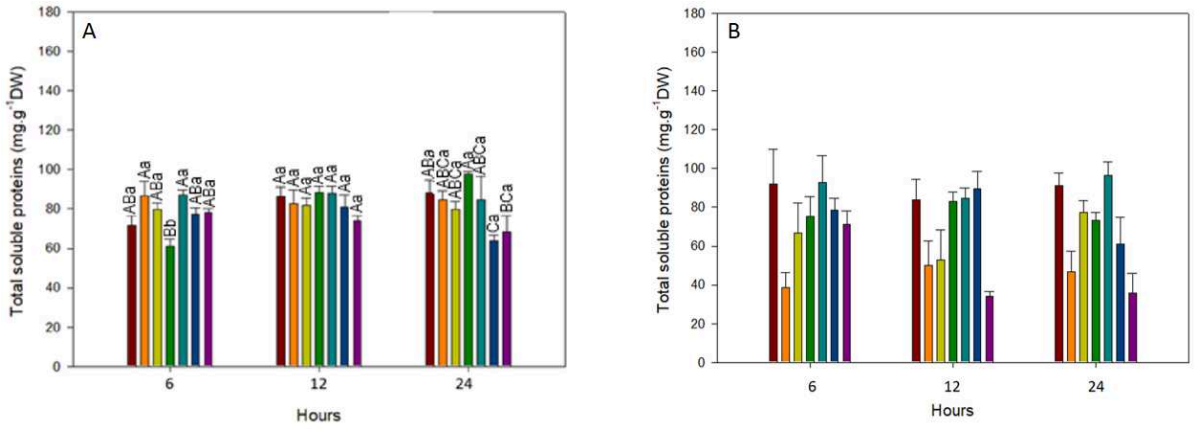

Figure 12. Variation in the total soluble proteins content for the strain Scytonemataceae CCM-UFV057 in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars; T2 (yellow bars); T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments, and lower-case letters demonstrate statistical differences along time *The samples in stationary did not present statistical differences.

4.4.4 Phycobiliproteins

For both strains, it was possible to note that the contents of phycoerythrin (PE) was lower when compared to the contents of phycocyanin (PC) and allophycocyanin (AP) within treatments (Figures 13 and 14). The strain *Neowestiellopsis* sp. CCM-UFV026 had higher values of PB than the strain Scytonemataceae CCM-UFV057 (Figures 13 and 14). Accordingly, in the log phase, total phycobiliprotein in the treatments for the strain CCM-UFV026 ranged between 16 and 22 $\mu g \cdot mg^{-1}$ DW whereas for the strain CCM-UFV057 it was from 6 to 9 $\mu g \cdot mg^{-1}$ DW (Figures 13A an 14A).

In the log phase, the *Neowestiellopsis* sp. CCM-UFV026 strain showed a decrease in total PB values (Figure 13A) with higher Ci concentrations. This pattern was not observed in the stationary phase of this strain (Figure 13B), in wich the treatments varied the total phycobiliprotein content, but without any clear (increased or decreased) pattern according to Ci concentration. Accordingly, in stationary phase, the strain *Neowestiellopsis* sp. CCM-UFV026 strain showed the lowest value of phycobiliproteins in T6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate) (Figure 13).

In the log phase, the Scytonemataceae strain CCM-UFV057 showed few significant differences among treatments, with treatments C (0.02 $g.L^{-1}$ of sodium carbonate), T3 (0.032 $g \cdot L^{-1}$ of sodium bicarbonate) and T4 (0.16 $g.L^{-1}$ of sodium bicarbonate) being statistically equal (Figure 14A), and T5 (0.8 $g \cdot L^{-1}$ of sodium bicarbonate) showed the lowest values of total PB. In the stationary phase, this strain showed a pattern of decreasing total phycobiliprotein content (Figure 14B) following an increased in Ci concentrations in the treatments, where treatment T1 (0 $g \cdot L^{-1}$ of sodium bicarbonate) and control showed the highest values.

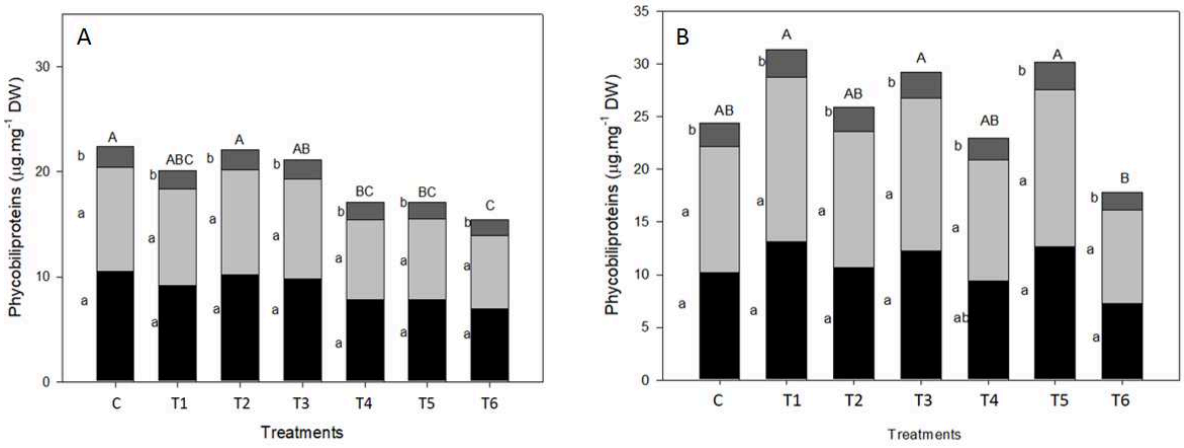

Figure 13. Variation in the phycobiliproteins (PBP) content for the strain *Neowestiellopsis* sp. CCM-UFV026 over log (A) and stationary (B) phases. The PBP content was determined at 12 hours (middle of day). Phycobiliproteins: Phycocyanin (black bars); Allophycocyanin (light grey bars); Phycoerythrin (dark grey bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments on total content of phycobiliproteins, and lower-case letters demonstrate statistical differences between Phycocyanin, Allophycocyanin and Phycoerythrin in the same treatment.

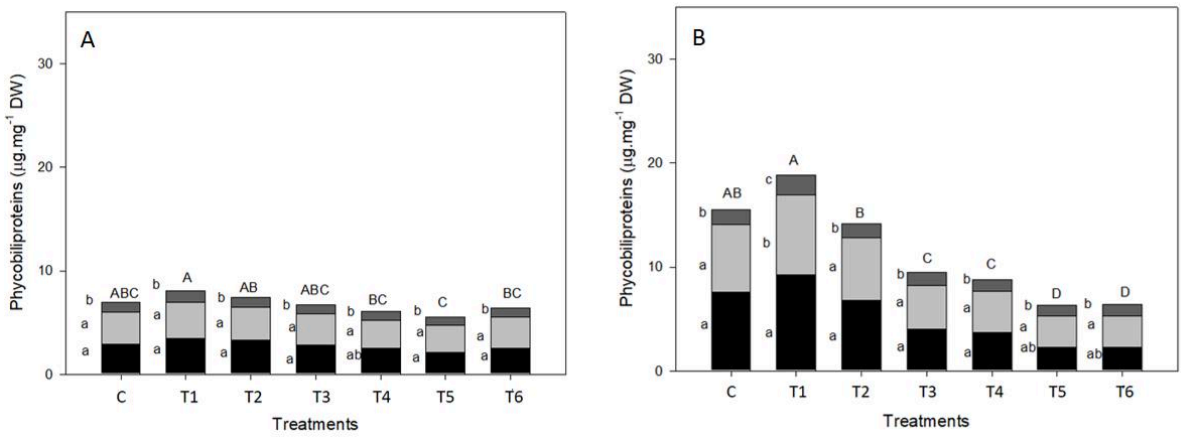

Figure 14. Variation in the phycobiliproteins (PBP) content for the strain Scytonemataceae CCM-UFV057 over log (A) and stationary (B) phases. The PBP content was determined at 12 hours (middle of day). Phycobiliproteins: Phycocyanin (black bars); Allophycocyanin (light grey bars); Phycoerythrin (drak grey bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments on total content of phycobiliproteins, and lower-case letters demonstrate statistical differences between Phycocyanin, Allophycocyanin and Phycoerythrin in the same treatment.

4.4.5 Glycogen

For both strains, no significant differences in the log phases between either treatments or collection times points (Figures 15A and 16A). The strain

Scytonemataceae CCM-UFV057 (Figure 16B) showed higher glycogen contents than the strain *Neowestiellopsis* sp. CCM-UFV026 (Figure 15 B).

Neowestiellopsis sp. CCM-UFV026 on stationary phase presented the lowest glycogen contents at the first time of collection (6 h) on treatments 5 (0.8 $g \cdot L^{-1}$ of sodium bicarbonate) and 6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate) (Figure 15B) which did not occur for the strain Scytonemataceae CCM-UFV057 (Figure 16B).

The strain Scytonemataceae CCM-UFV057 in the treatments 1 (0 $g \cdot L^{-1}$ of sodium bicarbonate), 2 (0.016 $g \cdot L^{-1}$ of sodium bicarbonate) and 3 (0.032 $g \cdot L^{-1}$ of sodium bicarbonate) presented the lowest glycogen content during the stationary phase in the points of 6 and 24h (Figure 16B), a pattern that was not be observed for *Neowestiellopsis* sp. CCM-UFV026 (Figure 15B).

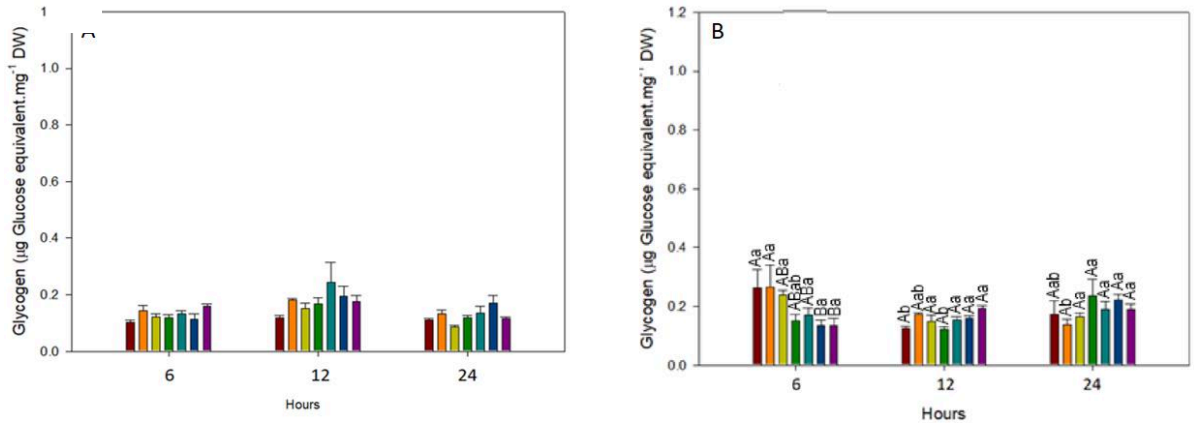

Figure 15. Variation in the glycogen content for the strain *Neowestiellopsis* sp. CCM-UFV026 in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars; T2 (yellow bars; T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments, and lower-case letters demonstrate statistical differences along time. *026 log phase did not show statistical differences.

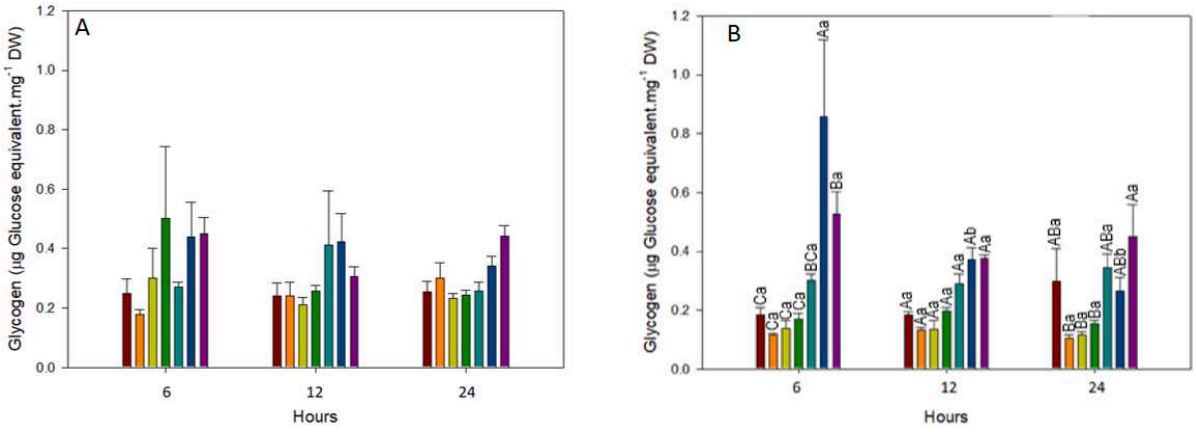

Figure 16. Variation in the glycogen content for the strain Scytonemataceae CCM-UFV057 in three sampling points over log (A) and stationary (B) phases. Treatments: Control (red bars); T1 (orange bars; T2 (yellow bars; T3 (green bars); T4 (Ciano bars); T5 (blue bars); T6 (purple bars). Values are presented as means ± error (n=4). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments, and lower-case letters demonstrate statistical differences along time. Log phase did not show statistical differences

4.4.6 Respiration

The strain *Neowestiellopsis* sp. CCM-UFV026 did not show statistical differences in the log phase for respiration (Figure 17A) unlike the stationary phase, where T3 (0,032 $g \cdot L^{-1}$ of sodium bicarbonate) showed the highest values of respiration (Figure 17B). It is also interesting to note that the treatments with higher concentrations of Ci did not differ statistically from the treatments with lower concentrations, indicating that within the gradient of Ci used in the work, this factor of metabolism did not show an evident pattern of response for strain *Neowestiellopsis* sp. CCM-UFV026 in none of the phases of its bacterial growth, but it seems that intermediary treatments stimulate the increase in the cell respiration.

For strain 057, there were no differences between treatments in any of the growth phases, which may indicate that for this strain, the variation used in the treatments did not significantly affect its respiration.

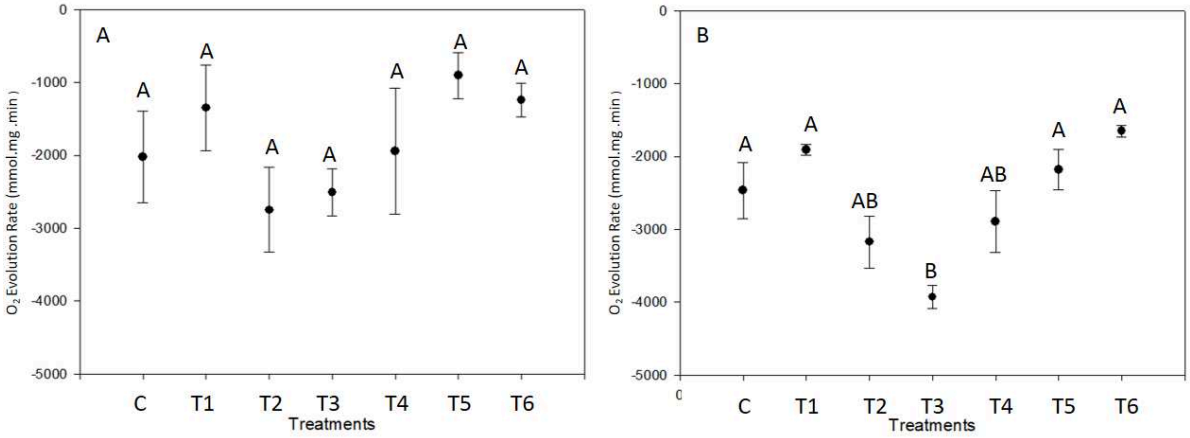

Figure 17. O_2 evolution rate at Clark electrode in 0 µmol photons·m^{-2}·s^{-1} (respiration) to strain *Neowestiellopsis sp.* CCM-UFV026 over log (A) and stationary (B) phases. The respiration was determined at 12 hours (middle of day). Values are presented as means ± error (n=3). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments on O_2 evolution rate at Clark electrode in 0 µmol photons·m^{-2}·s^{-1} (respiration). * log phase did not show statistical differences.

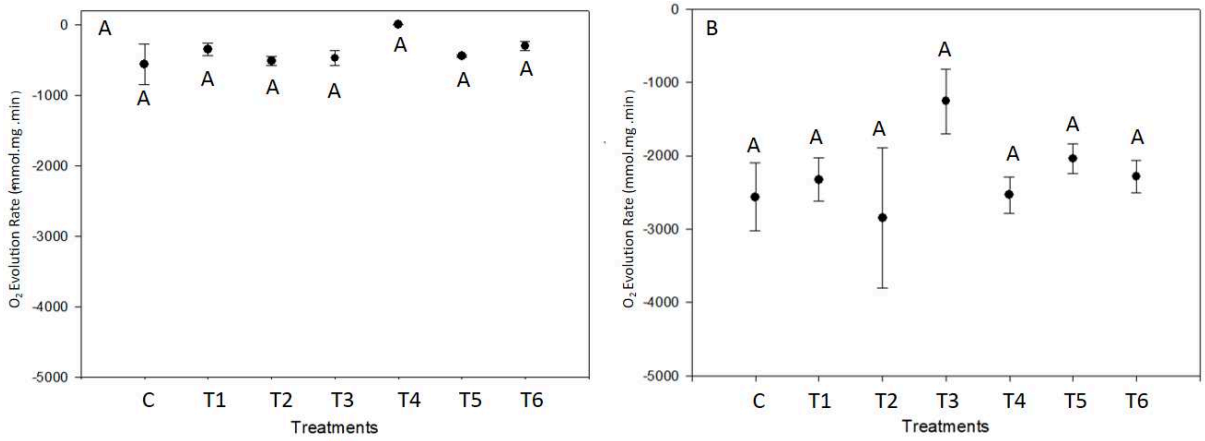

Figure 18. O_2 evolution rate at Clark electrode in 0 µmol photons·m^{-2}·s^{-1} (respiration) to strain Scytonemataceae CCM-UFV057 over log (A) and stationary (B) phases. The respiration was determined at 12 hours (middle of the day). Values are presented as means ± error (n=3). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments on O_2 evolution rate at Clark electrode in 0 µmol photons·m^{-2}·s^{-1} (respiration).* both phases did not show statistical differences.

4.4.7 Photosynthesis

For strain *Neowestiellopsis* sp. CCM-UFV026, the photosynthesis values did not differ statistically in the log phase between treatments (Figure 19A). In the stationary phase, it is possible to note that, as seen in respiration, intermediate treatments, such as T3 (0,032 g·L^{-1} of sodium bicarbonate), had higher photosynthesis rates compared to treatments with higher and lower Ci concentration for strain 026 (Figure 19B), treatment T4 (0,16 g·L^{-1} of sodium bicarbonate) also has higher photosynthesis rates than other treatments.

In the log phase, strain Scytonemataceae CCM-UFV057 presented lower values in treatments T5 (0,8 g·L^{-1} of sodium bicarbonate) and T6 (1,6 g·L^{-1} of sodium bicarbonate), and these treatments already showed photosynthesis negative values (Figure 20 A), also in the log phase, the absolute values of photosynthesis are lower for strain Scytonemataceae CCM-UFV057 compared to the values from strain *Neowestiellopsis* sp. CCM-UFV026 (Figure 19A). In the stationary phase, only the treatments with the smallest Ci concentration remained positive, since treatments C (0,02 g·L^{-1} of sodium carbonate), T5 (0,8 g·L^{-1} of sodium bicarbonate) and T6 (1,6 g·L^{-1} of sodium bicarbonate) presented negative photosynthesis rates, being that T6 (1,6 g·L^{-1} of sodium bicarbonate) were significantly inferior to the others (Figure 20B), presenting absolute value similar to the value it respiration (Figure 18B).

For the stationary phase, strain *Neowestiellopsis* sp. CCM-UFV026 also presented higher absolute values than strain Scytonemataceae CCM-UFV057, including positive values in all treatments.

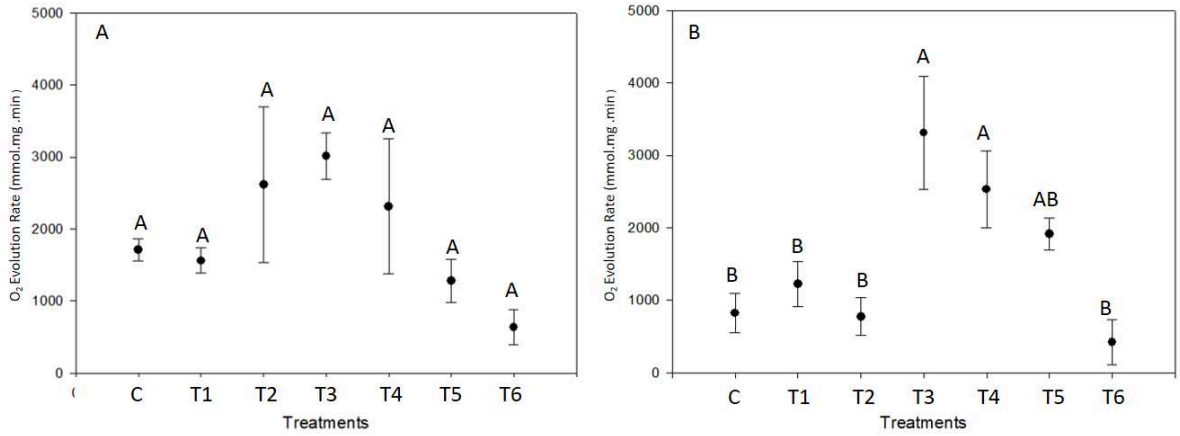

Figure 19. O_2 evolution rate at Clark electrode in 90 µmol photons·m^{-2}·s^{-1} (photosynthesis) to strain *Neowestiellopsis* sp. CCM-UFV026 over the log (A) and the stationary (B) phases. The photosynthesis was determined at 12 hours (middle of the day). Values are presented as means ± error (n=3). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments on O_2 evolution rate at Clark electrode in 90 µmol photons·m^{-2}·s^{-1} (photosynthesis).

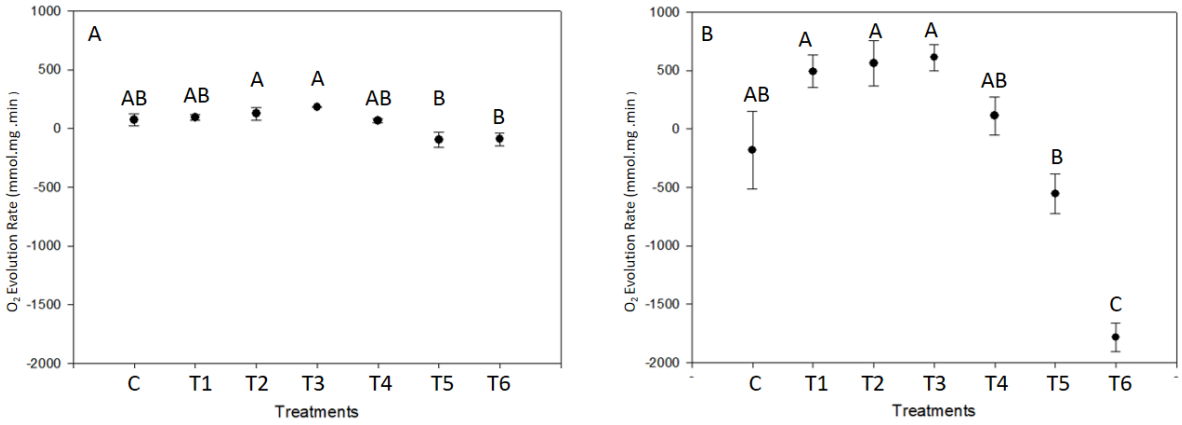

Figure 20. O_2 evolution rate at Clark electrode in 90 µmol photons·m^{-2}·s^{-1} (photosynthesis) to strain Scytonemataceae CCM-UFV057 over log (A) and stationary (B) phases. The photosynthesis was determined at 12 hours (middle of the day). Values are presented as means ± error (n=3). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences between Ci treatments on O_2 evolution rate at Clark electrode in 90 µmol photons·m^{-2}·s^{-1} (photosynthesis). * log phase did not show statistical differences

5. DISCUSSION

The effects of the Ci concentration on growth kinetics have been studied extensively at both cellular and molecular levels (Nguyen and Rittmann 2016). However, studies comparing microcystin-producer heterocytous cyanobacteria at different Ci concentrations are scarce (Castro 2019). To the best of our knowledge, this is the first work to compare growth parameters and the metabolism of two microcystin-producer and morphologically distinct filamentous heterocytous cyanobacterial strains at different Ci concentrations. Possibly more importantly, this study demonstrated that different Ci concentrations differentially affect growth within each strain and also differentially affect growth between the different strains (Tables 1 and 2). Although the highest Ci concentration treatment (1.6 g·L^{-1} of sodium bicarbonate) led to higher *Gt* in the strain Scytonemataceae CCM-UFV057, this was not observed for *Neowestiellopsis* sp. CCM-UFV026, for which treatment 2 (0.016 g·L^{-1} of sodium bicarbonate) led to higher *Gt*. Growth parameters in the log phase for the strain Scytonemataceae CCM-UFV057 are slightly different from the parameters presented by Castro (2019), who analyzed the strain Scytonemataceae CCM-UFV057 on the following Ci concentration: 0,02 g·L^{-1} of sodium carbonate , 0 g·L^{-1} of sodium bicarbonate (T1), 0,016 g·L^{-1} of sodium bicarbonate (T2) and 1,6 g·L^{-1} of sodium bicarbonate. Accordingly, the treatment with 0.016 g. L^{-1} of sodium bicarbonate was not different from the control and in the absence of sodium bicarbonate differed significantly from the others regarding *Gt* values (Castro 2019). In agreement with the

results described here, Vilar and Molica (2020) showed that increased Ci availability for the *Raphidiopsis raciborskii* ITEP-A1 caused a lower growth rate. In the paper, the authors mention that the pH factor can have an impact on the growth of the strain, but Castro (2019) did not observe any impact on the development of strain Scytonemataceae CCM-UFV057 when this factor was changed.

Besides the photosynthetic parameters, the distinct Ci concentrations also differentially affect the metabolic parameters of both strains as shown before by Castro (2019) with the strain CCM-UFV057, where, for example, there was a negative impact on the pigment content for the treatment with where, for example, there was a negative impact on the pigment content for the treatment. The decrease in chlorophyll *a* content was much more pronounced in treatments with high Ci concentrations (0.16, 0.8 and 1.6 $g \cdot L^{-1}$ of sodium bicarbonate). This response was more pronounced for the strain Scytonemataceae CCM-UFV057 in the stationary phase (Figure 8B) than for the strain *Neowestiellopsis* sp. CCM-UFV026. Furthermore, the strain Scytonemataceae CCM-UFV057 showed signs of chlorosis in treatment 6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate) (Figure 8B), which corroborates with the decrease in both growth and pigments (chlorophyll *a* and Phycobiliproteins) in this treatment. Remarkably, in treatments with lower Ci concentrations the strain Scytonemataceae CCM-UFV057 had a higher chlorophyll *a* content than the strain *Neowestiellopsis* sp. CCM-UFV026. The decrease in chlorophyll *a* in treatment 6 (1.6 g. L^{-1} of sodium bicarbonate) for both strains resembles with the data obtained by Castro (2019), which found significant differences between treatment 1.6 $g \cdot L^{-1}$ of sodium bicarbonate and the treatment whit less Ci supply.

The impact of treatment 6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate) on the decrease of phycobiliprotein content for both strains in the stationary phase (Figures 13B and 14B), most likely impacted photosynthetic parameters of both strains (Figures 7B and 8B). Phycobiliprotein and chlorophyll *a* content are directly related to light harvesting by the cyanobacteria (Pagels et al. 2019). In good agreement, Castro (2019) revealed that higher values of phycobiliproteins led to higher photosynthetic efficiency values for the strain Scytonemataceae CCM-UFV057. Furthermore, another indication for treatment 6 (1.6 g. L^{-1} of sodium bicarbonate) to cause a stress is that stressful conditions may cause reductions to the content of phycobiliproteins when compared to optimal cultivation conditions (Simeunović et al. 2013).

Phycobilisomes can act as nitrogen-storage reservoirs (Li et al. 2001). According to Pagels et al. (2019), these pigments are the main storage of nitrogen for the cyanobacterial cell which means in stress conditions, the levels of the use of nitrogen change, once the organism needs both nitrogen but also light harvesting. It is important to notice that in treatment 6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate) for the strain Scytonemataceae CCM-UFV057 in both growth phases exhibited a decrease in amino acid content with the time (Figure 10A and B), which may indicate a conversion of these amino acids into various proteins, supplying the cellular demand and possibly using phycobiliproteins as a source of nitrogen for synthesis It is worth to mention that despite being heterocytous strains, different stresses can negatively affect the rate of N fixation by heterocytes (Fernandes et al. 1993). Differently, a pattern of decrease in amino acid content during the day was not observed in the strain *Neowestiellopsis* sp. CCM-UFV026 for any treatment (Figures 11A and B). According to Flick and Kaiser (2012), protein repair and degradation are possible responses to stress, suggesting that besides the amino acid content being different in both strains under the treatments, the *Neowestiellopsis* sp. CCM-UFV026 is most likely under less stress than strain Scytonemataceae CCM-UFV057 in the higher Ci concentration treatment (1.6 $g \cdot L^{-1}$ of sodium bicarbonate), one of the evidences being the photosynthetic rate presented in the stationary phase by both strains (Figures 19B and 20B), *Neowestiellopsis* sp. CCM-UFV026 showed positive values of photosynthesis regardless of the treatment, while Scytonemataceae CCM-UFV057 showed negative values of photosynthesis in treatments with concentrations of Ci over 0.16 $g \cdot L^{-1}$ of sodium bicarbonate.

In the dark, it is common that there is a depletion in the content of glycogen (reserve carbohydrate) in cyanobacteria, due to the absence of photosynthesis and the need to remobilize energy in the form of ATP in the cell (Cano et al. 2018). Page-Sharp et al. (1998) showed in a *Scytonema* strain submitted to salt conditions that there is an increase in cellular glycogen content, with a decrease in the same when the medium was modified to standard conditions. In this study, the glycogen content varied little between treatments in the strain *Neowestiellopsis* sp. CCM-UFV026 (Figure 15B), indicates that there is cellular demand in these treatments and that excessive accumulation of glycogen is not taking place, this may also be another indication of the greater resistance of the *Neowestiellopsis* sp. CCM-UFV026 strain to the highest concentrations of Ci. The strain Scytonemataceae CCM-UFV057 presents higher glycogen content than under the treatments 4 (0,16 $g \cdot L^{-1}$ of sodium bicarbonate)

5 (0,8 g·L^{-1} of sodium bicarbonate) and 6 (1,6 g·L^{-1} of sodium bicarbonate) through the time on stationary phase (Figure 16B). It seems tempting to suggest that these results could indicate possible stressful conditions and a lower level of energy demand for the cyanobacterial cell, but, as analyzed by the respiratory rate, the treatments do not differ from each other, as it possibly does not corroborate the previously performed glycogen values (Figure 18).

In consonance with the respiration data presented in this work, Jeanjean et al. (1993) showed to strain *Synechocystis* PCC6803 under salt stress an increase on respiration and energy flux in cell.

Further studies are still required to fully elucidate the impact of Ci on the growth of heterocytous cyanobacteria and to what extent it impacts the carbon-nitrogen ratio, since these strains can fix atmospheric nitrogen.

6. CONCLUSION

Many studies have been conducted on microcystin-producing cyanobacteria in attempts to analyze their production or their toxic and harmful effects on mammals, humans and aquatic animals (Butler et al. 2009, Campos and Vasconcelos 2010, McLellan and Manderville 2017). However, the metabolic responses to different environmental conditions in these strains, especially in filamentous heterocytous ones, remain poorly studied. Our results indicate that growth kinetic parameters are not negatively affected in the *Neowestiellopsis* sp. strain CCM-UFV026 following increases in Ci concentrations, but its photosynthesis is affected in the stationary phase, although its pigment content tended to decrease in treatments 4 (0.16 $g \cdot L^{-1}$ of sodium bicarbonate), 5 (0.8 $g \cdot L^{-1}$ of sodium bicarbonate) and 6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate). On the other hand, Scytonemataceae CCM-UFV057 was characterized by a lower growth rate in treatment 6 (1.6 $g \cdot L^{-1}$ of sodium bicarbonate) coupled with intense signs of stress, as a decrease in pigments, high respiration rate and significantly decrease o photosynthesis. Based on the pattern proposed previously by Castro (2019) that already showed the growth parameters and metabolic responses of Scytonemataceae CCM-UFV057 under different Ci concentrations, it is reasonable to suggest that there is a differential response between the two investigated strains, as revealed with the same parameter investigated in this work. The results described here also indicate that coupling other analyses (e.g microcystin production) will provide an interesting approach to an in-depth understanding of the responses of microcystin-producing cyanobacteria strains to different Ci concentrations.

While previous studies indicate a potential involvement of microcystin in the regulation of distinct metabolic pathways, it remains to be determined how exactly the dynamic responses to fluctuations in Ci impact the overall carbon fluxes and how that contributes to the stress responses of microcystin-producer cyanobacteria. In consonance with previous studies, our work, however, provides compelling evidence that different strains of cyanobacteria may respond in a differential way under the sames conditions of inorganic carbon.

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