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PHYCOBILIPROTEINS ACCUMULATION IN A CYANOBACTERIUM ISOLATED FROM AN EXTREME ENVIRONMENT

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Mestrado em Biologia Marinha

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(Antonio Vargas Castelo)

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1. PORTUGUESE ABSTRACT

Desde o século passado, tem-se procurado formas alternativas e sustentáveis para minimizar os problemas relacionados ao desenvolvimento industrial, como o uso de substâncias tóxicas em diferentes aplicações e a escassez de recursos não renováveis. Por isso, a produção de moléculas bioativas através de processos biotecnológicos têm recebido crescente atenção. Essas substâncias são consideradas compostos notáveis, sintetizados por organismos em cultivos, ao final da fase de crescimento exponencial e início da fase estacionária.

Diferentes tipos de micro-organismos podem ser utilizados para a produção das moléculas bioativas, como cianobactérias e microalgas. Alguns deles são capazes de produzir metabólitos secundários, incluindo vitaminas, toxinas e compostos farmacêuticos, com demonstrada atividade antiviral, antibacteriana, antifúngica e anticarcinogênica. Além disso, cianobactérias também têm sido utilizadas como fontes alternativas de energia ou como fertilizantes. Nesse sentido, fica evidente o interesse econômico nesses micro-organimsos para a produção de colorantes naturais para alimentos e pigmentos para cosméticos, substituindo os compostos sintéticos usados em batons, delineadores e outros produtos.

Cianobactérias são considerados os mais antigos micro-organismos fotossintetizantes encontrados na Terra. Eles habitam diversos ambientes, como água salgada, salobra e doce, em fontes termais e frias, e ambientes que não podem ser colonizados por outras microalgas. Além disso, elas são encontradas em ambientes extremos, como vulcões, desertos e na Antártica. A sobrevivência das cianobactérias em tais ambientes inóspitos é possível graças a vias metabólicas singulares, que podem ter importantes aplicações biotecnológicas.

Chroococcidiopsis sp. é uma cianobactéria cuja filogenia ainda está sendo investigada, isolada do Deserto do Atacama, no Chile, pelo grupo de pesquisa da Universidade de Huelva. O Deserto do Atacama é caracterizado por condições ambientais extremas e é considerado o deserto mais árido do mundo, com condições semelhantes a Marte. Para se proteger da elevada radiação ultravioleta e dessecação *Chroococcidiopsis sp.* desenvolveu estratégias adaptativas de sobrevivência ao ambiente hostil. Nesse sentido,

Chroococcidiopsis sp. habita o interior de rochas calcárias, sendo então caracterizadas como cianobactérias endolíticas. Outra características tornam essa linhagem de cianobactéria interessante do ponto de vista biotecnológico, como halotolerância e a sua capacidade de sobreviver em ambientes com restrição de nutrientes e luz.

De uma forma geral, micro-organismos encontrados em ambientes extremos, com elevada dessecação e radiação ultravioleta, produzem substâncias como scitonemina, micosporina, exopolissacarídeos (EPS), carotenoides e ficobiliproteínas para se proteger dos danos associados a essas condições ambientais. Devido à importância das ficobiliproteínas no mercado mundial atual, o presente projeto focou na produção dessas moléculas pela *Chroococcidiopsis sp.* Ficobiliproteínas são pigmentos fluorescentes que ocorrem nos ficobilissomos. Existem três diferentes tipos de ficobiliproteínas: a ficocianina (PC), de cor azul; a aloficocianina (APC), de cor azul-esverdeada; a ficoeritrina (PE) e a ficoeritrocianina (PEC), ambas de cor vermelha. Todas elas apresentam picos diferentes de absorção e emissão no espectro de radiação fotossinteticamente ativa e a cianobactéria é capaz de alterar as suas abundâncias em resposta a diferentes fontes de estresse abiótico (luz, pH, nitrogênio, entre outros).

Considerando-se que: (1) fontes abióticas de estresse como luz, temperatura, nitrogênio, salinidade, pH e metais pesados (como Cu (II) e Fe(II)), influenciam o crescimento e a produção de metabólitos secundários de cianobactérias e microalgas; (2) o ambiente singular do qual a *Chroococcidiopsis* sp. foi isolada e o seu potencial para a produção de ficobiliproteínas; no presente trabalho, foram analisados os efeitos de fontes abióticas de estresse na cianobactéria em condições laboratoriais, com ênfase no efeito da luz e da salinidade na produção de ficobiliproteínas. Dessa forma, foi realizada uma abordagem inicial da avaliação do potencial biotecnológico da nova linhagem de cianobactéria *Chroococcidiopsis* sp.

Em um primeiro momento, foi avaliada a influência da intensidade luminosa no crescimento e conteúdo de ficobiliproteínas. Diferentes regimes de cultivo do micro-organismo foram usados para aumentar a produtividade e reduzir custos de processo. Os regimes mais comuns são cultivos contínuos e de batelada, devido à sua eficiência e simplicidade, respectivamente. Nesse sentido, o cultivo de batelada foi selecionado para a avliação do efeito da luz no crescimento e conteúdo de ficobiliproteínas da *Chroococcidiopsis* sp.. Entretanto, como o cultivo de batelada em larga escala pode

acarretar problemas de contaminação, também foram realizados cultivos semi-contínuos. Esse segundo experimento consistiu em manter a densidade celular dos diferentes cultivos em uma certa faixa de densidade óptica, através da reposição de parte do meio de cultivo consumido por meio novo.

Ademais, uma nova abordagem de cultivo de cianobactérias foi praticada no presente estudo, isto é, o uso de fertilizante como meio de cultivo. Essa alternativa reduz custos do processo e o tempo investido na preparação dos meios de cultivo tradicionais quando se trabalha em larga escala. Para tal substituição, três diferentes tipos de fertilizantes agrícolas (NPKs) foram analisados e os resultados foram comparados com outros trabalhos da literatura, principalmente utilizando microalgas, já que o uso de fertilizantes em cultivos de cianobactérias é escasso.

Finalmente, outro fator abiótico considerado no presente trabalho foi a salinidade. Conforme previamente explicado, *Chroococcidiopsis* sp. está adaptada ao Deserto do Atacama, onde a salinidade pode ser alta em determinadas condições, sendo esperado que esse fator exercesse influência no metabolismo da espécie. Logo, o seu efeito no crescimento e produção de ficobiliproteínas foi avaliado. Para esse experimento, fertilizantes agrícolas foram utilizados ao invés dos meios de cultivo tradicionais.

De acordo com os principais resultados, a produção de ficobiliproteínas em cultura em descontínuo sob o efeito da luz foi muito mais elevada quando foi utilizada uma intensidade de luz de 50 µmol photons·m^{-2}·s^{-1}, atingindo cerca de valores de 45 mg·g^{-1} para ficocianina, 120 mg·g^{-1} para a aloficocianina e 80 mg·g^{-1} para ficoeritrina. Para corroborar esse resultado, analisou-se um experimento em semi-contínuo, o que mostrou o mesmo resultado, mas os resultados mais baixos foram obtidos. Além disso, a utilização de fertilizantes mostrou melhor resultado quando foi adicionado ao meio de cultura, em que 80 mg·g^{-1} de ficobiliproteínas foi obtido durante a experiência. Para se obter um melhor conhecimento da presente estirpe, esta estirpe foi submetido sob diferentes concentrações de sal. Apesar disso, esta espécie só era capaz de crescer sob 0.2 M de NaCl. No entanto, a maior ficobiliproteínas accucumulation foi produzida quando foi cultivada em 0.4 M de NaCl, em que 14 mg·g^{-1} de ficocianina, 25 mg·g^{-1} de ficoeritrina e 35 mg·g^{-1} de aloficocianina foi produzido no fim da experiência.

2. ABSTRACT

Cyanobacteria are photosynthetic microorganisms that can be found in different environments. This type of microorganisms has been gaining attention because of its bio-technological potential. Cyanobacteria have been shown to produce secondary metabolites including vitamins, toxins and pharmaceutical compounds. Some of them have displayed antiviral, antibacterial, antifungal and anticancer activity. Moreover, cyanobacteria have also been used as source of alternative energy or as fertilizers. All this, confirm the commercial interest of such microorganisms.

Chroococcidiopsis sp. is a cyanobacterium originally isolated from Atacama Desert in Chile (phylogeny under study). Atacama Desert is characterized by extreme environmental conditions and it is considered the most arid desert in the world; with similar conditions to Mars. Under such environment, and in order to protect from the high UV radiation and the desiccation, *Chroococcidiopsis sp.* has developed adaptive strategies to survive under these harsh conditions. In this sense, *Chroococcidiopsis sp*. was found to live inside calcite rocks, therefore being characterized as an endolithic cyanobacterium.

Microorganisms found under extreme environments with high desiccation and high UV radiation are known to produce substances such as scytonemin, mycosporine, exopolysaccharides (EPS), phycobiliproteins and carotenoids to protect from the damage caused by this environment. Accordingly, and in order to evaluate the biotechnological potential of this novel strain as a phycobiliproteins producer, its growth and phycobiliproteins content will be analyzed under certain growth conditions such as light, nutrients and salt effect, under laboratory conditions, with special emphasis in phycobiliproteins, which have been already proven to have commercial applications.

According to the main results, the accumulation of phycobiliproteins in batch culture under light effect was much higher when was used a light intensity of 50 µmol photons$\cdot m^{-2}\cdot s^{-1}$, reaching approximately values of 45 $mg\cdot g^{-1}$ for phycocyanin, 120 $mg\cdot g^{-1}$ for allophycocyanin and 80 $mg\cdot g^{-1}$ for phycoerythrin. To corroborate this result, it was analyzed in a semi-continuous experiment, which showed the same result but lower results were obtained. Moreover, the use of fertilizer showed better result when it was added to the medium of culture, in which 80 $mg\cdot g^{-1}$ of phycobiliproteins were obtained during the experiment. To obtain a better knowledge of this strain, this strain was submitted under different concentrations of salt. In spite of it, this species was only able to grow under 0.2

M of NaCl. However, the higher phycobiliproteins accumulation was produced when it was cultured under 0.4 M of NaCl, in which 14 $mg \cdot g^{-1}$ of phycocyanin, 25 $mg \cdot g^{-1}$ of phycoerythrin and 35 $mg \cdot g^{-1}$ of allophycocyanin was produced at the end of the experiment.

3. INTRODUCTION

3.1 Atacama Desert as example of extreme environments.

In general, a desert is known to be a region where an extremely low amount of rain events takes place, even less than the required amount to support some plants life. However, the special category of "hyperarid" is attributed to those deserts which possess very low aridity index (AI) (lower than 0.05,) which is defined as the ratio between mean annual rainfall and evapotranspiration[1]. Among all the deserts, the Atacama Desert is known to be the driest desert on Earth and it extends across 1,000 km from 30ºS to 20ºS along the Pacific coast of South America, between the Pacific coastal range and the Andean Altiplano. Moreover, it has an extreme aridity -rainfall around 3-27 mm y^{-1}-^{2} due to the northward flowing Humbolt current and the strong Pacific anticyclone which prevents the movement of the air from further south, blocking most of air by the Andes[3]. However, many locations of this desert receive marine fog, which provides enough amount of water to allow some organisms to survive under those extreme conditions[4]. In addition, this desert has been characterized as the surface which receives the highest amount of UV radiation (UV index up to 43.3), and photosynthetic active radiation (PAR) (up to 2700 µmol photons·m^{-2}·s^{-1}) on the surface. In this sense, the annual mean surface solar radiation is around 312 W m^{-2} and extremely high temperatures are also found at the rock surfaces up to 68ºC. Because of these characteristics, together with its oligotrophic character and high salinity, make the Atacama Desert to be considered a poly-extreme environment[2]. By other hand, the analysis of its geology and soil characteristics suggest that these extreme conditions have been occurring for 10-15 million years and, because of this fact, it is considered as one of the oldest deserts on Earth. Moreover, Atacama presents no significant latitudinal movement since the late Jurassic, meaning 150 million years ago[5], as a consequence of its geographical location in the dry subtropical climate belt, as well as others facts, which results in the stability and the development of this arid desert[6]

Hyper-arid environments present common characteristics with the planet Mars, such as the cold and the aridity[7]. And specifically, it is said that the soil of the Atacama Desert share three characteristics as "Mars-like"[4]. The first one is that it has low levels of refractory organic material, which means that it does not decompose at the temperatures reached by the Viking Gas Chromatograph Mass Spectrometer (500ºC). The second is the presence of patches of soil with virtually no detectable soil bacteria either by culture or DNA amplification[8] nor by limulus amebocyte lysate. And the third is that the soil has the same

number of oxidized L and D amino-acids and L and D sugars[8], which can be explained by the chemical oxidation and not by the biological activity as it was thought[4]. In spite of sharing these harsh conditions with the planet Mars, many microorganisms have been able to colonize and tolerate the extreme environmental conditions of Atacama. For example, some of them have been able to adapt to live without water and to endure long time of desiccation in the soil crust. In these cases, organisms are in a microenvironment that retains more moisture than ambient conditions and which provides protection from temperature fluctuations and solar ultraviolet radiation[9].

According to the exact place where they live, microorganisms can be classified as endedaphic (in the soil), epedaphic (on the soil surface), hypolithic (under diaphanous rocks), chasmolithic (in cracks in the rocks) and endolithic (inside diaphanous rocks)[1]. In general, these types of organisms dominate the microalgal community of hot deserts, including non-vascular photosynthetic organisms, fungi and bacteria, and they all form intimate associations with the soil. These associations produce an alteration of the physiochemical composition of the soil and, as a consequence, they affect different characteristics of the soil such as development, stability, fertility and the water regime. Apart from that, these microorganisms are the main group of life which inhabits in hyper-arid hot and cold deserts and they support the primary productivity and nitrogen input in such extreme environmental conditions[6].

3.2 Cyanobacteria.

In general, Cyanobacteria have the capability to colonize many unknown places and they are considered to be one of the most diverse organisms as they comprise about 150 genera with more than 2,000 species. Moreover, they are considered to be the oldest group of Gram-negative organisms which are capable of doing the plant-like oxygenic photosynthesis[10]. And it has also been considered that plants' chloroplast come from them by endosymbiosis[11]. However, they are considered to be more efficient than plants from a photosynthetically point of view, because they require minimal amount of nutrients for growth, such as carbon dioxide, water and some salts while deriving energy from light. The cyanobacterial community of deserts has been described in many studies and 481 cultures of cyanobacteria have been already isolated from the Atacama Desert by culture-based approaches[1]. However, they are still in the process of being precisely identified by combination of morphological and molecular tools, although most of them are considered

to be novel forms[1,12]. One of the new novel strains is *Chroococcidiopsis* sp., an endolithic and thermophilic colonizer cyanobacteria that inhabits in rocks in the driest parts of the Atacama Desert where no visible life forms exist on rocks surfaces[6]. Members of this genus are very primitive, photosynthetic, coccoid cyanobacteria with the ability to tolerate extreme conditions such as high radiation, extreme temperatures, osmotic stress, nutrient deprivation and extreme pH values. Apart from being found in deserts, *Chroococcidiopsis* sp. has been found in many places, for example in freshwater, marine or hypersaline environments[13], in hot springs, nitrates caves[14,15], cold deserts, airspaces of porous rocks from Antarctic and valleys and in several liches as cyanobionts. All this shows their capability to colonize many unknown places[16] .

3.3 Influence of abiotic factors on cyanobacterial growth.

There is a wide range of essential environmental factors that control the cyanobacterial growth and their photosynthetic rates, such as appropriate nutrients availability, temperature, pH, buoyancy and light, between others[17]. Among all these abiotic factors, sunlight is the unique form of energy available and inexpensive on Earth for photosynthetic organisms[18]. In addition, light is considered to be an essential factor for photosynthetic organisms' growth and pigment accumulation. Cyanobacteria, as any plant, are able to convert light in the PAR range -from 400 to 700 nm- into biomass. However, cyanobacteria present higher conversion efficiencies of solar energy to biomass than plants, up to 3-9%[18] .It can be explained by the ability of cyanobacteria to control the amount of photosynthetic antenna pigments according to the spectrum of ambient light and they are also able to respond against the excess of light by modifying their physiological mechanisms to reduce light efficiency in order to result in optimal productivities. All this is known as chromatic adaptation and it happens as a result of the moderation of the red colored phycoerythrin and the blue-colored phycocyanin, with a predominance of phycoerythrin in green-light-grown cells and of phycocyanin in red-light grown cells[19].

As it is known, cyanobacteria, as other microorganisms, need a source of energy, water and carbon molecules that include some essential elements such as hydrogen, oxygen, nitrogen, phosphorus and sulfur (HONPS)[20]. These elements are essential for their growth. Nitrogen, an important factor on cyanobacterial growth[21], can be assimilated in different forms such as atmospheric nitrogen, nitrate, nitrite, ammonium, urea, cyanate, and some amino acids[22,23]. However, ammonium is, in general, the preferred nitrogen source for

many strains of cyanobacteria[24], as it is the most reduced inorganic form of that element[24]. Moreover, when ammonium is present in the growth medium together with other nitrogen forms, ammonium is normally used first due to the reduction of nitrogen assimilatory enzymes as a result of a process known as global nitrogen control in which it results in the inhibition of combined nitrogen transport systems[25,26]. In case of nitrite, it can be assimilated passively by diffusion through the cell membrane when it is protonated but essential transporters are necessary to concentrate them inside the cell[27]. Nitrate, in order to be assimilated, it is needed to be reduced to ammonium and this process is taken by nitrate and nitrite reductase[24]. These two processes are energetically costly as they consume up to 30 % of the reducing equivalents by the photosynthesis process[27]. Finally, there are organic nitrogen sources, such as urea, which can cross the lipidic bilayer passively[28,29]. The presence of such compounds is very limited in the oceans, ranging from 0.1 to 1 µmolar, but it increases in coastal zones and estuarine environments[30,31]. However, some cyanobacteria are able to take it up at lower concentrations than 1 µM as a result of possessing a special type of transporter which is known as urea ABC-type[24]. Still, before the urea can be assimilated, this substance needs to be hydrolyzed to ammonium and CO_2 by an enzyme known as Ni_{2+} urease[32]. Other cyanobacteria can fixate directly nitrogen molecules under what seems to be aerobic conditions. The ambient nitrogen molecules will be reduced to ammonium, but this process only takes place when the organisms are facing nitrogen deprivation. Nevertheless, this process is uncommon for many strains like *Arthrospira maxima* and *Synechocystis* [33,34]because it implies an expensive energetic metabolic reaction which is catalyzed by Nitrogenase. This enzyme is inhibited by the presence of O_2 and, because of that, this reaction is temporally separated from photosynthesis[35]. In this sense, many studies have found a peak of this enzyme's activity 12 hours after photosynthesis, when high rates of respiration occur, leading to the protection of cells from denaturation[24].

Under a stressful situation, such as nutrients limitation, different types of reserved substances can be produced[36]. In case of nitrogen limitation, cyanophycin is the most common nitrogen source for most of cyanobacteria[22] and, it is considered to be a more dynamic reserve than phycobiliproteins[37].

As it was mentioned previously, some cyanobacteria can live under very extreme conditions, for example in places with drastic variations of saline level[38]. Because of that,

the salinity is also considered to be an important factor for cyanobacteria growth. This abiotic element can be tolerated, until a certain extent, by many microorganisms. However, their capacity to tolerate it varies according to the presence or absence of physiological mechanisms, like the accumulation of inorganic or organic osmoregulators and the active extrusion of sodium from the cell interior[39,40]. In case of some cyanobacterial strains, such as *Spirulina*, salinity is experienced as a stress factor and it usually leads to photoinhibition of photosynthesis and to an increase of metabolized carbohydrates[41–43].Moreover, this stress results in other types of consequences such as the structural degradation of the center cores of photosystem I and II, and protein.

The basic physiological response of cyanobacteria cells to salt stress has been investigated in detail. The response takes place in three phases. Firstly, there is a sudden increase in the ambient concentration of NaCl which induces the influx of sodium and chloride ions to the cytoplasm. The second phase occurs in hours and it produces the replacement of sodium ions by potassium ions, reducing the toxic effect of a high concentration of sodium ions inside the cells. Finally, the third is the longest phase as it can last several hours. Moreover, it is considered to be an important phase as the cells have to adapt to high ions concentration. In order to mitigate the toxic effect of the ions, some compatible solutes are produced to conserve the structure of proteins and cell membranes apart from being activated the electron transport via PSI[44].

As a result of the adaptation of cells to different abiotic stress many substances are produced by cyanobacteria to mitigate different types of damage to the cells. Such substances could play an important role from a biotechnological point of view in diverse areas such as agriculture, aquaculture, pollution control (bioremediation), bioenergy and biofuels, and nutraceuticals. In this regard, bioactive pharmacological compounds (e.g. antibacterial, antifungal, antiviral, anticancer, muscle relaxants) and high-value products, such as polyunsaturated fatty acids (PUFA), phycobiliproteins, vitamins and enzymes of high demand are considered metabolites of interest produced by cyanobacteria[45–47].

3.4 Phycobiliproteins.

Phycobiliproteins (PBPs) are fluorescent antennae-protein pigments located on the phycobilisomes, the major light-harvesting complexes present in cyanobacteria (blue-green algae, prokaryotic), rhodophytes (red algae, eukaryotic), cryptomonas (biflagellate unicellular eukaryotic algae) and cyanelles (endosymbiotic plastid-like organelles)[48–50]. In case of cyanobacteria and red algae, four different types of phycobiliproteins exist: blue colored phycocyanin (PC), bluish green colored allophycocyanin (APC), red colored phycoerythrin (PE) and phycoerythrocyanin (PEC). Their corresponding maximum of light absorbance are 650-655 nm, 615-640 nm, 565-575 nm and 575 nm respectively. And their light emission peaks are 660 nm, 637 nm, 577 nm and 607 nm respectively[51].

Phycobiliproteins are covalently attached to open chain tetrapyrroles and comprised up to 60 percent of the total soluble protein content of the cells[51]. However, the composition and content of phycobiliproteins are influenced by different variables such as light and pH of the medium. For instance, variable spectral proportions of light like red:far red, blue:red, green:red and blue:green, affect the pigment composition and the photo-morphogenic signal in algae[52,53]. Moreover, it has been studied that Nitrogen has an important role as it alters the composition and abundance of these secondary light pigments since phycobiliproteins[45] are known to have a secondary role as intracellular nitrogen storage compounds[54,55]. For example, when cyanobacteria are grown under nitrogen starvation, phycobiliproteins are degraded in a process well known as chlorosis or bleaching[48]. This process consists of a proteolytic degradation of different types of pigments such as phycoerythrin, allophycocyanin and chlorophyll a, and it produces some alterations in the absorption and transference of energy for photosynthesis[53].

3.5 Applications of phycobiliproteins.

In general, phycobiliproteins and carotenoids have a high economic value since they can be used in different sectors such as Food, Nutraceuticals, Biotechnological industries (Fluorescent agent) and Biofertilizers[56]. In nutraceuticals and pharmaceutics these molecules are used in the prevention and treatment of certain diseases as a consequence of their role as antioxidants, anticancer, antituberculosis, antiviral, neuroprotective, anti-inflammatory, hepatoprotective and hypocholesterolemia[57].

The anti-inflammatory activity of carotenoids resides in their antioxidant properties against the reactive oxygen species. In addition, phycocyanin also inhibits the formation of leukotriene, an inflammatory metabolite of arachidonic acid[58]. In this sense, the cyanobacteria *Anizomenon flos-aquae* has been proposed as a good anti-inflammatory source as it contains high amounts of omega-3-alpha linolenic acid [59]. And finally, other pigments as Scytonemin has shown the same anti-inflammatory properties in human disorders like psoriasis and rheumatoid arthritis[60].

About the antiviral effect, certain extracts of the blue-green algae like *Phormidium tenue* has been used against the HIV virus[61]. These extracts are able to stimulate the production of sulfonic acid, which contains glycolipids, a new class of HIV inhibitors. In addition, other substances isolated from cyanobacteria has been used as inhibitors like Cyanoviridin-N and Calcium spirulan[62].

From an anticancer point of view, many types of cyanobacteria and microalgal extracts have been used such as the obtained with S*pirulina fusiformis* and *Dunaliella*. These extracts were found to inhibit the chemically induced carcinogenesis in model hamster buccal pouches. In addition, these microorganisms produce different types of molecules which have anticancer properties as they inhibit the tumour invasion and metastasis of melanoma cells. In the case of *Aphanizomenon flos-aqua,* such molecules include sulphated polysaccharides or Calcium spirulans[62].

From a commercial point of view, cyanobacterial phycobiliproteins have gained importance in different applications such as natural food colorants with non-toxic and non-carcinogenic activity in the pharmaceutical sector[45]. As example, phycobiliproteins are used in cosmetic preparations by replacing the synthetic dyes in lipstick and eyeliners. The prices of this substances vary from US$ 3-25 mg for food/cosmetic grade pigments but they can reach US$ 1500 mg for highly purified molecular markers (with antibodies or other fluorescent molecules). Although the cost of these substances have changed from 1997, the global market of phycobiliproteins was estimated to be more than US$ 50 million that time[63].

In case of Phycocyanin and Phycoerythrin, both are used as natural pigments and fluorescent proteins with applications as food colorants in chewing gum, jellies, health drinks, ice sherbets, popsicles, candies and dairy products[56]. Moreover, phycobiliproteins

have been used in clinical and immunological research laboratories as labels for antibodies, receptors, in immunolabeling experiments, in fluorescence microscopy and diagnostic, and as biological molecules in fluorescence-activated cell sorters[64].

3.6 Towards the commercial production of phycobiliproteins: new strains and the potential of agricultural fertilizers.

Commercial exploitation of the production of phycobiliproteins has only been achieved for few strains, such as *Spirulina* (cyanobacteria), *Porphyra* (seaweed) and *Porphyridium* sp. (red microalgae)[65]. Therefore, there is room for the evaluation of new organisms with the ability to produce phycobiliproteins. In this sense, to boost the production of phycobiliproteins new hyper-accumulating organisms are needed. But also, the production process can be optimized in terms of costs and easiness of operation. In this sense, the use of agricultural fertilizers (NPKs) as culture media, instead of the traditional chemicals, has been proposed as a new strategy. There are few studies related to the use of NPKs for the cultivation of microalgae[66,67]but little is known about their applicability in the cultivation of cyanobacteria. In this sense, it was decided to evaluate the potential use of NPKs for the cultivation of *Chroococcidiopsis* sp. For that, different NPKs, with different nitrogen source and N/P ratio, were used and the effect over growth and phycobiliproteins production was assessed.

4. PROJECT AIM

This study can be considered as a first approximation to the evaluation of the biotechnological use of a novel cyanobacterium for phycobiliproteins production. In this sense, the effect of different abiotic factors, light and salinity, in the growth and phycobiliproteins content of *Chroococcidiopsis* sp. was evaluated. Besides, and with the aim to contribute to a more sustainable production, the applicability of agricultural fertilizers as culture medium was also assessed.

5. MATERIAL AND METHODS

5.1 Sampling site

This novel strain was obtained from the Atacama Desert which is located in South America, concretely in Chile. The samples of calcite rocks were obtained by the components of a group of Bioprospection focused in extremophiles species. After that, the samples were surrendered to the Ciderta group to isolate the microorganisms and to perform the evaluation of the strain.

Figure 1: Sampling site in the Atacama Desert, where the samples were taken and (a) rock sample where *Chroococcidiopsis* sp. was isolated from.

5.2. Standard cultivation conditions.

The cells of the novel cyanobacteria isolated from the Atacama Desert (Figure 1), *Chroococcidiopsis* sp., were grown in 1L Erlenmeyer flasks in batch mode in a thermostated culture room at 25ºC. Unless otherwise indicated, the culture was grown at pH 7 in a Bold's Basal culture medium (BBM) whose composition is shown in Table 1.

Table 1. Composition of BBM medium[68] .

Components	$g \cdot L^{-1}$
$NaNO_3$	5
$Co\ (NO_3) \cdot 6\ H_2O$	$0.049 \cdot 10^{-2}$
KH_2PO_4	0.175
K_2HPO4	0.075
$FeSO_4 \cdot 7H_2O$	$0.498 \cdot 10^{-2}$
$CaCL_2$	$1.887 \cdot 10^{-2}$
$MgSO_4 \cdot 7H_2O$	0.153
$ZnSO_4 \cdot 7H_2O$	$0.088 \cdot 10^{-1}$
$CuSO_4 \cdot 5H_2O$	$0.157 \cdot 10^{-2}$
$MnCl_2 \cdot 4H_2O$	$0.144 \cdot 10^{-2}$
EDTA (acid)	0.05
$Na_2MoO_4 \cdot 2H_2O$	$0.108 \cdot 10^{-2}$
H_3BO_3	$0.114 \cdot 10^{-2}$

The pH of the cultures was adjusted to 7 by addition of 10% NaOH (v/v) or 3.7 % HCl (v/v). After that, the media were sterilized in an autoclave during 25 minutes at a temperature of 120ºC and 1 atm of pressure. The main purpose of this process is to avoid the contamination with other microorganisms.

During standard cultivation, but also during the different experiments unless otherwise indicated, initial inoculation of the different cultures was carried out from a mother culture maintained as explained above in media BBM. To do that, a certain volume of the mother culture, previously calculated based on the cell density, was transferred to centrifugation tubes. These tubes were centrifuged during 5 min at 3000 g (maximal speed). After that, the obtained pellets were dissolved in the corresponding volume (i.e. 500 ml) of fresh culture medium (BBM). During the experimental time, which normally lasted 15 days, different analyzes were performed, such as dry weight, optical density and phycobiliproteins content.

5.3 Culture medium prepared with fertilizers

In order to evaluate the applicability of fertilizers three different NPKs were used: 4-10-10, 18-6-6, and 8-6-6. The agricultural fertilizers present different composition regarding the Nitrogen, Phosphorus and Potassium components which are represented as percentage (%) of the fertilizers (NPK) which are representing in the table 2. Moreover, each of them were chosen depending on the nitrogen source, which can be in the form of urea, ammonium and nitrate. Moreover, the fertilizers have different N/P ratio, which is considered to be an essential factor on the growth of photosynthetic microorganisms.

Table 2. Concentration of the main components of fertilizers: nitrogen, phosphorus and potassium; N/P ratio and nitrogen source. Information regarding the reference media BBM is also present.

Fertilizers	N ($mol \cdot L^{-1}$)	P ($mol \cdot L^{-1}$)	K ($mol \cdot L^{-1}$)	N/P	
4-10-10	2.611	0.845	2.548	3.1	4% ammonium
18-6-6	8.009	0.524	1.580	15.3	15.9% urea, 2.1% ammonium
8-6-6	3.635	0.495	1.490	7.3	6% Urea, 2% ammonium
BBM	0.009	0.002	0.084	5.2	

The volume of fertilizers used to prepare the different media was adjusted for each culture medium in order to ensure the final nitrogen concentration was 9mM in every culture media, the same as in the reference media BBM. In this sense, 3.4, 1.1 and 2.5 $mL \cdot L^{-1}$ of 4-10-10, 18-6-6 and 8-6-6 were used to prepare the culture media. It resulted in the same N final content although the amount of phosphorus, another main nutrient for cyanobacterial growth, was different. In this sense, the final media presented 2.87 mM, 0.58 mM and 1.24 mM of phosphorous respectively. Finally, the different culture media were supplemented with a commercial solution of micronutrients in order to avoid growth limitations by main trace elements (Table 3).

Table 3. Composition of AG complex micronutrients solution added to the culture media prepared with fertilizers.

Micronutrients	% p/v	($mol \cdot L^{-1}$)
B	0.4	0.370
Cu	0.2	0.031

Fe	5.1	0.913
Mn	2.6	0.473
Mo	0.1	0.01
Zn	0.6	0.092

Finally, a solution of $CaCL_2$ and another one of $MgSO_4$ were added to the media prepared with the fertilizers with the purpose of avoiding the deficit of both elements. Noteworthy, these solutions can be avoided when using tap water in large-scale cultivation.

5.4. Culture media prepared at higher salinity

To obtain information regarding the effect of salinity in *Chroococcidiopsis* sp., different cultures were prepared in Erlenmeyer flasks at different salt concentration (NaCl), in particular 0.2, 0.4 and 0.6 M. The best NPK previously identified was used as culture media and a reference control without NaCl was used to compare the evolution of this strain during the experiment.

Firstly, some calculations were done to know the amount of NaCl that correspond to each Molarity. To do this, this equation was used:

$$M = \frac{n}{V} \; ; \; M = \frac{\frac{gr}{pm}}{V}$$

$$M_1 = \frac{\frac{gr}{pm}}{V} ; 0.2 = \frac{\frac{gr}{36.5}}{1} ; 0.2 = \frac{gr}{36.5} \rightarrow 36.5 \cdot 0{,}2 = gr_1 = 7.3 \text{ NaCl}$$

$$M_2 = \frac{\frac{gr}{pm}}{V} ; 0.4 = \frac{\frac{gr}{36.5}}{1} ; 0.4 = \frac{gr}{36.5} \rightarrow 36.5 \cdot 0{,}4 = gr_2 = 14.6 \text{ NaCl}$$

$$M_3 = \frac{\frac{gr}{pm}}{V} ; 0.6 = \frac{\frac{gr}{36.5}}{1} ; 0.6 = \frac{gr}{36.5} \rightarrow 36.5 \cdot 0{,}6 = gr_3 = 21.9 \text{ NaCl}$$

Once the amount of NaCl needed for each condition was calculated, it was added to one littler Erlenmeyer flask with water. After that, the flasks were autoclaved and the NPK was added to each bottle under sterile conditions.

5.5. Experimental conditions of the experiments with different light intensities.

Chroococcidiopsis sp. was cultivated in batch mode under different light intensities:10, 50, 100 and 150 µmol photons·m^{-2}·s^{-1} (Figure 2). The distance of the different Erlenmeyer flasks containing the cyanobacteria to the fluorescent lamps was adapted in order to supply the desired intensities at the surface of the flasks. A lux meter was used to measure the amount of light that reached the cultures. The media used for the experiments was normal BBM.

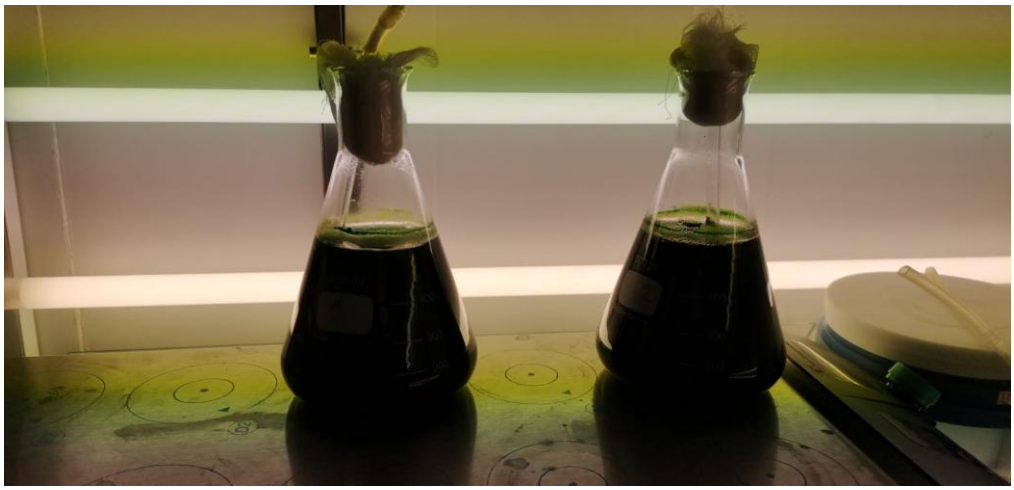


Figure 2: Picture taken of the different cultures at the beginning of the experiment with different ligth intensities (10, 50, 100 and 150 µmol photons·m^{-2}·s^{-1}).

5.6. Determination of dry weight

To determine the dry weight of *Chroococcidiopsis* sp. glass microfiber filters were used (Filter lab MFV5) with a diameter of 47 mm and a pore size of 0.7 µm (Figure 3A and 3B). Previously to the filtration of the culture broth, the filters were prewashed with deionized water and dried at 100ºC overnight. Once it was done, the dry empty filters were weighted in a precision scale.

The volume of the sample to be used (3- 5 mL) was adapted according to the culture's cell density. Once the filtration of the sample was performed, the filters were washed several times with deionized water to remove adhering inorganic salts. The wet filters containing the samples were dried at 100ºC overnight and, after that, were placed in a desiccator until they reached the ambient temperature. Finally, filters were weighted in a precision scale.

The dry weight is equivalent to the cell biomass contained in the filtrated volume. This data was obtained by calculating the difference between the initial weight of the empty filters and the weight of the filters after doing the filtration, all of them previously dried and cooled down.

Figure 3: Image of filters before (left) and after (right) their use for the dry weight determination.

5.7. Determination of optical density

The turbidity of the culture medium is proportional to the cell density, which allows to more quickly determine the growth of the culture if compared to the determination of dry weight. The difference in the light reaching the detector of a spectrophotometer between a sample and a blank, light which has not been absorbed but deflected or scattered by the cells in suspension, is an indirect way to estimate the biomass.

In this work these analyzes were performed in a Thermo Scientific–Evolution 201 UV spectrophotometer (Figure 4), set with a wavelength of 750 nm and 680 nm. Absorbance at 750 nm is widely used as an indicator of microalgal cell density and absorbance at 680 nm as indicator of the pigments density. Moreover, the absorbance ratio 680/750 can also be used as an indirect way to estimate the chlorophyll content of the biomass.

Finally, a correlation factor can also be calculated between the optical density at 750 nm and the dry weight of a certain experiment. That way, the determination of dry weight, which is time-consuming and expensive, can be avoided and absorbance data can be used to calculate the corresponding dry weight.

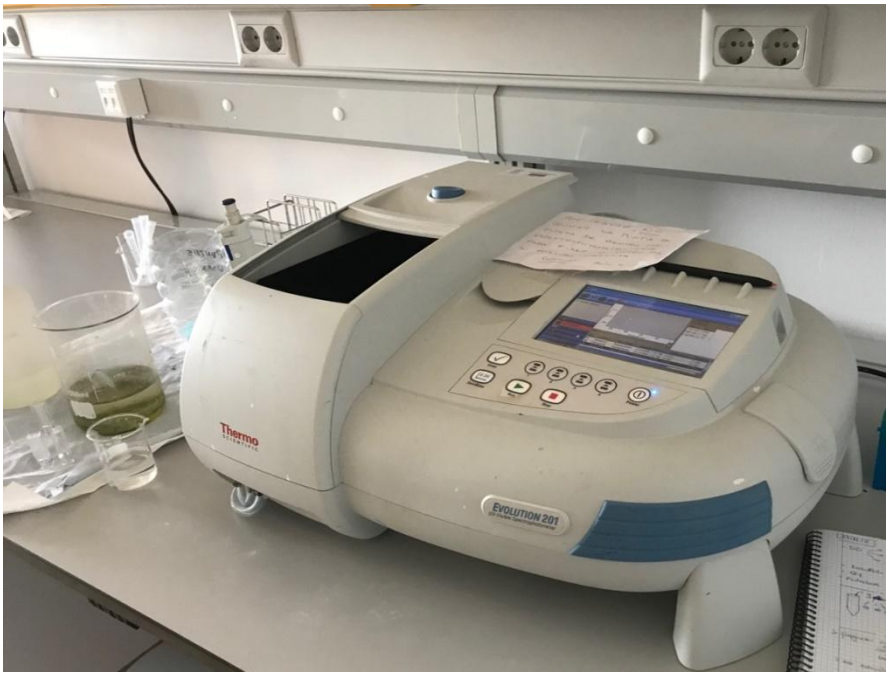


Figure 4: Thermo Scientific-Evolution 201 UV spectrophotometer used for the absorbance determination.

5.8. Calculation of Biomass productivity

The Biomass productivity was calculated as the increment of biomass, measured as dry weight, along a specific period of time. In the case of maximal biomass productivity, it was calculated during the exponential phase of growth.

5.9. Maximal photosynthetic efficiency of Photosystem II (Quantum yield, QY)

Chlorophyll fluorescence has been used as a potential tool to evaluate the photochemistry of photosystem II (PSII) and the cellular stress, considering the production of fluorescence is used as an alternative path in which the chloroplast dissipates the excess of energy.

Figure 5: Image of AquaPEN AP100.

The maximal efficiency of PSII, or quantum yield, was measured by the PAM (*Pulse Amplitude Modulation)* technique. To determine it, an AquaPEN AP100 (Photosystem Instruments (Figure 5), was used. An aliquot of the culture broth was incubated in the darkness in a cuvette during 15 minutes to ensure all the reaction centers of photosystems were oxidized. Subsequently, the sample was exposed to a saturating light pulse which resulted in the saturation of the reaction centers and the corresponding emission of fluorescence.

5.10. Pigments extraction and determination

The pigments extraction was performed in 2 steps. First, 2mL of cyanobacterial culture were placed into Eppendorf tubes (the amount of cell suspension required for analysis can vary according to the cell density of the culture). After that, the cells were centrifuged at 14,000 g for 5 min and supernatant was discarded. Glass beads of 0.25-0.5 mm and 1mL of methanol were added to the different samples and the cells were repeatedly disrupted in a bead miller (Restch MM400) during 5 cycles (5 min max speed -40Hx-; 30 seconds pause). Then, the cells were centrifuged again at 14,000 g for 10 min. After that, the supernatant was carefully removed with a glass Pasteur pipette and transferred into a new (clean) Eppendorf tube. These Eppendorf tubes were used to measure the chlorophyll *a* and total carotenoids content contained in the resulting methanolic solution.

Secondly, 1 mL of methanol was added to the resulting pellet and the mixture was vortexed for 10 seconds and centrifuged (14,000 g during 10 min). The supernatant was carefully discarded and, again, 1 mL of methanol was added to the pellet. Finally, the samples were

centrifuged at 14,000 g during 10 min and, after that, a blue pellet was obtained. This blue pellet was used to analyze the phycobiliproteins content. For that, it was necessary to add 1mL of phosphate buffer (0.1 M; pH= 7) to the blue cell pellet. Continuously, the samples were vortexed for 30 seconds and placed into a water bath with ultrasound where temperature was maintained between 27-30ºC. Incubation in the water bath was carried out for 2 hours with vortexing steps of 20sec every 20 min. Finally, the samples were centrifuged at 14,000 g during 10 min. Then, the supernatant was transferred into a clean Eppendorf tube by a glass Pasteur pipette and the phycobiliproteins content was analyzed

5.10.1. Determination of chlorophyll *a* and total carotenoids

The analysis of chlorophyll *a* and total carotenoids was done according to the reference of Zavrel and coworkers[69]. Firstly, the absorbance of the methanolic extracts (section 2.7) was measured at 470 nm, 665 nm and 720 nm (the final absorbance value at each wavelength should be in the linear absorbance range), using methanol as blank. Secondly, the concentration of chlorophyll *a* and total carotenoids was calculated according to the following equations:

$$Chl_a = 12.9447\ (A_{665} - A_{720})$$

$$Total\ carotenoids = \frac{[1{,}000\ (A_{470} - A_{720}) - 2.86\ (Chl_a[\mu g\ mL^{-1}])]}{221}$$

The final pigment concentration in the samples in µg mL^{-1} is obtained as the calculated pigment concentration multiplied by the dilution factor (volume of methanol/volume of sample.

5.10.2. Determination of Phycobiliproteins (PBS)

To analyze the phycobiliproteins content the absorbance of the aqueous extracts (section 2.10.) was measured at 565 nm, 620 nm and 650 nm (the final absorbance at each wavelength should be in linear absorbance range), using phosphate buffer as blank. After that, the concentration of phycobiliproteins content was calculated by Bryant equations, as described by Chapman and Kremer[70].

$$Phycocianin[CPC; mg\ mL^{-1}] = \frac{[A_{620} - (0.72\ A_{650})]}{6.29}$$

$$Allophycocianin\ [CAC; mg\ mL^{-1}] = \frac{[A_{650} - (0.191\ A_{620})]}{5.79}$$

$$Phycoerythrin\ [mg\ mL^{-1}] = \frac{\left[A_{565} - \left((2.41\ CPC) - (1.41\ CAPC)\right)\right]}{13.02}$$

The yield of phycocyanin is calculated as:

$$Phycocianin\ yield = \frac{(PC \cdot V)}{DB}$$

where *PC* is Phycocyanin concentration ($mg\ mL^{-1}$), *V* is the volume of solvent (mL) and *DB* is dry biomass weight (g).

5.11. Statistical analysis

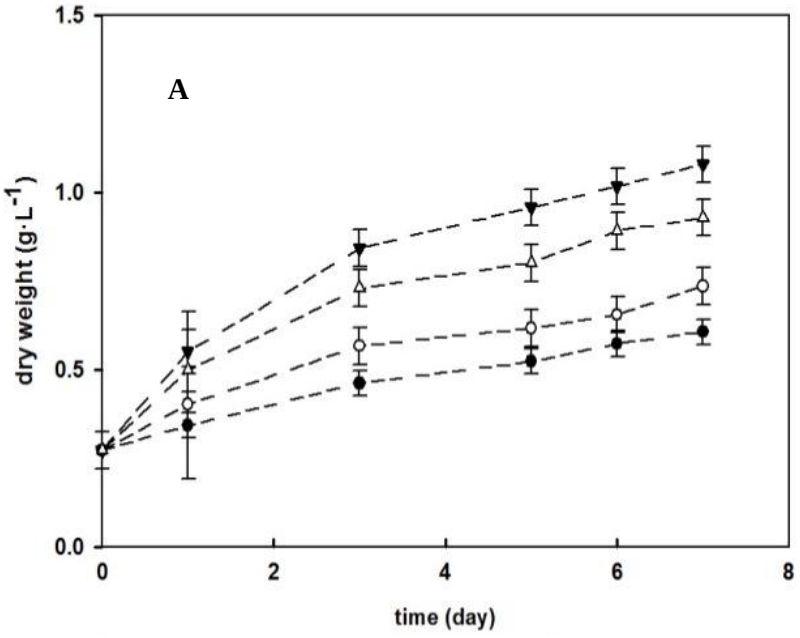
To determine if the different parameters (optical density, chlorophyll, carotenoids and phycobiliproteins content) show significant differences under the different culture conditions (light, fertilizers and salinity effect), the obtained results were evaluated by one-way ANOVA and the Turkey's test using the PRISM 7 software. Moreover, these values were considered statistically significant when $p < 0.05$.

6. RESULTS

6.1 Influence of light intensity in *Chroococcidiopsis* sp. during batch cultivation

During the first part of this Master Thesis (from November 2018 to January 2019), the assessment of growth and pigments concentration in *Chroococcidiopsis* sp. was carried out under different light intensity conditions. *Chroococcidiopsis* sp. was submitted to different light intensities (10, 50, 100 and 150 µmol photons·m^{-2}·s^{-1}) and batch cultivation was carried out in Erlenmeyer flasks in a cultivation room thermostated at 25ºC. Growth was daily analyzed along the experiment by measuring optical density of the samples at a wavelength of 750 nm (Figure 6A) and the dry weight (Figure 6B).

As it is shown in Figure 6, *Chroococcidiopsis* sp. was able to grow under all the light intensities employed although the best results were found at a light intensity of 100 µmol photons·m^{-2}·s^{-1} with significant difference among the rest. Moreover, below 100 µmol photons·m^{-2}·$s^{-1}$the growth was proportional to the intensity applied. However, above that intensity the growth was smaller. For all the conditions, the growth pattern was similar. No lag phase was observed during the cultivation and linear growth was found between the inoculation and day 3-5. At that moment, the growth seemed to level off although the stationary phase was not completely evident even after 7 days of cultivation.



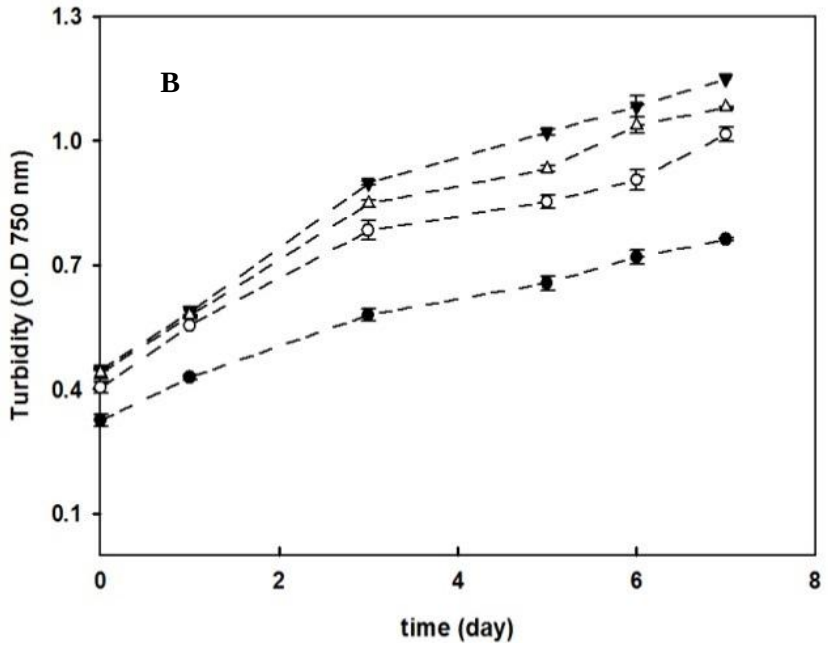


Figure 6: *Chroococcidiopsis* sp. growth evolution, measured as optical density at 750 nm (A) and biomass dry weight (B) along the experiment. This species was submitted to different light intensities (10 -•-, 50 -º-,100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

In order to get information about the relative chlorophyll content of the biomass the optical density of the samples was also measured at 680 nm (Figure 7). As it is shown in Figure 7, there was a continuous increment of chlorophyll content during the experiment. And, again, the culture exposed to a light intensity of 100 µmol photons·m^{-2}·s^{-1}showed the higher values with significance different regarding the rest light intensities. The same trend as commented previously for the growth pattern was observed: increasing chlorophyll content with increasing intensities below 100 µmol photons·m^{-2}·s^{-1}.

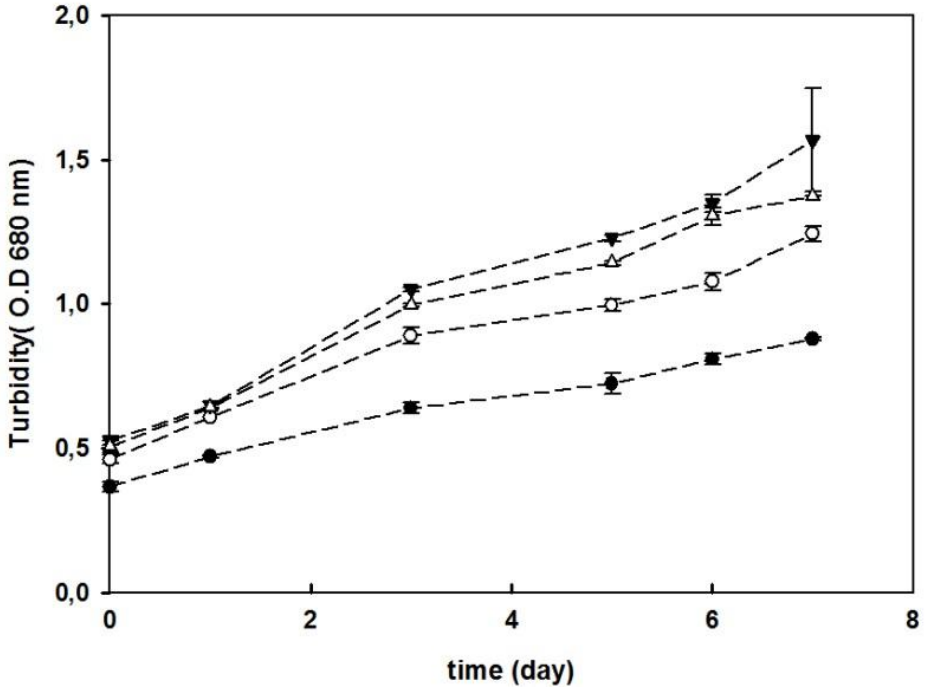


Figure 7: Chlorophyll content in *Chroococcidiopsis* sp., measured as optical density at 680 nm. This species was submitted to different light intensities (10 -•-, 50 - º-,100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

Regarding the photosynthetic efficiency of *Chroococcidiopsis* sp. it was analyzed during the experiment to discern if the biomass was subjected to any type of stress during the cultivation. As it is shown in Figure 8, the photosynthetic activity appeared to drop at the beginning on the experiment although the values were recovered after 3 days of cultivation and the efficiency remained more or less constant until the end. The initial drop in the photosynthetic efficiency was found for the highest intensity value (100 and 150 µmol photons·m^{-2}·s^{-1}) which implies a certain degree of photo-saturation/inhibition was experienced by the cells after the inoculation. However, *Chroococcidiopsis* sp. was able to cope with such conditions and its photosynthetic efficiency was recovered after 1-3 days of cultivation, although the values remained slightly lower than for the rest of intensities tested.

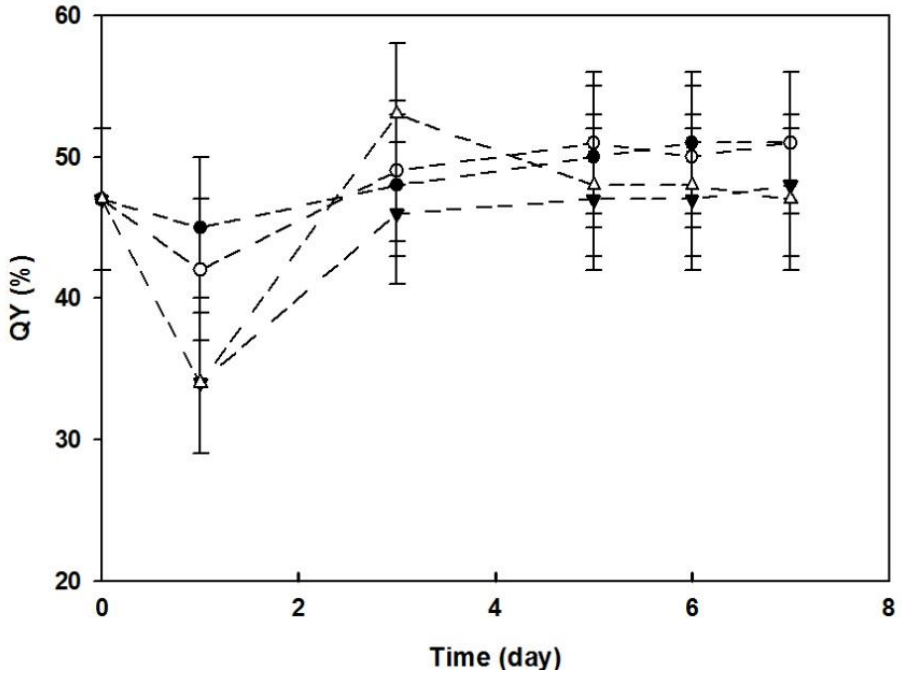


Figure 8: Variation of maximal photosynthetic efficiency of PSII, measured as maximal Quantum yield, of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different light intensities (10 -•-, 50 -º-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

In addition, the pigment content in *Chroococcidiopsis* sp. was analyzed. Figure 9 shows the chlorophyll and carotenoids content per volume of culture broth when submitted to different light intensities. As can be seen in Figure 9, there is a decrease in the first day although after that moment the pigment content increased progressively. The maximal values were found at 150 µmol photons·m^{-2}·s^{-1} in the case of chlorophyll, and at 100 µmol photons·m^{-2}·s^{-1} in the case of carotenoids. However, in terms of chlorophyll and carotenoids, there were statistical differences among the results obtained between 10 and 150 µmol photons·m^{-2}·s^{-1}.

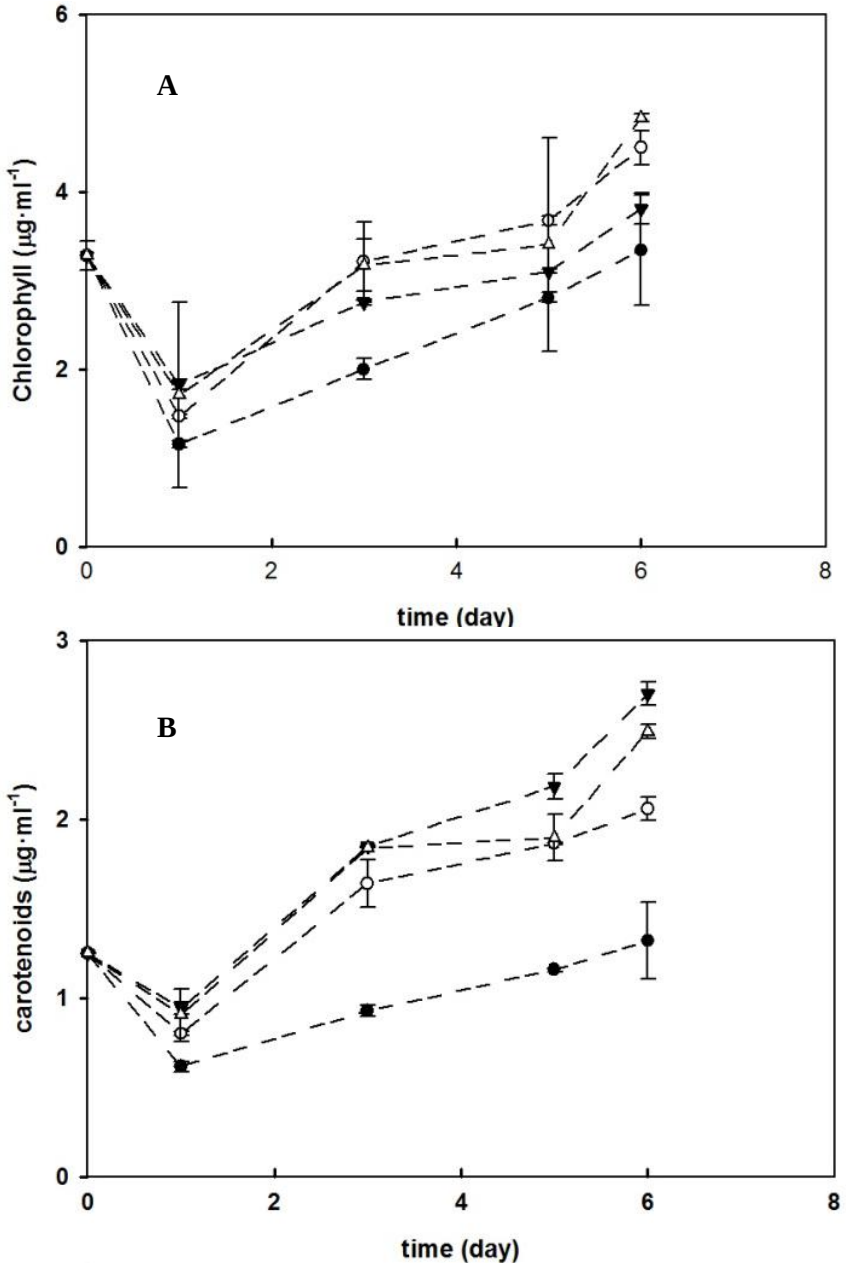


Figure **9**: Evolution of Chlorophyll (A) and total carotenoids (B), expressed per milliliters of culture of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different light intensities (10 -•-, 50 - º-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

A deeper analysis to the variation of these pigments was carried out in terms of milligrams per gram of biomass (Figure 10A and 10B). As it is shown in Figure 10, there was an increase in the cellular pigment content along the cultivation and the highest values were presented when the strain was submitted to 50 µmol photons·m^{-2}·s^{-1}. In spite of this, in terms of chlorophyll, the values had no significant differences among the rest light intensities, just when a light intensity of 50 µmol photons·m^{-2}·s^{-1} was used. Although, in terms of carotenoids, there were significant differences between the 50 µmol photons·m^{-2}·s^{-1} and the rest of light intensities.

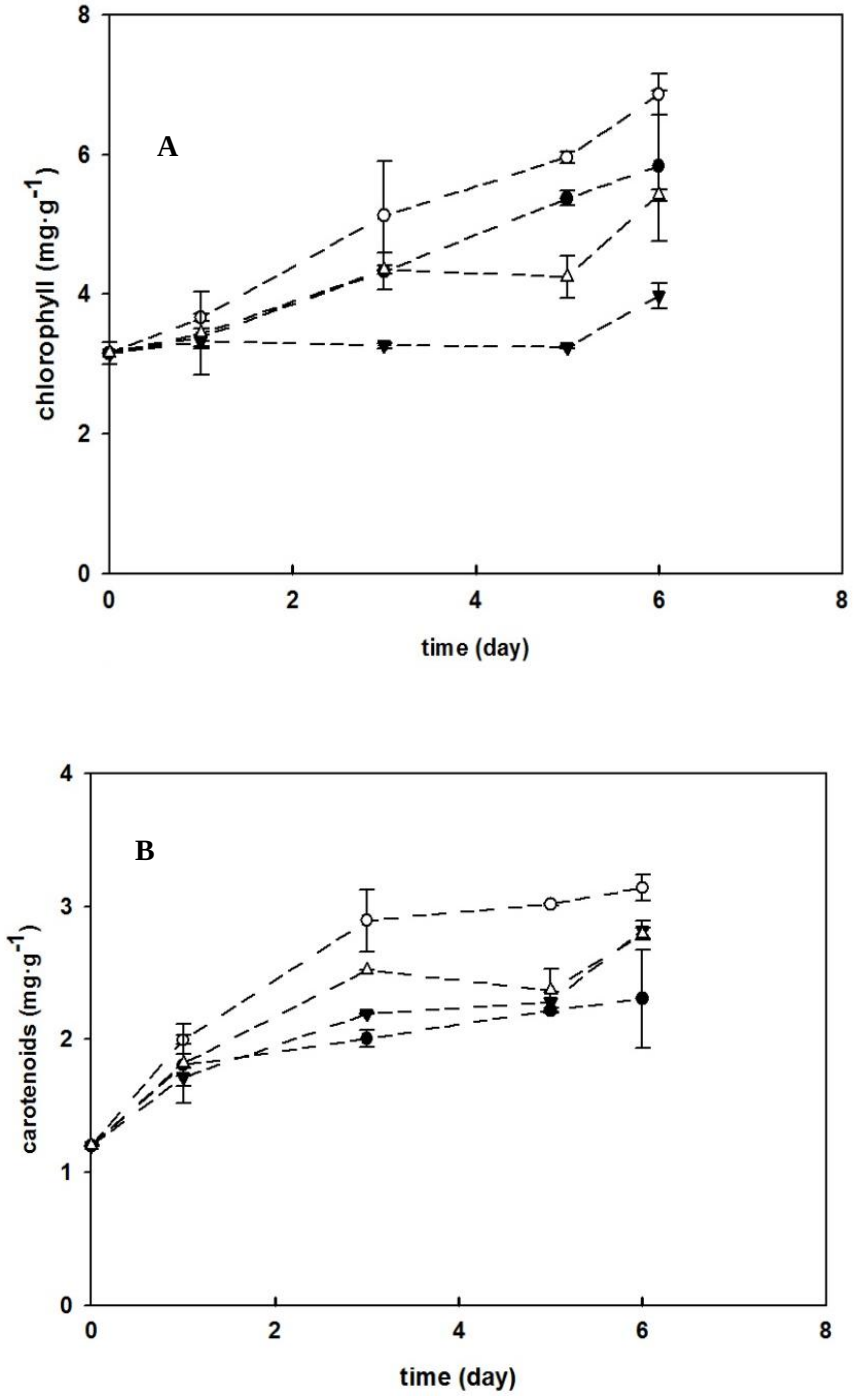


Figure 10: Evolution of Chlorophyll (A) and total carotenoids (B), expressed per milligram of biomass of culture of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different light intensities (10 -·-, 50 - º-, 100 -▾- and 150 -△- µmol photons·m^{-2}·s^{-1})

In the case of phycobiliproteins content per culture broth (Figures 11, 12 and 13), there was a progressive increase during the cultivation, as well as occurred with chlorophyll and carotenoids. In case of allophycocyanin, the maximal values were found when *Chroococcidiopsis* sp. was submitted at intensities equal or higher than 50 µmol photons·m^{-2}·s^{-1} while the lowest value was found at 10 µmol photons·m^{-2}·s^{-1}, with significant differences among them. In addition, the same pattern was found for phycocyanin and phycoerythrin and that trend was similar to the observed for the growth of *Chroococcidiopsis* sp. during the experiments.

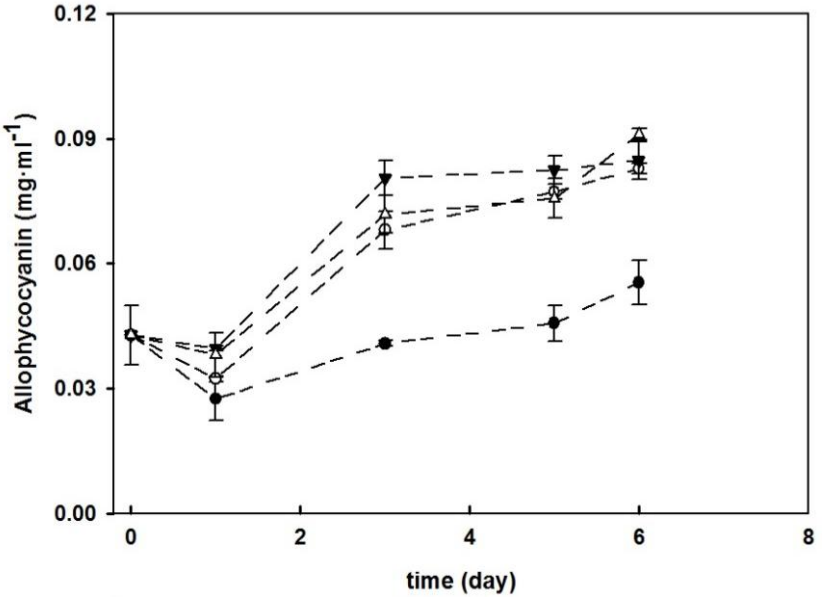


Figure 11: Evolution of Allophycocyanin expressed in milligram per milliliter of culture of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different light intensities (10 -•-, 50 -º-,100 -▼- and 150 -△-µmol photons·m^{-2}·s^{-1}).

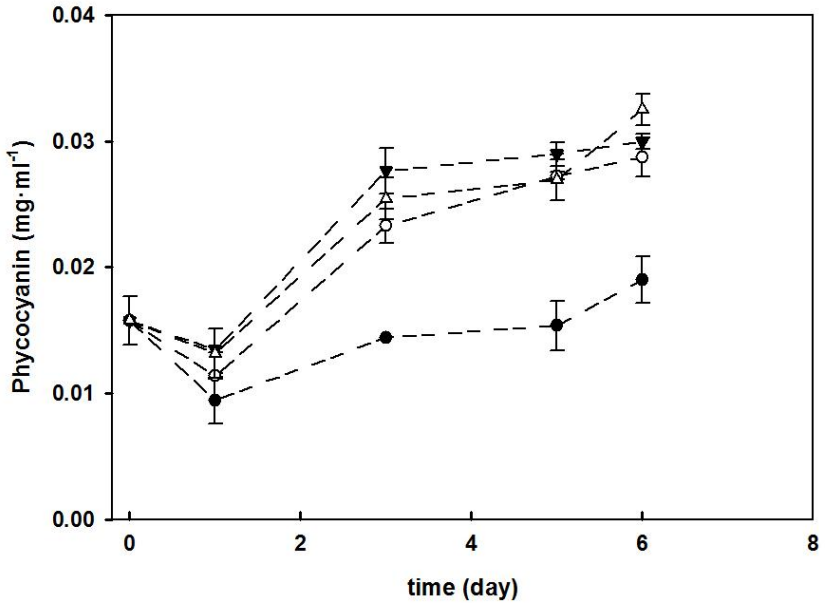


Figure 12: Evolution of Phycocyanin expressed in milligram per milliliter of culture of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different light intensities (10 -•-, 50 -º-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

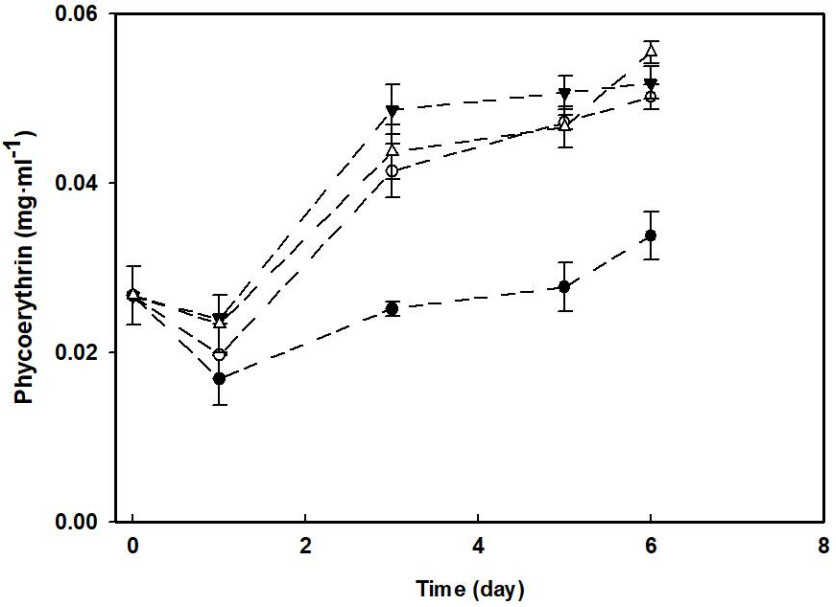


Figure 13**:** Evolution of Phycoerythrin expressed in milligram per milliliter of cult *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different light intensiti •-, 50 -º-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

Finally, the specific content in phycocyanin, allophycocyanin and phycoerythrin was also analyzed in terms of biomass accumulation. As can be seen in Figures 14, 15 and 16 the highest content of phycocyanin was produced when the cultures were submitted to a light intensity of 50 µmol photons·m^{-2}·s^{-1} and no differences were found between the rest of the intensities. And the same occurred with the rest of phycobiliproteins. However, from a statistical point of view, there was no statistical different among the 50 and 150 µmol photons·m^{-2}·s^{-1} in phycocyanin and phycoerythrin biomass. Although, in terms of allophycocyanin, there was statistical different between 50 and the rest of light intensities.

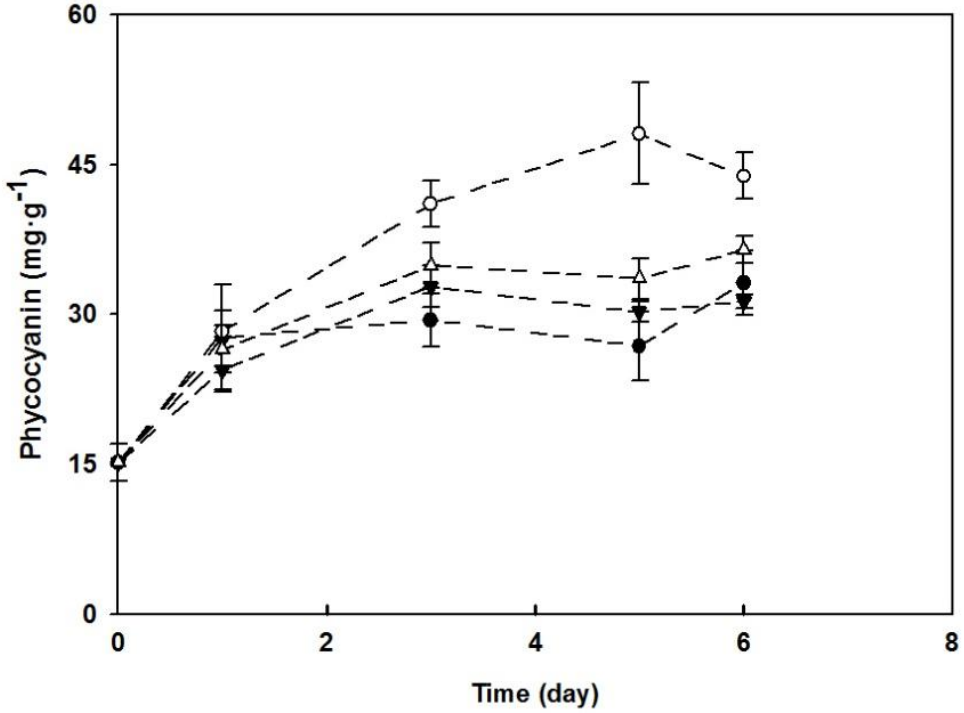


Figure 14: Evolution of Phycocyanin expressed in milligram per gram of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different light intensities (10 -•-, 50 - º-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

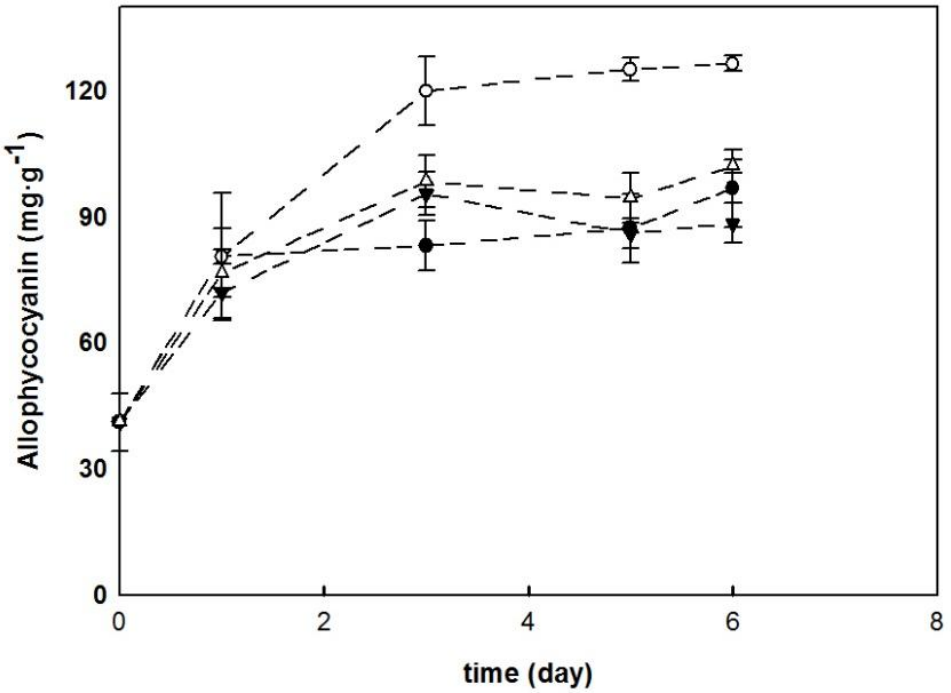


Figure 15: Evolution of Allophycocyanin expressed in milligram per gram of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different light intensities (10 -•-, 50 -º-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

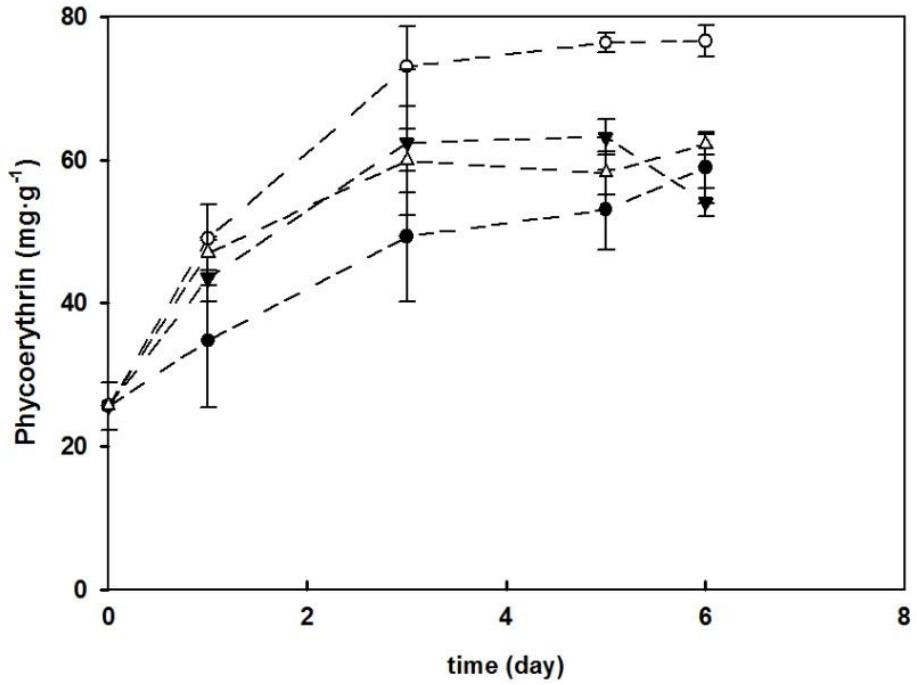


Figure 16**:** Evolution of Phycoerythrin expressed in milligram per gram of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different light intensities (10 -•-, 50 -º-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

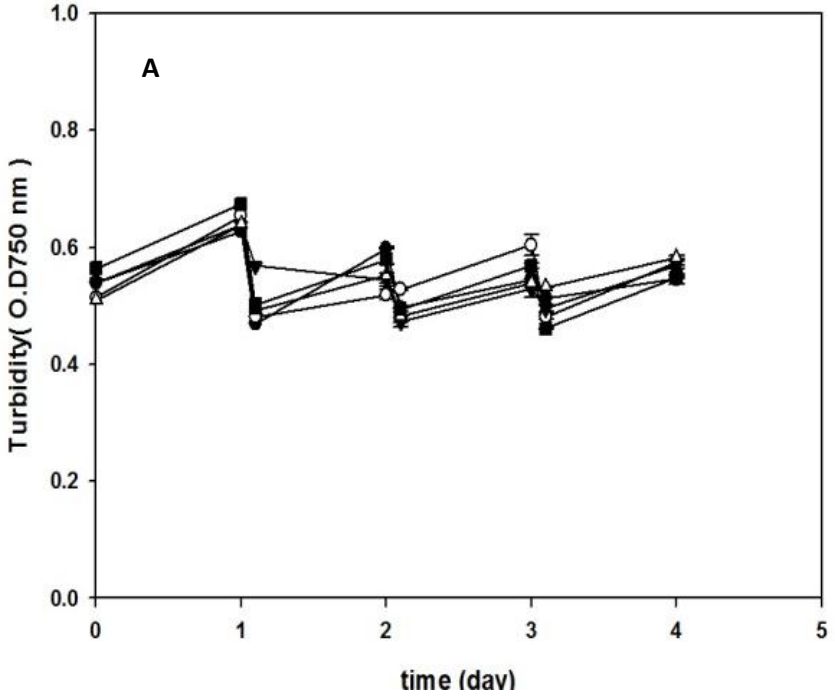
Moreover, the productivity was calculated for each light intensity in order to deduce which was the best light intensities that provide higher amount of biomass in shorter amount of time. As it is shown in the table 4, the highest value of productivity was obtained when it was cultured with a light intensity of 100 µmol photons·m^{-2}·s^{-1}.

Table 4: Productivity calculated during the linear growth phase (day 3) when *Chroococcidiopsis* sp. was submitted to different light intensities during batch cultivation.

Light intensities (µmol photons·m^{-2}·s^{-1})	Biomass productivity (mg·L^{-1}·d^{-1})	Allophycocyanin productivity (mg·L^{-1}·d^{-1})	Phycoerythrin productivity (mg·L^{-1}·d^{-1})	Phycocyanin productivity (mg·L^{-1}·d^{-1})
10	63.12	5.58	3.44	1.97
50	98.15	11.77	7.17	4.03
100	190.09	18.16	10.97	6.23
150	152.23	14.98	9.12	5.31

6.2 Influence of light intensity in *Chroococcidiopsis* sp. during semi-continuous cultivation

In order to evaluate the ability of *Chroococcidiopsis* sp. to adapt to the different light conditions and see their effect, the cyanobacterium was submitted to different light intensities: 10, 50, 70, 100 and 150 µmol photons·m^{-2}·s^{-1} during semi-continuous cultivation. For that, the cell density was maintained by periodical dilutions inside a range of 0.5-0.6 (optical density at 750 nm) as this optical density corresponds to the linear growth of this strain (data not shown). In order to get more knowledge about these conditions, growth and dry weight were daily analyzed along the experiment by measuring optical density of the samples at a wavelength of 750 nm (Figure 17 A) and weighting the samples before the dilution was carried out (Figure 17 B). Moreover, the samples were measured at a wavelength of 680 nm to get more information about the evolution of pigment content along the experiment (Figure 18).



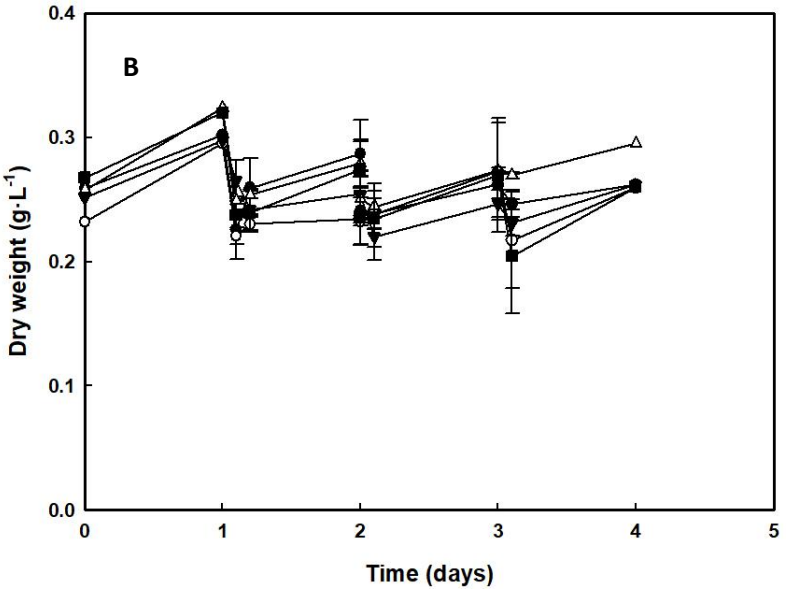


Figure 17: *Chroococcidiopsis* sp. growth evolution, measured as optical density at 750 nm (A) and biomass dry weight (B) along the experiment. This species was submitted to different light (10 -•-, 50 -º-.70 -▪-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

As can be seen in Figure 17 A, the semi-continuous cultivation ensured the biomass density to be kept inside a certain range previously defined. But it resulted in a constant dry weight along the experimental time.

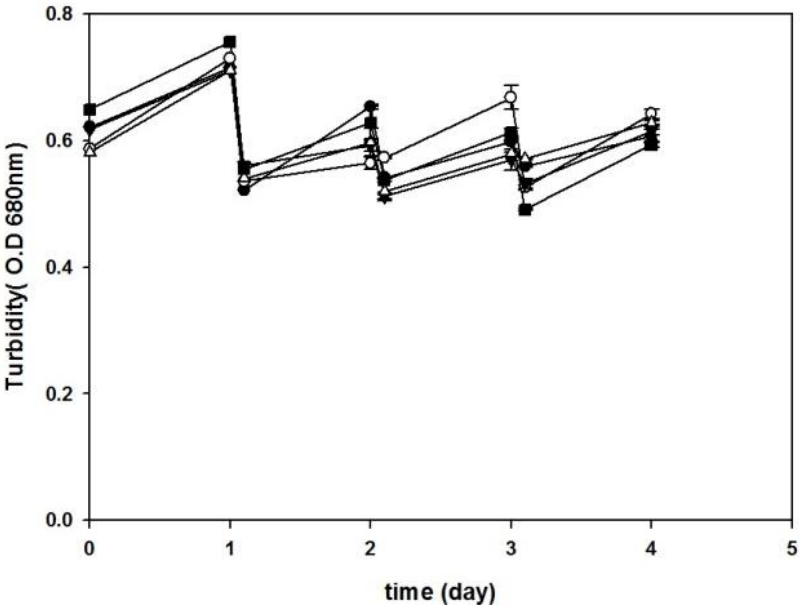


Figure 18: Chlorophyll content in *Chroococcidiopsis* sp., measured as optical density at 680 nm. This species was submitted to different light intensities (10 -•-, 50 -º-, 70 -▪-, 100 -▼- and 150 -△- µmol photons·m-2·s-1).

Regarding the photosynthetic efficiency of *Chroococcidiopsis* sp. it was analyzed during the experiment to discern if the biomass was subjected to any type of stress during the cultivation. The photosynthetic activity appeared to drop at the beginning on the experiment although the values were recovered after the first days of cultivation. The initial drop in the photosynthetic efficiency was found for the highest intensity values (50, 70, 100 and 150 µmol photons·m^{-2}·s^{-1}) which imply a certain degree of photoinhibition was experienced after the inoculation. However, *Chroococcidiopsis* sp. was able to cope with such conditions and its photosynthetic efficiency was recovered after the first day of cultivation. As it shown in the Figure 19, the highest values were present at a light intensity of 10 and 50 µmol photons·m^{-2}·s^{-1}.

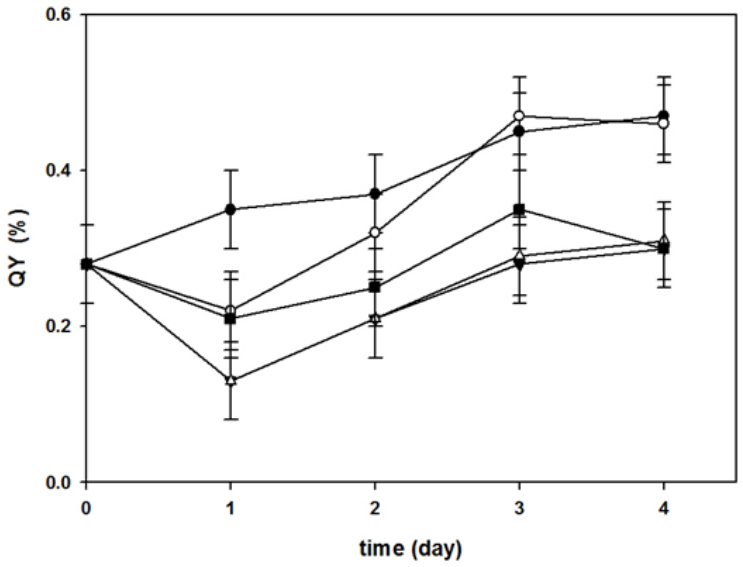


Figure 19: Evolution of Chlorophyll (A) and total carotenoids (B), expressed per milligram of biomass of culture of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different light (10 -•-, 50 -º-, 70 -▪-, 100 -▾- and 150 -△- µmol photons·m^{-2}·s^{-1}).

To obtain more information about the pigment content, the variation of these pigments was analyzed in terms of milligrams per gram of biomass (Figure 20 A and 20 B). In case of Chlorophyll and Carotenoids, there was an increase along the cultivation and the highest values were presented when the strain was submitted to 10 and 50 for chlorophyll with significant differences among the rest. While, in terms of carotenoids, the highest values were presented when it was submitted to 70 µmol photons·m^{-2}·s^{-1} with significant differences among the rest of light intensities.

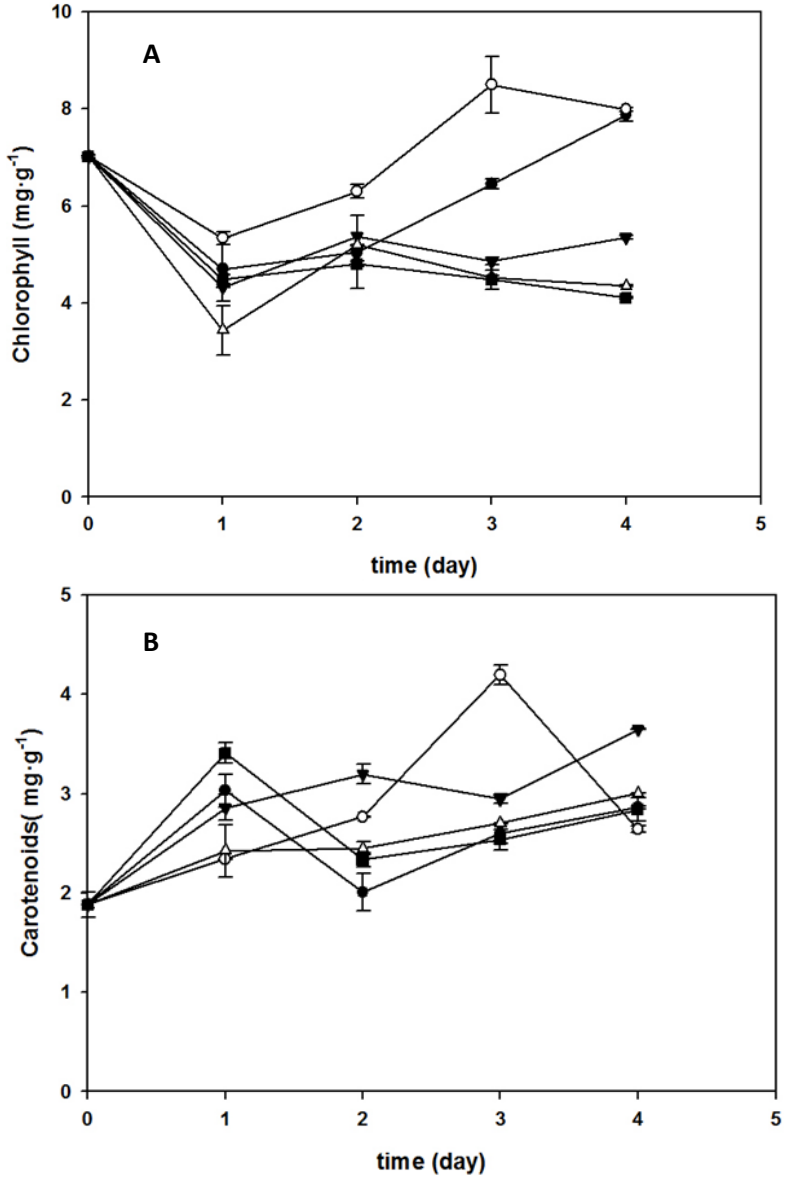


Figure 20: Chlorophyll (A) and carotenoids (B) content in *Chroococcidiopsis* sp., in milligram per gram of biomass. This specie was submitted to different light intensities (10 -•-, 50 -º-, 70 -▪-, 100 -▼- and 150 -△- µmol photons·m^{-2}·s^{-1}).

Finally, the specific biomass content of phycobiliproteins (allophycocyanin, phycocyanin and phycoerythrin) was also analyzed in terms of biomass concentration. As can be seen in the Figure 21, the highest content of allophycocyanin was produced when the cultures were submitted to a light intensity of 50 µmol photons·m^{-2}·s^{-1} with significant differences from the rest of light intensities. However, in case of phycoerythrin and phycocyanin, the highest values were present at 150 µmol photons·m^{-2}·s^{-1}. In terms of phycocyanin and phycoerythrin no significant differences were shown when the statistical analysis was

done. While in case of allophycocyanin, some significant differences were found between the light intensities.

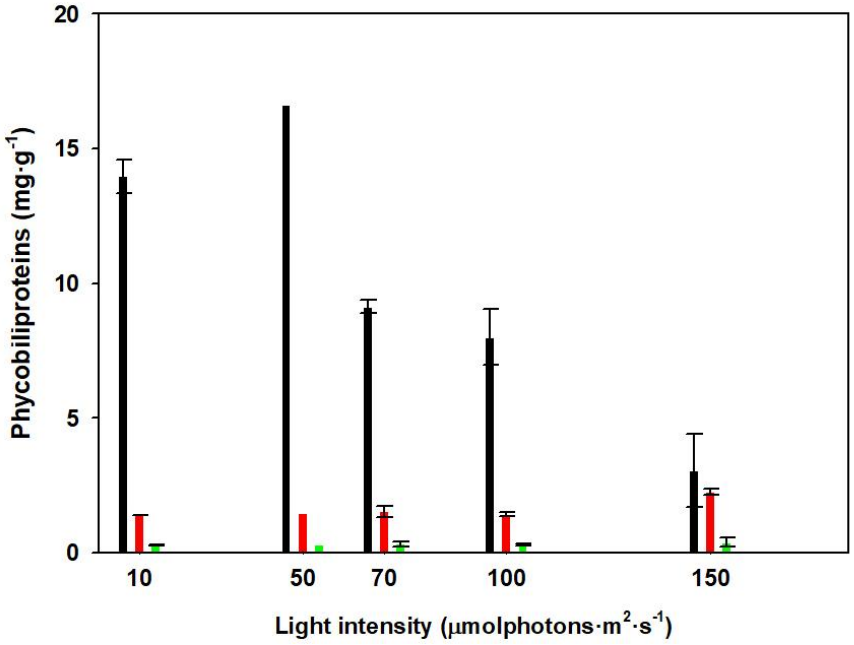


Figure 21: Evolution of Phycobiliproteins: Allophycocyanin (black), Phycocyanin (red) and Phycoerythrin (green), expressed per milligram of biomass of *Chroococcidiopsis* sp. at the end of the experiment. This specie was submitted to different light intensities (10, 50, 70, 100 and 150 µmol photons·m^{-2}·s^{-1}).

In addition, the productivity of the experiment was obtained during lineal growth parts of the experiment. This calculation was done in order to deduce which was the best light intensities regarding the production of biomass and phycobiliproteins in less time. As it is shown in the table 5, the highest values of productivity were obtained when it was cultured with a light intensity of 150 µmol photons·m^{-2}·s^{-1}.

Table 5: Average Productivity calculated during the different growth cycles when *Chroococcidiopsis* sp. was submitted to different light intensities during semi-continuous cultivation.

Light intensities (µmol photons·m^{-2}·s^{-1})	Biomass productivity (mg·L^{-1}·d^{-1})	Allophycocyanin productivity (mg·L^{-1}·d^{-1})	Phycoerythrin productivity (mg·L^{-1}·d^{-1})	Phycocyanin productivity (mg·L^{-1}·d^{-1})
10	41.80	0.58	0.012	0.058
50	43.79	0.73	0.014	0.065
70	38.44	0.35	0.013	0.059
100	48.02	0.38	0.014	0.069
150	44.43	0.14	0.017	0.100

6.3 Use of agricultural fertilizers in the cultivation of *Chroococcidiopsis* sp.

During the second part of this Master Thesis (from February 2019 to August 2019), the suitability of the use of commercial fertilizers in the cultivation of *Chroococcidiopsis* sp. was assessed. Growth and pigments concentration in *Chroococcidiopsis* sp. was evaluated by using different types of fertilizers under batch cultivation, and it was carried out in Erlenmeyer flasks in a cultivation room thermostated at 25ºC. This novel strain was cultured with different types of fertilizers, and the growth was daily analyzed along the experiment by measuring optical density of the samples at a wavelength of 750 nm (Figure 22). As it is shown in Figure 22, *Chroococcidiopsis* sp. was able to grow with all the employed fertilizers although the best results were found with the fertilizer 8-6-6. A lag phase was observed during the first days of cultivation and linear growth was found between the day 2-10th day. Then, the growth seemed to level off and the stationary phase was completely evident on the 11th days of cultivation. However, a totally different trend was found when the fertilizer 4-10-10 was used. After 7 days of cultivation depletion in the growth of this strain was observed until the end of the experiment.

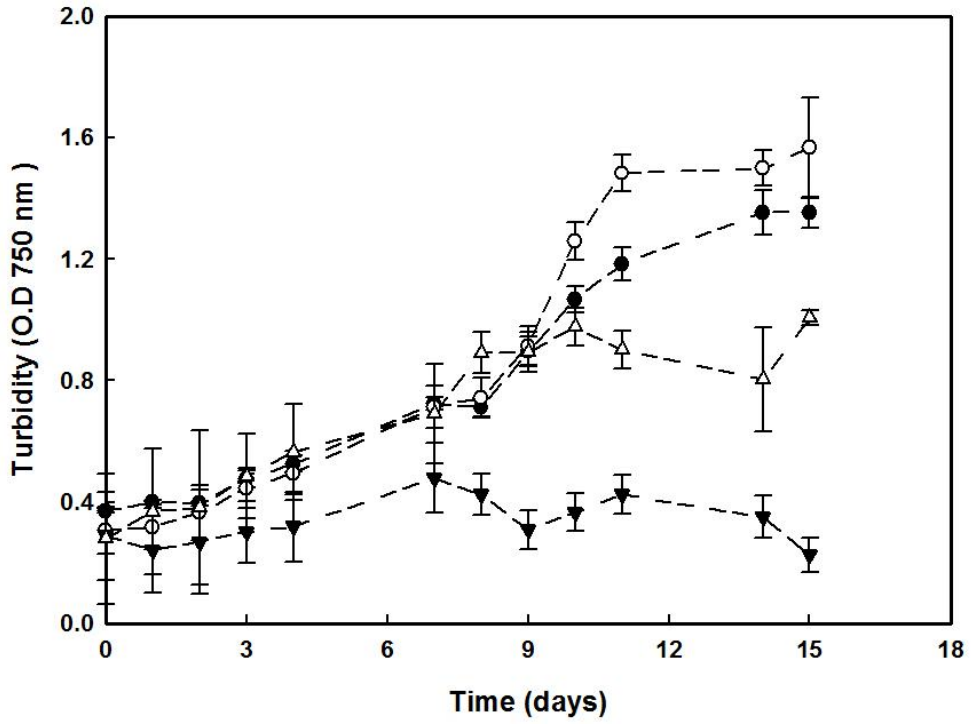


Figure 22**:** Growth evolution for *Chroococcidiopsis* sp. in Control with BBM media (-•-), 8-6-6 (-^{o}-)-, 4-10-10 (-▾-) and 18-6-6 (-△-) by measuring the optical density at 750 nm.

Regarding the photosynthetic efficiency of *Chroococcidiopsis* sp., it was analyzed during the cultivation without any remarkable deviation (data not shown). As example, the data at the end of the cultivation is shown in Figure 23 A. As can be seen in Figure 23 B, the photosynthetic activity of the Control seemed to be the highest, although no big differences were found. And between the fertilizers used, 8-6-6 also showed the highest efficiency. On the other hand, the fertilizer 4-10-10 showed the lowest photosynthetic efficiency, which was in accordance with the absence of growth observed at the end of the experiment.

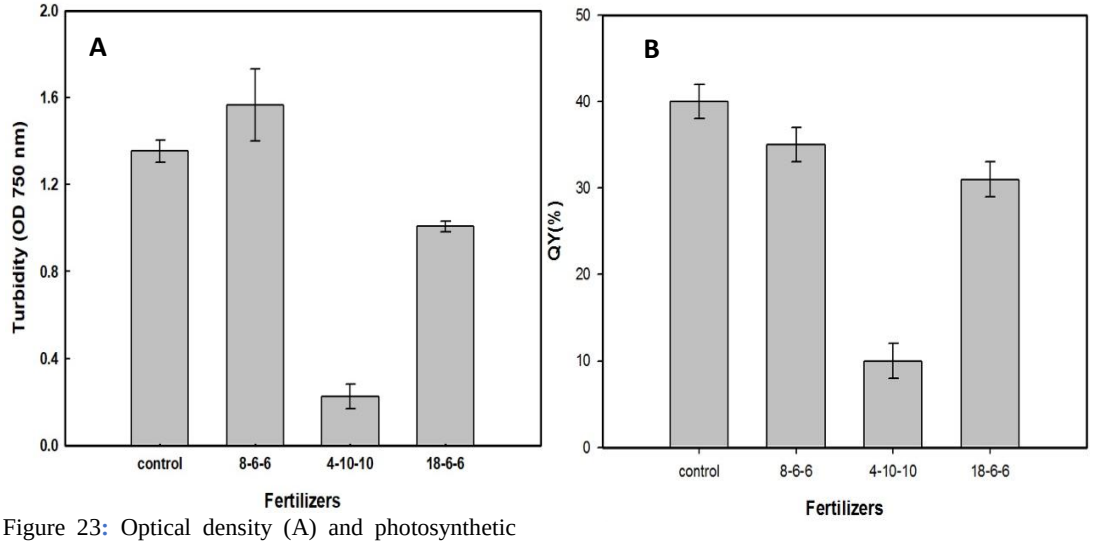


Figure 23: Optical density (A) and photosynthetic activity (B) of *Chroococcidiopsis* sp. at the end of the experiment with the different fertilizers: 8-6-6, 4-10-10 and 18-6-6. Control media with BBM was used as reference.

According to the photosynthetic activity, it appeared to have the same result like the optical density, presenting higher values when the 8-6-6- fertilizer was used on the experiment.

By other hand, the specific biomass content in phycocyanin, allophycocyanin and phycoerythrin was also analyzed regarding the use of fertilizers. As can be seen in Figure 24, the highest content of phycobiliproteins was produced when the cultures were cultured with the fertilizer 8-6-6-, although there were no significant differences regarding to the phycobiliproteins content between the Control and the fertilizer 18-6-6. However, the statistical analysis showed some significant differences among the Control and the fertilizer 8-6-6.

Information related to the content in the culture with the fertilizer 4-10-10 could not be obtained due to the lack of growth at the end of the experiment.

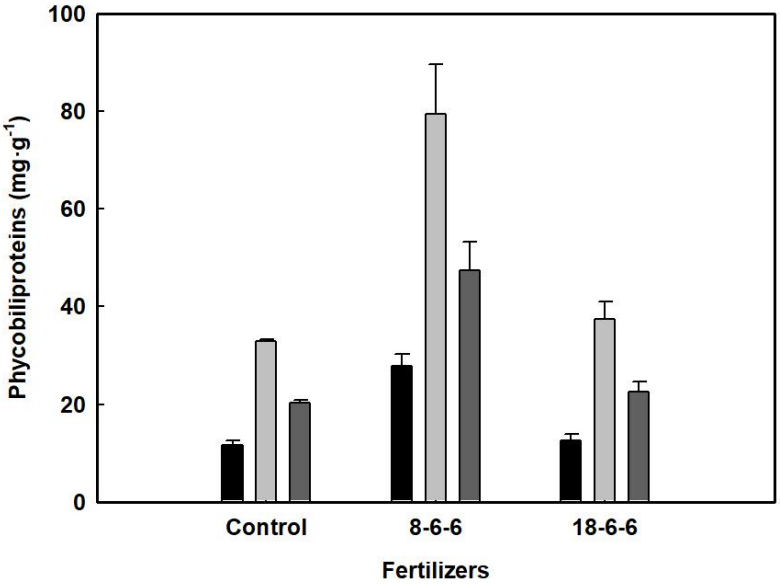


Figure 24: Phycobiliproteins content at the end of the cultivation with the different fertilizers. Culture media with BBM was used as reference. Each phycobiliproteins are representing by one color, "black – Phycocyanin", "brighter grey-Allophycocyanin "and "darker grey- Phycocyanin".

6.4 Effect of salinity in the cultivation of *Chroococcidiopsis* sp.

The last experiment carried out during the second part of this Master Thesis (from June 2019 to August 2019), was the assessment of growth and pigments concentration in *Chroococcidiopsis* sp. cultivated under different salt concentrations. Batch cultivation was carried out in Erlenmeyer flasks in a cultivation room thermostated at 25ºC and different salt concentrations were used (from 0 to 0.6 M of NaCl). In this case, the culture media was based in the fertilizer 8-6-6, which was the best fertilizer previously evaluated (section 6.3 of this thesis). And the reference media was 8-6-6, without any extra salt addition. Growth was daily analyzed along the experiment by measuring optical density of the samples at a wavelength of 750 nm (Figure 25). As it is shown in the Figures 25, *Chroococcidiopsis* sp. was able to grow under 0.2 M of NaCl although the best results were found with significant differences when it was cultured without salt (control). A lag phase was observed during the cultivation and linear growth was found between the inoculation and day 5-13. At that moment, the growth seemed to level off and the stationary phase was completely evident even after 13 days of cultivation. In addition, it seemed to be no growth

when this species was submitted to higher salt concentrations, specifically 0.4 and 0.6 M NaCl.

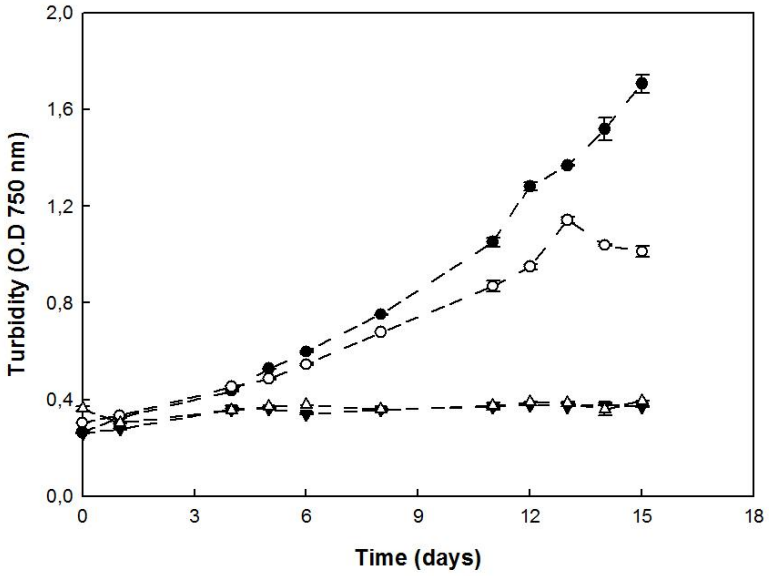


Figure 25: *Chroococcidiopsis* sp. growth evolution, measured as optical density at 750 nm along the experiment. This species was submitted to different salt concentrations (control -•-, 0.2 M - º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

In order to get information about the relative chlorophyll content of the biomass, the optical density of the samples was also measured at 680 nm (Figure 26). As it is shown in Figure 26, there was a continuous increment of chlorophyll content when it was submitted to 0.2 M of NaCl during the experiment, showing higher values than the optical density of samples at 750 nm. However, in case of being submitted to higher salt concentration such as 0.4 and 0.6 M of NaCl, there was no increment in the chlorophyll content of biomass along the experiment.

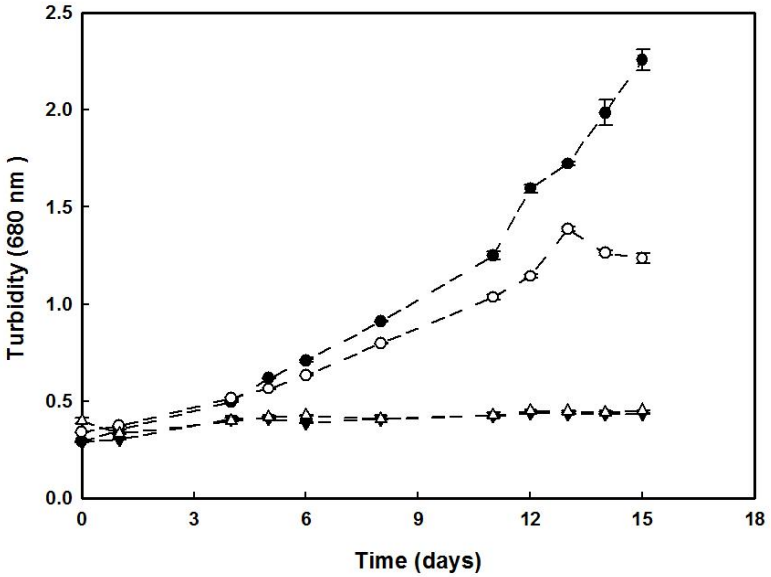


Figure 26: Chlorophyll content in *Chroococcidiopsis* sp., measured as optical density at 680 nm. This species was submitted to different salt concentrations (Control -•-, 0.2 M -º-, 0.4 M -▾- and 0.6 M -△- of NaCl).

Moreover, the growth was daily analyzed along the experiment by measuring the dry weight. As it is shown on the Figure 27, it exhibits the same patron as the optical density at 750 nm (Figure 25), meaning that a lag phase was observed during the cultivation and linear growth was found between the inoculation and day 5-13. After that moment, the growth seemed to level off, showing the stationary phase after 13 days of cultivation. Moreover, there was no growth when this strain was submitted to elevated salt concentration, concretely 0.4 and 0.6 M of NaCl.

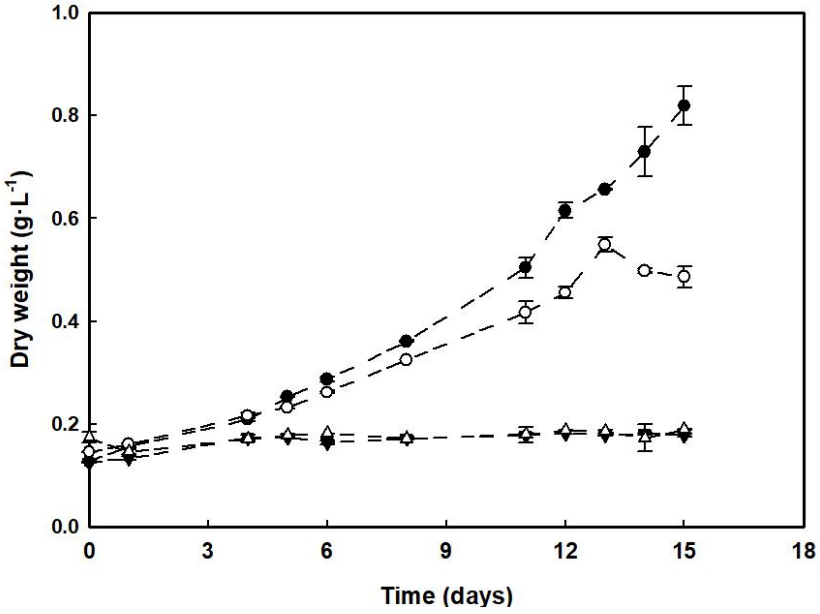


Figure 27**:** *Chroococcidiopsis* sp. biomass dry weight along the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▾- and 0.6 M -△- of NaCl).

Regarding the photosynthetic efficiency of *Chroococcidiopsis* sp., it was analyzed during the experiment to discern if the biomass was subjected to any type of stress during the cultivation. As it is shown in Figure 28, the photosynthetic activity for the control seemed to be mostly constant during the experiment although there was an increase during the first days. Regarding the cultures with salt, the cultures with 0.2 M and 0.4M of NaCl also showed an increase in the photosynthetic efficiency in the first days but it was followed by a drastical depletion until the end of the experiment. And the highest salinity, 0.6 M, showed a decreasing tendency since the first day of cultivation.

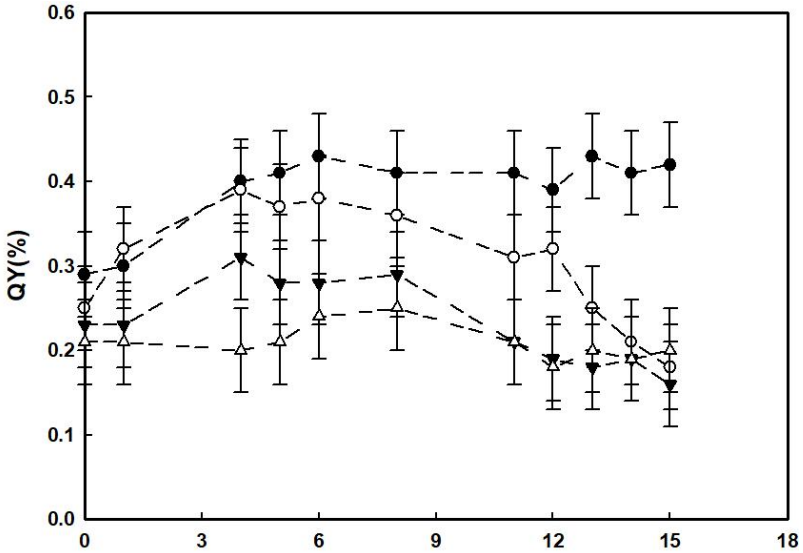


Figure 28**:** Variation of maximal photosynthetic efficiency of PSII, measured as maximal Quantum yield, of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

Besides, the pigment content in *Chroococcidiopsis* sp. was analyzed. The Figures 29 A and 29 B shows the carotenoids and chlorophyll content per volume of culture broth when submitted to different salt concentrations. As can be seen in the Figure 29, there is a continuous increase of the pigment content per volume of culture since the first day when it was submitted to 0.2 M of NaCl, but there was no increase when this strain was submitted to higher salt concentrations such as 0.4 and 0.6 M of NaCl. Moreover, the statistical analysis showed no significant differences between them.

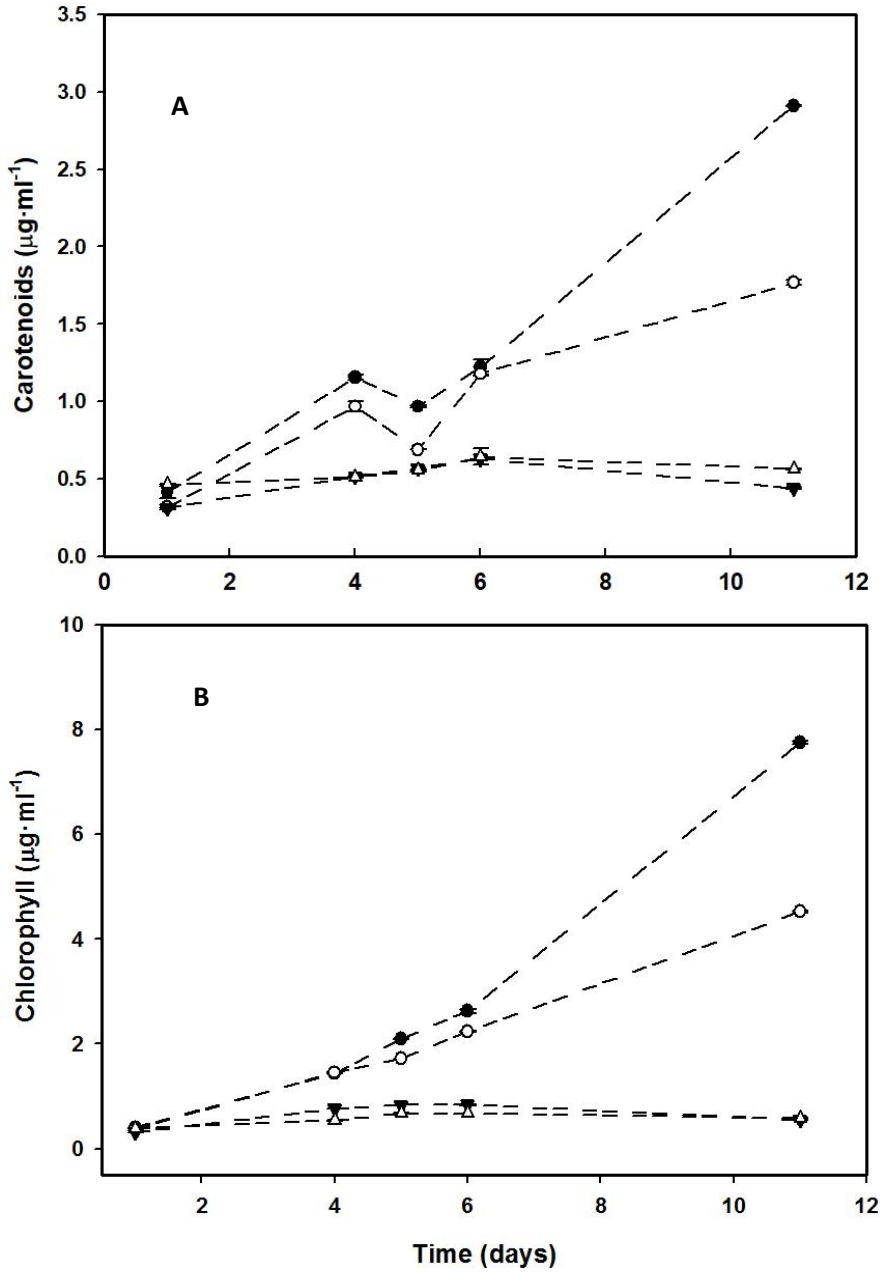


Figure 29: Evolution of Carotenoids (A) and total Chlorophyll (B), expressed per milliliter of culture of culture of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▾- and 0.6 M -△- of NaCl).

To obtain a deeper analysis regarding to the variation of these pigments, an examination was done in terms of milligrams per gram of biomass (Figure 30 A and 30 B). As it is shown in Figure 30 A, there was an increase in the chlorophyll content along the cultivation when the salt concentration was below 0.4 M. The highest salt concentrations, however, resulted in a constant concentration of chlorophyll which coincided with the absence of growth observed under such conditions. Moreover, the same trend was observed for the carotenoids content. An increasing concentration of carotenoids was found below

0.4 M of salt, while the carotenoids content was constant above that salinity value (Figure 30 B) but with no significant differences among them.

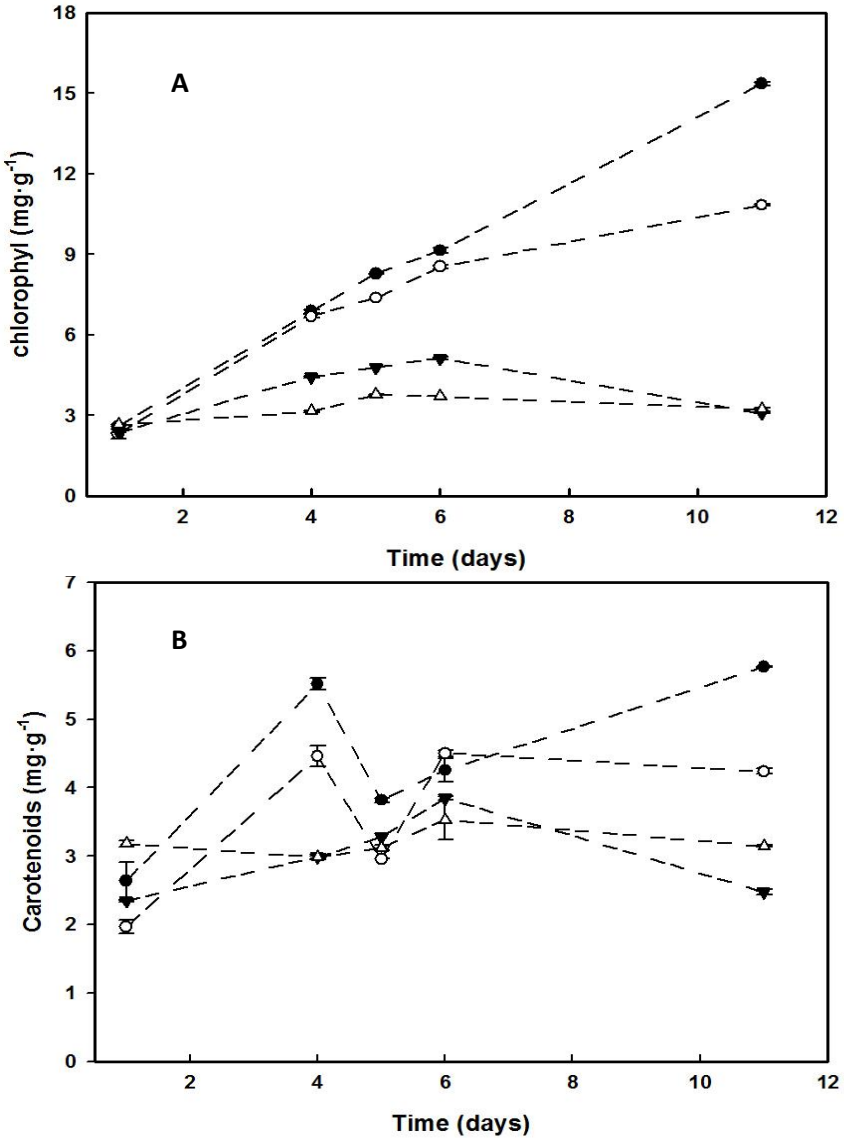


Figure 30: Evolution of Chlorophyll (A) and total carotenoids (B), expressed per gram of biomass of culture of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

In the case of phycobiliproteins content per culture broth (Figures 31, 32 and 33), there was a progressive variation during the cultivation, as well as occurred with the chlorophyll and carotenoids. Regarding the three different types of phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin), the same tendency was found during the cultivation, and the maximal values were found when the culture was submitted to 0.4 M of NaCl while the lowest value was found with 0.6 M of NaCl with significant differences among them. Besides, the allophycocyanin was the most abundant phycobiliproteins.

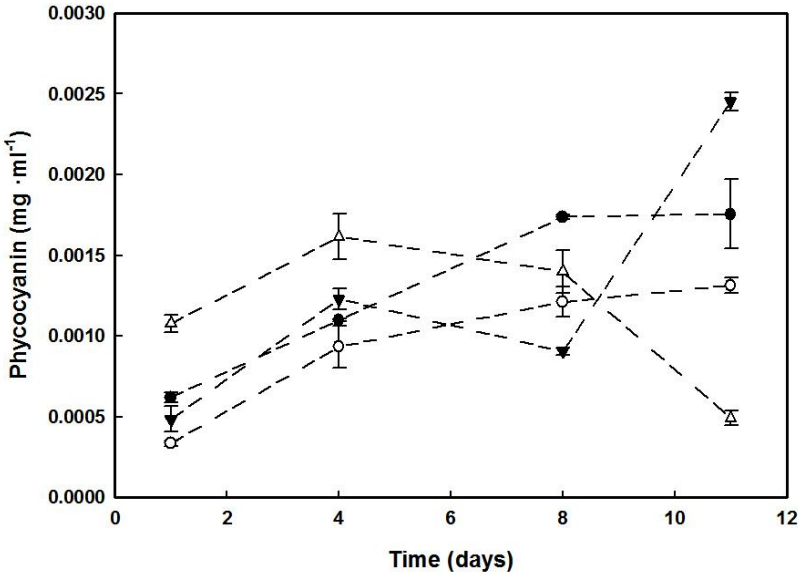


Figure 31: Evolution of Phycocyanin expressed in milligram per milliliter of culture of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

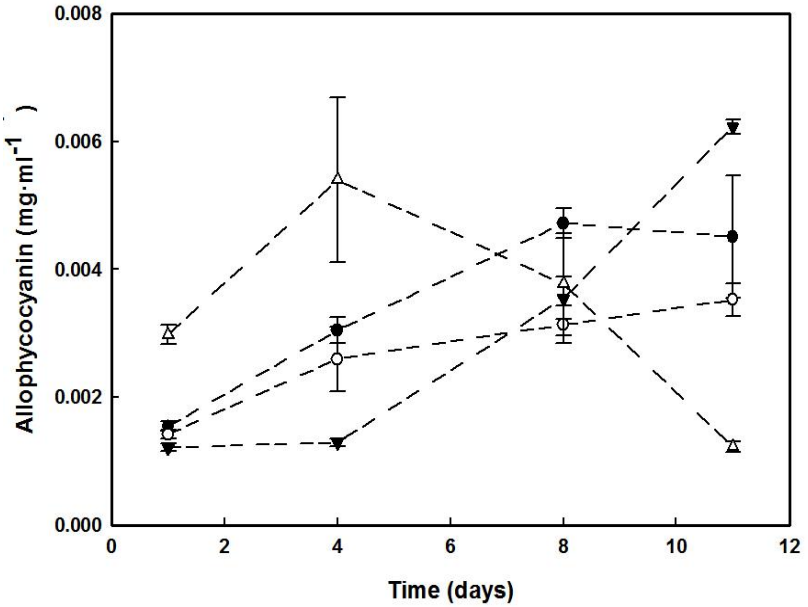


Figure 32: Evolution of Allophycocyanin expressed in milligram per milliliter of culture of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

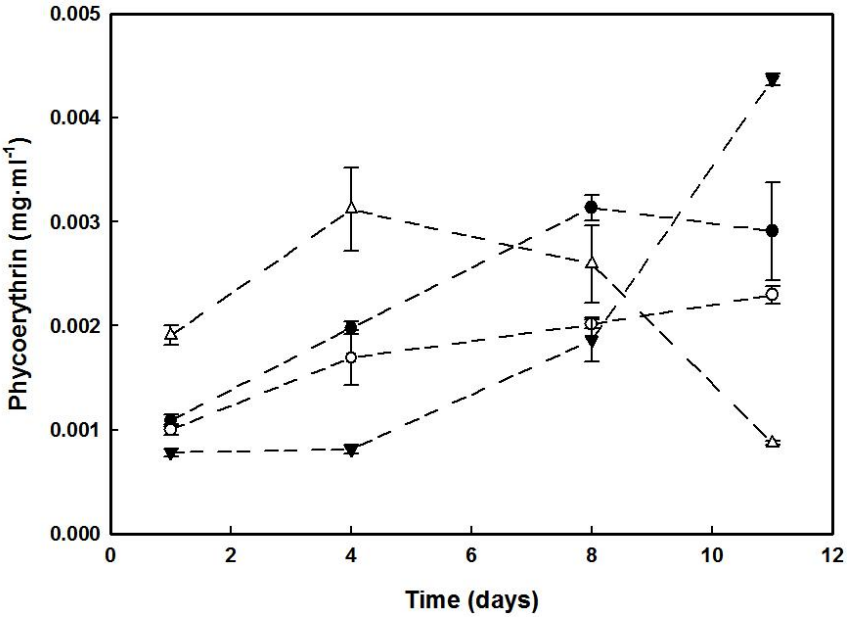


Figure 33: Evolution of Phycoerythrin expressed in milligram per milliliter of culture of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

Finally, the specific content in allophycocyanin, phycocyanin and phycoerythrin was also analyzed in terms of biomass accumulation. As can be seen in Figure 34, 35 and 36 the highest content of phycocyanin was produced with significant differences when the cultures were submitted to a salt concentration of 0.4 NaCl and no differences were found between the rest of the salinities. And the same occurred with the rest of phycobiliproteins. Moreover, it was found that the accumulation.

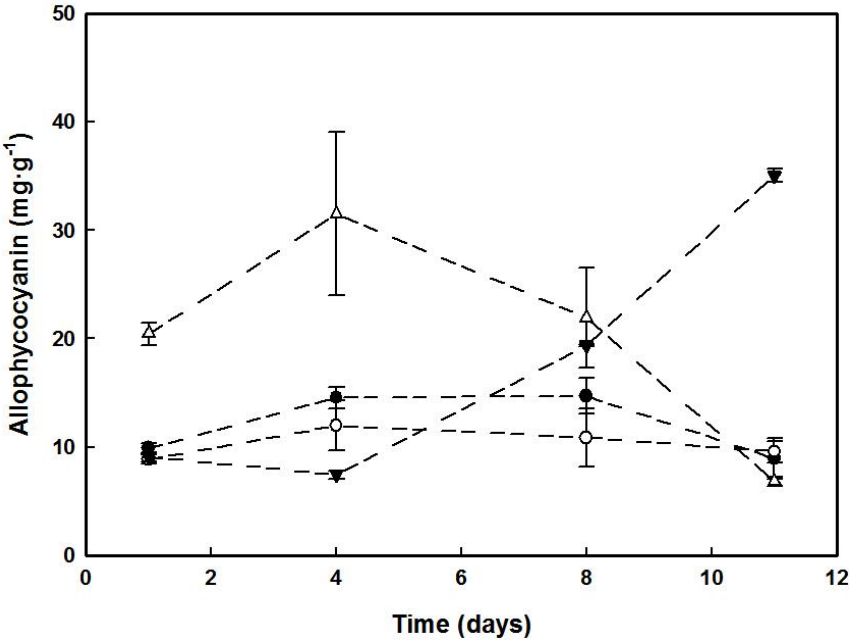


Figure 34: Evolution of Allophycocyanin expressed in milligram per gram of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

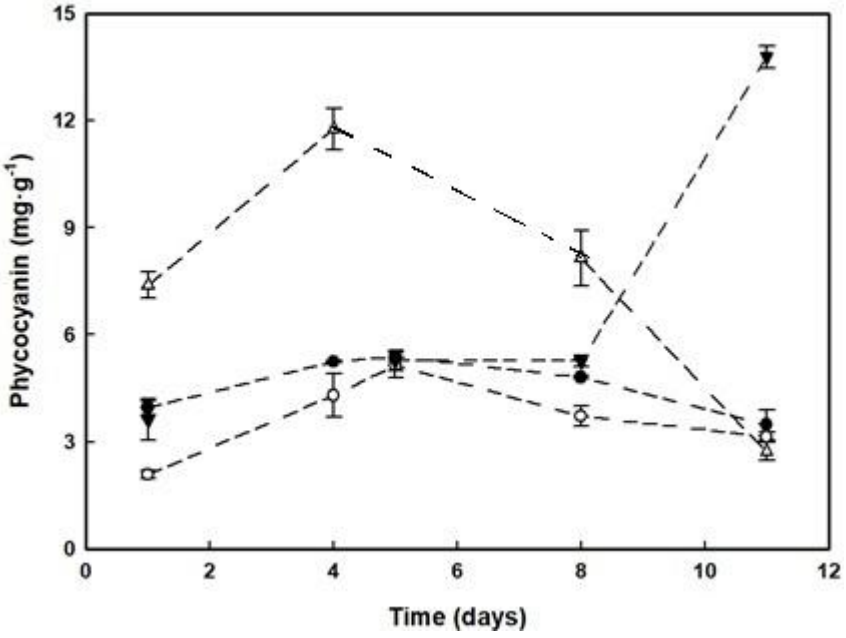


Figure 35: Evolution of Phycocyanin expressed in milligram per gram of biomass of *Chroococcidiopsis* sp. during the experiment. This species was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

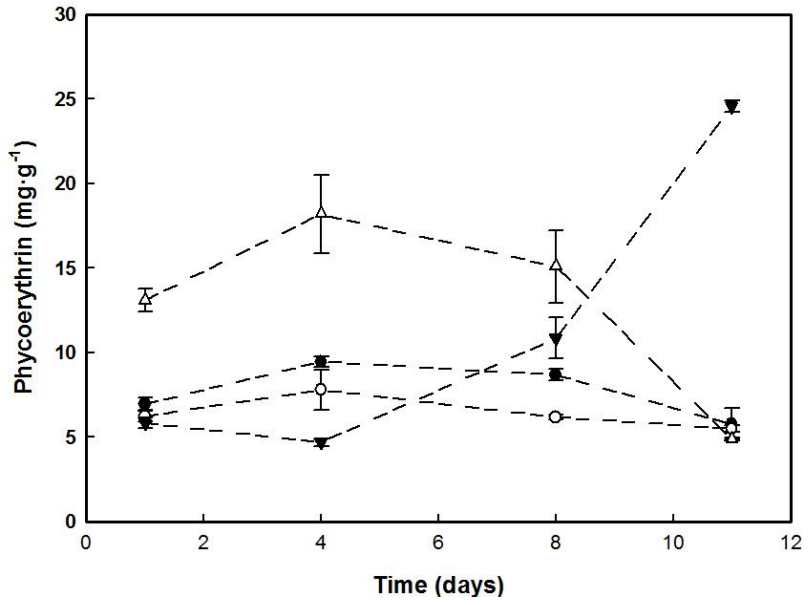


Figure 36: Evolution of Phycoerythrin expressed in milligram per biomass of *Chroococcidiopsis* sp. during the experiment. This specie was submitted to different salt concentrations (control -•-, 0.2 M -º-, 0.4 M -▼- and 0.6 M -△- of NaCl).

Moreover, the productivity was calculated for each treatment in order to deduce which was the condition of salt that provide higher amount of biomass in less time. As it is shown in

the table 6, the highest value of productivity was obtained when it was cultured without salt, although there was not much different with the treatment of 0.2 M of NaCl.

Table 6: Productivity calculated during the cultivation of *Chroococcidiopsis* sp. under different concentrations of salt.

Salt Concentration (M)	Biomass productivity (mg/L/d)	Allophycocyanin productivity (mg/L/d)	Phycoerythrin productivity (mg/L/d)	Phycocyanin productivity (mg/L/d)
Control	36	0.321	0.207	0.125
0.2	26	0.252	0.145	0.083
0.4	1	0.019	0.013	0.008
0.6	0	0.002	0.001	0.001

7 DISCUSSION

7.1. Influence of light intensity in *Chroococcidiopsis* sp. during batch and semi-continuous cultivation

The influence of different light intensities in *Chroococcidiopsis* sp. was assessed, ranging from 10 to 150 µmol photons·m^{-2}·s^{-1}, during batch cultivation. Growth was proportional to the light applied until the intensity of 100 µmol photons·m^{-2}·s^{-1} and the same growth pattern was found. However, the highest intensity (150 µmol photons·m^{-2}·s^{-1}) resulted in lower growth (Figure 6 B). Besides, the photosynthetic efficiency showed normal values for cyanobacteria[71],with slightly lower efficiencies in the cultures exposed to the highest intensities (100 and 150 µmol photons·m^{-2}·s^{-1}) and which also showed a steep drop after inoculation (Figure 8). All these seems to indicate that the cultures exposed to light intensities lower than 100 µmol photons·m^{-2}·s^{-1} might have been photo-limited and growth was proportional to the light supplied.

Regarding the pigment content, chlorophyll and carotenoids cellular content showed an increasing trend during the cultivation with the different light intensities. However, the maximal concentration was found when the strain was submitted to 50 µmol photons·m^{-2}·s^{-1} (Figure 10 A and 10 B). It could confirm that *Chroococcidiopsis* sp. was photo-limited at that light intensity and, therefore, stimulated in a major extent the synthesis of chlorophyll and carotenoids in order to enhance the light capture. On the other hand, at higher intensities, a certain degree of stress by excess of light could have been experienced and the chlorophyll content was then reduced and carotenoids slightly increased in the culture exposed to 150 µmol photons·m^{-2}·s^{-1} compared to 100 µmol photons·m^{-2}·s^{-1}.

Finally, the specific phycobiliproteins content showed the same trend as the total carotenoids content (Figure 14, 15 and 16), without remarkable differences between phycocyanin, allophycocyanin and phycoerythrin. A possible hypothesis could be that the cultures exposed to a light intensity between 10 and 50 µmol photons·m^{-2}·s^{-1} might experience a certain degree of photo-limitation and, therefore they stimulated the synthesis of the phycobiliproteins as accessory pigments to contribute to the light capture. However, when light intensity was between 100 and 150 µmol photons·m^{-2}·s^{-1}, a certain degree of stress imposed by light could be experienced, and it could explain the higher content of phycobiliproteins found at 150 compared to 100 µmol photons·m^{-2}·s^{-1}.

When semi-continuous cultivation was applied, the different cultures were able to adapt to the light conditions imposed and no remarkable differences were found between the growth of the cultures exposed to the minimal and maximal light intensities (Figure 17A and 17B). Moreover, the photosynthetic efficiency also showed acceptable values for cyanobacterial growth, although it was slightly higher in the cultures exposed to 10 and 50 µmol photons·m^{-2}·s^{-1}, which still indicated a better performance of *Chroococcidiopsis* sp. when submitted to lower light intensities.

Regarding the pigment concentration, the maximal values were also found when light intensity was 10 and 50 µmol photons·m^{-2}·s^{-1} for chlorophyll and 70 µmol photons·m^{-2}·s^{-1} for carotenoids (Figure 20 A and 20B). As occurred during the batch cultivation, *Chroococcidiopsis* sp. could have been photo-limited at that light intensities and, therefore, stimulated the synthesis of chlorophyll and carotenoids in order to enhance the light capture. But at higher intensities, a certain degree of stress by excess light could have been experienced and the pigment content was then reduced.

Finally, the specific biomass content of phycobiliproteins (Figure 21) showed allophycocyanin as main phycobiliproteins with a maximal cellular content when the cultures were submitted to a light intensity of 50 µmol photons·m^{-2}·s^{-1}. However, in case of phycoerythrin and phycocyanin, no big differences were found between the cellular content, although allophycocyanin showed a maximal content at 150 µmol photons·m^{-2}·s^{-1}. A possible hypothesis could be that the cultures exposed to a light intensity between 10 and 50 µmol photons·m^{-2}·s^{-1} might have experienced a certain degree of photo-limitation and, therefore they stimulated the synthesis of allophycocyanin as main secondary pigment for the light capture. However, when light intensity was between 70, 100 and 150 photons·m^{-2}·s^{-1}, a certain degree of stress imposed by light could have been experienced and phycocyanin could have been produced in order to protect from the "excess" of light.

There are different types of abiotic parameters that affect the growth rate and the biomass and pigment production of cyanobacterium, such as light, quality of it and temperature among others[72]. However, these factors affect differently according to the species. In general terms, although light intensity affects cyanobacteria in different ways it is said that an increase in phycobiliproteins will be produced as a consequence to the exposure to light. However, the optimal values for pigment production depends on the species. For example, the highest values of pigment production for *Spirulina platensis* were reported at a light

intensity of 47.25 µmol photons·m^{-2}·s^{-1}[173]. However, in the case of *Anabaena* NCCU-9, 25 µmol photons·m^{-2}·s^{-1} resulted in the best phycobiliproteins production[48]. As well, that intensity was reported to be optimal for other cyanobacteria such as *Synechococcus* NKBG 042902[74] and *Synechocystis*[75] . However, despite the differences in the optimal intensity, cyanobacteria in general prefer low light intensities to synthesize phycobiliproteins as a result of their low specific maintenance energy rates and their pigments composition. The same trend was observed for *Chroococcidiopsis* sp., with an optimal value for pigments production at 50 µmol photons·m^{-2}·s^{-1} (including phycobiliproteins) despite the regime of cultivation used (batch and semi-continuous) as the highest values of productivity was produced in batch cultivation at 100 µmol photons·m^{-2}·s^{-1} and 150 µmol photons·m^{-2}·s^{-1} in semi-continuous conditions.

In addition, light quality seems to play an important role in regulation of pigment synthesis[76]. For example, it was reported that *Pseudanabaena* sp. produce higher amount of biomass when it was cultured in blue light[10,46]. In spite of it, a higher production of phycoerythrin and carotenoids were obtained when it was cultured in green light and phycocyanin under red light[10]. However, a progressive depression in growth rate and production was detected when it was cultured in yellow light[10] . The same aspect was described in another cyanobacterium like *Anabaena ambigua* Rao, although in this case, the blue light was the best choice to obtain higher amount of phycocyanin content[77]. In spite of this effect, some cyanobacterium doesn't need any specific type of light to stimulate the synthesis of phycobiliproteins as Anabaena NCCU-9[48]. In this sense, light quality also needs to be specifically assessed for each particular strain and it could be interesting point to be evaluated with *Chroococcidiopsis* sp.

7.2 Use of agricultural fertilizers in the cultivation of *Chroococcidiopsis* sp.

The use of fertilizers in microalgal cultivation is gaining attention in the last years. However, little is known about their applicability in the cyanobacterium cultivation. In this sense, and with the aim to reduce the price and labour during a potential biotechnological application of *Chroococcidiopsis* sp., the use of 3 different NPKs (8-6-6, 18-6-6- and 4-10-10) was assessed. Growth trend was similar to the reference media (BBM) for 2 of the fertilizers, with a maximal growth found for the NPK 8-6-6. However, 4-10-10 did not

result in any growth even after 15 days of cultivation (Figure 22). And the same trend was observed for the photosynthetic efficiency (Figure 23B). Finally, regarding the specific biomass content of phycobiliproteins, the same fertilizer 8-6-6 showed the highest content (Figure 23). All these confirmed the potential of that fertilizer to be used in the cultivation of *Chroococcidiopsis* sp. without any negative impact in growth neither phycobiliproteins production.

One of the main factors that control the growth and productivity is nutrients composition[47]. In our case, three different types of fertilizers have been used during the experiment which are classified according to the concentration of the main elements: Nitrogen, Phosphorus and Potassium (NPKs). In the case of 4-10-10, fertilizer that seriously compromised growth, it is the fertilizer which presented the lowest N/P ratio (nitrogen was the same as the other fertilizers but phosphorous was in a higher concentration). In addition, nitrogen was present in the form of ammonium in this fertilizer. All these, might have compromised *Chroococcidiopsis* sp. growth. By other hand, 8-6-6 and 18-6-6 presented the same form of nitrogen –ammonium and urea- but with different N/P ratios, 7.26 and 15.5 respectively (Table 2).

In general, the growth and the synthesis of metabolites is affected by the composition of the culture medium. Although the components of 18-6-6 and 8-6-6 were the same but with different concentration, the growth was higher when 8-6-6 was used. Considering the amount of fertilizer used was adapted in each media to result in the same molar concentration of nitrogen in all the final media, there are two possible explanations for that difference: the lower N/P ratio in 8-6-6 (7.3 versus 15.5 in 18-6-6), or the lower amount of phosphorous in 18-6-6 (0.58mM versus 1.25 mM in 8-6-6) as a consequence of a lower amount of fertilizer used to prepare the final media with the same nitrogen concentration as BBM.

As previously commented, the growth of *Chroococcidiopsis* sp. in 8-6-6 and 18-6-6 was significantly higher than 4-10-10, and this could be explained by the composition of the fertilizers, although regarding to the statistical analysis , the fertilizer 8-6-6 was the only one that showed significant differences among the others. According to the nitrogen source, cyanobacteria normally prefer to use inorganic nitrogen forms for growth, although some strains are able to use organic nitrogen[24]. In our case, ammonia was found as unique

nitrogen form in 4-10-10 and *Chroococcidiopsis* sp. hardly grew. Although the other fertilizers also presented ammonia, the main nitrogen source was in the form of urea, which is the most significant organic form. This nitrogen source is actively transported into the cells and metabolized to ammonia and carbonic acid which both can be used by microalgae and cyanobacteria. Despite of the energy consuming process, many studies have shown a positive effect of this nitrogen source in the growth of certain species, for example *Arthrospira platensis*[78], *Chlorella* sp.[79] or *Coccomyxa acidophila*[80], where there were even higher rates than using another type of nitrogen source.

Among the essential nutrients, phosphorus is considered to be one of the main nutrients that control the development of natural populations of cyanobacteria in many environments due to the fact of being a limiting nutrient[81]. It is an essential component of many organic molecules such as DNA or RNA, phospholipid membrane and ATP. In case of phosphorous limitation, it can be accumulated as polyphosphate granules, which are used as a reserve to be consumed when the phosphate concentration depletes in the medium[47] . In our case, when phosphorus was present in an intermediate concentration (1.24 mM) the best results were obtained. But, as previously commented, the ratio N/P might have a more significant role in the performance of *Chroococcidiopsis* sp.

The Redfield ratio created by Atkison and Smith suggests that cyanobacteria cultivated with a N/P ratio higher than 30 show limited growth as a result of presenting low phosphorus levels, while when a nitrogen deficiency is produced, meaning that the N/P ratio is lower than 10, the growth is promoted[82]. This is in accordance with our findings, with the optimal growth found when the N/P ratio was 7.26 (fertilizer 8-6-6).

7.3 Effect of salinity in the cultivation of *Chroococcidiopsis* sp.

The effect of salinity over growth and phycobiliproteins accumulation in *Chroococcidiopsis* sp. was also assessed. The range of salinity used (0.2, 0.4 and 0.6 M) was selected based on previous data reported[83]. Only the culture exposed to the lowest salinity (0.2 M) was able to support growth. In this sense, the culture exposed to 0.2 M of NaCl showed a similar trend compared to the reference media although the final biomass concentration was considerably lower (0.5 versus 0.8 g/L approx.) (Figure 27). That trend

was confirmed by the photosynthetic efficiency data, where only the culture exposed to the lowest salinity showed acceptable photosynthetic efficiencies, although it was also compromised at the end of the cultivation (Figure 28). And the cultures with the highest concentrations of salt showed the lowest efficiencies since the beginning of the cultivation. All these seems to indicate that *Chroococcidiopsis* sp. was able to tolerate only moderate salt concentrations, although pre-adaptation to salinity might yield better results (not assessed in that thesis due to the lack of time).

Regarding the pigments content of *Chroococcidiopsis* sp., there was an increase in the cellular content of chlorophyll and carotenoids when the salt concentration was lower or equal to 0.2 M. Higher salinity values resulted in the absence of accumulation of these pigments (Figure 30A and 30B). However, the highest content of phycocyanin was produced when the cultures were submitted to a salt concentration of 0.4 NaCl and no differences were found between the rest of the salinities. And the same occurred with the rest of phycobiliproteins. A possible hypothesis could be that the cultures exposed to a high salt concentration experience certain degree of stress which results in the production of phycobiliproteins, with allophycocyanin as the major phycobiliprotein (Figures 34, 35 and 36).

Cyanobacteria have been found in many different light-exposed habitats on the Earth as they are able to adapt to a wide range of environmental factors such as salinity, which is consider to be an important abiotic factor on aquatic and terrestrial ecosystems. According to it, some cyanobacteria can inhabit in different hypersaline environments where the salt concentrations are higher than the seawater (3.5%) such as inland hypersaline lakes, coastal hypersaline lagoons, salter evaporation ponds, saline springs[84]. According to the resistance to salinity cyanobacteria can be grouped in three different salt-tolerance groups, such as salt sensitive, moderately halotolerant, and extremely halotolerant. Those cyanobacteria which are inside the hypersaline group are classified as moderately halophilic. Nevertheless, there are other cyanobacteria which cannot live in these environments as a result of the toxic effect of salts on their cellular metabolism.

Finally, there are other cyanobacterial strains that have been able to develop two different strategies to tolerate high salt concentrations which are known as “salt-in-strategy” and “salt-out-strategy”[85,86]. Most of cyanobacteria and all eukaryotic use the “salt-out-strategy” that consists in keeping the internal ion concentration low by accumulating small organic

molecules named as compatible solutes[87]. This strategy consists firstly in accumulating compatible solutes which do not modify the action of the metabolism and secondly, they actively export inorganic ions that steadily diffuse along their electrochemical gradients into the cytoplasm [87].

According to the salinity effect in cyanobacteria, a reduction in *Spirulina fusiformis* growth rate was observed when the salt concentration increased[88]. This caused the inhibition of photosynthesis and respiration system as a result of the high metabolic cost, leading to the inhibition of PSII activity and the detachment of phycobilisomes from the thylakoid membranes[44] . Moreover, a similar effect has been observed in *Microcystis aeruginosa, Synechococcus* sp, and *Anabena cylindrical* [89–91].

Regarding the production of phycobiliproteins, *Anabena* NCCU-9, it produced a higher amount of phycobiliproteins when it was submitted to a low concentration of NaCl [48]. In addition, this effect was also observed in *Spirulina platensis* which showed an increase of phycobiliproteins content when it was cultured with a concentration of 0.4 M of NaCl [92]. These studies are in accordance with our findings, where a higher content of phycobiliproteins was found at 0.4 M. However, the resulting productivity could not be optimal due to the poor growth observed under such conditions. In this sense, this effect depends on the halotolerant capacity of each strain and it has to be particularly addressed.

In general terms, the presence of salt produces a decrease in the Chlorophyll a, proteins and carbohydrate content while sometimes an increase of the lipid's contents could be detected[92] . Nevertheless, other strains as *Synechococcus* sp. and *Scytonema geitleri* showed a decrease in growth rate and lipids but an increase in proteins and carbohydrate content[93,94]

But, as previously commented, some species of cyanobacterium have been found in places with high salt concentration, above 25% up to NaCl saturation (4 M), such as *Chroococcidiopsis* sp., which could inhabit in halite evaporites in the Atacama Desert (Chile). However, our strain was obtained from a calcite rock which resulted in a different salt tolerance. As previously commented, although growth was compromised the best results in terms of phycobiliproteins were obtained when it was cultured with a concentration of 0.4 M of NaCl. However, considering the final productivity (combination of growth and phycobiliproteins content), 0.2 M should be proposed as the optimal to be

further investigated. Besides, preadaptation of the cultures to the different salinities might result in even better performances and optimize the process

7.4 State of the art in *Chroococcidiopsis* sp. cultivation.

Many environmental conditions influence cyanobacterial growth and phycobiliproteins content, such as nutrients, pH, light and temperature among others. In the specific case of *Chroococcidiopsis* sp., little information is available in this regard and only few studies are available. As example, the effect of pH and salt on its growth has been briefly characterized. In this sense, a cultivation pH range of 7.5 – 9.5 has been evaluated and no differences were found, which resulted in the definition of pH 8.5 as the optimal value[95]. In addition, in the case of salinity, it was proposed that the salt concentration in the media does not have any influence when used on a range of 4-8% (0.6 -1.3M). And finally, the effect of daily temperature on the biomass growth was assessed when it was submitted between 5.7-16 ºC[95].

Regarding the phycobiliproteins content in *Chroococcidiopsis* sp., only a study can be found where a higher amount of phycobiliproteins was produced when the culture medium was supplemented with nitrogen. However, there is little information about the effect of light in *Chroococcidiopsis* sp. For example, Das et al 2018, showed a change in the pigmentation of that strain (its color turned into reddish-brown) when it was exposed to light intensities above 100 µmol photons·m^{-2}·s^{-1})[95].

Considering the potential biotechnological value of *Chroococcidiopsis* sp., and that only few studies have been carried out with that species -and more specifically with that novel strain isolated from Atacama Desert- it is needed a deeper analysis of the environmental factors that could compromise growth and enhance pigment concentration in such strain. In this sense, that Master Thesis can be considered as a first approximation to the evaluation of the biotechnological potential of that strain and further work is needed in order to finally optimize the process for phycobiliproteins production.

8. CONCLUSIONS

Influence of light intensity as abiotic factor with biotechnological applications:

The exposure of *Chroococcidiopsis* sp. to different light intensities (10, 50, 100 and 150 µmol photons·m^{-2}·s^{-1}) allowed to evaluate its effect in growth and phycobiliproteins production. Biomass growth was proportional to the light intensity supplied below 100 µmol photons·m^{-2}·s^{-1}. Although the phycobiliproteins content was maximal at 50 µmol photons·m^{-2}·s^{-1}, probably due to a major light-harvesting role of phycobiliproteins, the phycobiliproteins volumetric productivity was also maximal at 100 µmol photons·m^{-2}·s^{-1}. Allophycocyanin was the most abundant phycobiliprotein, followed by phycoerythrin and phycocyanin (productivity values of 18.158, 10.972 and 6.234 mg/L/d respectively).

Chroococcidiopsis sp. seems to be naturally adapted to low light intensities, as endolithic species. Growth, photosynthesis and pigment content results indicate a certain degree of photo-limitation when it was cultivated at light intensities below 100 µmol photons·m^{-2}·s^{-1}. However, higher light intensities might be experienced as saturating and/or over-saturating (lower photosynthetic efficiency and higher carotenoid and phycobiliproteins content at the highest intensity), with a negative impact in final productivity values.

Semi-continuous cultivation of *Chroococcidiopsis* sp. under different light intensities (10, 50, 70, 100 and 150 µmol photons·m^{-2}·s^{-1}) allowed the biomass to adapt to the conditions imposed, resulting in similar growth at the different light intensities. However, a slightly different behavior was found in the phycobiliproteins content when compared to batch cultivation.

The production of allophycocyanin was maximal when the cultures were submitted to 50 µmol photons·m^{-2}·s^{-1}, while the rest of phycobiliproteins showed a higher production at 150 µmol photons·m^{-2}·s^{-1}. Allophycocyanin might play a role as accessory pigment to enhance light capture at low light intensities while phycoerythrin and phycocyanin might play a role as protectant at high light intensities.

Use of agricultural fertilizers for the cultivation of *Chroococcidiopsis* sp.:

Commercial agricultural fertilizers, with different nitrogen sources and N/P ratios, were used to cultivate *Chroococcidiopsis* sp. The fertilizer 4-10-10 seriously compromised growth, probably due to the lowest N/P ratio and the presence of ammonium as nitrogen source. The best results were obtained with the fertilizer 8-6-6, which showed higher values of phycobiliproteins cellular content than the control (approximately 80 mg/g of allophycocyanin, 55 mg/g of phycoerythrin and 35 mg/g of phycocyanin).

Fertilizers with a N/P ratio below 10, as it is the case of 8-6-6, seem to promote cyanobacterial growth and phycobiliproteins accumulation.

Influence of medium salinity as abiotic factor with biotechnological applications: *Chroococcidiopsis* sp. showed a low tolerance to salinity and it was able to reasonable grow only at the lowest concentration (0.2 M). Photosynthetic efficiency was dramatically compromised at moderate salinity values. However, phycobiliproteins content was higher at higher salinity values, 0.4 M, probably due to a role as protectants against the oxidative stress experienced by the cells under such conditions. Noteworthy, the poor growth observed at that salinity seriously compromised the applicability for phycobiliproteins production.

To sum up, selection of the optimal light intensity for *Chroococcidiopsis* sp. cultivation under batch and semi-continuous cultivation can improve the final phycobiliproteins productivity. The use of agricultural fertilizers in the culture medium could be employed as a useful tool to reduce labour and costs while increasing the productivity of phycobiliproteins. However, the nitrogen source and the N/P ratio might play an important role to be particularly addressed. And finally, salinity is an essential element that alters the growth and photosynthetic efficiency of *Chroococcidiopsis* sp. and adaptation to moderate salinity values might be needed in order to promote growth and phycobiliproteins accumulation.

9. ANNEX. Statistical analysis.

9.1 Influence of light intensity in *Chroococcidiopsis* sp during batch cultivation

9.1.1 Statistical analysis of optical density at 750 nm.

ANOVA SUMMARY	
F	675,7
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,998

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 100	Yes
50 VS. 150	Yes
100 VS. 150	Yes

9.1.2 Statistical analysis of Chlorophyll ($\mu g \cdot ml^{-1}$)

ANOVA SUMMARY	
F	7,94
P VALUE	0,0369
P VALUE SUMMARY	*
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,8562

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 100	Yes
50 VS. 150	Yes
100 VS. 150	Yes

9.1.3 Staticical analsysis of Carotenoids ($\mu g \cdot ml^{-1}$)

ANOVA SUMMARY	
F	53,76
P VALUE	0,0011
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9758

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 100	Yes
50 VS. 150	No
100 VS. 150	No

9.1.4 Statistical analysis of Cholorophyll ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	8,872
P VALUE	0,0306
P VALUE SUMMARY	*
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,8693

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	No
10 VS. 100	No
10 VS. 150	No
50 VS. 100	Yes
50 VS. 150	No
100 VS. 150	No

9.1.5 Statistical analysis of Carotenoids ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	6,137
P VALUE	0,056
P VALUE SUMMARY	ns
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	No
R SQUARE	0,8215

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	No
10 VS. 150	No
50 VS. 100	Yes
50 VS. 150	Yes
100 VS. 150	No

9.1.6 Statistical analysis of Phycocyanin ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	36,83
P VALUE	0,0023
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9651

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 100	No
50 VS. 150	No
100 VS. 150	No

9.1.7 Statistical analysis of Allophycocyanin ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	38,43
P VALUE	0,0021
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9665

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 100	No
50 VS. 150	No
100 VS. 150	No

9.1.8 Statistical analysis of Phycoerythrin ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	47,45
P VALUE	0,0014
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9727

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 100	No
50 VS. 150	No
100 VS. 150	No

9.1.9 Statistical analysis of Phycocyanin ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	13,52
P VALUE	0,0147
P VALUE SUMMARY	*
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9102

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	No
10 VS. 150	No
50 VS. 100	Yes
50 VS. 150	No
100 VS. 150	No

9.2. Statistical analysis of Allophycocyanin ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	18,94
P VALUE	0,0079
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9342

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	No
10 VS. 150	No
50 VS. 100	Yes
50 VS. 150	Yes
100 VS. 150	No

9.2.1 Statistical analysis of Phycoerythrin ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	11,78
P VALUE	0,0187
P VALUE SUMMARY	*
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,8983

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 100	No
10 VS. 150	No
50 VS. 100	Yes
50 VS. 150	No
100 VS. 150	No

9.3 Statistical analysis of Light experiment in *Chroococcidiopsis* sp. in Semi-continuous cultivation

ANOVA SUMMARY	
F	18,17
P VALUE	0,0035
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9356

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 70	Yes
10 VS. 100	Yes
10 VS. 150	No
50 VS. 70	No
50 VS. 100	No
50 VS. 150	Yes
70 VS. 100	No
70 VS. 150	No
100 VS. 150	Yes

9.3.1 Statistical analysis of chlorophyll content (µg·ml^{-1})

ANOVA SUMMARY	
F	1274
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,999

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 70	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 70	Yes
50 VS. 100	Yes
50 VS. 150	Yes
70 VS. 100	Yes
70 VS. 150	Yes
100 VS. 150	No

9.3.2 Statistical analysis of Carotenoids content (µg·ml^{-})

ANOVA SUMMARY	
F	63,36
P VALUE	0,0002
P VALUE SUMMARY	***
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9807

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 70	Yes
10 VS. 100	No
10 VS. 150	No
50 VS. 70	Yes
50 VS. 100	Yes
50 VS. 150	Yes

70 VS. 100	Yes
70 VS. 150	No
100 VS. 150	No

9.3.3 Statistical analysis of Chlorophyll content ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	1424
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P <0.05)?	Yes
R SQUARE	0,9991

TUKEY'S TEST	**SIGNIFICANT?**
10 VS. 50	No
10 VS. 70	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 70	Yes
50 VS. 100	Yes
50 VS. 150	Yes
70 VS. 100	Yes
70 VS. 150	Yes
100 VS. 150	No

9.3.4 Statistical analysis of Carotenoids content ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	98,77
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9875

TUKEY'S TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 70	Yes
10 VS. 100	No
10 VS. 150	No
50 VS. 70	Yes
50 VS. 100	Yes
50 VS. 150	No
70 VS. 100	Yes
70 VS. 150	Yes
100 VS. 150	No

9.3.5 Statistical analysis of Phycocyanin content ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	10,98
P VALUE	0,0108
P VALUE SUMMARY	*
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,8978

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	No
10 VS. 70	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 70	No
50 VS. 100	No
50 VS. 150	No
70 VS. 100	No
70 VS. 150	No
100 VS. 150	No

9.3.6 Statistical analysis of Allophycocyanin content ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	12,18
P VALUE	0,0086
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,907

TUKEY'S TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 70	No
10 VS. 100	No
10 VS. 150	No
50 VS. 70	Yes
50 VS. 100	Yes
50 VS. 150	No
70 VS. 100	No
70 VS. 150	No
100 VS. 150	Yes

9.3.7 Statistical analysis of Phycoerythrin content ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	9,629
P VALUE	0,0144
P VALUE SUMMARY	*
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,8851

TUKEY'S TEST	SIGNIFICANT?
10 VS. 50	No
10 VS. 70	Yes
10 VS. 100	No
10 VS. 150	No
50 VS. 70	Yes
50 VS. 100	No
50 VS. 150	No
70 VS. 100	No
70 VS. 150	Yes
100 VS. 150	No

9.3.8 Statistical analysis of Phycocyanin content ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	14,8
P VALUE	0,0056
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P <0.05)?	Yes
R SQUARE	0,9221

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	No
10 VS. 70	Yes
10 VS. 100	Yes
10 VS. 150	Yes
50 VS. 70	No
50 VS. 100	No
50 VS. 150	No
70 VS. 100	No
70 VS. 150	No
100 VS. 150	No

9.3.9 Statistical analysis of Allophycocyanin content ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	17,37
P VALUE	0,0039
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9329

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 70	No
10 VS. 100	No
10 VS. 150	No
50 VS. 70	Yes
50 VS. 100	Yes
50 VS. 150	No
70 VS. 100	No
70 VS. 150	No
100 VS. 150	No

9.4. Statistical analysis of Phycoerythrin content ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	16,28
P VALUE	0,0045
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9287

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
10 VS. 50	Yes
10 VS. 70	No
10 VS. 100	No
10 VS. 150	No
50 VS. 70	Yes
50 VS. 100	Yes
50 VS. 150	No
70 VS. 100	No
70 VS. 150	Yes
100 VS. 150	No

9.5. Statistical analysis from the Salinity experiment at the end of the experiment

9.5.1 Statistical analysis of turbidity at 750 nm

ANOVA SUMMARY	
F	63841
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	1

TUKEY'S MULTIPLE COMPARISONS TEST	**SIGNIFICANT?**
CONTROL VS. 0.2	Yes
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	No

9.5.2 Statistical analysis of Chlorophyll a ($\mu g \cdot ml^{-1}$)

ANOVA SUMMARY	
F	1664
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9992

TUKEY'S MULTIPLE COMPARISONS TEST	**SIGNIFICANT?**
CONTROL VS. 0.2	Yes
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	No

9.5.3 Statistical analysis of Carotenoids ($\mu g \cdot ml^{-1}$)

ANOVA SUMMARY	
F	28055
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	1

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
CONTROL VS. 0.2	Yes
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	Yes

9.5.4 Statistical analysis of Chlorophyll ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	28157
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	1

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
CONTROL VS. 0.2	Yes
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	No

9.5.5 Statistical analysis of carotenoids ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	4464
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9997

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
CONTROL VS. 0.2	Yes
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	Yes

9.5.6 Statistical analysis of Phycocyanin ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	72,07
P VALUE	0,0006
P VALUE SUMMARY	***
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9818

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
CONTROL VS. 0.2	No
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	Yes

9.5.7 Statistical analysis of Allophycocyanin ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	35,05
P VALUE	0,0025
P VALUE SUMMARY	**
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9634

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
CONTROL VS. 0.2	No
CONTROL VS. 0.4	No
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	Yes

9.5.8 Statistical analysis of Phycoerythrin ($mg \cdot mL^{-1}$)

ANOVA SUMMARY	
F	100,5
P VALUE	0,0003
P VALUE SUMMARY	***
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9869

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
CONTROL VS. 0.2	No
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	Yes
0.2 VS. 0.4	Yes
0.2 VS. 0.6	Yes
0.4 VS. 0.6	Yes

9.5.9 Statistical analysis of phycoyanin ($mg \cdot g^{-1}$)

ANOVA SUMMARY

F	641
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9979

TUKEY'S MULTIPLE COMPARISONS TEST	**SIGNIFICANT?**
CONTROL VS. 0.2	No
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	No
0.2 VS. 0.4	Yes
0.2 VS. 0.6	No
0.4 VS. 0.6	Yes

9.6 Statistical analysis of Allophycocyanin ($mg \cdot g^{-1}$)

ANOVA SUMMARY

F	276
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9952

TUKEY'S MULTIPLE COMPARISONS TEST	**SIGNIFICANT?**
CONTROL VS. 0.2	No
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	No
0.2 VS. 0.4	Yes
0.2 VS. 0.6	No
0.4 VS. 0.6	Yes

9.6.1 Statistical analysis of Allophycocyanin ($mg \cdot g^{-1}$)

ANOVA SUMMARY	
F	719,3
P VALUE	<0,0001
P VALUE SUMMARY	****
SIGNIFICANT DIFF. AMONG MEANS (P < 0.05)?	Yes
R SQUARE	0,9981

TUKEY'S TEST	SIGNIFICANT?
CONTROL VS. 0.2	No
CONTROL VS. 0.4	Yes
CONTROL VS. 0.6	No
0.2 VS. 0.4	Yes
0.2 VS. 0.6	No
0.4 VS. 0.6	Yes

9.7 Statistical analysis of Fertilizer experiment at the end of the experiment

TABLE ANALYZED	FERTILIZER				
TWO-WAY ANOVA	Ordinary				
ALPHA	0,05				
SOURCE OF VARIATION	% of total variation	P value	P value summary	Significant?	
EXPERIMENT	46,46	0,0214	*	Yes	
PHYCOBILIPROTEINS	45,57	0,0222	*	Yes	
ANOVA TABLE	SS	DF	MS	F (DFn, DFd)	P value
EXPERIMENT	1651	2	825,5	F (2, 4) = 11,66	P=0,0214
PHYCOBILIPROTEINS	1619	2	809,6	F (2, 4) = 11,43	P=0,0222
RESIDUAL	283,2	4	70,81		
NUMBER OF MISSING VALUES	0				

TUKEY'S MULTIPLE COMPARISONS TEST	SIGNIFICANT?
PHYCOCYANIN	
CONTROL VS. 8	No
CONTROL VS. 18	No
8 VS. 18	No
ALLOPHYCOCYANIN	
CONTROL VS. 8	Yes
CONTROL VS. 18	No
8 VS. 18	No
PHYCOERYTHRIN	
CONTROL VS. 8	No
CONTROL VS. 18	No
8 VS. 18	No

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